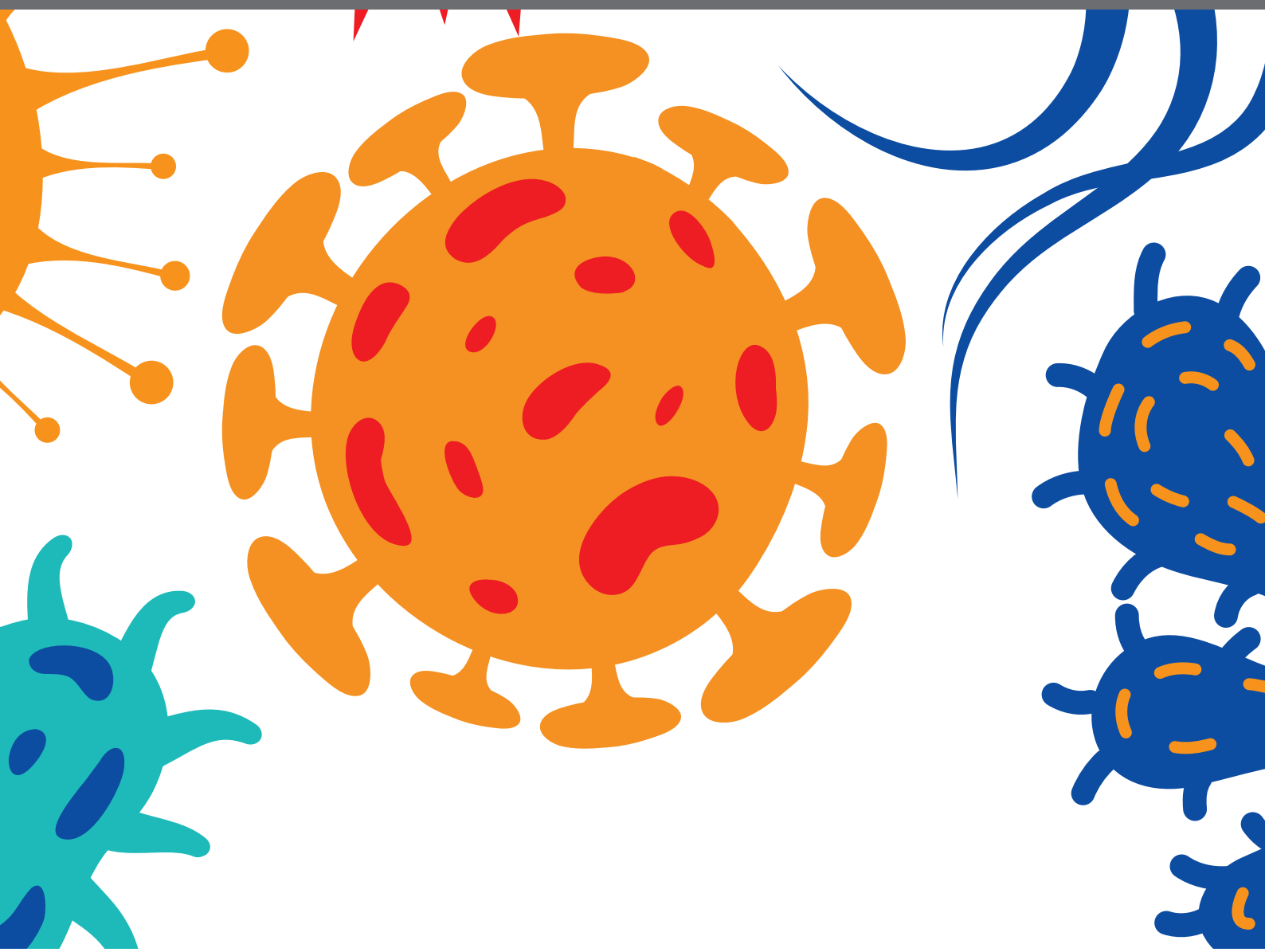
SALIVA AND ORAL MICROBIOTA: FROM PHYSIOLOGY TO DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS

EDITED BY: Andrea Santarelli, Lorenzo Lo Muzio and David Wong

PUBLISHED IN: Frontiers in Cellular and Infection Microbiology and Frontiers in Physiology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88966-495-5
DOI 10.3389/978-2-88966-495-5

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

SALIVA AND ORAL MICROBIOTA: FROM PHYSIOLOGY TO DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS

Topic Editors:
Andrea Santarelli, Marche Polytechnic University, Italy
Lorenzo Lo Muzio, University of Foggia, Italy
David Wong, University of California, Los Angeles, United States

Citation: Santarelli, A., Muzio, L. L., Wong, D., eds. (2021). Saliva and Oral Microbiota: From Physiology to Diagnostic and Therapeutic Implications. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-495-5

Table of Contents

05 ***Editorial: Saliva and Oral Microbiota: From Physiology to Diagnostic and Therapeutic Implications***
Andrea Santarelli, David T. W. Wong and Lorenzo Lo Muzio

07 ***Frequency of Tongue Cleaning Impacts the Human Tongue Microbiome Composition and Enterosalivary Circulation of Nitrate***
Gena D. Tribble, Nikola Angelov, Robin Weltman, Bing-Yan Wang, Sridhar V. Eswaran, Isabel C. Gay, Kavitha Parthasarathy, Doan-Hieu V. Dao, Katherine N. Richardson, Nadia M. Ismail, Iraida G. Sharina, Embriette R. Hyde, Nadim J. Ajami, Joseph F. Petrosino and Nathan S. Bryan

23 ***Evaluation of Sodium Hypochlorite Irrigant, Bingpeng Irrigant, and Fufang Bingpeng Irrigant as Endodontic Irrigants During Passive Ultrasonic Irrigation***
Yan Shi, Zhipeng Deng, Yulu Yang, Lanyue Cui, Tingtao Chen, Mingjing Hu, Lei Xie and Jian Yang

31 ***Efficacy of an All-Natural Polyherbal Mouthwash in Patients With Periodontitis: A Single-Blind Randomized Controlled Trial***
Scilla Sparabombe, Riccardo Monterubbianesi, Vincenzo Tosco, Giulia Orilisi, Andrell Hosein, Luigi Ferrante, Angelo Putignano and Giovanna Orsini

38 ***Beyond Head and Neck Cancer: The Relationship Between Oral Microbiota and Tumour Development in Distant Organs***
Marco Mascitti, Lucrezia Togni, Giuseppe Troiano, Vito Carlo Alberto Caponio, Davide Bartolomeo Gissi, Lucio Montebugnoli, Maurizio Procaccini, Lorenzo Lo Muzio and Andrea Santarelli

46 ***Unusual Conditions Impairing Saliva Secretion: Developmental Anomalies of Salivary Glands***
Lucrezia Togni, Marco Mascitti, Andrea Santarelli, Maria Contaldo, Antonio Romano, Rosario Serpico and Corrado Rubini

53 ***Do Changes in Oral Microbiota Correlate With Plasma Nitrite Response? A Systematic Review***
Khrystyna Zhurakivska, Giuseppe Troiano, Vito Carlo Alberto Caponio, Mario Dioguardi, Luigi Laino, Angela Bruna Maffione and Lorenzo Lo Muzio

61 ***Species-Level Salivary Microbial Indicators of Well-Resolved Periodontitis: A Preliminary Investigation***
Aneesha Acharya, Tsute Chen, Yuki Chan, Rory M. Watt, Lijian Jin and Nikos Mattheos

73 ***Salivary Biomarkers for Oral Squamous Cell Carcinoma Diagnosis and Follow-Up: Current Status and Perspectives***
Marta Cristaldi, Rodolfo Mauceri, Olga Di Fede, Giovanna Giuliana, Giuseppina Campisi and Vera Panzarella

85 ***Promising Treatment for Type 2 Diabetes: Fecal Microbiota Transplantation Reverses Insulin Resistance and Impaired Islets***
Hui Wang, Yuan Lu, Yan Yan, Shanshan Tian, Dongjie Zheng, Dongjing Leng, Cao Wang, Jingfeng Jiao, Zhiguo Wang and Yunlong Bai

95 ***Oral Microbiota Perturbations Are Linked to High Risk for Rheumatoid Arthritis***
Yanli Tong, Linlin Zheng, Pingying Qing, Hua Zhao, Yanhong Li, Linchong Su, Qiuping Zhang, Yi Zhao, Yubin Luo and Yi Liu

105 ***Interaction of Oral and Toothbrush Microbiota Affects Oral Cavity Health***
Qingyao Shang, Yuan Gao, Ting Qin, Shuai Wang, Yan Shi and Tingtao Chen

114 ***A Uniquely Altered Oral Microbiome Composition Was Observed in Pregnant Rats With* Porphyromonas gingivalis *Induced Periodontal Disease***
Molly S. Walkenhorst, Leticia Reyes, Gonzalo Perez, Ann Progulske-Fox, Mary B. Brown and Priscilla L. Phillips

134 ***Occlusional Modifications Reversibly Alter Aquaporin 5 Expression and Localization in Rat Salivary Glands***
Eri Saito, Ippei Watari, Mariko Mizumachi-Kubono, Sumire Hsu-Hayashi and Takashi Ono

141 **Bmal1 *Regulates Coagulation Factor Biosynthesis in Mouse Liver in* Streptococcus oralis *Infection***
Lili Chen, Shue Li, Jiaming Nie, Jiajia Zhao, Shaoling Yu, Yaoxu Li and Jinfeng Peng

frontiers in Physiology

EDITORIAL
published: 13 January 2021
doi: 10.3389/fphys.2020.637599

Editorial: Saliva and Oral Microbiota: From Physiology to Diagnostic and Therapeutic Implications

Andrea Santarelli[1]*, *David T. W. Wong*[2] and *Lorenzo Lo Muzio*[3]

[1] Department of Clinic Specialistic and Stomatological Sciences, Marche Polytechnic University, Ancona, Italy, [2] School of Dentistry, University of California, Los Angeles, Los Angeles, CA, United States, [3] Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy

Keywords: saliva, oral microbiota, omics sciences, point-of-care diagnosis, dysbiosis

Editorial on the Research Topic

Saliva and Oral Microbiota: From Physiology to Diagnostic and Therapeutic Implications

Saliva is a remarkably complex fluid with many properties and functions which are essential for both oral and general health. In the last years, technological advancements, especially in "-omics" studies and bioinformatics tools, recognized saliva as a pool of biological markers. Saliva represents a safe and non-invasive source of potentially useful information that could help to evaluate the state of health and the presence of disease (Dawes and Wong, 2019). Furthermore, the new developments in the field of cancer biology suggest that the so called "salivaomics" is a promising approach for early detection of cancer, first and foremost the oral cancer. However, the importance of the "classic" function of saliva is attested by the important consequences on quality of life suffered by subject with hyposalivation and xerostomia and by the efforts made in strategies for restoring salivary gland function in patients with parenchyma and ducts anomalies (Togni et al.). Moreover, the demographic transition in western countries is leading to the progressive aging of the population, increasing the number of elderlies that may experience occlusal and masticatory problems that in turns may imbalance water transportation in salivary gland as seen in the animal model (Saito et al.).

The extensive investigations of saliva highlight the specific characteristics of oral microenvironment with its bacterial population and its relationship with the whole body. In the last years, there has been a growing interest in understanding oral microbiota and its relationship with the oral health status and with local and systemic diseases. The study of oral microbial ecology represents another emerging connection between oral and systemic health, opening up the possibility for new diagnostic and therapeutic perspectives. Oral microbiota represents a heterogeneous group of microbial species colonizing all the oral cavity surfaces. About 700 bacterial species have been identified in oral cavity with 35% of them have not been cultured. To determine bacterial ecosystem, high-throughput sequencing methods have been developed, representing a leap toward characterization of oral microbial community and their host interaction (He et al., 2018). Recently, results from studies based on genome sequencing have started shedding light on the existence of site-specific microbial patterns that might be considered as "healthy oral microbiota." The main obstacle to this definition is the highly variable composition of microbial communities. Nevertheless, ecological imbalance of microbial community, called dysbiosis, has been extensively studied both in human and animal models. It is characterized by the loss of beneficial microbes, expansion of pathogenic microbes, and general loss of microbial diversity.

An increasing number of studies have shown that oral microbiota plays a role in the development of oral diseases, such as dental caries, periodontal disease, and oral stomatitis. The

OPEN ACCESS

Edited and reviewed by:
Thimios Mitsiadis,
University of Zurich, Switzerland

***Correspondence:**
Andrea Santarelli
andrea.santarelli@univpm.it

Specialty section:
This article was submitted to Craniofacial Biology and Dental Research, a section of the journal Frontiers in Physiology

Received: *03 December 2020*
Accepted: *10 December 2020*
Published: *13 January 2021*

Citation:
Santarelli A, Wong DTW and Lo Muzio L (2021) Editorial: Saliva and Oral Microbiota: From Physiology to Diagnostic and Therapeutic Implications. Front. Physiol. 11:637599. doi: 10.3389/fphys.2020.637599

salivary microbial species could help to discriminate patients with treated, well-maintained chronic periodontitis from healthy controls with similar gingival inflammation levels. Several species, such as *Treponema* spp., *Prevotella* spp., and *Capnocytophaga* spp., have been significantly associated with treated and well-maintained periodontitis. While *Leptotrichia buccalis, Corynebacterium matruchotii, Leptotrichia hofstadii,* and *Streptococcus intermedius*, resulted significant indicators of healthy periodontal condition (Acharya et al.). Moreover, the inoculation of *Porphyromonas gingivalis* in a *in vivo* mouse model showed the developmental of the periodontal disease, resulting in oral microbiome dysbiosis (Walkenhorst et al.).

Lately, is becoming evident that oral dysbiosis is associated with head and neck cancer development and with the pathogenesis of systemic diseases. Furthermore, it also seems to be involved in gastrointestinal neoplasms, especially esophageal, gastric, pancreatic, and colorectal cancers, paving the way for developing specific oral microbiota test to allow early cancer detection (Mascitti et al.). Liquid biopsy represents a non-invasive method for the detection of diagnostic and prognostic biomarkers in body fluids of oncologic patients. The salivary liquid biopsy provides several promising clinical uses in cancer management: it is painless, accessible, and low cost and represents a very helpful source of diagnostic and prognostic biomarker detection. Even if standardized protocols for isolation, characterization, and evaluation are needed, recent data suggest that saliva may be successfully included in future clinical diagnostic processes, with a considerable impact on early treatment strategies and a favorable outcome (Cristaldi et al.).

Regarding systemic diseases, the *Streptococcus oralis* has been recognized as a pathogen to cause multiorgan failure by contributing to the formation of microthrombus. Mice infected by Streptococcus oralis showed a downregulation of hepatic BMAL1 gene expression, a downregulation of the coagulation factor VII and an upregulation of the coagulation factor XII *in vitro* and *in vivo*. Thus, these results could improve the treatment of hepatic sepsis by the regulation of the BMAL1 gene expression (Chen et al.).

Oral microbial dysbiosis is known to increase susceptibility of an individual to develop the rheumatoid arthritis. It has been observed a characteristic compositional change of salivary microbes in individuals at high-risk for rheumatoid arthritis, suggesting oral microbiota dysbiosis occurs in the pre-clinical stage of this condition (Tong et al.). Moreover, the microbiota has been observed to have a growing relationship with diabetes. Microbiota imbalance has been hypothesized to be involved in the regulation of energy metabolism and the inflammatory immune response. In a type 2 diabetes mouse model, the insulin resistance was improved, and the pancreatic markers of inflammation were reduced after Fecal Microbiota Transplantation, providing a novel potential treatment strategy (Wang et al.).

Finally, studies pointed out that antibacterial toothpaste (Sparabombe et al.), all-natural polyherbal mouthwash (Shang et al.) and antibacterial mouthwashes could lead to an alteration of oral microbiota and of oral microbiota salivary nitrate, a substrate of oral and intestinal microbiota, metabolism. The consequences on the circulating Nitric Oxide levels are still completely unclear (Zhurakivska et al.). However, the alterations of oral microbiota could lead to modifications in plasmatic Nitric Oxide content, resulting in increases in systolic blood pressure. Moreover, the tongue cleaning frequency resulted as a predictor of chlorhexidine-induced changes in systolic blood pressure and tongue microbiome composition. These data suggest that management of the tongue microbiome together with adequate dietary intake of nitrate provide an opportunity for the improvement of resting systolic blood pressure (Tribble et al.).

In conclusion, this Research Topic has aimed to provide a complete and comprehensive overview of saliva, passing through oral microbiota interactions, and their role in health and disease, describing also point-of-care diagnostic and therapeutic possibilities.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Dawes, C., and Wong, D. T. W. (2019). Role of saliva and salivary diagnostics in the advancement of oral health. *J. Dent. Res.* 98, 133–141. doi: 10.1177/0022034518816961

He, X., Li, F., Bor, B., Koyano, K., Cen, L., Xiao, X., et al. (2018). Human tRNA-derived small RNAs modulate host-oral microbial interactions. *J. Dent. Res.* 97, 1236–1243. doi: 10.1177/0022034518770605

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 01 March 2019
doi: 10.3389/fcimb.2019.00039

Frequency of Tongue Cleaning Impacts the Human Tongue Microbiome Composition and Enterosalivary Circulation of Nitrate

Gena D. Tribble[1], Nikola Angelov[1], Robin Weltman[1], Bing-Yan Wang[1], Sridhar V. Eswaran[1], Isabel C. Gay[1†], Kavitha Parthasarathy[1], Doan-Hieu V. Dao[1], Katherine N. Richardson[1], Nadia M. Ismail[1], Iraida G. Sharina[2], Embriette R. Hyde[3], Nadim J. Ajami[4,5†], Joseph F. Petrosino[4,5] and Nathan S. Bryan[6]**

[1] Department of Periodontics, School of Dentistry, The University of Texas Health Science Center Houston, Houston, TX, United States, [2] Division of Cardiology, Department of Internal Medicine, McGovern Medical School, The University of Texas Health Science Center Houston, Houston, TX, United States, [3] SynBioBeta, Pleasant Hill, CA, United States, [4] Alkek Center for Metagenomics and Microbiome Research, Baylor College of Medicine, Houston, TX, United States, [5] Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, TX, United States, [6] Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, United States

The oral microbiome has the potential to provide an important symbiotic function in human blood pressure physiology by contributing to the generation of nitric oxide (NO), an essential cardiovascular signaling molecule. NO is produced by the human body via conversion of arginine to NO by endogenous nitric oxide synthase (eNOS) but eNOS activity varies by subject. Oral microbial communities are proposed to supplement host NO production by reducing dietary nitrate to nitrite via bacterial nitrate reductases. Unreduced dietary nitrate is delivered to the oral cavity in saliva, a physiological process termed the enterosalivary circulation of nitrate. Previous studies demonstrated that disruption of enterosalivary circulation via use of oral antiseptics resulted in increases in systolic blood pressure. These previous studies did not include detailed information on the oral health of enrolled subjects. Using 16S rRNA gene sequencing and analysis, we determined whether introduction of chlorhexidine antiseptic mouthwash for 1 week was associated with changes in tongue bacterial communities and resting systolic blood pressure in healthy normotensive individuals with documented oral hygiene behaviors and free of oral disease. Tongue cleaning frequency was a predictor of chlorhexidine-induced changes in systolic blood pressure and tongue microbiome composition. Twice-daily chlorhexidine usage was associated with a significant increase in systolic blood pressure after 1 week of use and recovery from use resulted in an enrichment in nitrate-reducing bacteria on the tongue. Individuals with relatively high levels of bacterial nitrite reductases had lower resting systolic blood pressure. These results further support the concept of a symbiotic oral microbiome contributing to human health via the enterosalivary nitrate-nitrite-NO pathway. These data suggest that management of the tongue microbiome by regular cleaning together with adequate dietary intake of nitrate provide an opportunity for the improvement of resting systolic blood pressure.

Keywords: oral microbiome, microbiome, microbial ecology, host-microbial symbiosis, nitrate, nitric oxide

OPEN ACCESS

Edited by:
Lorenzo Lo Muzio, University of Foggia, Italy

Reviewed by:
John Francis Brooks, University of Texas Southwestern Medical Center, United States
Janina P. Lewis, Virginia Commonwealth University, United States
Khrystyna Zhurakivska, University of Foggia, Italy

****Correspondence:***
Gena D. Tribble
gena.d.tribble@uth.tmc.edu
Nathan S. Bryan
nathan.bryan@bcm.edu

†Present Address:
Isabel C. Gay, East Carolina University College of Dentistry, Greenville, NC, United States
Nadim Ajami, MicrobiomeDX, Houston, TX, United States

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *31 October 2018*
Accepted: *07 February 2019*
Published: *01 March 2019*

Citation:
Tribble GD, Angelov N, Weltman R, Wang B-Y, Eswaran SV, Gay IC, Parthasarathy K, Dao D-HV, Richardson KN, Ismail NM, Sharina IG, Hyde ER, Ajami NJ, Petrosino JF and Bryan NS (2019) Frequency of Tongue Cleaning Impacts the Human Tongue Microbiome Composition and Enterosalivary Circulation of Nitrate. Front. Cell. Infect. Microbiol. 9:39. doi: 10.3389/fcimb.2019.00039

INTRODUCTION

The human oral cavity is an important habitat for microbes, and a healthy mouth can harbor upwards of ten billion bacteria (Loesche, 1993). Cardiovascular research has identified a potential role for the oral microbiome in human health via the conversion of dietary nitrate into nitrite, the bioactive storage pool available for spontaneous conversion to nitric oxide (NO) (Lundberg and Govoni, 2004; Lundberg et al., 2008). Continuous generation of NO is essential for the integrity of the cardiovascular system, and decreased production or bioavailability of NO is central to the development of many heart-related disorders (Lundberg et al., 2004, 2009; Bryan and Loscalzo, 2017). The human body is able to produce NO directly by the five-electron oxidation of L-arginine (Moncada and Higgs, 1993) by endothelial nitric oxide synthases (eNOS; **Figure 1A**). However, the eNOS gene is polymorphic, and the pathway can become dysfunctional with age (Niu and Qi, 2011). Failure to produce sufficient NO is causal for the onset and progression of a number of cardiovascular diseases, including hypertension and atherosclerosis (Taddei et al., 2001; Torregrossa et al., 2011). The enterosalivary nitrate-nitrite-NO pathway in humans appears to serve as an alternative pathway for production of bioactive NO, supplementing host endothelial NO production. This pathway functions via the bacterial conversion of dietary nitrate into nitrites that can then be converted to NO and participate in the regulation of endothelial vasodilation (**Figure 1B**) (Benjamin et al., 1994; Lundberg et al., 1994). This diet-dependent pathway relies upon commensal oral bacteria located on the tongue dorsum to perform the first step (nitrate reduction to nitrite) since mammals lack a functional nitrate reductase (Lundberg et al., 2004; Doel et al., 2005).

Although there is compelling evidence supporting the role of the tongue microbiome in the enterosalivary nitrate-nitrite-NO pathway, this remains a relatively unexplored area of human-microbial mutualism and many important questions must be addressed. Previous studies have demonstrated that nitrite-induced reductions in blood pressure are inhibited by antiseptic chlorhexidine (CHX) or other antibacterial mouthwashes (Tannenbaum et al., 1976; Govoni et al., 2008; Petersson et al., 2009; Kapil et al., 2013; McDonagh et al., 2015; Woessner et al., 2016; Mitsui and Harasawa, 2017). The focus of these studies has been from the perspective of cardiovascular physiology, and little information is available regarding the oral health status or habits of subjects in these studies. In this study we approach this symbiotic relationship from the perspective of oral health using a cohort of 27 oral health professionals with both excellent cardiovascular and oral health. These subjects used a CHX mouthwash for 7 days, and we assessed the response of resting systolic blood pressure and tongue microbiome community composition before and after exposure to this potent antimicrobial agent. The human oral microbiome is highly variable between individuals; therefore, we hypothesized that there would be inter-subject variability in response to CHX dependent upon the composition of the baseline tongue microbiota. In this study we provide the first assessment of the tongue microbiome in parallel with resting blood pressure in healthy individuals treated with CHX.

MATERIALS AND METHODS

Subject Recruitment

Subjects were recruited from the faculty, staff, dental and dental hygiene students of the University of Texas Health Science Center at Houston School of Dentistry. For inclusion in the study, subjects were over the age of 18 and capable of giving consent, were non-smokers, had not used antibiotics within the previous 3 months, had no history of bone loss, and no history of hypertension. Volunteers were evaluated for oral health, including the use of a standard periodontal exam, with spot probing for bleeding and loss of attachment, and an oral health subject history. During assessment, subjects were excluded upon discovery of bleeding on probing at more than 10% of sites, $<$24 teeth, attachment loss of more than 4 mm at any site, the presence of oral hard or soft tissue lesions, or a resting blood pressure of $>$130 mm/Hg. Thirty four subjects were screened, and 6 subjects excluded after oral exam and initial blood pressure measurements (**Supplemental Data Figure 1**). Of the 28 subjects initiating the study, one was discontinued due to antibiotic usage at the first follow-up visit, and one (subject NiOx38) was discontinued at the final visit. Data from the first three time points for NiOx38 were used for data analysis. Twenty-six subjects completed the final visit of the study. The study protocol was approved by the Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston (HSC-DB-14-0078). Subject demographic data is shown in **Table 1**.

Study Design And Procedures

The study uses a repeated measures linear mixed model to detect changes in systolic blood pressure over 14 days. Power analysis estimated that 24 subjects needed to complete the study in order to detect a medium effect size at 80% power. The study uses longitudinal repeated measures of resting systolic blood pressure as the primary outcome. The study protocol consisted of 4 visits: day 1 (baseline), day 7 (after 7 days CHX treatment), day 10 (3 days recovery) and day 14 (7 days recovery). Study visits were always scheduled at noon, and lasted for 1 h to allow repeated resting blood pressure measurements. For the duration of each visit, subjects were seated in a dental chair in the upright position, with ankles uncrossed. At the first visit, the subjects completed general demographics and medication questionnaires, and an oral hygiene survey.

At each visit blood pressure measurements were taken three times with an interval of 15 min between the measurements. The first blood pressure reading was taken after at least 10 min seated in the chair (Kallioinen et al., 2017). Blood pressure was taken on alternating arms at each time point, beginning with the right arm. Tongue scrapings and saliva were collected at each visit. Unstimulated whole saliva was taken using the passive drool method into a cryovial with a saliva collection aid (Salimetrics, LLC). Tongue samples

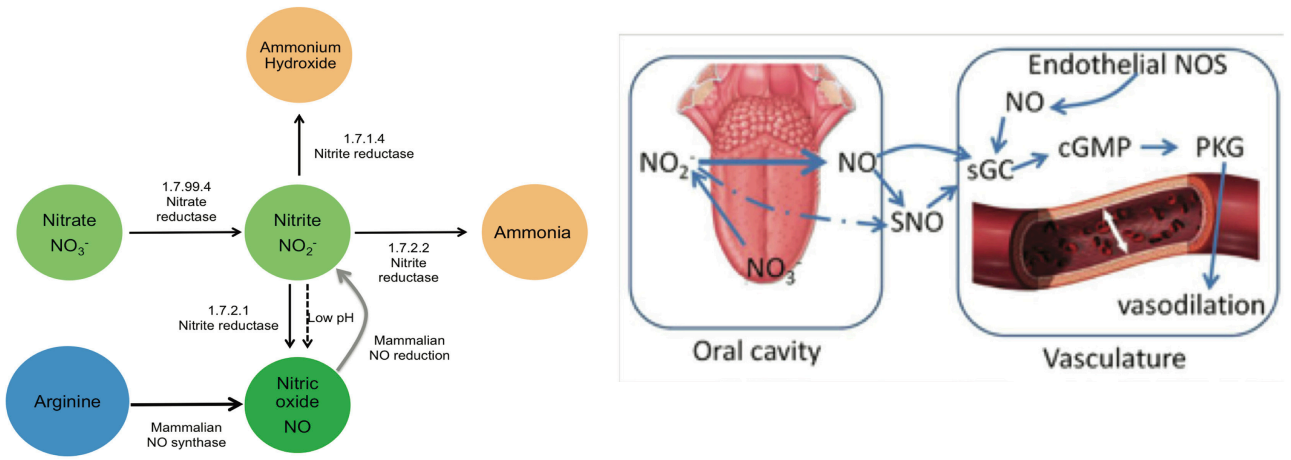


FIGURE 1 | Enterosalivary circulation of nitrate. **(A)** Bacterial and mammalian enzymes in nitrate metabolism. The bio-activation of nitrate from dietary or endogenous sources requires its initial reduction to nitrite, and because mammals lack nitrate reductase enzymes, this conversion is mainly carried out by commensal bacteria. The presence of bacteria encoding nitrate reductase (1.7.99.4) is predicted to be critical for enterosalivary circulation. Nitrite represents a major storage form of NO in blood and tissues: Nitrite- NO conversions can be driven by enzymatic and chemical mechanisms. 1.7.99.4—Nitrate reductase, 1.7.2.1—Nitrite reductase (NO-forming), 1.7.2.2—Nitrite reductase (cytochrome; ammonia-forming), 1.7.1.4—Nitrite reductase (NAD(P)H). **(B)** Interactions of oral nitrate metabolites with the host vasculature. Dietary nitrate converted to nitrite in the oral cavity may be converted to NO and/or react with endothelial and plasma proteins to form S-nitrosothiol (SNO). Nitrite along with NO and SNO has been shown to activate soluble guanylyl cyclase (sGC) and increase cGMP levels in tissue. Nitrite-NO-SNO, whether from dietary sources or endothelial production, improves vascular tone via cell signaling through cGMP/PKG and stimulation of smooth muscle relaxation.

were collected by drawing a sterile stainless steel tongue scraper with gentle pressure across the tongue dorsum from the posterior to the mid-anterior. 50 ul of tongue sample was transferred to MoBio PowerSoil tubes for bacterial DNA extraction and 16s rRNA community analysis. The remainder of the sample was transferred to cryovials containing freezing medium [(0.045% K_2HPO_4, 0.045% KH_2PO_4), 0.09% NaCl, 0.09% $(NH_4)_2SO_4$, 0.018% $MgSO_4$, 0.038% EDTA, 0.04% Na_2CO_3, 0.02% dithiothreitol, 0.2% Bacto-agar, 5% glycerol] and stored at −80°C for preservation of viable bacteria. At each visit, after collection of oral samples, subjects performed a nitrate rinse for 2 min with 50 ml of a solution of 1 mM sodium nitrate to assess *in vivo* nitrate reduction by oral bacteria. After 2 min, subjects spit the 50 ml nitrate rinse sample into a collection tube. Samples were frozen at −80°C, and subsequently analyzed for nitrate/nitrite content as described in Nitrate-Nitrite Quantification in Subject Samples.

The treatment in this study was use of 0.12% Chlorhexidine gluconate mouthwash (Peridex) twice a day for 7 days. At the completion of the first visit each subject was provided with a prescription oral mouthwash with 0.12% Chlorhexidine gluconate (CHX) and instructed to rinse with ½ ounce for 30 s twice a day as part of their normal oral hygiene in the morning and evening. Subjects were asked to discontinue use of any other mouthwash for the first 7 days, but otherwise maintain their normal oral care regimen, as documented by the oral hygiene survey (**Table 2**). At their second visit (day 7 time point) subjects were instructed to cease use of CHX and return to their normal oral health care products. Subjects returned at day 10 and 14 for blood pressure and oral sampling during the recovery from treatment phase.

Bacterial Viable Counts and Nitrate Reduction Assay

A subset of six subjects performed an 8-h time course to assess bacterial response to CHX. Tongue samples were obtained from the dorsum using a stainless steel tongue scraper hourly for 8 h after rinsing with CHX. A total of 10 samples were collected, at baseline, after CHX rinse, and hourly afterward for 8 h. Bacterial samples were collected from the posterior of the tongue dorsum by gently stroking the surface with a 0.6 cm diameter metal scraper three times, over a ∼0.6 cm^2 surface area.

A different section of the tongue was sampled at each time point. Bacterial sample was transferred to a cryovial containing freezing medium and stored immediately at −80°C for preservation of viable bacteria. For calculation of viable counts, samples were thawed on ice and serially diluted five times at 20-fold increments. The final three dilutions were dropped in 5 ul aliquots in triplicate onto Trypticase Soy Blood agar supplemented with 0.1%Yeast/7.5 uM hemin/3 uM vitamin K (TSBY-S) and incubated anaerobically at 37°C for 72 h. For calculation of *ex-vivo* nitrate reducing activity in tongue samples, samples were standardized to the same starting OD660 in sterile saline, and 100 ul aliquots were grown in 24 well tissue culture plates with 1 ml of TSBY-S broth supplemented with 5 mM nitrate solution. The plates were incubated for 24 h anaerobically and statically at 37°C. Biofilm supernatants were collected and processed for nitrite-nitrate measurements using the Nitrate reduction test (Sigma-Aldrich) as recommended.

Blood Pressure Statistical Analysis

Blood pressure data was analyzed using repeated measures two-way ANOVA, for both systolic and diastolic readings. The

TABLE 1 | Demographics.

	Count	Percent
GENDER		
Male	10	37%
Female	17	63%
AGE		
20–30	16	59%
30–40	6	22%
40–50	3	11%
50 and over	2	8%
RACE AND ETHNICITY		
African-American	3	10%
Caucasian	11	40%
Asian	5	17%
Hispanic	6	21%
Middle Eastern	1	3%
Asian-Caucasian	1	3%
EDUCATION		
High School Diploma	1	4%
Some college	5	19%
Associates Degree	1	4%
Bachelor's degree	11	41%
Professional degree	8	30%
No response	1	4%

27 subjects were enrolled in the study, and distribution of subjects by major demographic measures are shown.

TABLE 2 | Oral hygiene survey.

	Count	Percent
BRUSH TEETH		
Twice a day	22	81%
Three times a day	5	19%
FLOSS		
Several times per month	1	4%
Several times per week	8	30%
Once a day	16	67%
Twice a day	2	7%
CLEAN TONGUE		
Less than once a week	3	12%
Weekly	1	3%
Once a day	13	48%
Twice a day or more	10	37%
MOUTHWASH USE		
As needed	5	19%
Once a day	16	59%
Twice a day	6	22%
MOUTHWASH INGREDIENT		
Essential oils	11	41%
Cetylpyridinium chloride	10	37%
No response	6	22%
TYPE OF TOOTHBRUSH		
Manual	10	37%
Electric	12	44%
Both manual and electric	5	19%
VISITS TO DENTIST PER YEAR		
None	1	4%
Once	12	44%
Twice or more	14	52%

Subject responses to the oral hygiene survey are summarized.

first factor was time point, and the second factor was subject. Additional analysis was done with the second factor as gender, race-ethnicity, and other demographic variables. *Post-hoc* tests between time points and within individuals were done with Bonferroni analysis and Fishers Least Significant Difference. Association of blood pressure changes with subject metadata was assessed by Spearman's correlation. Differences between population distributions were assessed by the F-test for Variances. Power analysis for study design was done with G*Power. All other statistical analysis and graphing were performed with StatPlus for Excel (Microsoft).

Nitrate-Nitrite Quantification in Subject Samples

Nitrate and nitrite were determined by using HPLC, as described previously (Rassaf et al., 2002; Bryan and Grisham, 2007; Jiang et al., 2012b). This method employs ion chromatography with on-line reduction of nitrate to nitrite and subsequent post-column derivatization with the Griess reagent (ENO-20, EiCom, Kyoto, Japan).

Bacterial Community Analysis

DNA Extraction and 16S rRNA Gene V4 Amplification and Sequencing

Bacterial DNA was extracted from tongue samples using MoBio PowerSoil (Qiagen) protocol with 0.7 mm garnet beads, as recommended by the manufacturer. The V3-V4 region of the bacterial genomic DNA was amplified using barcoded primers 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806r (5′-GGACTACHVGGGTWTCTAAT-3′). The barcoding PCR reaction contained the following: 2 uL 4 uM barcoded primer stock, 5 uL DNA, 2 uL Taq Buffer II (Invitrogen), 0.15 uL Taq enzyme (Invitrogen), and 10.85 uL PCR. The reactions are 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 is used for each sample, allowing for pooling of samples for sequencing. All samples were pooled and sequenced on one lane of an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA) at the Baylor College of Medicine Human Genome Sequencing Center.

16S rRNA Gene Data Analysis

Reads 1 and 2 from the Illumina-sequenced amplicons were de-multiplexed and imported as paired reads into CLC Genomics Workbench v10 (Qiagen), trimming adaptors and barcodes. Reads were merged using alignment parameters with a mismatch

score of 2, a minimum total score of 8, and no tolerance of unaligned ends, and reads that failed to merge were discarded. Final reads were between 250 and 480 bp in length. Comparative quality of samples was assessed with the "filter samples" function, requiring all samples in the study to fall within 50% of the median number of reads to pass. All subject samples in the study passed the filter, with total reads per sample between 28,000 and 58,000. Sequences were clustered into OTUs against the HOMD 16s rRNA RefSeq database at 98% identity (version 14.5) (Chen et al., 2010). OTU clustering was set to allow creation of novel OTUs that failed to meet the similarity percentage required to match the selected database. The minimal occurrence to be defined as an OTU was set to 2. Novel OTUs were assigned identity by BLAST comparison to the bacterial 16s rRNA database at NCBI. Chimera detection was performed as part of OTU clustering with a chimera crossover cost of 3 using a Kmer size of 6. OTUs identified as chimeras were removed from the final OTU abundance table. Metadata was added to the final OTU abundance table to allow aggregation of data by time point, gender, subject, race, or oral hygiene frequency. Bar or pie charts were constructed to visualize the taxa present in each sample and across sample groups.

Calculation of Alpha and Beta Community Diversity and Significant Differences

Community diversity was assessed using the Microbial Genomics Diversity module of CLC Genomics Workbench. OTUs from the abundance table were aligned using MUSCLE with a required minimum abundance of 10. Aligned OTUs were used to construct a phylogenetic tree using Maximum Likelihood Phylogeny using the Neighbor Joining method and the Jukes Cantor substitution model. Alpha diversity measures were calculated for total OTUs, and Shannon's Entropy. Rarefication analysis was done by sub-sampling the OTU abundances in the different samples at a range of depths from 1 to 100,000; the number of different depths sampled was 20, with 100 replicates at each depth. Statistical significance in alpha diversity between cohorts and time points was calculated with one-way ANOVA and *post-hoc* tests by Bonferroni. PERMANOVA Analysis (Permutational Multivariate Analysis Of Variance) was used to detect significant differences in Beta diversity between groups. Differential abundance tests (non-parametric ANOVA) on the OTU frequency table were used to identify significant changes in the relative abundances of individual OTUs between groups. Differential abundance analysis values were calculated for: the max group means (maximum of the average RPKM's), –log2 fold change, fold change, standard *p*-value (significance at <0.05), and FDR *p*-value (false discovery rate corrected *p*-value).

Community Gene Content Using PICRUSt And LEFSE

Sequences were clustered into OTUs against the Greengenes database at 97% homology, and the minimal occurrence to be defined as an OTU was set to 2. OTU's that failed to match the database were discarded. Data tables were clustered by tongue cleaning frequency and time point, or dominant genus and time point, and relative abundance tables were imported into PICRUSt for estimation of community gene content (http://huttenhower.sph.harvard.edu/galaxy) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999). Significant enrichment of enzyme functions was identified using LEFSE (Segata et al., 2011).

Quantitative RT-PCR for Copper-Containing Nitrite Reductase (EC 1.7.2.1)

DNA sequences for the nitric oxide-forming nitrite reductase gene [KEGG orthology K00368; (Cantera and Stein, 2007)] were downloaded from 18 strains of *H. parainfluenza*, 1 strain of *H. pittmaniae*, 2 strains of *H. parahaemolyticus,* and 9 commensal strains of *Neisseria (N. mucosa/sicca/subflava/weaveri/cinerea)*. The genes were aligned with the "create alignment" module of CLC Genomics, with a gap open cost of 10 and a gap extension cost of 1.0. The resulting alignment is shown in **Supplemental Data Figure 8A**, and using the "design primers" module, multiple pairs of universal degenerate primers were designed. These universal primers failed specificity testing by BLAST against whole bacterial genomes. A second round of genus specific universal primers were designed for the *Haemophilus* nitrite reductase gene *nirK*, and the *Neisseria* nitrite reductase gene *aniA*. These primers passed both BLAST specificity and PCR cross-testing against genomic DNA of *Haemophilus* oral taxon 851 strain # F0397 and *N. mucosa* strain C102 (BEI Resources, Manassas, VA) by melting curve analysis and agarose gel analysis. Other quality control testing for was performed as recommended for qRT-PCR by the manufacturer (Taylor et al., 2010). Universal PCR primers for bacterial 16s rDNA were 907R and 357F as previously described (Martin et al., 2002). All primers were synthesized by Sigma-Aldrich, The Woodlands, TX. Quantitative RT-PCR reactions were carried out using SsoAdvanced Universal SYBR Supermix (Bio-rad, Hercules, CA). Reactions were performed on a StepOnePlus Real-Time PCR System (ThermoFisher Scientific), in 20 μl reactions with 10 μl 2× Supermix, 1–5 μl template, and 300 nM of each primer. Standards were 10-fold serial dilutions of genomic DNA from *Haemophilus* oral taxon 851 strain # F0397 (for *Haemophilus* nitrite gene *nirK* PCR), or genomic DNA from *N. mucosa* (for *Neisserria aniA* nitrite gene and 16s rRNA PCR). Genome equivalents per ng of genomic DNA were calculated on a genome size of 2.3 mB for *Neisseria* and 2.0 mB for *Haemophilus*. Standards were run with triple technical replicates, unknowns and negative controls were run in duplicate. Each primer set was run on separate 96 well plates, and each primer set was run at least twice. The relative ratios of nitrite gene counts normalized to bacterial cell number were calculated as (*Haemophilus* gene counts + *Neisseria* gene counts) divided by total 16s gene counts.

RESULTS

Blood Pressure Changes in Response to Treatment With and Recovery From Chlorhexidine

The subjects recruited for this study were all faculty, staff, or students at the University of Texas School of Dentistry

at Houston. The study design consisted of a baseline visit, a visit after 7 days treatment with CHX, a 3-days recovery from treatment follow-up, and a 7-days follow-up (**Supplemental Data Figure 1**). Twenty-seven subjects completed the first stage of the study, and 26 subjects completed the entire study. There were 17 females and 10 males; the average age was 31.8 years, with the youngest subject 22 and the oldest 71 years of age (**Table 1**). The average resting blood pressure at baseline in this cohort was 113/78 mmHg, and both systolic and diastolic data points conformed to a normal distribution. Two-way repeated measures ANOVA of systolic blood pressure readings indicate that significant differences exist between time points ($p = 0.017$), and between individual subjects ($p = 0.0001$), with a significant interaction component ($p = 0.005$). *Post-hoc* analysis identified significant changes in systolic blood pressure between the treatment and 3-days recovery time points (115 vs. 111.5 mm Hg), as well as the 3-days recovery and 7-days recovery (111.5 vs. 113.3 mm Hg; **Table 3**). Diastolic blood pressure was also significantly different at these same points.

Individual Blood Pressure Responses to Treatment With Chlorhexidine

The time point-subject interaction p-levels indicate that there were significant individual differences in response to CHX. Assessment of systolic blood pressure results on within-subject data revealed that 13 subjects had changes in blood pressure >5 mm/Hg in response to CHX; 9 subjects had an increase in resting blood pressure after treatment with CHX, while four had a decrease (**Figure 2**). This bimodal response to CHX treatment was confirmed by comparison of the blood pressure distribution at baseline to the other time points by an F-test for Variances. Only the CHX treatment time point has significantly different variance compared to baseline (p-level two-tailed = 0.04). Thus, in our study, there is a significant difference in individual response after 7 days of treatment with CHX, and an overall significant decrease in systolic and diastolic blood pressure average in the cohort after recovery from CHX for 3 days. We next determined if any of our demographic or hygiene data could account for the difference in individual response after 7 days of treatment with CHX.

Correlation Between Blood Pressure Response to CHX and Oral Hygiene Behaviors

At the first visit, subjects completed a demographic and oral hygiene survey. Subjects provided information on their age, gender, education level, number of times per day they brush their teeth, use dental floss, use mouthwash, clean their tongue, visit the dentist per year, and the types of oral care products used (**Table 2**). We utilized this data to investigate the different responses to treatment with CHX within our subject population. Both systolic and diastolic blood pressure was found to vary significantly by gender, with male subjects averaging 10 mm/Hg higher systolic and 5 mm/Hg higher diastolic than females. However, there was no significant interaction between gender and blood pressure response over the course of the study, indicating that there is no difference in response between males and females (**Supplemental Data Figure 2**). No significant variation was found between any other groups based on race/ethnicity, education, or other variables.

Subject data, represented by change in systolic blood pressure between baseline and after the 1 week CHX treatment, was correlated with metadata categories using Spearman's correlation. No significant correlations were found for any demographic or oral hygiene data, except for tongue cleaning frequency. An R of 0.45 for correlation with tongue cleaning frequency was determined to be significant with a p-value of 0.03 (**Figure 3A**). Subjects who cleaned their tongue twice or more per day as part of their normal oral hygiene were more likely to have an increase in systolic blood pressure during use of CHX for 1 week. Subjects who did not clean their tongue on a daily basis were more likely to have a decrease in systolic blood pressure. Separating the subjects into cohorts based on tongue-cleaning frequency, we performed one-way ANOVA on blood pressure data by cohort, and confirmed the cohort of subjects who clean twice a day had a significant increase in systolic BP after 7 days CHX treatment, followed by a significant decrease at the 3-days recovery time point (**Supplemental Data Figure 3**, p-value = 0.003). The zero cleaning cohort shows the opposite response, with a significant decrease after 7 days CHX (117. 5 mm/Hg to 111.0 mm/Hg, p-value = 0.03), and a subsequent increase at the 3 days recovery (111.0–117.4). To confirm that the frequency of tongue cleaning was not tied to other demographic variables, we used the Chi Square test for gender, age-range and race-ethnicity, with no significant associations.

From these observations, we hypothesized that the frequency of tongue cleaning as a significant effector of blood pressure could result from a combination of possible effects. First, regular tongue cleaning may result in a baseline tongue microbiome that has a greater ability to reduce nitrate, and conversely, failure to clean the tongue daily may result in a microbiome composition that is unfavorable to nitrate reduction. Further, cleaning the tongue disrupts the papillary surface and could allow increased penetration of CHX, resulting in a greater community disruption in the two cleaning cohort. To investigate these mechanisms, we used 16S rDNA community analysis of tongue samples to elucidate the dynamics of the tongue microbiome in our subjects, to compare differences between time points and tongue hygiene cohorts.

The Bacterial Microbiome of the Healthy Human Tongue

In tongue samples from 27 subjects at baseline, 272 unique OTUs were identified at the phylum level, representing eight different phyla of bacteria: Proteobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Actinobacteria, Candidate division SR1, Spirochaetes, and Candidate division TM7. Members of the first five phyla were found in every subject (**Supplemental Data Figure 4A**), while the remaining phyla were found as a minority component in some subjects, resulting in combined abundance values <1%. This is consistent with other studies in adults, which demonstrated the same major phyla on the tongue (Jiang et al., 2012a; Mark Welch et al., 2014). In our study, Proteobacteria account for 40% of the OTUs identified across the cohort; Bacteroidetes account for

TABLE 3 | Blood pressure values.

SYSTOLIC VALUES BY TIMEPOINT					
Timepoint	**Systolic mean**	**Minimum**	**Median**	**Maximum**	***N***
Baseline	113.0	99.0	113.0	129.0	27.0
Treatment CHX	115.0	91.7	114.0	135.7	27.0
Recovery 3 days	111.5	96.3	112.3	126.0	25.0
Recovery 7 days	113.3	94.7	113.7	132.0	26.0
DIASTOLIC VALUES BY TIMEPOINT					
Timepoint	**Diastolic mean**	**Minimum**	**Median**	**Maximum**	***N***
Baseline	78.1	69.3	77.7	89.7	27.0
Treatment CHX	78.4	65.0	78.3	95.7	27.0
Recovery 3 days	75.7	62.7	77.0	87.3	25.0
Recovery 7 days	79.0	68.3	78.5	96.0	26.0

Statistical comparisons	**Systolic blood pressure**		**Diastolic blood pressure**	
Group vs. Group (Contrast)	**Bonferroni *p*-level**	**Fisher LSD *p*-level**	**Bonferroni *p*-level**	**Fisher LSD *p*-level**
Baseline vs. Treatment 7 days CHX	0.508	0.085	1.000	0.751
Treatment 7 days CHX vs. Recovery 3 days	**0.014**	**0.002**	**0.025**	**0.004**
Recovery 3 days vs. Recovery 7 days	0.136	**0.023**	**0.003**	**0.000**
Baseline vs. Recovery 3 days	1.000	0.171	0.063	**0.010**
Baseline vs. Recovery 7 days	1.000	0.344	1.000	0.306
Treatment 7 days CHX vs. Recovery 7 days	1.000	0.444	1.000	0.477

Systolic and diastolic average values per time point are shown for all subjects in the study. Minimum, median, and maximum are also shown. N = number of data points per time point. Statistical comparisons for systolic and diastolic BP are also shown, including both Bonferroni and Fisher calculations. Significant p-values are shown in bold.

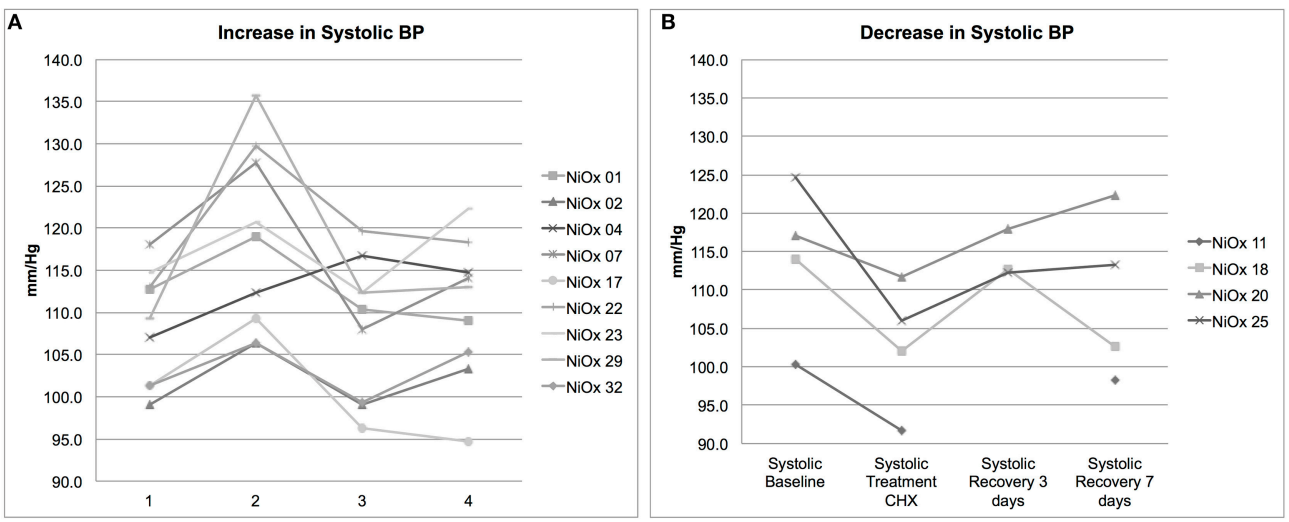


FIGURE 2 | Individual systolic blood pressure responses. Individuals with a >5 mm/Hg change in blood pressure after CHX treatment are shown **(A)** Nine subjects had an increase in systolic BP. **(B)** Four subjects had a decrease in systolic BP. Each point represents an average of three systolic readings of resting BP. Subject NiOX 11 did not have BP readings taken at the third time point.

23%, Firmicutes for 19% (**Table 4**). The most common OTU in our cohort at baseline was *Haemophilus parainfluenza*, a commensal bacterium of the oral-pharyngeal flora and a member of the Proteobacteria. This organism accounted for 22% of the combined abundance in the cohort, followed by *Neisseria subflava* at 12%. The genus *Haemophilus* was the most numerous in 14 of the 27 subjects at baseline (**Supplemental Data Figure 4B**). Six subjects had *Neisseria* as the most common genus, while four subjects had *Prevotella* and three had *Leptotrichia*. Other bacteria identified in this study are shown in **Supplemental Data Figure 4C**, and all species-level identifications are based on OTU comparisons to the HOMD 16s rRNA RefSeq database at 98% identity (Chen et al., 2010).

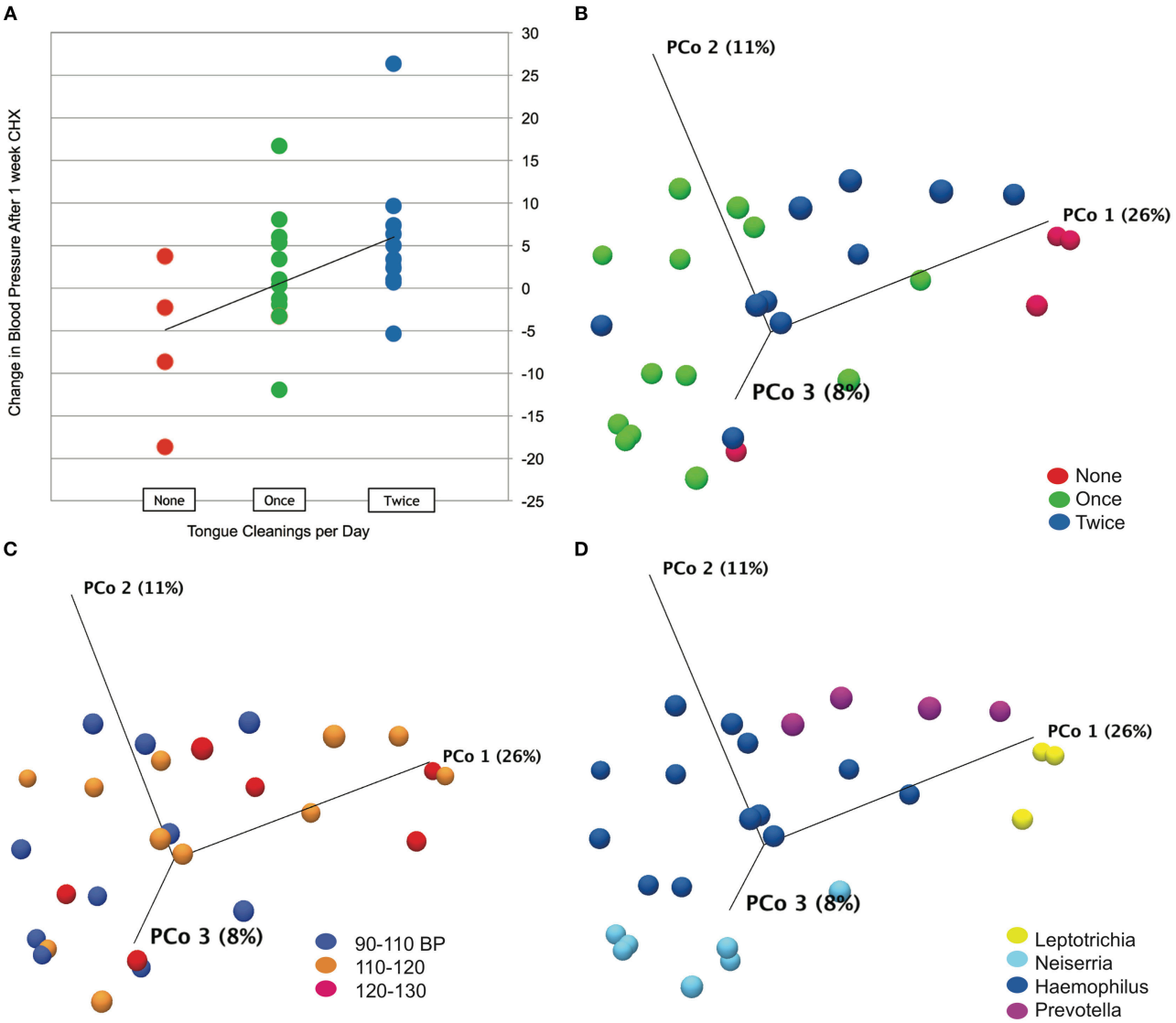


FIGURE 3 | Tongue cleaning impact on blood pressure response and microbiome composition. **(A)** Correlation plot for blood pressure response to chlorhexidine and frequency of tongue hygiene. Spearmans's correlation coefficient was used to determine the strength of association. **(B)** Bray-Curtis Principle component plot of bacterial communities at baseline, colorized by tongue-cleaning frequency. **(C)** Bray-Curtis Principle component plot of bacterial communities at baseline, colorized by resting systolic blood pressure. **(D)** Bray-Curtis Principle component plot of bacterial communities at baseline, colorized by dominant bacterial genus.

Effects of Tongue Hygiene and Demographics on Bacterial Community Composition

To test the effect of tongue cleaning behavior on microbiome composition, we generated Principle Component Plots for all samples at baseline. By PERMANOVA analysis, bacterial community distribution was significantly influenced by tongue cleaning frequency, with a Bray-Curtis ANOVA p-value of 0.00001, and Bonferroni *post-hoc* analysis indicated that all tongue-cleaning subgroups were significantly different from each other (**Figure 3B**). PERMANOVA values for systolic blood pressure ($p = 0.009$) and dominant genus ($p = 0.00001$) were also significant (**Figures 3C,D**). For systolic blood pressure, Bonferroni *post-hoc* analysis was significant only between groups 90–110 and 120–130 ($p = 0.006$). To further assess the demographic and behavioral influences on community composition, we used Chi Square to assess their correlations with the dominant bacterial genus per subject (**Supplemental Data Figure 5**). Frequency of tongue cleaning was found to have a significant correlation with the dominant bacterial genus on the tongue.

We next analyzed differences between hygiene cohorts at baseline with differential abundance analysis (**Supplemental Data Table 1**). The largest differences in composition were found between the zero cleanings cohort and

TABLE 4 | Average composition of the tongue microbiome across all subjects.

Phylum: Genus	Average tongue microbiome composition by phylum	Average tongue microbiome composition by genus	High-low range
Proteobacteria: Haemophilus	40%	25%	82–1%
Proteobacteria: Neisseria		13%	47–0.1%
Proteobacteria: Campylobacter		2%	9–0%
Bacteroidetes: Prevotella	23%	18%	46–1%
Bacteroidetes: Porphyromonas		3%	13–0%
Bacteroidetes: Flavobacteria		2%	3–0%
Firmicutes: Streptococcus	19%	9%	20–2%
Firmicutes: Veillonella		6%	15–1%
Firmicutes: Lachnospira		3%	18–0%
Firmicutes: Carnobacteria		1%	2–0%
Fusobacteria: Leptotrichia	12%	6%	34–0%
Fusobacteria: Fusobacteria		6%	19–0%
Actinobacteria: Actinomyces	5%	4%	13–0%
Actinobacteria: Micrococcus		1%	4–0%

The first and second column lists the distribution of bacteria by phylum, in order of most common to least common. The third column shows the average composition by genus, and the last column indicates the highest percentage and lowest percentage for individuals in the study.

the other two. There are significant differences in 33 different OTUs for subjects with zero cleanings/day compared to the other two groups, and only four OTUs different between the one and two-cleaning groups. Notably, the levels of *Leptotrichia sp*. are significantly higher in the zero-cleanings group, and the levels of two *Haemophilus* OTUs are significantly lower. Other OTUs lower in the zero-cleanings group are associated with *Veillonella parvula*, *Rothia muciliginosa*, and *Granulicatella adiacens*. Therefore, tongue-cleaning does have a significant impact on tongue bacterial community composition, and daily cleaning (either once or twice) results in an increased proportion of *H. parainfluenza* and other Proteobacteria, which are major nitrate and nitrite reducing species of the oral cavity (Hyde et al., 2014).

Bacterial Community Responses to CHX Treatment

We next tested the hypothesis that cleaning the tongue twice a day could influence blood pressure by causing a greater community disruption by CHX compared to the other cohorts. PERMANOVA analysis and Principle Component Plots for all time points demonstrated that samples continue to cluster by tongue-cleaning frequency (**Figure 4A**) after treatment with CHX. Further, there was no significant change in overall sample distribution for any tongue-cleaning cohort in response to CHX, based on PERMANOVA analysis (**Supplemental Data Figure 6**).

We further considered the impact of CHX exposure on community richness and diversity using Observed OTUs and Shannon Entropy calculations. Richness decreased upon CHX treatment for all three tongue-cleaning cohorts, but the only statistically significant decrease was in the two-cleaning group, between baseline and treatment with CHX (**Figure 4B**). Species diversity was also impacted in the two cleaning group after CHX exposure as assessed by Shannon Entropy, however this decrease was not statistically significant (**Figure 4C**).

Differential abundance analysis identified significant changes in some species in response to treatment with CHX (**Supplemental Data Table 2**). In the zero cleaning cohort three species had significant changes in abundance, while the one cleaning cohort had 10 and the two cleaning had 12. Of the species that increase in abundance after treatment, six are from the genus *Capnocytophaga*, which are known to be more resistant to CHX than other oral microbes (Wade and Addy, 1989). Collectively, this data demonstrates that twice-daily tongue cleaning in combination with CHX treatment has an impact on tongue microbiome richness, and a larger magnitude of impact relative to the other tongue-cleaning cohorts. The primary effect of tongue cleaning in general, however, appears to be on selection of a baseline community of bacteria enriched in nitrate-reducing species.

Microbiome Viability and Metabolism After CHX Treatment

We were surprised to discover that CHX treatment did not cause large-scale changes in microbiome community structure, as it is a potent antimicrobial. We next assessed the effect of CHX exposure on tongue microbiome viability and nitrate reductase activity for 8 h after a 30 s rinse, on six subjects (**Figure 5**). CHX caused a significant reduction in bacterial viability, but this effect was only a 10-fold reduction, detectable 6 h after treatment. The dynamics of recovery from CHX exposure are notable, in that there was a rapid recovery in viable counts between 6 and 8 h, which corresponded with a significant increase in nitrate reduction to nitrite in the tongue samples. Thus, CHX mouthwash does not eradicate viable bacteria on the tongue, but instead causes a transient loss of viable counts, and the recovery from CHX treatment is associated with increased bacterial metabolic activity.

Blood Pressure and CHX Responses by Dominant Genus Cohorts

We next revisited our blood pressure data and community responses to CHX from the perspective of the bacterial community type, and noted that bacterial communities associated with the lowest systolic blood pressure (90–110

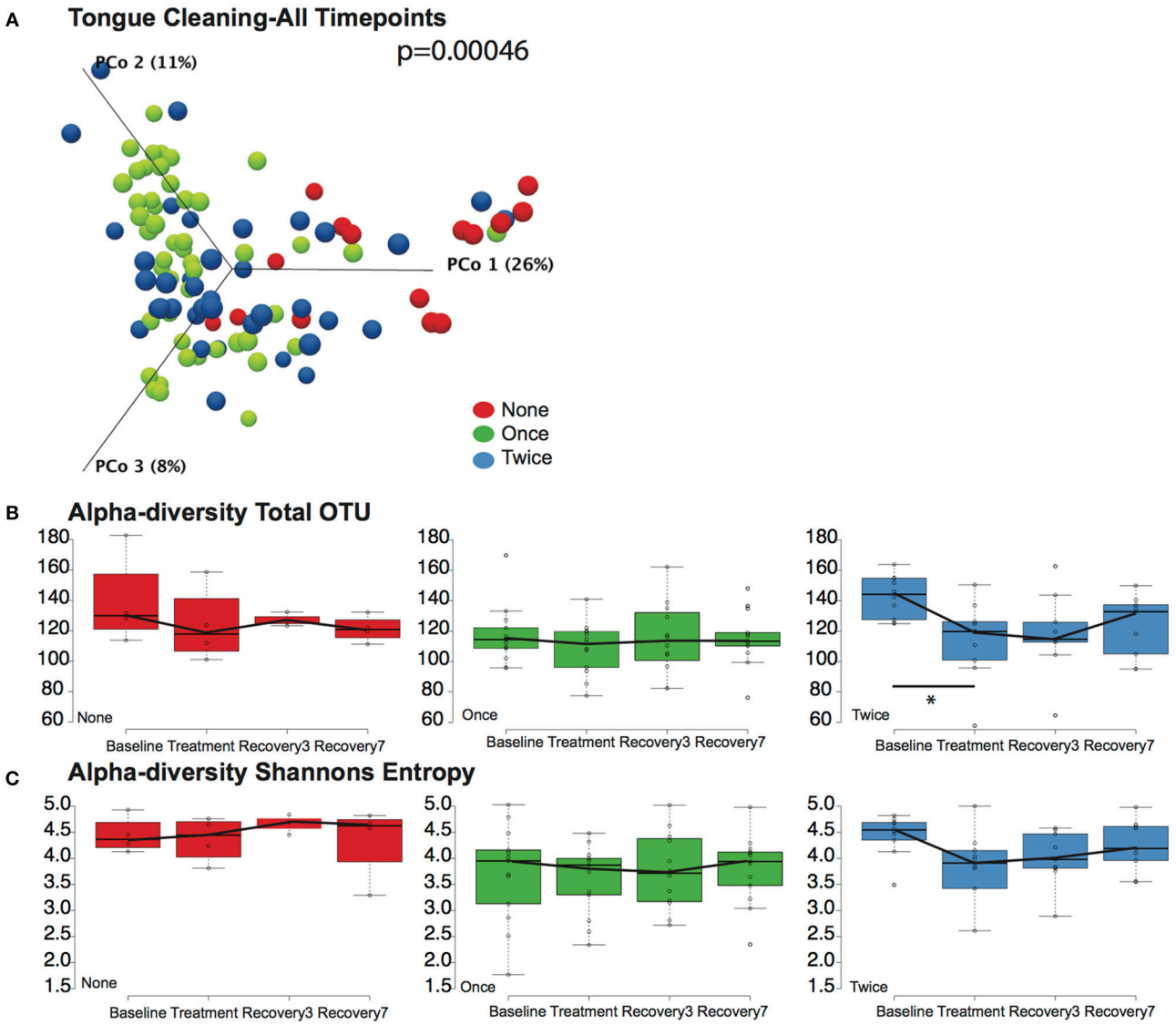


FIGURE 4 | Effects of tongue hygiene and chlorhexidine exposure on community diversity and composition. **(A)** Bray-Curtis Principle component plot of bacterial communities across all time points, colorized by tongue cleaning frequency. The distribution between cohorts remains distinct; the PERMANOVA *p*-value is 0.00046. **(B)** Community richness as measured by Total OTUs. Only the two-cleaning per day cohort, shown in blue, had a significant change in richness after CHX treatment as noted by the asterisk. **(C)** Community diversity as measured by Shannon entropy. No significant differences were noted for any cohort.

mM/Hg) were localized to the left side of the principle component plot, and superimposed more closely with the *Haemophilus* and *Neisseria* groups (**Figures 6A,B**). In support of this observation, we found that there were significant differences ($p = 0.005$) between bacterial genus groups in systolic blood pressure at baseline (**Figure 6C**). The three subjects with *Leptotrichia* tongue communities have an average systolic blood pressure at baseline of 123.2 mm/Hg, while the *Neisseria* defined-cohort has a systolic average of 110 mm/Hg. *Post-hoc* analysis identified the *Leptotrichia* cohort as having significantly higher systolic BP than *Haemophilus* and *Neisseria* groups. No groups had significant changes in BP over the time course, although the *Haemophilus* group had a five point increase in BP after CHX use.

Predicted Gene Content of the Human Tongue Microbiome

The PICRUSt program predicts gene family abundance (e.g., the metagenome) in microbial DNA samples for which only 16S rRNA gene data are available (Segata et al., 2011). Using PICRUSt, metagenomes were calculated for each tongue cleaning and bacterial genus cohort, and significant differences in relative abundance for gene families detected with linear discriminant analysis (LEfSe) with LDA score > 2 being considered significant.

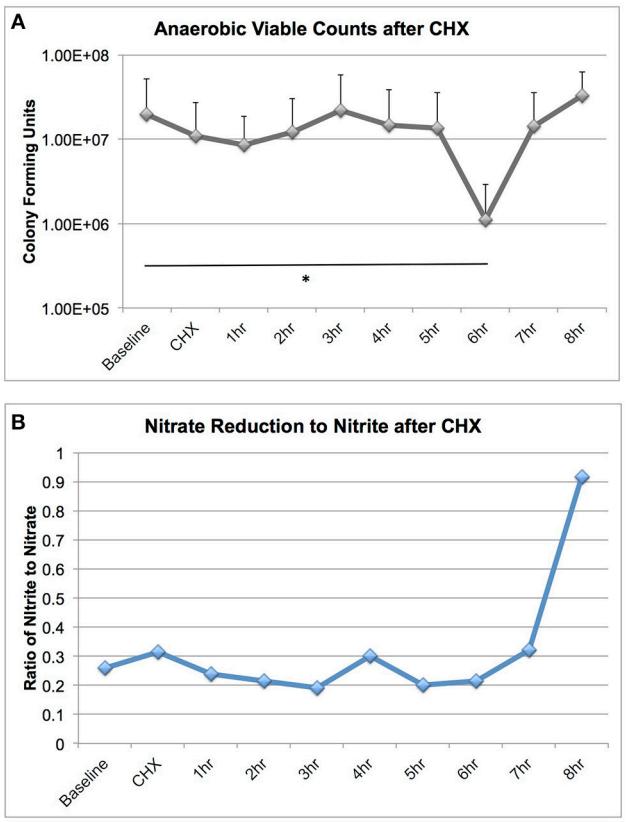


FIGURE 5 | Bacterial viable counts and nitrate reductase activity recovered from tongue scrapings after CHX exposure. Six subjects collected tongue scraping samples over an 8 h time course after a 30 s rinse with 2% Peridex. Tongue samples were adjusted to a standardized starting OD600 and: **(A)** Serially diluted and plated for enumeration of viable counts on blood agar plates incubated anaerobically for 7 days and **(B)** Assessed for nitrate and nitrite values after anaerobic overnight incubation in 5 mM nitrate broth. Nitrate and nitrite results are shown as a ratio. Significant differences in viable counts between time points is indicated by an asterisk.

Across all samples, PICRUSt identified 4,295 genes with assigned KEGG orthology identifiers (KO). Comparing cohorts, 173 genes were found to be significantly different between tongue cleaning groups (**Supplemental Data Table 3**), and 674 genes between bacterial genus groups (**Supplemental Data Table 4**).

Concerning the major bacterial enzymes associated with nitrate-nitrite metabolism (**Figure 1A**), NO-forming nitrite reductase [EC:1.7.2.1] varied significantly by both tongue-cleaning groups (**Supplemental Data Figure 7B**) and bacterial genus groups (**Figure 7A**); and [EC:1.7.2.2] ammonia-producing nitrite reductase was found to vary between the bacteria-genus cohorts (**Figure 7B**). Interestingly, the relative gene abundance for nitrate reductase did not significantly vary in either cohort (**Supplemental Data Figure 7A**). We confirmed the ability of all subjects to reduce nitrate to nitrite utilizing a 2-min *in vivo* oral rinse with 1 mM sodium nitrate. The average final concentration of nitrite for all subjects at baseline was 14 μM, and as predicted by the gene abundance, there was no significant difference in nitrite generation from nitrate between tongue-cleaning groups (**Supplemental Data Figure 7C**; $p = 0.3$) or bacterial-genus groups (data not shown).

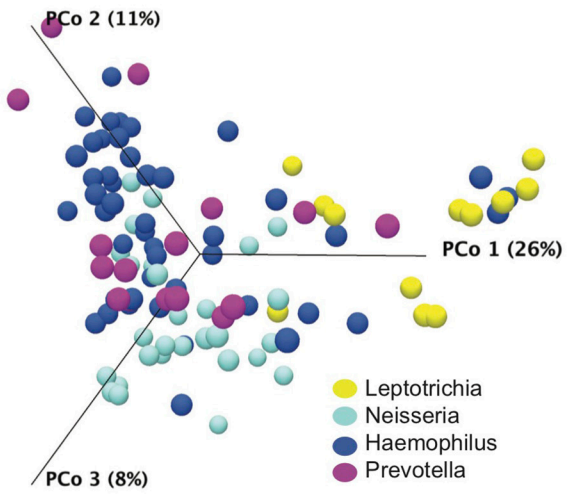
We next performed correlation analysis across all cohorts at all time points for the NO-forming nitrite reductase gene. The gene did not correlate with blood pressure changes in the tongue cleaning cohort (**Supplemental Data Figure 7D**), but had a significant inverse correlation in the bacterial-genus cohorts (**Figure 7C**; $R = -0.7$; $p = 0.0025$). Additionally, the ammonia-producing nitrite reductase [EC:1.7.2.2] had a significant positive correlation with systolic BP (**Figure 7D**; $R = 0.6$; $p = 0.013$). Interestingly, although the *Neisseria* cohort and the *Haemophilus* cohort both have high levels of NO-forming nitrite reductase, the *Haemophilus* group has the higher amount of ammonia-producing reductase. Thus, high levels of NO-forming reductase are not sufficient, but rather high levels of NO-forming and low-levels of ammonia-forming produce the most favorable conditions for lower systolic BP.

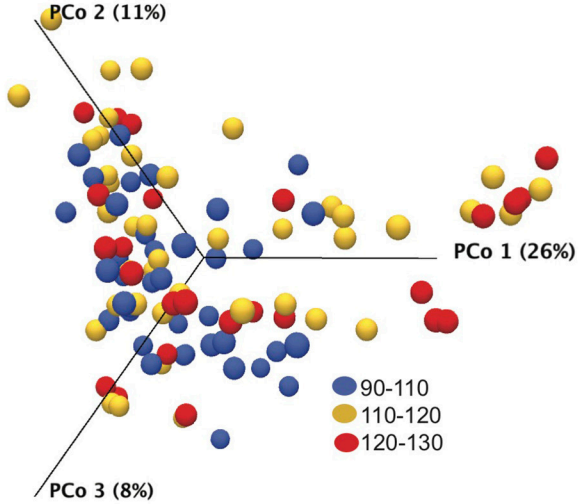
Quantitative PCR Detection of NO-Forming Nitrite Reductase in Individual Subjects

The correlation between increased NO-forming nitrite reductase and reduced systolic BP in the bacterial genus cohorts was further investigated in individual subject samples from the baseline time point. Primers specific for the NO-forming nitrite reductase of *Haemophilus* and *Neisseria* were designed to derive the Proteobacteria contribution to NO-forming gene content from the tongue bacterial DNA (**Supplemental Data Figures 8A–C**). Further, the total bacterial content of each sample was determined using quantitative RT-PCR primers for bacterial 16s rRNA, and the ratio of the total NO-forming gene to 16s rRNA in the sample was calculated (**Figure 7E**). We observed a significant inverse correlation of −0.44 (p-value = 0.02), further implicating a relationship between NO-forming bacterial nitrite reductase and resting systolic BP in individual subjects. We further note that not all subjects require a high concentration of NO forming nitrite reductase to achieve a healthy blood pressure. Five subjects in quadrant 2 have a NO-forming reductase/16s ratio of <0.05, but have a resting systolic BP of <110 mm/Hg. We predict that these subjects are producing sufficient endogenous NO, or have other BP regulation mechanisms in place that are bacterial NO-independent. In contrast, there are no subjects found in quadrant 4, implying that a high relative ratio of NO-forming nitrite reductase/16s ratio is contributing to a lower blood pressure.

DISCUSSION

This is the first longitudinal next-generation sequencing study demonstrating the impact of oral hygiene on the composition of the tongue microbiome. Tongue microbiome communities are of general interest in Eastern medicine because the appearance of the tongue coating is considered a manifestation of systemic health (Jiang et al., 2012a). In Western medicine interest has focused on the role of the microbiome in mucosal disorders (Docktor et al., 2012; de Paiva et al., 2016), and in dentistry the tongue microbiome has significant associations with halitosis (Ren et al., 2016). Regular tongue cleaning is recommended





C

Systolic values by Dominant Genus	Baseline	Treatment 7 days CHX	Recovery 3 days	Recovery 7 days	*p-level*
Leptotrichia systolic	**123.2**	**117.4**	**117.4**	**120.0**	0.18
StDev	6.3	9.8	5.5	11.2	
Neisseria systolic	**110.1**	**109.9**	**108.9**	**105.2**	0.49
StDev	8.4	12.0	10.2	11.5	
Haemophilus systolic	**111.8**	**116.4**	**110.6**	**114.7**	0.07
StDev	10.3	12.6	8.7	10.8	
Prevotella systolic	**114.4**	**117.2**	**113.3**	**115.5**	0.87
StDev	12.4	10.7	11.0	11.8	
p-level	0.005	0.193	0.140	0.003	
Bonferroni post-hoc p-values					
Haemophilus vs Leptotrichia	0.009				
Leptotrichia vs Neisseria	0.004				
Haemophilus vs Neisseria				0.013	
Leptotrichia vs Neisseria				0.007	
Neisseria vs Prevotella				0.057	

FIGURE 6 | Effects of bacterial community and chlorhexidine exposure on systolic blood pressure. **(A)** Bray-Curtis Principle component plot of bacterial communities across all time points, colorized by dominant bacterial genus. **(B)** Bray-Curtis Principle component plot of bacterial communities across all time points, colorized by resting systolic blood pressure. **(C)** Statistical comparisons of systolic blood pressure between subjects, grouped by dominant bacterial genus.

by the American Dental Association (http://www.mouthhealthy.org) based on evidence that cleaning can reduce the severity of halitosis (Pedrazzi et al., 2016), however there are no epidemiological data on tongue cleaning practices or frequency in the United States population. Based on this study, tongue cleaning assumes a new importance from the perspective of blood pressure regulation, as daily tongue cleaning appears to favor the increased abundance and metabolic activity of nitrate/nitrite metabolizing species, such as *H. parainfluenza* and commensal *Neisseria* spp. (Barth et al., 2009).

Consistent with the theory that the enterosalivary nitrate-nitrite-NO pathway is foundational to cardiovascular health, we demonstrated that our cohort of healthy subjects uniformly have tongue microbiomes encoding nitrate reductase activity. Interestingly, introduction of the antiseptic mouthwash CHX revealed different blood pressure responses, and a significant

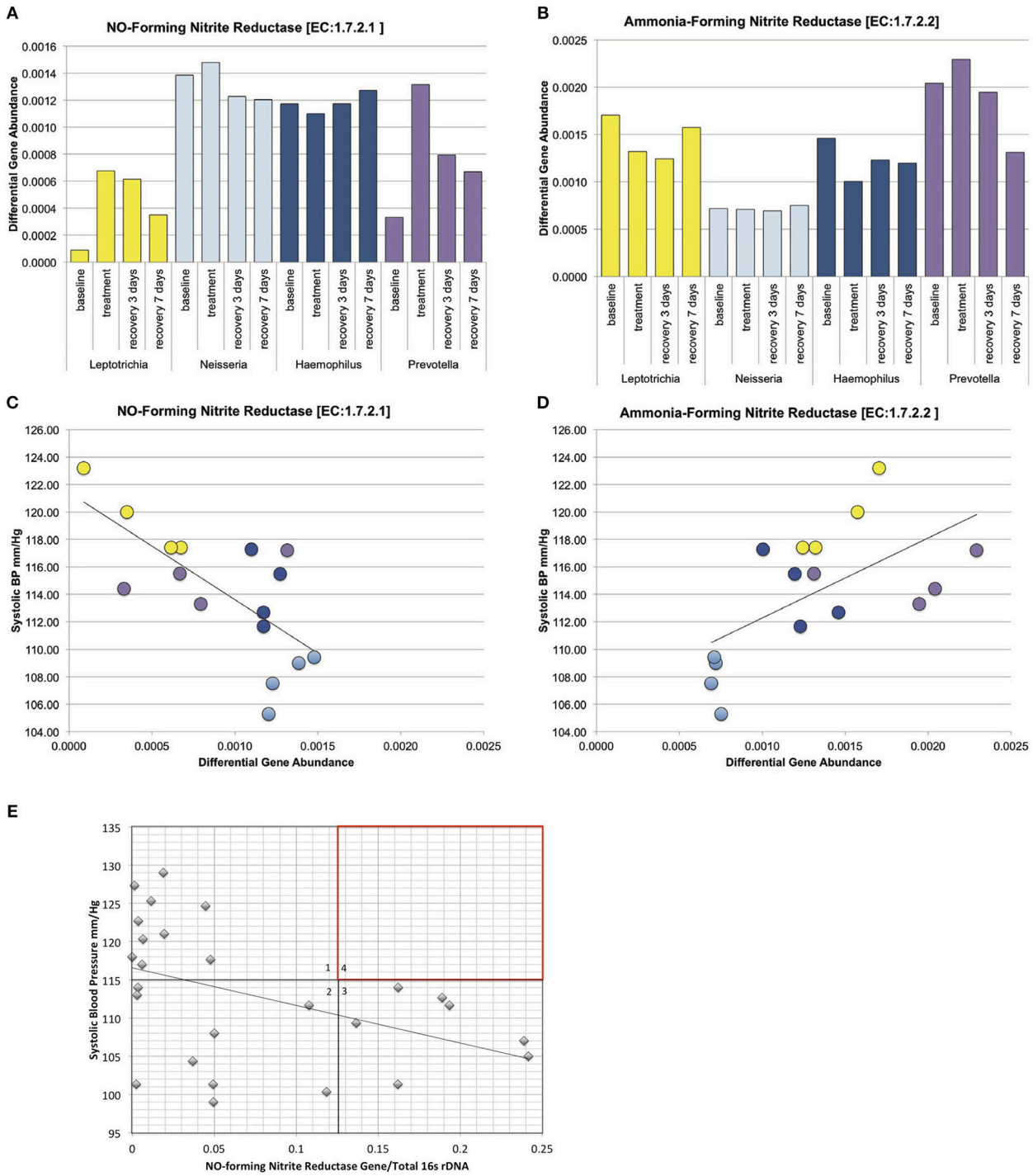


FIGURE 7 | Relative gene abundance in the dominant genus cohorts. Subjects were grouped into cohorts based on microbiome type, named by the dominant genus found in the microbiome. **(A)** Predicted relative gene abundance for NO-forming nitrite reductase, which is significantly different between groups as determined by Lefse analysis. Predicted relative gene abundance was estimated by PICRUSt and represented as the percent of gene present in the community metagenome. **(B)** Predicted relative gene abundance for ammonia-forming nitrite reductase, which is significantly different between groups as determined by Lefse analysis. **(C)** NO-forming nitrite reductase gene abundance negatively correlated with changes in systolic blood pressure, with an $R = -0.7$ and $p = 0.002$. **(D)** Ammonia-producing nitrite reductase positively correlated with systolic blood pressure, with an $R = 0.6$ and $p = 0.013$. **(E)** The ratio of NO-forming nitrite reductase gene abundance in individual subjects at baseline, correlated with systolic blood pressure. The trend seen in the bacterial genus cohorts is reproduced in individual samples. The absence of subjects in quadrant 4 (red) implies that high ratios of NO-forming nitrite reductase on the oral microbiome may supplement host NO production and contribute to lower resting blood pressure.

component of this response was determined to be the frequency of tongue cleaning. From our analysis, regular tongue cleaning results in a tongue microbiome that has a greater ability to reduce nitrite to NO, and conversely, failure to clean the tongue daily results in a microbiome composition that is less favorable to NO production, but instead appears to favor conversion of nitrite to ammonia. Introducing CHX to patients who clean their tongue twice a day results in a significant disruption of community alpha-diversity compared to the other groups, linking bacterial community disruption to changes in blood pressure. Our study also demonstrates a chemostat-like activity of the tongue microbiome, in that loss of population due to CHX exposure stimulates a rapid population recovery (Rai et al., 2019). Rapid recovery parallels an increase in nitrate reduction metabolic activity, and we propose that frequent tongue cleaning may activate bacterial metabolism and benefit the host through production of nitrite. Thus, regular tongue hygiene both selects for a favorable microbiome and "revs up" the activity of the community.

Although we did not survey host diet as a variable in our study, the composition of the human diet clearly has an impact on the composition of the microbiome, both in the gut and in the oral cavity. In a recent study on the salivary microbiome comparing vegans and omnivores (Hansen et al., 2018), the authors determine that the ratio of *Neisseria* to *Prevotella* is significantly higher in vegans. Further emphasizing the importance of dietary nitrate on the oral microbiome, Velmurugan et al. established in a randomized, double-blind study that sustained intake of nitrate-rich beetroot juice resulted in improved vascular function and also resulted in a significant increase in the percentage of *Neisseria flavscens* in the oral microbiome (Velmurugan et al., 2015). Both of these studies highlight the increase of commensal *Neisseria* spp. as a result of a diet high in nitrate-rich vegetables. Commensal Neisseria are part of the core oral microbiome in humans (Zaura et al., 2009), and reduction of nitrite to NO is obligatory for commensal Neisseria to survive in anaerobic conditions, such as those found in the crypts of the tongue (Bennett et al., 2014; Liu et al., 2015).

The data shown here provide static snapshots of what is undoubtedly a dynamic process. In our orally healthy cohort, the presence of nitrate reductase does not appear to be a rate-limiting component of the enterosalivary circulation of nitrate and subsequent reduction to nitrite. However, our experimental design has revealed a potential new aspect of enterosalivary circulation via the formation of NO in the oral cavity, in preference to formation of ammonia as the end product of nitrite. In subjects lacking other systemic health problems, the nitrite-reducing genetic content of the oral flora of the tongue significantly correlates with resting blood pressure values, with the putative mechanism through a supplemental contribution of nitrate reductase, and also nitric-oxide generating nitrite reductase. High levels of ammonia-forming nitrite reductase correlate with high blood pressure, and may result from "stealing" nitrite away from the NO pathway.

Although we have not established the mechanism by which generation of NO gas in the oral cavity could affect NO based vasodilation in the periphery, it is now well-established that NO generated in one biological compartment can affect NO homeostasis in distal tissue suggesting an endocrine function of NO (Elrod et al., 2008). Moreover, it is known that inhaled NO has systemic effects and can protect from myocardial ischemia reperfusion injury (Nagasaka et al., 2008) that is likely not due to NO itself but rather specific NO metabolites, nitrite and/or S-nitrosoglutatione (GSNO). Alternatively, NO gas produced in the lingual crypts could diffuse directly into the circulation through the highly vascularized tissue of the tongue.

Regulation of blood pressure is complex with multiple variables. There is a strong host component, including efficacy of endogenous NO production, weight, diet, gender, race, stress, and frequency of exercise (Rosendorff, 2013). We propose that the composition and metabolic activity of the oral microbiome should be considered an additional variable. Thirteen subjects on our study had changes of at least 5 mm/Hg in resting systolic blood pressure after CHX treatment, which is comparable to changes induced by manipulation of dietary salt intake (Graudal et al., 2017).

Manipulation of the human microbiome as a therapeutic target for disease management is on the near horizon. Screening the oral microbiome of resistant hypertensive patients may provide new insights into the etiology of their hypertension. The oral cavity is an attractive target for probiotic and/or prebiotic therapy because of the ease of access. The potential to restore the oral flora as a means to provide NO production is a completely new paradigm for NO biochemistry and physiology as well as to cardiovascular medicine and dentistry. These studies provide new insights into the host-oral microbiome symbiotic relationship. As NO is a ubiquitous signaling molecule, these systemic effects of these oral bacteria may have other significant effects on human health beyond maintenance of blood pressure.

DATA AVAILABILITY

16S rRNA gene data from this study is deposited at the Sequence Read Archive (SRA) maintained at https://trace.ncbi.nlm.nih.gov/Traces/sra under bioproject title "The Oral Microbiome in Blood Pressure Regulation" 2444628.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston under study protocol number HSC-DB-14-0078. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

GT, NA, and NB designed the study. NA, RW, B-YW, SE, IG, and KP recruited, screened, and sampled subjects. GT, D-HD,

KR, NI, IS, and NB performed sample processing, nitrate-nitrite analysis, and quantitative RT-PCR. NJA and JP supervised bacterial DNA processing. GT performed bacterial community analysis. GT, NA, RW, B-YW, IS, EH, NJA, and NB wrote the manuscript.

FUNDING

This work was supported by institutional funding to GT, NA, NB, and JP.

ACKNOWLEDGMENTS

The authors thank Stan Cron and Cameron Jeter for assistance in study design; and Emil Martin, Heidi Kaplan, and Charles Streckfus for helpful conversations and review of the data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00039/full#supplementary-material

REFERENCES

Barth, K. R., Isabella, V. M., and Clark, V. L. (2009). Biochemical and genomic analysis of the denitrification pathway within the genus Neisseria. *Microbiology* 155, 4093–4103. doi: 10.1099/mic.0.032961-0

Benjamin, N., O'Driscoll, F., Dougall, H., Duncan, C., Smith, L., Golden, M., et al. (1994). Stomach NO synthesis. *Nature* 368, 502–502. doi: 10.1038/368502a0

Bennett, J. S., Bratcher, H. B., Brehony, C., Harrison, O. B., and Maiden, M. C. J. (2014). "The genus Neisseria," in *The Prokaryotes*, eds E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, and F. Thompson (Berlin; Heidelberg: Springer), 881–900. doi: 10.1007/978-3-642-30197-1_241

Bryan, N. S., and Grisham, M. B. (2007). Methods to detect nitric oxide and its metabolites in biological samples. *Free Radic. Biol. Med.* 43, 645–657. doi: 10.1016/j.freeradbiomed.2007.04.026

Bryan, N. S., and Loscalzo, J. (2017). *Nitrite and Nitrate in Human Health and Disease*. New York, NY: Humana Press.

Cantera, J. J. L., and Stein, L. Y. (2007). Molecular diversity of nitrite reductase genes (nirK) in nitrifying bacteria. *Environ. Microbiol.* 9, 765–776. doi: 10.1111/j.1462-2920.2006.01198.x

Chen, T., Yu, W.-H., Izard, J., Baranova, O. V., Lakshmanan, A., and Dewhirst, F. E. (2010). The human oral microbiome database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database (Oxford)* 2010:baq013. doi: 10.1093/database/baq013

de Paiva, C. S., Jones, D. B., Stern, M. E., Bian, F., Moore, Q. L., Corbiere, S., et al. (2016). Altered mucosal microbiome diversity and disease severity in Sjögren syndrome. *Sci. Rep.* 6:23561. doi: 10.1038/srep23561

Docktor, M. J., Paster, B. J., Abramowicz, S., Ingram, J., Wang, Y. E., Correll, M., et al. (2012). Alterations in diversity of the oral microbiome in pediatric inflammatory bowel disease. *Inflamm. Bowel Dis.* 18, 935–942. doi: 10.1002/ibd.21874

Doel, J. J., Benjamin, N., Hector, M. P., Rogers, M., and Allaker, R. P. (2005). Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur. J. Oral Sci.* 113, 14–19. doi: 10.1111/j.1600-0722.2004.00184.x

Elrod, J. W. J., Calvert, J. W. J., Gundewar, S. S., Bryan, N. S. N., and Lefer, D. J. D. (2008). Nitric oxide promotes distant organ protection: evidence for an endocrine role of nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11430–11435. doi: 10.1073/pnas.0800700105

Govoni, M., Jansson, E. Å., Weitzberg, E., and Lundberg, J. O. (2008). The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* 19, 333–337. doi: 10.1016/j.niox.2008.08.003

Graudal, N. A., Hubeck-Graudal, T., and Jurgens, G. (2017). Effects of low sodium diet versus high sodium diet on blood pressure, renin, aldosterone, catecholamines, cholesterol, and triglyceride. *Cochrane Database Syst. Rev.* 85, 22–309. doi: 10.1002/14651858.CD004022.pub4

Hansen, T. H., Kern, T., Bak, E. G., Kashani, A., Allin, K. H., Nielsen, T., et al. (2018). Impact of a vegan diet on the human salivary microbiota. *Sci. Rep.* 8, 1–11. doi: 10.1038/s41598-018-24207-3

Hyde, E. R., Andrade, F., Vaksman, Z., Parthasarathy, K., Jiang, H., Parthasarathy, D. K., et al. (2014). Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: implications for nitric oxide homeostasis. *PLoS ONE* 9:e88645. doi: 10.1371/journal.pone.0088645

Jiang, B., Liang, X., Chen, Y., Ma, T., Liu, L., Li, J., et al. (2012a). Integrating next-generation sequencing and traditional tongue diagnosis to determine tongue coating microbiome. *Sci. Rep.* 2:936. doi: 10.1038/srep00936

Jiang, H., Parthasarathy, D., Torregrossa, A. C., Mian, A., and Bryan, N. S. (2012b). Analytical techniques for assaying nitric oxide bioactivity. *J. Vis. Exp.* e3722. doi: 10.3791/3722

Kallioinen, N., Hill, A., Horswill, M. S., Ward, H. E., and Watson, M. O. (2017). Sources of inaccuracy in the measurement of adult patients' resting blood pressure in clinical settings: a systematic review. *J. Hypertens.* 35, 421–441. doi: 10.1097/HJH.0000000000001197

Kapil, V., Haydar, S. M. A., Pearl, V., Lundberg, J. O., Weitzberg, E., and Ahluwalia, A. (2013). Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic. Biol. Med.* 55, 93–100. doi: 10.1016/j.freeradbiomed.2012.11.013

Liu, G., Tang, C. M., and Exley, R. M. (2015). Non-pathogenic neisseria: members of an abundant, multi-habitat, diverse genus. *Microbiology* 161, 1297–1312. doi: 10.1099/mic.0.000086

Loesche, W. J. (1993). *Dental Caries, A Treatable Infection*. Grand Haven, MI: ADQ Publications.

Lundberg, J. O., and Govoni, M. (2004). Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic. Biol. Med.* 37, 395–400. doi: 10.1016/j.freeradbiomed.2004.04.027

Lundberg, J. O., Weitzberg, E., Cole, J. A., and Benjamin, N. (2004). Nitrate, bacteria and human health. *Nat. Rev. Microbiol.* 2, 593–602. doi: 10.1038/nrmicro929

Lundberg, J. O., Weitzberg, E., and Gladwin, M. T. (2008). The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discov.* 7, 156–167. doi: 10.1038/nrd2466

Lundberg, J. O., Weitzberg, E., Lundberg, J. M., and Alving, K. (1994). Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* 35, 1543–1546. doi: 10.1136/gut.35.11.1543

Lundberg, J. O. J., Gladwin, M. T. M., Ahluwalia, A. A., Benjamin, N. N., Bryan, N. S. N., Butler, A. A., et al. (2009). Nitrate and nitrite in biology, nutrition and therapeutics. *Nat. Chem. Biol.* 5, 865–869. doi: 10.1038/nchembio.260

Mark Welch, J. L., Utter, D. R., Rossetti, B. J., Mark Welch, D. B., Eren, A. M., and Borisy, G. G. (2014). Dynamics of tongue microbial communities with single-nucleotide resolution using oligotyping. *Front. Microbiol.* 5:568. doi: 10.3389/fmicb.2014.00568

Martin, F. E., Nadkarni, M. A., Jacques, N. A., and Hunter, N. (2002). Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. *J. Clin. Microbiol.* 40, 1698–1704. doi: 10.1128/JCM.40.5.1698-1704.2002

McDonagh, S. T. J., Wylie, L. J., Winyard, P. G., Vanhatalo, A., and Jones, A. M. (2015). The effects of chronic nitrate supplementation and the use of strong and weak antibacterial agents on plasma nitrite concentration and exercise blood pressure. *Int. J. Sports Med.* 36, 1177–1185. doi: 10.1055/s-0035-1554700

Mitsui, T., and Harasawa, R. (2017). The effects of essential oil, povidone-iodine, and chlorhexidine mouthwash on salivary nitrate/nitrite and nitrate-reducing bacteria. *J. Oral Sci.* 59, 597–601. doi: 10.2334/josnusd.16-0593

Moncada, S., and Higgs, A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* 329, 2002–2012. doi: 10.1056/NEJM199312303292706

Nagasaka, Y., Fernandez, B. O., Garcia-Saura, M. F., Petersen, B., Ichinose, F., Bloch, K. D., et al. (2008). Brief periods of nitric oxide inhalation protect against myocardial ischemia–reperfusion injury. *Anesthesiology* 109, 675–682. doi: 10.1097/ALN.0b013e318186316e

Niu, W., and Qi, Y. (2011). An updated meta-analysis of endothelial nitric oxide synthase gene: three well-characterized polymorphisms with hypertension. *PLoS ONE* 6:e24266. doi: 10.1371/journal.pone.0024266

Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., and Kanehisa, M. (1999). KEGG: Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 27, 29–34. doi: 10.1093/nar/27.1.29

Pedrazzi, V., do Nascimento, C., Mardegan Issa, J. P., and Fedorowicz Z. (2016). Interventions for managing halitosis. *Cochrane Database System. Rev.* CD012213. doi: 10.1002/14651858.CD012213

Petersson, J., Carlström, M., Schreiber, O., Phillipson, M., Christoffersson, G., Jägare, A., et al. (2009). Gastroprotective and blood pressure lowering effects of dietary nitrate are abolished by an antiseptic mouthwash. *Free Radic. Biol. Med.* 46, 1068–1075. doi: 10.1016/j.freeradbiomed.2009.01.011

Rai, N., Huynh, L., Kim, M., and Tagkopoulos, I. (2019). Population collapse and adaptive rescue during long-term chemostat fermentation. *Biotechnol. Bioeng.* 116, 693–703. doi: 10.1002/bit.26898

Rassaf, T., Bryan, N. S., Kelm, M., and Feelisch, M. (2002). Concomitant presence of N-nitroso and S-nitroso proteins in human plasma. *Free Radic. Biol. Med.* 33, 1590–1596. doi: 10.1016/S0891-5849(02)01183-8

Ren, W., Xun, Z., Wang, Z., Zhang, Q., Liu, X., Zheng, H., et al. (2016). Tongue coating and the salivary microbial communities vary in children with halitosis. *Sci. Rep.* 6, 1–12. doi: 10.1038/srep24481

Rosendorff, C. (ed.). (2013). Rosendorff, C. (2013) "Hypertension, mechanisms and diagnosis," in *Essential Cardiology* (New York, NY: Springer), 543–559. doi: 10.1007/978-1-59259-918-9_32

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Taddei, S., Virdis, A., Ghiadoni, L., Salvetti, G., Bernini, G., Magagna, A., et al. (2001). Age-related reduction of NO availability and oxidative stress in humans. *Hypertension* 38, 274–279. doi: 10.1161/01.HYP.38.2.274

Tannenbaum, S. R., Weisman, M., and Fett, D. (1976). The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet. Toxicol.* 4, 549–552. doi: 10.1016/S0015-6264(76)80006-5

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., and Nguyen, M. (2010). A practical approach to RT-qPCR-publishing data that conform to the MIQE guidelines. *Methods* 50, S1–5. doi: 10.1016/j.ymeth.2010.01.005

Torregrossa, A. C., Aranke, M., and Bryan, N. S. (2011). Nitric oxide and geriatrics: implications in diagnostics and treatment of the elderly. *J. Geriatr. Cardiol.* 8, 230–242. doi: 10.3724/SP.J.1263.2011.00230

Velmurugan, S., Gan, J. M., Rathod, K. S., Khambata, R. S., Ghosh, S. M., Hartley, A., et al. (2015). Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study. *Am. J. Clin. Nutr.* 103, 25–38. doi: 10.3945/ajcn.115.116244

Wade, W. G., and Addy, M. (1989). *In vitro* activity of a chlorhexidine-containing mouthwash against subgingival bacteria. *J. Periodontol.* 60, 521–525. doi: 10.1902/jop.1989.60.9.521

Woessner, M., Smoliga, J. M., Tarzia, B., Stabler, T., Van Bruggen, M., and Allen, J. D. (2016). A stepwise reduction in plasma and salivary nitrite with increasing strengths of mouthwash following a dietary nitrate load. *Nitric Oxide* 54, 1–7. doi: 10.1016/j.niox.2016.01.002

Zaura, E., Keijser, B. J. F., Huse, S. M., and Crielaard, W. (2009). Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiol.* 9:259. doi: 10.1186/1471-2180-9-259

Conflict of Interest Statement: The authors of this manuscript declare the following competing interests: NB is a Founder and Shareholder of HumanN, a dietary supplement and functional nitric oxide nutrition company, a shareholder and consultant for SAJE Pharma, and he receives royalties on nitric oxide related patents from University of Texas. JP is President and Founder of Diversigen, a microbiome analysis service. NJA is Chief Scientific Officer at MicrobiomeDX, a microbiome analysis service. EH is Managing Editor at SynBioBeta.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 10 May 2019
doi: 10.3389/fcimb.2019.00145

Evaluation of Sodium Hypochlorite Irrigant, Bingpeng Irrigant, and Fufang Bingpeng Irrigant as Endodontic Irrigants During Passive Ultrasonic Irrigation

*Yan Shi[1], Zhipeng Deng[2], Yulu Yang[3], Lanyue Cui[3], Tingtao Chen[3], Mingjing Hu[2], Lei Xie[2] and Jian Yang[1]**

[1] Department of Conservative Dentistry and Endodontics, Stomatology Hospital of Nanchang University, Jiangxi Provincial Key Laboratory of Oral Biology, Nanchang, China, [2] Stomatology School of Nanchang University, Nanchang, China, [3] Institute of Translational Medicine, Nanchang University, Nanchang, China

OPEN ACCESS

Edited by:
Andrea Santarelli,
Polytechnical University of Marche, Italy

Reviewed by:
Prasanna Neelakantan,
The University of Hong Kong, Hong Kong
Dorina Lauritano,
University of Milano-Bicocca, Italy

***Correspondence:**
Jian Yang
jianyang399@hotmail.com.cn

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 02 November 2018
Accepted: 17 April 2019
Published: 10 May 2019

Citation:
Shi Y, Deng Z, Yang Y, Cui L, Chen T, Hu M, Xie L and Yang J (2019) Evaluation of Sodium Hypochlorite Irrigant, Bingpeng Irrigant, and Fufang Bingpeng Irrigant as Endodontic Irrigants During Passive Ultrasonic Irrigation. Front. Cell. Infect. Microbiol. 9:145. doi: 10.3389/fcimb.2019.00145

Given the increasing prevalence of antibiotic resistance among bacterial strains and the side effects caused by synthetic drugs, it is increasingly important to investigate potential herbal alternatives. In the present study, antimicrobial, cell cytotoxicity, and cleaning tests were performed to evaluate the potential of Fufang Bingpeng irrigant as a root canal irrigant, in addition to q-PCR and high-throughput sequencing analyses. Our *in vitro* results showed a low minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Fufang Bingpeng irrigant against *Porphyromonas gingivalis* ATCC 33277 (6.25 and 12.5%, respectively), *Prevotella intermedius* ATCC 25611 (6.25 and 6.25%, respectively), *Fusobacterium nucleatum* ATCC 25286 (6.25 and 6.25%, respectively), *Enterococcus faecalis* ATCC 19433 (25 and 25%, respectively), and *Bacteriodes fragilis* ATCC 25285 (12.5 and 12.5%, respectively). Furthermore, it effectively removed the remaining debris and increased the number of open dentinal tubules in root canals compared to the NaCl irrigant ($p < 0.05$). Fufang Bingpeng irrigant also presented low cytotoxicity to L929 cells compared to the NaClO irrigant. The *in vivo* results indicated that all irrigants used significantly reduced the number of bacteria compared to the number prior to treatment, and only 1/104.95 bacteria remained in the root canal following the use of Fufang Bingpeng irrigant ($p < 0.001$). Moreover, the high-throughput sequencing results indicated that all irrigants markedly enhanced the α diversity in the root canal compared to the before preparation control group, while Fufang Bingpeng maintained better microbial diversity than other groups. Therefore, Fufang Bingpeng irrigant presents a promising alternative for use as a root canal irrigant in clinical settings.

Keywords: root canal irrigant, NaClO irrigant, Fufang Bingpeng irrigant, cytotoxicity, cleaning efficacy, high-throughput sequencing

INTRODUCTION

The bacteria present in the root canal play an important role in the pathologies of pulpitis and periapical periodontitis, and the success of root canal therapy is mainly dependent on controlling bacterial infection inside the root canal (Rödig et al., 2010). Root canal irrigation represents one of the basic steps for root canal therapy, which can effectively control the bacterial infection inside the root canal (Vouzara et al., 2016).

Currently, NaClO irrigation is commonly used in the clinic due to its sound antibacterial properties, low cost and long period of action, while its shortcomings (e.g., high toxicity, unpleasant smell, potential corrosiveness, and allergy) hinder its use (Sabins et al., 2003; Rödig et al., 2010). Other irrigants, such as chlorhexidine and ardenia jasmine gargle, have also been used in the clinic; however, they have several drawbacks (antimicrobial-resistant strains and side effects), thus it is necessary to find more suitable irrigants (Stuart et al., 2006; Prabhakar et al., 2010; Sibley et al., 2012).

In the present study, Fufang Bingpeng irrigant, made by pure Chinese Medicines, was investigated by our group. Specifically, we assessed its effect on obligate anaerobic microorganisms, remaining debris and open dentinal tubules in the apical and middle thirds of the root canal, as well as the growth of L929 cells and microbial diversity in the root canal.

MATERIALS AND METHODS

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Irrigants

The pathogens *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedius* ATCC 25611, *Fusobacterium nucleatum* ATCC 25286, *Enterococcus faecalis* ATCC 19433, and *Bacteriodes fragilis* ATCC 25285 were cultured in brain/heart infusion (BHI) medium for 24 h in an incubator with an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. Then, Fufang Bingpeng irrigant and Bingpeng irrigant were added to the BHI plate to make final concentrations of 50, 25, 12.5, 6.25, 3.1, 1.6, 0.8, 0.4, 0.2, and 0.1%; and NaClO irrigant was added at 4, 2, 1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.016, and 0.008%. Pathogens were then inoculated onto the plates, and their MIC and MBC were determined using the viable cell counting method (Jiang et al., 2016).

Cytotoxicity of Tested Irrigants

L929 mouse fibroblasts cells were maintained in DMEM supplemented with 15% FBS at 37°C in a 5% CO_2 incubator. Different concentrations of irrigants were dissolved in cell culture media, and their effect on the viability of L929 cells was determined by MTT assay (Hu et al., 2018).

L929 cells were plated at a density of 10,000 cells per well in 100 μl of complete culture media. Cells were then treated with designated concentrations of NaClO irrigant (0.1%, 0.5%, 2.5% and 5%), Bingpeng irrigant (self-made; 6.25, 12.5, 25, and 50%) and Fufang Bingpeng irrigant (self-made; 6.25, 12.5, 25, and 50%) in 96-well microtiter plates for 30 min, 1 h, 6 h and 12 h at 37°C in a humidified incubator. After incubation until a specific stage, MTT reagent (20 μl, 5 mg/mL) was added and incubated for 3.5 h, then the MTT solution was carefully removed and 150 μl of DMSO was added. The absorbance was recorded on a microplate reader at a wavelength of 570 nm. Corresponding volumes of NaCl irrigant were used as the control.

Evaluation of the Cleaning Efficacy of the Tested Irrigants

Forty bicuspid teeth were collected from children (aged 11–13 years) undergoing orthodontic extraction. These children received no pulp canal therapy, and their apical foramen was completely developed and without caries. The collected teeth were then randomly divided into four groups and stored in physiological saline for further use.

Root canal preparation was performed using gradually deepening preparatory technology with a K-file. Between each instrument, the canals were irrigated with 5 ml NaCl irrigant (0.9%), NaClO irrigant (0.5% NaClO + 17% EDTA), Bingpeng irrigant (100%) or Fufang Bingpeng irrigant (100%), then 3 ml of physiological saline was used to terminate the effect of different irrigants on the root canal. The root canal was dried with paper points, and the root canal orifice was sealed with a cotton pellet and Cavit. A single operator with experience in this method and instrumentation prepared all canals.

With the aid of a surgical operating microscope, a longitudinal groove was cut in the root using the end of a diamond bur. The roots were removed from the crown and then split by placing a surgical blade in the groove and striking the blade with a small mallet. Images of the split roots were made using a digital camera set at 1:1. The images were transferred to a computer with Adobe Photoshop CS software and enlarged to 100× the original size. Lines were superimposed over the canals at 0, 3, and 6 mm from the apical constriction. The debris in each canal was traced, and the total number of pixels occupied by debris was determined using the histogram function in the software. The outline of the canal was then traced, and the same feature of the software reported the total pixels occupied by the canal. The percentage of debris was calculated by dividing the number of debris pixels by the total number of pixels, representing the entire area of the canal. The percentage of debris was calculated for the apical third and middle third of each canal.

Six specimens were randomly selected from each group. The teeth were split and their two halves were mounted in specimen holders. They were then placed in a vacuum chamber and coated with a 20-nm-thick gold-palladium layer for scanning electron microscopy (Hu et al., 2018). The apical and middle thirds of each root canal were selected, and photomicrographs of the central part were taken at 500× magnification on the longitudinal plane and then enlarged to 110 × 120 mm. A square 72-mm grid composed of 64 equal squares, of which 16 were randomly selected, was superimposed onto the scanning electron microscope (SEM) photomicrograph. The number of open dentinal tubules inside each square were counted by a single observer. The sum of the 16 squares of each grid was calculated, obtaining the total number of open dentinal tubules.

Extraction of Bacterial DNA

Between January 2018 and July 2018, 40 patients with periapical periodontitis were selected. Root canal preparation was performed using gradually deepening preparatory technology with K-files and S3 NiTi files. Between each instrument, the canals were irrigated with either 5 ml NaCl irrigant ($n = 10$), NaClO irrigant ($n = 10$), Bingpeng irrigant ($n = 10$), or Fufang Bingpeng irrigant ($n = 10$). Ultrasonic irrigation was performed for 10–20 s, followed by ultrasonic irrigation with 3 ml EDTA for 10–20 s. Physiological saline (5 ml) was then used to terminate the effect of the different irrigants on the root canal. Samples were collected before and after the preparation for further analysis.

All human tests were approved by the Committee on the Ethics of Human Experiments of Stomatology Hospital of Nanchang University (Jiangxi, China). Patient samples were obtained with written informed consent in accordance with the requirements of the Ethics Committee.

Real-Time PCR

The number of bacteria in the root canal samples were determined by real-time PCR using the ABI 7900HT fast real-time PCR system (Applied Biosystems, USA). The reaction buffer consisted of primers (338F, 5′-ACTCCTACGGGAGGCAGCAG-3′; 518R, 5′- ATTACCGCGGCTGCTGG-3′), ROX reference dye and SYBR® Primer EX Taq II (TaKaRa), and amplification began at 94°C for 8 min. The temperatures used for degeneration, annealing and extension were 94,

TABLE 1 | MIC and MBC of NaClO irrigant, Bingpeng irrigant, and Fufang Bingpeng irrigant against obligated anaerobic microorganisms.

Drugs	*P. gingivalis* ATCC 33277	*P. intermedius* ATCC25611	*F. nucleatum* ATCC 25286	*E. faecalis* ATCC 19433	*B. fragilis* ATCC25285
CONCENTRATION OF NACLO IRRIGANT (%)					
MIC	0.035	0.070	0.018	0.28	0.07
MBC	0.035	0.070	0.018	0.28	0.07
CONCENTRATION OF BINGPENG IRRIGANT (%)					
MIC	12.5	12.5	12.5	50	25
MBC	25	12.5	25	>50	25
CONCENTRATION OF FUFANG BINGPENG IRRIGANT (%)					
MIC	6.25	6.25	6.25	25	12.5
MBC	12.5	6.25	6.25	25	12.5

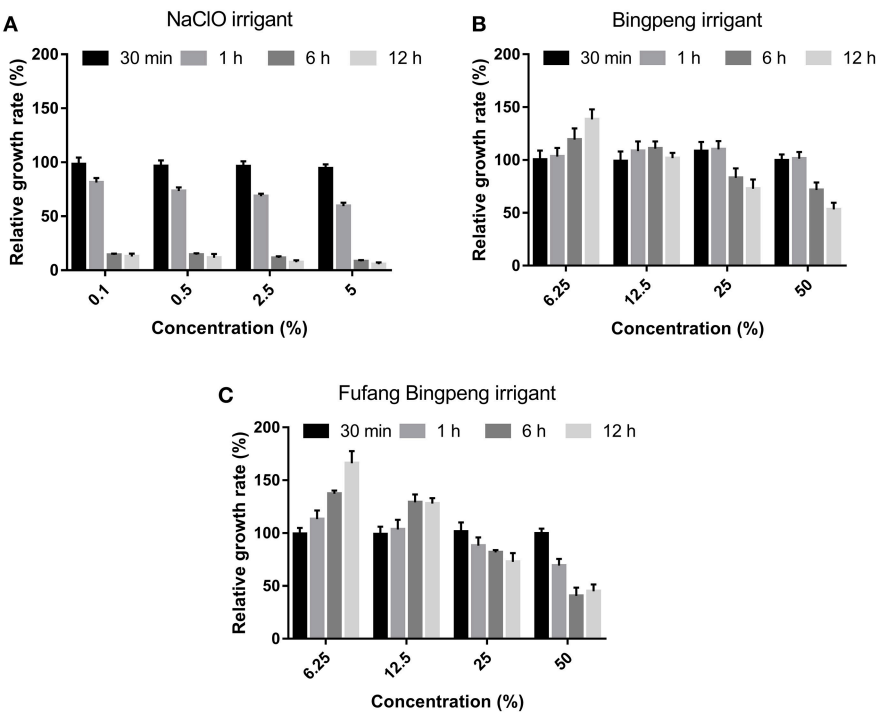


FIGURE 1 | Effect of different concentrations of NaClO irrigant **(A)**, Bingpeng irrigant **(B)** and Fufang Bingpeng irrigant **(C)** on the relative growth rate of L929 cells compared to the NaCl irrigant group.

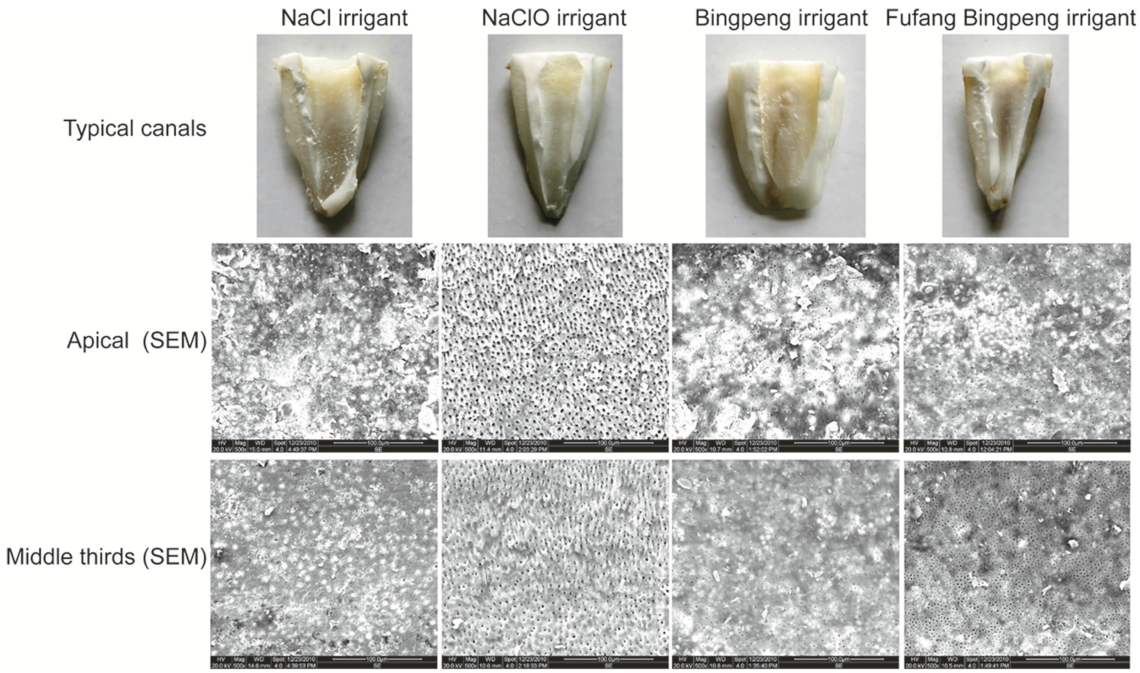


FIGURE 2 | Typical canals with debris and SEM (500*) of the apical and middle thirds of root canals treated with the NaCl irrigant, NaClO irrigant, Bingpeng irrigant, and Fufang Bingpeng irrigant.

TABLE 2 | Effect of NaCl irrigant, NaClO irrigant, Bingpeng irrigant, and Fufang Bingpeng irrigant on remaining debris and open dentinal tubules in the apical and middle thirds of the root canals.

Drugs	Remaining debris (%, $n = 10$)		Open dentinal tubule number ($n = 6$)	
	Apical thirds	Middle thirds	Apical thirds	Middle thirds
NaCl irrigant	20.02 + 6.02	12.41 + 5.61	9.83 + 7.86	13.33 + 5.24
NaClO irrigant	6.65 + 4.81*	4.55 + 6.02*	70.17 + 37.63*	85 + 26.33*
Bingpeng irrigant	10.11 + 6.03*	7.07 + 3.01*	40.03 + 16.43*	70.5 + 25.38*
Fufang Bingpeng irrigant	9.63 + 6.43*	6.86 + 2.81*	45.33 + 18.45	75.5 + 34.58*

*x+s, *p < 0.05 compared with NaCl irrigant.*

58, and 71°C, respectively. The relative expression levels of the target bacteria were analyzed using the $2^{-\Delta\Delta}$Ct method (Chen et al., 2018).

High-Throughput Sequencing

Primers (515F, 5′-GTG CCA GCM GCC GCG GTA A-3′; and 806R, 5′-GGA CTA CVS GGG TAT CTA AT-3′) were used to amplify the V4 region of the 16S rRNA genes of the extracted genomic DNA (GenBank accession number PRJNA496554) (Xin et al., 2016). FLASH was used to merge overlapped tags, the UPARSE software package was used to analyze sequences, and in-house Perl scripts were used to analyze the alpha (within samples) and beta (among samples) diversity. Sequences with high similarity (≥97%) were considered the same OTUs, and the QIIME software package was applied to analyze the weighted UniFrac distance.

Data Analysis

Data are presented as the mean ± standard deviation (SD), and Prism software version 7.0 (GraphPad Software, San Diego, CA, USA) was used to perform the statistical analyses. Error probabilities of $p < 0.05$ were considered statistically significant.

RESULTS

Antibacterial Effect of Irrigants

The MIC and MBC of the tested irrigants on *P. gingivalis* ATCC 33277, *P. intermedius* ATCC 25611, *F. nucleatum* ATCC 25286, *E. faecalis* ATCC 19433, and *B. fragilis* ATCC 25285 are shown in **Table 1**. All tested irrigants showed a significant antibacterial effect, and the NaClO irrigant possessed the lowest MIC and MBC values for *P. gingivalis* ATCC 33277 (0.035 and 0.035%, respectively), *P. intermedius* ATCC 25611 (0.070 and 0.070%, respectively), *F. nucleatum* ATCC 25286 (0.018 and 0.018%, respectively), *E. faecalis* ATCC 19433 (0.28 and 0.28%,

respectively) and *B. fragilis* ATCC 25285 (0.070 and 0.070%, respectively). Although the Fufang Bingpeng irrigant possessed a weaker effect on pathogens, its antibacterial effect was stronger than the Bingpeng and NaCl irrigants.

Cytotoxicity of Irrigants on L929 Cells

As shown in **Figure 1**, dose-dependent and time-dependent effects were observed when the cytotoxicity of irrigants was tested. After 30 min, all irrigants showed little cytotoxicity on L929 cells at all tested concentrations. After 1 h, the NaClO irrigant showed strong cytotoxicity, especially at concentrations >0.5%. At 6 and 12 h, few viable cells were obtained at all tested concentrations. For the Fufang Bingpeng irrigant and the Bingpeng irrigant, even low concentrations (6.25 and 12.5%, respectively) showed a growth-promoting effect, while high concentrations (25 and 50%, respectively) showed a serious cytotoxic effect on L929 cells, with the Fufang Bingpeng irrigant possessing higher cytotoxicity than the Bingpeng irrigant.

Effect of Irrigants on Cleaning Efficacy

Overall, the amount of debris in the NaCl irrigant group was markedly higher than other groups ($P < 0.05$), and no significant differences were observed among the NaClO, Fufang Bingpeng and Bingpeng irrigant groups in the apical part of the root canal (**Figure 2** and **Table 2**). Similarly, debris in the middle third of the root canal in the NaCl irrigant group was also higher than that in the NaClO irrigant group. Moreover, the SEM results indicated that there were fewer open dentinal tubules in the NaCl irrigant group than the other three groups ($p < 0.05$, apical part of the root canals), and the numbers in the middle third of the root canal were markedly lower than those in the NaClO and Fufang Bingpeng irrigant groups (**Figure 2** and **Table 2**). In summary, the cleaning efficacy of irrigants were as follows: NaClO irrigant > Fufang Bingpeng irrigant > Bingpeng irrigant > NaCl irrigant.

Effects of Irrigants on the Relative Bacterial Number in the Root Canal

Finally, we evaluated the effects of irrigants on bacterial diversity in the root canal. As shown in **Figure 3**, the NaCl, NaClO, Bingpeng, and Fufang Bingpeng irrigants all significantly reduced the number of bacteria compared to the number prior to treatment ($p < 0.001$). Compared to the bacterial number before the preparation, 99.59-fold and 104.95-fold reductions in bacterial numbers were obtained in the NaClO and Fufang Bingpeng irrigant groups, respectively.

Effects of Irrigants on the Microbial Composition of the Root Canal

High-throughput sequencing was used to explore the effects of the NaCl, NaClO, Bingpeng, and Fufang Bingpeng irrigants on the microbial composition of the root canal. In total, 3,547,824 filtered clean tags (73,913 tags/sample) and 44,834 OTUs were obtained from all samples, with an average of 934.04 OTUs per group (**Table S1**).

As shown in **Figure 4**, the irrigants used in the present study had an obvious effect on the microbial composition at both the phyla and genus levels, and the Fufang Bingpeng irrigant resulted in a marked reduction in *Fusobacterium*, *Enterococcus* and *Pseudoramibacter* at the genus level (**Figures 4A,B**). In addition, the Chao1 index and Shannon index indicated that all irrigants markedly enhanced the α diversity in the root canal compared to the before preparation control group (BC), especially for the NaCl irrigant, NaClO irrigant and Bingpeng irrigant groups ($p < 0.01$). When analyzed using the Venn method, 1142 core OTUs were found in all groups, with 468, 258, 228, 181, and 95 OTUs found in the BC group, the after preparation control group (AC), the after preparation NaClO irrigant group (ASH), the after preparation Bingpeng irrigant group (AB) and the after preparation Fufang Bingpeng irrigant group (AFB), respectively (**Figure 4E**). The principal coordinates analysis (PCoA) indicated that although irrigants changed the microbial diversity compared to the BC group, the AFB possessed the most similar bacterial composition to the BC group (**Figure 4F**). When the significant changes in bacteria were compared between the BC vs. AC, BC vs. ASH, BC vs. AB and BC vs. AFB groups, we found that *Prevotella* in the AC, ASF, AB, and AFB groups were significantly reduced compared to the BC group (**Figure 5**).

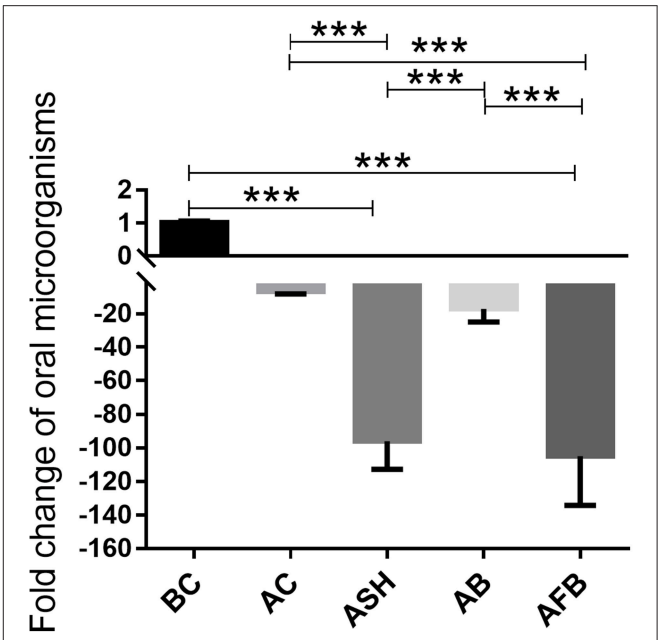


FIGURE 3 | Effects of the NaCl irrigant, NaClO irrigant, Bingpeng irrigant and Fufang Bingpeng irrigant on the elimination of bacteria in the oral cavity, assessed using q-PCR. BC, microbiota in the oral cavity of patients before treatment; AC, microbiota in the oral cavity of patients after treatment with the NaCl irrigant; ASH, microbiota in the oral cavity of patients after treatment with the NaClO irrigant; AB, microbiota in the oral cavity of patients after treatment with the Bingpeng irrigant; AFB, microbiota in the oral cavity of patients after treatment with the Fufang Bingpeng irrigant. ***$p < 0.001$.

DISCUSSION

Oral microorganisms, especially opportunistic pathogens, cause primary endodontic infections due to a gradual increase in number over long periods in the root canal (Prabhakar et al., 2010). As an adjunct, various irrigants have been applied to improve antimicrobial cleaning and shaping in endodontics and

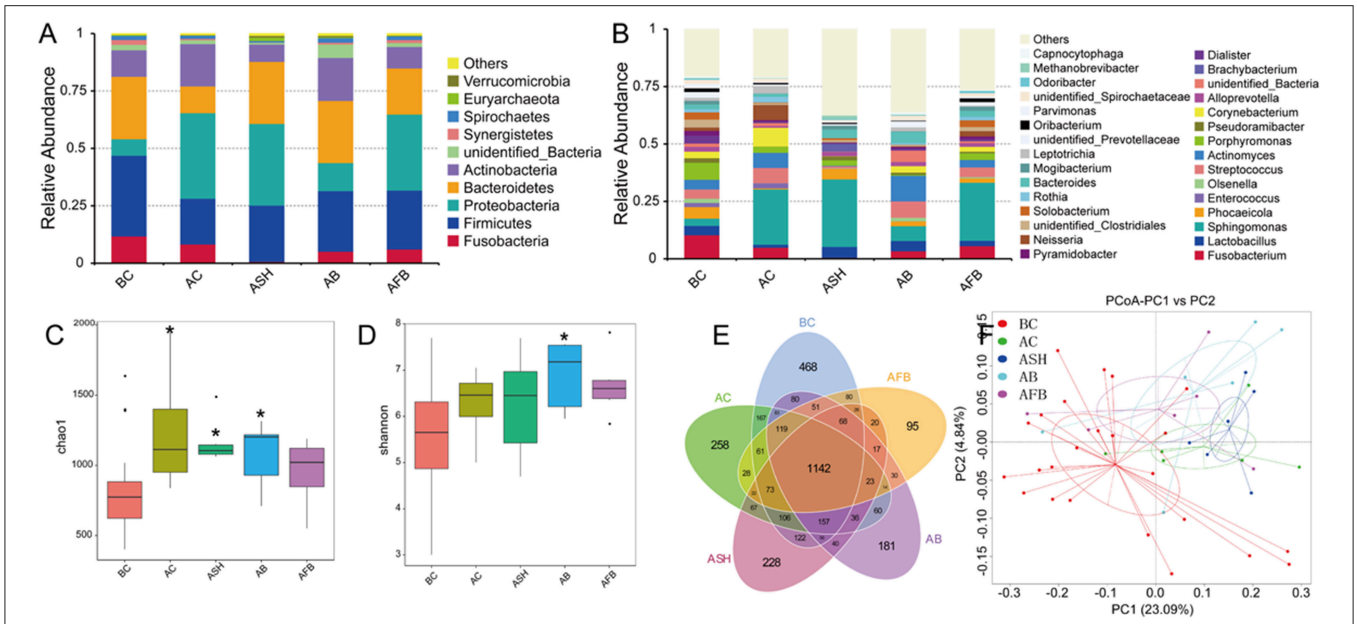


FIGURE 4 | Evaluation of the effects of the NaCl irrigant, NaClO irrigant, Bingpeng irrigant and Fufang Bingpeng irrigant on the oral microbiota. **(A)** Relative bacterial abundance at the phylum level, **(B)** relative bacterial abundance at the genus level, **(C)** the Chao1 index, **(D)** the Shannon index, **(E)** Scalar-Venn representation of the vaginal microbiota, and **(F)** principal coordinates analysis (PCoA). BC, microbiota in the oral cavity of patients before treatment; AC, microbiota in the oral cavity of patients after treatment with the NaCl irrigant; ASH, microbiota in the oral cavity of patients after treatment with the NaClO irrigant; AB, microbiota in the oral cavity of patients after treatment with the Bingpeng irrigant; AFB, microbiota in the oral cavity of patients after treatment with the Fufang Bingpeng irrigant. *$p < 0.05$ compared to the BC group.

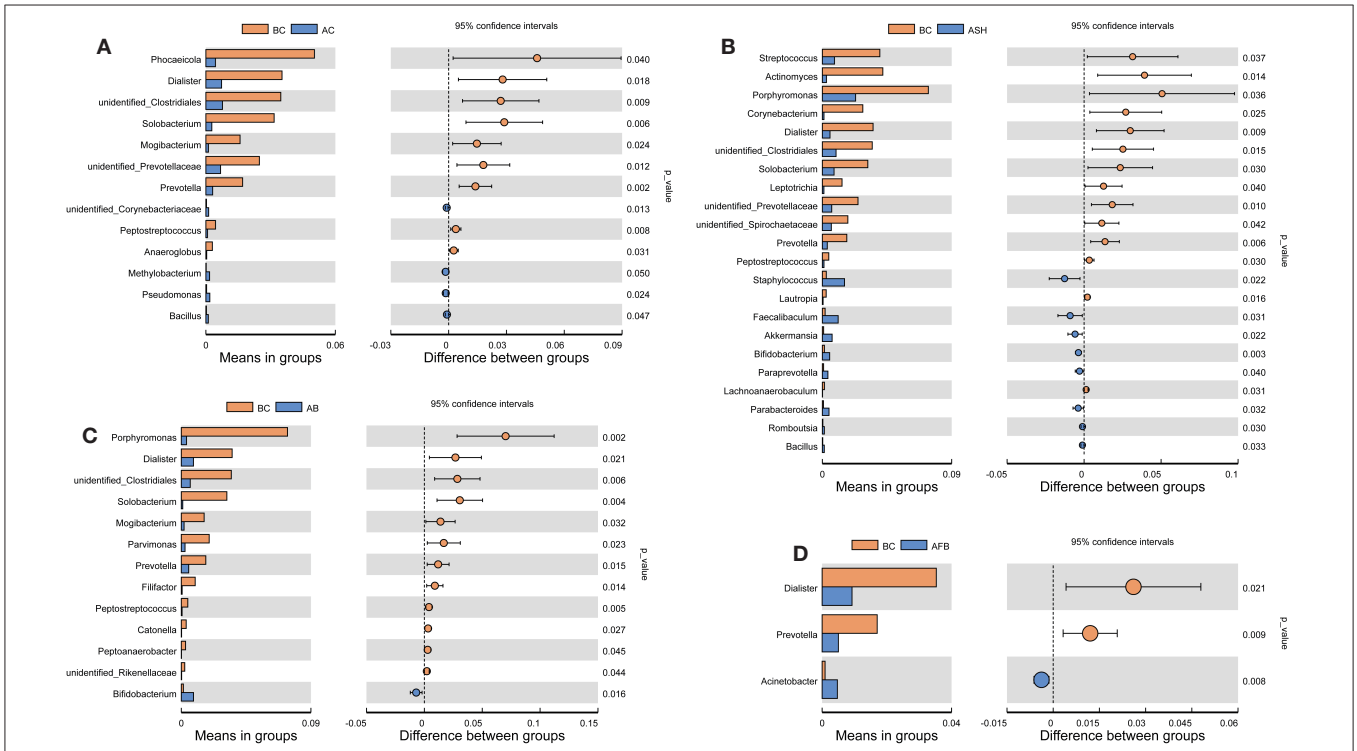


FIGURE 5 | Analysis of species differences among BC vs. AC **(A)**, BC vs. ASH **(B)**, BC vs. AB **(C)** and BC vs. AFB **(D)** groups. BC, microbiota in the oral cavity of patients before treatment; AC, microbiota in the oral cavity of patients after treatment with the NaCl irrigant; ASH, microbiota in the oral cavity of patients after treatment with the NaClO irrigant; AB, microbiota in the oral cavity of patients after treatment with the Bingpeng irrigant; AFB, microbiota in the oral cavity of patients after treatment with the Fufang Bingpeng irrigant.

to eliminate most of the bacteria. However, studies indicate that some bacteria can survive in the root canal (Gomes et al., 2010). The NaOCl irrigant is widely used, representing the most common irrigant used during endodontic treatment. However, its high toxicity, unpleasant taste and inability to dissolve dentin debris have hindered its use (Stuart et al., 2006; Da et al., 2008). Therefore, it is necessary to find a suitable irrigant with low toxicity and high efficiency.

The Bingpeng irrigant originates from "Bingpeng powder," which contains borneol, borax, cinnabar and weathered sodium sulfate, and has been suggested to effectively inhibit bacterial growth and eliminate inflammation (Shi et al., 2011; Wang, 2011). To enhance its antimicrobial effect and cleaning efficiency and decrease its toxicity, cinnabar was removed from the Bingpeng irrigant, and *Angelica archanglica* and *Asurum uropeum* were added. The resulting "Fufang Bingpeng" irrigant has greatly enhanced antibacterial and anti-inflammatory effects, and has an additional use for treating detumescence and abscesses, and as a pain reliever (Shi et al., 2011).

Anaerobic bacteria usually persist in treated root canals and are often resistant to traditional antibiotics. When anaerobic bacteria grow as a biofilm, antimicrobial agents cannot enter. Furthermore, a 1500-fold increase in the rate of antibiotic resistance had been observed compared to planktonic cells (Gordon et al., 1988; Mah and O'toole, 2001). As shown in **Table 1**, the NaClO irrigant effectively inhibited all tested pathogens and showed the lowest MIC and MBC values. Although the antibacterial effect of the Fufang Bingpeng irrigant was inferior to the NaClO irrigant, its MIC and MBC for *P. gingivalis* ATCC 33277, *P. intermedius* ATCC 25611, *F. nucleatum* ATCC 25286, *E. faecalis* ATCC 19433, and *B. fragilis* ATCC 25285 were as low as 6.25 vs. 12.5%, 6.25 vs. 6.25%, 6.25 vs. 6.25%, 25 vs. 25%, and 12.5% vs. 12.5%, respectively. The NaClO and EDTA irrigants showed a better effect on the amount of remaining debris and number of open dentinal tubules in the apical and middle thirds of the root canal (**Table 2**), while NaClO markedly inhibited the growth of L929 cells, even at low concentrations (**Figure 1**). The residual debris and smear layer in infected root canals harbor various microorganisms and their byproducts, which also hinders the ability of the irrigants to directly contact the entire root canal wall (Zehnder, 2006). The Fufang Bingpeng irrigant resulted in better removal of the smear layer, pulp tissue and dentin debris, and even enhanced the growth of L929 cells at low concentrations (**Figure 1** and **Table 2**).

The oral cavity contains various microorganisms that are associated with oral diseases. Bacterial infections within the root canal are usually polymicrobial in nature, and it is rare that a single species causes oral disease (Chen et al., 2017). Therefore, q-PCR and high-throughput sequencing were used to evaluate the effects of the NaCl, NaClO, Bingpeng, and Fufang Bingpeng irrigants on bacterial diversity in the root canal. As shown in **Figure 3**, all tested irrigants effectively reduced the bacterial number compared to the BC group, and 99.59- and 104.95-fold reductions were observed in the NaClO irrigant group and Fufang Bingpeng irrigant group, respectively (**Figures 3**, **5**). Moreover, the results of the high-throughput sequencing analysis indicated that although all tested irrigants greatly reduced the bacterial number (**Figure 3**) and changed the microbial composition compared with the BC group at the phyla and genus levels (**Figures 4A,B**), the α diversity analysis clearly showed that all tested irrigants enhanced the bacterial abundance (**Figures 4C,D**). Most importantly, both the α diversity analysis and PCoA results indicate that the Fufang Bingpeng irrigant could better sustain the microbial diversity in the BC and AC groups, and the similar bacterial diversity with formal ones will help improve oral health (**Figure 4**).

In the present study, the Fufang Bingpeng irrigant showed a sound antimicrobial effect, low cytotoxicity and high cleaning efficacy *in vitro*, and the *in vivo* results showed that the Fufang Bingpeng irrigant significantly reduced the bacterial number and sustained the microbial diversity, which helps to avoid oral infection and protect oral health. Therefore, the use of Fufang Bingpeng irrigant as a root canal irrigant might prove to be advantageous considering its desirable characteristics compared to the NaClO irrigant. However, as all PCR based technologies cannot differentiate viable or dead cells, the PMA-modified viability PCR technology should be introduced in our further work.

AUTHOR CONTRIBUTIONS

YS, TC, and JY designed the experiment. ZD, YY, LC, MH, and LX performed the experiments. TC and YS analyzed the data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

FUNDING

This work was supported by a grant from the Science and Technology plan of the Jianxi Health Planning Committee (Number: 2015BBG70239).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00145/full#supplementary-material

REFERENCES

Chen, T., Tian, P., Huang, Z., Zhao, X., Wang, H., Xia, C., et al. (2018). Engineered commensal bacteria prevent systemic inflammation-induced memory impairment and amyloidogenesis via producing GLP-1. *Appl. Microbiol. Biot.* 102:7565–7575. doi: 10.1007/s00253-018-9155-6

Chen, T., Yan, S., Wang, X., Xin, W., Meng, F., Yang, S., et al. (2017). High-throughput sequencing analyses of oral microbial diversity in healthy people and patients with dental caries and periodontal disease. *Mol. Med. Rep.* 16, 127–132. doi: 10.3892/mmr.2017.6593

Da, S. L., Sanguino, A. C., Rocha, C. T., Leonardo, M. R., and Silva, R. A. (2008). Scanning electron microscopic preliminary study of the efficacy of

SmearClear and EDTA for smear layer removal after root canal instrumentation in permanent teeth. *J. Endodont*. 34, 1541–1544. doi: 10.1016/j.joen.2008.08.007

Gomes, B. P. F. A., Lilley, J. D., and Drucker, D. B. (2010). Variations in the susceptibilities of components of the endodontic microflora to biomechanical procedures. *Int. Endod. J*. 29, 235–241. doi: 10.1111/j.1365-2591.1996.tb01375.x

Gordon, C. A., Hodges, N. A., and Marriott, C. (1988). Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived Pseudomonas aeruginosa. *J. Antimicrob. Chemoth*. 22, 667. doi: 10.1093/jac/22.5.667

Hu, H., Huang, G., Wang, H., Li, X., Wang, X., Feng, Y., et al. (2018). Inhibition effect of triptolide on human epithelial ovarian cancer via adjusting cellular immunity and angiogenesis. *Oncol. Rep*. 39, 1191–1196. doi: 10.3892/or.2017.6158

Jiang, M., Deng, K., Jiang, C., Fu, M., Guo, C., Wang, X., et al. (2016). Evaluation of the antioxidative, antibacterial, and anti-inflammatory effects of the aloe fermentation supernatant containing *Lactobacillus plantarum* HM218749.1. *Mediat. Inflamm*. 2016, 1–8. doi: 10.1155/2016/2945650

Mah, T. F., and O'toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends. Microbiol*. 9, 34–39. doi: 10.1016/S0966-842X(00)01913-2

Prabhakar, J., Senthilkumar, M., Priya, M. S., Mahalakshmi, K., Sehgal, P. K., and Sukumaran, V. G. (2010). Evaluation of antimicrobial efficacy of herbal alternatives (Triphala and green tea polyphenols), MTAD, and 5% sodium hypochlorite against Enterococcus faecalis biofilm formed on tooth substrate: an *in vitro* study. *J. Endod*. 36, 83–86. doi: 10.1016/j.joen.2009.09.040

Rödig, T., Döllmann, S., Konietschke, F., Drebenstedt, S., and Hülsmann, M. (2010). Effectiveness of different irrigant agitation techniques on debris and smear layer removal in curved root canals: a scanning electron microscopy study. *J. Endodont*. 36, 1983–1987. doi: 10.1016/j.joen.2010.08.056

Sabins, R. A., Johnson, J. D., and Hellstein, J. W. (2003). A comparison of the cleaning efficacy of short-term sonic and ultrasonic passive irrigation after hand instrumentation in molar root canals. *J. Endodont*. 29, 674–678. doi: 10.1097/00004770-200310000-00016

Shi, Y., Yang, J., and Chen, H. (2011). Cleaning efficacy of fufang bingpeng irrigant on root canals. *J. Oral Sci. Res.* 27, 1062–1065. doi: 10.13701/j.cnki.kqyxyj.2011.12.017

Sibley, C. D., Peirano, G., and Church, D. L. (2012). Molecular methods for pathogen and microbial community detection and characterization: current and potential application in diagnostic microbiology. *Infect. Genet. Evol*. 12, 505–521. doi: 10.1016/j.meegid.2012.01.011

Stuart, C. H., Schwartz, S. A., Beeson, T. J., and Owatz, C. B. (2006). Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. *J. Endodont*. 32, 93–98. doi: 10.1016/j.joen.2005.10.049

Vouzara, T., Koulaouzidou, E., Ziouti, F., and Economides, N. (2016). Combined and independent cytotoxicity of sodium hypochlorite, ethylenediaminetetraacetic acid and chlorhexidine. *Int. Endod. J*. 49, 764–773. doi: 10.1111/iej.12517

Wang, C. L. (2011). Determination of Boron and Mercury in Bingpeng San. *Chin. J. Exp. Trad. Med. Formulae*. 2011, 1005–9903. doi: 10.13422/j.cnki.syfjx.2011.06.032

Xin, F., Xin, W., Yang, S., Meng, F., Wang, X., Hua, W., et al. (2016). Evaluation of the microbial diversity in amyotrophic lateral sclerosis using high-throughput sequencing. *Front. Microbiol*. 7:1479.

Zehnder, M. (2006). Root canal irrigants. *J. Endodont*. 32, 389–398. doi: 10.1016/j.joen.2005.09.014

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 22 May 2019
doi: 10.3389/fphys.2019.00632

Efficacy of an All-Natural Polyherbal Mouthwash in Patients With Periodontitis: A Single-Blind Randomized Controlled Trial

Scilla Sparabombe[1†], Riccardo Monterubbianesi[1†], Vincenzo Tosco[1], Giulia Orilisi[1], Andrell Hosein[2], Luigi Ferrante[3], Angelo Putignano[1] and Giovanna Orsini[1]*

[1] Department of Clinical Sciences and Stomatology, Marche Polytechnic University, Ancona, Italy, [2] Department of Clinical and Molecular Sciences, Marche Polytechnic University, Ancona, Italy, [3] Department of Biomedical Sciences and Public Health, Marche Polytechnic University, Ancona, Italy

OPEN ACCESS

Edited by:
Lorenzo Lo Muzio,
University of Foggia, Italy

Reviewed by:
Victor E. Arana-Chavez,
University of São Paulo, Brazil
Franz E. Weber,
University of Zurich, Switzerland

***Correspondence:**
Giovanna Orsini
giovorsini@yahoo.com;
g.orsini@univpm.it

[†] These authors have contributed equally to this work as first authors

Specialty section:
This article was submitted to Craniofacial Biology and Dental Research, a section of the journal Frontiers in Physiology

Received: 06 March 2019
Accepted: 06 May 2019
Published: 22 May 2019

Citation:
Sparabombe S, Monterubbianesi R, Tosco V, Orilisi G, Hosein A, Ferrante L, Putignano A and Orsini G (2019) Efficacy of an All-Natural Polyherbal Mouthwash in Patients With Periodontitis: A Single-Blind Randomized Controlled Trial. Front. Physiol. 10:632. doi: 10.3389/fphys.2019.00632

Aim: This study aimed to evaluate the anti-inflammatory effect and the incidence of adverse effects of an all-natural polyherbal mouthwash in patients with periodontitis, after 3 months of use. These aims were accomplished by using full mouth bleeding score (FMBS), full mouth plaque score (FMPS), probing depth (PD) clinical attachment level (CAL) and a questionnaire recording any adverse events.

Methods: The present randomized controlled clinical study considered 40 patients with moderate or severe periodontitis, randomized in two groups: a test group (TG) and a control group (CG). TG was instructed to use a polyherbal mouthwash composed of *Propolis* resin extract, *Plantago lanceolata*, *Salvia officinalis* leaves extract, and 1.75% of essential oils and the CG was given a placebo mouthwash. Both groups were instructed to rinse for 2 min, twice daily after their routine oral home care with the different mouthwashes. Clinical measurements of FMBS, FMPS, PD and CAL were recorded at baseline (T0) and after 3 months (T1). The incidence of adverse outcomes was recorded at every follow-up. Mann–Whitney U test and Wilcoxon signed-rank test were used for the statistical analysis ($p < 0.05$).

Results: The final study sample consisted of 34 healthy individuals, 17 individuals in each of the two groups. TG and CG showed a statistically significant reduction in FMBS ($p = 0.001$ TG; $p = 0.002$ CG), FMPS ($p = 0.001$ TG; $p = 0.003$ CG), PD ($p = 0.001$ TG; $p = 0.011$ CG) and CAL ($p < 0.001$ TG; $p = 0.020$ CG) values from baseline to 3 months. The TG showed a statistically significant decrease in FMBS and FMPS compared with the CG. No adverse events or side effects were reported or observed in both groups.

Conclusion: The use of polyherbal mouthwash in patients with moderate or severe periodontitis has proved safe and effective in reducing bleeding score and plaque accumulation, after 3 months, compared with placebo, although no difference between the two groups were reported on PD and CAL (both improving at T1).

Keywords: mouthwash, all-natural polyherbal, periodontitis, full mouth bleeding score, full mouth plaque score, probing depth, clinical attachment level, randomized controlled trial

INTRODUCTION

Mechanical plaque control has an essential role in the prevention of periodontal disease, however, it is not sufficiently effective alone (Page and Eke, 2007; Sälzer et al., 2015; Tonetti et al., 2015). A meta-analysis provided strong evidence in favor of the use of antimicrobial agents as adjuncts to mechanical plaque control (Gunsolley, 2006). The author highlighted two types of mouthwashes with consistent antiplaque and anti-gingivitis effects: chlorhexidine gluconate and mouthwash containing essential oils, such as menthol (0.042%), thymol (0.064%), methyl salicylate (0.060%) and eucalyptol (0.092%), which are commercially available. Despite the potent bactericidal action of chlorhexidine, there have been many reported side effects, mainly when used for long periods, such as taste alteration, supragingival calculus formation, extrinsic tooth staining and desquamation of oral mucosa (Lang and Brecx, 1986; Eick et al., 2011). Furthermore, chlorhexidine has been shown to induce cytotoxic and genotoxic effects in cells (Liu et al., 2018).

The increasingly widespread use of the mouthwashes for long periods has led research into the direction of finding effective and safe products, with a greater focus on herbal drugs. Plant extracts can be used as an alternative to chlorhexidine digluconate as their polyphenols compounds exhibit antimicrobial effects. A recent systematic review and meta-analysis, out of a total of 9 articles, investigated the efficacy of daily rinsing with a green tea-based herbal mouthwash in terms of plaque index (PI) and/or gingival index (Mathur et al., 2018). They demonstrated that the herbal mouthwash was not significantly different compared to the standard chlorhexidine-based mouthwashes in reducing plaque and gingival inflammation. Three main groups of plant polyphenols (stilbenes, flavonoids, and proanthocyanidins) were found to exhibit activity against caries, periodontitis and candidiasis in pre-clinical studies, however, there was a lack of strong evidence, regarding randomized clinical trials (Varoni et al., 2012).

Another recently published meta-analyses and meta-regression selected 16 studies comparing a notorious mouthwash containing plant-derived essential oils to placebo solution, cetylpyridinium chloride (CPC) and flossing in the proximal area (Haas et al., 2016). The authors concluded that, in patients with gingivitis, a notorious mouthwash was more efficacious for the reduction of plaque and gingival inflammation than mechanical plaque control either alone or in combination with CPC mouthwash. Due to the proven effectiveness of plant extracts against periodontal disease, there are many commercially available mouthwashes which include one or more active ingredients derived from plants. However, most of them also include additional contents that are artificial and chemically synthesized in the laboratory, being therefore not all-natural (Springhouse, 2003). Indeed, some studies report that they are not entirely innocuous (Hammer and Heel, 2012; Azzimonti et al., 2015).

A recently made commercially available mouthwash includes, in addition to essential oils, a combination of other natural products such as *Propolis* resin extract, *Plantago lanceolata*, *Salvia officinalis* leaves extract. It contains no artificial or chemically synthesized ingredients and is therefore entirely natural. There exists no randomized controlled trial that supports the safety and effectiveness of its specific formulation.

The aim of this study was to evaluate the safety and anti-inflammatory effect of the latter all-natural, commercially available polyherbal mouthwash in patients with periodontitis, by comparing it with a placebo mouthwash, after 3 months of use. This was accomplished by measuring the differences, between the two groups, in the following clinical outcomes: full mouth bleeding score (FMBS), full mouth plaque score (FMPS), probing depth (PD) level $\geq$ 5 mm and clinical attachment level (CAL) $\geq$ 4 mm (O'Leary, 1967; Ainamo and Bay, 1975). The incidence of adverse outcomes (the probability of the occurrence of side effects must be the same in the two groups) was also recorded using a questionnaire.

MATERIALS AND METHODS

Forty (40) volunteers, age range 20–65 years, were recruited for the current single-blind randomized placebo-controlled clinical trial. This study was performed in the Outpatient Department of Clinical Sciences and Stomatology of the Polytechnic University of Marche, Ancona, (Italy) between February and June 2017. This study was carried out in accordance with the recommendations of the Ethics Committee of the Azienda Ospedaliero-Universitaria Ospedali Riuniti, Ancona (Protocol N. 2017-0087 UN) and was registered in the Australian New Zealand Clinical Trials Registry (number of trial: ACTRN12618001192279). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Study Population

After taking a detailed medical history and an initial clinical and radiologic examination, healthy individuals with a minimum of 20 teeth, smoker and no smoker, were selected. Clinical parameters for inclusion were: diagnosis of severe (at least 2 interproximal sites with CAL $\geq$ 6 mm and 1 interproximal site with PD $\geq$ 5 mm) or moderate (at least 2 interproximal sites with CAL $\geq$ 4 mm or 2 interproximal sites with PD $\geq$ 5 mm) periodontitis according to the Page and Eke classification (Page and Eke, 2007).

Exclusion criteria included: the use of antibiotics and anti-inflammatory drugs in previous 6 months; individuals with orthodontic or prosthetic appliances that could interfere with evaluation; individuals with an allergy to any ingredients used in the study; pregnant or lactating females; motor skills disorder.

All eligible volunteers were given oral and written information about the products and the purpose of the study and were asked to sign an informed consent.

Study Design

This clinical study was designed as a randomized, 3-month placebo-controlled, single-blind clinical trial. A single examiner (SS), with more than 8 years of practice, collected the following clinical parameters: bleeding index, using FMBS and PI, using FMPS, on 6 surfaces; PD obtained by counting the number of

pockets equal or greater than 5 mm; CAL recording the values equal to or greater than 4 mm, on 6 surfaces.

The subjects were randomly divided into two groups, through a computer-generated random table (Orsini et al., 2013). A CONSORT-type diagram explaining the design of this study is presented in **Figure 1**. At baseline (T0), after the clinical parameters assessment, all the participants received a thorough scaling and polishing to remove all plaque, stains and calculus, using ultrasonic scalers and hand instruments. For 3 months, a polyherbal mouthwash was prescribed to test group (TG) and a placebo mouthwash to the control group (CG). The polyherbal mouthwash (Pural Colluttorio, Fitomedical snc, Binasco, Milan, Italy) containing *Propolis* resin extract (1:3), *Plantago lanceolata* leaves extract (1:10), *Salvia officinalis* leaves extract (1:1) and 1.75% of essential oils from *Salvia officinalis*, *Syzygium aromaticum* buds, *Mentha piperita* leaves, *Commiphora myrrha* oleoresin and *Pistacia lentiscus* oleoresin, was made indistinguishable by the label's absence in 100 ml opaque brown bottles marked only with the patient's number. The placebo mouthwash was prepared with the following ingredients: 2 ml of glycerin (sweetening agent), cinnamon and vanilla flavoring agents, and brown food coloring, dissolved in 1 liter of distilled water and placed in 100 ml opaque brown bottles marked only with patient's number.

All participants were instructed to perform their routine oral home care (brushing, flossing or interproximal cleaning) twice daily and, immediately after, to rinse with 1 ml mouthwash diluted in a measuring cup (15 ml) of water for 2 min. All patients were instructed to not rinse/eat anything for 30 min after mouthwash use.

Participants were followed up every 20 days and were asked to bring the empty bottles of the tested mouthwash. Each participant was asked to complete a written questionnaire to assess for any adverse effects related to use of mouthwash at day 20, 40, 60, 80 and 3 months, such as a change in taste sensations, soreness and redness of oral mucosa/tongue/gingiva, feeling of dryness, burning or discoloration. After 3 months (T1), the FMBS, FMPS, PD and CAL parameters were re-collected.

Statistical Analyses

The differences of the single clinical parameters (FMBS, FMPS, PD level $\geq$ 5 mm, CAL $\geq$ 4 mm) were evaluated (Cosyn et al., 2013). For the same clinical score, the difference between TG and CG was carried out using the Mann–Whitney U test both at T0 and T1 ($p < 0.05$). Within each group, the differences in all clinical scores between T0 and T1 were evaluated using the Wilcoxon signed-rank test ($p < 0.05$). The difference in the rate of adverse events between the two groups were evaluated using the Fisher exact test for each time period.

RESULTS

Forty subjects were randomized based on the inclusion and exclusion criteria. There were six dropouts: one due to health reasons, four due to personal reasons and one excluded

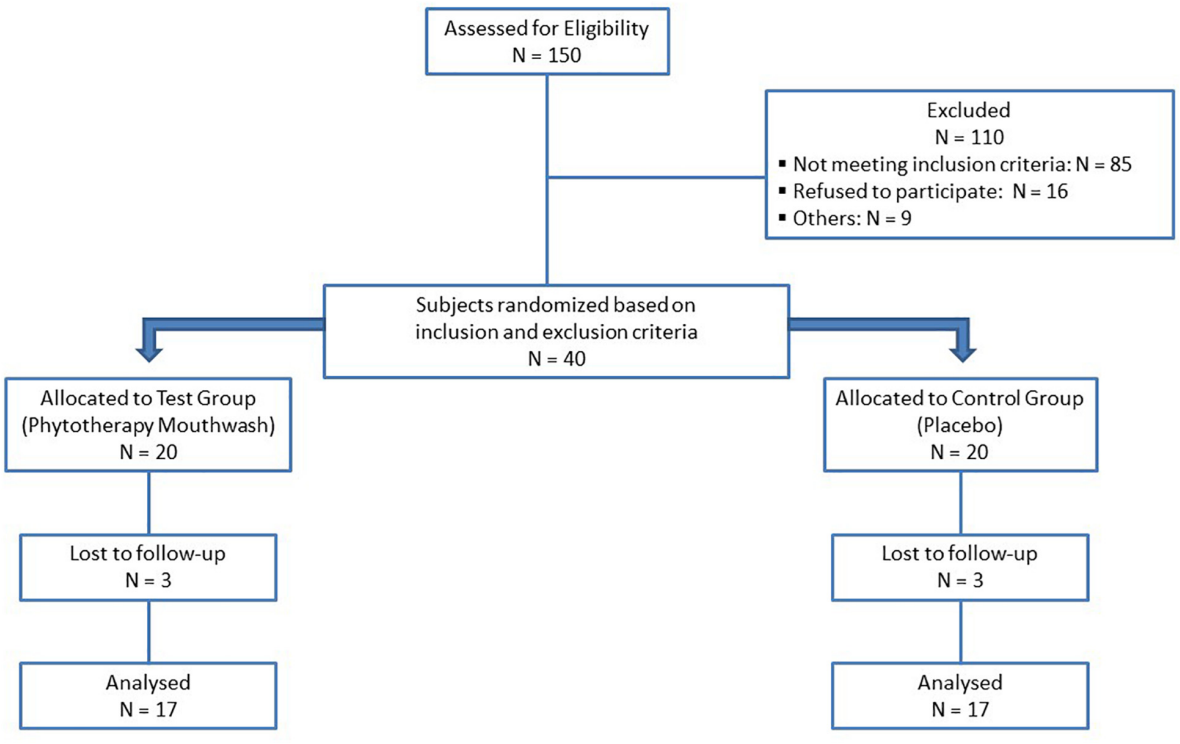


FIGURE 1 | CONSORT flow chart. N: Number of subjects.

because subsequently submitted to a drug therapy. In total, 34 subjects completed the trial (17 each group, see **Figure 1**). **Table 1** presents demographic data of the study population. There were no significant differences in mean age, sex and smoke of individuals between the two groups. At T0, there were no statistical differences in FMBS between the CG and TG; both groups showed a significant reduction in FMBS from T0 to T1. At T1, the TG group showed a statistically significant decrease in FMBS ($p = 0.002$) compared with the CG group (**Table 2**).

At T0, the two groups showed no significant differences in mean of FMPS values, full-mouth PD and CAL. At T1, the TG group showed a significant decrease in FMPS ($p = 0.005$) compared with the CG group. Moreover, there were no significant differences in mean of full-mouth PD and CAL between the two groups at T1: both groups showed a significant reduction in PD ($p = 0.001$ TG; $p = 0.011$ CG) and CAL ($p < 0.001$ TG; $p = 0.020$ CG) values from baseline to 3 months (**Table 2**). There were no subjective complaints, adverse outcomes or side effects reported or observed in both groups at day 20, 40, 60, 80 and 3 months (**Table 3**).

TABLE 3 | Side effects evaluation at each time period (day 20,40,60,80, 3 months) in the test (using polyherbal all-natural mouthwash) and Placebo groups.

SIDE effects		Test group ($n = 17$)	Control group ($n = 17$)
Soft tissue	Redness oral mucosa/tongue/gingiva	0	0
	Soreness oral mucosa/tongue/gingiva	0	0
	Mouth burning	0	0
	Desquamation	0	0
	Oral dryness	0	0
Hard tissue	Brown spots	0	0
Other	Taste perturbation (loss of taste)	0	0

DISCUSSION

The effectiveness of natural products and plant extracts is evidenced by several studies on its use in the treatment of oral diseases (Palombo, 2011; Škrovánková et al., 2012; Chinsembu, 2016). We conducted the first-ever randomized, controlled trial to investigate the effectiveness of a new combination of completely natural plant extracts (*Propolis* resin extract, *Plantago lanceolata*, *Salvia officinalis* leaves extract, and 1.75% of essential oils) in a single mouthwash available on the market, by comparing it with a placebo mouthwash.

The effectiveness of other herbal mouthwashes compared to a placebo mouthwash in patients with gingival or periodontal disease has been shown. Despite this, the topic is still controversial, due to some studies not using standardized methodology or having very short follow-ups. The effectiveness of three herbal extracts (*Juniperus communis*, *Urtica dioica*, *Achillea millefolium* on plaque and gingivitis) was studied by comparing it to a placebo mouthwash for 3 months, finding no beneficial effects (Weijden et al., 1998). Another study which investigated bacterial enumeration, plaque accumulation and gingival bleeding of a commercial herbal mouthwash containing *Salvadora persica* extract versus placebo over 3 weeks, found a statistically significant decrease in the PI of test subjects; a significant reduction in gingival bleeding was observed in both test and placebo groups (Khalessi et al., 2004).

Formulated in 2001, based on the traditional and historical uses of its functional ingredients and their components,

TABLE 1 | Basic demographic characteristics of study population.

Parameter	Test ($n = 17$)	Control ($n = 17$)
Mean age ± SD (years)	51,05 ± 7,7	51,29 ± 10,2
Age range (years)	30 to 62	30 to 62
Males/females	8/9	8/9
Smokers/no smokers	6/11	6/11

TABLE 2 | Values (mean ± SD) for Plaque and bleeding index and PD and CAL at Baseline and at 3 months.

Parameter	Interval	Test	Control	*P value*
FMBS	T0	12.23 ± 9.49	9.19 ± 6.23	*0.438*
	T1	1.63 ± 1.74	3.85 ± 2.68	*0.002*
	P value	*<0.001*	*0.002*	
FMPS	T0	44.70 ± 6.36	44.21 ± 16.04	*0.138*
	T1	17.64 ± 5.59	29.70 ± 14.38	*0.005*
	P value	*<0.001*	*0.003*	
PD ≥ 5 mm	T0	8.23 ± 14,42	2.36 ± 3.21	*0.068*
	T1	1.12 ± 1.79	1.37 ± 2.90	*0.820*
	P value	*0.001*	*0.011*	
CAL ≥ 4 mm	T0	43.25 ± 19.65	33.40 ± 16.28	*0.174*
	T1	29.17 ± 17.66	27.30 ± 17.39	*0.708*
	P value	*<0.001*	*0.020*	

Statistically significant at P < 0.05. FMBS, full mouth bleeding score; FMPS, full mouth plaque score; PD, probing depth; CAL, clinical attachment level.

the tested polyherbal mouthwash is all-natural. Numerous studies have highlighted essential oils antimicrobial effects even against multi-resistant bacteria (Mayaud et al., 2008; Chandra Shekar et al., 2015; Chinsembu, 2016). Because of their complex chemical composition, which includes more than 100 different therapeutic compounds, essential oils have a broad biological and antimicrobial activity spectrum: antibacterial, antifungal, and antiviral (Burt, 2004). *Plantago* species have considerable antiviral, anti-inflammatory, and antioxidant activities (Gálvez et al., 2005). *Plantago lanceolata* phenolic compounds seem to play a potential role in the control of bacterial growth and pathogenic oral flora virulence (Smullen et al., 2007). The antimicrobial effect of *sage* extract has been shown experimentally and clinically (Bozin et al., 2007; Geuenich et al., 2008). *Pistacia lentiscus* is an evergreen plant of the *Anacardiaceae* family, commonly found in the Mediterranean region; its fruits, galls, resin, and leaves have a long tradition in folk medicine. Recently, *Pistacia lentiscus* was found to exhibit an anti-inflammatory activity by reduction of inflammatory mediator production and inhibition of leukocyte recruitment to the inflammatory site (Ben Khedir et al., 2016). It also exhibited an antioxidant effect as a source of antioxidant molecules and indirectly as a stimulator of the activity and the expression of an antioxidant enzyme (Ben Khedir et al., 2016). *Propolis* is a well-known resinous material collected by bees; more than 300 components have been identified in *Propolis*, revealing that its composition is dependent upon the plant source and local flora. Several researchers have reported its antibacterial, antiviral, antitumor, anti-inflammatory property and immunomodulatory action (Sforcin, 2016; Oryan et al., 2018).

There is therefore strong beneficial evidence for these individual plant extracts in the tested mouthwash; however, no evidence exists for the combination of these plant extracts, formulated as a polyherbal mouthwash. This may be due to the quality of the substances and its delicate production process. Essential oils are unstable and fragile volatile compounds; they could be easily degraded by either oxidation, volatilization, heat or light if they are not protected from external factors, especially during the collection, storage and processing phase (Asbahani et al., 2015). *In vivo* studies and clinical trials on other herbal mouthwash have been performed (Khalessi et al., 2004; Haffajee et al., 2006), however, the need for clinical trials about the safety and efficacy of the combination of these herbal extracts in the tested mouth has been highlighted (Pan et al., 2013; Freires and Rosalen, 2016).

In the present study, the results are very encouraging; in both groups there was a significant reduction of inflammation from T0 to T1, confirming what is well described in the literature: mechanical therapy (scaling and polishing) improves clinical conditions by lowering the microbial load either by physical removal of plaque and by radical alteration of the subgingival habitat (Khalessi et al., 2004; Haffajee et al., 2006). However, in this study, 90 days after mechanical therapy, the TG had a greater reduction also in inflammation (FMBS) compared to the CG ($p = 0.002$).

A recent study investigated whether the adjunctive use of a mouthwash containing three natural essential oils (*Cymbopogon flexuosus* oil, *Thymus Zygis* oil, *Rosmarinus officinalis*) following sub-gingival debridement and scaling and root planning (SRP) could improve the clinical results regarding changes of the clinical parameters of PD, CAL and bleeding on probing (BoP) (Azad et al., 2016). Significant improvements of AL, PD, and BoP occurred in both groups after 3 months ($p < 0.001$); PD and BoP being significantly lower in the TG. In accordance with Azad study, our trial shows a significant improvement of PD ($P = 0.001$) and CAL ($P < 0.001$) in both groups, which can be explained because each subject in both groups had a preliminary scale and polishing at T0. Contrarily, no difference in PD and CAL between the two groups was revealed. This finding may be explained by the fact that PD reduction and gain in CAL are the expected result of mechanical instrumentation alone (Claffey et al., 1988; Haffajee et al., 1997). Therefore, mouthwashes do not seem crucial for these latter clinical outcomes. In support of our findings, another 3-month double-blind, randomized placebo-controlled trial studying clinical and microbial effects of an essential oil mouthwash in periodontal patients did not demonstrate any significant differences in full-mouth PD and full-mouth CAL between the test and placebo group (Cosyn et al., 2013). Indeed, plaque and bleeding indexes showed a significant reduction over time ($P = 0.029$); but, contrary to our data, there were no statically significant differences between the two groups (Cosyn et al., 2013).

The present single blind randomized controlled study shows a significant PI reduction in the TG and CG group. The considerable improvement of plaque control in both groups could be attributed to the Hawthorne effect; the subjects modified their oral hygiene routine due to the awareness of being observed (McCarney et al., 2007). However, FMPS has a significant difference between the two groups ($P = 0.005$), being significantly decreased in the TG. Our results are in agreement with other studies which investigated the anti-plaque and antigingivitis effects of a commercially available mouthwash containing essential oils with proven efficacy in individuals with and without periodontal disease (Sharma et al., 2004; Cortelli et al., 2009). However, although some herbal mouthwash showed side effects, mainly a mild mouth burning sensation (Manipal et al., 2016), the tested combination of all-natural polyherbal mouthwash has not demonstrated any discomfort or adverse events (**Table 3**). This study highlights the superior efficacy of the tested polyherbal mouthwash, over an essential oil mouthwash, as an agent without side effects, having considerable properties in controlling oral inflammation and microbiota. At this regard, a very recent comparative *in vitro* study demonstrated that chlorhexidine-based mouthwashes are still the most effective in regulating microbial homeostasis (Ardizzoni et al., 2018). However, chlorhexidine has several side effects that must be pondered when prescribing mouthwashes containing this molecule. Indeed, to the best of our knowledge, the need to develop alternative, innocuous but effective solutions are highly motivated. Therefore, despite the limitation that the polyherbal mouthwash was not compared to the "gold standard"

chlorhexidine mouthwash and was only compared to a placebo, its all-natural ingredients have to be kept in great attention, since the preliminary evidence of effectiveness and safety is very promising.

CONCLUSION

Common oral diseases such as gingivitis and periodontitis are based on microorganisms; herbal mouthwashes have an integral role in controlling the oral microbiota, and therefore are a useful adjunct in preventing periodontal disease. The use of the polyherbal mouthwash for 3 months significantly reduces inflammation (FMBS) and plaque accumulation (FMPS), showing a beneficial effect in patients with moderate or severe periodontitis. The most important finding is that, in bleeding and plaque score, participants using the all-natural polyherbal mouthwash showed significant improvement compared with the control. Noteworthy is that no adverse events or side effects were reported or observed in both groups at each time period. Further studies, in the near future, will be planned to compare it with the chlorhexidine mouthwash or other natural mouthwashes with proven efficacy.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the dataset is not publicly available as it is the property of the Polytechnic University of Marche prior to its publication. Requests to access the datasets should be directed to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee of the Azienda Ospedaliero-Universitaria Ospedali Riuniti, Ancona (Protocol N. 2017-0087 UN) and was registered in the Australian New Zealand Clinical Trials Registry (number of trial: ACTRN12618001192279). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SS and RM contributed to collection of data, conception and design, data analysis and interpretation, manuscript writing, critical reading and editing of the manuscript, and final revision and approval of the manuscript. VT and GuO assembled the data, analyzed and interpreted the data, wrote the manuscript, and approved the final version of the manuscript. GoO and AH interpreted the data, wrote and edited the manuscript, and approved the final version of the manuscript. LF, AP, and GoO contributed to conception and design, statistical analysis and interpretation, manuscript writing, and final approval and revision of the manuscript.

FUNDING

This work was supported by funds of the Polytechnic University of Marche (GoO and AP). The materials for the study were kindly donated by Fitomedical snc, Binasco, Milan, Italy.

REFERENCES

Ainamo, J., and Bay, I. (1975). Problems and proposals for recording gingivitis and plaque. *Int. Dent. J.* 25, 229–235.

Ardizzoni, A., Pericolini, E., Paulone, S., Orsi, C. F., Castagnoli, A., Oliva, I., et al. (2018). In vitro effects of commercial mouthwashes on several virulence traits of Candida albicans, viridans streptococci and Enterococcus faecalis colonizing the oral cavity. *PLoS One* 13:e0207262. doi: 10.1371/journal.pone.0207262

Asbahani, A. E., Miladi, K., Badri, W., Sala, M., Addi, E. H. A., Casabianca, H., et al. (2015). Essential oils: from extraction to encapsulation. *Int. J. Pharm.* 483, 220–243. doi: 10.1016/j.ijpharm.2014.12.069

Azad, M. F., Schwiertz, A., and Jentsch, H. F. R. (2016). Adjunctive use of essential oils following scaling and root planing –a randomized clinical trial. *BMC Complement. Altern. Med.* 16:171. doi: 10.1186/s12906-016-1117-x

Azzimonti, B., Cochis, A., Beyrouthy, M., Iriti, M., Uberti, F., Sorrentino, R., et al. (2015). Essential Oil from berries of lebanese juniperus excelsa M. Bieb displays similar antibacterial activity to chlorhexidine but higher cytocompatibility with human oral primary cells. *Molecules* 20, 9344–9357. doi: 10.3390/molecules20059344

Ben Khedir, S., Mzid, M., Bardaa, S., Moalla, D., Sahnoun, Z., and Rebai, T. (2016). In Vivo evaluation of the anti-inflammatory effect of *Pistacia lentiscus* fruit oil and its effects on oxidative stress. *Evid. Based Complement. Alternat. Med.* 2016, 1–12. doi: 10.1155/2016/6108203

Bozin, B., Mimica-Dukic, N., Samojlik, I., and Jovin, E. (2007). Antimicrobial and antioxidant properties of rosemary and sage (Rosmarinus officinalis L. and *Salvia officinalis L., Lamiaceae)* essential oils. *J. Agric. Food Chem.* 55, 7879–7885. doi: 10.1021/jf0715323

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* 94, 223–253. doi: 10.1016/j.ijfoodmicro.2004.03.022

Chandra Shekar, B. R., Nagarajappa, R., Suma, S., and Thakur, R. (2015). Herbal extracts in oral health care - a review of the current scenario and its future needs. *Pharmacogn. Rev.* 9, 87–92. doi: 10.4103/0973-7847.162101

Chinsembu, K. C. (2016). Plants and other natural products used in the management of oral infections and improvement of oral health. *Acta Trop.* 154, 6–18. doi: 10.1016/j.actatropica.2015.10.019

Claffey, N., Loos, B., Gantes, B., Martin, M., Heins, P., and Egelberg, J. (1988). The relative effects of therapy and periodontal disease on loss of probing attachment after root debridement. *J. Clin. Periodontol.* 15, 163–169. doi: 10.1111/j.1600-051x.1988.tb01563.x

Cortelli, S. C., Cortelli, J. R., Holzhausen, M., Franco, G. C. N., Rebelo, R. Z., Sonagere, A. S., et al. (2009). Essential oils in one-stage full-mouth disinfection: double-blind, randomized clinical trial of long-term clinical, microbial and salivary effects. *J. Clin. Periodontol.* 36, 333–342. doi: 10.1111/j.1600-051X.2009.01376.x

Cosyn, J., Princen, K., Miremadi, R., Decat, E., Vaneechoutte, M., and De Bruyn, H. (2013). A double-blind randomized placebo-controlled study on the clinical and microbial effects of an essential oil mouth rinse used by patients in supportive periodontal care. *Int. J. Dent. Hyg.* 11, 53–61. doi: 10.1111/idh.12000

Eick, S., Goltz, S., Nietzsche, S., Jentsch, H., and Pfister, W. (2011). Efficacy of chlorhexidine digluconate-containing formulations and other mouthrinses against periodontopathogenic microorganisms. *Quintessence Int.* 1985, 687–700.

Freires, I. A., and Rosalen, P. L. (2016). How natural product research has contributed to oral care product development? a critical view. *Pharm. Res.* 33, 1311–1317. doi: 10.1007/s11095-016-1905-5

Gálvez, M., Martín-Cordero, C., Houghton, P. J., and Ayuso, M. J. (2005). Antioxidant activity of Plantago bellardii All. *Phytother. Res.* 19, 1074–1076. doi: 10.1002/ptr.1797

Geuenich, S., Goffinet, C., Venzke, S., Nolkemper, S., Baumann, I., Plinkert, P., et al. (2008). Aqueous extracts from peppermint, sage and lemon balm leaves display potent anti-HIV-1 activity by increasing the virion density. *Retrovirology* 5:27. doi: 10.1186/1742-4690-5-27

Gunsolley, J. C. (2006). A meta-analysis of six-month studies of antiplaque and antigingivitis agents. *J. Am. Dent. Assoc.* 1939, 1649–1657. doi: 10.14219/jada.archive.2006.0110

Haas, A. N., Wagner, T. P., Muniz, F. W., Fiorini, T., Cavagni, J., and Celeste, R. K. (2016). Essential oils-containing mouthwashes for gingivitis and plaque: meta-analyses and meta-regression. *J. Dent.* 55, 7–15. doi: 10.1016/j.jdent.2016.09.001

Haffajee, A. D., Cugini, M. A., Dibart, S., Smith, C., Kent, R. L., and Socransky, S. S. (1997). The effect of SRP on the clinical and microbiological parameters of periodontal diseases. *J. Clin. Periodontol.* 24, 324–334. doi: 10.1111/j.1600-051x.1997.tb00765.x

Haffajee, A. D., Teles, R. P., and Socransky, S. S. (2006). The effect of periodontal therapy on the composition of the subgingival microbiota. *Periodontol.* 2000, 219–258. doi: 10.1111/j.1600-0757.2006.00191.x

Hammer, K. A., and Heel, K. A. (2012). Use of multiparameter flow cytometry to determine the effects of monoterpenoids and phenylpropanoids on membrane polarity and permeability in staphylococci and enterococci. *Int. J. Antimicrob. Agents* 40, 239–245. doi: 10.1016/j.ijantimicag.2012.05.015

Khalessi, A. M., Pack, A. R. C., Thomson, W. M., and Tompkins, G. R. (2004). An in vivo study of the plaque control efficacy of PersicaTM: a commercially available herbal mouthwash containing extracts of Salvadora persica. *Int. Dent. J.* 54, 279–283. doi: 10.1111/j.1875-595X.2004.tb00294.x

Lang, N., and Brecx, M. C. (1986). Chlorhexidine digluconate–an agent for chemical plaque control and prevention of gingival inflammation. *J. Periodontal Res.* 21, 74–89. doi: 10.1111/j.1600-0765.1986.tb01517.x

Liu, J. X., Werner, J., Kirsch, T., Zuckerman, J. D., and Virk, M. S. (2018). Cytotoxicity evaluation of chlorhexidine gluconate on human fibroblasts, myoblasts, and osteoblasts. *J. Bone Jt. Infect.* 3, 165–172. doi: 10.7150/jbji.26355

Manipal, S., Hussain, S., Wadgave, U., Duraiswamy, P., and Ravi, K. (2016). The mouthwash war - chlorhexidine vs. Herbal mouth rinses: a meta-analysis. *J. Clin. Diagn. Res.* 10, ZC81–ZC83. doi: 10.7860/JCDR/2016/16578.7815

Mathur, A., Gopalakrishnan, D., Mehta, V., Rizwan, S., Shetiya, S., and Bagwe, S. (2018). Efficacy of green tea-based mouthwashes on dental plaque and gingival inflammation: a systematic review and meta-analysis. *Indian J. Dent. Res.* 29, 225–232. doi: 10.4103/ijdr.IJDR_493_17

Mayaud, L., Carricajo, A., Zhiri, A., and Aubert, G. (2008). Comparison of bacteriostatic and bactericidal activity of 13 essential oils against strains with varying sensitivity to antibiotics. *Lett. Appl. Microbiol.* 47, 167–173. doi: 10.1111/j.1472-765X.2008.02406.x

McCarney, R., Warner, J., Iliffe, S., van Haselen, R., Griffin, M., and Fisher, P. (2007). The hawthorne effect: a randomised, controlled trial. *BMC Med. Res. Methodol.* 7:30. doi: 10.1186/1471-2288-7-30

O'Leary, T. (1967). The periodontal screening examination. *J. Periodontol.* 38(Suppl.), 617–624. doi: 10.1902/jop.1967.38.6_part2.617

Orsini, G., Procaccini, M., Manzoli, L., Sparabombe, S., Tiriduzzi, P., Bambini, F., et al. (2013). A 3-day randomized clinical trial to investigate the desensitizing properties of three dentifrices. *J. Periodontol.* 84, e65–e73. doi: 10.1902/jop.2013.120697

Oryan, A., Alemzadeh, E., and Moshiri, A. (2018). Potential role of propolis in wound healing: biological properties and therapeutic activities. *Biomed. Pharmacother.* 98, 469–483. doi: 10.1016/j.biopha.2017.12.069

Page, R. C., and Eke, P. I. (2007). Case definitions for use in population-based surveillance of periodontitis. *J. Periodontol.* 78, 1387–1399. doi: 10.1902/jop.2007.060264

Palombo, E. A. (2011). Traditional medicinal plant extracts and natural products with activity against oral bacteria: potential application in the prevention and treatment of oral diseases. *Evid. Based Complement. Alternat. Med.* 2011, 1–15. doi: 10.1093/ecam/nep067

Pan, S.-Y., Zhou, S.-F., Gao, S.-H., Yu, Z.-L., Zhang, S.-F., Tang, M.-K., et al. (2013). New perspectives on how to discover drugs from herbal medicines: cam's outstanding contribution to modern therapeutics. *Evid. Based Complement. Altern. Med.* 2013:627375. doi: 10.1155/2013/627375

Sälzer, S., Slot, D. E., Van der Weijden, F. A., and Dörfer, C. E. (2015). Efficacy of inter-dental mechanical plaque control in managing gingivitis – a meta-review. *J. Clin. Periodontol.* 42, S92–S105. doi: 10.1111/jcpe.12363

Sforcin, J. M. (2016). Biological properties and therapeutic applications of propolis: properties and applications of propolis. *Phytother. Res.* 30, 894–905. doi: 10.1002/ptr.5605

Sharma, N., Charles, C. H., Lynch, M. C., Qaqish, J., Mcguire, J. A., Galustians, J. G., et al. (2004). Adjunctive benefit of an essential oil–containing mouthrinse in reducing plaque and gingivitis in patients who brush and floss regularly. *J. Am. Dent. Assoc.* 135, 496–504. doi: 10.14219/jada.archive.2004.0217

Škrovánková, S., Mišurcová, L., and Machù, L. (2012). Antioxidant activity and protecting health effects of common medicinal plants. *Adv. Food Nutr. Res.* 67, 75–139. doi: 10.1016/B978-0-12-394598-3.00003-4

Smullen, J., Koutsou, G. A., Foster, H. A., Zumbé, A., and Storey, D. M. (2007). The antibacterial activity of plant extracts containing polyphenols against *Streptococcus mutans*. *Caries Res.* 41, 342–349. doi: 10.1159/000104791

Springhouse (2003). *Nursing 2004 Herbal Medicine Handbook for pda, Online Deliverable: Powered by Skyscape inc. Place of Publication not Identified*. Philadelphia, PA: Wolters Kluwer Health.

Tonetti, M. S., Chapple, I. L. C., Jepsen, S., and Sanz, M. (2015). Primary and secondary prevention of periodontal and peri-implant diseases. *J. Clin. Periodontol.* 42, S1–S4. doi: 10.1111/jcpe.12382

Varoni, E. M., Lodi, G., Sardella, A., Carrassi, A., and Iriti, M. (2012). Plant polyphenols and oral health: old phytochemicals for new fields. *Curr. Med. Chem.* 19, 1706–1720. doi: 10.2174/092986712799945012

Weijden, G. A., Timmer, C. J., Timmerman, M. F., Reijerse, E., Mantel, M. S., and Velden, U. (1998). The effect of herbal extracts in an experimental mouthrinse on established plaque and gingivitis. *J. Clin. Periodontol.* 25, 399–403. doi: 10.1111/j.1600-051X.1998.tb02462.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

MINI REVIEW
published: 26 June 2019
doi: 10.3389/fcimb.2019.00232

Beyond Head and Neck Cancer: The Relationship Between Oral Microbiota and Tumour Development in Distant Organs

Marco Mascitti[1], Lucrezia Togni[1], Giuseppe Troiano[2], Vito Carlo Alberto Caponio[2], Davide Bartolomeo Gissi[3], Lucio Montebugnoli[3], Maurizio Procaccini[1,4], Lorenzo Lo Muzio[2] and Andrea Santarelli[1,4]*

[1] Department of Clinical Sciences and Stomatology, Marche Polytechnic University, Ancona, Italy, [2] Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy, [3] Department of Biomedical and Neuromuscular Sciences, University of Bologna, Bologna, Italy, [4] Dental Clinic, National Institute of Health and Science of Aging, IRCCS INRCA, Ancona, Italy

OPEN ACCESS

Edited by:
Frederic Antonio Carvalho, INSERM U1107 Douleur et Biophysique Neurosensorielle (Neuro-Dol), France

Reviewed by:
Nele Brusselaers, Karolinska Institute (KI), Sweden
Mohamed Helmy, University of Toronto, Canada

***Correspondence:**
Marco Mascitti
marcomascitti86@hotmail.it

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *15 April 2019*
Accepted: *14 June 2019*
Published: *26 June 2019*

Citation:
Mascitti M, Togni L, Troiano G, Caponio VCA, Gissi DB, Montebugnoli L, Procaccini M, Lo Muzio L and Santarelli A (2019) Beyond Head and Neck Cancer: The Relationship Between Oral Microbiota and Tumour Development in Distant Organs. Front. Cell. Infect. Microbiol. 9:232. doi: 10.3389/fcimb.2019.00232

An altered oral microbiota has been linked with the development of several oral diseases, such as dental caries, periodontal disease, and oral stomatitis. Moreover, poor oral health has been linked to head and neck cancer, particularly oral cancer. In recent years a growing number of studies indicate that oral microbiota could be involved in the development of primary tumours outside of head and neck region. The aim of this article is to review the recent studies based on high-throughput technology to present evidences of a relationship between oral microbiota and "non-head and neck tumours." Oral dysbiosis seem to be more pronounced in patients with tumours of gastrointestinal tract, in particular oesophageal, gastric, pancreatic, and colorectal cancers, paving the way for developing specific oral microbiota test to allow early cancer detection. Regarding other tumour types, the results are promising but highly preliminary and still debated. Currently, there are several factors that limit the generalization of the results, such as the small sample size, the lack of adequate clinical information about patients, the different sequencing techniques used, and biological sample heterogeneity. Although only at the beginning, the analysis of oral microbiota could be the next step in the evolution of cancer therapy and will help clinicians to develop individualised approaches to cancer prevention and treatment.

Keywords: oral microbiota, oral microbiome, pancreatic cancer, gastrointestinal tract cancer, high-throughput sequencing

INTRODUCTION

The term "oral microbiota" refers to the heterogeneous group of microbial species colonizing all the oral cavity surfaces (Le Bars et al., 2017). In particular, about 700 bacterial species have been identified in oral cavity and, more importantly, 35% of them have not been cultured (Farrell et al., 2012). Indeed, the main limitation of microbial culture is the inability to identify the actual diversity of oral microbiota. To determine bacterial ecosystem as a whole, high-throughput sequencing methods have been developed, representing a leap toward characterization of oral microbial community (Benn et al., 2018). Currently, the most common approach to analyse oral microbiota

is genome sequencing, through the analysis of 16S rDNA gene sequences. Indeed, the identification of this conserved DNA sequence has provided a universal system for the identification of bacteria. Recently, results from studies based on genome sequencing have started shedding light on the existence of site-specific microbial patterns that might be considered as "healthy/normal oral microbiota" (García-Castillo et al., 2016). The main obstacle to this definition is the highly variable composition of microbial community due to multiple factors, such as diet, age, and site (Arweiler and Netuschil, 2016). Nevertheless, ecological imbalance of microbial community, called dysbiosis, has been extensively studied both in human and animal models. It is characterised by: loss of beneficial microbes, expansion of pathogenic microbes, and general loss of microbial diversity (Meurman and Uittamo, 2008; Petersen and Round, 2014). It is becoming evident that the dysbiosis can lead to cancer development (Schwabe and Jobin, 2013). Three paradigms were proposed to describe the pathogenetic processes involving the microbiota in cancer development: trigger of chronic inflammation and immune responses that promote tumorigenesis and tumour growth; altered metabolic activity leading to increased production of toxic metabolites; abrogation of virus latency leading to malignancies (Plottel and Blaser, 2011).

An increasing number of studies have shown that oral microbiota plays a role in the development of oral diseases, such as dental caries, periodontal disease, and oral stomatitis (Santarelli et al., 2015; Zhang et al., 2018). Poor oral hygiene and periodontal disease have been linked to oral cancer, and increasing evidences suggest that oral microbiota have a role in oral cancer development (García-Castillo et al., 2016). More broadly, is becoming evident that oral dysbiosis is associated with head and neck cancer development. Head and neck tumours originate from several anatomical sites, each associated with its own microbiota. Therefore, is possible that the crosstalk between microbial populations, combined with known risk factors, could drive head and neck carcinogenesis (Hayes et al., 2018). Oral dysbiosis has been also involved in the pathogenesis of systemic diseases. Indeed, recent studies indicate that oral microbiota seems to be involved in the tumours of distant organs, in particular "non-head and neck tumours" (**Table 1**; Klimesova et al., 2018).

The aim of this article is to review the recent studies based on genome sequencing to present evidences of a relationship between oral microbiota and tumours of distant organs.

PANCREATIC CANCER

Pancreatic cancer (PC) is a malignant tumour with a 5-year survival rate of 2–9% and a median survival time of 6 months (Mcguigan et al., 2018). Although localised PCs have better survival rate, these patients show absence or non-specific clinical manifestations. The diagnostic delay leads to a fatal outcome, with 80% of PCs that are unresectable at the time of presentation (Vincent et al., 2011). Despite the study of thousands of blood biomarkers for early detection of PC, none of them have been validated for clinical use, suggesting a continuous change of proteins expression over the course of disease evolution (Yachida et al., 2010).

Several reports have demonstrated an association between pathogenic microorganisms and PC, suggesting a role as diagnostic biomarkers. Furthermore, is possible that bacterial dysbiosis could contribute to the formation and progression of PC (Wang and Li, 2015). In addition to the well-known risk factors for PC, several studies have reported a positive association between PC and oral health status. In particular, the relationship between periodontal disease and tooth loss and an increased risk of PC was found, suggesting that toxins exposure and increase of systemic inflammation could promote tumour development (Hujoel et al., 2003; Stolzenberg-Solomon et al., 2003; Michaud et al., 2007). These findings led to the first studies on oral microbiota describing the possible link between oral dysbiosis and non-oropharyngeal cancers (**Tables 1**, **2**).

In 2012 was conducted the first microbiota profiling of salivary samples of patients affected by PC (Farrell et al., 2012). A marked reduction of *N. elongata* and *S. mitis* and a significant increase of *G. adiacens*, suggesting a link between variations in oral microbiota and PC. In another study, saliva from 108 subjects was evaluated to determine microbiota profiles (Torres et al., 2015). Although the overall microbiota diversity of the groups was similar, a significative higher ratio of *Leptotrichia* to *Porphyromonas* (LP ratio) was found in PC patients. Despite the small sample size, salivary LP ratio was suggested as a simple biomarker for PC. Moreover, this study confirmed previous results showing that patients with high levels of plasma antibodies to *P. gingivalis* were at higher risk of PC (Ahn et al., 2012b; Michaud et al., 2013). Oral microbiota of patients with intraductal papillary mucinous neoplasms (IPMN) and pancreatic ductal adenocarcinoma (PDAC) was recently studied (Olson et al., 2017). The results found similar overall microbiota profile in saliva of PDAC and IPMN patients, but the mean relative proportion of taxa among IPMN patients was between that for PDAC patients and the controls. Recently, Fan et al. conducted the first prospective case-control study with the aim to evaluate microbiota profile in mouthwash samples taken from PC patients and healthy controls (Fan et al., 2018). Although no significant differences in overall microbiota composition were found, *P. gingivalis* and *A. actinomycetemcomitans* were associated with a higher risk of PC, while *Fusobacteria* and *Leptotrichia* were associated with decreased tumour risk.

UPPER GASTROINTESTINAL TRACT CANCER

Upper gastrointestinal cancers are characterised by non-specific clinical manifestations, absence of effective treatments, and changing epidemiological trends. Oesophageal cancer is the sixth most common cause of cancer death, with a 5-year survival rate of 15–25%. Histologically, the two main types are oesophageal squamous cell carcinoma (SCC) and adenocarcinoma (AC) (Domper Arnal et al., 2015). Microbiota seems to play a role in oesophageal cancer aetiology, probably through the regulation of the barrier function, the modulation of inflammation, or

TABLE 1 | Published studies on the relationship between oral microbiota and tumours of distant organs, showing principal results.

References	Cancer	n° patients	Sequencing technique (instrument) [16S rDNA hypervariable regions]	Sample	Validated results	Main findings
Chen et al., 2015	EC	87 SCC, 63 dysplasia, 85 controls	Pyrosequencing (Roche 454 FLX Titanium) [V3–V4]	Saliva	No	Overall decreased microbial diversity in cancer patients. Oral microbiota of cancer and healthy subjects clustered in opposite directions, while dysplasia patients were located between the 2 groups.
Peters et al., 2017	EC	81 AC, 25 SCC, 210 controls	Synthesis (Illumina MiSeq) [V4]	Oral rinse	No	Overall microbial diversity of AC and SCC patients did not differ significantly from matched controls. *T. forsythia* and *P. gingivalis* were more abundant in AC and SCC cases, respectively.
Snider et al., 2018	EC	32 Barrett esophagus, 17 control	Synthesis (Illumina HiSeq 2500) [V4]	Oral swab	Yes	A 3-taxon model (*Lautropia, Streptococcus*, and an unspecified genus of the order *Bacteroidales*) discriminated between Barrett and controls (AUC = 0.94). Cancer patients with Barrett showed increased levels of *Enterobacteriaceae*.
Hu et al., 2015	GC	34 GC, 17 controls	Synthesis (Illumina MiSeq) [V2–V4]	Tongue coat	No	Overall diversity of tongue coating microbiota was reduced in "thick tongue" phenotype. Thick tongue coating was associated with gastric cancer.
Sun et al., 2018	GC	37 GC, 13 controls	Synthesis (Illumina MiSeq) [V4]	Saliva, plaque	No	Overall increased microbial diversity in cancer patients. A scoring system based on 11 different genera was used to screen gastric cancer (sensitivity = 97%, false positivity rate = 7.7%).
Wu et al., 2018	GC	57 GC, 80 controls	Pyrosequencing (Roche 454 GS-FLX) [V4]	Tongue coat	No	6 bacterial clusters were identified to distinguish cancer patients from controls. Cancer patients showed less abundant bacterial clusters 1–2 and more abundant bacterial cluster 3–6 (cluster 6 had AUC = 0.76).
Farrell et al., 2012	PC	10 PC, 10 controls	Microarray assay (HOMIM array)	Saliva	Yes	2-biomarker model (*N. elongata* and *S. mitis*) distinguished cancer patients from healthy controls (AUC = 0.90). 2-biomarker model (*G. adiacens* and *S. mitis*) distinguished cancer patients from chronic pancreatitis (AUC = 0.68).
Torres et al., 2015	PC	8 PC, 78 other disease, 22 controls	Synthesis (Illumina MiSeq) [V4]	Saliva	No	Overall microbiota diversity of the three groups was very similar. Significative higher ratio of *Leptotrichia* to *Porphyromonas* was found in cancer patients.
Olson et al., 2017	PC	40 PDAC, 39 IPMN, 58 controls	Synthesis (Illumina MiSeq) [V4–V5]	Saliva	No	Overall microbiota in PDAC and IPMN patients was very similar. PDAC cases showed higher proportion of *Firmicutes* and related taxa.
Fan et al., 2018	PC	361 PC, 371 controls	Pyrosequencing (Roche 454 FLX Titanium) [V3–V4]	Oral rinse	No	*P. gingivalis* and *A. actinomycetemcomitans* were associated with a higher risk of cancer. *Fusobacteria* and *Leptotrichia* were associated with decreased risk of cancer.
Han et al., 2014	CRC	47 CRC, 45 controls	Synthesis (Illumina MiSeq) [V2–V4]	Tongue coat	No	Thick tongue coating was associated with cancer patients and microbiota imbalance on the tongue was related to the changes in tongue coating.
Kato et al., 2016	CRC	68 CRC, 122 controls	Synthesis (Illumina MiSeq) [V3–V4]	Oral rinse	No	Colorectal cancer was associated with increased presence of *Lactobacillus* and *Rothia*. The differences of overall microbiota between smokers and non-smokers were higher in cancer patients.
Russo et al., 2017	CRC	10 CRC, 10 controls	Synthesis (Illumina MiSeq) [V3–V4]	Saliva	No	There were no significant differences in salivary microbiota between cancer patients and controls.
Flemer et al., 2018	CRC	45 CRC, 21 polyps, 25 controls	Synthesis (Illumina MiSeq) [V3–V4]	Oral swab	No	Overall microbiota differs between cancer patients and controls. A specific oral and faecal microbiota test discriminated healthy controls from patients with cancer and polyps (AUC = 0.94 and 0.98, respectively).
Yang et al., 2018	CRC	231 CRC, 461 control	Synthesis (Illumina HiSeq) [V4]	Oral rinse	No	*T. denticola* and *P. intermedia* were associated with increased cancer risk. Furthermore, 11 common and 16 rare taxa were also associated with cancer risk.
Wang et al., 2014	ALL	13 ALL, 12 controls	Pyrosequencing (Roche 454 GS-FLX) [V1–V3]	Plaque	No	ALL patients had reduced overall microbial diversity. Moreover, "leukemia-associated" taxa were detected.

(Continued)

TABLE 1 | Continued

References	Cancer	*n*° patients	Sequencing technique (instrument) [16S rDNA hypervariable regions]	Sample	Validated results	Main findings
Yan et al., 2015	LC	10 SCC, 10 AC, 10 controls	Synthesis (Illumina HiSeq 2000) [V3–V6]	Saliva	Yes	Overall microbiota differs between cancer patients and healthy controls. 2-biomarker model (*Veillonella* and *Capnocytophaga*) distinguished SCC and AC from controls (AUC = 0.80 and 0.86, respectively).
Lu et al., 2016	HC	35 HC, 25 controls	Synthesis (Illumina MiSeq) [V3–V4]	Tongue coat	No	Increased overall microbial diversity in HC patients. 2 microbial biomarkers (*Oribacterium* and *Fusobacterium*) discriminated between HC patients and controls (AUC = 0.81 and 0.77, respectively).
Wang et al., 2017	BC	55 BC, 21 controls	Synthesis (Illumina MiSeq) [V3, V4]	Oral rinse	No	There were no significant differences in overall oral microbiota between cancer patients and controls.
Galloway-Peña et al., 2017	AML	59 AML	Synthesis (Illumina MiSeq) [V4]	Oral swab	No	AML patients showed high intra-patient temporal instability of oral microbiota diversity. There was a significant relationship between microbiota changes and risk of infection after induction chemotherapy.

EC, oesophageal cancer; SCC, squamous cell carcinoma; AC, adenocarcinoma; GC, gastric cancer; PC, Pancreatic cancer; CRC, colorectal cancer; ALL, Acute lymphoblastic leukaemia; LC, lung cancer; HC, hepatic cancer; BC, breast cancer; AML, Acute myeloid leukaemia.

the direct metabolism of carcinogens (Peters et al., 2017). Furthermore, periodontal disease seems to be associated with increased cancer risk, suggesting a role of oral microbiota (Michaud et al., 2007).

Salivary microbiota of oesophageal SCC, oesophageal dysplasia, and healthy subjects were compared, showing reduction of several genera in cancer patients (Chen et al., 2015). Overall oral microbiota of oesophageal SCC and controls clustered in opposite directions, while dysplasia patients were located between the 2 groups. Oral microbiota profiling was prospectively studied in oesophageal SCC and AC, using pre-diagnostic oral wash samples from cancer and healthy cohorts, but lack of significant diversity between cancer patients and matched controls was found (Peters et al., 2017). Nevertheless, two periodontal pathogens, *T. forsythia* and *P. gingivalis*, were more abundant in oesophageal AC and SCC cases, respectively. A recent work evaluated the feasibility of early detection of Barrett's oesophagus through the assessment of oral microbiota (Snider et al., 2018). A 3-taxon model including *Lautropia*, *Streptococcus*, and a genus of the order *Bacteroidales* distinguished Barrett's oesophagus from controls with high sensitivity (96.9%) and specificity (88.2%) (**Tables 1**, **2**).

Gastric cancer (GC) is the third most common cause of cancer death, with a 5-year survival rate of 30%. Interestingly, 5-year survival rate in Japan is 70% for early stages of GC, suggesting the feasibility of mass screening programs (Karimi et al., 2014). The well-established role of *H. pylori* in GC development has given rise to the hypothesis that oral microbial dysbiosis could promote GC development (Ahn et al., 2012a). Several epidemiological studies corroborated such hypothesis showing the association between periodontal disease and tooth loss and an increased risk of GC (Stolzenberg-Solomon et al., 2003; Michaud et al., 2017). These findings led to the search for oral microbial biomarkers that allow an early and non-invasive diagnosis of GC (**Table 1**).

The relationship between tongue coating appearance and tongue microbiota profiling was investigated in GC patients and healthy controls (Hu et al., 2015). Tongue coating was significantly ticker in GC patients and was associated with reduced overall microbial diversity. Another study explored the alterations of tongue coating microbiota in patients with newly diagnosed GC and healthy controls, demonstrating a significative lower ratio of *Bacteroidetes* to *Firmicutes* in GC, especially in non-cardia GC patients (Wu et al., 2018). A recent case-control study was conducted with the aim to investigate the microbial community of both salivary and subgingival plaque in GC patients (Sun et al., 2018). Although saliva and plaque showed two different microbiotas, the overall microbial diversity was significantly increased in GC patients, and a screening system based on 11 different genera demonstrated high sensitivity (97%) (**Table 2**).

LOWER GASTROINTESTINAL TRACT CANCER

Colorectal cancer (CRC) is one of the most diagnosed malignancy and the second most common cause of cancer

TABLE 2 | Comparison of microbial composition at the genus level in cancer patients.

Cancer	Increased microbes (genus)	Reduced microbes (genus)	References
EC	*Streptococcus*, *Veillonella*	*Neisseria*, *Rothia*, *Haemophilus*	Chen et al., 2015; Peters et al., 2017; Snider et al., 2018
GC	*Streptococcus*, *Veillonella*	*Neisseria*, *Rothia*, *Leptotrichia*	Sun et al., 2018; Wu et al., 2018
PC	*Streptococcus*	*Neisseria*, *Haemophilus*, *Leptotrichia*	Torres et al., 2015; Olson et al., 2017; Fan et al., 2018
CRC	*Rothia*, *Actinomyces*, *Lactobacillus*	*Streptococcus*, *Neisseria*, *Haemophilus*	Han et al., 2014; Kato et al., 2016; Flemer et al., 2018; Yang et al., 2018
ALL/AML	*Veillonella*, *Streptococcus*	*Leptotrichia*	Wang et al., 2014; Galloway-Peña et al., 2017
LC	*Veillonella*	*Neisseria*	Yan et al., 2015
HC	*Leptotrichia*	*Streptococcus*, *Haemophilus*	Lu et al., 2016

*Only main microbial species were reported (for further details, see **Supplementary Table 1**). Interestingly, expression levels of genus Streptococcus change significantly depending on tumour type. EC, oesophageal cancer; GC, gastric cancer; PC, Pancreatic cancer; CRC, colorectal cancer; ALL/AML, Acute lymphoblastic leukaemia/acute myeloid leukaemia; LC, lung cancer; HC, hepatic cancer.*

death (Marley and Nan, 2016). A possible association between gut dysbiosis and CRC have been suggested. Indeed, both the whole colon microbiota and specific pathogens seems to contribute to the formation and progression of CRC (Klimesova et al., 2018). Findings of colonization of CRC tissues by typical oral bacteria raised the hypothesis of a possible involvement of oral microbiota in CRC development. The possible link between oral microbiota and CRC has been suggested by the fact that each day 10^{11} oral bacteria reach colorectal mucosa, influencing local microbiota (Klimesova et al., 2018). Furthermore, several epidemiological investigations suggested an association between periodontal disease and CRC (Hu et al., 2018). Although the interactions between CRC and the oral-gut microbiota axis are poorly understood, several studies have been conducted to investigate the role of oral bacteria as diagnostic biomarkers for CRC (**Table 1**).

Han et al. evaluated the relationship between tongue coating appearance and microbiota profiling in patients with CRC (Han et al., 2014). The results were similar to those obtained by the same research group in GC (Hu et al., 2015), showing a significant association between "thick tongue" phenotype and overall microbiota imbalance, especially in CRC patients. Another study investigated the association of smoking and CRC history with oral microbiota profile (Kato et al., 2016). The results failed to support earlier observations about the association between *F. nucleatum* and CRC, although increased presence of *Lactobacillus* and *Rothia* genera was found in cancer patients. Furthermore, smoking status was associated with a decrease in *Betaproteobacteria* class and *Veilonellaceae* family, especially in CRC patients. Microbial communities in a small group of CRC patients were investigated by comparing microbiota profiles from saliva, faeces and tissue samples (Russo et al., 2017). Although an altered microbial composition in faecal samples of CRC patients was noted, the differences in oral microbiota between cancer patients and healthy controls were inconsistent. A recent study compared microbiotas from faecal samples and colonic mucosa with oral microbiota in a larger cohort of patients (45 with CRC, 21 with colorectal polyps, and 25 healthy controls) (Flemer et al., 2018). Overall microbiota differs significantly between CRC patients and controls, suggesting that the colonic establishment of a putatively pathogenic oral-like community is more frequent in patients with CRC or polyps. Moreover, a specific oral and faecal microbiota test discriminated healthy controls from CRC patients with high sensitivity (76%) and specificity (94%). Recently, a large prospective case-control study investigated the association of specific periodontal pathogens and the overall oral microbiota with subsequent risk of CRC (Yang et al., 2018). The results showed a significant relationship between CRC risk and higher levels of *T. denticola* and *P. intermedia*. Furthermore, 11 common and 16 rare taxa seemed to be associated with cancer risk, especially among African-American and females.

OTHER TUMOURS

The promising results obtained by oral microbiota studies in gastrointestinal cancers led several research groups to investigate the diagnostic/prognostic applications of oral microbiota in other tumours. Although promising, these results are highly preliminary and far less clear than those reported in the aforementioned studies (**Tables 1**, **2**).

Among the blood cancers, leukaemia is frequently associated with oral manifestations, like periodontitis and candidiasis. Therefore, imbalance of commensal oral microflora could be related to higher risk of both oral and systemic infections (Păunică et al., 2009). Oral microbiota profiling was evaluated in paediatric patients affected by acute lymphoblastic leukaemia (Wang et al., 2014). In particular, a genome analysis was conducted on supragingival plaque microbiota in 13 ALL patients, showing a reduced overall microbial diversity and detecting a specific pool of leukaemia-associated taxa. A longitudinal study was conducted on patients with acute myeloid leukaemia, with the aim to evaluate the variability of oral and gut microbiota during induction chemotherapy (Galloway-Peña et al., 2017). The results highlighted a high intra-patient temporal variability of oral microbiota and a correlation with higher risk of infection after induction chemotherapy.

Among solid malignancies, oral microbiota profile was investigated in lung cancer (LC) patients (Yan et al., 2015). Salivary samples from patients with SCC and AC showed overall microbiota difference between cancer patients and healthy controls. Furthermore, scoring system based on 2 genera, *Veillonella* and *Capnocytophaga*, distinguished healthy controls from SCC and AC. Another high-throughput sequencing-based

study investigated tongue coating microbiota in cirrhotic patients with hepatic cancer (HC), finding an increased overall microbial diversity (Lu et al., 2016). Furthermore, 2 genera, *Oribacterium* and *Fusobacterium*, discriminated between HC patients and healthy controls. Finally, Wang et al. compared oral and urinary microbiota from 57 patients with breast cancer (BC) and 21 healthy subjects, but lack of significant relationship between oral microbiota and the presence of BC was found (Wang et al., 2017).

CONCLUSIONS

Overall, the results presented here showed a significant shift in oral microbiota composition between cancer patients and healthy controls. For example, *Firmicutes* and *Actinobacteria* were the most predominant phyla in cancer patients, while *Proteobacteria*, *Fusobacteria*, and *Bacteroides* were significantly more abundant in healthy controls (**Table 2**). Recently, the concept of resilience has been applied to the oral microbiota, defined as the capacity of this microbial ecosystem to deal with perturbation without shifting to potentially harmful states (Rosier et al., 2018). Strong and persistent perturbations to the oral environments can disrupt resilience, leading to a "regime shift" toward oral dysbiosis and, ultimately, to an increased risk of oral and systemic disease (Marsh, 2003).

Unfortunately, these results are heterogeneous among the studies, especially regarding specific cancer types, and do not allow drawing general conclusions. In particular, oral microbiota alterations seem to be more pronounced in patients with gastrointestinal tumours, paving the way for developing specific tests to allow early detection of cancer (Farrell et al., 2012; Torres et al., 2015; Flemer et al., 2018; Snider et al., 2018). The overlap between the microbiotas of oral cavity and gastrointestinal tract, probably due to colonization of gut mucosa by oral bacteria, could partially explain these results (Flynn et al., 2016).

There are several factors that currently limit the generalization of these results. First of all, the small sample size, mainly due to the high sequencing cost, limits the statistical power of the studies. Furthermore, lack of adequate information about patients (e.g., oral health status, active smoking, and antibiotic use) is a source of confounding factors (Snider et al., 2018). Most studies are conducted cross-sectionally, a design that is unable to infer causality between oral dysbiosis and tumour development. This aspect is particularly important, because the alternative hypothesis is the so-called "reverse causality," namely that modifications in the normal oral microbiota are the result of tumour progression, secondary to systemic processes (Fan et al., 2018). Indeed, although different pathogenic mechanisms have been proposed, direct evidence is not yet available and the reverse causality cannot be excluded. In particular, oral microbiota could be involved in cancer development, but specific tissue tropism for microbial translocation and molecular mechanisms of microbiota-driven carcinogenesis at distal sites are yet to be proven (Chen et al., 2017).

Regarding methodological aspects, different types of oral samples were evaluated making the comparison of these microbial communities difficult. Bacteria colonize all sites within the oral cavity, forming different habitats, each with different non-overlapping microbial populations (Jia et al., 2018). Therefore, it is important to develop a method that can access all oral sites and the microbial diversity inherent to them (Lim et al., 2017). Saliva is the most commonly studied sample, due to several features that make it ideal for research purposes. Thanks to its role in "ecological stability" of oral cavity, saliva allows to early detect an overall dysbiotic trend of oral microbiota (Rosier et al., 2018; Belibasakis et al., 2019). Although different collection methods can alter salivary microbiota (e.g., muscle movements from spitting action may provide larger microbial coverage compared with passive drooling), this seems to not significantly affect the overall microbial composition (Lim et al., 2017). The studies reported here used different sequencing techniques and analysed different 16S rDNA hypervariable regions, with an increased risk for ambiguous data for some rare bacterial species (**Table 1**; Verma et al., 2018). Furthermore, sequencing depth of these studies could not be sensitive enough to adequately describe differences in oral microbiota in patients with and without tumour (Teng et al., 2018).

Nevertheless, the evidences linking oral microbiota with different tumours are promising. Furthermore, metatranscriptomic and metabolomic analyses are making a significant contribution to the understanding of the relationship between oral microbiota metabolism and human health (García-Castillo et al., 2016). The demonstration of a direct causality between oral dysbiosis and risk of tumour development could lead to new prevention strategies. This could also lead to a broader definition of oral health, including also the presence of a stable microbial homeostasis. Furthermore, causal link between oral microbiota and cancer could contribute to the search for new biomarkers (e.g., markers of resilience) and new therapeutic anticancer strategies, such as the use of pre- and pro-biotics (Rosier et al., 2018).

Although only at the beginning, oral microbiota analysis could be the next step in the evolution of cancer therapy and will help clinicians to develop tailored prevention and treatment strategies.

AUTHOR CONTRIBUTIONS

MM, AS, and LL conceived the literature review. MM, LT, and GT described pancreatic studies. GT, VC, and LT described upper gastrointestinal tract studies. DG, LM, and AS described lower gastrointestinal tract studies. LL, MP, and LM wrote the concluding remarks. All authors discussed and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00232/full#supplementary-material

REFERENCES

Ahn, J., Chen, C. Y., and Hayes, R. B. (2012a). Oral microbiome and oral and gastrointestinal cancer risk. *Cancer Causes Control* 23, 399–404. doi: 10.1007/s10552-011-9892-7

Ahn, J., Segers, S., and Hayes, R. B. (2012b). Periodontal disease, *Porphyromonas gingivalis* serum antibody levels and orodigestive cancer mortality. *Carcinogenesis* 33, 1055–1058. doi: 10.1093/carcin/bgs112

Arweiler, N. B., and Netuschil, L. (2016). The oral microbiota. *Adv. Exp. Med. Biol.* 902, 45–60. doi: 10.1007/978-3-319-31248-4_4

Belibasakis, G. N., Bostanci, N., Marsh, P. D., and Zaura, E. (2019). Applications of the oral microbiome in personalized dentistry. *Arch. Oral Biol.* 104, 7–12. doi: 10.1016/j.archoralbio.2019.05.023

Benn, A., Heng, N., Broadbent, J. M., and Thomson, W. M. (2018). Studying the human oral microbiome: challenges and the evolution of solutions. *Aust. Dent. J.* 63, 14–24. doi: 10.1111/adj.12565

Chen, J., Domingue, J. C., and Sears, C. L. (2017). Microbiota dysbiosis in select human cancers: evidence of association and causality. *Semin. Immunol.* 32, 25–34. doi: 10.1016/j.smim.2017.08.001

Chen, X., Winckler, B., Lu, M., Cheng, H., Yuan, Z., Yang, Y., et al. (2015). Oral microbiota and risk for esophageal squamous cell carcinoma in a high-risk area of China. *PLoS ONE* 10:e0143603. doi: 10.1371/journal.pone.0143603

Domper Arnal, M. J., Ferrández Arenas, Á., and Lanas Arbeloa, Á. (2015). Esophageal cancer: risk factors, screening and endoscopic treatment in Western and Eastern countries. *World J. Gastroenterol.* 21, 7933–7943. doi: 10.3748/wjg.v21.i26.7933

Fan, X., Alekseyenko, A. V., Wu, J., Peters, B. A., Jacobs, E. J., Gapstur, S. M., et al. (2018). Human oral microbiome and prospective risk for pancreatic cancer: a population-based nested case-control study. *Gut* 67, 120–127. doi: 10.1136/gutjnl-2016-312580

Farrell, J. J., Zhang, L., Zhou, H., Chia, D., Elashoff, D., Akin, D., et al. (2012). Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. *Gut* 61, 582–588. doi: 10.1136/gutjnl-2011-300784

Flemer, B., Warren, R. D., Barrett, M. P., Cisek, K., Das, A., Jeffery, I. B., et al. (2018). The oral microbiota in colorectal cancer is distinctive and predictive. *Gut* 67, 1454–1463. doi: 10.1136/gutjnl-2017-314814

Flynn, K. J., Baxter, N. T., and Schloss, P. D. (2016). Metabolic and community synergy of oral bacteria in colorectal cancer. *mSphere* 1:e00102-16. doi: 10.1128/mSphere.00102-16

Galloway-Peña, J. R., Smith, D. P., Sahasrabhojane, P., Wadsworth, W. D., Fellman, B. M., Ajami, N. J., et al. (2017). Characterization of oral and gut microbiome temporal variability in hospitalized cancer patients. *Genome Med.* 9:21. doi: 10.1186/s13073-017-0409-1

García-Castillo, V., Sanhueza, E., Mcnerney, E., Onate, S. A., and García, A. (2016). Microbiota dysbiosis: a new piece in the understanding of the carcinogenesis puzzle. *J. Med. Microbiol.* 65, 1347–1362. doi: 10.1099/jmm.0.000371

Han, S., Chen, Y., Hu, J., and Ji, Z. (2014). Tongue images and tongue coating microbiome in patients with colorectal cancer. *Microb. Pathog.* 77, 1–6. doi: 10.1016/j.micpath.2014.10.003

Hayes, R. B., Ahn, J., Fan, X., Peters, B. A., Ma, Y., Yang, L., et al. (2018). Association of oral microbiome with risk for incident head and neck squamous cell cancer. *JAMA Oncol.* 4, 358–365. doi: 10.1001/jamaoncol.2017.4777

Hu, J., Han, S., Chen, Y., and Ji, Z. (2015). Variations of tongue coating microbiota in patients with gastric cancer. *Biomed Res. Int.* 2015:173729. doi: 10.1155/2015/173729

Hu, J. M., Shen, C. J., Chou, Y. C., Hung, C. F., Tian, Y. F., You, S. L., et al. (2018). Risk of colorectal cancer in patients with periodontal disease severity: a nationwide, population-based cohort study. *Int. J. Colorectal Dis.* 33, 349–352. doi: 10.1007/s00384-018-2965-2

Hujoel, P. P., Drangsholt, M., Spiekerman, C., and Weiss, N. S. (2003). An exploration of the periodontitis-cancer association. *Ann. Epidemiol.* 13, 312–316. doi: 10.1016/S1047-2797(02)00425-8

Jia, G., Zhi, A., Lai, P. F. H., Wang, G., Xia, Y., Xiong, Z., et al. (2018). The oral microbiota - a mechanistic role for systemic diseases. *Br. Dent. J.* 224, 447–455. doi: 10.1038/sj.bdj.2018.217

Karimi, P., Islami, F., Anandasabapathy, S., Freedman, N. D., and Kamangar, F. (2014). Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. *Cancer Epidemiol. Biomarkers Prev.* 23, 700–713. doi: 10.1158/1055-9965.EPI-13-1057

Kato, I., Vasquez, A. A., Moyerbrailean, G., Land, S., Sun, J., Lin, H. S., et al. (2016). Oral microbiome and history of smoking and colorectal cancer. *J. Epidemiol. Res.* 2, 92–101. doi: 10.5430/jer.v2n2p92

Klimesova, K., Jiraskova Zakostelska, Z., and Tlaskalova-Hogenova, H. (2018). Oral bacterial and fungal microbiome impacts colorectal carcinogenesis. *Front. Microbiol.* 9:774. doi: 10.3389/fmicb.2018.00774

Le Bars, P., Matamoros, S., Montassier, E., Le Vacon, F., Potel, G., Soueidan, A., et al. (2017). The oral cavity microbiota: between health, oral disease, and cancers of the aerodigestive tract. *Can. J. Microbiol.* 63, 475–492. doi: 10.1139/cjm-2016-0603

Lim, Y., Totsika, M., Morrison, M., and Punyadeera, C. (2017). The saliva microbiome profiles are minimally affected by collection method or DNA extraction protocols. *Sci. Rep.* 7:8523. doi: 10.1038/s41598-017-07885-3

Lu, H., Ren, Z., Li, A., Zhang, H., Jiang, J., Xu, S., et al. (2016). Deep sequencing reveals microbiota dysbiosis of tongue coat in patients with liver carcinoma. *Sci. Rep.* 6:33142. doi: 10.1038/srep33142

Marley, A. R., and Nan, H. (2016). Epidemiology of colorectal cancer. *Int. J. Mol. Epidemiol. Genet.* 7, 105–114.

Marsh, P. D. (2003). Are dental diseases examples of ecological catastrophes? *Microbiology* 149, 279–294. doi: 10.1099/mic.0.26082-0

Mcguigan, A., Kelly, P., Turkington, R. C., Jones, C., Coleman, H. G., and Mccain, R. S. (2018). Pancreatic cancer: a review of clinical diagnosis, epidemiology, treatment and outcomes. *World J. Gastroenterol.* 24, 4846–4861. doi: 10.3748/wjg.v24.i43.4846

Meurman, J. H., and Uittamo, J. (2008). Oral micro-organisms in the etiology of cancer. *Acta Odontol. Scand.* 66, 321–326. doi: 10.1080/00016350802446527

Michaud, D. S., Fu, Z., Shi, J., and Chung, M. (2017). Periodontal disease, tooth loss, and cancer risk. *Epidemiol. Rev.* 39, 49–58. doi: 10.1093/epirev/mxx006

Michaud, D. S., Izard, J., Wilhelm-Benartzi, C. S., You, D. H., Grote, V. A., Tjønneland, A., et al. (2013). Plasma antibodies to oral bacteria and risk of pancreatic cancer in a large European prospective cohort study. *Gut* 62, 1764–1770. doi: 10.1136/gutjnl-2012-303006

Michaud, D. S., Joshipura, K., Giovannucci, E., and Fuchs, C. S. (2007). A prospective study of periodontal disease and pancreatic cancer in US male health professionals. *J. Natl. Cancer Inst.* 99, 171–175. doi: 10.1093/jnci/djk021

Olson, S. H., Satagopan, J., Xu, Y., Ling, L., Leong, S., Orlow, I., et al. (2017). The oral microbiota in patients with pancreatic cancer, patients with IPMNs, and controls: a pilot study. *Cancer Causes Control* 28, 959–969. doi: 10.1007/s10552-017-0933-8

Păunică, S. C., Dumitriu, A., Mogoş, M., Georgescu, O., and Mogoş, I. (2009). The evaluation of the periodontium in patients with leukemia using thermographic imaging. *Hematology* 14, 341–346. doi: 10.1179/102453309X12473408860505

Peters, B. A., Wu, J., Pei, Z., Yang, L., Purdue, M. P., Freedman, N. D., et al. (2017). Oral microbiome composition reflects prospective risk for esophageal cancers. *Cancer Res.* 77, 6777–6787. doi: 10.1158/0008-5472.CAN-17-1296

Petersen, C., and Round, J. L. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cell. Microbiol.* 16, 1024–1033. doi: 10.1111/cmi.12308

Plottel, C. S., and Blaser, M. J. (2011). Microbiome and malignancy. *Cell Host Microbe* 10, 324–335. doi: 10.1016/j.chom.2011.10.003

Rosier, B. T., Marsh, P. D., and Mira, A. (2018). Resilience of the oral microbiota in health: mechanisms that prevent dysbiosis. *J. Dent. Res.* 97, 371–380. doi: 10.1177/0022034517742139

Russo, E., Bacci, G., Chiellini, C., Fagorzi, C., Niccolai, E., Taddei, A., et al. (2017). Preliminary comparison of oral and intestinal human microbiota in patients with colorectal cancer: a pilot study. *Front. Microbiol.* 8:2699. doi: 10.3389/fmicb.2017.02699

Santarelli, A., Mascitti, M., Rubini, C., Bambini, F., Zizzi, A., Offidani, A., et al. (2015). Active inflammatory biomarkers in oral lichen planus. *Int. J. Immunopathol. Pharmacol.* 28, 562–568. doi: 10.1177/0394632015592101

Schwabe, R. F., and Jobin, C. (2013). The microbiome and cancer. *Nat. Rev. Cancer* 13, 800–812. doi: 10.1038/nrc3610

Snider, E. J., Compres, G., Freedberg, D. E., Giddins, M. J., Khiabanian, H., Lightdale, C. J., et al. (2018). Barrett's esophagus is associated with a distinct oral microbiome. *Clin. Transl. Gastroenterol.* 9:135. doi: 10.1038/s41424-018-0005-8

Stolzenberg-Solomon, R. Z., Dodd, K. W., Blaser, M. J., Virtamo, J., Taylor, P. R., and Albanes, D. (2003). Tooth loss, pancreatic cancer, and *Helicobacter pylori*. *Am. J. Clin. Nutr.* 78, 176–181. doi: 10.1093/ajcn/78.1.176

Sun, J. H., Li, X. L., Yin, J., Li, Y. H., Hou, B. X., and Zhang, Z. (2018). A screening method for gastric cancer by oral microbiome detection. *Oncol. Rep.* 39, 2217–2224. doi: 10.3892/or.2018.6286

Teng, F., Darveekaran Nair, S. S., Zhu, P., Li, S., Huang, S., Li, X., et al. (2018). Impact of DNA extraction method and targeted 16S-rRNA hypervariable region on oral microbiota profiling. *Sci. Rep.* 8:16321. doi: 10.1038/s41598-018-34294-x

Torres, P. J., Fletcher, E. M., Gibbons, S. M., Bouvet, M., Doran, K. S., and Kelley, S. T. (2015). Characterization of the salivary microbiome in patients with pancreatic cancer. *PeerJ* 3:e1373. doi: 10.7717/peerj.1373

Verma, D., Garg, P. K., and Dubey, A. K. (2018). Insights into the human oral microbiome. *Arch. Microbiol.* 200, 525–540. doi: 10.1007/s00203-018-1505-3

Vincent, A., Herman, J., Schulick, R., Hruban, R. H., and Goggins, M. (2011). Pancreatic cancer. *Lancet* 378, 607–620. doi: 10.1016/S0140-6736(10)62307-0

Wang, C., and Li, J. (2015). Pathogenic microorganisms and pancreatic cancer. *Gastrointest. Tumors* 2, 41–47. doi: 10.1159/000380896

Wang, H., Altemus, J., Niazi, F., Green, H., Calhoun, B. C., Sturgis, C., et al. (2017). Breast tissue, oral and urinary microbiomes in breast cancer. *Oncotarget* 8, 88122–88138. doi: 10.18632/oncotarget.21490

Wang, Y., Xue, J., Zhou, X., You, M., Du, Q., Yang, X., et al. (2014). Oral microbiota distinguishes acute lymphoblastic leukemia pediatric hosts from healthy populations. *PLoS ONE* 9:e102116. doi: 10.1371/journal.pone.0102116

Wu, J., Xu, S., Xiang, C., Cao, Q., Li, Q., Huang, J., et al. (2018). Tongue coating microbiota community and risk effect on gastric cancer. *J. Cancer* 9, 4039–4048. doi: 10.7150/jca.25280

Yachida, S., Jones, S., Bozic, I., Antal, T., Leary, R., Fu, B., et al. (2010). Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* 467, 1114–1117. doi: 10.1038/natur e09515

Yan, X., Yang, M., Liu, J., Gao, R., Hu, J., Li, J., et al. (2015). Discovery and validation of potential bacterial biomarkers for lung cancer. *Am. J. Cancer Res.* 5, 3111–3122.

Yang, Y., Cai, Q., Shu, X. O., Steinwandel, M. D., Blot, W. J., Zheng, W., et al. (2018). Prospective study of oral microbiome and colorectal cancer risk in low-income and African American populations. *Int. J. Cancer*. 77:4931. doi: 10.1158/1538-7445.AM20 17-4931

Zhang, Y., Wang, X., Li, H., Ni, C., Du, Z., and Yan, F. (2018). Human oral microbiota and its modulation for oral health. *Biomed. Pharmacother.* 99, 883–893. doi: 10.1016/j.biopha.2018. 01.146

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

MINI REVIEW
published: 03 July 2019
doi: 10.3389/fphys.2019.00855

Unusual Conditions Impairing Saliva Secretion: Developmental Anomalies of Salivary Glands

*Lucrezia Togni[1], Marco Mascitti[1,2], Andrea Santarelli[1,2], Maria Contaldo[3], Antonio Romano[3], Rosario Serpico[3] and Corrado Rubini[4]**

[1] Department of Clinical Specialistic and Dental Sciences, Marche Polytechnic University, Ancona, Italy, [2] National Institute of Health and Science of Aging, IRCCS INRCA, Ancona, Italy, [3] Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania "Luigi Vanvitelli", Naples, Italy, [4] Department of Biomedical Sciences and Public Health, Marche Polytechnic University, Ancona, Italy

Salivary glands (SG) arise from ectodermal tissue between 6 and 12th weeks of intrauterine life through finely regulated epithelial-mesenchymal interactions. For this reason, different types of structural congenital anomalies, ranging from asymptomatic anatomical variants to alterations associated with syndromic conditions, have been described. Notable glandular parenchyma anomalies are the SG aplasia and the ectopic SG tissue. Major SG aplasia is a developmental anomaly, leading to variable degrees of xerostomia, and oral dryness. Ectopic SG tissue can occur as accessory gland tissue, salivary tissue associated with branchial cleft anomalies, or true heterotopic SG tissue. Among salivary ducts anomalies, congenital atresia is a rare developmental anomaly due to duct canalization failure in oral cavity, lead to salivary retention posterior to the imperforate orifice. Accessory ducts originate from the invagination of the developing duct in two places or from the premature ventral branching of the main duct. Heterotopic ducts may arise from glandular bud positioned in an anomalous site lateral to the stomodeum or from the failure of the intraoral groove development, hindering their proximal canalization. These anomalies require multidisciplinary approach to diagnosis and treatment. While ectopic or accessory SG tissue/ducts often do not require any treatment, patients with SG aplasia could benefit from strategies for restoring SG function. This article attempts to review the literature on SG parenchyma and ducts anomalies in head and neck region providing clinicians with a comprehensive range of clinical phenotypes and possible future applications of bioengineered therapies for next-generation of regenerative medicine.

Keywords: salivary glands, salivary gland aplasia, accessory salivary gland, accessory salivary ducts, facial developmental anomalies, stem cells

OPEN ACCESS

Edited by:
Lorenzo Lo Muzio,
University of Foggia, Italy

Reviewed by:
Amel Gritli-Linde,
University of Gothenburg, Sweden
Pier Paolo Claudio,
The University of Mississippi, United States

****Correspondence:***
Corrado Rubini
c.rubini@univpm.it

Specialty section:
This article was submitted to Craniofacial Biology and Dental Research, a section of the journal Frontiers in Physiology

Received: *25 March 2019*
Accepted: *20 June 2019*
Published: *03 July 2019*

Citation:
Togni L, Mascitti M, Santarelli A, Contaldo M, Romano A, Serpico R and Rubini C (2019) Unusual Conditions Impairing Saliva Secretion: Developmental Anomalies of Salivary Glands. Front. Physiol. 10:855. doi: 10.3389/fphys.2019.00855

INTRODUCTION

Salivary glands (SG) arise from ectodermal tissue during intrauterine life following a complex and well-timed sequence of epithelial-mesenchymal interactions (McDonald et al., 1986; Pham Dang et al., 2010). Parotid glands are the first to develop from the 6th week of intrauterine life, followed by submandibular (7th week), sublingual (8th week), and the other minor SGs (9–12th weeks) (Zhang et al., 2010; Berta et al., 2013; Chadi et al., 2017). Whereas parotid and minor SGs are certainly ectodermal in origin, submandibular, and sublingual glands arise from the mouth

floor in the junctional regions between ectoderm and endoderm (Amin and Bailey, 2001; Antoniades et al., 2006; Higley et al., 2010). From 18 to 25th week, SG acquire connective capsules and interlobular ducts, while glandular parenchyma shows a rapid growth, leading to highly increase in the number of lobules, and almost complete canalization of the tubules (Aronovich and Edwards, 2014). Although the parotid is the first to develop, it is encapsulated after the submandibular and sublingual glands. This delayed encapsulation is critical because lymphatic system develops within mesoderm after encapsulation of the submandibular and sublingual glands but prior to encapsulation of the parotid gland (Goldenberg et al., 2000). Noteworthy, distal perforation of the submandibular duct (SMD) or Wharton duct into the medial paralingual sulcus is believed to be one of the last stages in the SG development (Amin and Bailey, 2001; Aronovich and Edwards, 2014).

Parotid gland produces almost exclusively serous saliva, while submandibular and sublingual glands produce both serous and mucous secretions (Chadi et al., 2017). Parotid secretions are carried by the main parotid duct (PD) or Stensen duct into oral cavity: it arises from the anterior margin of the gland, from the confluence of two main tributaries, and opens on the parotid papilla into the oral vestibule. The PD mean length and diameter are 50.0 and 1.7 mm, respectively. An accessory PD may join the main duct as the latter passes over the masseter muscle (Kulkarni et al., 2011).

Submandibular duct begins by numerous branches from deep surface of the gland and it drains in a narrow orifice on the top of sublingual caruncle. The SMD mean length and diameter are 62.0 and 3.0 mm, respectively. Along its course, SMD bends at the posterior margin of mylohyoid muscle to form the genu of the SMD (with the angle ranging from 102.7° to 114.4°) (Horsburgh and Massoud, 2013). The major sublingual duct (Bartholin duct) originates from the center of the gland and is accompanied by 8–15 minor excretory ducts (Rivinus ducts). Whereas the minor ducts open into the sublingual fold, Bartholin duct opens alone or in conjunction with SMD at the sublingual caruncle (Zhang et al., 2010).

As previously mentioned, the SG development is a finely regulated process, involving well-timed sequences of cellular, and tissue events. For this reason, different types of structural congenital anomalies have been described so far, ranging from asymptomatic anatomical variants to alterations associated with syndromic conditions (**Table 1**). The aim of this article is to review the literature on SG parenchyma ad ducts anomalies in head and neck region (**Table 2** and **Supplementary Table 1**), providing clinicians with a comprehensive range of clinical phenotypes, and possible future applications of bioengineered therapies for SG repair and regeneration.

GLANDULAR PARENCHYMA ANOMALIES

Aplastic/Hypoplastic Gland Tissue

Aplasia/hypoplasia of the major SGs are rare developmental anomalies, either unilateral or bilateral, that can be isolated or part of a hereditary syndrome (McDonald et al., 1986; Yan et al., 2012). SG aplasia may occur alone or in association with accessory salivary tissue or with other developmental anomalies of the first pharyngeal arch. The first report describing major SGs aplasia was by Bradbury in 1879, while the earliest case of parotid glands aplasia was reported by Poirier in 1881 (Ferguson and Ponnambalam, 2005). The first genetic-related absence of SG was reported in 1924 by Ramsey, who described a pleotropic autosomal dominant disorder, with a high degree of penetrance, more commonly found in males (Matsuda et al., 1999; Goldenberg et al., 2000).

Parotid gland aplasia has an estimated incidence of 1:5000 live births, while only 49 cases of submandibular aplasia have been reported until now. The higher frequency of parotid gland aplasia could be related to its earlier morphogenesis compared to the other SG, exposing this gland to increased risk of developmental impairment (Goldenberg et al., 2000).

Clinical presentation varies depending on the number of missing SGs, their role in salivary flow, and the presence of compensatory hypertrophy of the remaining glands. SG aplasia leads to variable degrees of xerostomia and oral dryness, although some patients with hypoplasia and/or, unilateral aplasia are asymptomatic (McDonald et al., 1986; Matsuda et al., 1999).

Main symptoms include erythematous oral mucosa, glossitis, cheilitis, chronic erythematous candidiasis, and exfoliate lips. There is also an increased risk for dental caries, teeth erosion, periodontal disease, tongue papillary atrophy, pain, and oral ulcers. Furthermore, patients can report hoarseness, dysphagia, oropharyngeal infections, feeding/swallowing impairment, intolerance to acidic or spicy food, dysgeusia, and gastric acid reflux due to inadequate salivary buffering action (Antoniades et al., 2006; Herrera-Calvo et al., 2011; Santarelli et al., 2015).

The intraoral examination reveals the absence of parotid papillae and/or SMD orifices, lack of saliva production upon palpation and, in cases of unilateral aplasia, asymmetry of the parotid/submandibular region. The imaging techniques used to demonstrate SG anomalies include: Ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI), sialography, and scintigraphy (Matsuda et al., 1999). Ultrasonography is a primary step to assess anomalies of superficial parotid lobe and submandibular glands, while MRI is used for deeper lesions or sublingual glands. Sialography is prescribed to assess duct anomalies and ductal system of accessory/rudimentary SGs, while scintigraphy is useful to assess secretory function, including that of the minor SGs of nasal and oropharyngeal regions. Laboratory tests and/or SG biopsy can be obtained in order to determine if the underlying cause is related to systemic diseases, such as Sjögren syndrome (Antoniades et al., 2006; Higley et al., 2010; Herrera-Calvo et al., 2011).

Only topical therapies are available for SG aplasia, based on saliva substitutes. Other measures include lifestyle changes, such as increasing water intake and limiting irritating foods. Furthermore, regular dental examinations, low-sugar diet, and

TABLE 1 | Developmental anomalies and manifestation related to salivary gland aplasia/hypoplasia in syndromic and non-syndromic patients.

Developmental anomalies	Clinical features (head and neck)
1. Syndromes closely related to SG aplasia/hypoplasia	
Lacrimo-auriculo-dento-digital syndrome (LADD) or Levy-Hollister syndrome	Oral cavity: small and sharp lateral incisor, lateral upper incisor agenesis, and bifid uvula
	Lacrimal glands: aplasia/hypoplasia, duct obstruction, and absence of lacrimal punctum
	Other: cup-shaped ears, sensory, or mixed deafness.
Oculo-auriculo-vertebral spectrum (OAVS)	Oral cavity: micrognathia, macrostomia, oral facial cleft, mandibular hypoplasia/deformity, and tongue anomaly;
	Other: microtia, hemifacial macrosomia, facial palsy, branchial cyst, preauricular skin tag, and oral apraxia
Ectrodactyly ectodermal dysplasia cleft lip/palate syndrome (ECC)	Oral cavity: dental abnormalities, lip, and palate cleft Lacrimal glands: absence of lacrimal punctum Other: deformed ears, conductive hearing loss
2. Syndromes that can be associated with SG aplasia/hypoplasia	
Down syndrome	Oral cavity: dental agenesia, hypo/hyper/microdontia, delayed eruption, open bite, taurodontism, gingivitis/periodontitis, cheilitis-stomatitis, ogival vault, protruding tongue, and maxillary processes hypoplasia
	Head and neck: slanting palpebral fissures, epicanthic folds, brachycephaly, flat cranial base, and flattened nose bridge
Klinefelter syndrome	Oral cavity: shovel-shaped incisor, taurodontism, and delayed eruption
	Head and neck: brachycephaly
Treacher-Collins syndrome	Oral cavity: mandibular/maxillary dysostosis, cleft palate
3. Developmental anomalies associated with SG aplasia/hypoplasia in non-syndromic patients	
Lacrimal glands	Secretion disorders
Oral cavity	Hypo/oligo/anodontia, enamel hypoplasia, multiple caries, fissured tongue, lip, and palate cleft
Head and neck	Cranial deformity, mandibular ramus agenesia

SG, salivary gland.

TABLE 2 | Summary table of developmental disorders involving salivary glands and ducts.

Developmental disorders	Main features
Major SG aplasia	• Sporadic, familial, or syndromic forms (e.g., LADD, Down syndrome)
	• Isolated or in association with lacrimal gland disorders or other anomalies (e.g., cleft lip and palate)
Parotid gland aplasia	• Sporadic, familial, or syndromic forms (e.g., Klinefelter syndrome)
	• Unilateral or bilateral form
	• Isolated or in association with other gland anomalies (e.g., contralateral parotid gland hypoplasia/hypertrophy, parotid accessory tissue, lacrimal anomalies) or other diseases (e.g., pleomorphic adenoma, angioma)
Submandibular gland aplasia	• Sporadic or syndromic forms (e.g., ectodermal dysplasia)
	• Unilateral or bilateral form
	• Isolated or in association with other gland anomalies (e.g., sublingual gland hypoplasia/hypertrophy, cleft lip, and palate)
Ectopic SG tissue	• Accessory parotid gland (normal anatomical variant) and submandibular gland, with/without accessory salivary ducts
	• Gland tissue associated with branchial cleft anomalies (Huschke foramen)
	• Heterotopic SG tissue, with/without accessory salivary duct and other anomalies, sporadic, or familial form
SD atresia	• Unilateral or bilateral form
	• Atresia of submandibular or sublingual SD
Duplication or accessory SDs	• Unilateral or bilateral form
	• Duplicated or accessory SD (submandibular, sublingual, or parotid gland)
	• Isolated or associated with SG duplication

SG, salivary glands; SD, salivary ducts; LADD, Lacrimo-auriculo-dento-digital syndrome.

use of fluoride-based dental products are suggested in order to prevent dental caries (Chadi et al., 2017).

Salivary glands aplasia can occur with other multiple facial developmental anomalies (**Table 1**). The association of parotid and lacrimal aplasia raises the possibility of an ectodermal defect manifesting after the 6th week of fetal life. The absence of submandibular and sublingual glands supports their ectodermal origin although it is impossible to differentiate between ectodermal and endodermal-derived epithelium in the mouth floor (McDonald et al., 1986).

The mutation of FGF-10 or FGFR2b seems to decrease the survival of progenitor cells in a murine model, leading to aplasia/hypoplasia of major SGs (Berta et al., 2013). Although FGF-10 mutation has been reported in some patients, the diagnosis is usually based on clinico-radiological findings. The diagnostic genetic investigations are recommended only if they have a substantial impact on patient management. The mechanisms related to sporadic cases can be either a *de novo* mutation gene, especially FGF-10, or a multifactorial cause associated with genetic and/or environmental factors (e.g., infections during pregnancy) (Berta et al., 2013).

Ectopic Gland Tissue

The ectopic SG tissue has been reported in several body sites including hypophysis, middle and external ear, mandible, thyroglossal duct, thyroid and parathyroid gland capsules, lymph nodes, and neck region. Ectopic SG tissue can occur in three forms: (a) accessory gland tissue; (b) salivary tissue associated with branchial cleft anomalies; and (c) true heterotopic salivary gland tissue (HSGT).

Finally, stafne bone defect (SBD) should be mentioned as a special case of ectopic SG tissue. This is a bone cavity usually located on the posterior mandible, below the inferior alveolar canal (Philipsen et al., 2002). Several Authors suggested that SBD could be the result of hypertrophic SG lobe, an erosion from vascular tissue compression, or the incomplete Meckel cartilage calcification. Although SBD usually contains ectopic glandular tissue, other tissues can be found in the bone cavity, such as muscles, lymphoid tissue, blood vessels, fat, or connective tissue. Furthermore, even if present, glandular tissue is not directly connected to other SGs nor to independent ductal system (Hisatomi et al., 2019). For these reasons, this anomaly will not be further discussed.

Accessory Gland Tissue

Accessory SG tissue refers to lobules of SG tissue which are completely separated from the main gland at a variable distance, but which drain into the main duct. It may arise either from SG inclusions within lymph nodes or from anomalous migration of salivary lobules. The accessory parotid gland is present in about 21% of the population and is considered a normal anatomical variant. The histological features are usually identical to those of the main parotid gland, although 26% of accessory parotid tissue showed both mucous, and serous acini (Antoniades et al., 2006; Higley et al., 2010). The ectopic tissue is more susceptible to SG neoplasms: indeed, 1–7% of all parotid tumors arise from accessory glands, half of which is histologically malignant (Batsakis, 1986; Cannon et al., 2012). On the contrary, accessory submandibular gland is extremely rare; indeed, the first case was reported in 2000 and to date only 4 cases were reported (Koybasioglu et al., 2000).

Gland Tissue Associated With Branchial Cleft Anomalies

Branchial cleft anomalies result from persistence of the embryonic branchial cleft apparatus. The Huschke foramen (also known as foramen tympanicum) is a developmental defect of the first branchial cleft affecting 5–46% of the population, consisting in a connection between external acoustic canal and temporomandibular joint (De Zoysa et al., 2009). It has been associated with herniation of soft tissues from the temporomandibular joint into the external auditory meatus. In rare cases, this defect may result in fistula formation between the parotid gland and the external auditory meatus (Rushton and Pemberton, 2005). Since the first case in 1984, only 11 additional cases have been described in literature. Therefore, due to the rarity and non-specific clinical presentation of this anomaly, misdiagnosis is frequent (Rushton and Pemberton, 2005). The diagnosis is made by demonstrating the presence of amylase in the ear discharge, followed by a sialography. CT and MRI are useful to demonstrate bony anomalies and/or fistulous tract through the soft tissue (De Zoysa et al., 2009). Surgical treatment is the only option, consisting in closing the sialo-aural fistula by superficial parotidectomy (Cannon et al., 2012). Although the sialo-aural fistula is not a congenital abnormality *per se*, it is a spontaneous complication of a congenital malformation involving SG tissue.

True Heterotopic Salivary Gland Tissue

Heterotopic salivary gland tissue consists in salivary tissue with an independent ductal system located in an unusual anatomical site, isolated from the main SG, and clinically presenting as a saliva-draining skin fistula (Cannon et al., 2012). The external opening can be located in oral mucosa, retroauricular region, buccal, or cervical region (Dutta, 2017). Less extensive surgery is usually required for HSGT because the associated tract, if present, is usually short in contrast to branchial cleft anomalies, which may exist as complete fistulas (Cannon et al., 2012).

The ectopic parotid system refers to a specific anomaly in the parotid gland development, characterized by a congenital saliva-draining cheek fistula near the oral commissure. Congenital salivary fistula results from the partial failure in normal involution of the pharyngeal arches during embryogenesis. The external fistulous orifice typically opens near the mouth angle, at the fusion site of the maxillary and mandibular processes (Gadodia et al., 2008; Dutta, 2017). The constant presence of preauricular appendages and occasional mandibular hypoplasia suggests that this condition could be included in the Oculo-Auriculo-Vertebral spectrum (Dutta, 2017). In 2010 was reported a case of bilateral and symmetrical heterotopic submandibular glands, suggesting that epithelial cord forming the submandibular gland must have bifurcated and persisted to grow as separated SGs (Sanli et al., 2010).

Imaging techniques can be used to demonstrate the existence of accessory parotid gland with a separate ectopic duct, not communicating with the main gland, and duct (Kulkarni et al., 2011). Several surgical procedures have been proposed, mainly the excision of the ectopic accessory parotid with its ductal system or the intraoral transposition of the fistulous tract. Alternatively, less invasive technique consists of Ultrasonography-guided intraglandular injection of botulinum toxin and cauterization of fistula with trichloroacetic acid injection, but an higher risk of infection has been reported (Dutta, 2017).

SALIVARY DUCTS ANOMALIES

Congenital Atresia of Submandibular Duct

Submandibular duct congenital atresia, described for the first time in 1955, is a rare developmental anomaly due to canalization failure of the duct in oral cavity (Mandel and Alfi, 2012). The consequences of this condition are the salivary retention posterior to the imperforate orifice and the presence of cyst-like swelling in the mouth floor (Prosdocimo et al., 2018). Almost all cases observed in newborns, showing male predominance (73%), and unilateral clinical presentation (75%).

Clinical manifestation is a bluish cyst-like swelling containing translucent fluid in the mouth floor following the SMD course. The main differential diagnosis of SMD congenital atresia is oral ranula, and it can be difficult to clinically distinguish between them. However, the histological examination showed lack of epithelial wall, while SMD atresia results in a cystic cavity lined by pseudostratified columnar epithelium with brush borders and thin connective tissue consistent with a dilated duct (Amin and Bailey, 2001; Prosdocimo et al., 2018). The other differential diagnoses include dermal/epidermal inclusion cysts, thyroglossal cysts, bronchogenic cysts, lymphatic and vascular lesions, SG tumors, and sialolithiasis (Pownell et al., 1992; Rosow et al., 2009). MRI is mandatory for differential diagnosis, showing an elongated and dilated tubular structure, segmentally lobed in the mouth floor, consistent with the duct anatomic course. The sphincteric areas appear as localized duct narrowing (Gadodia et al., 2007; Mandel and Alfi, 2012).

Surgical treatment is indicated to avoid the sequelae associated with an imperforate orifice, such as glandular atrophy, permanent duct dilation, sialadenitis, airway compromise and feeding difficulties. The marsupialization is the treatment of choice, while other surgical options include simple incision, excision of terminal portion of the duct with sialodochoplasty, and stenting of the duct (Pownell et al., 1992; Gadodia et al., 2007).

Duplication and Accessory Salivary Ducts

Cases of PD anomalies are rare. Therefore, there are no adequate reports on its anatomic position and malformations. PD is formed by the confluence of two ducts near the posterior mandibular ramus border. When the two ducts merged with each other outside the parotid gland, the duplication of PD is determined. The histological sections allow to determine the existence of a lumen to confirm the presence of duplication ducts (Aktan et al., 2001; Hassanzadeh Taheri et al., 2015).

Accessory SMD originates from the invagination of the developing duct in two places or from the premature ventral branching of the main duct. It is usually smaller in caliber and runs parallel to the main duct. Only 9 case of SMD duplication were reported and most of them are unilateral and incidentally detected during sialography. As duplication anomalies are mostly asymptomatic, treatment is needed only for disease processes affecting the accessory duct (Gadodia et al., 2007).

Heterotopia of PD

The parotid bud develops at the ecto-endodermal junction of the developing stomodeum and opens into the oral cavity. If the parotid bud is positioned in an anomalous site lateral to the stomodeum, a cutaneous PD may develop (Grundfast et al., 1987). Another possibility is the formation of a premature communication with the skin, establishing an externally orifice and preventing the development of the parotid papilla into the oral vestibule. A further hypothesis is the failure of the intraoral groove development, hindering the proximal canalization of the duct, or the formation of the parotid papilla (Grundfast et al., 1987).

FUTURE PERSPECTIVES OF STEM/PROGENITOR CELL AND TISSUE-BASED THERAPIES FOR SG REPAIR AND/OR REGENERATION

Several strategies have been proposed to restoring SG functions: (a) autologous SG-derived epithelial stem/progenitor cells isolated from patients and transplanted to replace functionally damaged/lost cells; (b) non-epithelial cells or their bioactive lysates used to trigger the paracrine regenerative effects on remaining glandular cells or to generate new glandular cells; and (c) biomaterials loaded with glandular cells and/or bioactive lysates to mimic *in vivo* SGs (Lombaert et al., 2017).

The first study for transplanting autologous glandular cells was carried out in rodents using c-Kit+ (CD117) epithelial cells. This research showed that SGs contain cells with stem/progenitor properties that could maintain themselves and differentiate into multiple glandular cell types. Several c-Kit+ cell subpopulations (CD24+, CD49f+, and SCA1+) are located within the major ducts and possess higher levels of stem/progenitor activity. Furthermore, c-Kit+ cells are present in human SGs and can be isolated and cultured *ex vivo*; suggesting future applications for cell therapy. Salivary acinar cells could also be considered for cell therapy as they are able to duplicate themselves to repair local damage (Nanduri et al., 2013).

Salivary glands repair can also be driven by the remaining endogenous stem/progenitor cells. For this reason, any type of transplanted epithelial cell could enhance local endogenous repair if the appropriate molecular stimuli are presented to dormant stem/progenitor cells. Recent efforts to increase the number of c-Kit+ cells *ex vivo* using growth factors may be useful, although the number of cells needed to trigger SG regeneration remains unclear (Pringle et al., 2016).

Recently, the transplantation of a bioengineered germ, with or without mesenchyme, was proposed as a promising strategy to replace SGs in a mouse model with SG defects (Tanaka et al., 2018). Authors demonstrated that SGs orthotopical transplanting led to successful glandular development *in vivo*,

showing connection of the new SG to the main duct, and correct formation of ductal and acinar structures. Morphological, immunohistochemical, and genetic analyses confirmed the development of fully functional SG tissue. The orthotopically engrafted SGs were also functional in saliva secretion through the reconstruction of neural network. Another interesting finding was the non-essential role played by mesenchyme in inducing maturation of transplanted SG (Tanaka et al., 2018).

In conclusion, SG anomalies are a heterogeneous group of rare anomalies that requires a multidisciplinary approach to diagnosis and treatment. While ectopic or accessory SG tissue/ducts often do not require any treatment, patients with SG aplasia/hypoplasia could benefit from strategies for restoring SG function. From this perspective, restoring physiological functions with bioengineered organs is expected to be the next-generation of regenerative medicine.

AUTHOR CONTRIBUTIONS

LT, MM, AS, and AR conceived the literature review of the manuscript. MM, LT, and MC described the glandular parenchyma anomalies. RS, MC, and CR described the salivary duct anomalies. AS, RS, CR, and AR wrote the concluding remarks of the manuscript. All authors discussed and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00855/full#supplementary-material

REFERENCES

Aktan, Z. A., Bilge, O., Pinar, Y. A., and Ikiz, A. O. (2001). Duplication of the parotid duct: a previously unreported anomaly. *Surg. Radiol. Anat.* 23, 353–354. doi: 10.1007/s00276-001-0353-y

Amin, M. A., and Bailey, B. M. (2001). Congenital atresia of the orifice of the submandibular duct: a report of 2 cases and review. *Br. J. Oral Maxillofac. Surg.* 39, 480–482. doi: 10.1054/bjom.2001.0672

Antoniades, D. Z., Markopoulos, A. K., Deligianni, E., and Andreadis, D. (2006). Bilateral aplasia of parotid glands correlated with accessory parotid tissue. *J. Laryngol. Otol.* 120, 327–329. doi: 10.1017/s0022215106000338

Aronovich, S., and Edwards, S. P. (2014). A case of imperforate Wharton duct. *J. Oral Maxillofac. Surg.* 72, 744–747. doi: 10.1016/j.joms.2013.10.012

Batsakis, J. G. (1986). Heterotopic and accessory salivary tissues. *Ann. Otol. Rhinol. Laryngol.* 95, 434–435. doi: 10.1177/000348948609500422

Berta, E., Bettega, G., Jouk, P. S., Billy, G., Nugues, F., and Morand, B. (2013). Complete agenesis of major salivary glands. *Int. J. Pediatr. Otorhinolaryngol.* 77, 1782–1785. doi: 10.1016/j.ijporl.2013.07.025

Cannon, D. E., Szabo, S., and Flanary, V. A. (2012). Heterotopic salivary tissue. *Am. J. Otolaryngol.* 33, 493–496. doi: 10.1016/j.amjoto.2011.11.003

Chadi, M. J., Saint Georges, G., Albert, F., Mainville, G., Nguyen, J. M., and Kauzman, A. (2017). Major salivary gland aplasia and hypoplasia in down syndrome: review of the literature and report of a case. *Clin. Case Rep.* 5, 939–944. doi: 10.1002/ccr3.975

De Zoysa, N., Vasani, S., Kaniyur, S., and Frosh, A. (2009). Gustatory otorrhoea: a rare case of congenital external ear salivary fistula. *J. Laryngol. Otol.* 123, 1371–1374. doi: 10.1017/S0022215109005726

Dutta, M. (2017). The ectopic accessory parotid system with congenital cheek fistula: an overview and current update. *Laryngoscope* 127, 1351–1360. doi: 10.1002/lary.26343

Ferguson, M. M., and Ponnambalam, Y. (2005). Aplasia of the parotid gland in down syndrome. *Br. J. Oral Maxillofac. Surg.* 43, 113–117. doi: 10.1016/j.bjoms.2004.01.001

Gadodia, A., Seith, A., Neyaz, Z., Sharma, R., and Thakkar, A. (2007). Magnetic resonance identification of an accessory submandibular duct and gland: an unusual variant. *J. Laryngol. Otol.* 121:e18.

Gadodia, A., Seith, A., Sharma, R., and Thakar, A. (2008). Congenital salivary fistula of accessory parotid gland: imaging findings. *J. Laryngol. Otol.* 122:e11. doi: 10.1017/S0022215107001417

Goldenberg, D., Flax-Goldenberg, R., Joachims, H. Z., and Peled, N. (2000). Misplaced parotid glands: bilateral agenesis of parotid glands associated with bilateral accessory parotid tissue. *J. Laryngol. Otol.* 114, 883–885. doi: 10.1258/0022215001904257

Grundfast, K. M., Barber, C. S., and Kubicki, S. P. (1987). Congenital aberrantly located cutaneous Stensen's duct orifice–a newly described anomaly. *Int. J. Pediatr. Otorhinolaryngol.* 14, 197–202. doi: 10.1016/0165-5876(87)90031-0

Hassanzadeh Taheri, M. M., Afshar, M., and Zardast, M. (2015). Unilateral duplication of the parotid duct, its embryological basis and clinical significance: a rare cadaveric case report. *Anat. Sci. Int.* 90, 197–200. doi: 10.1007/s12565-014-0243-0

Herrera-Calvo, G., Garcia-Montesinos-Perea, B., Saiz-Bustillo, R., Gallo-Teran, J., and Lastra-Garcia-Baron, P. (2011). Unilateral submandibular gland aplasia with ipsilateral sublingual gland hypertrophy presenting as a neck mass. *Med. Oral Patol. Oral Cir. Bucal.* 16, e537–e540. doi: 10.4317/medoral.16.e537

Higley, M. J., Walkiewicz, T. W., Miller, J. H., Curran, J. G., and Towbin, R. B. (2010). Aplasia of the parotid glands with accessory parotid tissue. *Pediatr. Radiol.* 40, 345–347. doi: 10.1007/s00247-009-1443-y

Hisatomi, M., Munhoz, L., Asaumi, J., and Arita, E. S. (2019). Stafne bone defects radiographic features in panoramic radiographs: assessment of 91 cases. *Med. Oral Patol. Oral Cir. Bucal.* 24, e12–e19. doi: 10.4317/medoral.22592

Horsburgh, A., and Massoud, T. F. (2013). The salivary ducts of Wharton and Stenson: analysis of normal variant sialographic morphometry and a historical review. *Ann. Anat.* 195, 238–242. doi: 10.1016/j.aanat.2012.11.003

Koybasioglu, A., Ileri, F., Gencay, S., Poyraz, A., Uslu, S., and Inal, E. (2000). Submandibular accessory salivary gland causing Warthin's duct obstruction. *Head Neck* 22, 717–721. doi: 10.1002/1097-0347(200010)22:7<717::aid-hed12>3.3.co;2-t

Kulkarni, C. D., Mittal, S. K., Katiyar, V., Pathak, O., and Sood, S. (2011). Accessory parotid gland with ectopic fistulous duct–diagnosis by ultrasonography, digital fistulography, digital sialography and CT fistulography. a case report and review of current literature. *J. Radiol. Case Rep.* 5, 7–14. doi: 10.3941/jrcr.v5i7.680

Lombaert, I., Movahednia, M. M., Adine, C., and Ferreira, J. N. (2017). Concise review: salivary gland regeneration: therapeutic approaches from stem cells to tissue organoids. *Stem Cells* 35, 97–105. doi: 10.1002/stem.2455

Mandel, L., and Alfi, D. (2012). Diagnostic imaging for submandibular duct atresia: literature review and case report. *J. Oral Maxillofac. Surg.* 70, 2819–2822. doi: 10.1016/j.joms.2012.02.032

Matsuda, C., Matsui, Y., Ohno, K., and Michi, K. (1999). Salivary gland aplasia with cleft lip and palate: a case report and review of the literature. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 87, 594–599. doi: 10.1016/s1079-2104(99)70140-x

McDonald, F. G., Mantas, J., Mcewen, C. G., and Ferguson, M. M. (1986). Salivary gland aplasia: an ectodermal disorder? *J. Oral Pathol.* 15, 115–117. doi: 10.1111/j.1600-0714.1986.tb00588.x

Nanduri, L. S., Lombaert, I. M., Van Der Zwaag, M., Faber, H., Brunsting, J. F., Van Os, R. P., et al. (2013). Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. *Radiother. Oncol.* 108, 458–463. doi: 10.1016/j.radonc.2013.05.020

Pham Dang, N., Picard, M., Mondie, J. M., and Barthelemy, I. (2010). Complete congenital agenesis of all major salivary glands: a case report and review of the literature. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 110, e23–e27. doi: 10.1016/j.tripleo.2010.04.008

Philipsen, H. P., Takata, T., Reichart, P. A., Sato, S., and Suei, Y. (2002). Lingual and buccal mandibular bone depressions: a review based on 583 cases from a worldwide literature survey, including 69 new cases from Japan. *Dentomaxillofac. Radiol.* 31, 281–290. doi: 10.1038/sj.dmfr.4600718

Pownell, P. H., Brown, O. E., Pransky, S. M., and Manning, S. C. (1992). Congenital abnormalities of the submandibular duct. *Int. J. Pediatr. Otorhinolaryngol.* 24, 161–169. doi: 10.1016/0165-5876(92)90142-c

Pringle, S., Maimets, M., Van Der Zwaag, M., Stokman, M. A., Van Gosliga, D., Zwart, E., et al. (2016). Human salivary gland stem cells functionally restore radiation damaged salivary glands. *Stem Cells* 34, 640–652. doi: 10.1002/stem.2278

Prosdocimo, M. L., Barreto Nogueira, A. P., Aparecida De Albuquerque Cavalcante, M., Agostini, M., Benevenuto De Andrade, B. A., and Romanach, M. J. (2018). Congenital dilatation of the submandibular duct. *Int. J. Pediatr. Otorhinolaryngol.* 113, 16–18. doi: 10.1016/j.ijporl.2018.07.008

Rosow, D. E., Ward, R. F., and April, M. M. (2009). Sialodochostomy as treatment for imperforate submandibular duct: a systematic literature review and report of two cases. *Int. J. Pediatr. Otorhinolaryngol.* 73, 1613–1615. doi: 10.1016/j.ijporl.2009.03.007

Rushton, V. E., and Pemberton, M. N. (2005). Salivary otorrhoea: a case report and a review of the literature. *Dentomaxillofac. Radiol.* 34, 376–379. doi: 10.1259/dmfr/21133587

Sanli, E. C., Ozturk, N. C., Polat, A., and Ozturk, H. (2010). Bilateral and symmetrical heterotopic submandibular glands in the upper neck: case report. *Surg. Radiol. Anat.* 32, 979–982. doi: 10.1007/s00276-010-0641-5

Santarelli, A., Mascitti, M., Rubini, C., Bambini, F., Zizzi, A., Offidani, A., et al. (2015). Active inflammatory biomarkers in oral lichen planus. *Int. J. Immunopathol. Pharmacol.* 28, 562–568. doi: 10.1177/0394632015592101

Tanaka, J., Ogawa, M., Hojo, H., Kawashima, Y., Mabuchi, Y., Hata, K., et al. (2018). Generation of orthotopically functional salivary gland from embryonic stem cells. *Nat. Commun.* 9:4216. doi: 10.1038/s41467-018-06469-7

Yan, Z., Ding, N., Liu, X., and Hua, H. (2012). Congenital agenesis of all major salivary glands and absence of unilateral lacrimal puncta: a case report and review of the literature. *Acta Otolaryngol.* 132, 671–675. doi: 10.3109/00016489.2011.648273

Zhang, L., Xu, H., Cai, Z. G., Mao, C., Wang, Y., Peng, X., et al. (2010). Clinical and anatomic study on the ducts of the submandibular and sublingual glands. *J. Oral Maxillofac. Surg.* 68, 606–610. doi: 10.1016/j.joms.2009.03.068

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor is currently organizing a Research Topic with one of the authors, AS, and confirms the absence of any other collaboration.

frontiers in Physiology

SYSTEMATIC REVIEW
published: 13 August 2019
doi: 10.3389/fphys.2019.01029

Do Changes in Oral Microbiota Correlate With Plasma Nitrite Response? A Systematic Review

Khrystyna Zhurakivska[1], Giuseppe Troiano[1], Vito Carlo Alberto Caponio[1], Mario Dioguardi[1], Luigi Laino[2], Angela Bruna Maffione[1] and Lorenzo Lo Muzio[1]*

[1] Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy, [2] Multidisciplinary Department of Medical-Surgical and Odontostomatological Specialties, University of Campania "Luigi Vanvitelli", Naples, Italy

Background: Nitric Oxide (NO) has a role in immunitary defense, regulation of mucosal blood flow and mucus production, regulation of smooth muscle contraction, cerebral blood flow, glucose regulation, and mitochondrial function. NO can be synthetized endogenously through the L-arginine-NO pathway or it can be absorbed by the human intestine through the dietary intake. Most of the ingested NO is in the form of nitrate (NO_3^-). NO_3^- is a substrate of oral and intestinal microbiota and, at the end of the catabolic pathway, NO is released. Using antibacterial mouthwashes leads to an alteration of salivary NO_3^- metabolism, however, with unclear consequences on the circulating NO levels. The aim of this study is to perform a systematic review in order to elucidate if the alterations of oral microbiota lead to modifications in plasma NO content.

Methods: Electronic databases were screened, using the following terms: ["oral bacteria" and (nitrate OR nitrite OR nitric)]. Clinical studies reporting NO_3^- and NO_2^- measurements in blood and their correlation to oral microbiota variations were included. We focused on the correlation between the changes in oral microbiota and plasma concentrations of nitrites (primary outcome). Subsequently, we investigated if modifications in oral microbiota could lead to changes in blood pressure and salivary NO_2^- concentration (secondary outcome).

Results: Six studies, for a total of 82 participants were included in this review. In four studies, the use of mouthwash correlated to a reduction of plasma nitrite concentration ($p < 0.05$); Two studies did not find any difference in plasma nitrate or nitrite concentration. In five studies, a correlation between blood pressure (BP) changes and antibacterial mouthwashing emerged. Anyway, only three studies suggested a significant increase of systolic BP following mouthwashing compared with controls.

Conclusions: Although, the role of oral bacteria has been unequivocally demonstrated in the regulation of salivary NO_3^- metabolism, their influence on plasma concentration of NO species remains ambiguous. Further studies with larger sample size are required in order to demonstrate if an alteration in oral microbiota composition may influence the blood content of $NO_3^-/NO_2^-/NO$ and all the linked biological processes.

Keywords: oral microbiota, oral bacteria, nitrite (nitrate) [NO_2^- (NO_3^-)], mouthwash, nitric oxide—NO

OPEN ACCESS

Edited by:
Giovanna Orsini, Marche Polytechnic University, Italy

Reviewed by:
Heleni Vastardis, National and Kapodistrian University of Athens, Greece
Harald Osmundsen, University of Oslo, Norway

****Correspondence:***
Khrystyna Zhurakivska
khrystyna.zhurakivska@unifg.it

Specialty section:
This article was submitted to Craniofacial Biology and Dental Research, a section of the journal Frontiers in Physiology

Received: *27 March 2019*
Accepted: *25 July 2019*
Published: *13 August 2019*

Citation:
Zhurakivska K, Troiano G, Caponio VCA, Dioguardi M, Laino L, Maffione AB and Lo Muzio L (2019) Do Changes in Oral Microbiota Correlate With Plasma Nitrite Response? A Systematic Review. Front. Physiol. 10:1029. doi: 10.3389/fphys.2019.01029

INTRODUCTION

The specific contributions of oral microbiota in human physiopathology has not been explored yet, although different studies report how the composition of oral microbiota has a role in oral and systemic diseases (Santarelli et al., 2015; Sampaio-Maia et al., 2016; Aarabi et al., 2018; Cardoso et al., 2018).

In particular, some resident bacteria in the oral cavity are able to reduce the dietary intake of nitrates (NO_3^-), producing nitrites (NO_2^-). Among these, *Neisseria, Veillonella, Haemophilus, Porphyromonas, Fusobacterium, Prevotella, Leptotrichia, Brevibacillus, and Granulicatella* are mainly involved in this process (Doel et al., 2005; Hyde et al., 2014). The main dietary source of nitrate is represented by vegetables (Ysart et al., 1999). Once ingested, nitrate is absorbed in the gastrointestinal tract and enters in the bloodstream. Here, it mixes to the endogenous nitrate, which mainly derives from the L-arginine-NO pathway (Leaf et al., 1989). Most of the nitrate is then excreted in the urine, while up to 25% of plasma nitrate is taken up by the salivary glands and placed in the enterosalivary circulation (Spiegelhalder et al., 1976). Salivary nitrate is metabolized to nitrite by oral commensal bacteria and it can be further reduced to nitric oxide (NO). NO_2^- can be stored in blood and tissues and used when there is a decreased endogenous production of NO (Bryan and Loscalzo, 2011). Consequently, NO may contribute to a myriad of biological processes, among which: immunitary defense, regulation of mucosal blood flow and mucus production, regulation of smooth muscle contraction, cerebral blood flow, glucose regulation, and mitochondrial function (Bryan and Loscalzo, 2011). The above described processes are known as "nitrate-nitrite-nitric oxide pathway" (Zweier et al., 1995). Considering this physiological pathway, the oral microbiota gained a fundamental role; changes in its composition were assumed to affect the final NO production (Hyde et al., 2014). In particular, since the 1980s, it has been proposed that antimicrobial mouthwashing could inhibit the nitrite formation in saliva (Tannenbaum et al., 1976). Subsequently, it was supposed that such alterations could also lead to variations in the systemic availability of NO (Govoni et al., 2008; Kapil et al., 2013; Woessner et al., 2016). Plasma analysis may contribute to better understand the NO blood availability because of changes in the oral microbiota.

The aim of this study is to perform a systematic review in order to elucidate if alterations of oral microbiota lead to modifications in plasma content of nitrogen species.

MATERIALS AND METHODS

The protocol for this systematic review followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Liberati et al., 2009).

In addition, it was prospectively registered on the online database PROSPERO (International prospective register of systematic reviews) with the registration number CRD42019124473.

This systematic review was performed in order to answer the following question: "Do changes in oral microbiota influence variations in blood content of nitrogen species?"

Electronic databases PubMed, SCOPUS, and Web of Science were screened independently by two authors, KZ and GT, in order to select studies suitable for inclusion in this review. The following strategy of search was used: ["oral bacteria" AND (nitrate OR nitrite OR nitric)]. In addition, bibliographies of systematic reviews and included studies were manually revised in order to find other articles to be included in this study.

Only studies published in English language and fulfilling the following criteria were considered eligible for inclusion in this review:

- Original clinical studies reporting NO_3^- and NO_2^- blood measurements correlated with oral microbiota variations.

No restrictions were applied about the year of publication. Studies eligibility was independently assessed in a joint session by two authors (KZ and GT). Authors screened for articles by reading only title and abstract of the studies, according to the eligibility criteria. If the data reported in abstract were not sufficient to make a clear decision, the full text publication was examined. The selected papers were full-text evaluated in a second round and, if fulfilling the inclusion criteria, were included in the qualitative analysis. Any disagreement was solved in a discussion between reviewers in a joint session.

The following information was extracted from the included papers:

- Authors' names and year of publication, study design, number of participants, type of intervention, plasma NO_3^- and NO_2^- changes, salivary NO_3^- and NO_2^- changes, Blood Pressure Changes, Relative abundance of NO_3^- reducing species.

We focused on the correlation between the changes in oral microbiota and plasma concentrations of nitrites (primary outcome). Subsequently, we also investigated if modifications in oral microbiota could lead to changes in blood pressure and salivary NO_2^- concentration (secondary outcome).

RESULTS

A total of 200 records were retrieved after the application of search strategy. After the first screening and duplicates removing,

TABLE 1 | Summary of excluded studies.

Number	References	Reasons for exclusion
1	Clodfelter et al., 2015	Do not deal with oral microbiota changes.
2	Kapil et al., 2018	Do not deal with oral microbiota changes.
3	Doel et al., 2005	Do not report the blood content of NOx species.
4	Hyde et al., 2014	Do not report the blood content of NOx species.
5	Lundberg et al., 2004	Not original study.
6	Qu et al., 2016	Not original study.
7	Koch et al., 2017	Not original study.
8	Preshaw, 2018	Not original study.

84 records were chosen for title and abstract evaluation. Fourteen studies, resulting after this step, were selected for full text examination. Eight studies were excluded because did not meet inclusion criteria (Lundberg et al., 2004; Doel et al., 2005; Hyde et al., 2014; Clodfelter et al., 2015; Qu et al., 2016; Koch et al., 2017; Kapil et al., 2018; Preshaw, 2018). The reasons for exclusion are detailed in **Table 1**. The flow-chart in **Figure 1** summarizes the selection process of inclusion. At the end, six papers were included in the systematic review (**Table 2**; Govoni et al., 2008; Kapil et al., 2013; Bondonno et al., 2015; McDonagh et al., 2015; Sundqvist et al., 2016; Woessner et al., 2016). In these studies, the test groups (mouthwashing) were compared to control (no mouthwashing) and implications on the production of nitrogen species were evaluated. Other three studies with a different design were identified and deemed valid to be included in the present review (Burleigh et al., 2018; Vanhatalo et al., 2018; Liddle et al., 2019). They only investigated how changes in oral microbiota could lead to alterations in NO_3^-/NO_2^- concentrations in human fluids. They were considered relevant for the development of the topic and are showed separately in the results (**Table 3**).

The designs of the experiments were heterogeneous among studies, so it was not possible to perform a quantitative analysis. A total of 82 participants were enrolled in the included studies. The effect of various mouthwashes on NO_3^- metabolism was investigated, providing discordant results among the studies. Four studies (Govoni et al., 2008; Kapil et al., 2013; McDonagh et al., 2015; Woessner et al., 2016) detected a significant negative influence of mouthwashing on plasma nitrite concentration ($p < 0.05$), meanwhile other two studies (Bondonno et al., 2015; Sundqvist et al., 2016) found no significant alteration of plasma nitrate and nitrite, comparing mouthwash administration against placebo.

The types of mouthwashes used in the studies were different; nevertheless all of them contained chlorhexidine. In two studies more than one mouthwash was tested (McDonagh et al., 2015; Woessner et al., 2016), resulting in some differences in the outcomes (**Table 2**). Results regarding the secondary outcome:

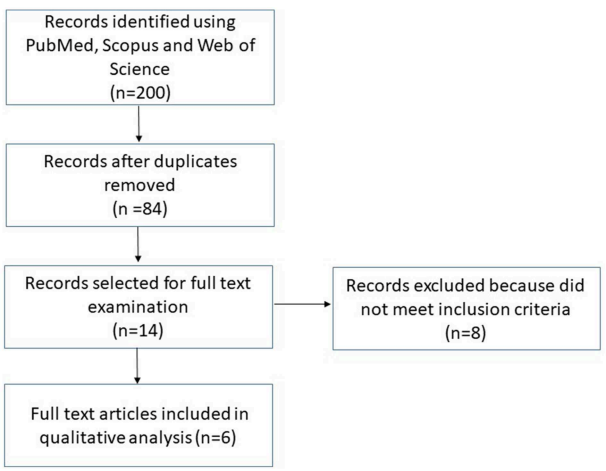


FIGURE 1 | Flow-chart summarizing the selection process.

- Effects of mouthwashing on the salivary NO_2^- changes: there were significant differences between the test and the control groups in all the studies (Govoni et al., 2008; Kapil et al., 2013; Bondonno et al., 2015; McDonagh et al., 2015; Sundqvist et al., 2016; Woessner et al., 2016).
- Effects of mouthwashing on Blood Pressure were evaluated in five studies: in three studies, Systolic Blood Pressure resulted significantly higher in the test groups compared to the controls (Kapil et al., 2013; Bondonno et al., 2015; Woessner et al., 2016). One study did not report any difference between test and control group (Sundqvist et al., 2016). In one study, the differences emerged only in some conditions (i.e., during treadmill walking), but not in others (McDonagh et al., 2015).

Three of the included studies, identified the types of oral nitrate reducing species and correlated their abundance with plasma NO_2^- changes (Burleigh et al., 2018; Vanhatalo et al., 2018; Liddle et al., 2019). The most abundant species emerged to be: *Prevotella melaninogenica*, *Veillonella dispar*, *Hemophilus parainfluenzae*, *Neisseria subflava*, *Veillonella parvula*, *Fusobacterium nucleatum* subsp. nucleatum, *Campylobacter concisus*, *Leptorichia buccalis*, *Prevotella* intermedia. In two of these studies no correlation emerged between the abundance of NO_3^- reducing species and plasma NO_2^- changes (Burleigh et al., 2018; Liddle et al., 2019). Furthermore, in one study emerged how NO_3^- supplementation influenced the salivary microbiome, leading to an increase in the relative abundance of *Proteobacteria* (+225%) and decrease of *Bacteroidetes* (−46%; $P < 0.05$; Vanhatalo et al., 2018). High relative quantities of *Rothia* and *Neisseria* and low presence of *Prevotella* and *Veillonella* were correlated with greater increases in plasma $[NO_2^-]$ in response to NO_3^- supplementation (Vanhatalo et al., 2018).

DISCUSSION

Numerous bacteria express nitrate reductase genes and are capable to reduce nitrate to nitrite *in vitro* (Torres et al., 2016). In humans a physiologically relevant nitrate reduction occurs by means of some facultative anaerobic bacteria localized in crypts of the tongue (Duncan et al., 1995; Doel et al., 2005). Among the highest nitrate-reducers species, identified in the oral cavity, there are: *Firmicutes* (*Staphylococcus*, *Streptococcus*, and *Veillonella*) and *Actinobacteria* (*Actinomyces*), followed by numerous other taxa, including *Pasteurella*, *Rothia*, *Neisseria*, *Haemophilus*, *Granulicatella* (Smith et al., 1999; Palmerini et al., 2003; Hyde et al., 2014). Using dietary nitrate supplementation as selective pressure for bacteria capable of nitrate reduction, several studies found proliferation of *Veillonella* (Koopman et al., 2016), *Neisseria* (Velmurugan et al., 2016), and *Rothia* species (Velmurugan et al., 2016), all previously identified as high nitrate reducers. These data, as well as those of the studies examined in this review, confirm a close link between the oral microbiota and the salivary circulation of nitrates. However, the scientific evidence of a systemic repercussion is still poor today.

In order to evaluate the efficacy and/or alteration of conversion of oral nitrate to estimate the availability of NO pool, most of the studies, including our analysis, used plasma

TABLE 2 | Summary of data extracted from the included studies.

References	N° of participants	Study design	Type of intervention		Salivary NO_3^- changes	Salivary NO_2^- changes	Plasma NO_3^- changes	Plasma NO_2^- changes	Blood pressure changes	Relative abundance of NO_3^- reducing species
			Test (T)	Control (C)						
Govoni et al., 2008	7	Cross-over	MW (10 ml of Corsodyl) before ingestion of sodium nitrate (10 mg/kg in 100 ml water)	Ingestion of sodium nitrate (10 mg/kg in 100 ml water)	No significant difference between groups	Abolished immediately after MW and remained below basal levels during the entire observation period	No significant difference between groups	The rise in test group was markedly attenuated	Not determined	The bacterial counts were reduced by a mean of 80% after the mouth wash
Kapil et al., 2013	19	Cross-over	MW (10 ml Corsodyl 0.2% chlorhexidine) twice daily for 7 days	No intervention	No changes in Control group; Increase in Test group (***p* < 0.001**)	No changes in Control group; Decrease in Test group (−282 ± 35 μmol/L, ***p* < 0.001)**	No changes in Control group; Increase in Test group (***p* = 0.0048**)	No changes in Control group; Decrease in Test group (−71 ± 15 nmol/L, ***p* = 0.001**)	Increasing of clinic SBP in Test group (***p* = 0.003**)	Not determined
McDonagh et al., 2015	12	Crossover	STRONG MW (Corsodyl®); WEAK; (Vademecum med® non-chlorexidine) 3 times daily, followed by BR consuming (70 mL containing ∼6.2 mmol of $NO3^-$) twice daily	Deionised water, followed by BR consuming (70 mL containing ∼6.2 mmol of $NO3^-$) twice daily	At 2 h: the increase was greater in STRONG compared to CON (***P* < 0.05**) but not WEAK ($P > 0.05$). At 4 h the increase was significantly larger in STRONG compared to CON and WEAK (***p* < 0.05**)	The increase was significantly lower at 2 h in STRONG compared to CON ($P <$ **0.05**). At 4 h, was lower after STRONG compared to CON and WEAK ($p <$ **0.05**)	The increase was not significantly different between CON, WEAK, and STRONG at 2 or 4 h post final BR ingestion	At 2 h, the elevation was lower in STRONG compared to CON (by 200 ± 174 nM) and WEAK (by 89 ± 112 nM) (***P* < 0.05**); change was lower (by 110 ± 157 nM) in WEAK compared to CON at 2 h (***P* < 0.05**). At 4 h was higher in CON and WEAK, compared to STRONG (***P* < 0.01**).	No differences in resting BP changes ($P > 0.05$). During treadmill walking, the increase in SBP and MBP was higher 4 h after the final nitrate bolus in STRONG compared with CON (***P* < 0.05**) but not WEAK	Not determined
Bondonno et al., 2015	15	Cross-over	Antibacterial MW 1.28 mg/mol chlorhexidine Gluconate twice daily for 3 days	Tap water	Increase (MW: 686 μmol/L; control: 252 μmol/L, ***p* < 0.001**)	Decrease (mouthwash: 41 μmol/L; control: 111 μmol/L, ***p* = 0.01**)	No difference (MW: 34.6 μmol/L, control: 30.0 μmol/L, $p = 0.2$)	A trend toward decreased not reaching statistical significance (MW: 150 nmol/L, control: 180 nmol/L, $p = 0.09$)	Test group compared to control: increase in SBP (2.3 mm Hg; ***p* = 0.01**) but did not increase DBP (0.7 mm Hg; $p = 0.4$)	Not determined

(Continued)

TABLE 2 | Continued

References	N° of participants	Study design	Type of intervention		Salivary NO_3^- changes	Salivary NO_2^- changes	Plasma NO_3^- changes	Plasma NO_2^- changes	Blood pressure changes	Relative abundance of NO_3^- reducing species
			Test (T)	**Control (C)**						
Sundqvist et al., 2016	17	Crossover	MW (2 g chlorhexidine, 0.55 g menthol, 70 ml 95% ethanol and 930 ml distilled water per 1,000 ml total volume) three times a day for 3 days	MW without clorexidine three times a day for 3 days	Higher in Test: 1,118 μM; placebo: 401 μM, ***p* < 0.01**	Lower in Test: 23 μM; placebo: 248 μM, ***p* < 0.001**	No difference (Test: 23 μM; placebo: 22 μM)	No difference: (Test 0.21 μM; placebo 0.22 μM)	No significant changes	Not determined
Woessner et al., 2016	12	Crossover	Two 70 mL bottles of BR juice for a total of 8.4 mmol nitrate intake, followed by 1) Listerine® antiseptic mouthwash (active ingredients: Eucalyptol 0.092%, Menthol 0.042%, Methyl salicylate 0.060%, Thymol 0.064%), 2) Cepacol ® antibacterial mouthwash (active ingredients: Cetylpyridinium chloride 0.05%), 3) Chlorhexidine mouthwash (active ingredient: chlorhexidine gluconate 0.12%)	Two 70 mL bottles of BR juice for a total of 8.4 mmol nitrate intake, followed by water	No differences among treatments ($p = 0.33$)	1) Antiseptic MW lower than control treatment (***p* < 0.05**); 2) Antibacterial MW lower than antiseptic (***p* < 0.05**); 3) Chlorexidine MW lower than antiseptic (***p*<0.05**), but not significantly lower than Antibacterial ($p = 0.07$)	No effect for MW treatments ($p = 79$)	1) antiseptic MW not significantly different than control ($p = 0.45$); 2) Antibacterial MW lower than control (***p* < 0.05**); 3) chlorhexidine MW lower than control (***p* < 0.01**)	SBP significantly higher in Antibacterial and Chlorexidine conditions than in control (***p* < 0.01**) and Antiseptic (***p* < 0.05**) conditions	Not determined

MW, mouthwash; BP, Blood Pressure; SBP, Systolic Blood Pressure; DBP, diastolic blood pressure; MBP, mean Blood Pressure. Statistically significant values are in bold.

TABLE 3 | Summary of data extracted from studies evaluating oral microbiota composition.

References	N° of participants	Study design	Type of intervention		Relative abundance of NO_3^- reducing species	Salivary NO_3^- changes	Salivary NO_2^- changes	Plasma NO_3^- changes	Plasma NO_2^- changes
			Test	**Control**					
Vanhatalo et al., 2018	18	Crossover	Two 10-day dietary supplementation periods with NO3$^-$ (NO3$^-$ -rich concentrated beetroot juice (BR) (2× 70 ml d−1, each 70 ml containing ∼6.2 mmol NO_3^-	Two 10-day supplementation periods with NO3$^-$ -depleted concentrated beetroot juice placebo (PL) (2× 70 ml d−1, each 70 ml containing ∼0.01 mmol NO_3^-)	NO_3^- supplementation altered the salivary microbiome compared to placebo by increasing the relative abundance of Proteobacteria (+225%) and decreasing the relative abundance of Bacteroidetes (−46%; $P < 0.05$). After NO^{3-}supplementation the relative abundances of Rothia (+127%) and *Neisseria* (+351%) were greater, and *Prevotella* (−60%) and *Veillonella* (−65%) were lower than in the placebo condition (all ***P* < 0.05**)	Not determined	Not determined	A significant correlation between plasma[NO_2^-]/NO_3^- dose and %relative abundance of taxonomic units: - *Campylobacter concisus*: −0.55 (***p* < 0.05**) - *Prevotella* (genus) −0.49 (***p* < 0.05**) - *Prevotella melaninogenica* −0.57 (***p* < 0.05**); - *Fusobacterium nucleatum* subsp. Vincentii: 0.55 (***p* < 0.05**)	
Burleigh et al., 2018	25		Ingestion of 2 × 70 ml of NO3$^-$ –rich beetroot juice (∼12.4 mmol NO3$^-$)		*Prevotella melaninogenica* 31.43 ± 10.33; *Veillonella dispar* 19.30± 11.97; *Hemophilus parainfluenzae* 2.78 ± 3.83; *Neisseria subflava* 2.57 ± 5.5.2; *Veillonella parvula* 0.24 ± 0.46; *Rothia mucilaginosa* 0.37 ± 0.49; *Rothia dentocarinosa* 0.003 ± 0.004	Not correlated with % relative bacteria abundance ($r = 0.30$; $p = 0.17$)	Correlated with % relative bacteria abundance ($r = 0.44$; $p = 0.03$)	Not correlated with % relative bacteria abundance ($r = −0.25$; $p = 0.25$)	Not correlated % relative bacteria abundance ($r = −0.06$; $p = 0.78$)
Liddle et al., 2019	10	Crossover	Ingestion of 2 × 70 ml of BR (total of ∼12.4 mmol NO_3^-		*Prevotella melaninogenica* 23.8 ± 6.4; *Veillonella dispar* 13.0 ± 4.0; *Haemophilus parainfluenzae* 6.5 ± 5.9; *Neisseria subflava* 1.7 ± 1.0; *Veillonella parvula* 0.9 ± 0.4; *Rothia mucilaginosa* 0.2 ± 0.1; *Rothia dentocariosa* <0.01 ± <0.01	Not determined	Not correlated with the sum of the NO3$^-$ –reducing bacteria ($P > 0.2$) Abundance of *Neisseria subflava* was negatively associated with peak salivary [NO2$^-$] ($R = −0.43$, $P = 0.03$)	Not determined	Not correlated with the sum of the NO_3^- reducing bacteria Abundance of *Neisseria subflava* was negatively associated with plasma [NO2$^-$] ($R = −0.43$, $P = 0.03$)

*[NO2$^-$]/NO3$^-$ dose = plasma [nitrite] relative to nitrate dose per kg body mass ingested; *p < 0.05. Statistically significant values are in bold.*

nitrite concentration (or changes in concentration), since the direct measurement of NO is difficult. This approach is justified by the relationships between plasma nitrite concentrations and physiological effects, as demonstrated by James et al. (2015). For this reason, only studies reporting blood measurements of nitrogen species were included in this review. Given this, the aim of the review was to verify even the alteration of oral microbiota could have a direct impact on plasma changes in nitrite concentration and, as proposed by some studies (Bondonno et al., 2015; Tribble et al., 2019), repercussions on Blood pressure or other biological processes (Joshipura et al., 2017).

From our analysis emerged some discordant results. Most of the evaluated studies report a correlation between oral microbiota and nitrites variations in plasma (Govoni et al., 2008; Kapil et al., 2013; McDonagh et al., 2015; Woessner et al., 2016). Two studies found no effect of mouthwashing on changes in plasma nitrites (Bondonno et al., 2015; Sundqvist et al., 2016). Govoni et al. (2008), despite having found a marked attenuation after mouthwash, noted that a significant rise in plasma nitrite occurred. Interestingly, this occurred despite the fact that the nitrite formation in the mouth has been completely abolished by the intervention, excluding its salivary origin. They proposed the existence of an alternative origin of nitrite, giving two possible explanations for this phenomenon: in the first hypothesis, they supposed a possible contribute of the gastrointestinal microflora to the nitrate reduction; in the second one, they hypothesized that mammalian cells in the gut wall o in another district are capable of nitrate reduction. However, despite this interesting hypothesis, results show that the sudden rise in plasma nitrite after nitrate ingestion is mainly deriving from bacterial nitrate reduction in the oral cavity and the use of antibacterial mouthwashes may have an impact on this chain. Even if, results of the interventions analyzed in the present review are statistically significant, the clinical repercussions need further investigation. Among these, the effects of the mouthwashes on Blood Pressure were those more analyzed, given the strong effect that the intake of rich in nitrates food has on blood pressure (Ashworth and Bescos, 2017). Following mouthwashing, a certain increase in blood pressure occurred in three studies (Kapil et al., 2013; Bondonno et al., 2015; Woessner et al., 2016), although, it should be noted that in one of these studies (Bondonno et al., 2015), the increase in BP was not associated with a statistically significant reduction in plasma nitrites. This controversial result could be linked to a bias in the kind of the patients included. The experiments were conducted in hypertensive men and women already in treatment with antihypertensive medication.

Another important risk of bias in interpreting the results, is given by the heterogeneity of the experiments conducted in various studies, in particular by the different timing of the fluid analysis, which could lead to mismatch in the comparison of the results, since the nitrate metabolism is subject to sudden changes in association with time.

CONCLUSIONS

Although, most of the included studies demonstrate a statistically significant correlation between an alteration of the oral microbiota due to the use of antibacterial mouthwash and plasma changes in nitrite concentration, this link has not been established yet. Further RCT studies with larger samples and well defined designs are necessary in order to demonstrate if an alteration in oral microbiota composition may influence the blood content of NO_3^-/NO_2^-/NO and, consequently, all the biological processes that depends on them.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and the supplementary files.

AUTHOR CONTRIBUTIONS

KZ and GT performed online research and collected data. VC analyzed data and results. LLa and MD contribute to write the manuscript. LLo and AM conceived and supervised the research.

REFERENCES

Aarabi, G., Schnabel, R. B., Heydecke, G., and Seedorf, U. (2018). Potential impact of oral inflammations on cardiac functions and atrial fibrillation. *Biomolecules* 8:E66. doi: 10.3390/biom8030066

Ashworth, A., and Bescos, R. (2017). Dietary nitrate and blood pressure: evolution of a new nutrient? *Nutr. Res. Rev.* 30, 208–219. doi: 10.1017/S0954422417000063

Bondonno, C. P., Liu, A. H., Croft, K. D., Considine, M. J., Puddey, I. B., Woodman, R. J., et al. (2015). Antibacterial mouthwash blunts oral nitrate reduction and increases blood pressure in treated hypertensive men and women. *Am. J. Hypertens.* 28, 572–575. doi: 10.1093/ajh/hpu192

Bryan, N. S., and Loscalzo, J. (2011). *Nitrite and Nitrate in Human Health and Disease.* Cham: Springer. doi: 10.1007/978-1-60761-616-0

Burleigh, M. C., Liddle, L., Monaghan, C., Muggeridge, D. J., Sculthorpe, N., Butcher, J. P., et al. (2018). Salivary nitrite production is elevated in individuals with a higher abundance of oral nitrate-reducing bacteria. *Free Radic. Biol. Med.* 120, 80–88. doi: 10.1016/j.freeradbiomed.2018.03.023

Cardoso, E. M., Reis, C., and Manzanares-Céspedes, M. C. (2018). Chronic periodontitis, inflammatory cytokines, and interrelationship with other chronic diseases. *Postgrad. Med.* 130, 98–104. doi: 10.1080/00325481.2018.1396876

Clodfelter, W. H., Basu, S., Bolden, C., Dos Santos, P. C., King, S. B., and Kim-Shapiro, D. B. (2015). The relationship between plasma and salivary NOx. *Nitric Oxide* 47, 85–90. doi: 10.1016/j.niox.2015.04.003

Doel, J. J., Benjamin, N., Hector, M. P., Rogers, M., and Allaker, R. P. (2005). Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur. J. Oral Sci.* 113, 14–19. doi: 10.1111/j.1600-0722.2004.00184.x

Duncan, C., Dougall, H., Johnston, P., Green, S., Brogan, R., Leifert, C., et al. (1995). Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat. Med.* 1, 546–551. doi: 10.1038/nm0695-546

Govoni, M., Jansson, E. A., Weitzberg, E., and Lundberg, J. O. (2008). The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* 19, 333–337. doi: 10.1016/j.niox.2008.08.003

Hyde, E. R., Andrade, F., Vaksman, Z., Parthasarathy, K., Jiang, H., Parthasarathy, D. K., et al. (2014). Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: implications for nitric oxide homeostasis. *PLoS ONE* 9:e88645. doi: 10.1371/journal.pone.0088645

James, P. E., Willis, G. R., Allen, J. D., Winyard, P. G., and Jones, A. M. (2015). Nitrate pharmacokinetics: taking note of the difference. *Nitric Oxide* 48, 44–50. doi: 10.1016/j.niox.2015.04.006

Joshipura, K. J., Muñoz-Torres, F. J., Morou-Bermudez, E., and Patel, R. P. (2017). Over-the-counter mouthwash use and risk of pre-diabetes/diabetes. *Nitric Oxide* 71, 14–20. doi: 10.1016/j.niox.2017.09.004

Kapil, V., Haydar, S. M., Pearl, V., Lundberg, J. O., Weitzberg, E., and Ahluwalia, A. (2013). Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic. Biol. Med.* 55, 93–100. doi: 10.1016/j.freeradbiomed.2012.11.013

Kapil, V., Rathod, K. S., Khambata, R. S., Bahra, M., Velmurugan, S., Purba, A., et al. (2018). Sex differences in the nitrate-nitrite-NO(*) pathway: role of oral nitrate-reducing bacteria. *Free Radic. Biol. Med.* 126, 113–121. doi: 10.1016/j.freeradbiomed.2018.07.010

Koch, C. D., Gladwin, M. T., Freeman, B. A., Lundberg, J. O., Weitzberg, E., and Morris, A. (2017). Enterosalivary nitrate metabolism and the microbiome: intersection of microbial metabolism, nitric oxide and diet in cardiac and pulmonary vascular health. *Free Radic. Biol. Med.* 105, 48–67. doi: 10.1016/j.freeradbiomed.2016.12.015

Koopman, J. E., Buijs, M. J., Brandt, B. W., Keijser, B. J., Crielaard, W., and Zaura, E. (2016). Nitrate and the origin of saliva influence composition and short chain fatty acid production of oral microcosms. *Microb. Ecol.* 72, 479–492. doi: 10.1007/s00248-016-0775-z

Leaf, C. D., Wishnok, J. S., and Tannenbaum, S. R. (1989). L-arginine is a precursor for nitrate biosynthesis in humans. *Biochem. Biophys. Res. Commun.* 163, 1032–1037. doi: 10.1016/0006-291X(89)92325-5

Liberati, A., Altman, D. G., Tetzlaff, J., Mulrow, C., Gøtzsche, P. C., Ioannidis, J. P., et al. (2009). The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *J. Clin. Epidemiol.* 62, e1–e34. doi: 10.1016/j.jclinepi.2009.06.006

Liddle, L., Burleigh, M. C., Monaghan, C., Muggeridge, D. J., Sculthorpe, N., Pedlar, C. R., et al. (2019). Variability in nitrate-reducing oral bacteria and nitric oxide metabolites in biological fluids following dietary nitrate administration: an assessment of the critical difference. *Nitric Oxide* 83, 1–10. doi: 10.1016/j.niox.2018.12.003

Lundberg, J. O., Weitzberg, E., Cole, J. A., and Benjamin, N. (2004). Nitrate, bacteria and human health. *Nat. Rev. Microbiol.* 2, 593–602. doi: 10.1038/nrmicro929

McDonagh, S. T., Wylie, L. J., Winyard, P. G., Vanhatalo, A., and Jones, A. M. (2015). The effects of chronic nitrate supplementation and the use of strong and weak antibacterial agents on plasma nitrite concentration and exercise blood pressure. *Int. J. Sports Med.* 36, 1177–1185. doi: 10.1055/s-0035-1554700

Palmerini, C. A., Palombari, R., Perito, S., and Arienti, G. (2003). NO synthesis in human saliva. *Free Radic. Res.* 37, 29–31. doi: 10.1080/1071576021000028398

Preshaw, P. M. (2018). Mouthwash use and risk of diabetes. *Br. Dent. J.* 225, 923–926. doi: 10.1038/sj.bdj.2018.1020

Qu, X. M., Wu, Z. F., Pang, B. X., Jin, L. Y., Qin, L. Z., and Wang, S. L. (2016). From nitrate to nitric oxide: the role of salivary glands and oral bacteria. *J. Dent. Res.* 95, 1452–1456. doi: 10.1177/0022034516673019

Sampaio-Maia, B., Caldas, I. M., Pereira, M. L., Pérez-Mongiovi, D., and Araujo, R. (2016). The oral microbiome in health and its implication in oral and systemic diseases. *Adv. Appl. Microbiol.* 97, 171–210. doi: 10.1016/bs.aambs.2016.08.002

Santarelli, A., Mascitti, M., Rubini, C., Bambini, F., Zizzi, A., Offidani, A., et al. (2015). Active inflammatory biomarkers in oral lichen planus. *Int. J. Immunopathol. Pharmacol.* 28, 562–568. doi: 10.1177/03946320155 92101

Smith, A., Benjamin, N., Weetman, D., Mackenzie, D., and Macfarlane, T. (1999). The microbial generation of nitric oxide in the human oral cavity. *Microb. Ecol. Health Dis.* 11, 23–27. doi: 10.3402/mehd.v11i1.7880

Spiegelhalder, B., Eisenbrand, G., and Preussmann, R. (1976). Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in vivo* formation of N-nitroso compounds. *Food Cosmet. Toxicol.* 14, 545–548. doi: 10.1016/S0015-6264(76)80005-3

Sundqvist, M. L., Lundberg, J. O., and Weitzberg, E. (2016). Effects of antiseptic mouthwash on resting metabolic rate: a randomized, double-blind, crossover study. *Nitric Oxide* 61, 38–44. doi: 10.1016/j.niox.2016.10.003

Tannenbaum, S. R., Weisman, M., and Fett, D. (1976). The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet. Toxicol.* 14, 549–552. doi: 10.1016/S0015-6264(76)80006-5

Torres, M. J., Simon, J., Rowley, G., Bedmar, E. J., Richardson, D. J., Gates, A. J., et al. (2016). Nitrous oxide metabolism in nitrate-reducing bacteria: physiology and regulatory mechanisms. *Adv. Microb. Physiol.* 68, 353–432. doi: 10.1016/bs.ampbs.2016.02.007

Tribble, G. D., Angelov, N., Weltman, R., Wang, B. Y., Eswaran, S. V., Gay, I. C., et al. (2019). Frequency of tongue cleaning impacts the human tongue microbiome composition and enterosalivary circulation of nitrate. *Front. Cell. Infect. Microbiol.* 9:39. doi: 10.3389/fcimb.2019. 00039

Vanhatalo, A., Blackwell, J. R., L'heureux, J. E., Williams, D. W., Smith, A., Van Der Giezen, M., et al. (2018). Nitrate-responsive oral microbiome modulates nitric oxide homeostasis and blood pressure in humans. *Free Radic. Biol. Med.* 124, 21–30. doi: 10.1016/j.freeradbiomed.2018.05.078

Velmurugan, S., Gan, J. M., Rathod, K. S., Khambata, R. S., Ghosh, S. M., Hartley, A., et al. (2016). Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study. *Am. J. Clin. Nutr.* 103, 25–38. doi: 10.3945/ajcn.115. 116244

Woessner, M., Smoliga, J. M., Tarzia, B., Stabler, T., Van Bruggen, M., and Allen, J. D. (2016). A stepwise reduction in plasma and salivary nitrite with increasing strengths of mouthwash following a dietary nitrate load. *Nitric Oxide* 54, 1–7. doi: 10.1016/j.niox.2016.01.002

Ysart, G., Miller, P., Barrett, G., Farrington, D., Lawrance, P., and Harrison, N. (1999). Dietary exposures to nitrate in the UK. *Food Addit. Contam.* 16, 521–532. doi: 10.1080/026520399283669

Zweier, J. L., Wang, P., Samouilov, A., and Kuppusamy, P. (1995). Enzyme-independent formation of nitric oxide in biological tissues. *Nat. Med.* 1, 804–809. doi: 10.1038/nm0895-804

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 11 October 2019
doi: 10.3389/fcimb.2019.00347

Species-Level Salivary Microbial Indicators of Well-Resolved Periodontitis: A Preliminary Investigation

Aneesha Acharya[1,2], *Tsute Chen*[3,4], *Yuki Chan*[1], *Rory M. Watt*[1], *Lijian Jin*[1] and *Nikos Mattheos*[1]*

[1] Faculty of Dentistry, The University of Hong Kong, Hong Kong, China, [2] Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pune, India, [3] Department of Microbiology, The Forsyth Institute, Cambridge, MA, United States, [4] Department of Oral Medicine, Infection & Immunity, Harvard School of Dental Medicine, Harvard Medical School, Boston, MA, United States

Objective: To profile the salivary microbiomes of a Hong Kong Chinese cohort at a species-level resolution and determine species that discriminated clinically resolved periodontitis from periodontally healthy cases.

Methods: Salivary microbiomes of 35 Hong Kong Chinese subjects' under routine supportive dental care were analyzed. All subjects had been treated for any dental caries or periodontal disease with all restorative treatment completed at least 1 year ago and had ≤3 residual pockets. They were categorized based on a past diagnosis of chronic periodontitis into "healthy" (H) or "periodontitis" (P) categories. Unstimulated whole saliva was collected, genomic DNA was isolated, and high throughput Illumina MiSeq sequencing of 16S rRNA (V3-V4) gene amplicons was performed. The sequences were assigned taxonomy at the species level by using a BLASTN based algorithm that used a combined reference database of HOMD RefSeqV14.51, HOMD RefSeqExtended V1.1 and GreenGeneGold. Species-level OTUs were subjected to downstream analysis in QIIME and R. For P and H group comparisons, community diversity measures were compared, differentially abundant species were determined using DESeq2, and disease indicator species were determined using multi-level pattern analysis within the R package "indicspecies."

Results: P subjects were significantly older than H subjects ($p = 0.003$) but not significantly different in their BOP scores ($p = 0.82$). No significant differences were noted in alpha diversity measures after adjusting for age, gender, and BOP or in the beta diversity estimates. Four species; *Treponema* sp. *oral taxon 237, TM7* sp. *Oral Taxon A56, Prevotella* sp. *oral taxon 314, Prevotella* sp. *oral taxon 304*, and *Capnocytophaga leadbetteri* were significantly more abundant in P than in the H group. Indicator species analysis showed 7 significant indicators species of P group. *Fusobacterium* sp *oral taxon 370* was the sole positive indicator of P group (positive predictive value = 0.9, $p = 0.04$). Significant indicators of the H category were *Leptotrichia buccalis, Corynebacterium matruchotii, Leptotrichia hofstadii,* and *Streptococcus intermedius.*

OPEN ACCESS

Edited by:
Andrew T. Gewirtz, Georgia State University, United States

Reviewed by:
Mark Buijs, VU University Amsterdam, Netherlands
Andrea Santarelli, Marche Polytechnic University, Italy

***Correspondence:**
Nikos Mattheos
nikos@mattheos.net

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *14 November 2018*
Accepted: *27 September 2019*
Published: *11 October 2019*

Citation:
Acharya A, Chen T, Chan Y, Watt RM, Jin L and Mattheos N (2019) Species-Level Salivary Microbial Indicators of Well-Resolved Periodontitis: A Preliminary Investigation. Front. Cell. Infect. Microbiol. 9:347. doi: 10.3389/fcimb.2019.00347

Conclusion: This exploratory study showed salivary microbial species could discriminate treated, well-maintained chronic periodontitis from healthy controls with similar gingival inflammation levels. The findings suggest that certain salivary microbiome features may identify periodontitis-susceptible individuals despite clinical disease resolution.

Keywords: saliva, salivary microbiota, oral microbiome, periodontal diseases, 16S rRNA gene sequencing

INTRODUCTION

Salivary microbiomes show temporal stability in health (Belstrøm et al., 2016). Discernible microbial community patterns at baseline are shown to predict increased risk of inflammatory disease onset in a skin model (van Rensburg et al., 2015). Similarly, whether some microbial "traits" in the oral microbiome are associated with past disease experience or future risk is not established. Subgingival microbiota is most relevant to periodontitis. Distinguishable subgingival microbial clusters have been associated with active chronic periodontitis (Kirst et al., 2014), but not consistently (Koren et al., 2013). Others found subgingival plaque microbiomes did not vary measurably after periodontal therapy (Schwarzberg et al., 2014). Several investigations have shown that salivary microbial profiles can also differentiate both active caries and periodontal disease (Yang et al., 2011; Belstrøm et al., 2015; Chen H. et al., 2015). One plausible reason for these findings is the passage of microbiota from periodontal pockets and carious lesions into saliva. In salivary flora, the proportion of taxa shared with dental plaque is minor but seems sufficient to discriminate disease. As such, the salivary microbiome composition has been found comparable to pooled subgingival plaque in terms of its "representativeness" of the subgingival microbiota (Belstrøm et al., 2017). Others, however, have noted no significant impact of periodontal disease or periodontal therapy on salivary microbiota and attributed this to a low volume of microbial ingress from the subgingival niche (Yamanaka et al., 2012; Takeshita et al., 2016). A previous study from our group found no significant differences in the salivary burden of specific periodontal pathogen abundances between treated, clinically resolved chronic periodontitis and healthy groups (Acharya et al., 2015). However, if no differences existed in the salivary microbiome assemblages between these two groups was unknown. The host-microbiome link is bi-directional, and as inherent features of oral microbiota affect immune programming (Madhwani and McBain, 2016), they may also associate with susceptibility to infection. A stable "core" oral microbial community structure that persists over years has been demonstrated (David et al., 2014) and such ecological stability is linked closely to an individual's salivary composition (Rosier et al., 2018). Plaque biofilm accumulation and its consequent compositional shift to a dysbiotic state is key to the initiation of periodontitis-associated inflammation in susceptible individuals. In resilient individuals, host-immune traits such as salivary nitrate levels and microbial community traits such as bacteriocin levels or anti-inflammatory cytokine stimulation may sufficiently counter disease-related shifts (Rosier et al., 2018). In a carbohydrate challenge experiment, Benítez-Páez et al. (2014) found a caries-resistant individual did not show microbial community shifts similar to caries-susceptible individuals. Arguably, inherent microbial susceptibility "traits" may be reflected in states of clinically resolved periodontal disease. Salivary microbiome differences between well-treated periodontitis and healthy subjects with comparable periodontal health could potentially reflect stable ecological patterns that predict past or future periodontal disease.

Any analysis of microbiome features is highly sensitive to the level of taxonomic resolution achieved. The standard OTU based taxonomy assignment pipelines for 16s rRNA sequence data provide genus-level resolution and have comprised the dominant approach in saliva microbiome studies using 16s rRNA sequencing (Schloss and Westcott, 2011). A novel pipeline reported a taxonomic resolution to the species–level using oral 16S rRNA reads (Al-Hebshi et al., 2015, 2016). This approach was based on the extended human oral microbiome database (HOMD) (Chen et al., 2010). Improved taxonomic feature resolution may enable better discrimination of microbial community fingerprints. In the present study, we profiled the salivary microbiomes of a Hong Kong Chinese cohort using a species-level resolution approach and sought the discriminant species of treated, well-maintained periodontitis from periodontally healthy cases.

MATERIALS AND METHODS

Subject Selection

39 Hong Kong Chinese (HK) adult subjects enrolled in a routine dental maintenance program as a part of a previous multicenter study were (Acharya et al., 2015). All study procedures were conducted at The Prince Philip Dental Hospital, Hong Kong after obtaining Institutional Ethical Committee Approval (HKU/HA: UW 13359) and were in accordance with the Declaration of Helsinki (World Medical Association, 2013). Subjects who had been treated for any caries and periodontal disease, and had completed all active therapies, with the restorative phase completed for at least 1 year, were eligible for recruitment. Inclusion criteria were: age $>$ 30 years and good general health. Exclusion criteria were: presence of $>$3 residual pockets with PPD $>$ 4 mm, past or current smoking or tobacco chewing, pregnancy, regular use of any medication, having a history of trauma, dental extractions, oral infections, antibiotic, steroidal or csteroidal anti-inflammatory drug consumption within the

past 3 months, known systemic diseases (HIV, tuberculosis, hepatitis, known history of any other infectious disease, diabetes mellitus, ischemic heart disease, hypertension, thyroid or other hormonal disorders, autoimmune disease, and cancer) or history of radiation therapy. Subjects were categorized based on their past dental records. A past diagnosis of chronic periodontitis diagnosis at their initial visit according to clinical criteria by Armitage (1999) was categorized as periodontitis (P). Subjects were categorized as healthy (H) if diagnosed as periodontally healthy at the initial visit.

Saliva Collection, DNA Purification, and Sequencing

Unstimulated whole saliva was collected during the morning as described before (Acharya et al., 2015). In brief, unstimulated saliva was collected before 11 a.m. in the morning. Subjects had refrained from eating or drinking for a minimum of 1 h before saliva collection. Saliva was allowed to passively pool intraorally and was expectorated into sterile collection tubes, which were kept on ice and immediately transported for storage at −80°C until further analysis. Saliva samples were thawed, equilibrated to room temperature before DNA extraction. Salivary cells were pelleted by centrifugation at 14,000 rpm for 10 min and the supernatant was carefully removed. Salivary genomic DNA was extracted using the column extraction method with the QIAamp DNA Blood Mini Kit®, following the manufacturer's instructions. The DNA concentration was assessed using a NanoDrop® spectrophotometer and a Qubit® Fluorometer, while its integrity was determined using agarose gel electrophoresis. 16S rRNA V3-4 gene amplification and MiSeq (300 base-paired end) sequencing was performed using the standard Illumina dual-indexing protocol (Fadrosh et al., 2014). The primer sequences used were; Forward: CCTACGGGNGGCWGCAG Reverse: TACNVGGGTATC TAATCC.

Data Analysis

Sequencing data were quality filtered. Demultiplexing, barcode, linker-primer sequences trimming, and raw reads quality filtering (minimum Q-value = 20, with removal of reads with 20% or higher of read original length with low quality, removal of reads contaminated by adapter, with ambiguous or N basecall, and those with low complexity as indicated by 10 consecutive same bases) was done using MISEQ reporter (v 2.0) software. Raw FASTQ sequences were deposited in the SRA BioProject database (BioProject ID Number PRJNA337949). Samples with <500 read counts were excluded in all analysis. Read pairs were merged to single sequences with a script (join_paired_ends.py) provided by the Quantitative Insights into Microbial Ecology (QIIME) package version 1.9.1 (Caporaso et al., 2010), using default settings. The merged reads were then taxonomically assigned to the species-level based on a previously published algorithm (Al-Hebshi et al., 2015), with additional steps to further identify potential novel species. The taxonomy assignment pipeline is depicted in **Figure 1**. Briefly, merged sequence reads were BLASTN-searched against a panel of full-length 16S rRNA sequences. These consisted of 889 sequences from HOMD RefSeqV14.51, 495 from HOMD RefSeqExtended V1.1, 3,940 from GreenGeneGold, and 18,620 from NCBI 16S rRNA Reference. The version of the 16S rRNA gene reference sequence database used by this manuscript is dated 20170422 and is available online together with the documentation: http://www.homd.org/ftp/NGS_Pipeline/Species_Level_BLASTN/. This combined reference set has a total 20,699 sequences and represents a total of 13,640 oral and non-oral microbial species. The first step was a BLASTN-search with a matching criteria of ≥98% identity and ≥98% read length coverage. Reads matching multiple species or unmatched reads were further processed where *de novo* Chimera were filtered using USEARCH (Edgar, 2010) with (98% identity cutoff), short reads (<200 nt), and singletons were removed. *De-novo* OTUs (operational taxonomy units) were clustered at a 98% identity cutoff. USEARCH (version v8.1.1861_i86linux32) was used for taxonomy assignment and those with <98% match were discarded. If sequences with the same scientific name had different upper-level taxonomy nomenclature in the combined reference set, we choose a single taxonomy with the following priority: HOMD+HOME-EXT, GreenGene Gold, and then NCBI Taxonomy. Manual curation was also performed to resolve issues including mis-spelling, non-alpha numeric characters, and missing information. The algorithm for taxonomy assignment is illustrated in **Figure 1** and documented in the online resource: http://www.homd.org/ftp/NGS_Pipeline/Species_Level_BLASTN/20170422/Species_Level_BLASTN_QIIME_Pipeline_Doc_20170422.pdf.

After the taxonomy assignment, species-level OTUs were subject to down-stream bioinformatics analyses, including alpha and beta diversity analyses using the QIIME pipeline. Species-level taxonomic plots were generated. Alpha diversity measures were computed, rarefaction curves were drawn and comparisons made between P and H groups with linear regression controlling for Age, Gender, and BOP as co-predictors. Beta diversity measures, phylogenetic (weighted and unweighted unifrac) and Bray-Curtis metrics were determined, Principal coordinate analysis plots were drawn and comparisons between P and H categories made using permutational multivariate analysis of variance using "Adonis" (R package "vegan" version 2.2.1) (p-values generated at 5,000 permutations). Similar comparisons were made to analyse community differences related to Gender, Age group, and BOP.

To determine species associated with subject characteristics, 2 approaches were adopted using the species-level OTU abundance table as input. Firstly, the differential abundance (DA) of species between sample groupings (Periodontal status; P vs. H, Gender: Male vs. Female, Age: >50 years vs. ≤ 50 years, BOP: >25% vs. ≤ 25%) were each determined using DESeq2 with p-value adjusted for multiple testing using the Benjamini–Hochberg method (cut-off = adjusted $p < 0.05$) (Love et al., 2014). Secondly, "indicator species" were determined by multi-level pattern analysis. For this purpose, the "multipatt" and "indval.g" functions in the R package "Indicspecies" were used (De Caceres

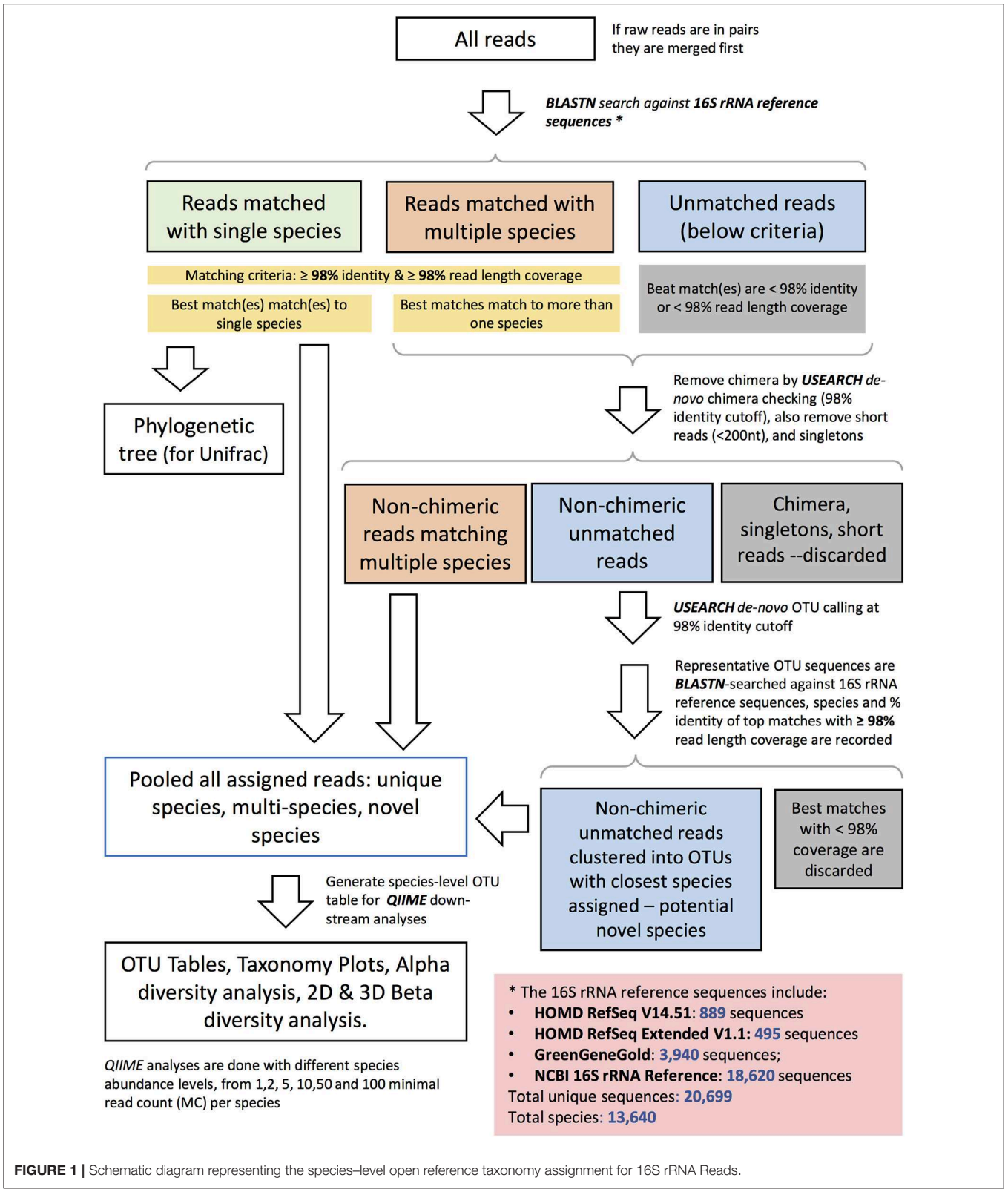


FIGURE 1 | Schematic diagram representing the species–level open reference taxonomy assignment for 16S rRNA Reads.

and Legendre, 2009). The indicator approach measures the predictive ability of community features for a group category (McGeoch and Chown, 1998). The indicator statistic represents the probability of group fidelity. Ideal indicator species are characterized by high exclusivity and fidelity. The function "indVal.g" accounts for unbalanced between-

group size variances. Indicator species with significant *p*-values (<0.05, computed using 5,000 permutations) were determined (De Cáceres et al., 2012).

RESULTS

Clinical Data, Sequencing Output, Processing and Taxonomy Assignment

Salivary microbiomes of 35 subjects were analyzed after 4 subjects were excluded due to having <500 reads per sample. The read count details are presented in **Table S1**. The clinical and demographic data for these 35 subjects are summarized in **Table 1**. A total of 63,834 assigned reads and 418 species-level taxa were determined at ≥98% sequence identity and ≥98% read length coverage to the reference sequences. Of these, 56,399 reads were assigned to 358 single species, 7,413 reads were assigned to 51 taxa that contain multiple species due to tied sequence identity to reference sequences of multiple sequences, and 22 were assigned to 6 potential novel species due to <98% sequence identity to all the reference sequences (**Table S2**). The genera with the most number of taxa assigned with multiple species were *Fusobacterium* (11 species, ranging from 2 to 5 species), *Streptococcus* (11 species, ranging from 2 to 15 species), and *Prevotella* (6 species, ranging from 2 to 4 species). Six novel species were assigned to a very small number (22) of reads: *Flavobacriia* genera, *Bergeyella* sp. *Oral taxon 907* (97.42% identity) and *Capnocytophaga; multispecies* (96.35% identity), *Clostridiales* multispecies (96.21% identity), *Sporomusaceae multispecies* (87.50% identity), *Leptotrichia multispecies* (97.99% identity), *Neisseria* sp. *Oral taxon 018* (97.67% identity). The number of species observed per sample ranged from 43 to 186 (mean = 89.1, sd = 29.0). Species shared among individuals were considered as representing a "core microbiome." Seventy-nine species were shared by 50% individuals and 26 by 80% individuals including species from *Streptococcus, Gemella, Rothia, Actinomyces, Fusobacterium, Leptotrichia, Prevotella, Porphyromonas, Neisseria, Capnocytophaga, Haemophilus and Lautropia* (**Figure 2**). Five species were present in 95% individuals; *Rothia mucilaginosa, Prevotella melaninogenica, Streptococcus multispecies spp24_14, Veillonella parvula, Haemophilus parainfluenzae* (**Table S3**).

TABLE 1 | Clinical and demographic characteristics.

Group	H ($n = 20$)	P ($n = 15$)	Comparison P vs. H	Total ($n = 35$)
Mean Age (st.dev) (years)	47.3 (14.5)	60.9 (7.0)	$W = 62.5$, $p = 0.003$*	53.8 (13.6)
Gender	15F, 5M	11F, 4M	$X^2 = 0.013$, $p = 1$	26F, 9M
Mean % BOP positive sites (st.dev)	15.4 (13.8)	16.7 (20.1)	$W = 157.5$, $p = 0.82$	16.0 (15.5)

*W, Mann Whitney Wilcoxson's test statistic; X^2, Chi-squared test statistic; *p < 0.05, significant.*

Community Differences Between Periodontitis and Health Groups

There were no significant differences observed in mean alpha diversity measures for the P and H groups, which was reflected in the rarefaction plots (**Figures 3A,B**). However, the P group showed lower alpha diversity measures compared to the H

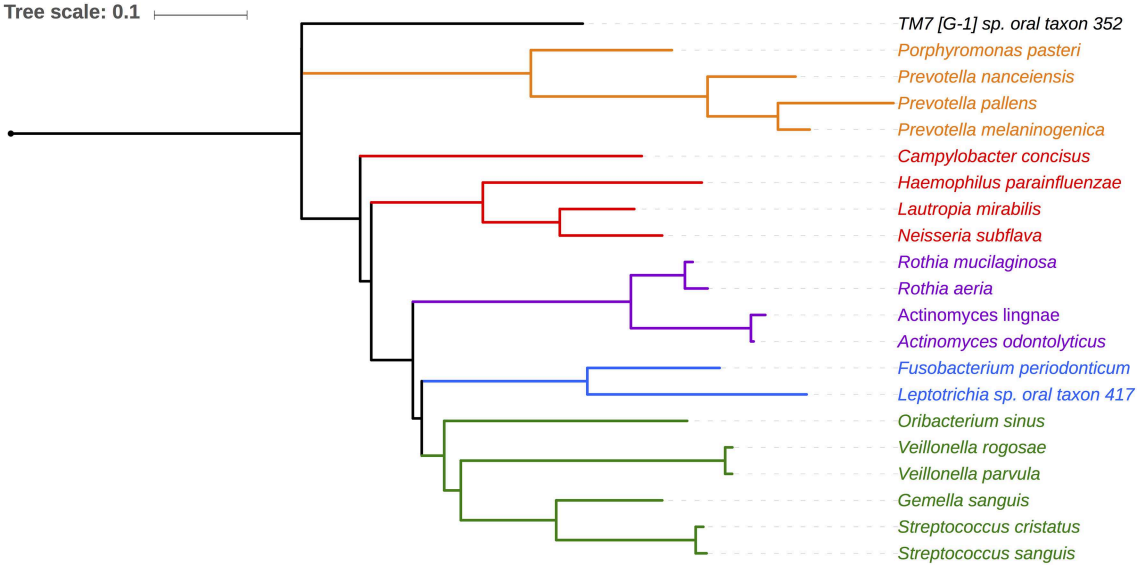


FIGURE 2 | Phylogenetic relationship of 21 species shared by 80% of the subjects. Phylotypes assigned to single HOMD entity are shown. Five phylotypes assigned to multiple species are not shown (*Granulicatella multispecies spp49 2, Streptococcus, multispecies spp1 2, Streptococcus multispecies spp24 14, Streptococcus multispecies spp32 2*).

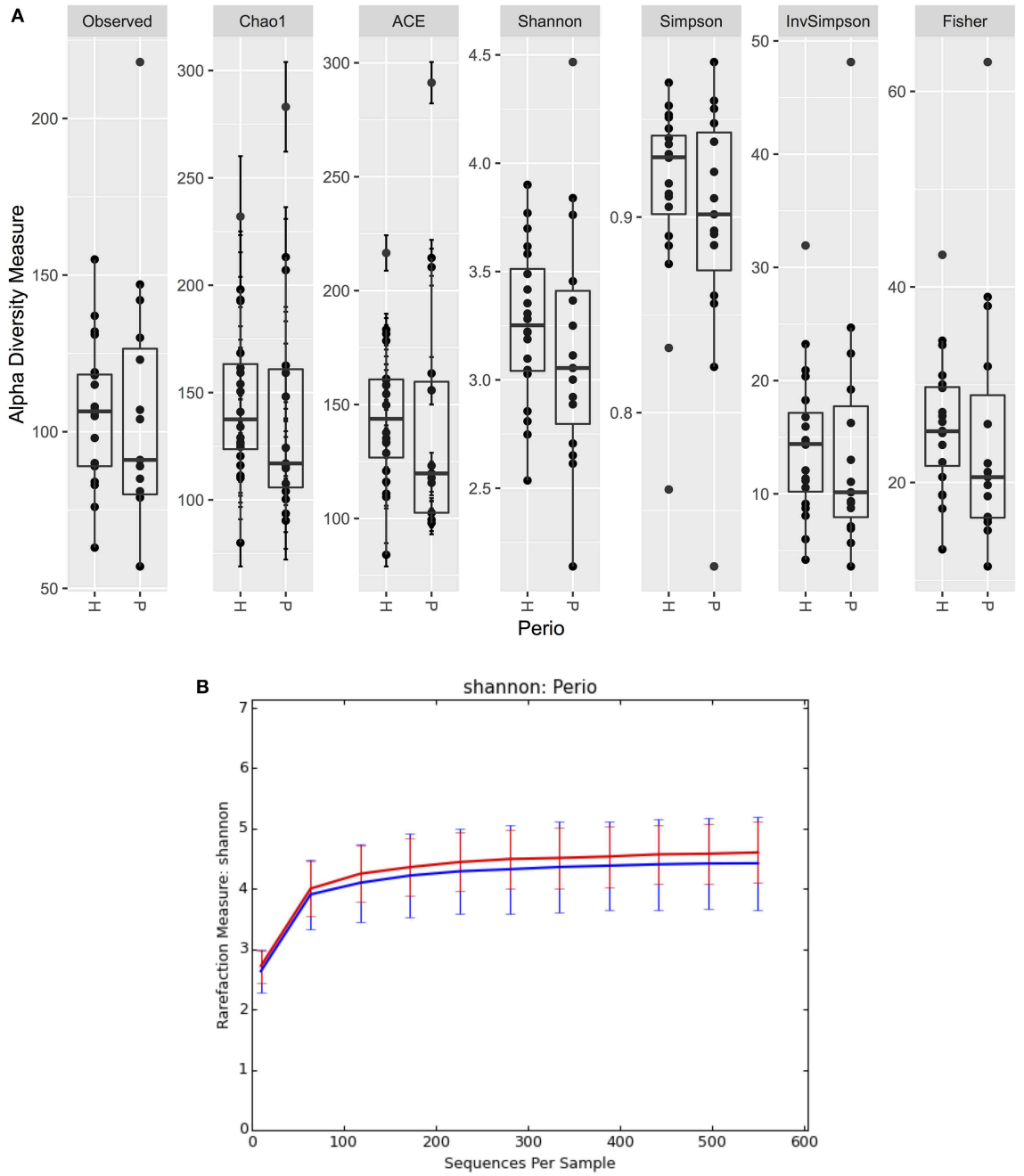


FIGURE 3 | (A) Boxplots of alpha diversity measures in P and H groups. **(B)** Rarefaction plot of Shannon alpha diversity in P and H groups. P, blue color; H, red color.

group. Linear regression analysis (controlling for the effects of age, gender, and BOP) showed that a periodontal disease status did not have a significant impact on salivary alpha diversity measures (**Table 2**, **Table S4**). Comparison of beta diversity measures; Bray–Curtis ($f = 0.56$, $p = 0.93$) and Unifrac measures (unweighted Unifrac: $f = 0.78$, $p = 0.66$; weighted Unifrac: $f = 0.25$, $p = 0.97$) revealed no significant differences in community composition between the P and H groups. Correspondingly, no evident clustering of P or H cases was noted in the ordination plots (**Figure 4**). Similarly, no significant differences in beta diversity were associated with gender, BOP or Age categories (age $>$ 50 years, age $\leq$ 50 years).

Figure 5 shows the mean relative abundances and phylogenetic relationships of the indicator and DA species that discriminated the P and H groups. Using DeSeq2, 4 species were found to be significantly more abundant in the P group as compared to the H group (**Table 3**, **Table S5**). These were *Treponema* sp. *oral taxon 237, TM7* sp. *Oral Taxon A56,*

Prevotella sp. *oral taxon 314, Prevotella* sp. *oral taxon 304, Capnocytophaga leadbetteri*. However, no species had significant differences in abundance between males and females, or between the two age categories. A BOP value of >25% was associated with a significantly higher abundance of *Fusobacterium multispecies spp48_2* (log2 fold change = 2.32, adjusted $p < 0.001$). Indicator analysis showed 7 species as significant indicators of the P group (**Table 4**, **Table S6**). Among these, 6 were negative indicators whereas *Fusobacterium* sp *oral taxon 370* was the only positive indicator. Four species emerged as significant H group indicators (**Table 4**). *TM7 G.1* sp *oral taxon 347* and *Fusobacterium* sp *oral taxon 370* had low sensitivity but high positive predictive values (>0.9) for P group.

DISCUSSION

Here, we conducted an exploratory investigation to assess the salivary microbiome in P and H subjects within a Hong Kong Chinese cohort. The pattern of shared "core" species was similar to past reports (Takeshita et al., 2016; Mason et al., 2018) and particularly resonated with a previous report in Chinese subjects (Huang et al., 2011). The use of a smaller, curated reference sequence set may have enabled better accuracy of species-level taxonomical assignment. Arguably, higher taxonomic resolution may improve the discrimination of host trait-microbiota associations. We noted significant differences in the relative abundances of select salivary species and identified several indicator species discriminatory of P and H groups.

The ordination or beta diversity analysis of our data appeared to be in agreement with Takeshita et al. (2016) who found no association between salivary microbiome composition and periodontal disease. However, unconstrained beta diversity ordinations reflect only the major sources of variation in composition. Salivary community diversity measures were not significantly linked to any of the clinical and demographic variables, although a trend for lower alpha diversity was notable in the P group. The small sample and restrictive inclusion criteria very likely limit the detection of differences in microbiome composition related to subject background. As such, most subject characteristics have small effect-sizes on microbiome composition and very few factors like geographical location and lifestyle are known to have large effect sizes (Lazarevic et al., 2010).

P group subjects were significantly older but did not differ in levels of BOP from H subjects and had 3 or less periodontal pockets, reflecting well-resolved periodontitis with low levels of residual disease activity. Treated periodontitis cases can vary in the level of residual periodontal disease. As influx from inflamed subgingival niches is likely to be a major source of periodontitis-associated bacteria in saliva (Saygun et al., 2011; Belstrøm et al., 2015), the selection criteria were designed to exclude P subjects with significant numbers of residual periodontal pockets. Similar levels of residual inflammation and lack of residual pockets in the P group could be one factor why no major ecological differences from H subjects were noted. The most dominant factor in microbiome composition is interpersonal variation (Lazarevic

TABLE 2 | Adjusted linear regression coefficients for alpha diversity indices between P vs. H group.

Diversity metric	Coefficient[a]	95% CI[a]	*P*-value[a]	Variance explained (Model R squared)
Observed species	1.1	−23.0 to 25.7	0.93	0.03
Chao 1	−2.7	−39.4 to 34.0	0.88	0.03
ACE	−8.7	−42.7 to 25.4	0.61	0.04
Shannon	−0.13	−0.5 to 0.3	0.53	0.04
Simpson	−0.01	−0.1 to 0.0	0.73	0.03
Inverse Simpson	−2.3	−8.6 to 4.1	0.47	0.05
Fisher's alpha	−1.5	−9.1 to 6.1	0.68	0.05

Simple linear regression with predictors Age, BOP, Gender (reference level = F), and Periodontal Status (reference level = H). [a]Values for predictor "Periodontal Status".

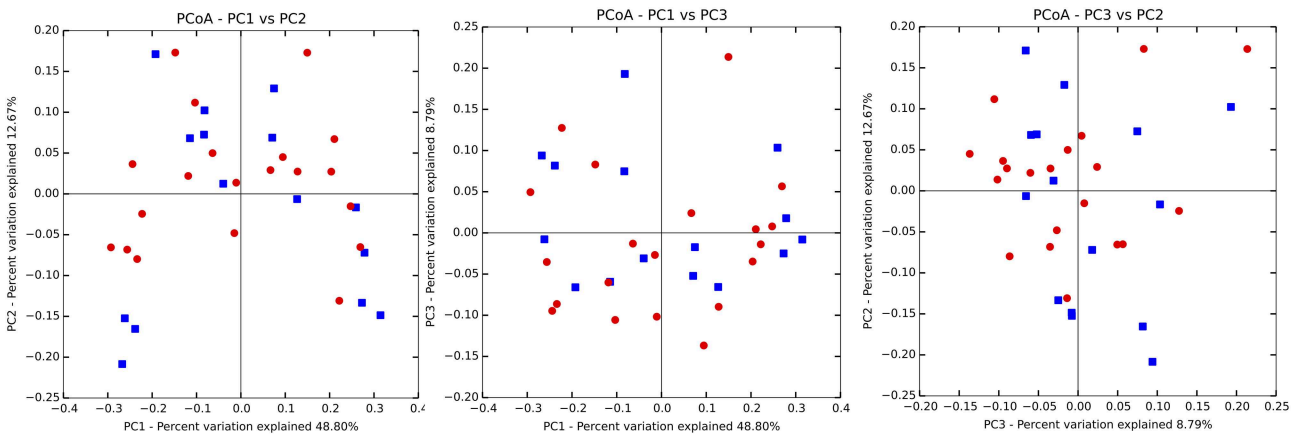


FIGURE 4 | Principal coordinate ordination plots based on the weighted Unifrac beta diversity distance. P, blue color; H, red color. No clustering of P and H samples was evident.

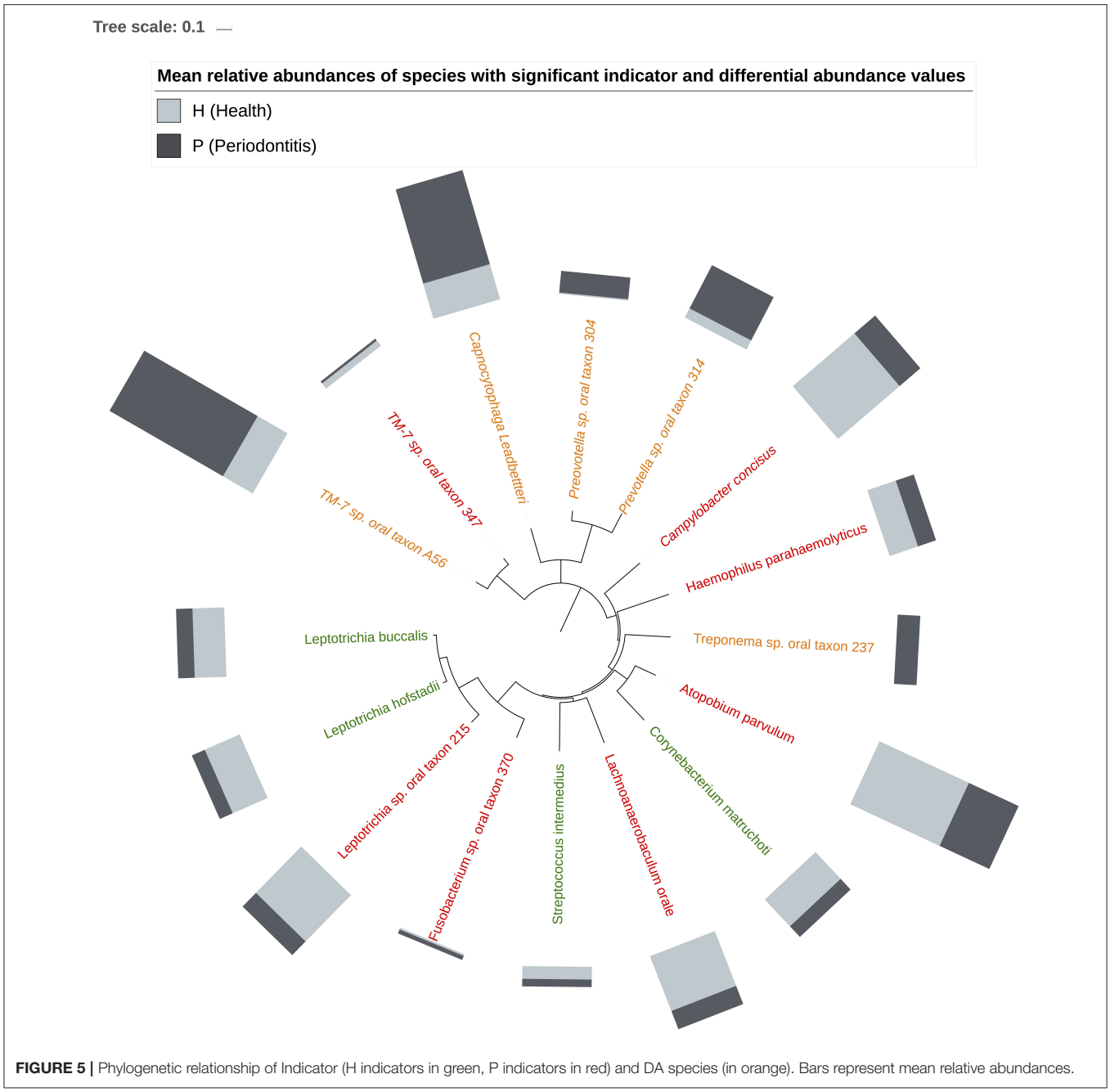


FIGURE 5 | Phylogenetic relationship of Indicator (H indicators in green, P indicators in red) and DA species (in orange). Bars represent mean relative abundances.

et al., 2010). A large prospective study analyzed microbial communities in periodontal disease and health (Chen et al., 2018) and found disease-linked microbiomes are more heterogeneous in both pre- and post-treatment situations. Further studies are needed to understand the extent to which salivary microbiomes may be stable and resistant to periodontal treatment.

We used two approaches to analyse microbial features that distinguished the P and H categories. The DESeq2 approach utilized here is more robust in small sample sizes as compared to non-parametric approaches. The "indicator species" analysis is based on the "Indicator Value" index (De Caceres and Legendre, 2009) which reflects the association of a feature with grouping category. None of the significant indicator species were "ideal" or had sensitivity and specificity values close to 1. While these would need validation in larger cohorts, this finding may also reflect the difficulty of individual microbial features which strongly reflect a periodontitis phenotype, considering the complex, polymicrobial nature of the disease and variations in both health and disease linked microbiome compositions. Despite comparable levels of gingival health, the P group was distinguishable by specific salivary microbial species, which largely aligned with past reports (Abusleme et al., 2013; Pérez-Chaparro et al., 2014). Among the

significantly raised species in P, *Treponema* sp. *oral taxon 237* has been implicated as a periodontal pathogen (Abusleme et al., 2013). Two *Preovtella* species *Prevotella* sp. *oral taxon 314* and *Prevotella* sp. *oral taxon 304* were significantly more abundant in P subjects. *Prevotella* species have previously been associated with both periodontal health and disease. A considerable amount of species and strain level genomic diversity (Ibrahim et al., 2017), as well as variability in their response to periodontal therapy, is documented (Schwarzberg et al., 2014). An increase in salivary anaerobic species in treated periodontitis may imply greater potential systemic risk due to extraoral translocation (Endo et al., 2015; Vieira Colombo et al., 2016). Some *Prevotella* species, in particular, have been linked to new-onset rheumatoid arthritis (Scher et al., 2012). A past study, reported the anaerobic bacillus *Capnocytophaga leadbetteri* in periodontitis and hematological infections but not in periodontally healthy mouths (Ehrmann et al., 2013).

Fusobacterium sp *oral taxon 370* has been linked to fermentative function, and has been found to be raised in periodontal disease (Dabdoub et al., 2016). Furthermore, *Fusobacterium multispecies spp48_2* species was significantly more abundant in subjects with high BOP, independent of past periodontal status. With the exception of *Fusobacterium* sp *oral taxon 370*, all other P state indicator species were "negative" indicators characterized by greater mean abundance in H subjects. Indicator analysis identified several species that were lower in P as having significant indicator value. Notably, several of these negative indicator species were from genera previously identified as associated with periodontitis (Pérez-Chaparro et al., 2014), which seems somewhat paradoxical considering they were found in lower relative abundance in resolved disease compared to health. Hypothetically, it may reflect differences in microbiome assembly in plaque and saliva caused by periodontal therapy (Chen et al., 2018). *Campylobacter concisus* emerged as a negative indicator for P cases. This species is recognized as oral commensal and implicated in gastric and inflammatory bowel disease (Liu et al., 2018). The oral cavity is considered a potential reservoir (Zhang et al., 2010). It can act as an opportunistic pathogen in immune-inflammatory and epithelial cell dysregulation (Deshpande et al., 2016; Brunner et al., 2018). A rise in its abundance after successful periodontitis therapy has been reported (Tanner et al., 1987), again supporting the premise that periodontal treatment may result in specific differences in microbiome composition. The indicator species *Lachnoanaerobaculum orale* lies within *Lachnospiraceae* which were previously reported by Chen L. et al. (2015) as inversely linked to caries in Chinese subjects. Some *Lachnospiraceae* members are newly recognized in periodontitis and some species correlate with gingival inflammation (Abusleme et al., 2013; Kistler et al., 2013). *Lachnospiraceae* contains many genera that are butyrate producers, a trait associated with anti-inflammatory properties in the gut (Lopetuso et al., 2013) but also with periodontitis progression (Tsuda et al., 2010). The low-abundance species *Atopobium parvulum* has been linked to halitosis (Kazor et al., 2003). Several *Haemophulis* species are members of core oral microbiome (Mok et al.,

TABLE 3 | Differential abundance of significantly different species between P and H groups (DeSEQ2 test).

Species	Log2 fold change P vs. H (se)	Test statistic	Adjusted *p*-value	Total reads assigned to species
Treponema sp. *oral taxon 237*	1.68 (0.41)	4.15	<0.01*	56
TM7 sp. *Oral Taxon A56*	1.80 (0.45)	4.01	<0.01*	519
Prevotella sp. *oral taxon 314*	1.54 (0.39)	3.94	<0.01*	143
Prevotella sp. *oral taxon 304*	1.57 (0.41)	3.86	<0.01*	62
Capnocytophaga leadbetteri	1.45 (0.42)	3.43	0.02 *	357

*$p < 0.05$, significant.

TABLE 4 | Indicator species of P and H groups.

	Test-statistic	*p*-value	Positive predictive value	Sensitivity	Total reads assigned to species
Group H (number of indicator species = 4)					
Leptotrichia buccalis	0.80	0.01*	0.85	0.75	107
Corynebacterium matruchotii	0.79	0.03*	0.82	0.75	128
Leptotrichia hofstadii	0.72	0.04*	0.87	0.60	126
Streptococcus intermedius	0.71	0.03 *	0.77	0.65	43
Group P (number of indicator species = 7)					
Campylobacter concisus	0.86	0.02*	0.74	1.00	291
Atopobium parvulum	0.79	0.03*	0.71	0.87	416
Leptotrichia sp *oral taxon 215*	0.76	0.04*	0.79	0.73	213
Lachnoanaerobaculum orale	0.76	0.04*	0.86	0.67	193
Haemophilus parahaemolyticus	0.74	0.03*	0.82	0.67	127
TM7 G.1 sp *oral taxon 347*	0.56	0.04*	0.94	0.33	26
Fusobacterium sp *oral taxon 370*	0.55	0.04*	0.90	0.33	16

*$p < 0.05$, significant.

2017). *TM7 G.1* sp *oral taxon 347* was increased in subgingival plaque of active periodontal disease (Griffen et al., 2011). Overall, the indicator analysis seemed in particular agreement with a past report that found subgingival *Capnocytophaga, Corynebacterium, Haemophilus, Leptotrichia,* and *Streptococcus* had significant negative associations with clinical periodontal measures (Camelo-Castillo et al., 2015).

The present findings must be considered in light of the major limitations of the study; the subject groups were small, not balanced for size, demographics and past periodontal disease parameters including disease severity, which limit the generalizability of these results. Importantly, the small sample size of 35 allows only preliminary findings and these require validation in large sampled studies. Because of the primarily exploratory focus of this study, the downstream analysis was based on a minimum read count per species of 1 and aimed to capture the maximal ecological variation feasible. On the same lines, the 6 novel species reported were based on a very small number of reads. These findings may be considered as a basis for a hypothesis that variations in the oral microbiome related to a periodontitis phenotype are retained even after clinical disease resolution. Another question that arises is whether oral species can discriminate periodontally susceptible individuals before clinical disease onset or during very early disease. Longitudinal and interventional investigations in larger cohorts are essential to test such hypotheses. Translational implications of such findings could be the development of improved microbiome-based risk-markers or therapeutics, considering saliva is an attractive sampling biofluid. Other implications include an improved understanding of host-microbiome interactions in the context of periodontal disease. We did not analyse how the salivary ecological composition represented that of the subgingival niche, which should be addressed in future studies. The issues of sampling variance and compositional nature of microbiome data need to be considered when viewing the generalisability of the particular signature taxa, which were based on a single replicate in our study. Technical differences in saliva collection methods, storage, handling, DNA extraction, choice of sequencing technology or 16s rRNA gene hypervariable region, sequencing depth, and bioinformatic pipeline can each cause induce variation in the outcomes (Mascitti et al., 2019) and precludes a generalization of these specific findings. Here, we collected unstimulated saliva, which varies in its microbial profile from stimulated saliva and is shown to harbor a greater diversity of species (Gomar-Vercher et al., 2018). Besides, other influencing factors such as diet, body-mass index, and multiple lifestyle factors which impact microbiome assemblages were not considered in this preliminary analysis and could be a source of confounding. In addition, a single time point sampling precludes the differentiating between stable and dynamic salivary species. The exact biological relevance of the discriminant microbial features also remains unclear. While taxonomical information is valuable, functional aspects of the host-microbiome interaction are central to disease processes. Whole-genome, transcriptome, and metabolome approaches may better answer how saliva microbiome is linked to periodontal susceptibility.

CONCLUSION

Species-level analysis of the salivary microbiome in well-resolved periodontitis cases revealed no differences in global community diversity metrics as compared with periodontally healthy subjects. However, differentially abundant and indicator species were discernible between these groups. These findings suggested differences in oral microbiota may persist despite acceptable clinical resolution of periodontitis. The nature and implication of these differences warrant further investigations.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Declaration of Helsinki, World Medical Association with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB) (ref: IRB number UW 13359).

AUTHOR CONTRIBUTIONS

AA aided the study design, collected the samples and clinical data, performed the laboratory steps, statistical analysis, and compiled the study findings as a manuscript. TC performed the sequence data processing, taxonomy assignments to the species-level and edited the manuscript. YC contributed to the study design and strategy for the statistical analysis. RW contributed to the study concept and design, supervision of laboratory steps, manuscript preparation and revision. NM contributed to the study concept and design, overall supervision, clinical management, and revised the manuscript. LJ contributed to the study concept and manuscript editing.

FUNDING

This study was funded by The University of Hong Kong.

ACKNOWLEDGMENTS

The authors acknowledge The University of Hong Kong for financial support for this study and Dr. Mei Leng Koh for assistance in the clinical data collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00347/full#supplementary-material

REFERENCES

Abusleme, L., Dupuy, A. K., Dutzan, N., Silva, N., Burleson, J. A., Strausbaugh, L. D., et al. (2013). The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J.* 7, 1016–1025. doi: 10.1038/ismej.2012.174

Acharya, A., Koh, M. L., Kheur, S., Watt, R. M., Jin, L., and Mattheos, N. (2015). Salivary IL-1β and red complex bacteria as predictors of the inflammatory status in sub-peri-implant niches of subjects with peri-implant mucositis. *Clin. Oral Implants Res.* 27, 662–667. doi: 10.1111/clr.12713

Al-Hebshi, N. N., Abdulhaq, A., Albarrag, A., Basode, V. K., and Chen, T. (2016). Species-level core oral bacteriome identified by 16S rRNA pyrosequencing in a healthy young Arab population. *J. Oral Microbiol.* 8:31444. doi: 10.3402/jom.v8.31444

Al-Hebshi, N. N., Nasher, A. T., Idris, A. M., and Chen, T. (2015). Robust species taxonomy assignment algorithm for 16S rRNA NGS reads: application to oral carcinoma samples. *J. Oral Microbiol.* 7:28934. doi: 10.3402/jom.v7.28934

Armitage, G. C. (1999). Development of a classification system for periodontal diseases and conditions. *Ann. Periodontol.* 4, 1–6. doi: 10.1902/annals.1999.4.1.1

Belstrøm, D., Fiehn, N.-E., Nielsen, C. H., Klepac-Ceraj, V., Paster, B. J., Twetman, S., et al. (2015). Differentiation of salivary bacterial profiles of subjects with periodontitis and dental caries. *J. Oral Microbiol.* 7:27429. doi: 10.3402/jom.v7.27429

Belstrøm, D., Holmstrup, P., Bardow, A., Kokaras, A., Fiehn, N.-E., and Paster, B. J. (2016). Temporal stability of the salivary microbiota in oral health. *PLoS ONE* 11:e0147472. doi: 10.1371/journal.pone.0147472

Belstrøm, D., Sembler-Møller, M. L., Grande, M. A., Kirkby, N., Cotton, S. L., Paster, B. J., et al. (2017). Microbial profile comparisons of saliva, pooled and site-specific subgingival samples in periodontitis patients. *PLoS ONE* 12:e0182992. doi: 10.1371/journal.pone.0182992

Benítez-Páez, A., Belda-Ferre, P., Simón-Soro, A., and Mira, A. (2014). Microbiota diversity and gene expression dynamics in human oral biofilms. *BMC Genomics* 15:311. doi: 10.1186/1471-2164-15-311

Brunner, K., John, C. M., Phillips, N. J., Alber, D. G., Gemmell, M. R., Hansen, R., et al. (2018). Novel *Campylobacter concisus* lipooligosaccharide is a determinant of inflammatory potential and virulence. *J. Lipid Res.* 59, 1893–1905. doi: 10.1194/jlr.m085860

Camelo-Castillo, A., Novoa, L., Balsa-Castro, C., Blanco, J., Mira, A., and Tomás, I. (2015). Relationship between periodontitis-associated subgingival microbiota and clinical inflammation by 16S pyrosequencing. *J. Clin. Periodontol.* 42, 1074–1082. doi: 10.1111/jcpe.12470

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7 335–336. doi: 10.1038/nmeth.f.303

Chen, C., Hemme, C., Beleno, J., Shi, Z. J., Ning, D., Qin, Y., et al. (2018). Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. *ISME J.* 12, 1210–1224. doi: 10.1038/s41396-017-0037-1

Chen, H., Liu, Y., Zhang, M., Wang, G., Qi, Z., Bridgewater, L., et al. (2015). A Filifactor alocis-centered co-occurrence group associates with periodontitis across different oral habitats. *Sci. Rep.* 5:9053. doi: 10.1038/srep09053

Chen, L., Qin, B., Du, M., Zhong, H., Xu, Q., Li, Y., et al. (2015). Extensive description and comparison of human supra-gingival microbiome in root caries and health. *PLoS ONE* 10:e0117064. doi: 10.1371/journal.pone.0117064

Chen, T., Yu, W.-H., Izard, J., Baranova, O. V., Lakshmanan, A., and Dewhirst, F. E. (2010). The human oral microbiome database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database* 2010:baq013. doi: 10.1093/database/baq013

Dabdoub, S. M., Ganesan, S. M., and Kumar, P. S. (2016). Comparative metagenomics reveals taxonomically idiosyncratic yet functionally congruent communities in periodontitis. *Sci. Rep.* 6:38993. doi: 10.1038/srep38993

David, L. A., Materna, A. C., Friedman, J., Campos-Baptista, M. I., Blackburn, M. C., Perrotta, A., et al. (2014). Host lifestyle affects human microbiota on daily timescales. *Genome Biol.* 15:R89. doi: 10.1186/gb-2014-15-7-r89

De Caceres, M., and Legendre, P. (2009). Associations between species and groups of sites: indices and statistical inference. *Ecology* 90, 3566–3574. doi: 10.1890/08-1823.1

De Cáceres, M., Legendre, P., Wiser, S. K., and Brotons, L. (2012). Using species combinations in indicator value analyses. *Methods Ecol. Evol.* 3, 973–982. doi: 10.1111/j.2041-210x.2012.00246.x

Deshpande, N. P., Wilkins, M. R., Castaño-Rodríguez, N., Bainbridge, E., Sodhi, N., Riordan, S. M., et al. (2016). *Campylobacter concisus* pathotypes induce distinct global responses in intestinal epithelial cells. *Sci. Rep.* 6:34288. doi: 10.1038/srep34288

Edgar, R. C. (2010), Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Ehrmann, E., Handal, T., Tamanai-Shacoori, Z., Bonnaure-Mallet, M., and Fosse, T. (2013). High prevalence of -lactam and macrolide resistance genes in human oral Capnocytophaga species. *J. Antimicrob. Chemother.* 69, 381–384. doi: 10.1093/jac/dkt350

Endo, S., Mishima, E., Takeuchi, Y., Ohi, T., Ishida, M., Yanai, M., et al. (2015). Periodontitis-associated septic pulmonary embolism caused by Actinomyces species identified by anaerobic culture of bronchoalveolar lavage fluid: a case report. *BMC Infect. Dis.* 15:552. doi: 10.1186/s12879- 015-1286-0.

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., et al. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2:6. doi: 10.1186/2049-2618-2-6

Gomar-Vercher, S., Simón-Soro, A., Montiel-Company, J. M., Almerich-Silla, J. M., and Mira, A. (2018). Stimulated and unstimulated saliva samples have significantly different bacterial profiles. *PLoS ONE* 13:e0198021. doi: 10.1371/journal.pone.0198021

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., et al. (2011). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J.* 6, 1176–1185. doi: 10.1038/ismej.2011.191

Huang, S., Yang, F., Zeng, X., Chen, J., Li, R., Wen, T., et al. (2011). Preliminary characterization of the oral microbiota of Chinese adults with and without gingivitis. *BMC Oral Health* 11:33. doi: 10.1186/1472-6831-11-33

Ibrahim, M., Subramanian, A., and Anishetty, S. (2017). Comparative pan genome analysis of oral Prevotella species implicated in periodontitis. *Funct. Integr. Genomics* 17, 513–536. doi: 10.1007/s10142-017-0550-3

Kazor, C. E., Mitchell, P. M., Lee, A. M., Stokes, L. N., Loesche, W. J., Dewhirst, F. E., et al. (2003). Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. *J. Clin. Microbiol.* 41, 558–563. doi: 10.1128/jcm.41.2.558-563.2003

Kirst, M. E., Li, E. C., Alfant, B., Chi, Y.-Y., Walker, C., Magnusson, I., et al. (2014). Dysbiosis and alterations in predicted functions of the subgingival microbiome in chronic periodontitis. *Appl. Environ. Microbiol.* 81, 783–793. doi: 10.1128/aem.02712-14

Kistler, J. O., Booth, V., Bradshaw, D. J., and Wade, W. G. (2013). Bacterial community development in experimental gingivitis. *PLoS ONE* 8:e71227. doi: 10.1371/journal.pone.0071227

Koren, O., Knights, D., Gonzalez, A., Waldron, L., Segata, N., Knight, R., et al. (2013). A guide to enterotypes across the human body: meta-analysis of microbial community structures in human microbiome datasets. *PLoS Comput. Biol.* 9:e1002863. doi: 10.1371/journal.pcbi.1002863

Lazarevic, V., Whiteson, K., Hernandez, D., Francois, P., and Schrenzel, J. (2010). Study of inter- and intra-individual variations in the salivary microbiota. *BMC Genomics* 11:523. doi: 10.1186/1471-2164-11-523

Liu, F., Ma, R., Wang, Y., and Zhang, L. (2018). The clinical importance of *Campylobacter concisus* and other human hosted Campylobacter Species. *Front. Cell. Infect. Microbiol.* 8:243. doi: 10.3389/fcimb.2018.00243

Lopetuso, L. R., Scaldaferri, F., Petito, V., and Gasbarrini, A. (2013). Commensal Clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathog.* 5:23. doi: 10.1186/1757-4749-5-23

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15:550. doi: 10.1186/s13059-014-0550-8

Madhwani, T., and McBain, A. J. (2016). The application of magnetic bead selection to investigate interactions between the oral microbiota and salivary immunoglobulins. *PLoS ONE* 11:e0158288. doi: 10.1371/journal.pone.0158288

Mascitti, M., Togni, L., Troiano, G., Caponio, V., Gissi, D. B., Montebugnoli, L., et al. (2019). Beyond head and neck cancer: the relationship between oral microbiota and tumour development in distant organs. *Front. Cell. Infect. Microbiol.* 9:232. doi: 10.3389/fcimb.2019.00232

Mason, M. R., Chambers, S., Dabdoub, S. M., Thikkurissy, S., and Kumar, P. S. (2018). Characterizing oral microbial communities across dentition states and colonization niches. *Microbiome* 6:67. doi: 10.1186/s40168-018-0443-2

McGeoch, M. A., and Chown, S. L. (1998). Scaling up the value of bioindicators. *Trends Ecol. Evol.* 13, 46–47.

Mok, S. F., Karuthan, C., Cheah, Y. K., Ngeow, W. C., Rosnah, Z., Yap, S. F., et al. (2017). The oral microbiome community variations associated with normal, potentially malignant disorders and malignant lesions of the oral cavity. *Malays. J. Pathol.* 39, 1–15.

Pérez-Chaparro, P. J., Gonçalves, C., Figueiredo, L. C., Faveri, M., Lobão, E., Tamashiro, N., et al. (2014). Newly identified pathogens associated with periodontitis. *J. Dent. Res.* 93, 846–858. doi: 10.1177/0022034514542468

Rosier, B. T., Marsh, P. D., and Mira, A. (2018). Resilience of the oral microbiota in health: mechanisms that prevent dysbiosis. *J. Dent. Res.* 97, 371–380. doi: 10.1177/0022034517742139

Saygun, I., Nizam, N., Keskiner, I., Bal, V., Kubar, A., Açikel, C., et al. (2011). Salivary infectious agents and periodontal disease status. *J. Periodont. Res.* 46, 235–239. doi: 10.1111/j.1600-0765.2010.01335.x

Scher, J. U., Ubeda, C., Equinda, M., Khanin, R., Buischi, Y., Viale, A., et al. (2012). Periodontal disease and the oral microbiota in new-onset rheumatoid arthritis. *Arthritis Rheum.* 64, 3083–3094. doi: 10.1002/art.34539

Schloss, P. D., and Westcott, S. L. (2011). Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. *Appl. Environ. Microbiol.* 77, 3219–3226. doi: 10.1128/aem.02810-10

Schwarzberg, K., Le, R., Bharti, B., Lindsay, S., Casaburi, G., Salvatore, F., et al. (2014). The personal human oral microbiome obscures the effects of treatment on periodontal disease. *PLoS ONE* 9:e86708. doi: 10.1371/journal.pone.0086708

Takeshita, T., Kageyama, S., Furuta, M., Tsuboi, H., Takeuchi, K., Shibata, Y., et al. (2016). Bacterial diversity in saliva and oral health-related conditions: the Hisayama Study. *Sci. Rep.* 6:22164. doi: 10.1038/srep22164

Tanner, A. C., Dzink, J. L., Ebersole, J. L., and Socransky, S. S. (1987), Wolinella recta, *Campylobacter concisus*, Bacteroides gracilis, and *Eikeneila corrodens* from periodontal lesions. *J. Periodontal Res.* 22, 327–330. doi: 10.1111/j.1600-0765.1987.tb01593.x

Tsuda, H., Ochiai, K., Suzuki, N., and Otsuka, K. (2010). Butyrate, a bacterial metabolite, induces apoptosis and autophagic cell death in gingival epithelial cells. *J. Periodont. Res.* 45, 626–634. doi: 10.1111/j.1600-0765.2010.01277.x

van Rensburg, J. J., Lin, H., Gao, X., Toh, E., Fortney, K. R., Ellinger, S., et al. (2015). The human skin microbiome associates with the outcome of and is influenced by bacterial infection. *MBio* 6:e01315-15. doi: 10.1128/mbio.01315-15

Vieira Colombo, A. P., Magalhães, C. B., Hartenbach, F. A. R. R., Martins do Souto, R., and Maciel da Silva-Boghossian, C. (2016). Periodontal-disease-associated biofilm: a reservoir for pathogens of medical importance. *Microb. Pathog.* 94, 27–34. doi: 10.1016/j.micpath.2015.09.009

World Medical Association (2013). World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *JAMA* 310, 2191–2194. doi: 10.1001/jama.2013.281053

Yamanaka, W., Takeshita, T., Shibata, Y., Matsuo, K., Eshima, N., Yokoyama, T., et al. (2012). Compositional stability of a salivary bacterial population against supragingival microbiota shift following periodontal therapy. *PLoS ONE* 7:e42806. doi: 10.1371/journal.pone.0042806

Yang, F., Zeng, X., Ning, K., Liu, K.-L., Lo, C.-C., Wang, W., et al. (2011). Saliva microbiomes distinguish caries-active from healthy human populations. *ISME J.* 6, 1–10. doi: 10.1038/ismej.2011.71

Zhang, L., Budiman, V., Day, A. S., Mitchell, H., Lemberg, D. A., Riordan, S. M., et al. (2010). Isolation and detection of *Campylobacter concisus* from saliva of healthy individuals and patients with inflammatory bowel disease. *J. Clin. Microbiol.* 48, 2965–2967. doi: 10.1128/jcm.02391-09

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 10 December 2019
doi: 10.3389/fphys.2019.01476

Salivary Biomarkers for Oral Squamous Cell Carcinoma Diagnosis and Follow-Up: Current Status and Perspectives

Marta Cristaldi[1], Rodolfo Mauceri[1,2], Olga Di Fede[1], Giovanna Giuliana[1], Giuseppina Campisi[1] and Vera Panzarella[1]*

[1] Department of Surgical, Oncological and Oral Sciences, University of Palermo, Palermo, Italy, [2] Department of Biomedical and Dental Sciences and Morphofunctional Imaging, University of Messina, Messina, Italy

OPEN ACCESS

Edited by:
Lorenzo Lo Muzio, University of Foggia, Italy

Reviewed by:
Pier Paolo Claudio, University of Mississippi, United States
Marco Mascitti, Marche Polytechnic University, Italy

***Correspondence:**
Giuseppina Campisi
campisi@odonto.unipa.it

Specialty section:
This article was submitted to Craniofacial Biology and Dental Research, a section of the journal Frontiers in Physiology

Received: *24 September 2019*
Accepted: *18 November 2019*
Published: *10 December 2019*

Citation:
Cristaldi M, Mauceri R, Di Fede O, Giuliana G, Campisi G and Panzarella V (2019) Salivary Biomarkers for Oral Squamous Cell Carcinoma Diagnosis and Follow-Up: Current Status and Perspectives. Front. Physiol. 10:1476. doi: 10.3389/fphys.2019.01476

Oral cancer is the sixth most common cancer type in the world, and 90% of it is represented by oral squamous cell carcinoma (OSCC). Despite progress in preventive and therapeutic strategies, delay in OSCC diagnosis remains one of the major causes of high morbidity and mortality; indeed the majority of OSCC has been lately identified in the advanced clinical stage (i.e., III or IV). Moreover, after primary treatment, recurrences and/or metastases are found in more than half of the patients (80% of cases within the first 2 years) and the 5-year survival rate is still lower than 50%, resulting in a serious issue for public health. Currently, histological investigation represents the "gold standard" of OSCC diagnosis; however, recent studies have evaluated the potential use of non-invasive methods, such as "liquid biopsy," for the detection of diagnostic and prognostic biomarkers in body fluids of oral cancer patients. Saliva is a biofluid containing factors such as cytokines, DNA and RNA molecules, circulating and tissue-derived cells, and extracellular vesicles (EVs) that may be used as biomarkers; their analysis may give us useful information to do early diagnosis of OSCC and improve the prognosis. Therefore, the aim of this review is reporting the most recent data on saliva biomarker detection in saliva liquid biopsy from oral cancer patients, with particular attention to circulating tumor DNA (ctDNA), EVs, and microRNAs (miRNAs). Our results highlight that saliva liquid biopsy has several promising clinical uses in OSCC management; it is painless, accessible, and low cost and represents a very helpful source of diagnostic and prognostic biomarker detection. Even if standardized protocols for isolation, characterization, and evaluation are needed, recent data suggest that saliva may be successfully included in future clinical diagnostic processes, with a considerable impact on early treatment strategies and a favorable outcome.

Keywords: liquid biopsy, salivary biomarkers, circulating tumor DNA, extracellular vesicles, microRNAs, early diagnosis, prognosis, oral squamous cell carcinoma

INTRODUCTION

Oral cancer is one of the most common cancers in the world, representing a serious problem for global health (Shah and Gil, 2009). Roughly 90% of oral cancers histologically originate from squamous cells and are classified as oral squamous cell carcinoma (OSCC) (Rivera, 2015; Mascitti et al., 2018).

Despite current advancements in cancer prevention strategies and therapeutic approaches, the prognosis of OSCC-affected patients is still poor; this mainly depends on late diagnosis in an advanced stage (i.e., III or IV) (Rivera, 2015; Mascitti et al., 2018). Moreover, after primary treatment, recurrences and/or metastases are found in more than half of the patients (80% of cases within the first 2 years), and the 5-year survival rate is still lower than 50%, resulting in a serious issue for public health; as a result, in the latest decades, the annual mortality rate has remained unchanged at 145,000 cases (Grobe et al., 2013; Panzarella et al., 2014; Rivera, 2015).

To date, the "gold standard" for OSCC diagnosis is represented by a clinical oral examination integrated by a histological investigation on tissue biopsy of suspicious lesions (Fuller et al., 2015). However, cancer research is currently focusing on finding less invasive and cost-effective methods to provide a more comprehensive view of the cancer profile, also to more easily monitor its evolution and therapeutic response (Siravegna et al., 2017; Wang J. et al., 2017). In this regard, many non-invasive techniques such as liquid biopsy have been recently proposed as supportive tools for diagnosis, prognosis, and follow-up of OSCC (Dionne et al., 2015; Fuller et al., 2015; Aro et al., 2017).

As is widely known, cancers developed from different types of DNA alterations which are classified based on their effects on structure: small-scale genetic alterations, which comprise different types of point mutations (e.g., DNA base insertions, substitutions, and deletions), and large-scale gene alterations such as DNA rearrangements (e.g., gene duplications/deletions, chromosomal translocations/inversions, and loss of heterozygosity) (Stadler et al., 2010). It is noteworthy that the changes in DNA genetic and epigenetic structure (i.e., altered function of factors implicated in gene expression) can be detected by several biomarkers as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), microRNAs (miRNAs), and extracellular vesicles (EVs), which can be found in several biological fluids such as blood, serum, plasma, pleural fluid, urine, and saliva (Kaczor-Urbanowicz et al., 2017). The evaluation of these cancer biomarkers in liquid biopsy has the advantage of providing a real-time picture of primary and metastatic tumors at different time points, giving information about tumor and tumor burden and early evidence of drug resistance and tumor recurrence (Di Meo et al., 2017). Moreover, liquid biopsy analysis allows defining of the DNA molecular profile of cancer patients, whose inclusion in the tumor, node, and metastasis staging system may help to develop more highly personalized therapies and decrease risks of inappropriate treatments (Xu et al., 2017).

Oral squamous cell carcinoma is strictly associated with the oral microenvironment, resulting in a direct contact with saliva, an acidic biological fluid derived from secretion of major and minor salivary glands, and involved in many physiological and pathological processes (Kaufman and Lamster, 2002; Nieuw Amerongen and Veerman, 2002; Chiappin et al., 2007; Jankowska et al., 2007; Lee and Wong, 2009). In recent decades, saliva has been widely investigated as a promising source of OSCC biomarkers for liquid biopsy (Kaczor-Urbanowicz et al., 2017). Employing saliva in liquid biopsy for OSCC management has many advantages over other specimens: (i) it is considered "the mirror of the body," reflecting any physiological and pathological change on local and distant sites of the body; (ii) it represents an easier, quicker, and more accessible screening tool (Kaczor-Urbanowicz et al., 2017); and (iii) it gives the opportunity to collect larger volumes of samples for examination, to easily repeat analysis, and to monitor OSCC over time (Kaczor-Urbanowicz et al., 2017; Gai et al., 2018; Khurshid et al., 2018).

In light of the above, this narrative review aims to describe the recent data regarding the employment of different salivary biomarkers in liquid biopsy for OSCC diagnosis, prognosis, follow-up, and patient response to therapy. Moreover, we discuss the current challenges and future perspectives concerning the use of salivary biomarkers in OSCC management.

SALIVARY BIOMARKERS AND OSCC

Circulating Tumor DNA

During physiological cellular turnover or in particular pathological conditions, apoptotic and necrotic cells release debris and DNA/RNA molecules into body fluids. In physiologic conditions, cell-derived molecules and debris are removed by phagocytes; instead, in cancer patients, this mechanism results in impairment, inducing the accumulation of cell-free DNA (cfDNA) in the tissue microenvironment and biological fluids (Jahr et al., 2001; Chan et al., 2004; Mouliere et al., 2011). As a result, cancer patients have increased levels of cfDNA in body fluids (Diehl et al., 2008; Kohler et al., 2011). cfDNA released from cancer cells, also known as ctDNA, can be distinguished from cfDNA physiologically released from non-cancer cells through several features: biofluid concentration, somatic mutations, and overall size. Because of random partial digestion of genomic DNA, cfDNA from apoptotic cells is measured at 180–200 base pairs; necrosis or autophagy during the cancer process usually generates larger DNA molecules, from 100 to 400 base pairs (Wang et al., 2003; Diehl et al., 2008; Kohler et al., 2011).

It is noteworthy that many studies also showed that ctDNA in cancer patients reflects the genetic and epigenetic alterations found in tissue samples of malignant lesions (Ignatiadis et al., 2015; Cheng et al., 2016; Qin et al., 2016); in addition, many other cancer characteristics such as size, cellular turnover, stage, vascularity, and drug response are associated with ctDNA concentrations (Chang et al., 2017).

Several mechanisms have been suggested to explain the release of ctDNA in body fluids of cancer patients, even if they remain dubious. It could be the result of accelerated metabolism of cancer cells, responsible for the increased number of apoptotic/necrotic cells (Shukla et al., 2013; Francis and Stein, 2015; Volik et al., 2016; Payne et al., 2018). Cancer cells released from primary

tumor mass and disseminating throughout body fluids (e.g., CTCs) could actively release fragments of cellular nucleic acids, which, when internalized from non-cancer cells, induce neoplastic transformation (Ilie et al., 2014; Cheng et al., 2016). Cancer transformation can be also the result of the internalization of vesicles (e.g., EVs), containing ctDNA and actively released from cancer cells, from healthy cells (Lau et al., 2013; Kaczor-Urbanowicz et al., 2017).

ctDNA is mainly released into the bloodstream; however, it can also be found in other body fluids. For instance, by means of salivary gland ultrafiltration, passive diffusion, or active transport, ctDNA can easily reach saliva from a local site and bloodstream, carrying information regarding primary tumors and/or metastasis (Pu et al., 2016; Kaczor-Urbanowicz et al., 2017; Wang X. et al., 2017). Thanks to less dilution and contamination, the analysis of the ctDNA in saliva is much more sensitive than that in the bloodstream (Peng et al., 2017).

As described in **Table 1**, many studies have applied salivary ctDNA in the management of head and neck squamous cell carcinoma (HNSCC) (Liao et al., 2000; El-Naggar et al., 2001; Viet and Schmidt, 2008; Sethi et al., 2009; Demokan et al., 2010; Carvalho et al., 2011; Guerrero-Preston et al., 2011; Sun et al., 2012; Rettori et al., 2013; Ramadoss et al., 2015; Wang et al., 2015; Ferlazzo et al., 2017; Lacombe et al., 2017). When HNSCC patients with tumors at the oral cavity, oropharynx, larynx, and hypopharynx were enrolled at the early stages (I and II) and late stages (III and IV), ctDNA was detected, respectively, in 100% of HNSCC patients enrolled at the early stages and in 95% enrolled at the late stages, with an enrichment in saliva of all patients affected by OSCC; this result is very specific for OSCC detection. Moreover, being postsurgically found in 100% of patients who subsequently had a clinical recurrence and in any patients without recurrence, it could be also a valuable biomarker for oral cancer follow-up and surveillance (Wang et al., 2015). Interestingly, when the OSCC-leading genetic alterations in ctDNA of saliva and tissue samples were analyzed, a correlation in loss of heterozygosity and p53 mutations was found, demonstrating that saliva can be a reliable and non-invasive alternative to tissue biopsy for OSCC detection (Liao et al., 2000; El-Naggar et al., 2001; Ramadoss et al., 2015).

Among the epigenetic alterations frequently found in salivary ctDNA, gene promoter methylation has been extensively investigated in cancer. Numerous studies, comparing the methylation rate of genes involved in the cell cycle, proliferation, and apoptosis, found a difference in salivary samples of HNSCC patients and healthy subjects, demonstrating that salivary ctDNA methylation could be considered a very promising biomarker for HNSCC management and specifically OSCC diagnosis, prognosis, and follow-up (Viet et al., 2007; Viet and Schmidt, 2008; Sethi et al., 2009; Puttipanyalears et al., 2014). Epigenetic alterations such as DNA methylation rate can be easily detected in salivary ctDNA, proving to be a valuable sensitive and specific marker for OSCC diagnosis and prognosis (Demokan et al., 2010; Pattani et al., 2010; Guerrero-Preston et al., 2011; Ferlazzo et al., 2017). Moreover, the evaluation of the methylation status of genes, involved in OSCC development at diagnosis, seems to be useful as a predictive factor to develop a personalized therapy and assess the patient response during surveillance (Viet and Schmidt, 2008; Lacombe et al., 2017).

Extracellular Vesicles

As is widely known, EVs represent one of the main mechanisms of intercellular communication (Deutsch et al., 2008). The discovery of cell-derived EVs in saliva able to selectively "package" factors such as DNAs, RNAs, miRNAs, and proteins recently attracted the attention of researchers, being promising additional sources of biomarkers (Valadi et al., 2007; Krief et al., 2011; El Andaloussi et al., 2013; Lötvall et al., 2014; Wong, 2015; Yáñez-Mó et al., 2015). To date, the most investigated vesicles in cancer development and progression are exosomes and microvesicles, small membrane vesicles similar in some structural and functional elements but distinguished by means of size and cellular release processes (Wong, 2006; Pfaffe et al., 2011; Corrado et al., 2013; Kastelowitz and Yin, 2014).

Many studies demonstrated that EVs, transferring protein and nucleic acids able to support or inhibit tumorigenesis, are implicated in tumor microenvironments (Kalluri, 2016; Ruivo et al., 2017); additionally, it has been recently shown that EVs may have a role in oral cancers (Gai et al., 2018; Han et al., 2018). Differences in relative size, proteome signature, and expression of exosomal markers have been highlighted between OSCC parental and metastatic cells; moreover, OSCC parental cells release exosomes with oncogenic markers able to influence the surrounding tumor microenvironment. The evaluation of exosomal markers from OSCC patients can support diagnosis, prognosis, and assessment of patient response to therapy, detecting potential tumor recurrence (Byun et al., 2015; Languino et al., 2016; Li et al., 2016; Liu et al., 2017; Ono et al., 2018).

Nevertheless, few studies have evaluated the role of salivary EVs in OSCC management (**Table 2**; Winck et al., 2015; Zlotogorski-Hurvitz et al., 2016). Salivary tumor-derived exosomes have different morphological and molecular features compared to healthy saliva samples and can detect cancer and malignant transformations early in high-risk patients (Zlotogorski-Hurvitz et al., 2016; Malonek et al., 2018). In the comparison of the proteomic analysis of whole saliva from healthy and OSCC patients, it was also shown that the qualitative and quantitative features of saliva EVs may support OSCC diagnosis and give information regarding the prognosis of cancer patients. The proteome of salivary EVs shows the presence of proteins implicated in cancer inflammatory response, transport of metals, and cellular growth and proliferation, allowing the classification of OSCC patients and the ability to give prognosis information with a high percentage of accuracy (Winck et al., 2015).

MicroRNAs

MicroRNAs are small single-strand RNA molecules (19–21 nucleotides), transcribed by cellular polymerase RNA and submitted to a double sequential cut: in the nucleus (primary miRNA) and cytoplasm (precursor miRNA). Then, depending on the complementarity level with the target, they cleave a messenger RNA (mRNA) or inhibit its translation (Bartel, 2004; Perron and Provost, 2008).

TABLE 1 | Diagnostic, prognostic, and follow-up applications of ctDNA in OSCC, based on population study.

Authors (year)	Title	Cases	Controls	Type of alteration	Application	Results
Liao et al., 2000	Mutation of p53 gene codon 63 in saliva as a molecular marker for oral squamous cell carcinomas	10 OSCC	27	DNA point mutation	Diagnosis	Of the OSCCs, 62.5% harbor C-deletion in exon 4 codon 63 from preoperative saliva samples. TSG p53 exon 4 codon 63 mutation is a good biomarker for OSCC diagnosis.
El-Naggar et al., 2001	Genetic heterogeneity in saliva from patients with oral squamous carcinomas: implications in molecular diagnosis and screening	40 OSCC	10	DNA microsatellite	Diagnosis	A correlation on DNA microsatellite alterations between saliva and tumor specimens was found. The saliva ctDNA can be used as a genetic biomarker for early detection of OSCC.
Viet and Schmidt, 2008	Methylation array analysis of preoperative and postoperative saliva DNA in oral cancer patients	13 OSCC	10	DNA hypermethylation	Diagnosis and prognosis	Sites highly methylated in the tissue and preoperative saliva samples, but not methylated in the postoperative saliva samples or in normal subjects, were found. A genetic classifier consisting of specifically methylated gene loci was generated and can be used as a composite biomarker for early diagnosis and prognosis of OSCC.
Sethi et al., 2009	Non-invasive molecular detection of head and neck squamous cell carcinoma: an exploratory analysis	27 HNSCC (7 OSCC)	10	CNV (loss and gain)	Diagnosis	Gain of PMAIP1 (18q21.31) solely or in conjunction with gain of PTPN1 (20q13.13), detected in saliva DNA, differentiated 100% of HNSCC cases from normal controls with high sensitivity and specificity, with no tumor site differentiation. However, it has clinical utility for non-invasive HNSCC diagnosis and screening, including OSCC.
Demokan et al., 2010	KIF1A and EDNRB are differentially methylated in primary HNSCC and salivary rinses	101 HNSCC (39 OSCC)	76	DNA hypermethylation	Diagnosis	Promoter hypermethylation of K1F1A and EDNRB genes, detected in saliva DNA, is preferentially methylated in salivary rinses from HNSCC compared with healthy patients. In addition, patients with OSCC had higher methylation on the K1F1A promoter. KIF1A and EDNRB are potential biomarkers for HNSCC detection, with K1F1A being more specific for OSCC.
Carvalho et al., 2011	Detection of promoter hypermethylation in salivary rinses as a biomarker for head and neck squamous cell carcinoma surveillance	61 HNSCC (30 OSCC)	–	DNA hypermethylation	Prognosis and follow-up	Of the HNSCC patients analyzed, 54.1% showed methylation in the promoter of at least one of the studied genes (i.e., DAPK, DCC, MINT-31, TIMP3, p16, MGMT, and CCNA1) in saliva ctDNA, with a significantly lower local disease control and overall survival in patients with recurrence. In addition, among all sites analyzed, a higher percentage of patients with OSCC, with respect to the other sites, showed DNA hypermethylation. Detection of DNA hypermethylation in saliva ctDNA can potentially predict local recurrence and overall survival in OSCC patients.
Guerrero-Preston et al., 2011	NID2 and HOXA9 promoter hypermethylation as biomarkers for prevention and early detection in oral cavity squamous cell carcinoma tissues and saliva	16 OSCC	19	DNA hypermethylation	Diagnosis	The promoter hypermethylation of HOXA9 and NID2 genes detected in tissue, saliva, and serum ctDNA of OSCC patients has a moderate to significant agreement with clinical diagnosis, distinguishing healthy from OSCC patients at the moment of pretreatment. They are potentially useful for OC early detection and prevention.
Sun et al., 2012	Detection of TIMP3 promoter hypermethylation in salivary rinse as an independent predictor of local recurrence-free survival in head and neck cancer	197 HNSCC (53 OSCC)	–	DNA hypermethylation	Prognosis	Promoter hypermethylation of TIMP3 detected in pretreatment salivary rinse ctDNA of HNSCC patients is associated with disease-free survival; even if without tumor site differentiation, hypermethylation of TIMP3 can be considered a salivary independent prognostic factor of HNSCC recurrence, including OSCC.
Rettori et al., 2013	Prognostic significance of TIMP3 hypermethylation in post-treatment salivary rinse from head and neck squamous cell carcinoma patients	146 HNSCC (70 OSCC)	60	DNA hypermethylation	Diagnosis, prognosis, and follow-up	DNA hypermethylation in CCNA1, DAPK, DCC, MGMT, and TIMP3 is strictly correlated to the presence of OSCC tumor. Moreover, 100% of HNSCC patients with TIMP3 DNA hypermethylation, 6 months after treatment, had lower local recurrence-free survival. DNA hypermethylation on CCNA1, DAPK, DCC, MGMT, and TIMP3 genes is a specific OSCC diagnostic biomarker; TIMP3 DNA hypermethylation is an independent prognostic factor to recognize HNSCC patient subgroups with high risk of local recurrence.

(Continued)

TABLE 1 | Continued

Authors (year)	Title	Cases	Controls	Type of alteration	Application	Results
Ramadoss et al., 2015	C-deletion in exon 4 codon 63 of p53 gene as a molecular marker for oral squamous cell carcinoma: a preliminary study	20 OSCC	5	DNA point mutation	Diagnosis	PCR data showed the presence of C-deletion in exon 4 of p53 gene in saliva of 100% OSCC. Mutation in exon 4 codon 63 of the p53 gene, evaluated by PCR, is a fast, reliable, accurate, and sensitive molecular method that may be employed as a potential molecular diagnostic marker for OSCC.
Wang et al., 2015	Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas	93 HNSCC (3 OSCC)	–	DNA somatic mutations and HPV genes	Diagnosis, prognosis, and follow-up	After evaluation of ctDNA somatic mutations or HPV genes from saliva and plasma of HNSCC patients, saliva ctDNA mutations resulted in 100% specificity for OSCC cancer detection. Moreover, any mutation was identified after surgery in the patients whose tumors did not recur, being considered a valuable diagnostic, prognostic, and follow-up biomarker.
Ferlazzo et al., 2017	Influence of MTHFR genetic background on p16 and MGMT methylation in oral squamous cell cancer	58 OSCC	90	DNA hypermethylation	Diagnosis	There is a higher frequency of p16 and MGMT promoter methylation in OSCC patients than in normal controls. The assessment of DNA methylation rate could represent a powerful diagnostic approach for early detection of cancer.
Lacombe et al., 2017	Analysis of saliva gene expression during head and neck cancer radiotherapy: a pilot study	8 HNSCC (1 OSCC)	–	Aberrant expression	Follow-up	This pilot study shows that salivary gene biomarkers can be detected and their expression modified during head and neck radiotherapy. The increasing expression of the genes CCDKN1A and DDB2, during radiation treatment, is correlated to the cumulative received dose. Saliva is a minimally invasive means of biomarker collection to directly measure radiation dose escalation during treatment.

OSCC, oral squamous cell carcinoma; ctDNA, circulating tumor DNA; TSG, tumor suppressor gene; HNSCC, head and neck squamous cell carcinoma; CNV, copy number variation; miRNA, microRNA; PCR, polymerase chain reaction; HPV, human papillomaviruses; MTHFR, methylenetetrahydrofolate reductase.

TABLE 2 | Diagnostic and prognostic application of EVs in OSCC, based on population study.

Authors (year)	Title	Cases	Controls	Type of alteration	Application	Results
Winck et al., 2015	Insights into immune responses in oral cancer through proteomic analysis of saliva and salivary extracellular vesicles	24 OSCC	10	Aberrant expression	Prognosis	The proteomic data from whole saliva and saliva EVs of OSCC and healthy patients allow us to classify OSCC patients with 90% accuracy, being considered valuable additional parameters/biological biomarkers to define the prognosis in OSCC patients.
Zlotogorski-Hurvitz et al., 2016	Morphological and molecular features of oral fluid-derived exosomes: oral cancer patients versus healthy individuals	36 OC (OSCC not specified)	25	Altered exosome morphology and protein composition	Diagnosis	Saliva from OC patients contains morphologically and molecularly different exosomes with respect to healthy subjects. In particular, the decrease in the expression of CD9 and CD81 rather than the increase of CD63 expression can be an indicator of OC, even in the very early stages of the disease, in high-risk patients without clear clinical sign.

EV, extracellular vesicle; OC, oral cancer; OSCC, oral squamous cell carcinoma.

MicroRNAs can be released into body fluids as cell-free miRNAs, associated with RNA-binding proteins or selectively packaged into EVs (Larrea et al., 2016). Their involvement in tumor microenvironments has been widely investigated, demonstrating that the expression of miRNAs could be associated with the deregulation of oncosuppressors or oncogenes, contributing to tumor development or inhibition. Moreover, they were unchanged in tissues and body fluids, and their expression is frequently tumor specific; for these reasons, the possibility to analyze miRNA profiles associated with cancer patients represents a new paradigm in biomarker discovery for cancer clinical diagnostics. Specifically, with regard to HNSCC cancers strictly associated with saliva, the evaluation of miRNAs released by OSCC cells in saliva represents a promising perspective for OSCC management (Momen-Heravi et al., 2014).

Over the years, many studies and reviews summarized the updates on evaluation of miRNA expression alteration for diagnosis and risk stratification of oral cancers, finding that many miRNAs are differentially expressed between healthy and OSCC patients (**Table 3**; Park et al., 2009; Wiklund et al., 2011; Liu et al., 2012; Yang et al., 2013; Momen-Heravi et al., 2014; Al-malkey and Abbas, 2015; Zahran et al., 2015; Duz et al., 2016; Greither et al., 2017). The different salivary

TABLE 3 | Diagnostic and follow-up application of miRNAs in OSCC, based on population study.

Authors (year)	Title	Cases	Controls	Type of alteration	Application	Results
Park et al., 2009	Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection	10 OSCC	10	Expression downregulation	Diagnosis	miRNAs are present in both whole saliva and supernatant saliva. Two of evaluated miRNAs, miR-125a and miR-200a, are downregulated in the saliva of the OSCC patients compared to healthy controls, being useful in detecting OSCC.
Wiklund et al., 2011	MicroRNA alterations and associated aberrant DNA methylation patterns across multiple sample types in oral squamous cell carcinoma	15 OSCC	7	Aberrant expression and DNA hypermethylation	Diagnosis	Compared to healthy subjects, OSCC patients had deregulated miRNAs with associated DNA methylation patterns. Particularly, repression of miR-375 and methylation on miR-137, miR-200c-141, and miR-200 s/miR-205 loci were found in OSCC patients vs. healthy patients, being promising candidates to develop OSCC-specific miRNA signatures.
Liu et al., 2012	Exploiting salivary miR-31 as a clinical biomarker of oral squamous cell carcinoma	45 OSCC	24	Expression upregulation	Diagnosis and follow-up	Salivary miR-31 was significantly increased in patients with OSCC at all clinical stages, including very early stages. In addition, it was shown to be more abundant in saliva than in plasma, and after tumor surgical removal, its expression was reduced. Salivary miR-31 is a promising specific biomarker for early detection and postoperative follow-up of OSCC.
Yang et al., 2013	Progress risk assessment of oral premalignant lesions with saliva miRNA analysis	45 OSCC potentially progressing LGD leukoplakia	-	Aberrant expression	Diagnosis	A specific miRNA aberrant profile was found in saliva samples of progressing oral LGD leukoplakia; it allows monitoring of cancer precursor lesions and early detection of progression toward OSCC, before clinical evidence.
Momen-Heravi et al., 2014	Genomewide study of salivary microRNAs for detection of oral cancer	9 OSCC-bt, 8 OSCC-r	9	Aberrant expression	Diagnosis	The miRNA profiles derived from OSCC, OSCC-r, and healthy controls were distinctively different. In particular, overexpression of miRNA-27b was found in OSCC saliva samples and not in the saliva of the other two groups; it can be potentially considered a promising OSCC diagnosis salivary biomarker.
Zahran et al., 2015	Salivary microRNAs in oral cancer	100 OC (20 OSCC)	20	Aberrant expression	Diagnosis	Salivary miRNA-21, miRNA-145, and miRNA-184 were differentially expressed in OSCC and healthy saliva samples. They can be considered non-invasive, rapid diagnostic biomarkers for oral malignant transformation and early detection of OSCC, with miRNA-184 having the best diagnostic value.
Al-malkey and Abbas, 2015	Expression analysis of salivary microrna-31 in oral cancer patients	35 OC (OSCC not specified)	20	Expression upregulation	Diagnosis and follow-up	Salivary miR-31 expression increased in OC patients, with enrichment in saliva compared with plasma, allowing the proposal of miR-31 as a sensitive biomarker for early detection and postsurgical follow-up of OC.
Duz et al., 2016	Identification of miR-139-5p as a saliva biomarker for tongue squamous cell carcinoma: a pilot study	25 OSCC	25	Aberrant expression	Diagnosis	Salivary miR-139-5p was significantly reduced in TSCC patients compared to controls, and its level turned back to normal after surgery. It is a feasible and promising diagnostic marker.
Greither et al., 2017	Salivary miR-93 and miR-200a as post-radiotherapy biomarkers in head and neck squamous cell carcinoma	17 HNSCC (5 OSCC)	–	Aberrant expression	Follow-up	MiR-93 and miR-200a were significantly increased 12 months after radiotherapy, demonstrating to be valuable biomarkers for treatment monitoring post-radiation of HNSCC.

miRNA, microRNA; LGD, low-grade dysplasia; OSCC, oral squamous cell carcinoma; OSCC-bt, oral cancer cell carcinoma before treatment; OSCC-r, oral cancer cell carcinoma in remission; OC, oral cancer; TSCC, tongue squamous cell carcinoma.

expression of miRNAs, as miR-125, miR- 200a, miR-21, miR-145, miR-200a, miR-93, miR-375, and miR-184 in OSCC patients compared to the healthy subjects, has been demonstrated to be a reliable method to detect OSCC and potentially malignant oral lesions (Park et al., 2009; Wiklund et al., 2011; Zahran et al., 2015; Greither et al., 2017; Maheswari et al., 2018). Salivary miR-139-5p has also demonstrated to be a valuable biomarker for response to therapy and evaluation of OSCC recurrence; it was downregulated in saliva samples from OSCC patients compared to the saliva of healthy subjects, and its expression increased in OSCC patient saliva after surgery (Duz et al., 2016).

Finally, other studies also focused on the potential of salivary miRNAs to assess the progression risk of oral premalignant lesions to OSCC, distinguishing progressing from non-progressing oral low-grade dysplasia; for example, the increased expression of miR-708, miR-10b, miR-19a, miR-30e, miR-26a, and miR-660 and decreased expression of miR-99, miR-15a, miR-197, miR-145, and miR-150 in saliva of OSCC patients represent biomarkers for neoplastic malignant transformation of oral lesions as oral low-grade dysplasia; some miRNAs, notably acting in cancer as oncosuppressors and found upregulated in patients with oral low-grade dysplasia, could represent elements of protection against the malignant transformation (Yang et al., 2013). Even though current evidence on the role of miRNAs in OSCC is encouraging, it is suggested that a combination of salivary miRNAs and proteomic data could help to have greater coverage and increased robustness of results (Yakob et al., 2014; Zahran et al., 2015).

Other Potential Biomarkers: CTCs

Circulating tumor cells have been widely investigated in the bloodstream as other potential OSCC biomarkers. They are released from primary tumor mass, disseminating via the lymphatic and blood vessels throughout the body. Their presence in blood circulation is correlated to metastasis, recurrences, and worse prognosis (Jatana et al., 2010; Hristozova et al., 2011; Alix-Panabieŕes and Pantel, 2013; Grobe et al., 2013). They can both promote the development of metastasis and colonize the primary tumor site, supporting the primary tumor growth in a process called tumor self-seeding (Ilie et al., 2014; Zhang et al., 2016). Many studies highlighted the correlation between increased levels of CTCs and OSCC diagnosis (Nichols et al., 2012; He et al., 2013); however, it seems that the increased number of CTCs is more correlated to prognosis and locoregional relapse (Jatana et al., 2011; Buglione et al., 2012; Gröbe et al., 2014). Therefore, analysis of CTCs in blood samples of cancer patients may really support clinician decisions, helping to phenotypically and genetically characterize the primary tumor (Jatana et al., 2010; Ilie et al., 2014; Lianidou et al., 2014; Rolfo et al., 2014; Tinhofer et al., 2014; Wu et al., 2016; Zhou et al., 2016), but being more frequently found in late-stage cancers, it seems to be more informative as a tool for cancer prognosis; moreover, the investigation of drug molecular targets on the CTC surface could help to develop immunotherapies (Nonaka and Wong, 2018). All studies performed so far derived from analysis of blood samples; the investigation of CTC on saliva remains unexplored. We conceive that future studies on salivary CTCs from OSCC patients may provide relevant information about tumor prognosis and genetic alterations potentially associated with tumor recurrence, contributing to development of targeted therapies.

DISCUSSION

With a frequency of 2–4% of all worldwide cancer cases, OSCC is the most common epithelial neoplasia affecting the head-and-neck area (Markopoulos, 2012). Many efforts have been done so far to improve the strategies of OSCC diagnosis, prognosis, and development of therapies; however, it still is a cancer with poor prognosis (Moore et al., 2000). This primarily depends on diagnostic delay and insufficiently informative techniques to do prognosis and uncover tumor recurrence after surgery and/or therapy establishment. Given the intra-tumoral and inter-tumoral heterogeneity and the dynamic behavior with modification over time on the molecular profile, also depending on the therapeutical response, the traditional strategies of cancer screening are not sufficient for the effective management of OSCC; we urgently need novel and more effective time-saving strategies (Bellairs et al., 2017). In light of the above, the latest body of literature has focused on understanding the tumor-leading molecular basis, to develop more precise and advanced methodologies for early detection, prognosis, and establishment of successful therapies, decreasing the rate of recurrences (Woolgar, 2006).

Liquid biopsy is currently one of the most investigated techniques to easily outline the molecular identity of cancers in both early and late stages, and due to its accessibility, ease of employment, and natural feature of being in close contact with OSCC cells, saliva is considered one of the most indicative body fluids for liquid biopsy in OSCC (Kaczor-Urbanowicz et al., 2017; Gai et al., 2018; Khurshid et al., 2018).

Saliva has been proposed as a valuable means for early diagnosis, definition of cancer patient molecular profile, and development of a personalized therapy and allows monitoring of response and cancer relapse; indeed, it contains bioactive elements such as ctDNAs, miRNAs, and EVs derived from OSCC cells, where genetic and epigenetic alterations can be easily detected and give relevant information. In particular, salivary ctDNA, miRNAs, and EVs seem to be the preferred biomarkers for early detection and diagnosis; instead, CTCs, even if more widely investigated in plasma, seem to be more informative to predict prognosis (Nonaka and Wong, 2018).

The results obtained so far lead to the term "salivaomics" to indicate all the translational and clinical tools based on salivary biomarkers and considered very supportive for cancer management (Wong, 2014).

CONCLUSION AND FUTURE PERSPECTIVES

There is an increasing interest in employing saliva samples as a source of biomarkers for OSCC liquid biopsy analysis. However, there are still several challenges that are yet to be overcome which are briefly indicated below and summarized in **Table 4** for each of the mentioned and revised biomarkers.

ctDNA

Salivary ctDNA holds cancer DNA genetic and epigenetic alterations; it can reflect the complex molecular profile deriving from tumor spatial and intratumoral heterogeneity, and its alterations in sequence can primarily support diagnosis and

TABLE 4 | Pros and cons of salivary biomarkers in OSCC diagnosis, prognosis and follow-up.

Salivary biomarkers	Pros	Cons
ctDNA	• ctDNA holds cancer DNA genetic and epigenetic alterations	• Inter-patient (e.g., age, gender, diet, and smoking) and intra-patient variability and tumoral and temporal heterogeneity
		• Non-tumoral circulating free DNA contamination of sample
	• ctDNA can reflect the complex molecular profile deriving from tumor spatial and intratumoral heterogeneity	• High costs of isolation and detection technologies (e.g., NGS and RT-qPCR)
	• DNA alterations in ctDNA and/or relative levels are useful for cancer diagnosis, prognosis, and follow-up	• Little coverage of ctDNA alteration detection platform
		• No standardized and reproducible protocols
EVs	• EVs allow detection of low-expression biomarkers otherwise not detectable in saliva	• Complex and expensive isolation (i.e., ultracentrifugation, ExoQuick-TC, and an aqueous two-phase system), characterization (e.g., electron microscopy, dynamic light scattering, and western blotting), and analysis technologies (e.g., RT-qPCR)
	• EV different relative levels, morphology, and composition (e.g., lipids, proteins, DNA, and miRNAs) are useful for cancer diagnosis and prognosis	• No standardized and reproducible protocols
miRNAs	• The aberrant expression of miRNAs is informative for cancer diagnosis and follow-up	• Expensive detection and analysis (e.g., RT-qPCR) technologies
		• Absence of valid endogenous RT-qPCR control
		• Inter-patient variability (e.g., age and inflammation)
		• No standardized and reproducible protocols

ctDNA, circulating tumor DNA; EV, extracellular vesicle; miRNA, microRNA; NGS, next-generation sequencing; OSCC, oral squamous cell carcinoma; RT-qPCR, quantitative reverse-transcription PCR.

secondly prognosis and assessment of patient response to therapy; nevertheless, several challenges still need to be overcome for ctDNA implementation in clinics (Van Ginkel et al., 2017; Payne et al., 2018). Firstly, it is necessary to improve the ctDNA isolation methodologies and sensibility of detection technologies to remove the noise due to cfDNA molecules physiologically released from non-tumoral cells and detect cancer even in the presence of an almost undetectable amount of ctDNA. In addition, we need to reduce the bias derived from inter-patient (e.g., age, gender, diet, and smoking) and intra-patient variability and tumoral and temporal heterogeneity, for a higher specificity of analysis, and it is necessary to improve the genetic coverage of ctDNA detection platforms to detect multiple molecular markers (Ignatiadis et al., 2015).

EVs

Extracellular vesicles allow detection of low-expression biomarkers, and the relative levels, morphology, and composition (e.g., lipids, proteins, and nucleic acids) were informative for oral cancer diagnosis and prognosis. Nevertheless, the use of EVs in OSCC management still has limitations related to the complex and expensive isolation, characterization, and analysis technologies. Among all methods to isolate salivary EVs, ultracentrifugation and ExoQuick-TC (a chemical-based agent kit used to precipitate EVs) represent the most currently physical-based used methods. Ultracentrifugation is a widely used isolation methodology able to give a minimally contaminated EV pellet; however, it is a complex and prolonged process that needs specialized and expensive equipment as well as large samples. In contrast, ExoQuick-TC can isolate and purify EVs from small samples, even if with more impurities compared to ultracentrifugation (Théry et al., 2006; Zlotogorski-Hurvitz et al., 2015; Boriachek et al., 2018). Another technique to isolate salivary EVs, named the aqueous two-phase system, has been recently developed by Kim et al. (2017) and seems to be more efficient in terms of yield and purity compared to ultracentrifugation and ExoQuick-TC. With regard to characterization, the following techniques are worth mentioning: electron microscopy; an ultra-sensitive technique of low-force microscopy to investigate shape, structure, chemistry, and mechanics of vesicles; dynamic light scattering to perform a size distribution analysis; and western blotting analysis to evaluate EV-specific markers (Théry et al., 2006; Byun et al., 2015). However, despite their widespread use in daily laboratory activity, the above-described techniques are still too expensive and time-consuming to employ in clinical routine. The use of size and surface markers for selection is urgently necessary to increase yield and quality of isolated vesicles (Witwer, 2015; Barger et al., 2016); besides, the development of new and innovative strategies is essential to more easily characterize EVs. Last, but not least, the validation and standardization of protocols are essential to safely move from the bench to the bedside; develop an easy, cost-effective, and robust clinical strategy; and obtain reproducible data (Witwer et al., 2013; Zahran et al., 2015; Han et al., 2018).

MiRNAs

The aberrant expression of miRNAs is very supportive of OSCC diagnosis, but as previously described for other salivary biomarkers, we still need to face many challenges. The protocols used to analyze and quantify isolated miRNAs need to be normalized and standardized. In the case of RT-qPCR analysis, global mean expression and exogenous (spiked-in) miRNAs and endogenous controls are currently used to normalize RNAs (Mestdagh et al., 2009; Yoshizawa and Wong, 2013). Current research identified miR-191 and miR-16 as unique suitable endogenous controls; however, they need to be validated in larger cohorts of patients (Momen-Heravi et al., 2014). Another variable that influences miRNA investigation in OSCC is the inter-patient variability (e.g., age and inflammation). Age represents

a continuous variable affecting the investigations; many studies demonstrate that miRNAs are involved in the regulation of aging processes and are often upregulated in older individuals. This may be limiting and decrease the robustness, sensitivity, and specificity of miRNA investigation in OSCC patients (Wan et al., 2017). Inflammation is a typical condition of cancer development and influences the expression of miRNAs; possible fluctuations of miRNA expression may not be directly linked to cancer features, but to body inflammatory response to cancer presence, perturbing miRNA expression and decreasing reproducibility of data (Kulkarni et al., 2017). Standardization of protocols should be defined to store and handle isolated miRNAs, making them available for clinical routine (Witwer et al., 2013).

CONCLUSION

In line with the current status and perspectives of liquid biopsy and saliva analysis, saliva has promising implications in OSCC management; it is conceivable that future studies on employment of ctDNAs, EVs, miRNAs, and CTCs, as salivary biomarkers in OSCC clinical routine, will certainly help to establish consistent strategies for early diagnosis of cancer lesions, facilitate advance prevention, and support the development of targeted therapies; this will improve treatment outcomes of OSCC patients, also reducing chemotherapy/radiotherapy side effects.

AUTHOR CONTRIBUTIONS

MC, VP, and RM conceptualized the manuscript, reviewed the literature, and drafted the manuscript. OD, GG, and GC reviewed the manuscript, critically edited, and intellectually contributed.

FUNDING

This research was supported by the Italian Ministry of University and Research (PON Industrial Ph.D. 2016-2017 – prot. DOT1320875), Doctoral Course of Experimental Oncology and Surgery, Cycle XXXII to MC (Ph.D. student).

REFERENCES

Alix-Panabieŕes, C., and Pantel, K. (2013). Circulating tumor cells: liquid biopsy of cancer. *Clin. Chem.* 59, 110–118. doi: 10.1373/clinchem.2012.194258

Al-malkey, M. K., and Abbas, A. A. H. (2015). Original research article expression analysis of salivary microrna-31 in oral cancer patients. *Int. J. Curr. Microbiol. Appl. Sci.* 4, 375–382.

Aro, K., Wei, F., Wong, D. T., and Tu, M. (2017). Saliva liquid biopsy for point-of-care applications. *Front. Public Heal.* 5:77. doi: 10.3389/fpubh.2017.00077

Barger, J. F., Rahman, M. A., Jackson, D., Acunzo, M., and Nana-Sinkam, S. P. (2016). Extracellular miRNAs as biomarkers in cancer. *Food Chem. Toxicol.* 98(Pt A), 66–72. doi: 10.1016/j.fct.2016.06.010

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297. doi: 10.1016/S0092-8674(04)00045-5

Bellairs, J. A., Hasina, R., and Agrawal, N. (2017). Tumor DNA: an emerging biomarker in head and neck cancer. *Cancer Metastasis Rev.* 36, 515–523. doi: 10.1007/s10555-017-9685-x

Boriachek, K., Islam, M. N., Möller, A., Salomon, C., Nguyen, N. T., Hossain, M. S. A., et al. (2018). Biological functions and current advances in isolation and detection strategies for exosome nanovesicles. *Small* 14. doi: 10.1002/smll.201702153

Buglione, M., Grisanti, S., Almici, C., Mangoni, M., Polli, C., Consoli, F., et al. (2012). Circulating tumour cells in locally advanced head and neck cancer: preliminary report about their possible role in predicting response to non-surgical treatment and survival. *Eur. J. Cancer* 48, 3019–3026. doi: 10.1016/j.ejca.2012.05.007

Byun, J. S., Hong, S. H., Choi, J. K., Jung, J. K., and Lee, H. J. (2015). Diagnostic profiling of salivary exosomal microRNAs in oral lichen planus patients. *Oral Dis.* 21, 987–993. doi: 10.1111/odi.12374

Carvalho, A. L., Henrique, R., Jeronimo, C., Nayak, C. S., Reddy, A. N., Hoque, M. O., et al. (2011). Detection of promoter hypermethylation in salivary rinses as a biomarker for head and neck squamous cell carcinoma surveillance. *Clin. Cancer Res.* 17, 4782–4789. doi: 10.1158/1078-0432.CCR-11-0324

Chan, K. C. A., Zhang, J., Hui, A. B. Y., Wong, N., Lau, T. K., Leung, T. N., et al. (2004). Size distributions of maternal and fetal DNA in maternal plasma. *Clin. Chem.* 50, 88–92. doi: 10.1373/clinchem.2003.024893

Chang, Y., Tolani, B., Nie, X., Zhi, X., Hu, M., and He, B. (2017). Review of the clinical applications and technological advances of circulating tumor DNA in cancer monitoring. *Ther. Clin. Risk Manag.* 13, 1363–1374. doi: 10.2147/TCRM.S141991

Cheng, F., Su, L., and Qian, C. (2016). Circulating tumor DNA: a promising biomarker in the liquid biopsy of cancer. *Oncotarget* 7, 48832–48841. doi: 10.18632/oncotarget.9453

Chiappin, S., Antonelli, G., Gatti, R., and De Palo, E. F. (2007). Saliva specimen: a new laboratory tool for diagnostic and basic investigation. *Clin. Chim. Acta* 383, 30–40. doi: 10.1016/j.cca.2007.04.011

Corrado, C., Raimondo, S., Chiesi, A., Ciccia, F., De Leo, G., and Alessandro, R. (2013). Exosomes as intercellular signaling organelles involved in health and disease: basic science and clinical applications. *Int. J. Mol. Sci.* 14, 5338–5366. doi: 10.3390/ijms14035338

Demokan, S., Chang, X., Chuang, A., Mydlarz, W. K., Kaur, J., Huang, P., et al. (2010). KIF1A and EDNRB are differentially methylated in primary HNSCC and salivary rinses. *Int. J. Cancer* 127, 2351–2359. doi: 10.1002/ijc.25248

Deutsch, O., Fleissig, Y., Zaks, B., Krief, G., Aframian, D. J., and Palmon, A. (2008). An approach to remove alpha amylase for proteomic analysis of low abundance biomarkers in human saliva. *Electrophoresis* 29, 4150–4157. doi: 10.1002/elps.200800207

Di Meo, A., Bartlett, J., Cheng, Y., Pasic, M. D., and Yousef, G. M. (2017). Liquid biopsy: a step forward towards precision medicine in urologic malignancies. *Mol. Cancer* 16:80. doi: 10.1186/s12943-017-0644-5

Diehl, F., Schmidt, K., Choti, M. A., Romans, K., Goodman, S., Li, M., et al. (2008). Circulating mutant DNA to assess tumor dynamics. *Nat. Med.* 14, 985–990. doi: 10.1038/nm.1789

Dionne, K. R., Warnakulasuriya, S., Zain, R. B., and Cheong, S. C. (2015). Potentially malignant disorders of the oral cavity: current practice and future directions in the clinic and laboratory. *Int. J. Cancer* 136, 503–515. doi: 10.1002/ijc.28754

Duz, M. B., Karatas, O. F., Guzel, E., Turgut, N. F., Yilmaz, M., Creighton, C. J., et al. (2016). Identification of miR-139-5p as a saliva biomarker for tongue squamous cell carcinoma: a pilot study. *Cell. Oncol.* 39, 187–193. doi: 10.1007/s13402-015-0259-z

El Andaloussi, S., Mäger, I., Breakefield, X. O., and Wood, M. J. A. (2013). Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat. Rev. Drug Discov.* 12, 347–357. doi: 10.1038/nrd3978

El-Naggar, A. K., Mao, L., Staerkel, G., Coombes, M. M., Tucker, S. L., Luna, M. A., et al. (2001). Genetic heterogeneity in saliva from patients with oral squamous carcinomas: implications in molecular diagnosis and screening. *J. Mol. Diagn.* 3, 164–170. doi: 10.1016/S1525-1578(10)60668-X

Ferlazzo, N., Currò, M., Zinellu, A., Caccamo, D., Isola, G., Ventura, V., et al. (2017). Influence of MTHFR genetic background on p16 and MGMT

methylation in oral squamous cell cancer. *Int. J. Mol. Sci.* 18, E724. doi: 10.3390/ijms18040724

Francis, G., and Stein, S. (2015). Circulating cell-free tumour DNA in the management of cancer. *Int. J. Mol. Sci.* 16, 14122–14142. doi: 10.3390/ijms160614122

Fuller, C., Camilon, R., Nguyen, S., Jennings, J., Day, T., and Gillespie, M. B. (2015). Adjunctive diagnostic techniques for oral lesions of unknown malignant potential: systematic review with meta-analysis. *Head Neck* 37, 755–762. doi: 10.1002/hed.23667

Gai, C., Camussi, F., Broccoletti, R., Gambino, A., Cabras, M., Molinaro, L., et al. (2018). Salivary extracellular vesicle-associated miRNAs as potential biomarkers in oral squamous cell carcinoma. *BMC Cancer* 18:439. doi: 10.1186/s12885-018-4364-z

Greither, T., Vorwerk, F., Kappler, M., Bache, M., Taubert, H., Kuhnt, T., et al. (2017). Salivary miR-93 and miR-200a as post-radiotherapy biomarkers in head and neck squamous cell carcinoma. *Oncol. Rep.* 38, 1268–1275. doi: 10.3892/or.2017.5764

Grobe, A., Blessmann, M., Hanken, H., Friedrich, R. E., Schon, G., Wikner, J., et al. (2013). Prognostic relevance of circulating tumor cells in blood and disseminated tumor cells in bone marrow of patients with squamous cell carcinoma of the oral cavity. *Clin. Cancer Res.* 20, 425–433. doi: 10.1158/1078-0432.CCR-13-1101

Gröbe, A., Blessmann, M., Hanken, H., Friedrich, R. E., Schön, G., Wikner, J., et al. (2014). Prognostic relevance of circulating tumor cells in blood and disseminated tumor cells in bone marrow of patients with squamous cell carcinoma of the oral cavity. *Clin. Cancer Res.* 20, 425–433. doi: 10.1158/1078-0432.CCR-13-1101

Guerrero-Preston, R., Soudry, E., Acero, J., Orera, M., Moreno-López, L., Macía-Colón, G., et al. (2011). NID2 and HOXA9 promoter hypermethylation as biomarkers for prevention and early detection in oral cavity squamous cell carcinoma tissues and saliva. *Cancer Prev. Res.* 4, 1061–1072. doi: 10.1158/1940-6207.CAPR-11-0006

Han, Y., Jia, L., Zheng, Y., and Li, W. (2018). Salivary exosomes: emerging roles in systemic disease. *Int. J. Biol. Sci.* 14, 633–643. doi: 10.7150/ijbs.25018

He, S., Li, P., He, S., Long, T., Zhang, N., Fang, J., et al. (2013). Detection of circulating tumour cells with the cellsearch system in patients with advanced-stage head and neck cancer: preliminary results. *J. Laryngol. Otol.* 127, 788–793. doi: 10.1017/S0022215113001412

Hristozova, T., Konschak, R., Stromberger, C., Fusi, A., Liu, Z., Weichert, W., et al. (2011). The presence of circulating tumor cells (CTCs) correlates with lymph node metastasis in nonresectable squamous cell carcinoma of the head and neck region (SCCHN). *Ann. Oncol.* 22, 1878–1885. doi: 10.1093/annonc/mdr130

Ignatiadis, M., Lee, M., and Jeffrey, S. S. (2015). Circulating tumor cells and circulating tumor DNA: challenges and opportunities on the path to clinical utility. *Clin. Cancer Res.* 21, 4786–4800. doi: 10.1158/1078-0432.CCR-14-1190

Ilie, M., Hofman, V., Long, E., Bordone, O., Selva, E., Washetine, K., et al. (2014). Current challenges for detection of circulating tumor cells and cell-free circulating nucleic acids, and their characterization in non-small cell lung carcinoma patients. What is the best blood substrate for personalized medicine? *Ann. Transl. Med.* 2:107. doi: 10.3978/j.issn.2305-5839.2014.08.11

Jahr, S., Hentze, H., Englisch, S., Hardt, D., Fackelmayer, F. O., Hesch, R. D., et al. (2001). DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res.* 61, 1659–1665.

Jankowska, A. K., Waszkiel, D., and Kowalczyk, A. (2007). Saliva as a main component of oral cavity ecosystem. Part I. Secretion and function. *Wiad. Lek.* 60, 148–154.

Jatana, K. R., Balasubramanian, P., Lang, J. C., Yang, L., Jatana, C. A., White, E., et al. (2010). Significance of circulating tumor cells in patients with squamous cell carcinoma of the head and neck: initial results. *Arch. Otolaryngol. Head Neck Surg.* 136, 1274–1279. doi: 10.1001/archoto.2010.223

Jatana, K. R., Lang, J. C., and Chalmers, J. J. (2011). Identification of circulating tumor cells: a prognostic marker in squamous cell carcinoma of the head and neck? *Futur. Oncol.* 7, 481–484. doi: 10.2217/fon.11.19

Kaczor-Urbanowicz, K. E., Martin Carreras-Presas, C., Aro, K., Tu, M., Garcia-Godoy, F., and Wong, D. T. W. (2017). Saliva diagnostics – current views and directions. *Exp. Biol. Med.* 242, 459–472. doi: 10.1177/1535370216681550

Kalluri, R. (2016). The biology and function of exosomes in cancer. *J. Clin. Invest.* 126, 1208–1215. doi: 10.1172/JCI81135

Kastelowitz, N., and Yin, H. (2014). Exosomes and microvesicles: identification and targeting by particle size and lipid chemical probes. *Chem. Biochem.* 15, 923–928. doi: 10.1002/cbic.201400043

Kaufman, E., and Lamster, I. B. (2002). The diagnostic applications of saliva - a review. *Crit. Rev. Oral Biol. Med.* 13, 197–212. doi: 10.1177/154411130201300209

Khurshid, Z., Zafar, M. S., Khan, R. S., Najeeb, S., Slowey, P. D., and Rehman, I. U. (2018). Role of salivary biomarkers in oral cancer detection. *Adv. Clin. Chem.* 86, 23–70. doi: 10.1016/bs.acc.2018.05.002

Kim, J., Shin, H., and Park, J. (2017). RNA in salivary extracellular vesicles as a possible tool for systemic disease diagnosis. *J. Dent. Res.* 96, 938–944. doi: 10.1177/0022034517702100

Kohler, C., Barekati, Z., Radpour, R., and Zhong, X. Y. (2011). Cell-free DNA in the circulation as a potential cancer biomarker. *Anticancer Res.* 31, 2623–2628.

Krief, G., Deutsch, O., Gariba, S., Zaks, B., Aframian, D. J., and Palmon, A. (2011). Improved visualization of low abundance oral fluid proteins after triple depletion of alpha amylase, albumin and IgG. *Oral Dis.* 17, 45–52. doi: 10.1111/j.1601-0825.2010.01700.x

Kulkarni, V., Uttamani, J. R., Naqvi, A. R., and Nares, S. (2017). MicroRNAs: emerging players in oral cancers and inflammatory disorders. *Tumor Biol.* 39:1010428317698379. doi: 10.1177/1010428317698379

Lacombe, J., Brooks, C., Hu, C., Menashi, E., Korn, R., Yang, F., et al. (2017). Analysis of saliva gene expression during head and neck cancer radiotherapy: a pilot study. *Radiat. Res.* 188, 75–81. doi: 10.1667/RR14707.1

Languino, L. R., Singh, A., Prisco, M., Inman, G. J., Luginbuhl, A., Curry, J. M., et al. (2016). Exosome-mediated transfer from the tumor microenvironment increases TGFβ signaling in squamous cell carcinoma. *Am. J. Transl. Res.* 8, 2432–2437.

Larrea, E., Sole, C., Manterola, L., Goicoechea, I., Armesto, M., Arestin, M., et al. (2016). New concepts in cancer biomarkers: circulating miRNAs in liquid biopsies. *Int. J. Mol. Sci.* 17:E627. doi: 10.3390/ijms17050627

Lau, C., Kim, Y., Chia, D., Spielmann, N., Eibl, G., Elashoff, D., et al. (2013). Role of pancreatic cancer-derived exosomes in salivary biomarker development. *J. Biol. Chem.* 288, 26888–26897. doi: 10.1074/jbc.M113.452458

Lee, Y. H., and Wong, D. T. (2009). Saliva: an emerging biofluid for early detection of diseases. *Am. J. Dent.* 22, 241–248. doi: 10.1016/j.bbi.2008.05.010

Li, L., Li, C., Wang, S., Wang, Z., Jiang, J., Wang, W., et al. (2016). Exosomes derived from hypoxic oral squamous cell carcinoma cells deliver miR-21 to normoxic cells to elicit a prometastatic phenotype. *Cancer Res.* 76, 1770–1780. doi: 10.1158/0008-5472.CAN-15-1625

Lianidou, E. S., Strati, A., and Markou, A. (2014). Circulating tumor cells as promising novel biomarkers in solid cancers. *Crit. Rev. Clin. Lab. Sci.* 51, 160–171. doi: 10.3109/10408363.2014.896316

Liao, P. H., Chang, Y. C., Huang, M. F., Tai, K. W., and Chou, M. Y. (2000). Mutation of p53 gene codon 63 in saliva as a molecular marker for oral squamous cell carcinomas. *Oral Oncol.* 36, 272–276. doi: 10.1016/S1368-8375(00)00005-1

Liu, C. J., Lin, S. C., Yang, C. C., Cheng, H. W., and Chang, K. W. (2012). Exploiting salivary miR-31 as a clinical biomarker of oral squamous cell carcinoma. *Head Neck*. 34, 219–224. doi: 10.1002/hed.21713

Liu, T., Chen, G., Sun, D., Lei, M., Li, Y., Zhou, C., et al. (2017). Exosomes containing miR-21 transfer the characteristic of cisplatin resistance by targeting PTEN and PDCD4 in oral squamous cell carcinoma. *Acta Biochim. Biophys. Sin.* 49, 808–816. doi: 10.1093/abbs/gmx078

Lötvall, J., Hill, A. F., Hochberg, F., Buzás, E. I., Vizio, D., Di Gardiner, C., et al. (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for extracellular vesicles. *J. Extracell. Vesicles.* 3:26913. doi: 10.3402/jev.v3.26913

Maheswari, T. U., Venugopal, A., Sureshbabu, N., and Ramani, P. (2018). Salivary micro RNA as a potential biomarker in oral potentially malignant disorders: a systematic review. *Tzu Chi Med. J.* 30, 55–60. doi: 10.4103/tcmj.tcmj-114-17

Malonek, D., Vered, M., Yahalom, R., Dekel, B., and Zlotogorski Hurvitz, A. (2018). PO-082 Salivary extracellular vesicles from oral cancer patients: exclusive signature of the infrared spectrum. *ESMO Open* 2018:3. doi: 10.1136/esmoopen-2018-eacr25.611

Markopoulos, A. K. (2012). Current aspects on oral squamous cell carcinoma. *Open Dent. J.* 6, 126–130. doi: 10.2174/1874210601206010126

Mascitti, M., Orsini, G., Tosco, V., Monterubbianesi, R., Balercia, A., Putignano, A., et al. (2018). An overview on current non-invasive diagnostic devices in oral oncology. *Front. Physiol.* 9:1510. doi: 10.3389/fphys.2018.01510

Mestdagh, P., Van Vlierberghe, P., De Weer, A., Muth, D., Westermann, F., Speleman, F., et al. (2009). A novel and universal method for microRNA RT-qPCR data normalization. *Genom. Biol.* 10:R64. doi: 10.1186/gb-2009-10-6-r64

Momen-Heravi, F., Trachtenberg, A. J., Kuo, W. P., and Cheng, Y. S. (2014). Genomewide study of salivary MicroRNAs for detection of oral cancer. *J. Dent. Res.* 93(7 Suppl.), 86S–93S. doi: 10.1177/0022034514531018

Moore, S. R., Johnson, N. W., Pierce, A. M., and Wilson, D. F. (2000). The epidemiology of tongue cancer: a review of global incidence. *Oral Dis.* 6, 75–84. doi: 10.1111/j.1601-0825.2000.tb00105.x

Mouliere, F., Robert, B., Peyrotte, E., Del Rio, M., Ychou, M., Molina, F., et al. (2011). High fragmentation characterizes tumour-derived circulating DNA. *PLoS One* 6:e23418. doi: 10.1371/journal.pone.0023418

Nichols, A. C., Lowes, L. E., Szeto, C. C. T., Basmaji, J., Dhaliwal, S., Chapeskie, C., et al. (2012). Detection of circulating tumor cells in advanced head and neck cancer using the cellsearch system. *Head Neck* 34, 1440–1444. doi: 10.1002/hed.21941

Nieuw Amerongen, A. V., and Veerman, E. C. I. (2002). Saliva - The defender of the oral cavity. *Oral Dis.* 8, 12–22. doi: 10.1034/j.1601-0825.2002.1o816.x

Nonaka, T., and Wong, D. T. W. (2018). Liquid biopsy in head and neck cancer: promises and challenges. *J. Dent. Res.* 97, 701–708. doi: 10.1177/0022034518762071

Ono, K., Eguchi, T., Sogawa, C., Calderwood, S. K., Futagawa, J., Kasai, T., et al. (2018). HSP-enriched properties of extracellular vesicles involve survival of metastatic oral cancer cells. *J. Cell Biochem.* 119, 7350–7362. doi: 10.1002/jcb.27039

Panzarella, V., Pizzo, G., Calvino, F., Compilato, D., Colella, G., and Campisi, G. (2014). Diagnostic delay in oral squamous cell carcinoma: the role of cognitive and psychological variables. *Int. J. Oral Sci.* 6, 39–45. doi: 10.1038/ijos.2013.88

Park, N. J., Zhou, H., Elashoff, D., Henson, B. S., Kastratovic, D. A., Abemayor, E., et al. (2009). Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. *Clin. Cancer Res.* 15, 5473–5477. doi: 10.1158/1078-0432.CCR-09-0736

Pattani, K. M., Zhang, Z., Demokan, S., Glazer, C., Loyo, M., Goodman, S., et al. (2010). Endothelin receptor type B gene promoter hypermethylation in salivary rinses is independently associated with risk of oral cavity cancer and premalignancy. *Cancer Prev. Res.* 3, 1093–1103. doi: 10.1158/1940-6207.CAPR-10-0115

Payne, K., Spruce, R., Beggs, A., Sharma, N., Kong, A., Martin, T., et al. (2018). Circulating tumor DNA as a biomarker and liquid biopsy in head and neck squamous cell carcinoma. *Head Neck*. 40, 1598–1604. doi: 10.1002/hed.25140

Peng, M., Chen, C., Hulbert, A., Brock, M. V., and Yu, F. (2017). Non-blood circulating tumor DNA detection in cancer. *Oncotarget* 8, 69162–69173. doi: 10.18632/oncotarget.19942

Perron, M. P., and Provost, P. (2008). Protein interactions and complexes in human microRNA biogenesis and function. *Front. Biosci.* 13, 2537–2547. doi: 10.1016/j.clinph.2011.06.006.A

Pfaffe, T., Cooper-White, J., Beyerlein, P., Kostner, K., and Punyadeera, C. (2011). Diagnostic potential of saliva: current state and future applications. *Clin. Chem.* 57, 675–687. doi: 10.1373/clinchem.2010.153767

Pu, D., Liang, H., Wei, F., Akin, D., Feng, Z., Yan, Q. X., et al. (2016). Evaluation of a novel saliva-based epidermal growth factor receptor mutation detection for lung cancer: a pilot study. *Thorac. Cancer* 7, 428–436. doi: 10.1111/1759-7714.12350

Puttipanyalears, C., Subbalekha, K., Mutirangura, A., and Kitkumthorn, N. (2014). Alu hypomethylation in smoke-exposed epithelia and oral squamous carcinoma. *Asian Pac. J. Cancer Prev.* 14, 5495–5501. doi: 10.7314/apjcp.2013.14.9.5495

Qin, Z., Ljubimov, V. A., Zhou, C., Tong, Y., and Liang, J. (2016). Cell-free circulating tumor DNA in cancer. *Chin. J. Cancer* 35, 36. doi: 10.1186/s40880-016-0092-4

Ramadoss, R., Krishnan, R., Sukhija, H., Balachander, N., Raghavendhar, K., and Sen, S. (2015). C-deletion in exon 4 codon 63 of p53 gene as a molecular marker for oral squamous cell carcinoma: a preliminary study. *Contemp. Clin. Dent.* 6(Suppl. 1), S227–S234. doi: 10.4103/0976-237x.166840

Rettori, M. M., De carvalho, A. C., Bomfim longo, A. L., De oliveira, C. Z., Kowalski, L. P., Carvalho, A. L., et al. (2013). Prognostic significance of TIMP3 hypermethylation in post-treatment salivary rinse from head and neck squamous cell carcinoma patients. *Carcinogenesis* 34, 20–27. doi: 10.1093/carcin/bgs311

Rivera, C. (2015). Essentials of oral cancer. *Int. J. Clin. Exp. Pathol.* 8, 11884–11894. doi: 10.1111/anae.12918

Rolfo, C., Castiglia, M., Hong, D., Alessandro, R., Mertens, I., Baggerman, G., et al. (2014). Liquid biopsies in lung cancer: the new ambrosia of researchers. *Biochim. Biophys. Acta Rev. Cancer* 1846, 539–546. doi: 10.1016/j.bbcan.2014.10.001

Ruivo, C. F., Adem, B., Silva, M., and Melo, S. A. (2017). The biology of cancer exosomes: Insights and new perspectives. *Cancer Res.* 77, 6480–6488. doi: 10.1158/0008-5472.CAN-17-0994

Sethi, S., Benninger, M. S., Lu, M., Havard, S., and Worsham, M. J. (2009). Noninvasive molecular detection of head and neck squamous cell carcinoma: an exploratory analysis. *Diagn. Mol. Pathol.* 18, 81–87. doi: 10.1097/PDM.0b013e3181804b82

Shah, J. P., and Gil, Z. (2009). Current concepts in management of oral cancer - Surgery. *Oral Oncol.* 45, 394–401. doi: 10.1016/j.oraloncology.2008.05.017

Shukla, D., Kale, A. D., Hallikerimath, S., Yerramalla, V., and Subbiah, V. (2013). Can quantifying free-circulating DNA be a diagnostic and prognostic marker in oral epithelial dysplasia and oral squamous cell carcinoma? *J. Oral Maxillofac. Surg.* 71, 414–418. doi: 10.1016/j.joms.2012.04.03

Siravegna, G., Marsoni, S., Siena, S., and Bardelli, A. (2017). Integrating liquid biopsies into the management of cancer. *Nat. Rev. Clin. Oncol.* 14, 531–548. doi: 10.1038/nrclinonc.2017.14

Stadler, Z. K., Thom, P., Robson, M. E., Weitzel, J. N., Kauff, N. D., Hurley, K. E., et al. (2010). Genome-wide association studies of cancer. *J. Clin. Oncol.* 28, 4255–4267. doi: 10.1200/JCO.2009.25.7816

Sun, W., Zaboli, D., Wang, H., Liu, Y., Arnaoutakis, D., Khan, T., et al. (2012). Detection of TIMP3 promoter hypermethylation in salivary rinse as an independent predictor of local recurrence-free survival in head and neck cancer. *Clin. Cancer Res.* 18, 1082–1091. doi: 10.1158/1078-0432.CCR-11-2392

Théry, C., Amigorena, S., Raposo, G., and Clayton, A. (2006). Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr. Protoc. Cell Biol.* 30, 3.22.1–3.22.29. doi: 10.1002/0471143030.cb0322s30

Tinhofer, I., Konschak, R., Stromberger, C., Raguse, J.-D., Dreyer, J. H., Jöhrens, K., et al. (2014). Detection of circulating tumor cells for prediction of recurrence after adjuvant chemoradiation in locally advanced squamous cell carcinoma of the head and neck. *Ann. Oncol.* 25, 2042–2047. doi: 10.1093/annonc/mdu271

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. doi: 10.1038/ncb1596

Van Ginkel, J. H., Huibers, M. M. H., Noorlag, R., De Bree, R., Van Es, R. J. J., and Willems, S. M. (2017). Liquid biopsy: a future tool for posttreatment surveillance in head and neck cancer? *Pathobiology*. 84, 115–120. doi: 10.1159/000452861

Viet, C. T., Jordan, R. C., and Schmidt, B. L. (2007). DNA promoter hypermethylation in saliva for the early diagnosis of oral cancer. *J. Calif. Dent. Assoc.* 35, 844–849.

Viet, C. T., and Schmidt, B. L. (2008). Methylation array analysis of preoperative and postoperative saliva DNA in oral cancer patients. *Cancer Epidemiol. Biomark. Prev.* 17, 3603–3611. doi: 10.1158/1055-9965.EPI-08-0507

Volik, S., Alcaide, M., Morin, R. D., Collins, C., Centre, V. P., and Centre, G. S. (2016). Cell-free DNA clinical significance and utility in cancer shaped by emerging technologies. *Mol. Cancer Res.* 14, 898–908. doi: 10.1158/1541-7786.MCR-16-0044

Wan, Y., Punyadeera, C., Perry, C., Kenny, L., Vagenas, D., Salazar, C., et al. (2017). Salivary miRNA panel to detect HPV-positive and HPV-negative head and neck cancer patients. *Oncotarget* 8, 99990–100001. doi: 10.18632/oncotarget.21725

Wang, B. G., Huang, H. Y., Chen, Y. C., Bristow, R. E., Kassauei, K., Cheng, C. C., et al. (2003). Increased plasma DNA integrity in cancer patients. *Cancer Res.* 63, 3966–3968.

Wang, J., Chang, S., Li, G., and Sun, Y. (2017). Application of liquid biopsy in precision medicine: opportunities and challenges. *Front. Med.* 11, 522–527. doi: 10.1007/s11684-017-0526-7

Wang, X., Kaczor-Urbanowicz, K. E., and Wong, D. T. W. (2017). Salivary biomarkers in cancer detection. *Med. Oncol.* 34:7. doi: 10.1007/s12032-016-0863-4

Wang, Y., Springer, S., Mulvey, C. L., Silliman, N., Schaefer, J., Sausen, M., et al. (2015). Detection of somatic mutations and HPV in the saliva and plasma of patients with head and neck squamous cell carcinomas. *Sci. Transl. Med.* 7:293ra104. doi: 10.1126/scitranslmed.aaa8507

Wiklund, E. D., Gao, S., Hulf, T., Sibbritt, T., Nair, S., Costea, D. E., et al. (2011). MicroRNA alterations and associated aberrant DNA methylation patterns across multiple sample types in oral squamous cell carcinoma. *PLoS One* 6:e27840. doi: 10.1371/journal.pone.0027840

Winck, F. V., Ribeiro, A. C. P., Domingues, R. R., Ling, L. Y., Riaño-Pachón, D. M., Rivera, C., et al. (2015). Insights into immune responses in oral cancer through proteomic analysis of saliva and salivary extracellular vesicles. *Sci. Rep.* 5:16305. doi: 10.1038/srep16305

Witwer, K. W. (2015). Circulating MicroRNA biomarker studies: pitfalls and potential solutions. *Clin. Chem.* 61, 56–63. doi: 10.1373/clinchem.2014.221341

Witwer, K. W., Buzás, E. I., Bemis, L. T., Bora, A., Lässer, C., Lötvall, J., et al. (2013). Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J. Extracell. Vesicles* 2:20360. doi: 10.3402/jev.v2i0.20360

Wong, D. T. (2006). Salivary diagnostics powered by nanotechnologies, proteomics and genomics. *J. Am. Dent. Assoc.* 137, 313–321. doi: 10.14219/jada.archive.2006.0180

Wong, D. T. W. (2014). Salivaomics. *J. Am. Dent. Assoc.* 143(10 Suppl.), 19S–24S. doi: 10.14219/jada.archive.2012.0339

Wong, D. T. W. (2015). Salivary extracellular noncoding RNA: emerging biomarkers for molecular diagnostics. *Clin. Ther.* 37, 540–551. doi: 10.1016/j.clinthera.2015.02.017

Woolgar, J. A. (2006). Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncol.* 42, 229–239. doi: 10.1016/j.oraloncology.2005.05.008

Wu, X., Mastronicola, R., Tu, Q., Faure, G. C., De Carvalho Bittencourt, M., and Dolivet, G. (2016). A rare case of extremely high counts of circulating tumor cells detected in a patient with an oral squamous cell carcinoma. *BMC Cancer* 16:552. doi: 10.1186/s12885-016-2591-8

Xu, L., Li, J. H., Ye, J. M., Duan, X. N., Cheng, Y. J., Xin, L., et al. (2017). A retrospective survival analysis of anatomic and prognostic stage group based on the American Joint committee on cancer 8thedition cancer staging manual in luminal B human epidermal growth factor receptor 2-negative breast cancer. *Chin. Med. J.* 130, 1945–1952. doi: 10.4103/0366-6999.211896

Yakob, M., Abemayor, E., Wong, D. T. W., Fuentes, L., and Wang, M. B. (2014). Salivary biomarkers for detection of oral squamous cell carcinoma: current state and recent advances. *Curr. Oral Heal. Rep*. 1, 133–141. doi: 10.1007/s40496-014-0014-y

Yáñez-Mó, M., Siljander, P. R. M., Andreu, Z., Zavec, A. B., Borràs, F. E., Buzas, E. I., et al. (2015). Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* 4:27066. doi: 10.3402/jev.v4.27066

Yang, Y., Li, Y. X., Yang, X., Jiang, L., and Zhou, Z. (2013). Progress risk assessment of oral premalignant lesions with saliva miRNA analysis. *BMC Cancer* 13:129. doi: 10.1186/1471-2407-13-129

Yoshizawa, J. M., and Wong, D. T. W. (2013). Salivary micrornas and oral cancer detection. *Methods Mol. Biol.* 936, 313–324. doi: 10.1007/978-1-62703-83-0-24

Zahran, F., Ghalwash, D., Shaker, O., Al-Johani, K., and Scully, C. (2015). Salivary microRNAs in oral cancer. *Oral Dis.* 936, 313–324. doi: 10.1111/odi.12340

Zhang, Y., Ma, Q., Liu, T., Guan, G., Zhang, K., Chen, J., et al. (2016). Interleukin-6 suppression reduces tumour self-seeding by circulating tumour cells in a human osteosarcoma nude mouse model. *Oncotarget* 7, 446–458. doi: 10.18632/oncotarget.6371

Zhou, J., Huang, A., and Yang, X. R. (2016). Liquid biopsy and its potential for management of hepatocellular carcinoma. *J. Gastrointest. Cancer* 47, 157–167. doi: 10.1007/s12029-016-9801-0

Zlotogorski-Hurvitz, A., Dayan, D., Chaushu, G., Korvala, J., Salo, T., Sormunen, R., et al. (2015). Human saliva-derived exosomes: comparing methods of isolation. *J. Histochem. Cytochem.* 63, 181–189. doi: 10.1369/0022155414564219

Zlotogorski-Hurvitz, A., Dayan, D., Chaushu, G., Salo, T., and Vered, M. (2016). Morphological and molecular features of oral fluid-derived exosomes: oral cancer patients versus healthy individuals. *J. Cancer Res. Clin. Oncol.* 142, 101–110. doi: 10.1007/s00432-015-2005-3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with one of the authors, GC.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 17 January 2020
doi: 10.3389/fcimb.2019.00455

Promising Treatment for Type 2 Diabetes: Fecal Microbiota Transplantation Reverses Insulin Resistance and Impaired Islets

Hui Wang[1], Yuan Lu[1], Yan Yan[1], Shanshan Tian[1], Dongjie Zheng[1], Dongjing Leng[1], Cao Wang[1], Jingfeng Jiao[1], Zhiguo Wang[1] and Yunlong Bai[1,2]**

[1] Department of Pharmacology (State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Medicine Research, Ministry of Education), College of Pharmacy, Harbin Medical University, Harbin, China, [2] Translational Medicine Research and Cooperation Center of Northern China, Chronic Disease Research Institute, Heilongjiang Academy of Medical Sciences, Harbin, China

Type 2 diabetes is a common metabolic disorder related to insulin resistance, or deficiency of insulin secretion, caused by decreased insulin sensitivity and destruction of islet structure and function. As the second human genome, the microbiota has been observed to have a growing relationship with diabetes in recent years. Microbiota imbalance has been hypothesized to be involved in the regulation of energy metabolism and the inflammatory immune response in diabetes. The present study aimed to investigate whether fecal microbiota transplantation (FMT) could alleviate the symptoms associated with type 2 diabetes. To this end, a type 2 diabetes mouse model was first established through the consumption of a high-fat diet combined with streptozotocin (100 mg/kg), and FMT was used to rebuild the gut microbiota of diabetic mice. Fasting blood glucose, oral glucose tolerance tests, and HbA1c levels were monitored, while the hypoglycemic effects of FMT were also observed. Insulin levels were tested by ELISA and related indexes such as HOMA-IR, HOMA-IS, and HOMA-β were calculated. We found that insulin resistance and pancreatic islet β-cells were improved after FMT treatment. Meanwhile, the markers of inflammation in the pancreatic tissue were detected by ELISA and immunohistochemistry, which indicated that inflammatory response decreased following FMT treatment. Furthermore, flow cytometry and western blot results revealed that FMT inhibited the β-cell apoptosis. Here, the effect of FMT on hypoglycemia in type 2 diabetes was addressed by improving insulin resistance and repairing impaired islets, thereby providing a potential treatment strategy for type 2 diabetes.

Keywords: diabetic, microbiota, FMT, apoptosis, inflammation

OPEN ACCESS

Edited by:
Lorenzo Lo Muzio, University of Foggia, Italy

Reviewed by:
Natalia Shulzhenko, Oregon State University, United States
Vito Carlo Alberto Caponio, University of Foggia, Italy

****Correspondence:***
Zhiguo Wang
441427302@qq.com
Yunlong Bai
baiyunlong@ems.hrbmu.edu.cn

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *22 August 2019*
Accepted: *13 December 2019*
Published: *17 January 2020*

Citation:
Wang H, Lu Y, Yan Y, Tian S, Zheng D, Leng D, Wang C, Jiao J, Wang Z and Bai Y (2020) Promising Treatment for Type 2 Diabetes: Fecal Microbiota Transplantation Reverses Insulin Resistance and Impaired Islets. Front. Cell. Infect. Microbiol. 9:455. doi: 10.3389/fcimb.2019.00455

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is the most common endocrine and metabolic disease, which is characterized by high blood glucose, insulin resistance, and a relative deficiency of insulin. Clinical epidemiological data show that the number of patients with T2DM is increasing rapidly worldwide; the number is estimated to exceed 439 million by 2030 (Shaw et al., 2010). Sustained hyperglycemia and metabolic disorders can lead to tissues and organs dysfunction, particularly the eyes, kidneys,

cardiovascular and nervous systems (Koopal et al., 2018). Presently, the main causes of T2DM are peripheral insulin resistance and pancreatic islet β-cell injury and dysfunction, which leads to abnormal blood glucose regulation and serious complications (Ruiz et al., 2018; Sun et al., 2018). However, the currently available drugs, diet control, insulin injections, and other treatments do not prevent the occurrence of diabetes or the development of various chronic complications.

The intestinal microflora is a general term for the various microorganisms that are located in the intestinal environment (Kerr et al., 2015). The number of intestinal microorganisms is more than 10^{14}/mL in the human body, which forms a complex and relatively independent micro-ecosystem; microorganisms participate in numerous functions such as substance transformation and energy metabolism (Jia et al., 2017). When the ratio of beneficial bacteria to harmful bacteria is disturbed, the balance of microbiota is broken, and metabolic disorders occur in the human body, leading to other related diseases. It is believed that T2DM is the result of impaired islet β-cells function due to genetic and environmental factors. Owing to in-depth research on intestinal function, the role of microbiota in the progression of type 2 diabetes is becoming increasingly clear and acknowledged (Kang et al., 2018). Studies have shown that intestinal microflora is significantly different between patients with type 2 diabetes and healthy individuals (Savilahti et al., 2018). The proportion of *Firmicutes* and *Clostridium* was significantly reduced in T2DM patients, and the levels of *Bacteroides*, *Escherichia coli* and other conditional pathogenic bacteria were increased (Larsen et al., 2010; Qin et al., 2012; Wang et al., 2018). In contrast, probiotics have been shown to improve intestinal microflora disturbances and effectively alleviate the symptoms of diabetes. Due to the complexity of the intestinal microflora, its structure and function have not yet been fully understood yet, and its specific role in T2DM has not been completely elucidated.

Apoptosis is the programmed cell death that occurs in multicellular organisms (Curti et al., 2017). Previous studies have shown that impaired islet β-cell function is associated with decreased β-cells due to apoptosis (Butler et al., 2003; Curti et al., 2017). Imbalanced intestinal microflora activates low-grade chronic inflammation of islets, which may cause damage and dysfunction of islet β-cells and promote β-cell apoptosis. In the current study, we aimed to evaluate whether fecal microbiota transplantation (FMT) could alleviate the symptoms in a high-fat diet (HFD) and streptozotocin-induced diabetes. Our results showed that rebuilding gut microbiota could be decreased fasting blood glucose (FBG) levels and improves insulin resistance in T2DM mice by relieving islets destruction. From the perspective of gut microbiota, our study aimed to support the theory that remodeling intestinal microflora could improve T2DM, which elucidates the capability of FMT to control diabetes mellitus and offer a new consideration for FMT as a valuable treatment strategy in metabolic diseases.

Abbreviations: AUC, Areas under the curve of glucose; FBG, Fasting blood glucose; FMT, Fecal microbiota transplantation; HE, Hematoxylin and eosin; HFD, High-fat diet; HOMA-IR, Homeostasis model assessment-Insulin resistance; HOMA-IS, Homeostasis model assessment-Insulin sensitive; HOMA-β, Homeostasis model assessment-β; IHC, Immunohistochemistry; IL-10, Interleukin-10; IL-6, Interleukin-6; OGTTs, Oral glucose tolerance tests; PBS, Phosphate-buffered saline; SDS, Sodium dodecyl sulfate; T2DM, Type 2 diabetes mellitus; TNF-α, Tumor necrosis factor-alpha.

MATERIALS AND METHODS

Animals and Ethics Approval

Kunming (KM) mice, weighing 20–22 g, were provided by the Animal Center of the Second Affiliated Hospital of Harbin Medical University. The methods were performed in accordance with the National Guidelines for Experimental Animal Welfare (The Ministry of Science and Technology, People's Republic of China, 2006). All experimental protocols were pre-approved by the Experimental Animal Ethics Committee of Harbin Medical University, China (No. HMUIRB 20180025).

Type 2 diabetes was induced by a high-fat diet for 6 weeks and combined with a single intraperitoneal injection of streptozotocin (100 mg/kg) (Sigma, St. Louis, MO) dissolved in 0.01 mol/L citric acid solution (pH = 4.3). One week later, FBG was measured through a tail vein. T2DM mouse models were selected in which the FBG value was higher than 11.1 mmol/L. The mice were randomly divided into control, T2DM, and T2DM+FMT groups. All groups were raised under standard temperature (23 ± 1°C), humidity (55% ± 5%), and a normal diet for 8 weeks. Healthy KM mice (22–25 g) with FBG lower than 6.0 mmol/L were randomly selected as the normal control group. Mice in the T2DM+FMT group were intragastrically administered with 0.3 ml fecal suspension daily for 8 weeks; the control and T2DM mice received equivalent volumes of sterilized distilled water. The FBG and OGTTs levels were observed during the experiment in all groups.

Fecal Microbiota Transplantation (FMT)

To serve as donor mice, KM mice were obtained and housed under constant temperature and humidity conditions for 1 week. Animals were placed in an empty autoclaved cage a day before collecting the fecal samples. The mice is defecated normally, and the first three fecal pellets of each animal were collected in an empty 1.5 ml tube using a sterile toothpick. The tube is quickly closed, placed in liquid nitrogen, and then transferred to a refrigerator at −80°C for storage until preparation of the fecal suspension (Ericsson et al., 2017). Fecal samples (300 mg) were dissolved in 10 ml sterilized distilled water and mixed well for use.

ELISA

Blood samples were collected from the medial canthus vein of each mouse. Pancreas tissue homogenate was obtained by sonication. Plasma HbA1c and serum insulin were measured using the mouse ELISA Kit (Cloud-Clone Corp, Wuhan, China) after the blood samples were centrifuged (1,000 × g) for 15 min at 4°C. Pancreas tissue IL-6, IL-10, and TNF-α were detected using the mouse ELISA Kit (Cloud-Clone Corp, Wuhan, China). The ELISAs were performed according to the manufacturer's protocols.

Hematoxylin and Eosin (HE) Staining

Mice were administered 2% Tribromoethanol (0.01 ml/g) by intraperitoneal injection. After anesthetizing, pancreatic tissue was removed and immediately immobilized for 24 h in 4% paraformaldehyde. Following fixation, the tissue was processed by dehydration with a gradient concentration of ethanol, cleared in xylene, embedded in paraffin, and sliced at 4 μm thickness. HE staining was performed according to the manufacturer's protocols (Solarbio, Beijing, China) (He et al., 2018).

Immunohistochemistry (IHC)

Tissue sections were deparaffinized and antigen retrieval is performed using the pressure cooker method. The sections were blocked with normal sheep serum at room temperature for 30 min, followed by incubation at 4°C overnight with primary antibodies for IL-6, IL-10, TNF-α (All antibody, Bioss, Beijing, China, 1:100). The sections were washed three times with PBS and then incubated with secondary antibodies (ZSGB, Beijing, China) at 37°C for 1 h. DAB solution was added to cover tissue sections and incubated at room temperature for 2 min. Hematoxylin stained for 15 s, dehydrated, transparent and mounted for paraffin section. A "quick score" method for IHC semiquantitation validation was used. The score range is 0–18 that is positively correlated to the positive intensity and the expression quantity. The formula is: Quick Score = $A_{\text{the percentage of positive cells}} \times B_{\text{the average intensity}}$. A is assigned into 0 to 6 (1 = 0–4%; 2 = 5–19%; 3 = 20–39%; 4 = 40–59%; 5 = 60–79%; 6 = 80–100%). B is assigned into negative, weak, intermediate and strong staining that is represented by 0, 1, 2, and 3, respectively.

Western Blot

Total protein was extracted from the pancreatic tissue and separated by electrophoresis using 12% sodium dodecyl sulfate (SDS) polyacrylamide gels. The proteins are transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 4% milk for 2 h and then incubated at 4°C overnight with primary antibodies for Bcl-2, Bax, Caspase-3, and GAPDH (Bcl-2, SANTA CRUZ, 1:1000; Bax, proteintech, 1:1000; Caspase-3, Millipore, 1:200; GAPDH, ZSGB, Beijing, China, 1:1000). The membranes were washed three times with PBS containing 0.5% Tween 20 (PBS-T) and then incubated with secondary antibodies (ZSGB, Beijing, China, 1:10000) at room temperature for 1 h. The images are captured on Odyssey 1.2 software (LI-COR Biosciences, Valencia, CA). Relative band densities were quantified using Image Studio software with GAPDH as an internal control protein.

Flow Cytometry

Pancreatic islet single-cell suspensions are prepared from the pancreatic tissue through a sieve. The cells were washed three times with p0hosphate-buffered saline (PBS), centrifuged (1,000 × g) for 5 min at 4°C, and the cell concentration is maintained at approximately 1×10^5 cells/ml. The flow cytometry is performed with the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocols (Vazyme, Nanjing, China). The cells were suspended in 100 μl 1× Binding Buffer solution, stained with 5 μl Annexin V-FITC and 5 μl PI Staining Solution, and incubated at room temperature for 10 min. Next, 400 μl 1× Binding Buffer is added and apoptosis is assessed by flow cytometry (BD LSRFortessaTM, Franklin Lakes, NJ, USA).

16S Sequencing and Bioinformatics Analysis

First, the fecal samples of each mouse are collected in a sterilized tube. The samples are stored at −80°C refrigerator until use. The genomic DNA of each fecal sample was extracted based on the SDS method. The purity and concentration of DNA are detected by agarose gel electrophoresis and the samples are then diluted to 1 ng/μL with sterile water. Using the diluted genomic DNA as the template, the V3–V4 region is amplified by the enzyme and PCR buffer (New England Biolabs, Massachusetts, USA). According to the concentration of the PCR products, the samples were mixed and purified on the 2% agarose gel electrophoresis, and then target bands were cut and recycled by GeneJET Kit (Thermo Scientific, Waltham, USA). Libraries are constructed by the Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific, Waltham, USA) and finally the raw data were obtained according to manufacturer's protocols of Ion S5^{TM}XL (Thermo Scientific, Waltham, USA). Cluster analysis of OTU is performed using Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) (Edgar, 2013) and alpha diversity is calculated using Qiime software.

Statistical Analysis

All measurement data were presented as mean ± SEM and analyzed by GraphPad Prism 5. For normal distribution data, differences between groups are analyzed by One-way variance analysis (ANOVA). For non-normal distribution data, we performed a Kruskal-Wallis analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

FMT Alleviated Hyperglycemia in T2DM Mice

To explore the therapeutic effects of FMT against hyperglycemia in T2DM mice, fecal samples or normal saline were administered to mice for 8 weeks. We first characterized the changes in microbiota that occurred after FMT using 16S sequencing. According to the OTUs clustering analysis results, the species number was significantly increased after FMT treatment for 8 weeks as shown in **Figure S1A**. Concurrently, the Alpha Diversity of Shannon index and Simpson index indicated that the distribution of intestinal flora had a certain recovery in the T2DM+FMT group (**Figures S1B,C**). The above results showed that after FMT treatment, the intestinal flora of T2DM mice was more uniformly. The blood glucose level was monitored every 2 weeks until the eighth week. As shown in **Figure 1A**, the control group and T2DM+FMT groups had stable FBG levels stable during the experiment period, while the T2DM group was sustained hyperglycemia. Compared with the T2DM group, the FBG level in the T2DM+FMT group declined

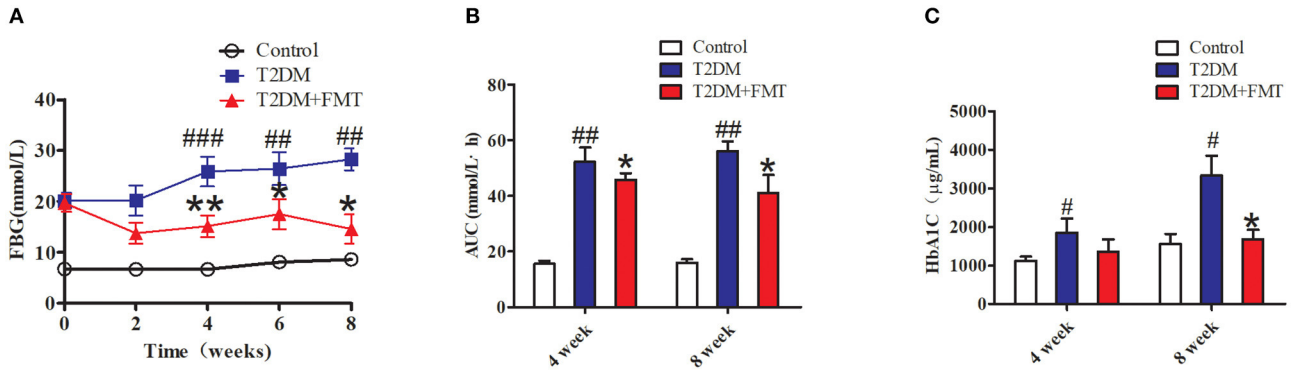


FIGURE 1 | The effect of FMT on hyperglycemia inT2DM mice. **(A)** Relative levels of FBG on different groups during the experiment period. **(B)** Effect on the areas under the curve of glucose (AUC). Mice were administrated 20% glucose (2 g/kg) and blood glucose was tested in 0, 30, 60, 120 min. AUC = 1/2 × [0.5 × (FBG_0 + FBG_{30}) + 0.5 × (FBG_{30} + FBG_{60}) + (FBG_{60} + FBG_{120})]. **(C)** Effect on plasma HbA1c level was detected by Elisa $^*P < 0.05$ vs. T2DM, $^{**}P < 0.01$ vs. T2DM, $^{\#}P < 0.05$ vs. Control, $^{\#\#}P < 0.01$ vs. Control, $^{\#\#\#}P < 0.001$ vs. Control, $n = 4$–6.

from the second week and was significantly reduced by the fourth week after FMT treatment. Meanwhile, the OGTTs were tested and the areas under the curve of glucose were calculated. It was observed that OGTTs also improved from the fourth week in the T2DM+FMT group (**Figure 1B**). We also collected blood samples and detected plasma HbA1c levels during the fourth and eighth weeks, and expectedly, the HbA1c level decreased in the FMT group over time (Kruskal-Wallis test, $p < 0.05$) (**Figure 1C**). These results suggest that FMT could stablish and decrease blood glucose and improve glucose tolerance.

FMT Improved Insulin Resistance and Repaired Injured Islets

Since insulin resistance is the main cause of T2DM (Fraulob et al., 2010), the insulin-related indicators were tested after 8 weeks of treatment. Blood samples were obtained from the medial canthus vein and the serum insulin levels were measured using an ELISA kit. As shown in **Figure 2A**, the serum insulin level increased in the T2DM group compared with the control group, and it was reversed following FMT treatment. We also calculated the index of insulin resistance and insulin sensitivity. The T2DM group had the highest index of HOMA-IR and lowest HOMA-IS, but the T2DM+FMT group had largely ameliorated insulin resistance and increased insulin sensitivity (**Figures 2B,C**). Type 2 diabetes is characterized by insulin resistance, reduced insulin sensitivity, and impaired pancreatic β-cell function. To identify the effect of FMT on the function of islets, the HOMA-β and pancreas histology was performed. The results of HOMA-β indicated that the FMT group had greatly reversed pancreatic β-cell function compared with the T2DM group (**Figure 2D**). Simultaneously, HE staining revealed that the islets of the T2DM group were seriously damaged; however, the number and the size of islets was obviously increased in the FMT group (Kruskal-Wallis test, $p < 0.05$) (**Figures 2E–G**). Thus, FMT could improve insulin resistance and alleviate islet damage.

FMT Inhibited Chronic Inflammation in Pancreatic Tissue

Generally, secretion of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) (De Taeye et al., 2007) and interleukin-6 (IL-6) increases, or secretion of anti-inflammatory cytokines including interleukin-10 (IL-10) decreases, this causes chronic inflammation in tissues leading to islet structure damaged and dysfunction in diabetic patients. To elucidate the mechanism underlying FMT-induced improvement of islet structural damage, we further detected the expression level of pro-inflammatory cytokines and anti-inflammatory. After 8 weeks of blood glucose monitoring, mice were sacrificed and pancreas samples were stored for testing inflammatory cytokines. The quick score results of IHC shown that pro-inflammatory factors of IL-6 and TNF-α were concentrated in the range of 4–9, and anti-inflammatory of IL-10 was between 0 and 3 in the T2DM group. However, the score was reversed after FMT treatment (**Tables S1–S3**). IHC results indicated that IL-6 and TNF-α in the T2DM+FMT group were lower than in the T2DM group; IL-10 was much higher in the T2DM group (**Figure 3**). Moreover, the inflammatory cytokines in pancreatic tissues were also detected by ELISA. Similarly, the results were consistent with the IHC data (Kruskal-Wallis test, $p < 0.05$) (**Figure 4**). FMT may improve damaged islets through decreased the secretion of pro-inflammatory cytokines and increased secretion of anti-inflammatory cytokines.

FMT Protected Islet Structure and Function by Decreasing Pancreatic β-Cell Apoptosis

The increase in β-cell apoptosis is a fundamental reason for impaired islet structure and function induced by the inflammatory response (Butler et al., 2003). To investigate whether FMT could protect islets by inhibiting pancreatic β-cell apoptosis, pancreatic islet single-cell suspensions were prepared from the pancreatic tissue and apoptosis was assessed by flow cytometry. The results showed that cell apoptosis in the T2DM group significantly increased, compared to

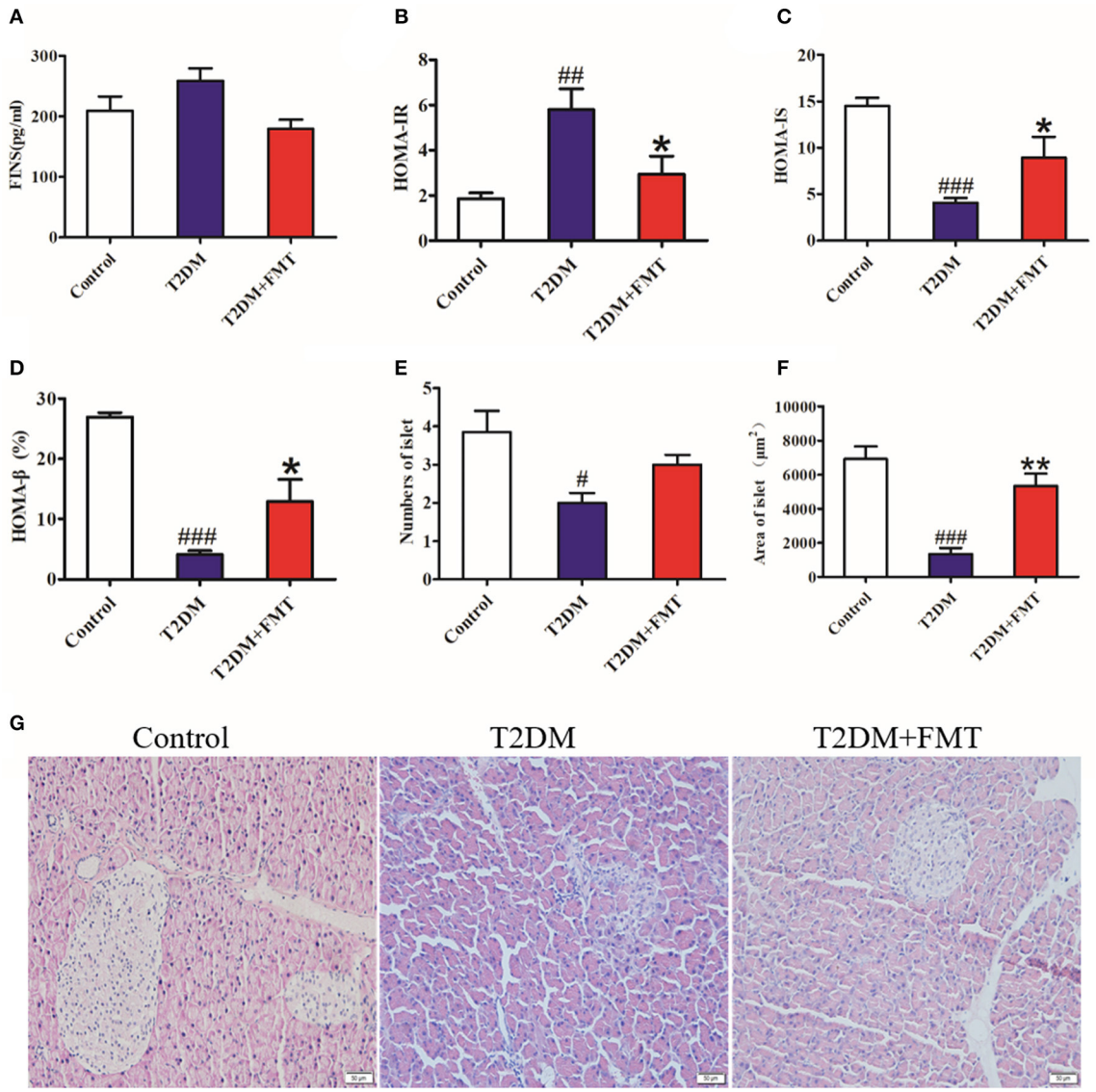


FIGURE 2 | The effect of FMT on insulin resistance and islet of the pancreas. **(A)** Fasting serum insulin level in different group. **(B)** HOMA-IR index in different group. HOMA-IR index = FBG × FINS/22.5 **(C)** HOMA-IS index in different group. HOMA-IS index = 1/HOMA-IR. **(D)** HOMA-β index in different group. HOMA-β index = 20 × FINS/(FBG-3.5) (%) **(E)** Relative amount of the islet in each group observed by HE staining image. **(F)** Relative area of the islet in each group observed by HE staining image. **(G)** Representative HE images of pancreas. $^{*}P < 0.05$ vs. T2DM, $^{**}P < 0.01$ vs. T2DM, $^{\#}P < 0.05$ vs. Control, $^{\#\#}P < 0.01$ vs. Control, $^{\#\#\#}P < 0.001$ vs. Control, $n = 4$–6.

the control group, whereas cell apoptosis was attenuated in the T2DM+FMT group (**Figures 5A,B**). Meanwhile, we also checked the expression levels of apoptosis-related indicators, including the pro-apoptotic proteins Caspase-3 and Bax, and anti-apoptotic protein Bcl-2. The western blotting results showed that the expression level of cleaved Caspase-3 and Bax were notably upregulated in the T2DM group compared with the control group and reversed by FMT (**Figures 5C,D**). The anti-apoptotic protein Bcl-2 was downregulated in the T2DM group and upregulated after treatment with FMT (**Figure 5E**). Hence, we drew the conclusion that diabetes promotes islet cell apoptosis, while FMT promotes islet cell regeneration by suppressing cell apoptosis.

DISCUSSION

Gut microbiota primarily colonizes in the host intestinal mucosa, and its effects are conferred to human pathophysiology and metabolism (Kau et al., 2011). Microflora disorder is a general characteristic found in diabetes and other metabolic diseases (Aydin et al., 2018). Numerous studies have shown that the disorder of gut microbiota has the potential to affect the progression of diabetes (Saad et al., 2016; Nie et al., 2019). It has been shown that patients with T2DM have a moderate dysbiosis between the butyrate-producing bacteria and *Lactobacillus* species (Qin et al., 2012). Furthermore, various metabolites produced by microbiota,

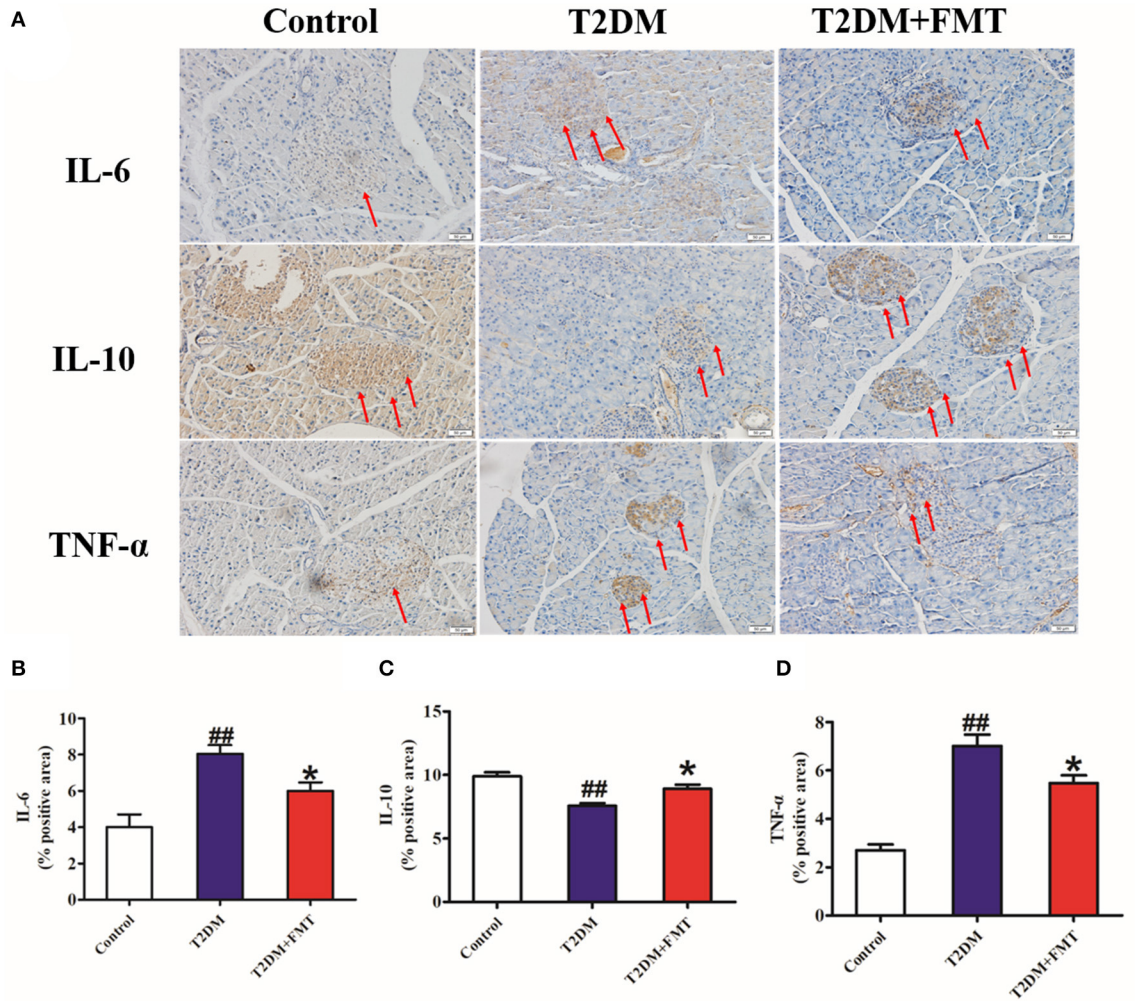


FIGURE 3 | The effect of FMT on pro-inflammatory and anti-inflammatory cytokines. **(A)** Representative IHC images of IL-6, IL-10, and TNF-α in pancreas. **(B)** The positive expression rate of IL-6 in pancreas detected by IHC. **(C)** The positive expression rate of IL-10 in pancreas detected by IHC. **(D)** The positive expression rate of TNF-α in pancreas detected by IHC. $^{*}P < 0.05$ vs. T2DM, $^{\#\#}P < 0.01$ vs. Control, $n = 8$.

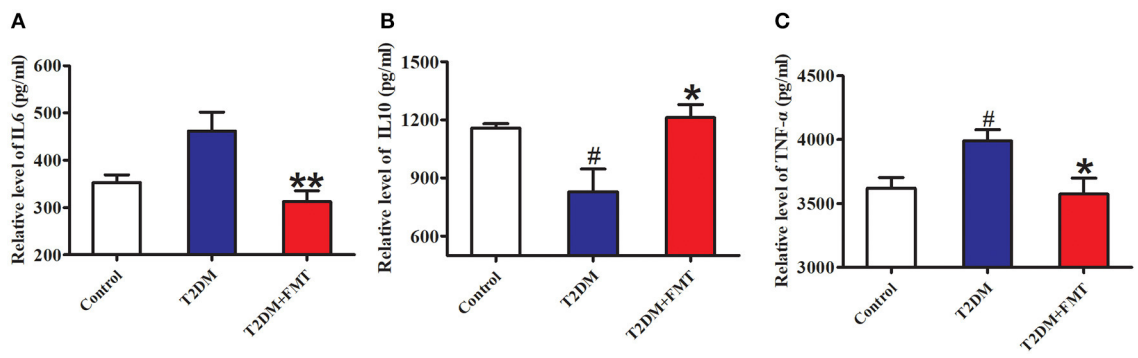


FIGURE 4 | The content of pro-inflammatory and anti-inflammatory cytokines. **(A)** The content of IL-6 in pancreas detected by ELISA. **(B)** The content of IL-10 in pancreas detected by ELISA. **(C)** The content of TNF-α in pancreas detected by ELISA. $^{*}P < 0.05$ vs. T2DM, $^{**}P < 0.01$ vs. T2DM, $^{\#}P < 0.05$ vs. Control, $n = 4$–6.

such as short-chain fatty acids, were significantly different between type 2 diabetic and normal hosts (Salek et al., 2007). Many intervention studies have shown that the intake of intestinal probiotics could effectively improve intestine microecology disorder, and relieve the symptoms of diabetic patients.

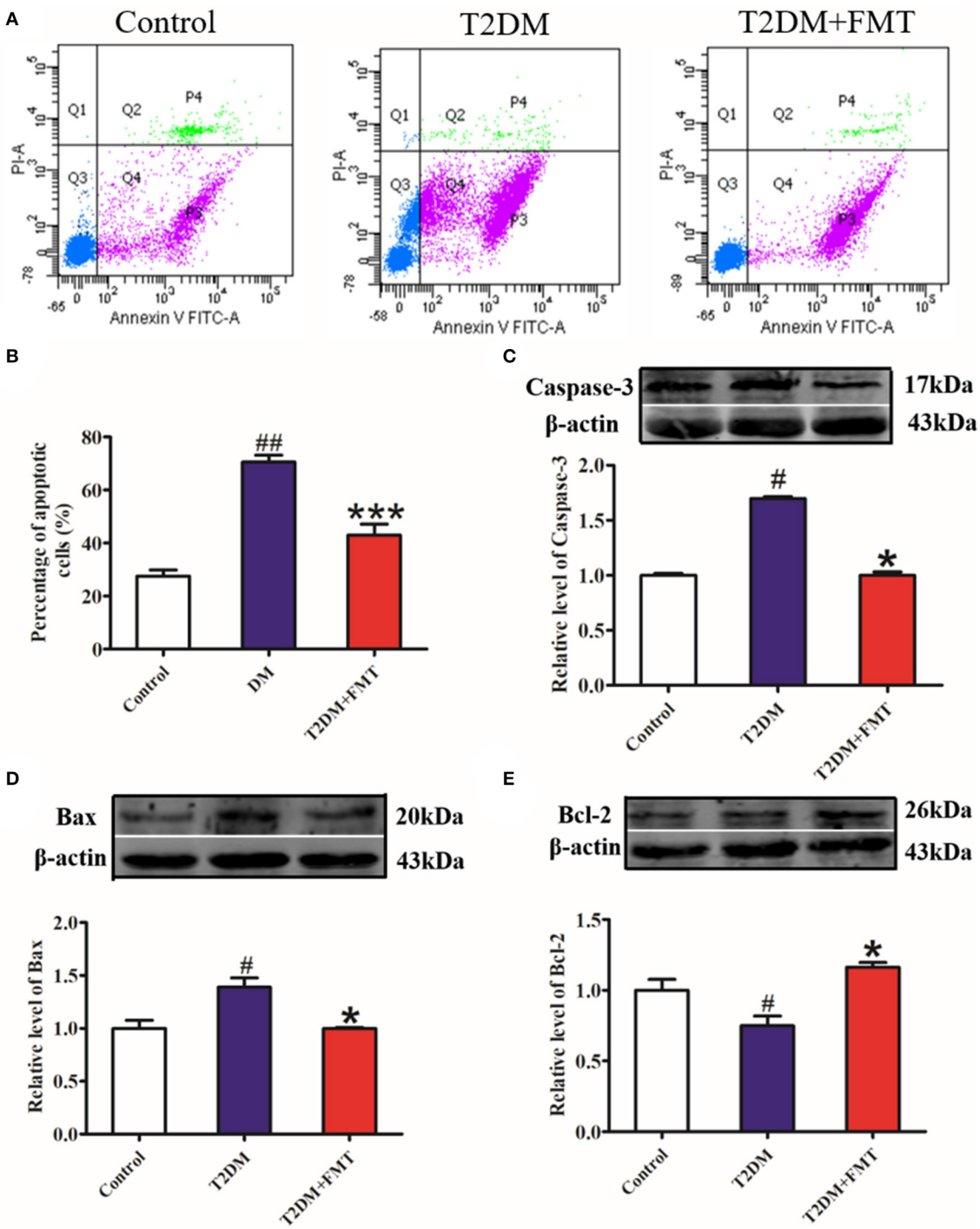


FIGURE 5 | The effect of FMT on apoptosis in pancreas tissue. **(A)** Representative flow cytometry images for pancreatic islet single cell. **(B)** Relative percentage of apoptosis cells in pancreas tissue calculated by flow cytometry. **(C)** The expression level of cleaved Caspase-3 in pancreas detected by western blot. **(D)** The expression level of Bax in pancreas detected by western blot. **(E)** The expression level of Bcl-2 in pancreas detected by western blot. $^{*}P < 0.05$ vs. T2DM, $^{***}P < 0.001$ vs. T2DM, $^{\#}P < 0.05$ vs. Control, $^{\#\#}P < 0.01$ vs. Control, $n = 4$–6.

A few data have demonstrated that the formation of gut microbiota could be reshaped to improve or control disease status (Shen et al., 2015). Fecal microbiota transfer (FMT) is a method to treat diseases by reconstructing the microbiota (Lee et al., 2018). There is increasing evidence regarding the treatment potential of FMT based on an already developed clinical plan that has been the first-line therapy for recurrent *Clostridium difficile* infections (Cammarota

et al., 2017). However, understanding the influence of the intestinal microbiota on metabolic diseases is in the initial stage and the data regarding the function of FMT on type 2 diabetes are still scarce. In our study, we established T2DM animal models by feeding mice high-fat diet combined with streptozotocin and observed that FMT treatment successfully reduced FBG and improved glucose tolerance on diabetic mice (**Figures 1A,B**). It was possible to restore the balance of intestinal microflora to promote host homeostasis (Burrello et al., 2018). According to a recent study, in metabolic syndrome, when commensal bacteria from various phyla stay in a certain range, they contribute to control the ratio of pathogenic species and support optimal physical conditions (Burrello et al., 2018). Glycated hemoglobin test usually reflects the patient's blood glucose control in the last 8–10 weeks and our ELISA results of HbA1c further provided evidence of the hypoglycemic effect of FMT (**Figure 1C**). These results showed that FMT had a therapeutic effect on hyperglycemia. As reported in the literature, FMT could display a recovered phenotype upon transfer donor's intestinal flora to the receptor, which was consonant with our current experimental results (de Groot et al., 2017).

T2DM is characterized by insulin resistance, which has also been confirmed in our study (**Figure 2B**). Alterations of gut microbiota composition are highly linked with adiposity and insulin resistance, which has been considered to be an environmental factor for type 2 diabetes (Borody et al., 1989; de Groot et al., 2017). After T2DM mice reconstructed their microbiota through the feces received from normal mice, fasting insulin levels were decreased and insulin resistance was improved (**Figures 2A,B**). At the same time, the insulin sensitivity index (HOMA-IS) was increased in the T2DM+FMT group. Similarly, in a clinical trial, obese patients received FMT treatment from lean healthy individuals showed a positive change in insulin sensitivity (Aron-Wisnewsky et al., 2019). For the current research, dysbiosis of gut microbiota is associated with the development of insulin resistance and diabetes (de Groot et al., 2017). The mechanism underlying the gut microbiota-induced amelioration of insulin resistance may be through changes in body energy balance or by alleviating obesity caused by a high-fat diet. However, the exact mechanism needs to be explored comprehensively.

To the best of our knowledge, microbiota imbalance induces more gram-negative bacteria, which produce high amounts of LPS and activate low-grade chronic inflammation of the islets. When the microbiota of normal mice was transplanted into diabetic mice, the secretion of pro-inflammatory factors decreased and anti-inflammatory secretion increased in pancreatic tissues (**Figures 3**, **4**). Studies have shown that FMT is involved in the low-grade inflammation characterized by metabolic disorders. It has been reported that therapeutic FMT could decrease the secretion of inflammatory factors and triggered several immune-mediated signal-pathways in colitis (Burrello et al., 2018). As mentioned in another study, transplantation of gut microbiota like *F. prausnitzii* prevented the inflammation damage in the pancreas (Ganesan et al., 2018). These findings were similar to our HE staining and HOMA-β index results, which indicated that the size of the islet and the function of pancreatic islet β cell was recovered after FMT treatment in diabetic mice (**Figures 2D–G**). IL-6 and TNF-α are pro-inflammatory cytokines with multiple functions that can directly act on islet cells, causing pancreatic islet β cell injuries (Park et al., 2018). Low-grade chronic inflammation caused by microbiota imbalance usually leads to the destruction of islet structure and impaired pancreatic islet β cell function, and islet β-cells apoptosis is the fundamental reason for the destruction of islet structure. Nonetheless, islet injury and dysfunction were reversed when the inflammatory responses and the apoptosis of islet beta cells were relieved through FMT treatment (**Figure 5**).

Although there are several studies about the function of FMT, the mechanism underlying FMT-induced alleviation of the disease remains largely unelucidated. Diabetes symptoms may be relieved through the synergistic effects between the commensal gut microbiota after FMT treatment, or possibly triggered by multiple immune-inflammatory processes and pathways. Future research should focus on the bacterial taxonomic and functional changes affiliated with FMT treatment in diabetic patients, as well as how FMT affects the metabolism of other organs in long-term improvement. Due to the complexity of intestinal flora, further research should explore whether the particular microflora species or communities in FMT make an effort on preventing and treating diabetes. This may provide a novel perspective and reference that could be verified in population-based studies about the effect of FMT on diabetes for the next work. Long-term restoration of gut microbiota through FMT may be used as a promising therapeutic application for diabetes.

In conclusion, this study demonstrated that high-fat diet induced T2DM can be treated through FMT by improving the insulin resistance and attenuating pancreatic islet β-cell destruction. We discovered that FMT could alleviate hyperglycemia, which provided a bacterial-based treatment strategy for the management of T2DM.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental Animal Ethics Committee of Harbin Medical University, China (No. HMUIRB 20150034).

AUTHOR CONTRIBUTIONS

HW conceptualized and designed the experiments in this study. YL, YY, ST, DL, and CW performed the experimental

procedures related to the study. ST, DZ, and JJ revised the article. ZW proofread and discussed articles related to this research. YB provided the financial support and reviewed the experimental directions. All authors contributed to the generation of experimental data for this study.

FUNDING

This project was supported by the National Natural Science Foundation of China (Grant Nos. 81674326 and 81730012).

ACKNOWLEDGMENTS

We would like to extend our gratitude to Syeda Madiha Zahra for modifying the language used in this article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00455/full#supplementary-material

REFERENCES

Aron-Wisnewsky, J., Clement, K., and Nieuwdorp, M. (2019). Fecal microbiota transplantation: a future therapeutic option for Obesity/Diabetes? *Curr. Diab. Rep.* 19:51. doi: 10.1007/s11892-019-1180-z

Aydin, O., Nieuwdorp, M., and Gerdes, V. (2018). The gut microbiome as a target for the treatment of Type 2 Diabetes. *Curr. Diab. Rep.* 18:55. doi: 10.1007/s11892-018-1020-6

Borody, T. J., George, L., Andrews, P., Brandl, S., and Moore-Jones, D. (1989). Bowel-flora alteration: a potential cure for inflammatory bowel disease and irritable bowel syndrome? *Med. J. Aust.* 150:604. doi: 10.5694/j.1326-5377.1989.tb136704.x

Burrello, C., Garavaglia, F., Cribiu, F. M., Ercoli, G., Lopez, G., Troisi, J., et al. (2018). Therapeutic faecal microbiota transplantation controls intestinal inflammation through IL10 secretion by immune cells. *Nat. Commun.* 9:5184. doi: 10.1038/s41467-018-07359-8

Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., and Butler, P. C. (2003). β-Cell deficit and increased β-cell apoptosis in humans with Type 2 Diabetes. *Diabetes* 52, 102–110. doi: 10.2337/diabetes.52.1.102

Cammarota, G., Ianiro, G., Tilg, H., Rajilić-Stojanović, M., Kump, P., Satokari, R., et al. (2017). European consensus conference on faecal microbiota transplantation in clinical practice. *Gut* 66, 569–580. doi: 10.1136/gutjnl-2016-313017

Curti, V., Di Lorenzo, A., Dacrema, M., Xiao, J., Nabavi, S. M., and Daglia, M. (2017). *In vitro* polyphenol effects on apoptosis: an update of literature data. *Semin. Cancer Biol.* 46, 119–131. doi: 10.1016/j.semcancer.2017.08.005

de Groot, P. F., Frissen, M. N., de Clercq, N. C., and Nieuwdorp, M. (2017). Fecal microbiota transplantation in metabolic syndrome: history, present and future. *Gut Microbes* 8, 253–267. doi: 10.1080/19490976.2017.1293224

De Taeye, B. M., Novitskaya, T., McGuinness, O. P., Gleaves, L., Medda, M., Covington, J. W., et al. (2007). Macrophage TNF-alpha contributes to insulin resistance and hepatic steatosis in diet-induced obesity. *Am. J. Physiol. Endocrinol. Metab.* 293, E713–E725. doi: 10.1152/ajpendo.00194.2007

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

Ericsson, A. C., Personett, A. R., Turner, G., Dorfmeyer, R. A., and Franklin, C. L. (2017). Variable colonization after reciprocal fecal microbiota transfer between mice with low and high richness microbiota. *Front. Microbiol.* 8:196. doi: 10.3389/fmicb.2017.00196

Fraulob, J. C., Ogg-Diamantino, R., Fernandes-Santos, C., Aguila, M. B., and Mandarim-De-Lacerda, C. A. (2010). A mouse model of metabolic syndrome: insulin resistance, fatty liver and non-alcoholic fatty Pancreas Disease (NAFPD) in C57BL/6 mice fed a high fat diet. *J. Clin. Biochem. Nutr.* 46, 212–223. doi: 10.3164/jcbn.09-83

Ganesan, K., Chung, S. K., Vanamala, J., and Xu, B. (2018). Causal relationship between diet-induced gut microbiota changes and diabetes: a novel strategy to transplant *Faecalibacterium* prausnitzii in preventing diabetes. *Int. J. Mol. Sci.* 19:3720. doi: 10.3390/ijms19123720

He, X., Lai, Q., Chen, C., Li, N., Sun, F., Huang, W., et al. (2018). Both conditional ablation and overexpression of E2 SUMO-conjugating enzyme (UBC9) in mouse pancreatic beta cells result in impaired beta cell function. *Diabetologia* 61, 881–895. doi: 10.1007/s00125-017-4523-9

Jia, L., Li, D., Feng, N., Shamoon, M., Sun, Z., Ding, L., et al. (2017). Anti-diabetic effects of clostridium butyricum CGMCC0313.1 through promoting the growth of gut butyrate-producing *Bacteria* in Type 2 Diabetic Mice. *Sci. Rep.* 7:7046. doi: 10.1038/s41598-017-07335-0

Kang, Y., Li, Y., Du, Y., Guo, L., Chen, M., Huang, X., et al. (2018). Konjaku flour reduces obesity in mice by modulating the composition of the gut microbiota. *Int. J. Obes.* 43, 1631–1643. doi: 10.1038/s41366-018-0187-x

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., and Gordon, J. I. (2011). Human nutrition, the gut microbiome and the immune system. *Nature* 474, 327–336. doi: 10.1038/nature10213

Kerr, C. A., Grice, D. M., Tran, C. D., Bauer, D. C., Li, D., Hendry, P., et al. (2015). Early life events influence whole-of-life metabolic health via gut microflora and gut permeability. *Crit. Rev. Microbiol.* 41, 326–340. doi: 10.3109/1040841X.2013.837863

Koopal, C., Visseren, F. L. J., Westerink, J., van der Graaf, Y., Ginsberg, H. N., and Keech, A. C. (2018). Predicting the effect of fenofibrate on cardiovascular risk for individual patients with Type 2 Diabetes. *Diabetes Care* 41, 1244–1250. doi: 10.2337/dc17-0968

Larsen, N., Vogensen, F. K., van den Berg, F. W., Nielsen, D. S., Andreasen, A. S., Pedersen, B. K., et al. (2010). Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLoS ONE* 5:e9085. doi: 10.1371/journal.pone.0009085

Lee, P., Yacyshyn, B. R., and Yacyshyn, M. B. (2018). Gut microbiota and obesity: an opportunity to alter obesity through fecal microbiota transplant (FMT). *Diabetes Obes. Metab.* 21, 479–490. doi: 10.1111/dom.13561

Nie, Q., Chen, H., Hu, J., Fan, S., and Nie, S. (2019). Dietary compounds and traditional Chinese medicine ameliorate type 2 diabetes by modulating gut microbiota. *Crit. Rev. Food Sci. Nutr.* 59, 848–863. doi: 10.1080/10408398.2018.1536646

Park, J., Decker, J. T., Smith, D. R., Cummings, B. J., Anderson, A. J., and Shea, L. D. (2018). Reducing inflammation through delivery of lentivirus encoding for anti-inflammatory cytokines attenuates neuropathic pain after spinal cord injury. *J. Control. Release* 290, 88–101. doi: 10.1016/j.jconrel.2018.10.003

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., et al. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490, 55–60. doi: 10.1038/nature11450

Ruiz, L., Gurlo, T., Ravier, M. A., Wojtusciszyn, A., Mathieu, J., Brown, M. R., et al. (2018). Proteasomal degradation of the histone acetyl transferase p300 contributes to beta-cell injury in a diabetes environment. *Cell Death Dis.* 9:600. doi: 10.1038/s41419-018-0603-0

Saad, M. J., Santos, A., and Prada, P. O. (2016). Linking gut microbiota and inflammation to obesity and insulin resistance. *Physiology.* 31, 283–293. doi: 10.1152/physiol.00041.2015

Salek, R. M., Maguire, M. L., Bentley, E., Rubtsov, D. V., Hough, T., Cheeseman, M., et al. (2007). A metabolomic comparison of urinary changes in type 2 diabetes in mouse, rat, and human. *Physiol. Genomics* 29, 99–108. doi: 10.1152/physiolgenomics.00194.2006

Savilahti, E., Harkonen, T., Savilahti, E. M., Kukkonen, K., Kuitunen, M., and Knip, M. (2018). Probiotic intervention in infancy is not associated with development of beta cell autoimmunity and type 1 diabetes. *Diabetologia* 61, 2668–2670. doi: 10.1007/s00125-018-4738-4

Shaw, J. E., Sicree, R. A., and Zimmet, P. Z. (2010). Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res. Clin. Pract.* 87, 4–14. doi: 10.1016/j.diabres.2009.10.007

Shen, T. C., Albenberg, L., Bittinger, K., Chehoud, C., Chen, Y. Y., Judge, C. A., et al. (2015). Engineering the gut microbiota to treat hyperammonemia. *J. Clin. Invest.* 125, 2841–2850. doi: 10.1172/JCI 79214

Sun, Y., Shi, H., Yin, S., Ji, C., Zhang, X., Zhang, B., et al. (2018). Human mesenchymal stem cell derived exosomes alleviate type 2 diabetes mellitus by reversing peripheral insulin resistance and relieving beta-Cell Destruction. *ACS Nano* 12, 7613–7628. doi: 10.1021/acsnano.7b 07643

The Ministry of Science and Technology, People's Republic of China (2006). Guiding opinions on treating experimental animals. *Sci. Technol. Inform. Anim. Veter.* 2007, 35–36.

Wang, S., Huang, M., You, X., Zhao, J., Chen, L., Wang, L., et al. (2018). Gut microbiota mediates the anti-obesity effect of calorie restriction in mice. *Sci. Rep.* 8:13037. doi: 10.1038/s41598-018-31353-1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 22 January 2020
doi: 10.3389/fcimb.2019.00475

Oral Microbiota Perturbations Are Linked to High Risk for Rheumatoid Arthritis

Yanli Tong[1], *Linlin Zheng*[1], *Pingying Qing*[1], *Hua Zhao*[1], *Yanhong Li*[1], *Linchong Su*[1,2], *Qiuping Zhang*[1], *Yi Zhao*[1], *Yubin Luo*[1]* *and Yi Liu*[1]

[1] *Department of Rheumatology and Immunology, West China Hospital, Sichuan University, Chengdu, China,* [2] *Hubei Provincial Key Laboratory of Occurrence and Intervention of Rheumatic Diseases, Enshi, China*

Oral microbial dysbiosis is known to increase susceptibility of an individual to develop rheumatoid arthritis (RA). Individuals at-risk of RA may undergo different phases of disease progression. In this study, we aim to investigate whether and whereby the oral microbiome communities alter prior to symptoms of RA. Seventy-nine saliva samples were collected from 29 high-risk individuals, who were positive for anti-citrullinated protein antibodies (ACPA) and have no clinical arthritis, 27 RA patients and 23 healthy controls (HCs). The salivary microbiome was examined using 16S ribosomal RNA gene sequencing. Alpha and beta diversity analysis and the linear discriminant analysis were applied to examine the bacterial diversity, community structure and discriminatory taxa between three groups, respectively. The correlation between salivary bacteria and autoantibodies were analyzed. In the "pre-clinical" stages, salivary microbial diversity was significantly reduced comparing to RA patients and HCs. In contrast to HCs, like RA patients, individuals at high-risk for RA showed a reduction in the abundance of genus *Defluviitaleaceae_UCG-011* and the species *Neisseria oralis*, but an expansion of *Prevotella*_6. Unexpectedly, the relative abundance of *Porphyromonas gingivalis*, reported as opportunistic pathogens for RA development, was significantly decreased in high-risk individuals. Additionally, we identified four genera in the saliva from high-risk individuals positively correlated with serum ACPA titers, and the other two genera inversely displayed. In summary, we observed a characteristic compositional change of salivary microbes in individuals at high-risk for RA, suggesting that oral microbiota dysbiosis occurs in the "pre-clinical" stage of RA and are correlated with systemic autoimmune features.

Keywords: oral microbiome, rheumatoid arthritis, high risk, anti-citrullinated protein autoantibodies, dysbiosis

OPEN ACCESS

Edited by:
Lorenzo Lo Muzio,
University of Foggia, Italy

Reviewed by:
Marco Mascitti,
Marche Polytechnic University, Italy
J. Christopher Fenno,
University of Michigan, United States

***Correspondence:**
Yubin Luo
luoyubin2016@163.com

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *24 November 2019*
Accepted: *24 December 2019*
Published: *22 January 2020*

Citation:
Tong Y, Zheng L, Qing P, Zhao H, Li Y, Su L, Zhang Q, Zhao Y, Luo Y and Liu Y (2020) Oral Microbiota Perturbations Are Linked to High Risk for Rheumatoid Arthritis. Front. Cell. Infect. Microbiol. 9:475. doi: 10.3389/fcimb.2019.00475

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disease that primarily involves the joints. Over the past years, research focusing on the earliest stage of RA has led to the discovery of RA-related systemic inflammation and autoimmunity in the pre-clinical stage. The presence of circulating autoantibodies, elevation of cytokines and chemokines levels, and increase of acute phase reactants precede clinical arthritis (Rantapaa-Dahlqvist et al., 2003; Berglin et al., 2004; Nielen et al., 2004; Jorgensen et al., 2008; Sokolove et al., 2012). Prospective studies define

ACPA-positive individuals as populations at risk for developing RA, and the chance in subjects with seropositivity and arthralgia can be as high as nearly 30% within 1 year (van de Stadt et al., 2011). In view of potential preventive strategies, the pre-clinical and earliest stages of RA are likely to represent important therapeutic windows within which disease outcomes can be dramatically modulated. Exploring risk factors and biomarkers for RA, especially in the earliest stage of disease is definitely an urgent need.

Complex interplay between genetic and environmental factors contributes to RA etiopathogenesis. An ancient theory of infectious etiology of RA has now been re-emphasized. Recent studies have further corroborated this theory by indicating mucosal origins of the disease. Data from epidemiological and translational studies suggest that environmental exposure and dysbiosis in mucosal sites (lung, gastrointestinal tract, and oral cavity) have causative roles in the development of RA (Scher et al., 2012, 2013, 2016). Of note, *Porphyromonas gingivalis*, a periodontal pathogen, was capable of producing peptidylarginine deiminase (PAD) enzyme to citrullinated antigens. The presence of antibodies to *P. gingivalis* was positively correlated with the increased titer of ACPA (Mikuls et al., 2012; Lappin et al., 2013). Additionally, a recent study revealed a new species in the oral sites of RA patients which promoted citrullinated antigens production (Konig et al., 2016). This species, *Aggregatibacter actinomycetemcomitans*, led to dysregulated PAD function and release of hypercitrullinated proteins through inducing neutrophil migration and neutrophil extracellular traps (NETs) formation (Hirschfeld et al., 2016; Konig et al., 2016). Animal studies further showed that two periodontal pathogens, *P. gingivalis* and *Prevotella nigrescens* aggravated collagen-induced arthritis in mice (de Aquino et al., 2014). These observations support the theory that oral dysbiosis may be an origin of autoantigen production and a risk factor for RA.

Although the oral microbiome is substantially altered in RA patients (Scher et al., 2012; Zhang et al., 2015), little is known about its status in the initial stages of disease development. Whether this alteration precedes clinically evident arthritis and associated with disease development or merely represents a resultant or concomitant phenomenon of the disease is unclear. As ACPA is highly specific (Schellekens et al., 2000), detectable early and predictive of rapid progression and erosion of RA (Nielen et al., 2004; Ronnelid et al., 2005; Syversen et al., 2008; Rombouts et al., 2015), individuals with ACPA positivity are at increased risk for RA, especially those with arthralgia (van de Stadt et al., 2011). We therefore sought to test whether the oral microbiome exhibits distinct taxonomic features in seropositive individuals at high-risk for RA and provide a potential avenue for early detection, intervention or prevention.

MATERIALS AND METHODS

Participants and Study Design

Individuals at high-risk for RA were recruited from West China Hospital, Sichuan University, China. These subjects had a positive serum antibody for ACPA, with or without arthralgia at the time of enrollment. Absence of arthritis was confirmed by physical examination of 44 joints. RA patients were diagnosed according to the American College of Rheumatology (ACR) 2010 classification for RA. Most RA patients were receiving oral disease-modifying anti-rheumatic drugs (DMARDs) and/or corticosteroids at the time of enrollment. Patients receiving biological agents were excluded. Age, gender and ethnicity-matched healthy controls (HCs) with no personal history of inflammatory arthritis were recruited. ACPA-negative profiles for HCs were obtained from the health management center. Subjects from all three study groups were $\geq$18 years old. Individuals having a history of antibiotics treatment or surgery in the last 3 months, current extreme diet, major organ dysfunction, cancer, other rheumatic or autoimmune diseases including osteoarthritis, systemic lupus erythematosus, Sjögren syndrome, diabetes were excluded. A total of 79 participants who met the inclusion and exclusion criteria were enrolled, including 29 high-risk individuals, 27 RA patients and 23 HCs. The study procedure was approved by the Biomedical Research Ethics Committee, West China Hospital of Sichuan University (ChiCTR1900022605), and the written consents were obtained from all the participants according to the Declaration of Helsinki. Sociodemographic factors and clinical activity are summarized in **Table 1**.

Periodontal Health Evaluation and Saliva Collection

Periodontitis was assessed using a self-reported questionnaire involving bleeding on brushing teeth, non-traumatic loose or missing teeth, or periodontal disease diagnosed by a dentist. Individuals reporting any of these issues were recorded positive. Participants were asked to refrain from eating, drinking or smoking for 30 min prior to sample collection. For saliva collection, participants were first asked to rinse mouth with bottled water to remove food debris, keep lips shut for 3 min, and then spit saliva directly into a 50ml sterile Falcon tube (Becton). After collection, samples were immediately frozen and stored at −80°C.

Bacterial DNA Extraction

Microbial DNA was isolated from saliva using the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA) according to manufacturer's protocols. Concentration and purification of the final DNA were determined by NanoDrop 2000 (Thermo Scientific, USA), and quality checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by thermocycler PCR system (GeneAmp 9700, ABI, USA). The resulted PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol.

TABLE 1 | Demographic and clinical features among RA patients, high-risk individuals (Pre) and healthy controls.

Characteristic	RA ($n = 27$)	High-risk for RA ($n = 29$)	Healthy controls ($n = 23$)
Age, mean (median) years	51.1 (52)	48.1 (50)	49.5 (49)
Female, %	59	41	57
Disease duration, mean (median) months	17.9 (12.5)	–	–
Disease activity parameter			
ESR, mean (median) mm/h	36.05 (27.50)	–	–
CRP, mean (median) mg/L	14.99(3.18)	–	–
DAS28, mean (median)	4.98 (4.61)	–	–
Autoantibody status			
ACPA positive, *n* (%)	25 (93)	29 (100)	0
IgM-RF positive, *n* (%)	23 (85)	4 (14)	0
ACPA titer, mean (median) U/ml	320.3 (384.3)	173.4 (89.6)	–
IgM-RF titer, mean (median) IU/ml	281.4 (126.0)	47.1 (20.0)	–
Medication use, %			
DMARDs (MTX, LEF, HCQ)	81	0	0
Prednisone	74	0	0
Biologic agent	0	0	0
Smoking status, *n* (%)			
Current	8 (30)	12 (42)	8 (35)
Former	3 (12)	2 (7)	1 (4)
Never	16 (58)	15 (51)	14 (61)
Periodontitis, %*	63	62	57

*ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, Disease Activity Score in 28 joints; ACPA, anti–citrullinated protein antibody; IgM-RF, IgM rheumatoid factor; DMARDs, Disease-modifying antirheumatic drugs; MTX, Methotrexate; LEF, Leflunomide; HCQ, hydroxychloroquine. *Periodontitis was assessed using a self-reported questionnaire involving bleeding on brushing teeth, non-traumatic loose or missing teeth, or periodontal disease diagnosed by a dentist. Individuals reporting any of these issues were recorded positive.*

Illumina MiSeq Sequencing and Processing of Sequencing Data

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA). Raw fastq files were quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) the reads were truncated at any site receiving an average quality score <20 over a 50 bp sliding window, (ii) sequences whose overlap being longer than 10 bp were merged according to their overlap with mismatch no more than 2 bp, and (iii) sequences of each sample were separated according to barcodes (exactly matching) and Primers (allowing 2 nucleotide mismatching), and reads containing ambiguous bases were removed. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1) with a novel "greedy" algorithm that performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU132) 16S rRNA database using confidence threshold of 70%. The fastq files were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA578951).

Statistical Analysis

To determine statistically different bacterial taxa among the three groups, we applied the Kruskal-Wallis H-test, with multiple test corrected by Benjamini-Hochberg false discovery rate (FDR) test. *Post-hoc* test was applied to further determine the difference between each group-pair if multiple test among the three groups was significantly different. The linear discriminant analysis (LDA) effect size (LefSe) analysis was applied to detect the most discriminatory taxa among groups. Different features with an LDA score cut-off of 3.0 were identified. For cross-sectional analyses of baseline characteristics and comparison of diversity indexes among three groups, differences were evaluated using the one-way ANOVA test, corrected by FDR. The ANOSIM test was applied to the binary euclidean distance matrix containing all analyzed samples to define if the overall structure of the microbiota was significantly different between the groups. Spearman's correlation analyses were used to assess potentially clinically relevant associations on all taxa. The correlation network between the genera was plotted using cytoscape. Significant correlations with absolute value of Spearman correlation coefficient (rho) >0.5 were plotted. Two-tailed $P < 0.05$ were considered significant.

RESULTS

Characteristic of Participants

Detailed demographic characteristics of the participants enrolled in the study are given in **Table 1**. Age and gender were comparable among the three groups. The mean disease duration in RA patients was 17.9 months (median 12.5 months) and the mean DAS28 was 4.98, reflecting the presence of moderate disease. Nine out of 29 individuals reported having a history of arthralgia in high-risk group, although no arthritis based on physical examination of 44 joints was detected at the time of enrollment. Among RA patients, ACPA and IgM-RF positivity were 93 and 85%, respectively, compared to 100 and 14% in high-risk individuals (ACPA, $P = 0.23$; IgM-RF, $P < 0.0001$). Compared to the high-risk individuals, the sera antibodies titers in RA patients were 1.8-fold higher for ACPA (median level 320.3 vs. 173.4 U/ml) and 6-fold higher for IgM-RF (median level 281.4 vs. 47.1 IU/ml), respectively. Most of the patients with RA were on DMARDs (81%) and/or prednisone (74%) treatment. This study enrolled patients who were naïve to biological agent treatment. Smoking status and periodontitis were not significantly different between groups.

Distinct Features of Oral Microbial Community in Individuals at High-Risk for RA

Overall, 79 saliva samples yielded 16S rRNA V3-V4 gene sequences with a median depth of sequencing of 53,183 reads per sample (IQR = 45,928–60,223). Using a distance-based similarity of ≥97% for species level OTU assignment, a total of 1,088 OTUs were identified. The number of OTUs from

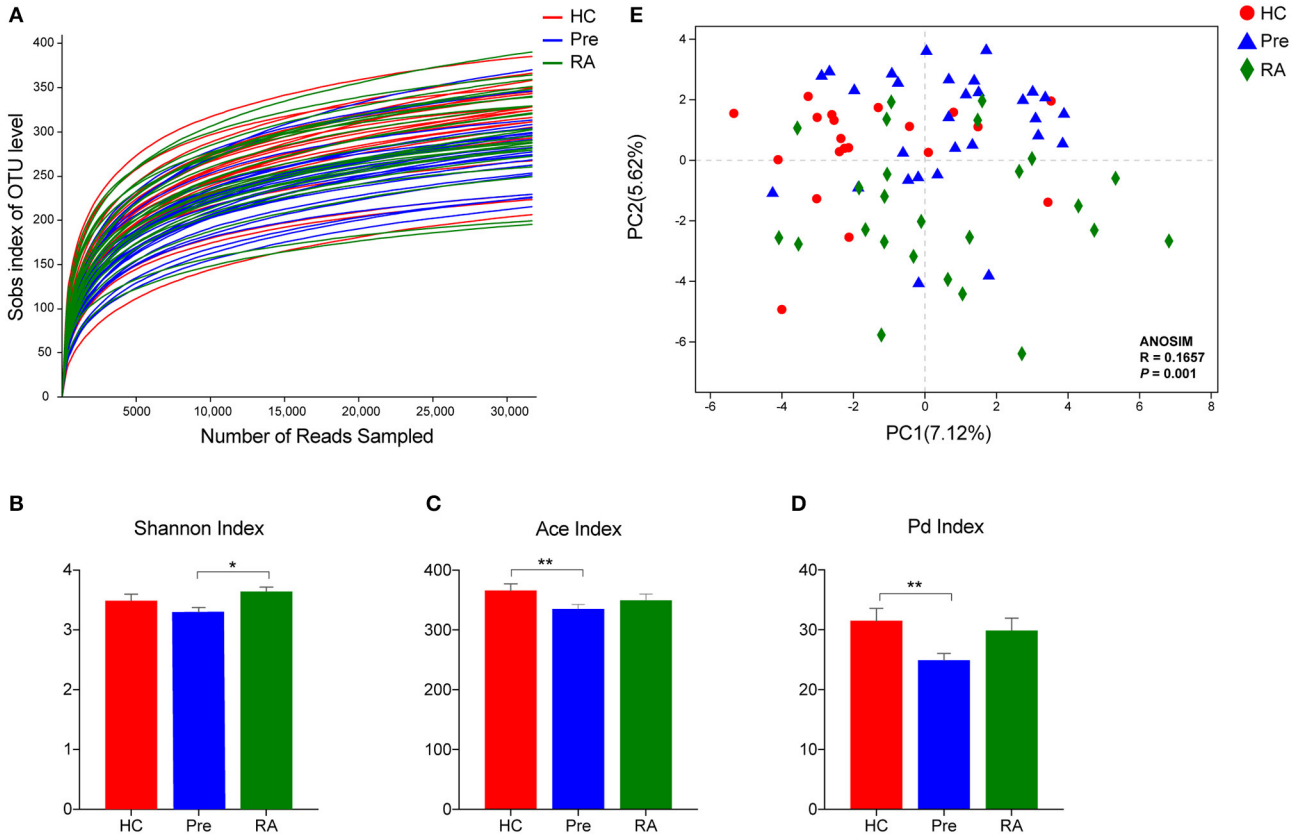


FIGURE 1 | Oral microbiota community characteristics in high-risk individuals (Pre), RA patients and healthy controls (HCs). **(A)** Rarefaction curves showing the number of bacterial sequences obtained are saturated. Alpha diversity was calculated using Shannon diversity index **(B)**, Ace community richness index **(C)**, and the Faith's phylodiversity index **(D)**, revealing a distinct feature in high-risk individuals. **(E)** Beta diversity demonstrated that samples clustered showed a tendency of gradual change from healthy subjects, high-risk individuals to RA patients. $^{*}p < 0.05$ and $^{**}p < 0.01$.

each sample increased sharply before reaching a plateau, which indicates that the number of bacterial sequences obtained represented the bacterial communities well, as the rarefaction curves tended toward saturation (**Figure 1A**). When compared to HCs and RA patients, oral microbial alpha-diversity was significantly reduced in high-risk individuals (designated "Pre"), as shown by the Shannon diversity index (Pre vs. RA), the ace community richness index and Faith's phylodiversity index (Pre vs. HCs) (**Figures 1B–D**). Subsequently, we analyzed whether the overall structure of the oral microbiota of HCs differed from that of RA and at-risk individuals based on binary euclidean distance. We further applied PCoA analysis to cluster samples along orthogonal axes of maximal variance. As shown in **Figure 1E**, beta-diversity plots differentiated the oral microbiota of HCs, RA patients and high-risk individuals (ANOSIM test; $R = 0.1657$, $P = 0.001$).

Alteration of Specific Taxa Abundance in High-Risk Individuals

To further probe the distinct bacterial taxa among groups, we first analyzed the relative abundance of the most abundant taxa. The pie charts revealed that the dominant phyla across all subjects were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Actinobacteria*, which together accounted for more than 95% of bacterial sequences (**Figure 2A**). We then analyzed the differential phyla among the three groups. Notably, the relative abundance of *Firmicutes* increased gradually from HCs, high-risk individuals to RA patients (**Figures 2A,B**, $P = 0.0421$ for RA vs. HCs), while *Proteobacteria* showed an inverse trend of transition (**Figures 2A,B**, $P = 0.0003199$ for RA vs. HCs). The ratio of *Firmicutes* to *Proteobacteria* significantly increased from HCs to high-risk individuals to RA patients as evaluated by Chi-square test (**Supplementary Figure 1**, $P = 0.0083$). Other taxa including *Actinobacteria* and *Patescibacteria* showed a similar trend of enrichment in high-risk individuals and RA patients vs. HCs (**Figures 2A,B**).

We then applied Kruskal-Wallis followed by FDR correction and the LEfSe method to analyze more specific differences in microbiota composition among three groups. At the genus level, *Defluviitaleaceae_UCG-011* was significantly decreased in both RA and high-risk individuals compared to HCs (**Figure 2C**). Characteristic genera changes were found in high-risk individuals, with *Rothia* genus increased (Pre vs. RA) and *Filifactor* decreased (Pre vs. HCs, **Figure 2C**). The abundance

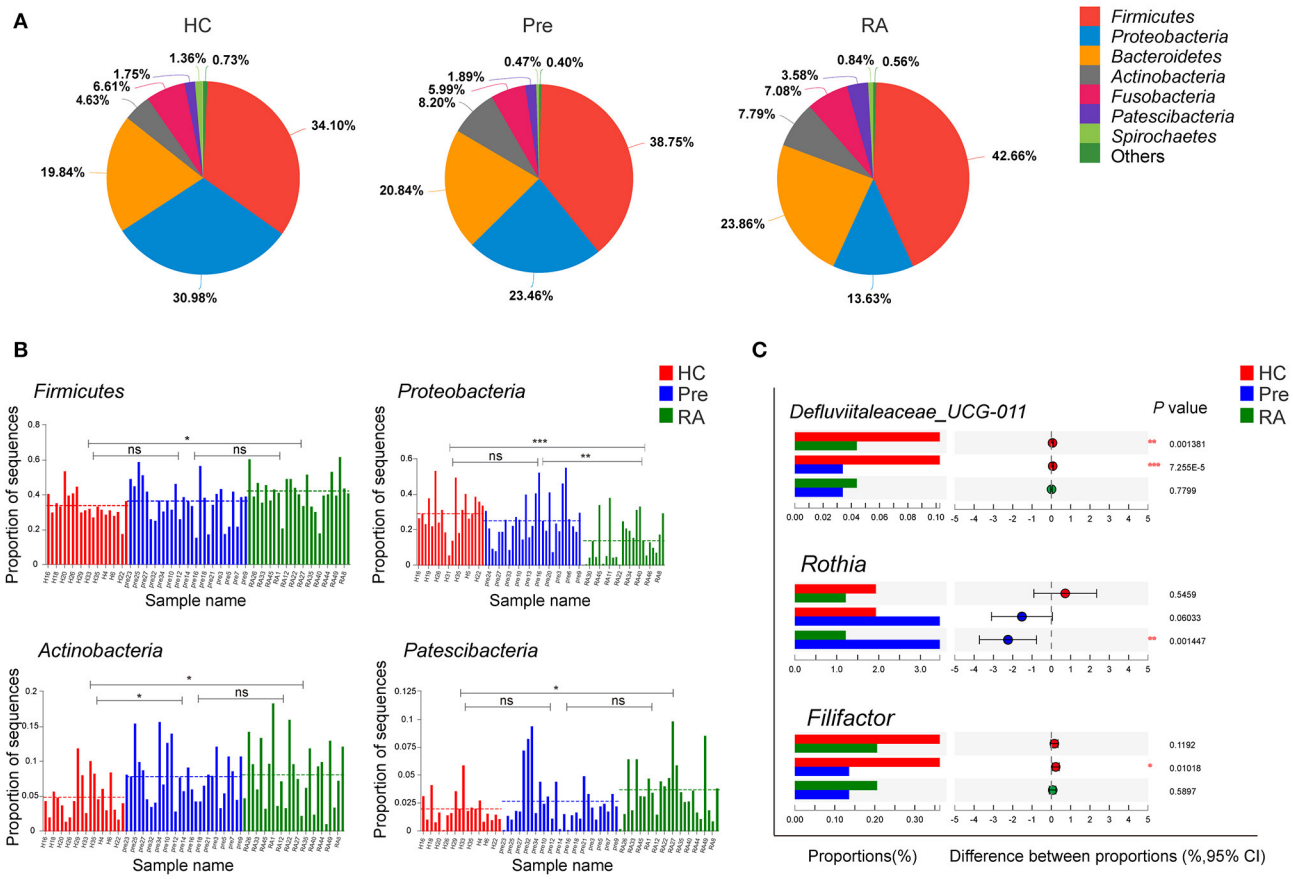


FIGURE 2 | Differentially abundant taxa at phylum and genus level. **(A,B)** The relative abundance of dominant phyla dynamically altered at different stages of RA as represented by healthy controls (HCs), high-risk for RA individuals (Pre), and RA patients (RA). **(C)** Characteristic genera changes were found in high-risk individuals. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$; ns, non-significant.

of three genera, *Actinomyces*, *Prevotella_6,* and *Parvimonas* showed tendencies of gradual change in different stages of disease represented by the three groups, although significant differences were achieved only between RA patients and HCs. The genera enriched in RA patients compared to HCs included *Actinomyces*, *Prevotella_6*, and *Selenomonas_3*, whilst *Neisseria, Haemophilus, Parvimonas,* and *Eubacterium_yurii_group* were diminished in RA patients (**Supplementary Figure 2**). These findings were further verified by the LEfSe analysis, as shown in the cladogram and the contributory discriminate taxa with LDA score >3 was plotted for each group (**Figures 3A,B**).

The titres of antibodies to a periodontopathic species, *P. gingivalis,* are associated with the presence of RA-related autoantibodies in at-risk individuals (Mikuls et al., 2012; Johansson et al., 2016). A further study indicates *P. gingivalis* not only induced periodontitis, but also worsened the concurrent T cell-dependent arthritis in mice (de Aquino et al., 2014). We, therefore, assumed an elevated abundance of *P. gingivalis* in the saliva of high-risk individuals and RA patients. Contrary to our initial hypothesis, there was no significant difference in its relative abundance in RA patients compared to HCs. Moreover, the relative abundance of *P. gingivalis* was significantly decreased in high-risk individuals (Pre vs. HCs, **Figure 3C**). We also found two uncultured species within the *Rothia* genus and *Porphyromonas* genus more abundant in high-risk individuals than in RA patients. Intriguingly, among other differentially abundant species in RA patients (**Supplementary Figure 3**), an unclassified species annotated within the *Prevotella_6* genus was elevated in the saliva of RA patients (RA vs. HCs, $P = 0.009$). The relative abundance of *Neisseria oralis* was decreased in both RA patients and high-risk individuals (**Figure 3C**).

Systemic Autoimmune Signature in High-Risk Individuals and RA Patients Is Associated With Characteristic Saliva Taxa

To further investigate whether the observed changes in saliva microbiota was associated with the autoimmune characteristics in high-risk individuals and with other disease parameters in RA, we analyzed the correlations of the relative abundance of bacterial genera with (1) serum concentrations of ACPA and RF in high-risk and RA individuals, (2) disease activity parameters including CRP, ESR, DAS28, and (3) course of the disease in RA patients.

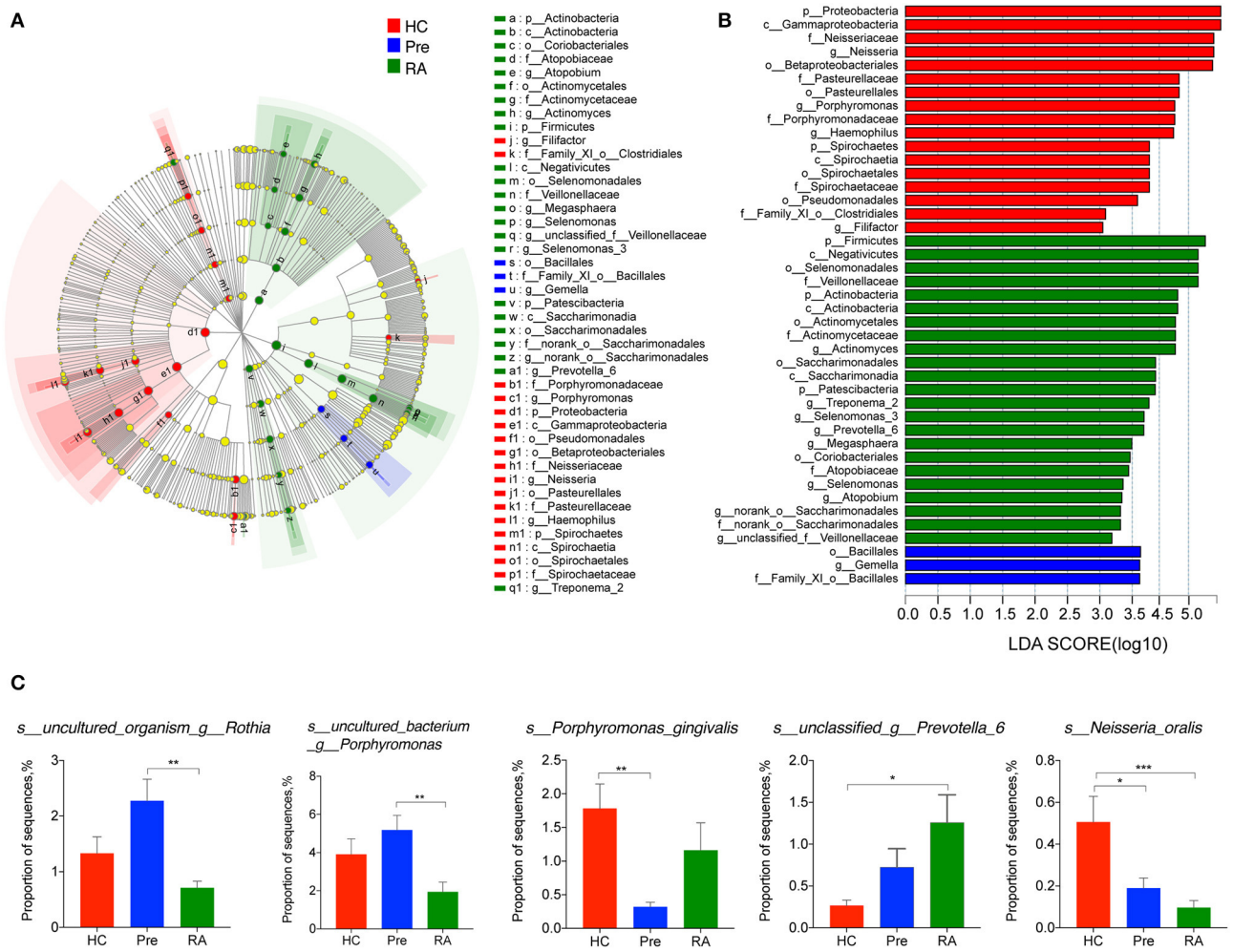


FIGURE 3 | LEfSe analysis revealed the specific taxa changes in high-risk individuals (Pre) and RA patients. LefSe analysis was applied to identify differentially abundant taxa, which are highlighted on the phylogenetic tree in cladogram format **(A)** and for which the LDA scores more than 3 are shown **(B)**. **(C)** Species abundance changes unique to at-risk individuals, and those consistent with changes in RA patients. LDA, linear discriminant analysis; LefSe, the LDA effect size. $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

The study revealed that in high-risk individuals, serum ACPA concentration was positively correlated with the relative abundance of *Eubacterium nodatum_group, Peptostreptococcus, Tannerella, norank_o__Absconditabacteriales_SR1*, while conversely associated with *Haemophilus* and *Neisseria* (**Figure 4A**), both of which were significantly decreased in RA patients (**Supplementary Figure 2**). A negative correlation with *Pseudomonas* was found for RF in high-risk individuals (**Figure 4A**).

In RA patients, however, no genus was correlated with the disease course, which indicates a relatively stable saliva bacterial community in the time spans (**Figure 4B**). Contrary to the study in high-risk individuals, only *norank_o__Absconditabacteriales_SR1* was positively associated with serum ACPA concentration in RA patients. Notably, the concentration of RF was significantly associated with the relative abundance of *Abiotrophia, Corynebacterium, Fretibacterium,* and *Prevotella_1*. Clinical disease activity parameter DAS28 positively correlated with *Ruminococcaceae_UCG-014*, which was also associated with CRP and ESR. In addition, *Lactobacillus* and *norank_f__Saccharimonadaceae*, both increased in RA patients (**Supplementary Figure 2**), positively correlated with ESR (**Figure 4B**). Finally, CRP value at the time of enrollment was also positively associated with *Lactobacillus* and negatively with *Oribacterium*.

Examination of Interactions Among Differentially Abundant Microbes

A correlation network was constructed to investigate the co-abundance and co-exclusion interactions between the differentially abundant microbes. The correlated genera were from eight phyla as indicated in **Figure 5A**. Most of the correlations within the community were positive, with only a few negative correlations. *Gemella* genus was negatively associated with *Megasphaera* and *Prevotella_6*. *Prevotella_6*, on the other side, was also negatively associated with *Porphyromonas*.

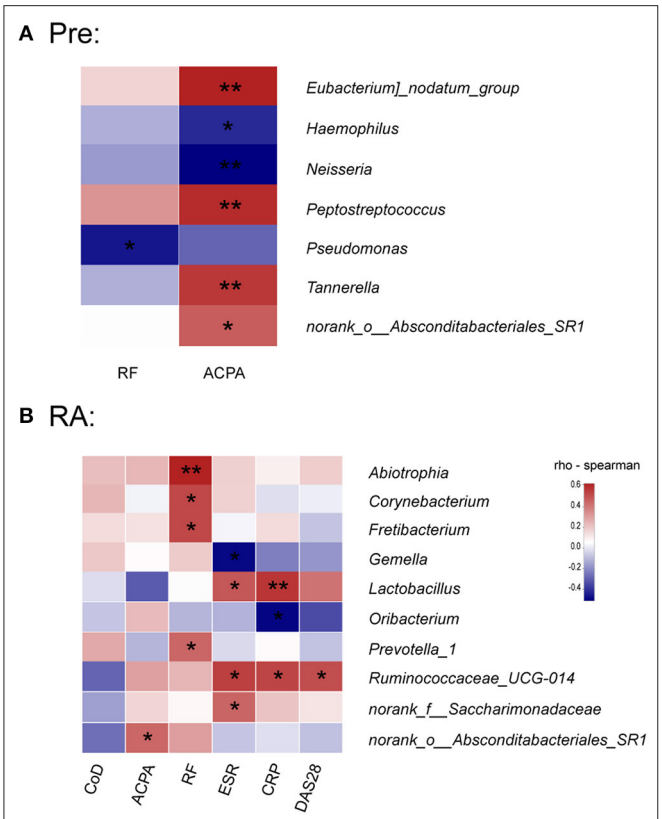


FIGURE 4 | Association between saliva microbiota abundance and systemic autoimmune signature in high-risk individuals and RA patients. **(A,B)** The correlations between the relative abundance of saliva bacterial genera with serum concentrations of ACPA and RF in high-risk individuals and RA patients. **(B)** Correlations between the relative abundance of specific genera and serum ACPA and RF concentrations, disease activity score (DAS28), serum acute phase reactants levels, and course of the disease (CoD) in RA patients. The color scale represents the magnitude of correlation. Red scale indicates positive correlations; blue scale indicates negative correlations. Pre, "pre-clinical" at risk for RA individuals; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibodies; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate. $^{*}p < 0.05$ and $^{**}p < 0.01$.

Additionally, significant negative correlation was found between *Atopobium* and *Neisseria*.

Models of Saliva Bacterial Biomarkers Profile and Predicted Function in RA Patients

We next used the machine learning random forests algorithm to construct a prediction model (Breiman, 2001). A panel of 11 genera was selected based on the model (**Figures 5B,C**). The efficacy of these differentially expressed bacteria in discriminating between RA patients and HCs was calculated using a receiver operator characteristic (ROC) curve. The area under the ROC curve (AUC) was 80.0%, and the 95% confidence interval (CI) was 67–93% (**Figure 5D**).

We then applied PICRUSt (Langille et al., 2013) to infer the functional content of the microbiota. The associations between differentially abundant taxa with predicted functional pathways KEGG (Kyoto Encyclopedia of Genes and Genomes) was illustrated in **Supplementary Figure 4A**. Specifically, an increase in OTUs functional representatives of the bacterial toxins pathway, carbohydrate digestion and absorption pathway, starch, and sucrose metabolism pathway was observed in RA patients compared to HCs. By comparison, OTUs involved in fatty acid biosynthesis, glutathione metabolism, glycan biosynthesis and metabolism, inorganic ion transport and metabolism, and phosphatidylinositol signaling system were decreased in RA patients (**Supplementary Figure 4B**).

DISCUSSION

Our study identified, for the first time, compositional alterations of oral microbiota in individuals at high-risk for RA, who have developed systemic autoimmunity associated with RA. Importantly, several saliva taxa changes were associated with serum autoantibody levels or clinical disease parameters in high-risk individuals and/or RA patients. A bacterial biomarker panel was constructed in discriminating RA patients from healthy individuals. Putative markers involved in bacterial toxins pathway, carbohydrate digestion and absorption were upregulated while pathways involved in fatty acid biosynthesis, glycan biosynthesis and metabolism, and phosphatidylinositol signaling were under-represented in RA patients. These findings support the hypothesis that microbiome changes occurring in mucosal sites such as the oral cavity might contribute to disease pathogenesis in the initial stages of RA.

We found that the microbial diversity in the oral cavity was comparable between HCs and RA patients. This is in line with a prior study that reported similar microbial richness and diversity in subgingival samples from new-onset RA patients (NORA) and HCs (Scher et al., 2012). Similar results were also reported in stool samples from RA patients and HCs (Zhang et al., 2015). Noticeably, we identified that in "pre-clinical" high-risk individuals, microbial diversity and richness was significantly reduced compared to RA patients and HCs. The overall composition of saliva microbial communities was also different amongst the three groups, suggesting oral microbiota perturbations already exist in the "pre-clinical" stage of RA.

Although some genera were significantly altered in RA patients (discussed later), only a few genera characterized the oral microbiome of high-risk individuals, including *Rothia* and *Filifactor*. Of note, some species of the genus *Rothia* have been identified as opportunistic pathogens and can cause bacteremia, endocarditis, joint infections, and pneumonia (Boudewijns et al., 2003; Verrall et al., 2010; Ramanan et al., 2014; de Steenhuijsen Piters et al., 2016). We found that the *Rothia* genus, and an uncultured species annotated within the genera were enriched in high-risk individuals compared to RA patients. Interestingly, the *Filifactor* genus (including *Filifactor alocis* and an unclassified species) was diminished in high-risk individuals. In fact, *F. alocis* has been associated with periodontitis and periodontal biofilm formation. Similarly, even though suspicions exist that the PAD-producing *P. gingivalis* might be involved

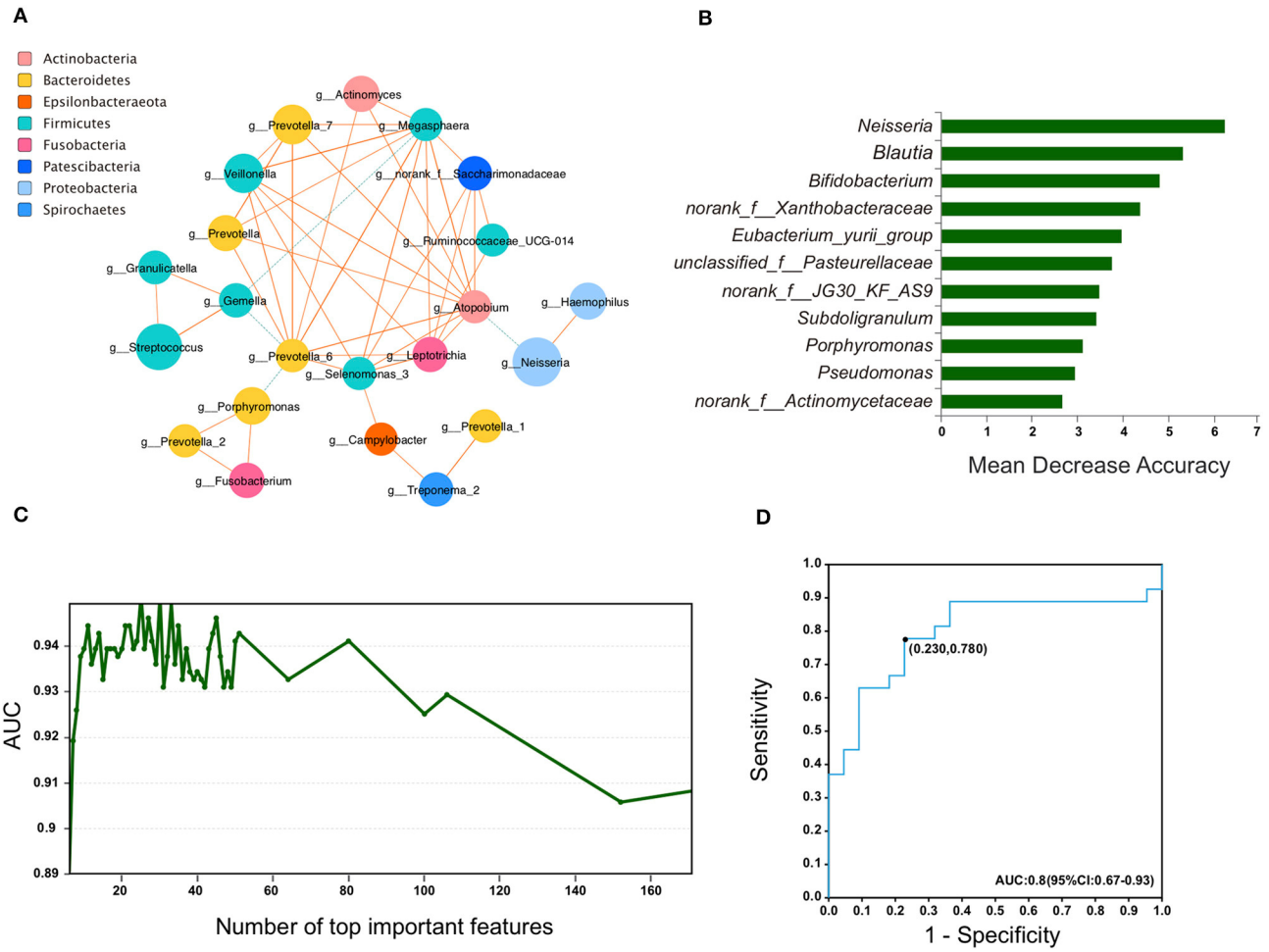


FIGURE 5 | Saliva bacterial biomarkers characterizes RA patients. **(A)** Plots of co-abundance and co-exclusion association networks between differentially abundant genera. Each node represents one genus. The node size is proportional to the mean relative abundance of the genus in all samples. Node color indicates the phylum it belongs to. Lines between nodes show positive correlations (solid orange lines) or negative correlations (dashed green lines). Line width is proportional to the value of Spearman correlation coefficient and reflects the magnitude of association. **(B–D)** A random forest model was applied to identify bacterial biomarkers for RA patients. Ranked lists of genera in order of random forests reporting feature importance scores were obtained **(B)** and an AUC-validation method was used to determine the optimal genera set **(C)**. The built ROC curve based on the selected panel of 11 genera yield an AUC of 0.8 **(D)**.

in RA pathogenesis (Mikuls et al., 2012; de Aquino et al., 2014), our study found comparable level of *P. gingivalis* in RA patients and HCs and no association of this species with autoantibody titer was observed. This finding is in agreement with recent studies that did not find an association between *P. gingivalis* or its PAD and RA (Scher et al., 2012; Konig et al., 2015). Interestingly, we found decreased level of *P. gingivalis* in high-risk individuals, even though periodontitis prevalence was similar to that in HCs. The unique oral microbial features in high-risk individuals reflect the characteristics of the pre-clinical stage.

As expected, only a minority of taxa changes characterized the high-risk stage while most of the significant discrepancies were only found between the diagnosed RA patients and HCs. Importantly, some taxa exhibited a tendency of gradual change during the development stages of the disease, including three genera *Actinomyces, Prevotella_6, Parvimonas* and an unclassified species within *Prevotella_6*. Indeed, the enrichment of *Actinomyces* and *Prevotella* have been reported in RA patients' oral samples (Scher et al., 2012; Zhang et al., 2015). Recently, much attention has been paid to the potential pathogenic role of *Prevotella* spp., especially an intestinal species *Prevotella* copri, in RA. Presence of *P. copri* in fecal samples was strongly correlated with disease in NORA (Scher et al., 2013). A recent study further highlighted the association of the *Prevotella* spp. with RA pathogenesis by revealing its enrichment in fecal sample of "pre-clinical" RA individuals (Alpizar-Rodriguez et al., 2019). Our study identified the increase of *Prevotella_6* in saliva samples with disease progression, which further indicates that *Prevotella*, not limited to the gut but also other mucosal sites, may be involved in RA pathogenesis. Additionally, consistent with a previous study in DMARDs naïve RA patients (Zhang

et al., 2015), both *Haemophilus* spp. and *Neisseria* spp. were depleted in our group of RA patients. Intriguingly, although not significantly reduced compared to HCs, the relative abundance of both genera negatively correlated with the level of serum ACPA in high-risk individuals. Further, in line with the study by Zhang et al. (2015), *Lactobacillus* spp. was elevated in saliva of our RA patients, and its level was positively correlated with acute inflammation makers such as CRP and ESR. Given these tantalizing clues indicating a contribution of microbial dysbiosis at various mucus sites to RA pathogenesis, it is likely that complex microbiome network in multiple mucus sites align together in this process.

However, our study has limitations. The oral cavity is home to one of the most diverse microbial communities of human body, with the flowing saliva containing the "whole-mouth" bacterial community and most commonly studied (Mascitti et al., 2019). However, bacteria colonize all sites within the oral cavity, forming different habitats with non-overlapping microbial populations. Regional variations, such as the bacterial profiles in the supragingival plaque of tooth surfaces and subgingival plaque, etc., were not investigated. Samples from multiple oral sites may help build a panoramic view of the oral microbiome association with the disease (Mascitti et al., 2019). Additionally, relatively small number of participants limits the statistical power of the study, given that samples from the individuals in pre-clinical stages is difficult to acquire. A larger study including more participants would help draw a more concrete conclusion. Another limitation is that most of the involved established RA patients were under treatment, which may affect the salivary microbiome of RA patients. Even though we have a main study focus on the high-risk individuals, who were treatment-naïve, an also treatment-naïve group of RA patients would help improve the study. Finally, the assessment of periodontitis was based on self-reported questionnaires regarding periodontitis symptoms and medical history. Periodontitis status might be underestimated or overestimated in an individual. However, as the questionnaire was applied to all individuals independently, a presumably equal readout would be expected. Further, our study didn't find an association between the abundance of specific periodontitis pathogen with autoantibody titer in either high-risk individuals or RA patients. The seemingly paradoxical finding of lower abundance of periodontitis associated pathogen *P. gingivalis* in high-risk individuals complicates what is known about the relationship between periodontitis and RA pathogenesis. Further studies involving larger number of pre-clinical individuals and graded severity of periodontitis assessment would help better clarify this association.

CONCLUSIONS

In summary, we demonstrate that the pre-clinical stage of RA is characterized by oral dysbiosis, some of which are correlated with autoantibody titer. Common and unique microbial features exist in the high-risk stage compared to established RA. Our findings support the mucosal origin hypothesis in the development of RA. Further mechanistic insights into possible causation through well-designed prospective human studies and evidence derived from *in vivo* experiments from animal models are warranted.

DATA AVAILABILITY STATEMENT

The fastq files were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA578951).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Biomedical Research Ethics Committee, West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YLuo and YLiu designed and supervised the study, reviewed and edited the manuscript. YT, LZ, PQ, HZ, QZ, and YZ collected the samples and sociodemographic, and pathological data. YT, YLi, and LS curated the data. YT, LZ, and YLuo conducted analyses and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This research was funded by National Natural Science Foundation of China (Nos. 81770101 and 81403041) for performing experiment and data analysis and publish fee; Outstanding interdisciplinary project of West China Hospital, Sichuan University (No. ZYJC18024) and Post-Doctoral Research Project, West China Hospital, Sichuan University (No. 2019HXBH037) for the cost used for enrolling individuals in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00475/full#supplementary-material

REFERENCES

Alpizar-Rodriguez, D., Lesker, T. R., Gronow, A., Gilbert, B., Raemy, E., Lamacchia, C., et al. (2019). *Prevotella* copri in individuals at risk for rheumatoid arthritis. *Ann. Rheum. Dis.* 78, 590–593. doi: 10.1136/annrheumdis-2018-214514

Berglin, E., Padyukov, L., Sundin, U., Hallmans, G., Stenlund, H., Van Venrooij, W. J., et al. (2004). A combination of autoantibodies to cyclic citrullinated peptide (CCP) and HLA-DRB1 locus antigens is strongly associated with future onset of rheumatoid arthritis. *Arthritis Res. Ther.* 6, R303–R308. doi: 10.1186/ar1187

Boudewijns, M., Magerman, K., Verhaegen, J., Debrock, G., Peetermans, W. E., Donkersloot, P., et al. (2003). Rothia dentocariosa, endocarditis and mycotic

aneurysms: case report and review of the literature. *Clin. Microbiol. Infect.* 9, 222–229. doi: 10.1046/j.1469-0691.2003.00503.x

Breiman, L. (2001). Random forests. *Mach. Learn.* 45, 5–32. doi: 10.1023/A:1010933404324

de Aquino, S. G., Abdollahi-Roodsaz, S., Koenders, M. I., van de Loo, F. A., Pruijn, G. J., Marijnissen, R. J., et al. (2014). Periodontal pathogens directly promote autoimmune experimental arthritis by inducing a TLR2- and IL-1-driven Th17 response. *J. Immunol.* 192, 4103–4111. doi: 10.4049/jimmunol.1301970

de Steenhuijsen Piters, W. A., Huijskens, E. G., Wyllie, A. L., Biesbroek, G., van den Bergh, M. R., Veenhoven, R. H., et al. (2016). Dysbiosis of upper respiratory tract microbiota in elderly pneumonia patients. *ISME J.* 10, 97–108. doi: 10.1038/ismej.2015.99

Hirschfeld, J., Roberts, H. M., Chapple, I. L., Parcina, M., Jepsen, S., Johansson, A., et al. (2016). Effects of *Aggregatibacter actinomycetemcomitans* leukotoxin on neutrophil migration and extracellular trap formation. *J. Oral Microbiol.* 8:33070. doi: 10.3402/jom.v8.33070

Johansson, L., Sherina, N., Kharlamova, N., Potempa, B., Larsson, B., Israelsson, L., et al. (2016). Concentration of antibodies against *Porphyromonas gingivalis* is increased before the onset of symptoms of rheumatoid arthritis. *Arthritis Res. Ther.* 18:201. doi: 10.1186/s13075-016-1100-4

Jorgensen, K. T., Wiik, A., Pedersen, M., Hedegaard, C. J., Vestergaard, B. F., Gislefoss, R. E., et al. (2008). Cytokines, autoantibodies and viral antibodies in premorbid and postdiagnostic sera from patients with rheumatoid arthritis: case-control study nested in a cohort of Norwegian blood donors. *Ann. Rheum. Dis.* 67, 860–866. doi: 10.1136/ard.2007.073825

Konig, M. F., Abusleme, L., Reinholdt, J., Palmer, R. J., Teles, R. P., Sampson, K., et al. (2016). Aggregatibacter actinomycetemcomitans-induced hypercitrullination links periodontal infection to autoimmunity in rheumatoid arthritis. *Sci. Transl. Med.* 8:369ra176. doi: 10.1126/scitranslmed.aaj1921

Konig, M. F., Paracha, A. S., Moni, M., Bingham, C. O. 3rd, and Andrade, F. (2015). Defining the role of *Porphyromonas gingivalis* peptidylarginine deiminase (PPAD) in rheumatoid arthritis through the study of PPAD biology. *Ann. Rheum. Dis.* 74, 2054–2061. doi: 10.1136/annrheumdis-2014-205385

Langille, M. G., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Lappin, D. F., Apatzidou, D., Quirke, A. M., Oliver-Bell, J., Butcher, J. P., Kinane, D. F., et al. (2013). Influence of periodontal disease, *Porphyromonas gingivalis* and cigarette smoking on systemic anti-citrullinated peptide antibody titres. *J. Clin. Periodontol.* 40, 907–915. doi: 10.1111/jcpe.12138

Mascitti, M., Togni, L., Troiano, G., Caponio, V. C. A., Gissi, D. B., Montebugnoli, L., et al. (2019). Beyond head and neck cancer: the relationship between oral microbiota and tumour development in distant organs. *Front. Cell. Infect. Microbiol.* 9:232. doi: 10.3389/fcimb.2019.00232

Mikuls, T. R., Thiele, G. M., Deane, K. D., Payne, J. B., O'Dell, J. R., Yu, F., et al. (2012). *Porphyromonas gingivalis* and disease-related autoantibodies in individuals at increased risk of rheumatoid arthritis. *Arthritis Rheum.* 64, 3522–3530. doi: 10.1002/art.34595

Nielen, M. M., van Schaardenburg, D., Reesink, H. W., van de Stadt, R. J., van der Horst-Bruinsma, I. E., de Koning, M. H., et al. (2004). Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum.* 50, 380–386. doi: 10.1002/art.20018

Ramanan, P., Barreto, J. N., Osmon, D. R., and Tosh, P. K. (2014). Rothia bacteremia: a 10-year experience at Mayo Clinic, Rochester, Minnesota. *J. Clin. Microbiol.* 52, 3184–3189. doi: 10.1128/JCM.01270-14

Rantapaa-Dahlqvist, S., de Jong, B. A., Berglin, E., Hallmans, G., Wadell, G., Stenlund, H., et al. (2003). Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum.* 48, 2741–2749. doi: 10.1002/art.11223

Rombouts, Y., Ewing, E., van de Stadt, L. A., Selman, M. H., Trouw, L. A., Deelder, A. M., et al. (2015). Anti-citrullinated protein antibodies acquire a pro-inflammatory Fc glycosylation phenotype prior to the onset of rheumatoid arthritis. *Ann. Rheum. Dis.* 74, 234–241. doi: 10.1136/annrheumdis-2013-203565

Ronnelid, J., Wick, M. C., Lampa, J., Lindblad, S., Nordmark, B., Klareskog, L., et al. (2005). Longitudinal analysis of citrullinated protein/peptide antibodies (anti-CP) during 5 year follow up in early rheumatoid arthritis: anti-CP status predicts worse disease activity and greater radiological progression. *Ann. Rheum. Dis.* 64, 1744–1749. doi: 10.1136/ard.2004.033571

Schellekens, G. A., Visser, H., de Jong, B. A., van den Hoogen, F. H., Hazes, J. M., Breedveld, F. C., et al. (2000). The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum.* 43, 155–163. doi: 10.1002/1529-0131(200001)43:1<155::AID-ANR20>3.0.CO;2-3

Scher, J. U., Joshua, V., Artacho, A., Abdollahi-Roodsaz, S., Ockinger, J., Kullberg, S., et al. (2016). The lung microbiota in early rheumatoid arthritis and autoimmunity. *Microbiome* 4:60. doi: 10.1186/s40168-016-0206-x

Scher, J. U., Sczesnak, A., Longman, R. S., Segata, N., Ubeda, C., Bielski, C., et al. (2013). Expansion of intestinal *Prevotella* copri correlates with enhanced susceptibility to arthritis. *Elife* 2:e01202. doi: 10.7554/eLife.01202

Scher, J. U., Ubeda, C., Equinda, M., Khanin, R., Buischi, Y., Viale, A., et al. (2012). Periodontal disease and the oral microbiota in new-onset rheumatoid arthritis. *Arthritis Rheum.* 64, 3083–3094. doi: 10.1002/art.34539

Sokolove, J., Bromberg, R., Deane, K. D., Lahey, L. J., Derber, L. A., Chandra, P. E., et al. (2012). Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. *PLoS ONE* 7:e35296. doi: 10.1371/annotation/2e462817-ab93-4d78-95a4-1d8b9d172971

Syversen, S. W., Gaarder, P. I., Goll, G. L., Odegard, S., Haavardsholm, E. A., Mowinckel, P., et al. (2008). High anti-cyclic citrullinated peptide levels and an algorithm of four variables predict radiographic progression in patients with rheumatoid arthritis: results from a 10-year longitudinal study. *Ann. Rheum. Dis.* 67, 212–217. doi: 10.1136/ard.2006.068247

van de Stadt, L. A., van der Horst, A. R., de Koning, M. H., Bos, W. H., Wolbink, G. J., van de Stadt, R. J., et al. (2011). The extent of the anti-citrullinated protein antibody repertoire is associated with arthritis development in patients with seropositive arthralgia. *Ann. Rheum. Dis.* 70, 128–133. doi: 10.1136/ard.2010.132662

Verrall, A. J., Robinson, P. C., Tan, C. E., Mackie, W. G., and Blackmore, T. K. (2010). Rothia aeria as a cause of sepsis in a native joint. *J. Clin. Microbiol.* 48, 2648–2650. doi: 10.1128/JCM.02217-09

Zhang, X., Zhang, D., Jia, H., Feng, Q., Wang, D., Liang, D., et al. (2015). The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment. *Nat. Med.* 21, 895–905. doi: 10.1038/nm.3914

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 04 February 2020
doi: 10.3389/fcimb.2020.00017

Interaction of Oral and Toothbrush Microbiota Affects Oral Cavity Health

Qingyao Shang[1,2], *Yuan Gao*[1], *Ting Qin*[1], *Shuai Wang*[1], *Yan Shi*[1,2,3]* and *Tingtao Chen*[1,3]*

[1] The Key Laboratory of Oral Biomedicine, Department of Conservative Dentistry and Endodontics, The Affiliated Stomatological Hospital of Nanchang University, Nanchang, China, [2] School of Stomatology, Nanchang University, Nanchang, China, [3] National Engineering Research Centre for Bioengineering Drugs and the Technologies, Institute of Translational Medicine, Nanchang University, Nanchang, China

Tooth brushing is necessary to maintain oral health. Little research has been carried out to explore microbial diversity in toothbrushes and to study the potential impact of these bacteria on human health. In the present study, 20 participants were enrolled, and the microbial diversity in their oral cavity and toothbrushes was investigated using high-throughput sequencing. Our results indicate that 1,136 and 976 operational taxonomic units (OTUs) were obtained from groups CB (samples from toothbrushes of participants using traditional Chinese medicinal toothpaste) and AB (samples from toothbrushes of those using antibacterial toothpaste), respectively. The pathogens *Acinetobacter baumannii*, *Staphylococcus aureus*, and *Candida albicans* were identified on toothbrushes. The presence of these pathogens increases the chance for the host to get infectious diseases, neurodegenerative diseases, cardiovascular diseases, and cancers. Moreover, our *in vitro* results indicate that traditional Chinese medicinal toothpaste and antibacterial toothpaste can not only inhibit the growth of pathogens but also markedly inhibit the growth of probiotics *Lactobacillus salivarius* and *Streptococcus salivarius*. Therefore, the inhibitory effect of toothpaste on probiotics, together with the existence of pathogens in toothbrushes, indicates a potential risk of tooth brushing for people in a sub-healthy state.

Keywords: brushing teeth, toothbrush, high-throughput sequencing, pathogens, oral microbiota

OPEN ACCESS

Edited by:
Lorenzo Lo Muzio, University of Foggia, Italy

Reviewed by:
Janina P. Lewis, Virginia Commonwealth University, United States
Vito Carlo Alberto Caponio, University of Foggia, Italy

***Correspondence:**
Tingtao Chen
chentingtao1984@163.com
Yan Shi
2641041552@qq.com

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *16 August 2019*
Accepted: *14 January 2020*
Published: *04 February 2020*

Citation:
Shang Q, Gao Y, Qin T, Wang S, Shi Y and Chen T (2020) Interaction of Oral and Toothbrush Microbiota Affects Oral Cavity Health. Front. Cell. Infect. Microbiol. 10:17. doi: 10.3389/fcimb.2020.00017

INTRODUCTION

Host to the second largest human microbial library, the oral cavity is composed of teeth, gingival sulcus, tongue, tonsils, and soft and hard palates, and provides a suitable growth environment for a large number of microorganisms (up to 700 species) (Xie et al., 2010). The oral microbiota is mainly composed of Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, and Proteobacteria, which are indispensable for human health due to their ability to inhibit the proliferation of exogenous microorganisms and promote host homeostasis and defense (Bo et al., 2012).

Factors such as smoking, antibiotic abuse, mouthwash, and toothpaste can cause an imbalance in the oral microflora and eventually result in dental caries, gingivitis, periodontitis, candidiasis, endodontic infections, orthodontic infections, and oral cancer (Lin et al., 2008; Chen and Jiang, 2014). Dental caries and periodontal disease are common oral diseases which are caused by the dominance of acidogenic and acid-tolerating species (e.g., *Streptococcus mutans*) or the inflammation produced by these cariogenic bacteria (Aas et al., 2005; Lin et al., 2008). Worse, these oral diseases can lead to other systemic diseases. Pritchard et al. (2017) found that periodontal

disease is an important causes of Alzheimer's disease (AD) due to epithelial cell barrier disruption and chronic infections. Whitmore and Lamont (2014) found that oral squamous cell carcinoma (OSCC) is closely related to an imbalance of oral bacteria.

Since the nineteenth century, toothbrushes have been widely used to suppress the growth of harmful bacteria, remove food residue, keep the breath fresh, protect enamel, and prevent and alleviate gingival inflammation (Fischman, 1997). However, the residual moisture and food debris residing in toothbrushes provide a suitable environment for pathogen growth, rendering them a potential risk for various oral diseases (Hays, 2006; Bik et al., 2010).

In the present study, a high-throughput sequencing method was applied to compare microbial diversity in the oral cavity and toothbrushes (used with traditional Chinese medicinal toothpaste and antibacterial toothpaste), to explore the potential risk of pathogens in toothbrushes and provide useful suggestions on oral health.

MATERIALS AND METHODS

Ethical Statement

The study was approved by the Ethical Committee of the Stomatological Hospital of Nanchang University, and all participants provided written informed consent. All experiments were conducted in accordance with the approved guidelines.

Selection of Participants

Participants aged between 18 and 30 were enrolled from the Medical College of Nanchang University. Their physical conditions, including age, weight, medical history, recent antibiotic use, smoking history, alcohol drinking, and oral health situation, were investigated by questionnaire survey. Twenty participants (10 males, 10 females), who were in good health, had not used antibiotics for a year, had no smoking history, did not drink alcohol, and practiced standard oral hygiene without the use of mouthwash, aged 20–27 (average 22 years old), were ultimately enrolled (**Table 1**).

Sample Collection

The most representative types of toothpaste in China—traditional Chinese medicinal toothpaste [main active components: *Chondrus crispus*, *Coptis chinensis*, *Sarcandra glabra* (does not contain fluoride); no. 20160005, Cao Shan Hu Oral Care Products Co., Ltd, Jiang Xi] and antibacterial toothpaste (Crest Seven Good Effects toothpaste; main active components: sodium fluoride, stannous chloride; no. 20160024, Procter & Gamble Co., Ltd. in Guangzhou)—were used (Pinducciu et al., 1996; Mojon, 2002). The 20 participants were randomly divided into two groups—group C [brushed teeth using traditional Chinese medicinal toothpaste twice a day for 15 days (after breakfast and before sleep)] and group A [brushed teeth using antibacterial toothpaste twice a day for 15 days (after breakfast and before sleep)]. Bacteria were collected from the oral cavity and toothbrushes. Oral bacteria were collected by gargling using 10 ml of sterile saline solution, and toothbrush microorganisms were collected by vibrating the toothbrush in 10 ml of sterile saline solution in a 50 ml sterile Eppendorf (EP) tube using a vortex. Samples were divided into groups CO (oral samples from participants in group C, $n = 10$), CB (toothbrush samples from participants in group C, $n = 10$), AO (oral samples from participants in group A, $n = 10$) and AB (toothbrush samples from participants in group A, $n = 10$).

TABLE 1 | Information of participants enrolled in the present study including gender, age, and body weight.

	Total	C	A	*p*-value
Participants (n)	20	10	10	—
Gender [n (%)]	10 (50)	5 (50)	5 (50)	1
Age (years)	22.65 (21.69–23.61)	22.00 (21.00–24.25)	21.50 (21.00–24.50)	0.74
Body weight (kg)	64.85 ± 10.36	65.57 ± 11.83	64.13 ± 9.26	0.77

In total, 20 participants, who were in good health, had not used antibiotics for a year, had no smoking history, and did not drink alcohol, aged 20–27 (average 22 years old), were ultimately enrolled. Participants in group C brushed their teeth using traditional Chinese medicinal toothpaste. Participants in group A brushed their teeth using antibacterial toothpaste. The p-value was calculated by an independent-samples T-test using SPSS 17.0 software. $P < 0.05$ showed homogeneity of variance.

DNA Extraction and High-Throughput Sequencing

Samples in each group were collected for bacterial DNA extraction by a bead beating method (Okpalugo et al., 2009) using genomic DNA kits (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. Then, the concentration and quality of extracted DNA were detected via a spectrophotometer at 230 nm (A 230) and 260 nm (A 260) (NanoDrop; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then, the V4 region of the 16S rDNA genes of each sample was amplified using 515F/806R primers (515F, 5′-GTGCCAGCMGCCGCGGTAA-3′; 806R, 5′-GGACTACVSGGGTATCTAAT-3′), and PCR products were sequenced with an Illumina MiSeq platform (GenBank accession number PRJNA553856; Fang et al., 2016).

Bioinformatics and Multivariate Statistics

Fast Length Adjustment of Short reads (FLASH) software (v1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoc and Salzberg, 2011) was used to pair sequences according to the overlapping bases. The UCLUST sequence alignment tool (Edgar, 2010) of Quantitative Insights into Microbial Ecology (QIIME) software was used to merge the previously obtained sequences and for partitioning of operational taxonomic units (OTUs) with 97% sequence similarity. R software was used to calculate the number of OTUs shared by each group and the proportion of unique OTUs. QIIME software was used to obtain a composition and abundance distribution table for each sample at the five classification levels. Using R software, a partial least squares discrimination analysis (PLS-DA) discriminant model was constructed based on the species abundance and sample

TABLE 2 | Sequencing results of isolates from oral rinse solution.

Strain no.	Closest relative	Aerobic condition	Source	Similarity (%)	GeneBank no.
1	*E. hormaechei*	Facultative anaerobic	Oral	99	HQ238708.1
2	*S. salivarius*	Facultative anaerobic	Oral	99	MH447009.1
3	*S. aureus*	Aerobic	Toothbrush	99	LR134351.1
4	*E. cloacae*	Facultative anaerobic	Oral	100	KT441077.1
5	*E. faecalis*	Facultative anaerobic	Oral	99	KE351689.1
6	*L. salivarius*	Facultative anaerobic	Oral	99	FJ384627.1
7	*C. albicans*	Aerobic	Toothbrush	99	BD267550.1

Seven strains were isolated and identified from oral rinse solution: E. hormaechei, S. salivarius, S. aureus, E. cloacae, E. faecalis, L. salivarius, and C. albicans. The sequences were blasted with the NCBI database.

grouping data. The variable importance in projection (VIP) coefficient was calculated for each species.

Bacterial Isolation From the Oral Cavity and Toothbrushes

Bacteria from the oral rinse solution and toothbrushes were cultivated using brain heart infusion (BHI) broth on solid plates (37°C, 12 h), and bacteria were isolated by comparing bacterial colony size, color, and bacterial morphology. In the end, all isolates were further identified via molecular biological identification (Wang et al., 2015).

Antibacterial Effect of Toothpaste

Toothbrushes were mixed with sterile water at a ratio of 1:3 (vol:vol) in a 50 ml sterile EP tube, and the supernatant was transferred to a new 50 ml sterile EP tube for further use.

Then, the tested strains—*Enterobacter hormaechei*, *Streptococcus salivarius*, *Staphylococcus aureus*, *E. cloacae*, *Enterococcus faecalis*, *L. salivarius*, and *Candida albicans*—were co-incubated with traditional Chinese medicinal toothpaste solution or antibacterial toothpaste solution for 4 h at 37°C, and a viable cell counting method was used to count the number of bacteria (**Table 2**; Deng et al., 2015).

Statistical Analysis

Statistical analysis was carried out using R software. PLS-DA analysis was carried out according to the species abundance matrix and sample grouped data, and a discriminant model was constructed. Differences among groups were analyzed based on the Kruskal–Wallis test using Statistical Product and Service Solutions 20.0 (SPSS 20.0) software. The statistical significance was set at $P < 0.05$ for significant difference.

RESULTS

Alpha Diversity of Microbiota in the Oral Cavity and Toothbrushes

To compare the microbes in the oral cavity and toothbrushes, 16S rDNA amplicon sequencing analysis was used to sequence the V4 hypervariable region, and the sequencing data were filtered to obtain valid data. The effective tags of all samples were clustered, and sequences with more than 97% similarity were considered as one OTU. In total, 1,680,600 usable raw sequences and 4,283 OTUs were obtained from all the samples, and the average number of OTUs in each group was 1,070 (data not shown).

As shown in **Figure 1A**, the Shannon index showed that the use of traditional Chinese medicinal toothpaste increased the microbial abundance in toothbrushes (CB) compared with the oral cavity (CO); microbial diversity in the oral cavity (AO) and toothbrushes (AB) using antibacterial toothpaste was almost the same. **Figure 1B** shows that use of traditional Chinese medicinal toothpaste also increased the number of bacterial species in toothbrushes (CB), while an obvious reduction was observed for toothbrushes (AB) compared with the oral cavity when antibacterial toothpaste was used (400 vs. 344).

The Venn diagram in **Figure 1C** indicates that 1,136, 976, 1,130, and 1,041 OTUs were obtained from groups CB, AB, CO, and AO, and the common OTUs (666) represented 58.63% (666/1,136) of CB, 68.24% (666/976) of AB, 58.94% (666/1,130) of CO, and 63.98% (666/1,041) of AO, respectively. In addition, the opportunistic pathogens *Burkholderia cepacia* and *Pseudomonas pseudoalcaligenes* were identified from groups CB and AB, and a large number of the opportunistic pathogens *Stenotrophomonas maltophilia* and *Acinetobacter baumannii* (reported as clinically drug-resistant) were identified from group AB (Moore et al., 1999; Gales et al., 2001).

In **Figure 1D**, we can see that the dots in groups CO and CB are far away from each other, while those in AO and AB are gathered together, indicating that the strong antimicrobial activity of antibacterial toothpaste had an impactful convergent effect on microbial diversity.

Beta Diversity of Microbiota in Oral Cavity and Toothbrush

Based on the weighted UniFrac distance at phylum level, data for the top 12 microorganism populations were analyzed to check the similarity among different groups (24). As presented in **Figure 2A**, Firmicutes, Proteobacteria, and Bacteroidetes were the three dominant phyla and accounted for >85% of the total sequencing number in all groups; the percentage of Firmicutes, Proteobacteria, and Bacteroidetes was 42.84% vs. 41.46% vs. 43.14% vs. 33.70%, 27.34% vs. 40.33% vs. 31.61% vs. 48.17%, and 18.34% vs. 4.84% vs. 14.80% vs.

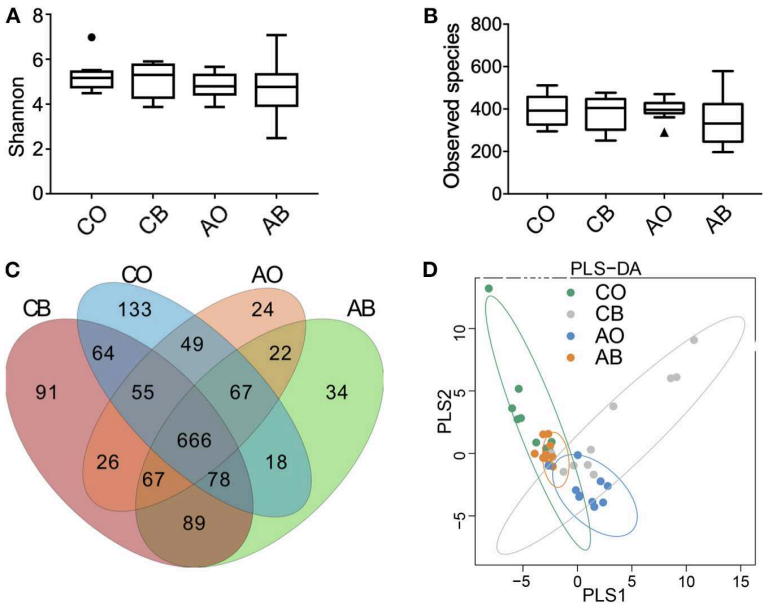


FIGURE 1 | Indices of oral bacterial diversity. **(A)** Calculations of Shannon indices among groups CO, CB, AO, and AB; **(B)** calculations of species observed among groups CO, CB, AO, and AB; **(C)** scalar Venn diagrams among groups CO, CB, AO, and AB; **(D)** PLS-DA analysis of groups CO, CB, AO, and AB. CO, oral microbiota of people using traditional Chinese medicinal toothpaste ($n = 10$); CB, microbiota of toothbrushes corresponding to group CO ($n = 10$); AO, oral microbiota of people using antimicrobial toothpaste ($n = 10$); AB, microbiota of toothbrushes corresponding to group AO ($n = 10$).

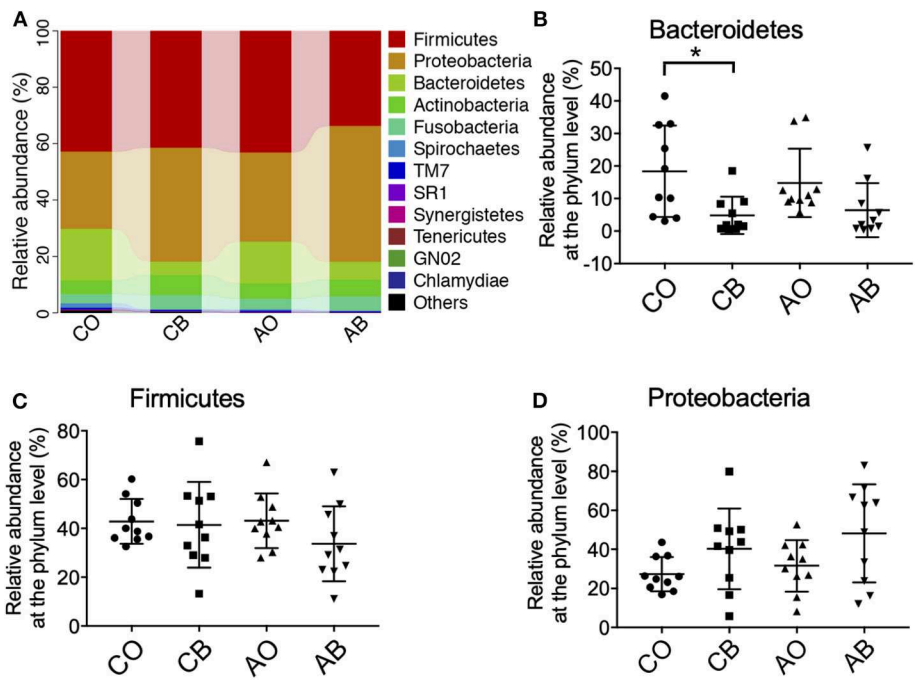


FIGURE 2 | Ratios of the known bacteria in groups CO, CB, AO, and AB at the phylum level **(A)**. Relative abundance of Bacteroidetes **(B)**, Firmicutes **(C)**, and Proteobacteria **(D)** in groups CO, CB, AO, and AB. CO, oral microbiota of people using traditional Chinese medicinal toothpaste ($n = 10$); CB, microbiota of toothbrushes corresponding to group CO ($n = 10$); AO, oral microbiota of people using antimicrobial toothpaste ($n = 10$); AB, microbiota of toothbrushes corresponding to group AO ($n = 10$). Data are presented as means ± SD; $^*P < 0.05$ by Kruskal–Wallis.

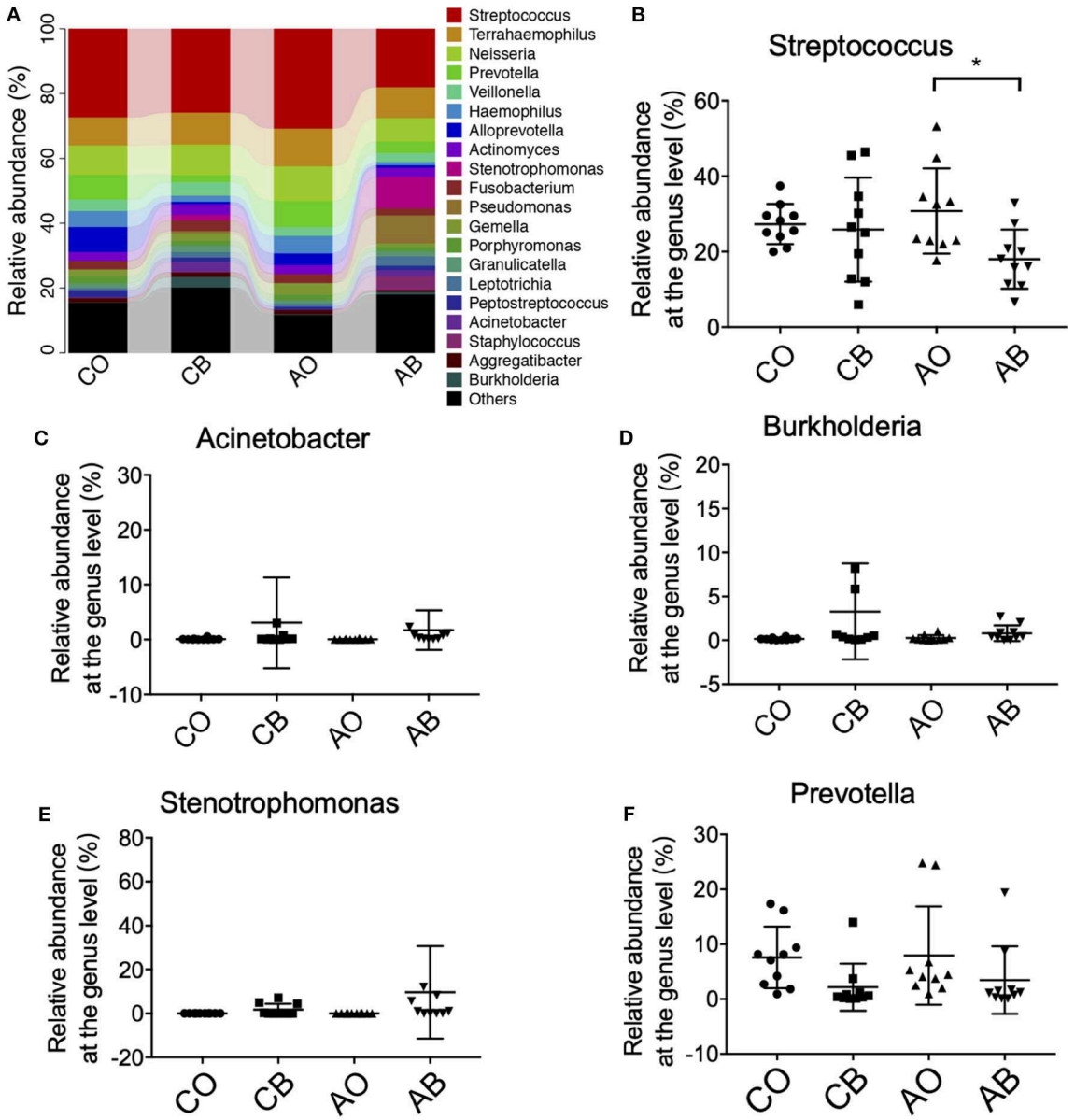


FIGURE 3 | Ratios of the known bacteria in groups CO, CB, AO, and AB at the genus level. **(A)** Relative abundance of *Streptococcus* **(B)**, *Acinetobacter* **(C)**, *Burkholderia* **(D)**, *Stenotrophomonas* **(E)**, and *Prevotella* **(F)** in groups CO, CB, AO, and AB. CO, oral microbiota of people using traditional Chinese medicinal toothpaste ($n = 10$); CB, microbiota of toothbrushes corresponding to group CO ($n = 10$); AO, oral microbiota of people using antimicrobial toothpaste ($n = 10$); AB, microbiota of toothbrushes corresponding to group AO ($n = 10$). Data are presented as means ± SD; $^*P < 0.05$ by Kruskal–Wallis.

6.40% in groups CO, CB, AO, and AB, respectively. Use of traditional Chinese medicinal toothpaste reduced the abundance of Bacteroidetes (CB vs. CO = 4.84 vs. 18.34%) and increased the abundance of Proteobacteria (CB vs. CO = 40.33 vs. 27.34%; **Figures 2B,D**). Antibacterial toothpaste obviously reduced the abundance of Bacteroidetes (AB vs. AO = 6.40 vs. 14.80%) and Firmicutes (AB vs. AO = 33.70 vs. 43.13%), while it increased the abundance of Proteobacteria (AB vs. AO = 48.16 vs. 31.61%) compared with group AO (**Figures 2B–D**).

At genus level, *Streptococcus*, *Terrahaemophilus*, *Neisseria*, and *Prevotella* were dominant in all groups (**Figure 3A**), and traditional Chinese medicinal toothpaste and antibacterial toothpaste hardly affected the abundance of *Neisseria*, *Actinomyces*, *Stenotrophomonas*, *Acinetobacter*, or *Burkholderia* in all groups (**Figures 3B–F**). However, it seems that use of antibacterial toothpaste in group AO could increase the number of *Streptococcus* in the oral cavity compared with traditional Chinese medicinal toothpaste (**Figure 3**).

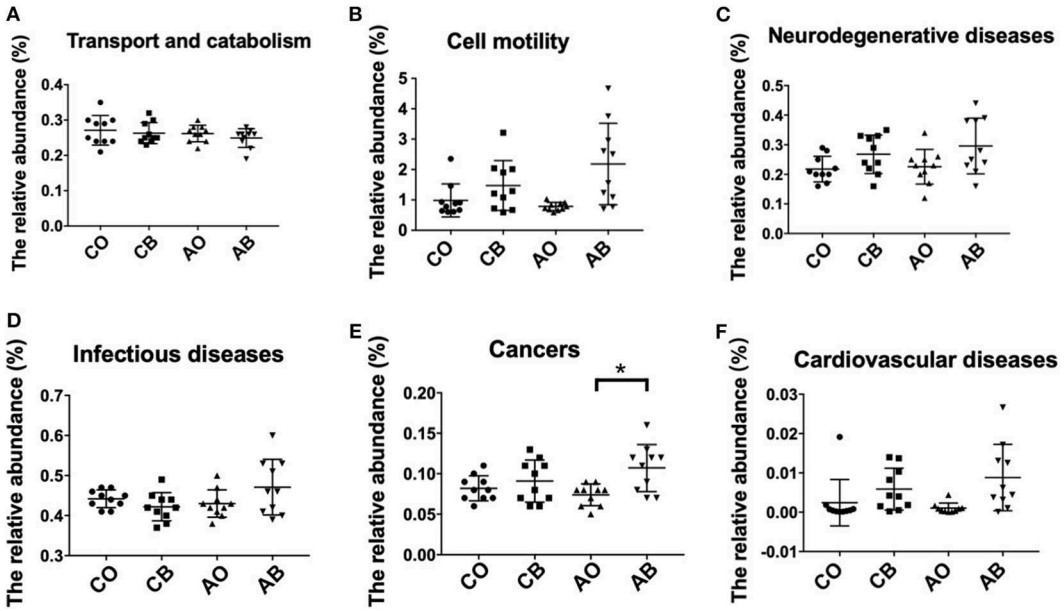


FIGURE 4 | Relative abundance of groups CO, CB, AO, and AB using PICRUSt based on the KEGG database. Classification based on cellular process—transport and catabolism **(A)**, cell motility **(B)**. Classification based on human disease—neurodegenerative diseases **(C)**, infectious diseases **(D)**, cancers **(E)**, cardiovascular diseases **(F)**. CO, oral microbiota of people using traditional Chinese medicinal toothpaste ($n = 10$); CB, microbiota of toothbrushes corresponding to group CO ($n = 10$); AO, oral microbiota of people using antimicrobial toothpaste ($n = 10$); AB, microbiota of toothbrushes corresponding to group AO ($n = 10$). Data are presented as means ± SD; $^{*}P < 0.05$ by Kruskal–Wallis.

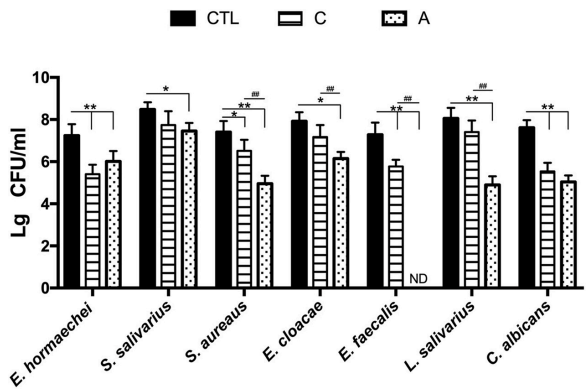


FIGURE 5 | Inhibitory action of traditional Chinese medicinal toothpaste and antimicrobial toothpaste on isolated strains. CTL: PBS buffer; C, 20% concentration of traditional Chinese medicinal toothpaste; A, 20% concentration of antimicrobial toothpaste. CO, oral microbiota of people using traditional Chinese medicinal toothpaste ($n = 10$); CB, microbiota of toothbrushes corresponding to group CO ($n = 10$); AO, oral microbiota of people using antimicrobial toothpaste ($n = 10$); AB, microbiota of toothbrushes corresponding to group AO ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$. Data are presented as means ± SD; $^{*}P < 0.05$ vs. control group (CTL group) by ANOVA, $^{\#}P < 0.05$ vs. C group by Kruskal–Wallis.

Potential Risk of Microbial Changes in the Oral Cavity and Toothbrushes for Human Diseases

We evaluated the potential risk of microbial diversity in the oral cavity and toothbrushes for human diseases based on the KEGG database. As shown in **Figure 4**, there were no significant differences in transport and catabolism capability among groups CO, CB, AO, and AB (**Figure 4A**), while bacteria in toothbrushes (from both traditional Chinese medicinal toothpaste and antibacterial toothpaste groups) greatly enhanced cell motility in the host (**Figure 4B**) and the chance of the host suffering from neurodegenerative diseases, infectious diseases, cancers, and cardiovascular diseases (**Figures 4C–F**). It seems that the high abundance of pathogenic bacteria in toothbrushes obviously increased the potential risk for human health.

Bacterial Isolation and Antibacterial Effect of Toothpastes on Isolates

To further study the antibacterial effect of toothpastes on oral bacteria, the plate method and molecular biological identification were used to isolate bacteria from the oral cavity and toothbrushes. As shown in **Table 1**, *E. hormaechei*, *S. salivarius*, *S. aureus*, *E. cloacae*, *E. faecalis*, *L. salivarius*, and *C. albicans* were screened, of which *E. hormaechei*, *S. aureus*, *E. cloacae*, *E. faecalis*, and *C. albicans* are pathogenic bacteria, and *L. salivarius* and *S. salivarius* are beneficial bacteria.

When testing the antibacterial effect of toothpastes, we found that antibacterial toothpaste significantly inhibited the growth of *E. hormaechei* (A vs. CTL = Lg 6.01 vs. Lg 7.23, $p < 0.01$), *S. salivarius* (A vs. CTL = Lg 7.45 vs. Lg 8.48, $p < 0.05$), *S. aureus* (A vs. CTL = Lg 4.95 vs. Lg 7.40, $p < 0.01$), *E. cloacae* (A vs. CTL = Lg 6.14 vs. Lg 7.92, $p < 0.01$), *E. faecalis* (A vs. CTL = ND vs. Lg 7.28, $p < 0.01$), *L. salivarius* (A vs. CTL = Lg 4.89 vs. Lg

8.05, $p < 0.01$), and *C. albicans* (A vs. CTL = Lg 5.04 vs. Lg 7.61, $p < 0.01$). Although the antibacterial effect of traditional Chinese medicinal toothpaste was inferior, it also obviously inhibited the growth of *E. hormaechei* (C vs. CTL = Lg 5.40 vs. Lg 7.23, $p < 0.01$), *S. aureus* (C vs. CTL = Lg 6.51 vs. Lg 7.40, $p < 0.05$), *E. faecalis* (C vs. CTL = Lg 5.77 vs. Lg 7.28, $p < 0.01$), and *C. albicans* (C vs. CTL = Lg 5.53 vs. Lg 7.61, $p < 0.01$; **Figure 5**).

DISCUSSION

The long-term use of mouthwash, antibacterial toothpaste, and throat tablets can cause dysbacteriosis and further endanger oral health (Hays, 2006; Bik et al., 2010). Research has indicated that microorganisms in toothbrushes might have a potential risk to cause a variety of diseases (Hays, 2006), but little research has been carried out to explore the interaction of microbiota between the oral cavity and toothbrush.

In the present study, high-throughput sequencing was used to explore microbial diversity in the oral cavity and toothbrushes. The oral cavity and toothbrushes had a similar Shannon index and species observed, and the common OTUs (666) represented 58.63, 68.24, 58.94, and 63.98% of the population in groups CB, AB, CO, and AO. Therefore, the abundant nutrients and frequent microbial communication resulted in a high number and variety of species of bacteria in toothbrushes (**Figure 1**). Although some ingredients (e.g., honeysuckle, *Sarcandra glabra*, pseudo-ginseng, mint) contained in traditional Chinese medicinal toothpaste possess and anti-inflammatory effects, it seems these ingredients allow for a greater abundance of bacteria compared with antibacterial toothpaste containing antibacterial ingredients (e.g., sodium fluoride, stannous chloride; **Figure 1D**). In addition, some opportunistic pathogens, e.g., *A. baumannii* (which causes wound infection, sepsis, and even leads to death), *B. cepacia* (which causes cystic fibrosis in human lungs), and *S. maltophilia* (which causes iatrogenic infection and respiratory infections), were identified in groups AB and CB (Moore et al., 1999; Gales et al., 2001). This means these pathogens in toothbrushes may cause serious infectious diseases in low-immunity populations.

At phylum level, we found greater abundance of Proteobacteria in groups CB and AB. The phylum Proteobacteria contains various human pathogens, e.g., *Helicobacter*, *Neisseria*, *Brucella*, and *Rickettsia*, and they are reported to have a strong connection with metabolic disorders, inflammatory bowel disease, and lung disease (Wade, 2013; Chen and Jiang, 2014; Rizzatti et al., 2017; Vester-Andersen et al., 2017). At genus level, higher levels of *Actinomyces*, *Burkholderia*, and *Stenotrophomonas* were identified in groups CB and AB. *Acinetobacter* species are ubiquitous organisms widely distributed in nature as important opportunistic pathogens causing a range of nosocomial infections, including pneumonia, bacteraemia, secondary meningitis, urinary tract infections, and surgical wound infections (Gales et al., 2001). *Burkebacteria* were originally part of the *pseudogencin*, and *B. cepacia* and *B. pseudomallei* in this genus have harmful effects on human health because of their characteristics of resistance to antibiotics and high motility (Moore et al., 1999). *Stenotrophomonas* is famous for *S. maltophilia* which is an important nosocomial pathogen associated with infections of compromised individuals (Gales et al., 2001). *S. maltophilia* is associated with infections of the respiratory tract and causes bacteraemia, endocarditis, and urinary tract infections. It is intrinsically resistant to multiple antibiotics and disinfectants, and clinical isolates often

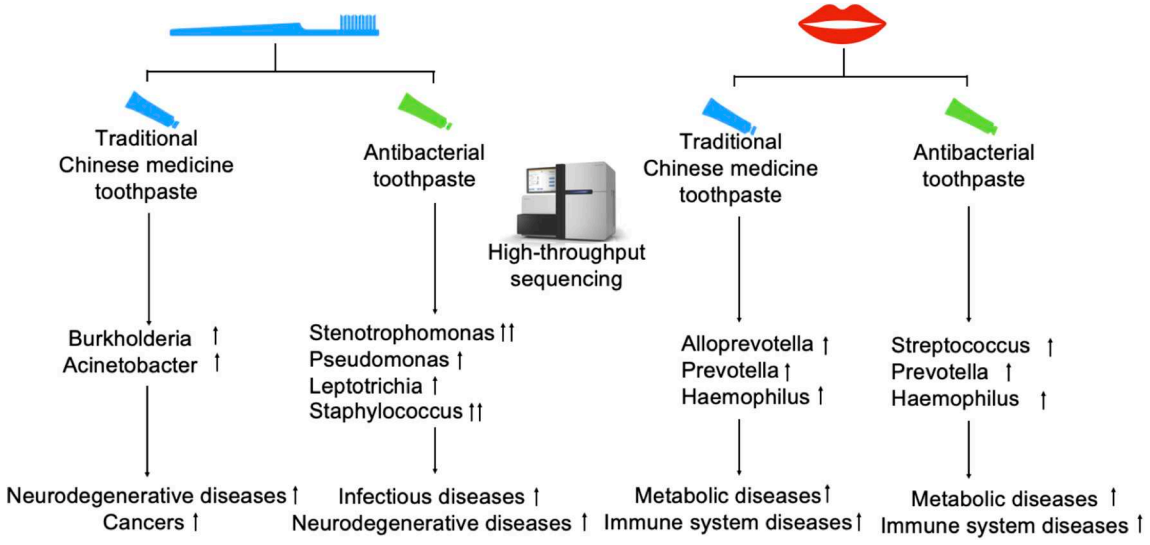


FIGURE 6 | Flow chart of the present study. Toothbrush and oral samples were obtained from the participants treated with traditional Chinese medicinal toothpaste and antibacterial toothpaste, respectively. A high-throughput sequencing method was used to analyse species richness. Genera showing a significant increase compared with other groups are shown with ↑; those showing a highly significant increase are shown with ↑↑. The increased risks of human disease for each group are shown.

display high-level multidrug resistance (Zhang et al., 2000). Interestingly, we also found a lower abundance of *Streptococcus* and *Prevotella* in toothbrushes than in the oral cavity. As we know, *Streptococcus* and *Prevotella* are dominant pathogens for inducing oral diseases; therefore, the residual ingredients in toothbrushes can better inhibit their growth because there are more antimicrobial ingredients in toothbrushes than in the oral cavity due to the high fluidity of the oral cavity. Furthermore, our bioinformatics prediction results indicate that the bacteria in toothbrushes greatly enhanced cell motility, neurodegenerative diseases, cancers, and cardiovascular diseases when traditional Chinese medicinal toothpaste was used, and the antimicrobial toothpaste greatly enhanced the potential for cell motility, neurodegenerative diseases, infectious diseases, cancers, and cardiovascular diseases compared with the bacteria in the oral cavity (**Figure 4**). However, as limited data are provided by high-throughput sequencing, the shotgun metagenomic method is needed to further confirm these results.

To further discuss the potential effects of toothpastes on bacterial growth, the viable counting method was applied and *E. hormaechei*, *S. salivarius*, *S. aureus*, *E. cloacae*, *E. faecalis*, *L. salivarius*, and *C. albicans* were isolated from the oral cavities and toothbrushes. Our results indicate that traditional Chinese medicinal toothpaste and antimicrobial toothpaste possess sound inhibitory effects on all tested strains (**Figure 5**). *E. hormaechei* is a nosocomial pathogen that can infect vulnerable hospitalized patients and can be transmitted from patient to patient when infection control techniques are inadequate (Wenger et al., 1997). The traditional Chinese medicinal toothpaste had a better inhibitory effect on *E. hormaechei*. *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), is an important food-borne pathogenic bacterium associated with significant morbidity and mortality. *C. albicans* is primarily a commensal organism in the oral cavity, digestive tract, and genital regions of healthy individuals and yet is responsible for mucosal candidiasis, such as thrush and angular keratitis, as well as visceral candidiasis, often deadly infections (Rai et al., 2019). We also tested two oral probiotics, *L. salivarius,* and *S. salivarius*. As a typical anti-allergic probiotic, *L. salivarius* helps reduce the production of specific IgE antibodies in serum, promotes the secretion of interferon (IFN-γ) in spleen cells, and enhances the TH1-type immune response to regulate the TH2-type immune response which is overactive due to allergies (Iwamoto et al., 2010; Li et al., 2010). *S. salivarius* can produce bacteriocin, which has an inhibitory effect on bad breath-related bacteria, especially anaerobic bacteria which decompose to produce sulfides, emitting a foul odor and producing bad breath (Volpe et al., 1969; Iwamoto et al., 2010). Studies have shown that people with bad breath have less *S. salivarius* in the mouth (Iwamoto et al., 2010). Although both traditional Chinese medicinal toothpaste and antibacterial toothpaste effectively reduced pathogenic bacteria, they also suppressed the oral probiotics which destroyed the stability of the oral microbiota and damaged oral health, especially the antibacterial toothpaste.

CONCLUSION

In the present study, we compared microbial diversity in the oral cavities and toothbrushes, and found a large number of bacteria in toothbrushes, including some pathogens. Pathogens existing in toothbrushes may transfer to the oral cavity and enhance the risk of neurodegenerative diseases, cancers, and cardiovascular diseases (**Figure 6**). Moreover, the strong antimicrobial effect of toothpaste on beneficial oral bacteria may also disturb the oral microbial system. Here, we compared the microbiota between oral cavities and toothbrushes, and explored the potential risk of bacteria in toothbrushes, which provides useful data on oral health. However, sampling by gargling carries a risk of contamination from the throat or upper aerodigestive ways; therefore, a more suitable sampling method for oral bacteria should be used (Santigli et al., 2017).

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: GenBank accession number PRJNA553856.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the Stomatological Hospital of Nanchang University and all participants provided written informed consent. All experiences were conducted in accordance with the approved guidelines. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TC designed the experiments. TC, YS, QS, YG, TQ, and SW analyzed the data and wrote the manuscript. QS, YG, TQ, and SW performed the experiments. All authors discussed the results and commented on the final manuscript.

FUNDING

This work was supported by grants from the National Natural Science Foundation of China (No. 31560264), the Major Academic Discipline and Technical Leader of JiangxiProvince (No. 20194BCJ22032), Key R&D plan of JiangXi Science and Technology Agency (No. 20181BBG70028), JiangXi Provincial Science and technology innovation team (No. 20181BCB24003).

REFERENCES

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., and Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *J. Clin. Microbiol.* 43, 5721–5732. doi: 10.1128/JCM.43.11.5721-5732.2005

Bik, E. M., Long, C. D., Armitage, G. C., Loomer, P., Emerson, J., Mongodin, E. F., et al. (2010). Bacterial diversity in the oral cavity of 10 healthy individuals. *ISME J.* 4, 962–974. doi: 10.1038/ismej.2010.30

Bo, L., Faller, L. L., Niels, K., Varun, M., Mohammad, G., Sommer, D. D., et al. (2012). Deep sequencing of the oral microbiome reveals signatures of periodontal disease. *PLoS ONE* 7:e37919. doi: 10.1371/journal.pone.0037919

Chen, H., and Jiang, W. (2014). Application of high-throughput sequencing in understanding human oral microbiome related with health and disease. *Front. Microbiol.* 5:508. doi: 10.3389/fmicb.2014.00508

Deng, K., Chen, T., Wu, Q., Xin, H., Wei, Q., Hu, P., et al. (2015). *In vitro* and *in vivo* examination of anticolonization of pathogens by *Lactobacillus paracasei* FJ861111.1. *J. Dairy Sci.* 98, 6759–6766. doi: 10.3168/jds.2015-9761

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics

Fang, X., Wang, X., Yang, S., Meng, F., Wang, X., Wei, H., et al. (2016). Evaluation of the microbial diversity in amyotrophic lateral sclerosis using high-throughput sequencing. *Front. Microbiol.* 7:1479. doi: 10.3389/fmicb.2016.01479

Fischman, S. L. (1997). The history of oral hygiene products: how far have we come in 6000 years? *Periodontol 2000* 15, 7–14. doi: 10.1111/j.1600-0757.1997.tb00099.x

Gales, A. C., Jones, R. N., Forward, K. R., Liñares, J., Sader, H. S., and Verhoef, J. (2001). Emerging importance of multidrug-resistant acinetobacter species and *Stenotrophomonas maltophilia* as pathogens in seriously Ill patients: geographic patterns, epidemiological features, and trends in the SENTRY antimicrobial surveillance program (1997–1999). *Clin. Infect. Dis.* 32, S104–S113. doi: 10.1086/320183

Hays, C. J. (2006). Bacteria in the oral flora: a study of antimicrobial agents in toothpastes on toothbrushes. *Saint Martin's Univ. Biol. J.* 1, 167–173.

Iwamoto, T., Suzuki, N., Tanabe, K., Takeshita, T., and Hirofuji, T. (2010). Effects of probiotic *Lactobacillus salivarius* WB21 on halitosis and oral health: an open-label pilot trial. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* 110, 201–208. doi: 10.1016/j.tripleo.2010.03.032

Li, C. Y., Lin, H. C., Hsueh, K. C., Wu, S. F., and Fang, S. H. (2010). Oral administration of *Lactobacillus salivarius* inhibits the allergic airway response in mice. *Can. J. Microbiol.* 56, 373–379. doi: 10.1139/W10-024

Lin, C. K., Tsai, H. C., Lin, P. P., Tsen, H. Y., and Tsai, C. C. (2008). *Lactobacillus acidophilus* LAP5 able to inhibit the Salmonella choleraesuis invasion to the human Caco-2 epithelial cell. *Anaerobe* 14, 251–255. doi: 10.1016/j.anaerobe.2008.07.003

Magoc, T., and Salzberg, S. L. (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Mojon, P. (2002). Oral health and respiratory infection. *J. Can. Dent. Assoc.* 68, 340–345.

Moore, R. A., Deshazer, D., Reckseidler, S., Weissman, A., and Woods, D. E. (1999). Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. *Antimicrob. Agents Chemother.* 43, 465–470. doi: 10.1128/AAC.43.3.465

Okpalugo, J., Ibrahim, K., and Inyang, U. S. (2009). Toothpaste formulation efficacy in reducing oral flora. *Trop. J. Pharm. Res.* 8, 71–77. doi: 10.4314/tjpr.v8i1.14714

Pinducciu, G., Micheletti, L., Piras, V., Songini, C., Serra, C., Pompei, R., et al. (1996). Periodontal disease, oral microbial flora and salivary antibacterial factors in diabetes mellitus type 1 patients. *Eur. J. Epidemiol.* 12, 631–636. doi: 10.1007/BF00499463

Pritchard, A. B., Crean, S., Olsen, I., and Singhrao, S. K. (2017). Periodontitis, microbiomes and their role in Alzheimer's disease. *Front. Aging Neurosci.* 9:336. doi: 10.3389/fnagi.2017.00336

Rai, L. S., Singha, R., Sanchez, H., Chakraborty, T., Chand, B., Bachellier-Bassi, S., et al. (2019). The *Candida albicans* biofilm gene circuit modulated at the chromatin level by a recent molecular histone innovation. *PLoS Biol.* 17:e3000422. doi: 10.1371/journal.pbio.3000422

Rizzatti, G., Lopetuso, L. R., Gibiino, G., Binda, C., and Gasbarrini, A. (2017). Proteobacteria: a common factor in human diseases. *Biomed. Res. Int.* 2017:9351507. doi: 10.1155/2017/9351507

Santigli, E., Koller, M., and Klug, B. (2017). Oral biofilm sampling for microbiome analysis in healthy children. *J. Vis. Exp.* 2017, 130:e56320. doi: 10.3791/56320

Vester-Andersen, M., Mirsepasi-Lauridsen, H. C., Vind, I., Prosberg, M., Mortensen, C., Träger, C., et al. (2017). In Crohn's disease, an aggressive disease course is related to an increased abundancy of proteobacteria; a seven year follow up study. *Gastroenterology* 152:S625. doi: 10.1016/S0016-5085(17)32219-9

Volpe, A. R., Kupczak, L. J., Brant, J. H., King, W. J., Kestenbaum, R. C., and Schlissel, H. J. (1969). Antimicrobial control of bacterial plaque and calculus and the effects of these agents on oral flora. *J. Dental Res.* 48, 832–841. doi: 10.1177/00220345690480053701

Wade, W. G. (2013). The oral microbiome in health and disease. *Pharmacol. Res.* 69, 137–143. doi: 10.1016/j.phrs.2012.11.006

Wang, X., Wu, Q., Deng, K., Wei, Q., Hu, P., He, J., et al. (2015). A novel method for screening of potential probiotics for high adhesion capability. *J. Dairy Sci.* 98, 4310–4317. doi: 10.3168/jds.2015-9356

Wenger, P. N., Tokars, J., Brennan, P., Samel, C., Bland, L., Miller, M., et al. (1997). An outbreak of Enterobacter hormaechei infection and colonization in an intensive care nursery. *Clin. Infect. Dis.* 24, 1243–1244. doi: 10.1086/513650

Whitmore, S. E., and Lamont, R. J. (2014). Oral bacteria and cancer. *PLoS Pathog.* 10:e1003933. doi: 10.1371/journal.ppat.1003933

Xie, G., Chain, P. S. G., Lo, C. C., Liu, K. L., Gans, J., Merritt, J., et al. (2010). Community and gene composition of a human dental plaque microbiota obtained by metagenomic sequencing. *Mol. Oral Microbiol.* 25, 391–405. doi: 10.1111/j.2041-1014.2010.00587.x

Zhang, L., Li, X. Z., and Poole, K. (2000). Multiple antibiotic resistance in *Stenotrophomonas maltophilia*: involvement of a multidrug efflux system. *Antimicrob. Agents Chemother.* 44, 287–293. doi: 10.1128/AAC.44.2.287-293.2000

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 06 March 2020
doi: 10.3389/fcimb.2020.00092

A Uniquely Altered Oral Microbiome Composition Was Observed in Pregnant Rats With *Porphyromonas gingivalis* Induced Periodontal Disease

*Molly S. Walkenhorst[1], Leticia Reyes[2], Gonzalo Perez[2], Ann Progulske-Fox[3], Mary B. Brown[4] and Priscilla L. Phillips[1]**

[1] Department of Microbiology and Immunology, Kirksville College of Osteopathic Medicine, A.T. Still University of Health Sciences, Kirksville, MO, United States, [2] Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI, United States, [3] Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, FL, United States, [4] Department of Infectious Diseases and Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL, United States

OPEN ACCESS

Edited by:
Lorenzo Lo Muzio,
University of Foggia, Italy

Reviewed by:
Marco Mascitti,
Marche Polytechnic University, Italy
Davide Sartini,
Marche Polytechnic University, Italy

***Correspondence:**
Priscilla L. Phillips
pphillips@atsu.edu

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 17 December 2019
Accepted: 21 February 2020
Published: 06 March 2020

Citation:
Walkenhorst MS, Reyes L, Perez G, Progulske-Fox A, Brown MB and Phillips PL (2020) A Uniquely Altered Oral Microbiome Composition Was Observed in Pregnant Rats With Porphyromonas gingivalis Induced Periodontal Disease. Front. Cell. Infect. Microbiol. 10:92. doi: 10.3389/fcimb.2020.00092

Porphyromonas gingivalis is an anaerobic bacterium commonly found in the oral cavity and associated with the development of periodontal disease. *P. gingivalis* has also been linked to several systemic vascular and inflammatory diseases including poor pregnancy outcomes. Little is known about the changes in the oral flora during pregnancy in connection to *P. gingivalis* infection. This pilot study aims to explore changes in the oral microbiome due to *P. gingivalis* inoculation and pregnancy in an *in vivo* rat model of periodontal disease. A metagenomic sequencing analysis targeting seven of the 16S rRNA gene variable regions was performed for oral samples collected at the following time points: baseline control (week 0), *P. gingivalis* inoculated (week 11), *P. gingivalis* inoculated and pregnant rat at necropsy (week 16). A second set of animals were also sampled to generate a sham-inoculated (week 11) control group. We found that the rat oral microbiome profiles were more similar to that of the human oral cavity compared to previous reports targeting one or two 16S variable regions. Overall, there appears to be a relatively stable core microbiome in the oral cavity. As expected, *P. gingivalis* induced periodontal disease resulted in oral microbiome dysbiosis. During pregnancy, some aspects of the oral microbiome shifted toward a more baseline-like profile. However, population analyses in terms of dissimilarity measures and especially metagenomic based predictions of select characteristics such as cell morphology, oxygen requirement, and major metabolite synthesis showed that pregnancy did not restore the composition of the oral microbiome. Rather, a uniquely altered oral microbiome composition was observed in pregnant rats with pre-established periodontal disease.

Keywords: oral microbiome, oxygen requirement, microbial metabolites, pregnancy, periodontal disease, rat model

INTRODUCTION

Periodontitis is currently considered a disease of dysbiotic polymicrobial etiology in which oral microbial species capable of thriving in inflamed microenvironments predominate (Abusleme et al., 2013; Hajishengallis, 2015). Although the pathogenesis of periodontal disease is complex, one view is that dysbiosis is initiated by the presence of keystone species that subverts the host immune defense, and this allows secondary pathogens to thrive and ultimately dominate. *Porphyromonas gingivalis* has gained notoriety as a keystone pathogen of periodontal disease because even as a low abundance species, it has profound effects on the oral microbial community structure (Hajishengallis, 2011; Olsen et al., 2017). *P. gingivalis* is armed with an array of virulence factors that subvert host innate antimicrobial defenses without blocking inflammatory pathways; referred to as "non-productive inflammation" (Zenobia and Hajishengallis, 2015). This not only supplies *P. gingivalis* the nutrient source it needs for growth, but also provides a selective advantage for co-colonizing microbes able to tolerate and thrive in an inflammatory environment (Zenobia and Hajishengallis, 2015).

Metagenomic studies of the oral microbiome also show that pregnancy perturbs the composition of the oral microbial community (Paropkari et al., 2016; Lin et al., 2018). In pregnant women without periodontal disease, the supragingival community structure is reported to shift toward a predominance of bacteria within the genera *Neisseria*, *Porphyromonas*, and *Treponema* (Lin et al., 2018). In particular, the increased abundance of *Prevotella* and *Treponema* spp. is linked to increasing concentrations of sex hormones associated with pregnancy (Lin et al., 2018). Moreover, pregnancy can modify the impact of environmental risk factors on the oral microbiome. For instance, the subgingival microbiome of women who smoke during pregnancy are reported to have significantly less Gram-negative and Gram-positive anaerobes than non-pregnant smokers (Paropkari et al., 2016). To date, it is unknown if or in what manner pregnancy affects the dysbiotic oral microbiome associated with periodontal disease in patients with preexisting oral disease.

For this pilot study, we chose *P. gingivalis* as our model organism since it has been implicated in promoting adverse pregnancy outcomes (Barak et al., 2007; Chaparro et al., 2013; Vanterpool et al., 2016). Our objective was to investigate the interaction of pregnancy and the oral dysbiosis of periodontal disease in a rat model of *P. gingivalis*-induced periodontitis (Kesavalu et al., 2007; Verma et al., 2010) using 16S metagenomic analysis.

MATERIALS AND METHODS

Periodontitis Model and Sample Collection

All procedures were conducted after approval from the University of Wisconsin–Madison, Institutional Animal Care and Use Committee. Specific pathogen free CD-IGS rats obtained from the same colony (Charles River International Laboratories, Inc., Kingston, NY; RGD ID 734476) were housed in the same room under barrier conditions. Oral swabs and blood were collected prior to antibiotic treatment and animals were randomly assigned to sham control (inoculation with sterile vehicle) or *P. gingivalis* strain A7UF, a lab adapted strain expressing Type IV *fim* operon and with 99.2% whole genome DNA sequence identity to A7436 (data not shown). At the onset of the inoculation phase of the study and thereafter control animals were always handled before infected animals.

Periodontitis was induced as previously described (Phillips et al., 2018). Specifically, 7–8 week old female rats first received kanamycin (20 mg) and ampicillin (20 mg) daily for 4 days in the drinking water to reduce the number of commensal bacteria. The oral cavity was then swabbed with 0.12% chlorhexidine gluconate (Peridex: 3M ESPE Dental Products, St. Paul, MN, USA) mouth rinse to inhibit endogenous organisms and to facilitate subsequent colonization with *P. gingivalis*. Rats were switched back to antibiotic free water and rested for 3 days to allow clearance of antibiotics from their system before beginning the inoculation phase of the study. An equal number of rats were randomly assigned to the control or *P. gingivalis* group. Each inoculate was prepared from an overnight culture of *P. gingivalis* grown in supplemented Tryptic Soy Broth (Phillips et al., 2018). Bacterial concentrations in broth were determined by optical density readings taken at 550 nm with a UV-6300 PC double beam spectrophotometer (VWR, Chicago, IL, USA). Bacteria were pelleted by centrifugation at 3,000 × g for 5 min and resuspended in sterile 2% (w/v) low viscosity carboxymethylcellulose (CMC; Sigma, St. Louis, MO, USA) to achieve a final concentration of 1×10^{10} CFU per ml. Each animal received an oral inoculation containing 1×10^{9} CFU for 4 consecutive days per week on 6 alternate weeks totaling 24 inoculations over a 12-week period. Control animals received sterile 2% CMC. In order to minimize disruption of bacterial plaque, animals were fed a gamma-irradiated powdered rodent diet (Teklad Global 18% protein rodent diet, Envigo, Madison, WI) during the inoculation phase of the study. At the end of the inoculation period (week 12), oral swabs were taken, blood was collected for serology, and rats were switched back to the same diet in a pelleted form. Animals were rested for 1 week before breeding. Breeding was confirmed by the presence of sperm in vaginal lavages that were performed the following morning, which was considered gestation day GD 0. Dams were euthanized at GD 18 oral swabs were taken, skulls were collected for morphometric analysis of alveolar bone loss, and serum was collected for detection of *P. gingivalis* specific IgM and IgG.

For the purposes of 16S metagenomic analysis, we were interested in the oral samples collected from sham and *P. gingivalis* treated CD rats. Specifically, we processed oral specimens collected from the *P. gingivalis* (PG) treatment group (six animals) at three time points for sequencing: baseline, 3-months post *P. gingivalis* inoculation, and at necropsy (pregnant at GD 18). In contrast, only oral specimens collected from the sham treatment group (six animals) at the 3-months post sham inoculation time point were used for sequencing. The animals and specimen descriptions, including the sample IDs for the 24 sequencing libraries we generated and used for this 16S metagenomic analysis pilot study, are listed in **Table S1**.

Assessment of Alveolar Bone Resorption

Mandibles were disarticulated from the skull and most of the tissue was removed by dissection. Remaining tissue was removed by Dermestid beetles. Rat mandibles were then immersed in a 3% (v/v) hydrogen peroxide solution overnight, washed with deionized water and air-dried. In order to delineate the cemento-enamel junction (CEJ), jaws were stained with a 0.1% (W/V) methylene blue for 1 min, washed and air-dried. Specimens were coded to prevent bias and calibrated images of both the buccal and lingual side of each jaw were captured with an EOS 650D RebelT41 camera (Canon, USA, Inc., Long Island, NY). Morphometric measurements were performed by two independent investigators that were blinded to treatment. Calibrated digital images were analyzed with Image J 1.50b analysis software (Rasband, National Institutes of Health, USA). Area measurements (mm^2) of both the lingual and buccal surface of each mandible were determined by using the freehand tool to manually trace the surface perimeter of the CEJ and alveolar bone crest (ABC). The sum of both area measurements was recorded and values from two independent investigators were averaged.

Maxillae were fixed in 10% buffered formalin, washed and decalcified for 48 h before processing for histology. Excess tissue was removed and the row of molars were transected through the center of each molar so that interdental bone loss could be evaluated. Calibrated images of maxillary tissue were analyzed with Image J software as previously described (Phillips et al., 2018). Briefly, a line was drawn to connect the mesial to the distal aspect of each molar at the CEJ. A central line perpendicular to this CEJ was drawn from the CEJ to the top of the alveolar bone crest and the distance was recorded in mm. The distance measurements of each interdental region were averaged.

Detection of *P. gingivalis*-Specific IgM and IgG

Humoral responses to *P. gingivalis* were determined by ELISA as previously described (Phillips et al., 2018) with the following modifications. Briefly, a whole cell lysate of *P. gingivalis* was sonicated (Sonic Dismembrator, Thermo Fisher Scientific, Waltham, MA) and the total protein concentration of the lysate was measured by Bradford Protein Assay (Thermo Fisher Scientific, Waltham, MA). Stock aliquots of the lysate were stored at a concentration of 2 mg/ml at −20 C. For the ELISA assay, whole cell lysates were diluted with 50 mM bicarbonate coating buffer pH 9.4 (Thermo Fisher Scientific, Waltham, MA) to yield a final concentration of 20 μg/ml. Goat anti-rat IgM (Southern Biotech, Birmingham, AL) and donkey anti-rat IgG (Thermo Fisher Scientific, Waltham, MA) conjugated to horse radish peroxidase (HRP) were used for detection (1:4,000 and 1:2,500, respectively). Pooled sera from *P. gingivalis* infected rats and uninfected animals were used to establish serum dilutions that fell within the linear range of a dilution curve. Subsequent ELISAs were batched and each plate contained a positive and negative control. In order to minimize plate to plate variation, each 96 well plate contained serum samples from each group. All samples were run in duplicate and readings were obtained with a Model 680 microplate reader (Biorad Laboratories, Hercules, CA).

Statistical analysis of alveolar bone loss measurements and serology data were performed with unpaired student's *t*-test using Prism 7.03 Software (GraphPad Software Inc.). For all testing $P < 0.05$ was considered significantly different.

DNA Isolation and Enrichment for Bacterial DNA

The specimens for each sampled time point were collected on either rayon swabs (sham-inoculated) or cytological brushes (baseline, PG-inoculated, pregnant) and flash frozen and stored at −80°C until they were batch processed. At the time of processing, collection devices were thawed, immersed in sterile 200 μL of 1× phosphate buffered saline (PBS; pH 7.4), vortexed for 30 s, and the solution was transferred into sterile 2 mL sample extract tube. The swab/brush was centrifuged at 15,000 × *g* for 5 min to collect the residual liquid that was trapped in the collection device, then pipetting it into the appropriate sample extract tube. This washing process was repeated twice more for a total of three rinses for each swab. All extracted liquids from each sample were combined into one tube per specimen. Each sample extract tube was centrifuged at 21,000 × *g* for 10 min to pellet cells and detritus. The PBS was removed from the pellet and 40 μL of resuspended Dynabeads from the Dynabeads DNA DIRECT Universal Kit (Invitrogen: Cat. No. 63006) was added to each sample pellet and mixed. The Dynabead/sample mixtures were processed according to the manufacturers' protocol and the concentration of the isolated total DNA in the eluent was estimated using a NanoDrop 2000C Spectrophotometer (Thermo Scientific: Cat. No. ND2000C). The total DNA isolated from each oral specimen contains a large proportion of rat DNA, thus it is essential to enrich for bacterial DNA to optimize downstream 16S metagenomic analysis. The chemistry provided in the NEBNext Microbiome DNA Enrichment Kit (New England BioLabs: Cat. No. E2612L) enriches for bacterial DNA by selectively binding and removing CpG-methylated host DNA while maintaining microbial diversity after enrichment. Cytosine methylation occurs in bacteria in very low frequency across bacterial genomes while it is common across eukaryotic genomes. Validation studies, referenced by the manufacturer, using this enrichment method have shown that bacterial species with unusual methylation density of its DNA are rare and bind at a very low level to the enrichment beads (e.g., *Neisseria flavescens*). The enriched microbial DNA purified by the ethanol precipitation and the DNA concentration was estimated using a NanoDrop 2000c spectrophotometer. Semi-quantitative verification of enrichment was performed using qPCR (CFX-Connect Real-Time PCR detection system; BioRad Laboratories Inc.), SsoAdvanced Universal SYBR Green Supermix (BioRad Laboratories Inc.: Cat. No. 1725270), and NEBNext 16S Universal Control Primers (New England BioLabs).

PCR Amplification of Microbial 16S rRNA Gene Variable Regions

16S rRNA libraries of each microbial DNA enriched sample were generated by PCR using the Ion 16S Metagenomics Kit (ThermoFisher Scientific: Cat. No. A26216) and a PCR

thermocycler (Mastercycler nexus gradient; Eppendorf AG). Briefly, two primer sets are used to generate amplicons from six different regions of the 16S rRNA genes. Primer Set One targets variable regions 2, 4, 8 and Primer Set Two targets variable regions 3, 6–7, 9. PCR amplification was performed according to the manufacturers' protocol for each sample, along with negative and positive controls. The amplicons from 20 μL of each PCR reaction were then purified using the Axygen AxyPrep Mag PCR Clean-up Kit (Fisher Scientific: Cat. No. 14-223-151) and eluted in elution buffer (EB; 10 mM Tris-Cl, pH 8.5). The amplicons generated for each pair of reactions (two primer sets) for each sample were pooled and their DNA concentration was estimated with a NanoDrop 2000c spectrophotometer until confirmation that each sample pool consisted of 100 ng−1 μg of DNA, as required for adaptor ligation. The final concentration of amplicons in each sample pool was determined using a Qubit dsDNA hs Assay Kit (Invitrogen: Cat. No. Q32851) according to the manufacturers' protocol.

16S Barcoded Library Preparation for Multiplexed Sequencing

Sequencing-ready16S rRNA gene libraries were prepared using the NEBNext Fast DNA Library Prep Set for Ion Torrent (New England BioLabs: Cat. No. E6270S) and DNA barcode-adapter oligonucleotides specific for the Ion Torrent system. All 16s rRNA libraries were prepared according to the manufactures' recommendations with modifications to maximize yield. Briefly, purified 16S amplicons were first end repaired and blunt-end ligated to sample specific barcode-adapters (**Table S2**) in order to generate three groups for downstream pooling. We estimated that the maximum number of libraries to load in equal-molar concentrations per 318™ Ion Chip in order to generate a sufficient number of reads for metagenomic analysis was 13. Adaptor ligated DNA was size selected to obtain fragments between 200 and 400 base pairs long using the Agencourt AMPure XP PCR Purification system (Beckman Coulter: Cat. No. A63880). DNA fragments below 200 base pairs in length would likely consist of free adaptors and empty adaptor-adaptor products, whereas fragments over 400 base pairs would likely have multiple copies of the adapters ligated to their ends.

The size selected amplicons were PCR amplified using primers to the ION torrent P1 adapter and A adapter sequences, to generate at least 1 μg of unbiased amplicons of all the barcoded-adapter-ligated DNA fragments. PCR amplifications were performed using the following parameters: (1) initial denaturing at 98°C for 30 s; (2) 8–11 cycles of 98°C for 10 s, 58°C for 30 s, and 65°C for 30 s; (3) 65°C for 5 min; and (4) a final hold at 4°C. After amplification, the amplicons in each sample were purified using Agencourt AMPure XP and the DNA concentration was determined by qPCR using the Ion Universal Library Quantitation Kit (ThermoFisher Scientific: Cat. No. A26217) according to the Ion 16S Metagenomics Kit User Guide (pgs. 18–22). Each sample was assayed in replicates of three. The calculated average concentration for each sample was used for subsequent library pooling calculations.

Each 16S barcoded library were diluted and pooled in equal molar concentrations into one of three pools: Pools A, B, and C (**Table S2**). Each pool was PCR amplified using the P1 and A adaptor primers from the NEBNext Fast DNA Library Prep Set for Ion Torrent with the following modified PCR parameters: (1) initial denaturing at 98°C for 30 s; (2) 30 cycles of 98°C for 10 s, 58°C for 30 s, and 65°C for 30 s; (3) 65°C for 5 min; and (4) a final hold at 4°C. After amplification, DNA in each pool was purified using the Agencourt AMPure XP. Our yield was ∼1,000× higher than needed and contained a higher proportion of short fragments, which led to the need to modify the templating procedure. We created Pool D to re-sequence a subset of sample libraries which had the fewest number of reads (**Table S2**), described in more detail in the supplementary sequencing pool section below. For that run, we shortened the number of cycles at this amplification step to 25, significantly improving the library quality (proportion of full-length fragments). We also modified our dilution method to generate more accurate equal-molar pooling. The final concentrations of the pooled libraries were verified by qPCR using the 16S Metagenomics Kit User Guide protocol as already described.

Template Preparation, Enrichment, and Sequencing

The first step required to sequence our DNA amplicons using the Ion Torrent platform (Thermo Fisher Scientific Inc., Waltham, MA USA) is to template each of our pooled DNA libraries. Templating involves loading appropriately diluted pooled libraries onto ion sphere particles (ISPs) using the Ion PGM Hi-Q View OT2 Kit (ThermoFisher Scientific: Cat. No. A29900) and the Ion OneTouch-2 and OneTouch-ES Instruments (Thermo Fisher Scientific: Cat. No. 4474779) according to the manufactures' protocol for 400 base pair read length templating. The manufactures' protocol for templating lists a suggested concentration for each type of library in order to acquire a target range of template positive particles. Successful templating is achieved when the sample passes a quality control test, which is explained in more detail below. The concentration of the freshly diluted pooled libraries that were successfully templated and subsequently sequenced in this study were as follows: Pools A and C at 26 pM each, Pool B at 23 pM, and Pool D at 10 pM.

To determine the percentage of ISPs with bound template DNA (pooled library), a PCR thermocycler, a Qubit 3.0 Fluorometer (Thermo Fisher Scientific: Cat. No. Q33216), and the Ion Sphere Quality Control Kit (Thermo Fisher Scientific: Cat. No.4468656) were used according to the manufactures' protocol found in the Ion PGM Hi-Q View OT2 Kit manual. The acceptable level of templated ISPs is 10–30% with an ideal range from 20 to 25%. The percent templated ISPs for each pool were determined to be as follows: 31% for Pool A; 18% for Pool B; 22% for Pool C; and 16% for Pool D. Though the percent templated ISPs for Pool A was 1% higher than the acceptable level, we have found that the percentages generated tend to have a variation of a few percentage points when performed in replicate, so we deemed Pool A to be sufficiently close to

acceptable levels. Templating preparations of pooled libraries that passed the quality control assay were then enriched for template positive ISPs using the Ion OneTouch ES Instrument and Ion PGM Enrichment Beads (Dynabead MyOne Streptavidin C1 beads; Thermo Fisher Scientific: Cat. No. 4478525) which function to selectively bind to the template positive ISPs.

The Ion Torrent PGM system preparation, sequencing run, and post-run cleanup were performed using the chemistry and supplies provided in the Ion PGM Hi-Q Sequencing Kit (Thermo Fisher Scientific: Cat. No. A25592) and Ion PGM Hi-Q Wash 2 Bottle Kit (Thermo Fisher Scientific: Cat. No. A25591) according to the sequencing protocol in the Ion PGM Hi-Q Sequencing User Guide as follows. First, the sequencing run plan was created on the Ion Torrent Server with the following criteria: (1) Application—Metagenomics; (2) Target technique−−16S Targeted Sequencing; (3) Library Kit—Ion Plus Fragment Library Kit; (4) Template Kit—Ion PGM Hi-Q View OT2 Kit−−400; (5) Sequencing Kit—Ion PGM Hi-Q Sequencing Kit; (6) Flows −850; (7) Chip Type—Ion 318™ Chip v2; and (8) Barcoded Set—IonXpress. Second, the sequencing primers were annealed to the template positive ISPs using a PCR thermocycler (95°C 2 min, 37°C 2 min). Third, the sequencing polymerase was added to the primer annealed template positive ISPs and loaded onto an Ion 318™ Chip v2 (Thermo Fisher Scientific: Cat. No. 4484354). Fourth, the loaded Ion chip was placed into the cleaned and initialized Ion Torrent PGM instrument (Thermo Fisher Scientific: Cat. No. 4462921) and the sequencing run was initiated.

After sequencing Pools A, B, and C, and preliminary analyses, we found that some of the individual libraries within each pool had low numbers of mapped reads. This was not unexpected considering other studies using the equal-molar pooling method of combining multiple samples into one sequencing run have also reported widely varying numbers of read sequences generated among samples within a pool (Lemos et al., 2011). To address this potentially problematic issue, an additional sequencing run was performed. Using the following predetermined criteria, specific samples were chosen for a supplementary sequencing pool (Pool D): Samples that generated 2,500 mapped reads or less and generated a metagenomic profile with low species diversity compared to other samples of the same treatment group. The samples meeting these criteria were C31, C35, 44, 46, N43, N45, and N47 (**Table S2**). Though meeting the above criteria, one sample, C34, had no mapped reads despite using all of the P1/A adaptor PCR reaction when pooling (pool A), thus this sample was not reamplified. After preliminary analysis of Pool D, the new sequencing data were combined with the previous data collected for each sample.

16S Metagenomic and Statistical Analysis

Using the Ion Reporter Software, the sequencing data for each individual sample were organized into their appropriate test group: Baseline, PG-inoculated, sham-inoculated, or pregnant. These groups were then analyzed using a stringently designed workflow to generate the 16S Oral Microbiome Profiles. The workflow included the following criteria: (1) Application—Metagenomics; (2) Sample Groups—Single/Multi; (3) References—Curate MicroSEQ® 16S Reference Library v2013.1 and Curate Greengenes v13.5; (4) Primers—Default; (5) Primer(s) Detected—Both ends; (6) Minimum Alignment Coverage −90.0%; (7) Read Abundance Filter −10 copy minimum; (8) Genus Cutoff −97.0%; (9) Species Cutoff −99.0%; and (10) Slash ID Reporting Percentage −0.2%. This workflow was used to map the sequencing data (i.e., assign reads to Operational Taxonomic Units) for each individual sample, as well as the collective (consensus) data for each group, against multiple curated reference microbial genome databases to generate individual microbiome population profiles (OTU data sets) for each sample as well as consensus profiles for each treatment group. The similarity BP (base-pair) cutoff was set to 150 for all data reported here. Each individual sample was also analyzed to determine if there were any microbiome profiles that appeared unusual or as outliers when compared to the rest of the profiles within their experimental group. The consensus microbiome profiles were then compared between treatment groups and/or time points to identify shifts in the microbial population in response to *P. gingivalis* inoculation or pregnancy and for analyses of other descriptors associated with the identified community members at the family, genus, or species level (i.e., Gram stain characteristic, oxygen requirements, predictive metabolic profiles). R software (R Core Team, 2013) was used to graphically display the population percentage of identified community members within in each microbiome profile in context of the selected group descriptor.

The mapped output data (microbiome population profiles) for each treatment group were assessed and graphically displayed by performing alpha and beta diversity analysis using the QIIME bioinformatics pipeline (Caporaso et al., 2010) on the Ion Reporter Software Suite (Thermo Fisher Cloud). These analyses produce descriptive community statistics. We report alpha diversity intra-group analysis of the consensus microbiomes, comprising of all samples belonging to a group, for each of our four groups. The alpha diversity pipeline generates rarefaction curves which reflect the species (community) abundance (number of OTUs) and evenness (population proportion) within the group, and thus if the sequencing data output was deep enough to accurately characterize the microbiota present at the time of sampling they will generate rarefaction curves (phylogenetic diversity plots) that plateau when only the rarest species remain to be sampled. Four different nonparametric statistical analyses were completed to create rarefaction curves: (1) Observed Species, (2) Simpson index, (3) Shannon index, and (4) Chao1 index. The Observed Species and Choa1 analyses measure sample/community richness. Simplistically, the output based on these measures depends on the presence or absence of species observed in samples within a group. Chao1 index also reflects population proportion (evenness) to a degree by taking in account the number of times a species occurs. However, these methods are strongly affected by sample size, which can lead to an underestimation of sample richness (Colwell and Coddington, 1994), and thus are not as popular a measure when performing analyses on 16S metagenomic data generated using short read platforms like Ion Torrent PGM or MiSeq when the number of OTUs identified are low in a study. That said, after

reaching plateau, the estimation of diversity becomes relatively independent of sample size (Hughes et al., 2001). Moreover, using six 16S regional targets rather than one or two would compensate for small sample size leading to poor coverage common to 16S metagenomic analysis performed on these small read platforms that commonly use only one or two regional targets. Using six regional targets would also diminish the occurrence of false positive that reportedly (Edgar, 2017) plagues QIIME analysis of 16S metagenomic data generated from short read platforms when using a single variable region target. The Simpson and Shannon analyses reflect population diversity (abundance and evenness). More precisely, the Simpson index reflects species dominance and the probability of two individual organisms that belong to the same species being randomly chosen. Similar to the Chao1 index, it is weighted toward the abundance of the most common species. For the Simpson Index, a measure of 1 indicates complete evenness in species proportions within that sample or group. In contrast, the Shannon Index measures the average degree of uncertainty in predicting as to what species an individual organism will belong when chosen at random, thus this measure is affected by both the number of species identified and their population proportion (evenness).

Bray-Curtis beta diversity analyses was used to compare individual sample or group microbiome profiles to each other to identify community dissimilarities. This is a distance based statistical analysis used to identify sources of variance within individual sample communities or among groups. This method uses abundance of an OTU but not the phylogeny it belongs to, unlike the UniFrac method to estimate beta diversity. To visualize beta diversity, Principal Coordinate Analysis (PCoA) plots were used. PCoA is a multidimensional scaling computation that converts the microbiome population profile data into correlated similarities variables, then transforms them into a dissimilarity or distance matrix that can then be spatially plotted on a set of three orthogonal axes as a data point such that a maximum amount of variation for that data point is explained by the first principal coordinate axes, the second largest amount of variation is explained by the second principal coordinate axes, etc. (Caporaso et al., 2010).

Predicted Microbial Metabolome Profiles

A predictive analysis of morphological classification, oxygen requirement, and major metabolite synthesis profiles was performed. To identify the capacity to produce major metabolites in the oral cavity in response to *P. gingivalis* inoculation and then pregnancy, the consensus microbiome profiles were analyzed using KEGG mapper (www.genome.jp/kegg/) and further reviewed using the Substrata database (www.datapunk.net/substrata) and STRING (string-db.org). In order to be classified as belonging to a select metabolite group, the metabolite must be identified as a major end product rather than just having the genetic capacity to generate the metabolite in question. Bergey's manual of systematics of archaea and bacteria was also used to support appropriate metabolite classification of each genus (Whitman, 2015). When classification was unclear due to lack of strong evidence to support inclusion, or if strong evidence supported being excluded as a major producer of any one of these select metabolites, these identified members were classified as Other. All predictive qualitative metabolic outcome designations were plotted as percent OTU at the genus level.

RESULTS

Impact of *P. gingivalis* Infection on Alveolar Bone Loss and Humeral Immunity

All baseline and sham-inoculated samples were negative for *P. gingivalis* by PCR. All specimens from the PG and pregnant groups were positive for *P. gingivalis* by PCR. All *P. gingivalis* inoculated animals had significantly greater alveolar bone loss than aged-matched sham-inoculated controls confirming that these animals had periodontal disease (**Figure 1**).

We also measured *P. gingivalis* specific IgM and IgG before (baseline), after inoculation, and during pregnancy to assess microbial exposure (**Figure 2**). None of the animals had detectable *P. gingivalis* specific IgM at any time point in the study (data not shown). There was no difference in the level of *P. gingivalis* specific IgG between baseline and sham control groups. Both PG inoculated and pregnant groups had greater amounts of *P. gingivalis* specific IgG than the baseline group ($P < 0.0001$).

The Effect of *P. gingivalis* Infection and Pregnancy on the Oral Microbiome

We first looked for shifts in the native oral ecology by evaluating the intra-group richness, diversity, and relative depth of coverage at each collection time point and condition. Sequences (reads) with more than 10 copies (abundance) and a sequence match that mapped at 90% or greater accuracy to the reference genomes are assigned a specific OTU. These are standard criteria to maximize the confidence that the identified bacteria are all significant contributors to microbiome community. For our data, the specific mapping cutoff was 97% sequence match at the genus level and 99% sequence match at the species level for all qualifying reads. All specimens, with the exception of one sham-inoculated control sample (ID# 34), generated OTUs that met these criteria. To visualize our data, consensus microbiome profiles, graphed as OTUs at the family level are shown in the **Figure S1**. Consensus microbiome profiles at the family, genus, and species levels, sorted by 16S variable region, were also visualized using Krona charts generated using the Ion Torrent software suite and are shown in the **Figures S2**, **S3**.

Alpha diversity was evaluated by four different statistical analyses including: Observed Species, Simpson Index, Shannon Index, Chao1 index (**Figure 3**). The rarefaction curves plateaued for all groups, indicating that our data sets had adequate coverage and only the rarest species may not have been identified. Both Observed Species and Choa1 rarefaction curves showed that abundance was similar among PG-inoculated, pregnant, and baseline groups, while the sham-inoculated group, which had the lowest number of OTUs, had the least abundance. In contrast, the Shannon and Simpson rarefaction curves, which also reflect population evenness, showed that PG-inoculated group had the greatest diversity. Specifically, the average Shannon and Simpson diversity indices at the species level were, respectively, 3.24 and

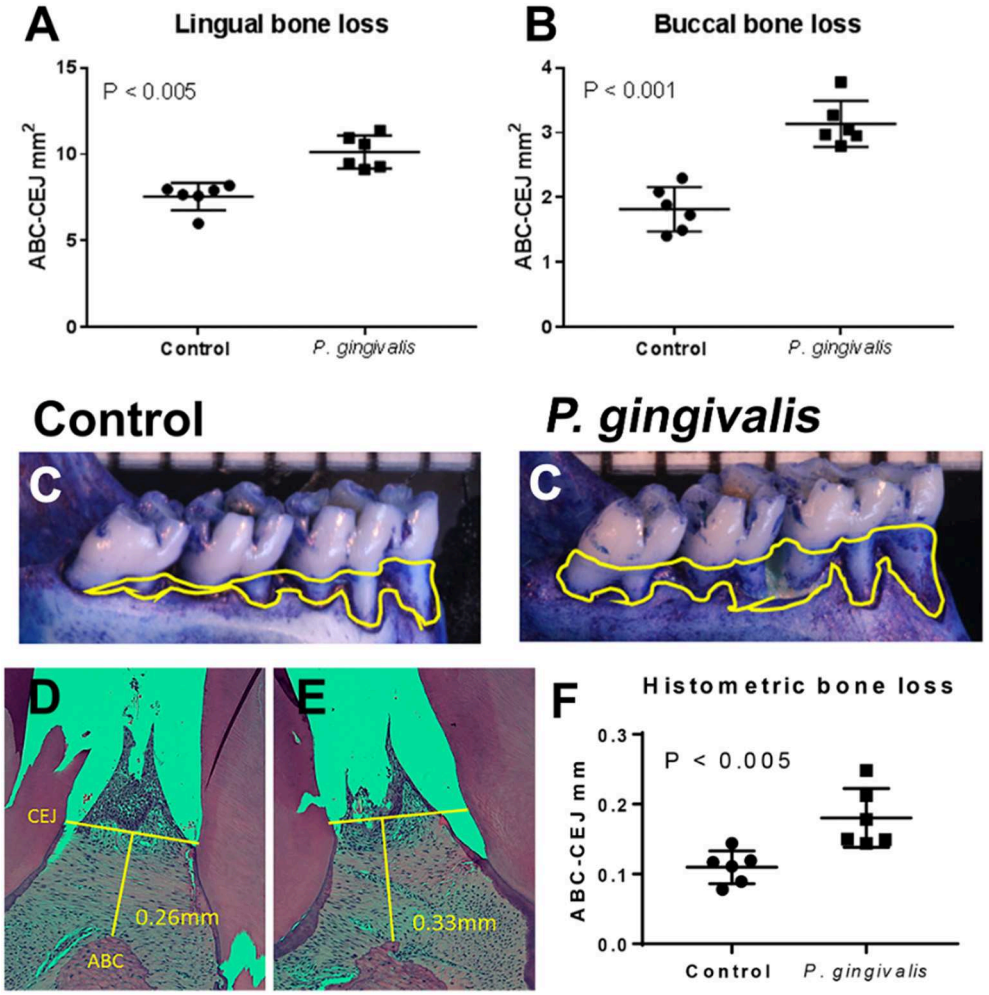


FIGURE 1 | All *P. gingivalis* inoculated animals had significantly greater alveolar bone loss than aged-matched sham-inoculated controls (baseline) confirming that these animals had periodontal disease. Morphometric assessment of mandibular and maxillary bone loss in pregnant control and PG-inoculated rats. Values in each graph represent the extent of horizontal bone loss, labeled as CEJ-ABC area measurements (mm^2), on the lingual **(A)** and buccal **(B)** sides from each mandible. **(C)** Representative images of the lingual aspect of the rat mandible illustrating the extent of horizontal bone loss. Yellow lines demarcate the CEJ-ABC junction that was used to determine extent of bone loss. Representative images of maxillary interdental papilla from control **(D)** and PG-inoculated rats **(E)** demonstrating how CEJ and ABC measurements were taken for histomorphometry **(F)**. Bars in all graphs show the mean ± SD. Data were analyzed by unpaired student's t test.

0.84 (PG-inoculated), 2.45 and 0.67 (pregnant), 2.47 and 0.63 (baseline), and 2.35 and 0.66 (sham-inoculated), where a higher value indicates greater diversity in that group.

Community variance was visualized though 3-dimensional Bray Curtis PCoA plots. Each data point was sorted and color coded by experimental group, oral infection status, or pregnancy status, and plotted across three primary variation distance measures. The lack of clustering along PC1 indicated that experimental treatment was not the primary source of variation (**Figure 4**). However, experimental groups did contribute to community variance at the family, genus, and species level. For example, at the species level, baseline, PG-inoculated, and pregnant groups clearly clustered within their group along PC2, which accounted for 16.55% of community variance (**Figure 5**). These distance measures data also showed that the pregnancy group was positioned most distant from zero along PC2, indicating that pregnancy in each infected animal exerted a greater degree of oral microbial community variance than *P. gingivalis* infection alone at the species level. At the genus and family level, the baseline, PG-inoculated, and pregnant groups clustered within their groups along PC3: 10.91% at the genus level and 10.90% at the family level (**Figure 6**). When sorted by infection status (**Figure 7**), the data sets clustered within their groups along PC5 at the species level (8.31%), PC6 at the genus level (6.14%), and PC9 at the family level (3.86%).

Beta diversity analysis was also performed to determine group dissimilarity between each collective consensus microbiome at the family, genus, or species level and plotted as four data points in PCoA plots. By merging data sets within each group, more rare members identified in each sample have less impact on

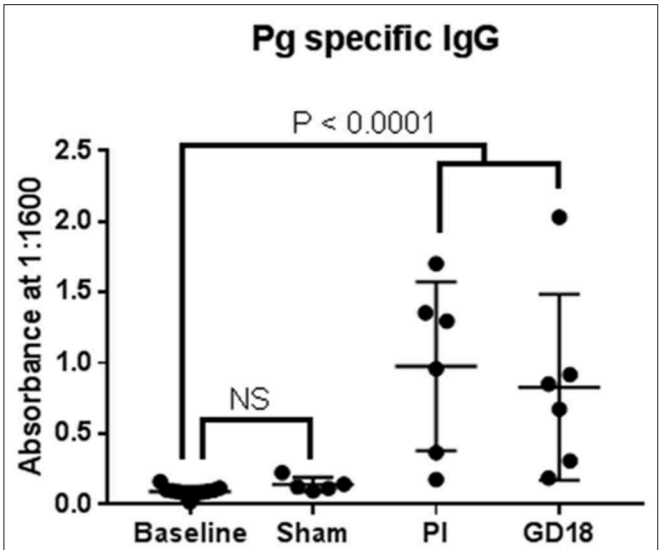


FIGURE 2 | We also measured *P. gingivalis* specific IgM and IgG before (baseline), after inoculation, and during pregnancy to assess microbial exposure.

the distance measures, thus generating plots in an alternative context, similar to alpha diversity measures. Example graphs are shown in **Figure 8**. When Bray Curtis metrics were used and the data points were visualized along the first three axes of variation, both baseline and pregnant group plotted positions were found close together while PG-inoculated and sham-inoculated were both positioned away. Though PG-inoculated and sham-inoculated clustered together along PC2, they were the most distantly plotted points from each other when viewed with respect to PC1. In contrast, when Euclidian metrics of community dissimilarity were used, which only take in account presence-absence and not abundance, sham-inoculated, baseline, and pregnant microbiomes clustered closely together while PG-inoculated microbiome plotted distantly.

Similarities and Differences in the Oral Microbial Composition in Healthy Non-pregnant Uninfected Rats

Specimens collected prior to inoculation (baseline) as well as specimens collected from sham-inoculated animals were used as uninfected controls for this study. Baseline profiles represent the normal oral flora in 2-month old female rats prior to antibiotic treatment, whereas the sham-inoculated group represents age-related 5-month old socially mature rats previously treated with antibiotics and sterile vehicle. When comparing the microbiome profiles between baseline and sham-inoculated groups, the two dominant families identified were *Pasteurellaceae* and *Streptococcaceae* (**Figure S1**). *Pasteurellaceae* made up 39.55% of the population observed for the baseline group, and 44.32% of the population in the sham-inoculated group. The dominant species identified within the *Pasteurellaceae* (75–85%) was *Haemophilus parainfluenzae* at 75–85%.

Notable differences at the family level between the sham and baseline groups were a relatively larger population of *Lactobacillaceae* in the sham-inoculated group (15.81%) compared to the baseline group (1.13%) and the relatively larger population of *Staphylococcaceae* in the baseline group (43.56%) compared to the sham group (17.83%). A lesser but notable difference was also observed for *Veillonellaceae* (8.14% sham-inoculated; 2.95% baseline) and *Micrococcaceae* (0.37% sham-inoculated; 6.20% baseline).

Roughly half of all mapped reads (OTUs) in each test group derived from the same animals could be identified down to the species level (i.e., 50.0% of baseline; 54.6% of PG-inoculated; 58.0% of pregnant) while a larger percentage of Sham-inoculated OTUs could be mapped to the species level (68.8%).

It was difficult to evaluate changes in the *Streptococcus* at the species level among all groups due to the high level of species level slash calls for this genus (**Figures S2**, **S3**). Slash calls are sequences (OTUs) that aligned with more than one possible reference sequence, and where the percentage difference between these top hits is $\leq$0.2% of the sequence. For example, only 11.04% of the *Streptococcus* population could be further identified to the species level in the baseline group while 53.73% of the *Streptococcus* population in the sham-inoculated group could be identified to the species level. However, we could specifically review select species, for example: 3.52% of the microbial population in the baseline group and 1.67% in the sham-inoculated group were identified as *Streptococcus sanguinis*. Overall, our data illustrates that *Streptococcus* species are typically diverse in the rat oral cavity and that the majority of the OTUs that mapped to *Streptococcaceae* family are currently unmappable to the species level.

Similarities and Differences in the Oral Microbial Composition in Healthy Non-pregnant Rats in Response to *P. gingivalis* Inoculation

To determine *P. gingivalis*-induced changes in the normal oral microbiome, we compared their consensus microbiome profiles collected post-inoculation (PG-inoculated) to samples collected prior to infection (baseline). We found that *Porphyromonadaceae* was present in the PG-inoculated group, albeit a at 0.02% of the total mapped microbiome population, confirming animals were indeed colonized.

At the family level (**Figure S1**), *Streptococcaceae* and *Pasteurellaceae* were the most dominant; however, the proportion of *Pasteurellaceae* decreased after *P. gingivalis* inoculation from 39.5% at baseline to 14.4% in the infected non-pregnant animals (PG-inoculated). In contrast, the *Streptococcaceae* population slightly increased from 43.6% at baseline to 55.4% in the PG-inoculated group.

Again, comparative analyses of *Streptococcus* species were incomplete due to the high level of slash calls that mapped to this genus (**Figures S2**, **S3**). Nevertheless, our data indicate that the population proportion distribution of *Streptococcus* species altered in response to *P. gingivalis* colonization. Specifically, we identified the following species in the baseline group, in order

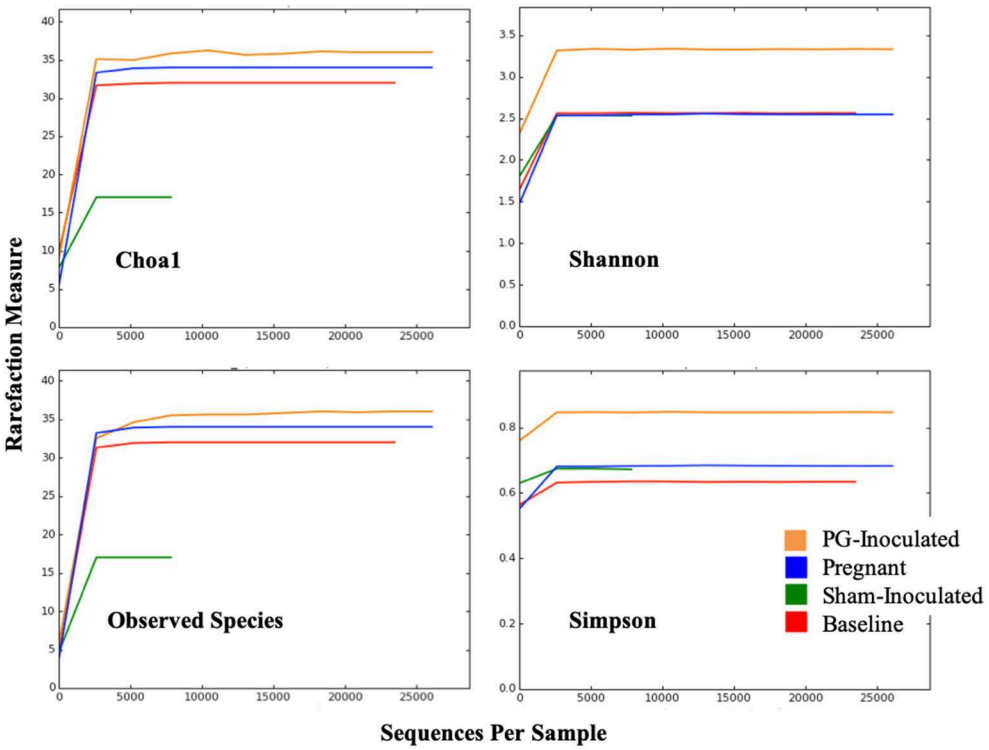


FIGURE 3 | Alpha diversity was evaluated by four different statistical analyses including: Observed Species, Simpson Index, Shannon Index, Chao1 index.

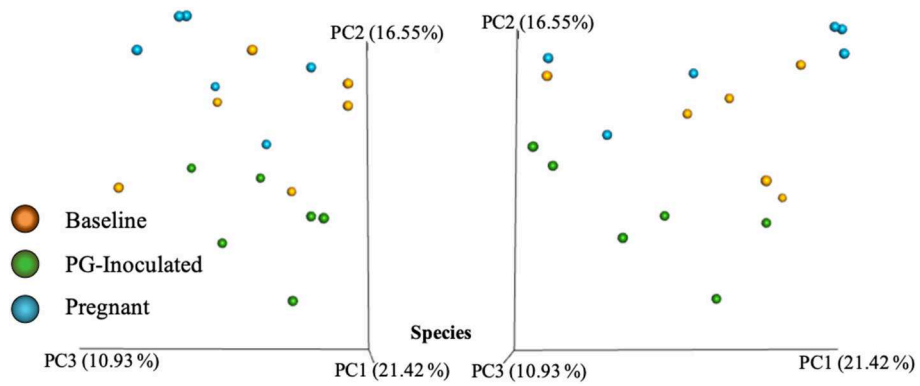


FIGURE 4 | Bray Curtis PCoA plot of beta diversity measures of dissimilarity shows at the species level, baseline, PG-inoculated, and pregnant groups clearly clustered within their group along PC2 not PC1.

of decreasing population proportion: *S. sanguinis, S. mutans, S. hyointestinalis, S. infantis, S. oralis, S. australis, S. danieliae, S. suis, S. lactarius,* and *S. pneumoniae.* Whereas, the species that could be specifically identified in the PG-inoculated group, in order of decreasing population proportion, were *S. mutans, S. sanguinis, S. infantis, S. oralis, S. lactarius, S. australis, S. sinensis, S. pneumoniae, S. merionis, S. parasanguinis, S. anginosus,* and *S. pseudopneumoniae.*

Other changes noted in the PG-inoculated microbiome profile were an increase in proportion of *Proteus mirabilis* and *Enterococcus faecalis* species as compared to controls. Overall, these data suggest that *P. gingivalis* inoculation perturbed the oral microbiome, leading to a shift in the microbiome profile with increased microbial diversity, which corroborates with our alpha and beta diversity statistical analyses.

Shifts in the Oral Microbial Composition of *P. gingivalis* Infected Rats in Response to Pregnancy

In order to evaluate the interaction of pregnancy and periodontal disease, we compared the consensus oral microbiome profiles prior to inoculation (baseline), at the end of the inoculation phase

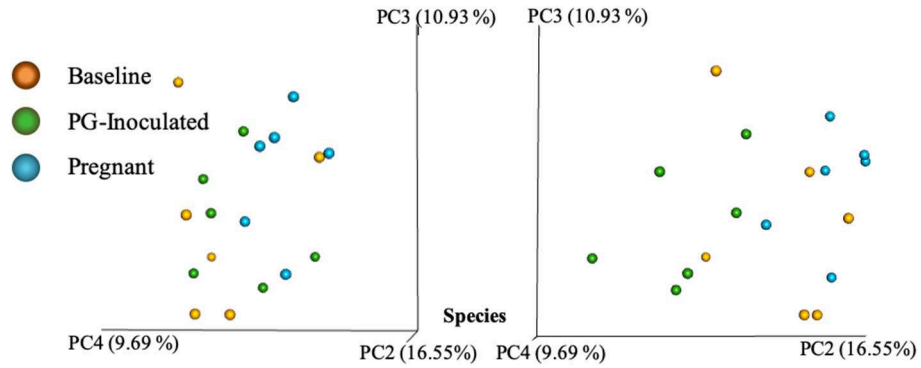


FIGURE 5 | Bray Curtis PCoA plot of beta diversity measures of dissimilarity shows at the species level, baseline, PG-inoculated, and pregnant groups clearly clustered within their group along PC2 and PC4 but not PC3.

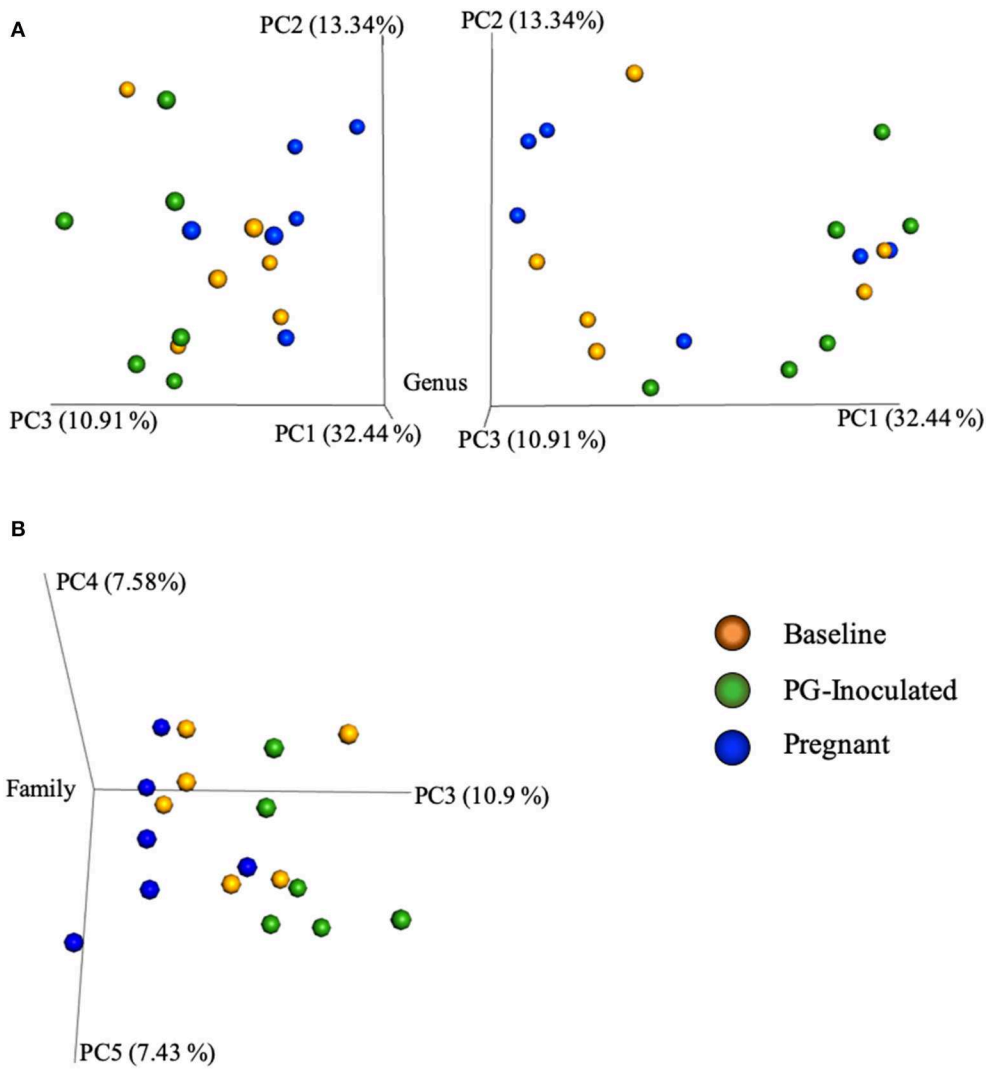


FIGURE 6 | Bray Curtis PCoA plot of beta diversity measures of dissimilarity shows that at the genus level, the baseline, PG-inoculated, and pregnant groups clustered within their groups along PC3 at the **(A)** genus level and **(B)** family level.

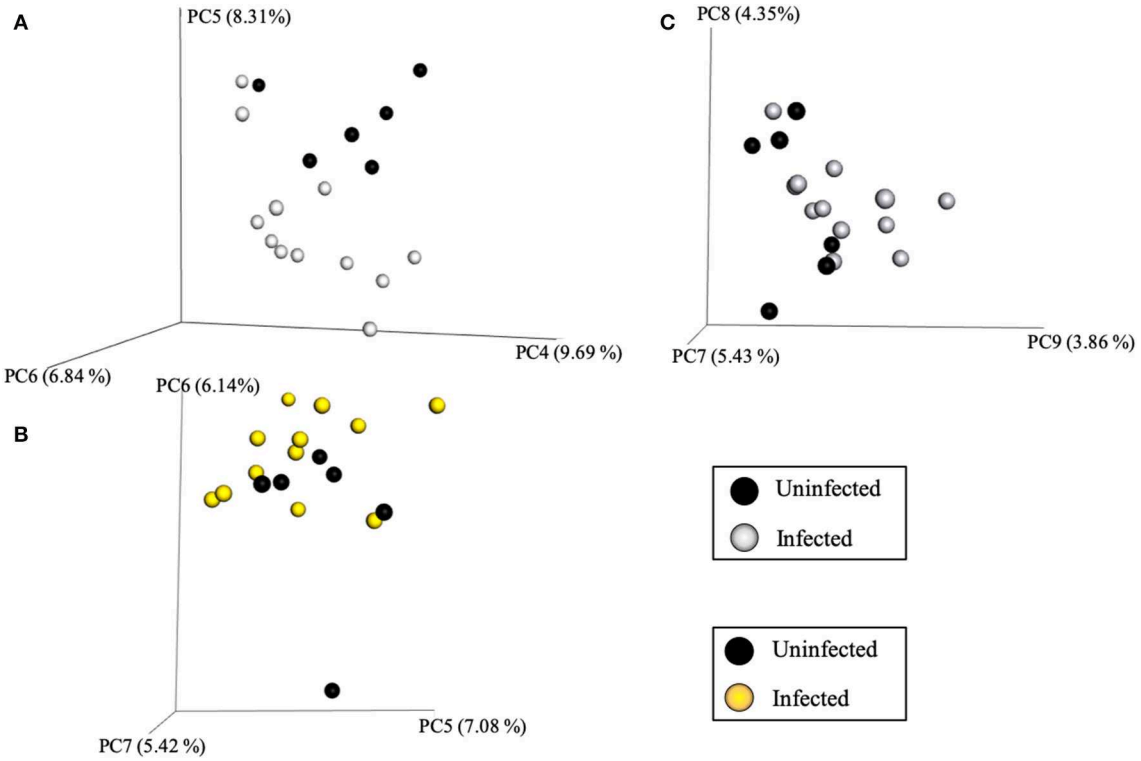


FIGURE 7 | Bray Curtis PCoA plot of beta diversity measures of dissimilarity shows, when the data sets were sorted by infection status, they clustered within their groups along PC5 at the species level **(A)**, PC6 at the genus level **(B)**, and PC9 at the family level **(C)**.

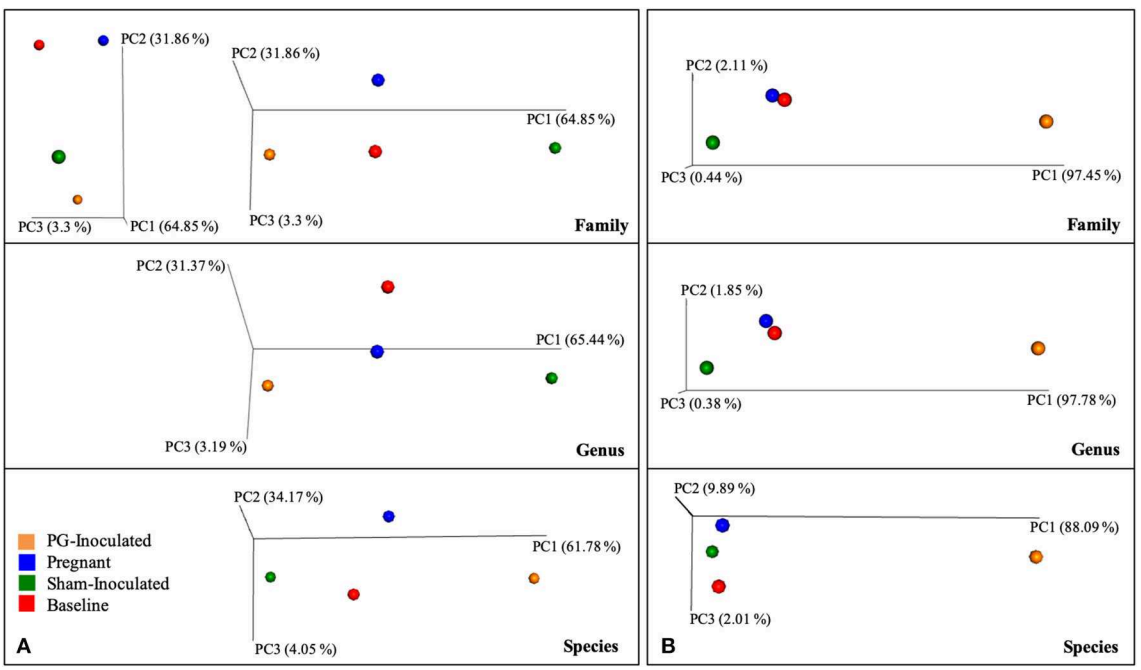


FIGURE 8 | Collective consensus microbiome at the family, genus, or species level and plotted as four data points in PCoA plots using **(A)** Bray Curtis or **(B)** Eucledian of beta diversity measures of dissimilarity.

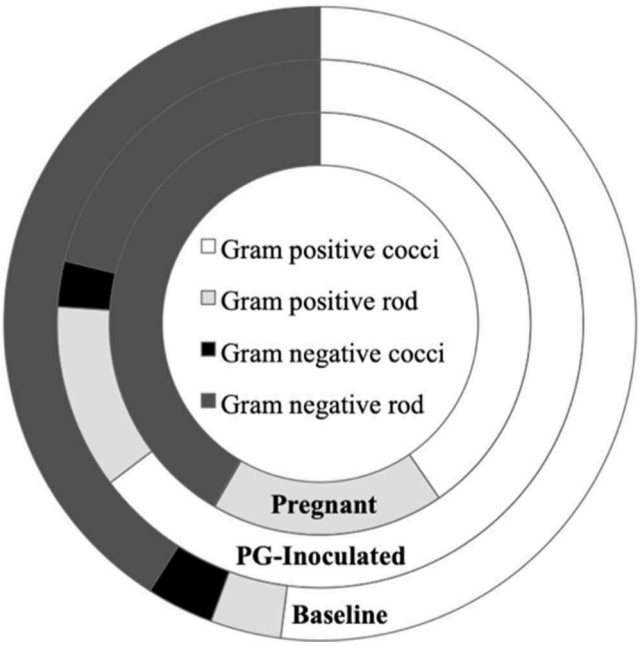


FIGURE 9 | Population proportion of identified community members in terms of predicted Gram stain classification and cell morphology for baseline, PG-inoculated, and pregnant groups.

(PG-inoculated), and at GD 18 (pregnant). As in the baseline and PG-inoculated groups, *Streptococcaceae* and *Pasteurellaceae* remained the two most dominant families in the pregnant group microbiome (**Figure S1**). There were however, some distinctive differences and notable shifts in the pregnant group. For example, *Streptococcaceae* and *Pasteurellaceae*, appear to be reverting toward baseline levels. A similar trend was observed among the other less common families with the following notable exceptions. First, *Porphyromonadaceae,* which was present only in PG-inoculated and pregnant groups, increased from 0.02% in the PG-inoculated group to 0.74% in the pregnant group. Second, *Corynebacteriaceae* population notably increased to 13.49% in the pregnant group microbiome from 1.93% in the baseline group and 3.13% in the PG-inoculated group.

At the species level (**Figures S2**, **S3**), *Haemophilus parainfluenzae* rebounded and became the dominant single species identified in the pregnant group. Similarly, many of the minor species that appeared in the PG-inoculated time point reverted to near baseline levels during pregnancy. Notable exceptions included *Corynebacterium mastitidis* which made up 98.96% of identified *Corynebacterium* species. Again, changes in *Streptococcus* at the species level could not be fully assessed due to the high level of slash calls. The fraction of the population within the *Streptococcus* genus that could be positively identified down to the species level in the pregnant group was 10.78%, which was similar to the 11.04% at baseline, but less than the 47.49% of the PG-inoculated group. Though a large proportion of *Streptococcus* OTUs could not be mapped down to single species, the standardized methodology used prohibits group specific bias so species comparisons among groups are valid. To illustrate how consistent the data was, allowing group profile comparisons, we assessed relative population proportions of *S. mutans* relative to all OTU categories. For example, *S. mutans* made up 2.18% at baseline, 28.61% at PG-inoculated, and 2.75% at pregnancy of all *Streptococcaceae* family OTUs. These numbers correlate with the population proportion of *S. mutans* relative to all identified species level OTUs (1.83% baseline; 29.05% PG-inoculated; 1.63% pregnant), as well as the percent proportion of all OTUs (0.91% baseline; 15.85% PG-inoculated; 0.95% pregnant) that mapped to any microbial family. Overall, this genus appears to be consistently diverse with *S. sanguinis*, *S. mutans*, and *S. infantis* being the most dominant members identified.

Metagenomic Shifts Relative to Morphological Classification, Oxygen Requirement, and Major Metabolite Profiles

In addition to assessing the changes in microbiome profiles in terms of microbial identity, we also performed predictive analyses of select microbial cellular functions. The oral microbiome has classically been sorted by morphological characteristics and Gram's classification so the first step in our functional analysis was to sort our consensus data in these terms. Though no spirochetes were detected, shifts were observed in the consensus profiles of Gram-positive and Gram-negative bacteria (**Figure 9**). For example, when the conditions in the host shifted from baseline to PG-inoculated, there was an increase in Gram-positive bacteria, most notably, there was over a 3-fold increase in Gram-positive rods and a 2-fold decrease in Gram-negative rods. The pregnant group showed an even larger increase in Gram-positive rods, reflecting an almost 5-fold increase relative to baseline. In contrast, the population proportion of Gram-negative rods shifted back toward baseline levels during pregnancy. The population proportion of Gram-negative cocci was similar between the baseline and PG-inoculated groups, but decreased from ∼3% down to 0.1% in the pregnant group. The observed changes become more interesting when the oxygen requirements of the detected members of the consensus population were considered in our following analysis.

We then profiled microbial populations based on their oxygen requirement (**Figure 10A**). While the proportion of facultative anaerobic bacteria remained relatively constant between baseline and PG-inoculated, the proportion of strictly anaerobic bacteria shifted down and the proportion of aerobes shifted up by 0.6% of the total population (**Figure 10B**). In contrast, the population proportion of facultative anaerobes shifted down from 93.7 to 81.5% in response to pregnancy. This change was accompanied by a decrease in strict anaerobes (2.8–1.7%) and an increase in strict aerobes (3.4–16.8%), reflecting an overall increase in oxygen requirement of the oral microbiota.

We next profiled the predicted microbial metabolomes of all groups. Among the wide variety of metabolites that could be identified, 2, 3-butanediol, butyrate, lactic acid, and propionate were found to be the most population defining products by which most members of each microbiome could be differentially sorted. *Anaerostripes* is the only genus that we determine should

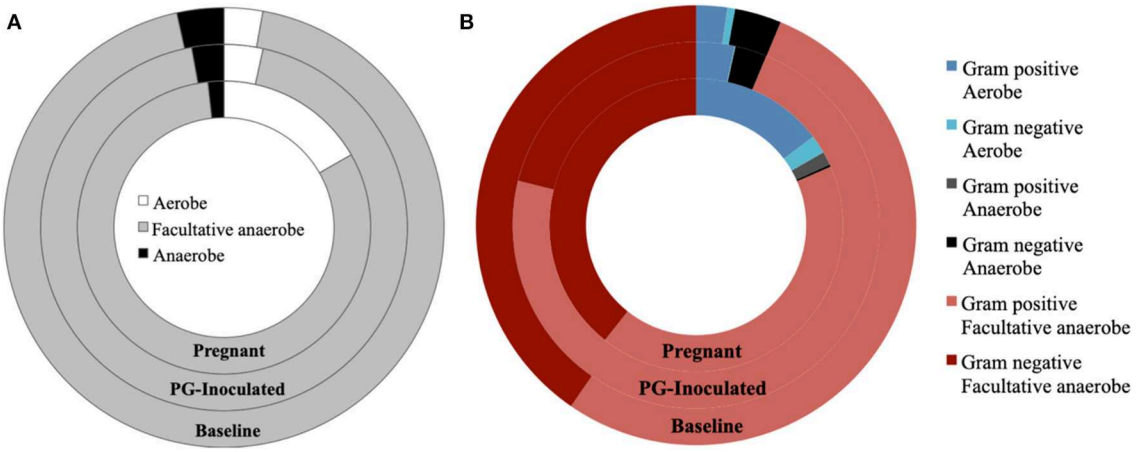


FIGURE 10 | Population proportion of identified community members in terms of predicted **(A)** oxygen requirements alone, or **(B)** oxygen requirements in context of Gram stain classification, for baseline, PG-inoculated, and pregnant groups.

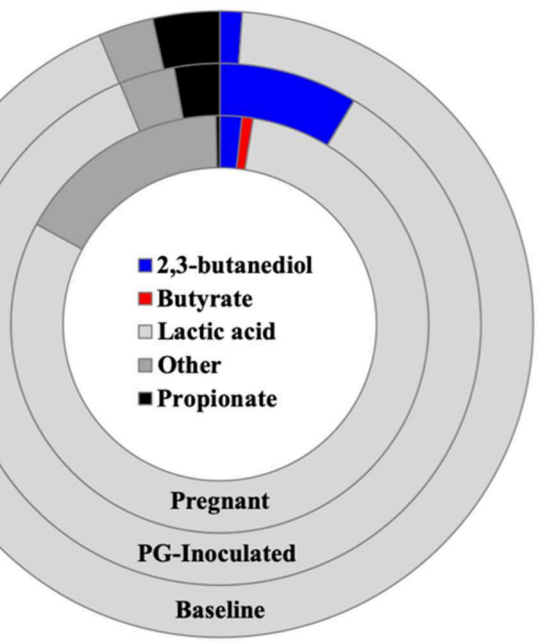


FIGURE 11 | Population proportion of identified community members in terms of predicted metabolic characteristics as a major generator of target microbial metabolites for baseline, PG-inoculated, and pregnant groups.

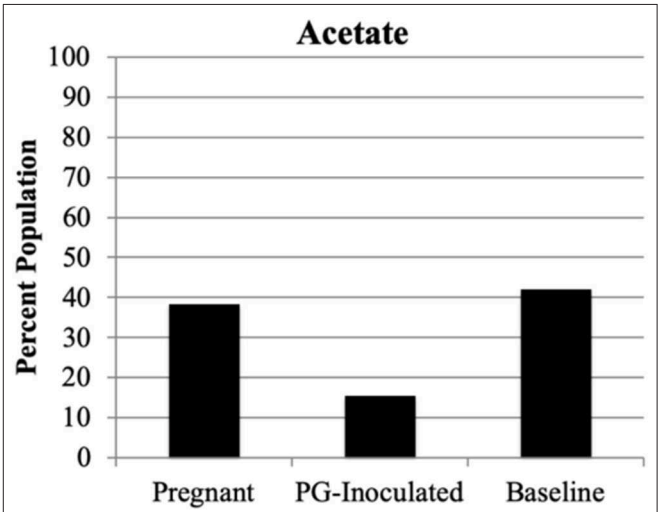


FIGURE 12 | Population proportion of identified community members in terms of predicted metabolic characteristic as a major generator of acetate for baseline, PG-inoculated, and pregnant groups.

be categorized as belonging to both the butyrate and lactic acid major metabolite groups. All others could be sorted into one of these four metabolite groups or placed into the "Other" group as not a major producer of these metabolites (**Figure 11**). The predicted population proportion of lactic acid producers decreased from 92.57% (baseline) to 85.15% (PG-inoculated) to 80.50% (pregnant). The proportion of propionate producers decreased from 3.44%, (baseline) to 2.81% (PG-inoculated) to 0.30% (pregnant). In contrast, the proportion of butyrate producers progressively increased from 0%, (baseline) to 0.02% (PG-inoculated) to 0.86% (pregnant). The proportion of 2, 3-butanediol producers increased from 1.15% (baseline) to 8.59% (PG-inoculated) in response to PG-infection but then decreased to 1.70% during pregnancy. The remaining population portion of bacteria designated as "Other" increased over 5-fold during pregnancy, reflecting the increase in obligate aerobes in the oral cavity of pregnant rats at GD 18. The ability to produce acetate as a major metabolite was also assessed but plotted independently because it was typically co-produced with one of the select four metabolites (**Figure 12**). The proportion of acetate producers decreased from 42.03% (baseline) to 15.34% (PG-inoculated) but increased in response to pregnancy to 38.38%.

Because the ability to synthesize riboflavin (Vitamin B2) is associated with gastrointestinal (GI) health (Yoshii et al.,

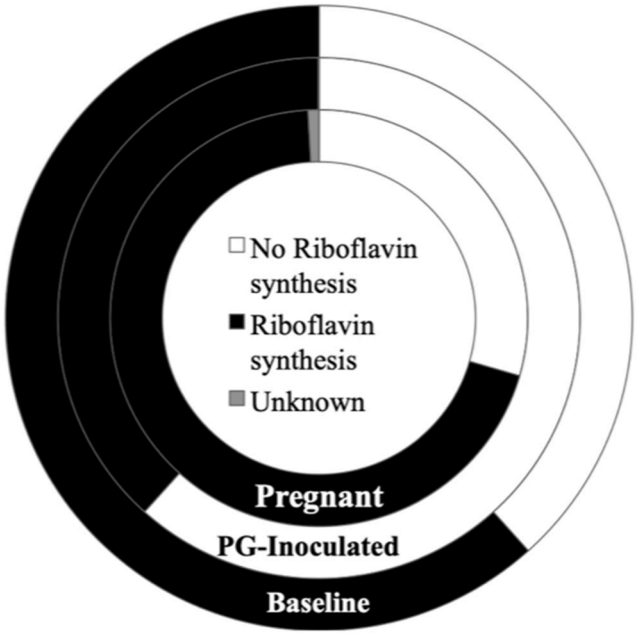


FIGURE 13 | Population proportion of identified community members in terms of predicted ability to synthesize riboflavin for baseline, PG-inoculated, and pregnant groups.

2019) and many of the species identified in the oral cavity also colonize the GI, we performed an independent assessment of riboflavin producers within the consensus microbiome profile of each group (**Figure 13**). Riboflavin biosynthesis capability was determined by the genomic presence of the complete pathway, using the KEGG pathways database. The oral microbial population's predicted collective capability to synthesize riboflavin dramatically decreased from 85.4% (baseline) to 43.1% (PG-inoculated) in response to infection, but this ability recovered during pregnancy by GD 18, increasing to 95.8% (pregnant). Notably, a small proportion of the population (∼1% of the pregnant group and 0.1% of the PG-inoculated group) could not be positively or negatively categorized due to limitations in the reference KEGG database at the time of analysis. A cursory analysis of all groups showed that ≤0.2% of each consensus microbiome profile had the predicted ability to feed into the porphyrin metabolism pathway, using riboflavin as an early precursor metabolite, to ultimately synthesize vitamin B12 (data not shown).

DISCUSSION

The Oral Microbiome of Healthy Rats Compared to Previously Published Human and Rat Profiles

The Forsyth Institute created a publicly available human oral microbiome database (www.homd.org). Using whole 16S rRNA gene sequencing, they reported that 96% of the human oral microbiome consists of six phyla (*Firmicutes* 36.7%, *Bacteroidetes* 17.3%, *Proteobacteria* 17.1%, *Actinobacteria* 11.6%, *Spirochaetes* 7.9%, *Fusobacteria* 5.2%) (Dewhirst et al., 2010). In contrast, we found that the bacteria we identified at baseline in the oral cavity of rats belong to only three phyla (*Firmicutes* 48.10%, *Proteobacteria* 43.64%, *Actinobacteria* 8.26%). The undetected phyla of *Bacteroidetes, Spirochaetes*, and *Fusobacteria* include key periodontopathogenic bacteria associated with human periodontal disease and implicated in adverse pregnancy outcomes (e.g., *P. gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Fusobacterium nucleatum*) (Aagaard et al., 2014; Prince et al., 2016; Lin et al., 2018). After *P. gingivalis* inoculation, the consensus oral microbiome included four phyla (*Firmicutes* 72.22%, *Proteobacteria* 24.61%, *Actinobacteria* 3.15%, *Bacteroidetes* 0.02%) with the family *Porphyromonadaceae* making up 100% of the identified *Bacteroidetes* phylum population, consistent with experimentally induced oral infection. By GD 18, the orally infected pregnant rat oral microbiome consisted of the same four phyla, but with notable population shifts (*Firmicutes* 41.69%, *Proteobacteria* 40.11%, *Actinobacteria* 15.54%, *Bacteroidetes* 2.66%). The *Bacteroidetes* phylum in the pregnant group now consisted of four families, including *Porphyromonadaceae, Bacteroidaceae, Cytophagaceae*, and *Flavobacteriaceae*.

We found that the most abundant genus in the oral cavity of our rat model at baseline was *Streptococcus*, similar to the reported findings of the human oral microbiota (Dewhirst et al., 2010). With regards to the two most abundant genera at baseline, *Streptococcus* and *Haemophilus* are known to specifically adhere to each other (co-aggregate) during biofilm development due to outer membrane adhesions found on some *Streptococcus* species that recognize specific polysaccharide receptors on *Haemophilus parainfluenzae* (Lai et al., 1990; Heller et al., 2016). Interestingly, the large percentage of highly diverse *Streptococcus* species and the high percentage of *Haemophilus parainfluenzae* observed in these rat oral microbiomes was also observed in the development of early human dental biofilm (Heller et al., 2016), suggesting that the periodontitis and pregnancy rat model used in this study could be considered an appropriate translational model for oral pathology as it represents a relatively similar oral flora to that of a human, but with the notable absence of periodontopathogenic bacteria. Rather than a shortcoming, we view this absence as a critical advantage of this model in that it allows controlled colonization of the oral cavity with periodontopathogenic bacteria to investigate their role in oral and extra-oral disease development.

Due to few previously completed studies in the area of oral microbiomes of rats, it was difficult to compare our results to other published data. The only study we believe to be somewhat equivalent to ours for comparison was the Manrique et al. study that characterized the normal oral flora of healthy Sprague-Dawley rats by collecting the supragingival plaque of the upper molars (Manrique et al., 2013). However, the Manrique study found that *Rothia* dominated the oral cavity at 74.43% and the next most abundant bacterial genus were *Streptococcus* (4.67%). Though OTUs that mapped to the *Micrococcaceae* family was identified in all of our experimental groups (6.2–0.02%), *Rothia* was not universally found in our study. *Rothia* OTUs were found in the baseline consensus microbiome of our study at a

very low proportion (1.4% total oral microbial population). The proportion found in the other groups were even lower, with 0.5% in the pregnant group and 0% (undetected) in both the PG-inoculated and sham-inoculated groups. Instead, our study found that the two most dominant genus at baseline, representing normal oral floral of healthy rats, were *Streptococcus* (41.77%) and *Haemophilus* (32.69%).

Though microbiome profiles reflect population variation among different animal groups, the observed differences between studies are also impacted by differences in animal colonies, sampling technique and differences in microbiome profile generation analysis parameters, such as stringency criteria and the reference databases used. We believe a major source of variation between our outcomes relative to recent publications are the differences in how the 16S libraries were generated. Most published studies that have reported loci specific 16S microbiomes of rats typically use sequencing libraries targeting only one or two variable regions of the 16S rRNA gene. The Manrique et al. study, for example specifically targeted the 5-6 variable region of the 16S rRNA gene, while we had more complete coverage of the target gene by sequencing seven of the nine variable region amplicons in each library (Manrique et al., 2013). Although they had a larger depth of read by using the 454 pyrosequencing platform, and thus were likely able to identify very rare species (Manrique et al., 2013), we had much better coverage of the target gene. Targeting seven sites of the 16S gene generates microbiome profiles that are reportedly comparable to full length 16S gene sequencing microbiomes, while targeting a single or only a few variable regions results in incomplete microbiome profiles (Bolchakova et al., 2015). Our data supports this observation when we viewed primer specific microbiome profiles generated from our data sets. For example, when viewing the consensus baseline microbiome profile with respect to each primer set, OTUs that could positively map to *Rothia* species only occurred for sequences derived from the V4, V6-7, and V8 primers among the seven sets used. Similarly, we found that *Streptococcaceae* dominated the oral microbiome profile (66.74–85.29%) when using the same technique in Sprague Dawley rats (Reyes et al., 2018). This suggests that our consensus profiles more accurately reflect the population proportions of the identified family, genus, and species than studies using only one or two variable regions for analysis.

Oral Community Diversity in Pregnant Rats With Periodontal Disease Was Relatively Similar to Uninfected by GD 18 in Terms of Abundance and Evenness

While the relative diversity of an individual's microbiome does not necessarily indicate disease (He et al., 2015), there is reported evidence that the oral microbiome from patients with periodontal disease typically display greater diversity (Griffen et al., 2012; He et al., 2015), while data reported for other diseases such as data reported for dental caries typically display a reduction in diversity as a consequence of an increase in abundance of certain species in the flora isolated from the affected sites that are contributing to pathology (He et al., 2015). Among methods used to determine diversity, alpha diversity measures that take into account both abundance and evenness are reported to be better predictors of biologically relevant diversity (McCoy and Matsen, 2013). In our study, the Shannon and Simpson indices seem to indicate that the alpha diversity between the two control groups (baseline and sham-inoculated) were very similar. However, the Shannon and Simpson diversity indices estimated for the pregnant group was likewise similar to the controls. In other words, collectively, the population proportion of positively identified species were similar between pregnant, baseline, and sham-inoculated groups, despite sham-inoculated group having the fewest number of positively identified species, while PG-inoculated group had the greatest diversity. Considering the pregnant animals had periodontal disease, as indicated by their level of alveolar bone loss, our data illustrates the limitations of using alpha diversity analysis as a strict independent indicator of disease. A possible explanation for our observations that would be consistent with previous reports is that by GD 18, periodontal disease progression may have arrested and was on a trajectory of healing and perhaps the diversity indices reflect a state in which the oral microbiome is shifting back toward health.

While alpha diversity rarefaction curves reaching plateau reportedly indicate that that all but the rarest species were identified, this interpretation is based on some assumptions with respect to sample collection and processing. Due to some uncertainty that there was enough template sequenced for some of our libraries to comfortably claim that the data collected accurately represent the oral community found since there were differences between the number of reads each sample generated, we chose to create Pool D for a subset of samples identified as having the fewest number of reads from Pools A, B, and C. When comparing the data sets generated from each repeated sample, we found that their individual microbiome profiles, as analyzed by Bray Curtis beta diversity and visualized in PCoA plots, remained tightly clustered to each other and within their experimental group, thus all consensus data reported here included all the collected sequence data from all four pools A–D. This outcome also supports our belief that our samples, including those samples that had the fewest OTUs after their first sequence run in pools A–C, were of adequate sample size to create representative profiles of the microbiome found in the oral cavity of each animal, within the limits of the reference databases used.

Oral Community Diversity in Terms of Species Composition Dissimilarities Were Conditionally Dependent, Shifting in Response to Both PG-Inoculated and Pregnancy

The advantage of using beta diversity analysis, as our approach to community analysis, is that PCoA allows discovery of the most important axes along which samples vary (dissimilarity). When using an appropriate beta diversity metric, PCoA plots also identifies similar samples as clustered points along an ax(s) of variance through visual examination of the plots. Bray-Curtis dissimilarity was selected because it is one of the most well-known approaches to quantify the difference (distance) between samples. This metric is also based on OTU abundance rather than just presence-absence data; thus the "size" and "shape"

of the count vectors are taken into account in this statistical measure, which ranges from zero to one with zero defined as identical. Because the potential sources of variance (e.g., variables of interest) are not pre-defined before performing this type of statistical analysis, identifying the source of the data variance (why they cluster) may not be obvious. It can be particularly challenging for complex, multifactorial, non-categorical, or longitudinal data.

Intra-animal Bray-Curtis beta diversity analysis between baseline, PG-inoculated, and pregnancy appear widely scattered along PC1 when each animal's microbiome was plotted individually (**Figure 4**), but when the consensus data of each group was plotted as single points, this metric suggests that *P. gingivalis* infection was the greatest source of population variation in this study (**Figure 8**). Each individual sample point from baseline, PG-inoculated, and pregnant groups did clearly cluster within their groups along the PC2 axis at the species level, indicating that collectively, the experimental group conditions were the second greatest source of species variation (**Figure 4**). At both the genus and family level, however, the ability to distinctly discriminate between experimental groups shifted from PC2 to PC3, indicating that the core population proportions of member family and genus were more resistant to perturbations at each collection time-point over the course of the study than were specific species identity. Together, all distance measures data reported in our results (**Figures 4**–**8**) suggest that community variance in terms of member composition was most influenced by oral infection. This outcome is supported by the alpha indexes, which showed that the greatest diversity in terms of abundance and community evenness was observed in the PG-inoculated group prior to breeding and that the population became more even during pregnancy by GD 18.

When Bray-Curtis beta diversity analysis of consensus data included the sham-inoculated group, this point was positioned furthest from the center and was most distant from the baseline control point relative to PC1 (**Figure 8**), suggesting that the core consensus communities were most similar between groups sampled from the same animal set. In contrast, Euclidean beta diversity analysis showed that PG-inoculated group was the most dissimilar among the four groups along PC1 and PC3 axes, while the consensus points of the other three groups clustered together. Along PC2, however, the Sham-inoculated point was more dissimilar, particularly at the species level. Reported comparisons of different beta-diversity metrics used to compare community data (Legendre and de Caceres, 2013) suggest that Euclidean matrix is not appropriate when visualizing dissimilarity between individually plotted communities derived from complex samples (e.g., microbiomes from animals) since absence-presence data (without abundance) may put too high or low a value on each community member identified within each individual sample; however, it is useful to visualize dissimilarities between collective consensus community group data. It is important to recall that all of the animals used in this study came from the same colony and were housed the same way.

These data suggest that health and *P. gingivalis* inoculation induced periodontal disease guides microbial composition at the species level, that species level shifts are often reflective of their condition, and that species level dissimilarities typically become more pronounced during disease, irrespective of whether the samples were collected from the same animals (intra-group) or from a different set of animals. These data also support the concept that there are common core site-specific microbiomes among host species and that established microbiomes within an individual animal are resistant to gross dysbiotic shifts in response to host conditions when reviewed at higher taxonomic levels.

Analyzing Baseline vs. Sham-Inoculated Groups Suggest a Less Diverse but Recovering Core Microbiome 12-Weeks Post-antibiotic Treatment

The standard protocol for inducing periodontitis in rats is to first prophylactically treat animals with antibiotics, then allow for a short microbial recovery time before starting inoculations (Kesavalu et al., 2007; Verma et al., 2010; Phillips et al., 2018). Prophylactic antibiotics are known to cause a chemically induced dysbiosis (Rogers et al., 2010; Manrique et al., 2013). Hence, we included pre-antimicrobial treatment samples in our study to potentially identify such perturbations. Despite there being overall similarities in the oral communities of baseline and sham-inoculated groups in terms of alpha diversity, we identified a larger population proportion of *Staphylococcaceae* and *Lactobacillaceae* in both the baseline and PG-inoculated group animals relative to the non-pregnant sham group animals, suggesting intra-animal microbiome stability and inter-animal differences would make it difficult to perform meaningful inter-animal group profile comparisons. Consequently, we primarily focused on intra-animal time course microbiome comparisons in this report. However, we did draw some basic conclusions from our inter-animal comparisons as follows.

Baseline was used as our pre-antibiotic, uninfected group while sham-inoculated was our time-matched vehicle control of an oral community recovering from antibiotic induced dysbiosis since it had 12 weeks of recovery time without addition of further stressors. As shown in **Figure S1**, *Pasteurellaceae* populations were similar between our control groups but the *Staphylococcaceae* family shifted from 43.56% at baseline to 55.4% at PG-inoculated but was only at 17.83% in the sham-inoculated group. Moreover, there was altered *Streptococcus* species diversity in the sham-inoculated group. As stated in our results, our alpha diversity findings as measured by Shannon and Simpson indices for overall community species richness (abundance and evenness) indicate that sham-inoculated and baseline were more similar to each other than to PG-inoculated group consensus profiles. This result agrees with the findings of Manrique et al. which indicated that while antibiotics reduce the overall abundance of bacteria, the core microbial community structure remains the same when given time to recover from the perturbation (Manrique et al., 2013). Overall, our data suggests that the oral flora in the sham-inoculated group, while not the same as baseline group was likely recovering its "normal" microbiome core profile characteristics by the end of the 12-week sham inoculation period.

Infection and Pregnancy Produced an Altered Community Metabolic Profile Unique to Each Condition

When alpha and beta diversity analysis of the collective consensus microbiome of each group were compared (**Figures 3**, **8**), we observed a shift in response to *P. gingivalis* inoculation that induced periodontal disease that seemed to then revert toward baseline during pregnancy. A broad survey of population proportions among a wide variety of identified families, including the top 5 most dominant families, supported this trend. Thus overall, this data suggests that the rat oral microbiome has a family level commonality at its core. However, between animal variability, particularly at lower taxonomic levels, indicates that the oral microbiome at pregnancy was actually not that similar to baseline, making comparative analyses challenging.

Notable trends, microbiome shifts, and predicted associations were discovered that suggest certain broad characteristics (e.g., Gram classification, cell morphology) traditionally used to describe oral microbial populations "typical" of health or disease were relatively inadequate indicators overall of dysbiosis when compared to our assessment of the metagenomic changes in the population in context of predicted metabolic shifts. For example, the oral community progressively became more populated with Gram-positive rods and less populated with Gram-negative cocci. In contrast, Gram-positive cocci and Gram-negative rod population proportions shifted in response to infection then shifted back in response to pregnancy. Altered microbiome diversity and dysbiosis was still evident in the pregnant group when assessed in context of oxygen requirements and predicted major metabolite production, the latter of which is covered in more detail in the next section. Although the population proportion of the obligate anaerobe *P. gingivalis* was larger in the pregnancy group, we observed that by GD 18 that the now orally infected and pregnant rats had a more aerobic oral community overall (**Figure 10**). We also identified oral colonization of previously undetected opportunistic and commensal bacteria (**Supplementary Material**). Considering both pregnancy and periodontal disease impact the inflammatory and immune response (Guncu et al., 2005; Wu et al., 2015), these conditions would likely result in the host being much more vulnerable to colonization by these previously undetected organisms.

Insights and Limitations of the Predicted Morphological Classification, Oxygen Requirement, and Major Metabolite Production Profiles of Baseline, Infected, and Pregnant Animals

We found that the predicted population proportion of both lactic acid producers and propionate producers progressively decreased while butyrate producers progressively increased over time in response to *P. gingivalis* inoculation then in response to pregnancy by GD 18. Lactic acid production is generally associated with carbohydrate fermentation by oral *Streptococci* and other genera of the oral microbiome (McLean et al., 2012) which, combined with diet, contributes to excessive biofilm formation, low pH, and caries development in humans. When lactic acid production is reviewed in context of periodontal disease, our findings agree with consensus observations reported in the literature. Specifically, in a normal oral cavity during health, oral *Streptococci* typically dominate the oral microbial population but lose their foothold to periodontal pathogens, especially Gram-negative anaerobes (Takahashi, 2015).

Acetate, propionate, and butyrate are all short chain fatty acids (SCFAs), are produced by many bacteria by a variety of mechanisms, and have complex contextual associations with both health and disease. For example, propionate is a weaker acid than lactic acid, often generated though conversion of lactic acid, and is typically produced in the oral cavity by genera such as *Veillonella* and *Lactobacillus* (found in all of our groups), and *Clostridium* (absent in our control groups). Lactic acid conversion to propionate contributes to acid neutralization, and is believed to facilitate dominance of the more acid sensitive *Streptococci* species associated with dental health as well as facilitating growth of acid sensitive periodontal pathogens such as *Porphyromonas* (Takahashi, 2015). Both butyrate and propionate are major metabolites produced by oral bacteria such as *Porphyromonas* and *Clostridium* that use proteins and amino acids as a primary carbon source, typically co-generating ammonia though amino acid deamination, further contributing to acid neutralization (Takahashi, 2015). SCFA production is generally associated with health when produced in the gut by colonic bacteria, typically through fermentation of dietary fiber. Specific metabolites such as butyrate, when provided in a dietary supplement, have shown beneficial health effects in the host (van Immerseel et al., 2010; Herrema et al., 2017). In contrast, butyrate production in the oral cavity is reported to be cytotoxic in patients with oral disease, such as periodontal infection, and has been shown to be responsible for the release of reactive oxygen species in chronic periodontitis (Anand et al., 2016). These reported associations highlight the importance of site-specific studies in context of host-pathogen interactions. When delving into the particulars of microbial butyrate production among pathogens and commensals, Anand et al. showed that these organisms have evolved distinct pathways where, unlike typical commensal species, species that are recognized as pathogens typically co-generate harmful byproducts like ammonia along with butyrate, which explains how butyrate production could be associated with both health and disease (Anand et al., 2016).

The microbial metabolite 2, 3-butanediol is typically produced during carbohydrate fermentation. Major production of this metabolite by certain commensal oral *Streptococci*, when in association with the opportunistic environmental pathogen *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients, is associated with chronic disease and it is generally considered to be cytotoxic to human cells at high concentrations and long term exposure (Whiteson et al., 2017). Interestingly, *in vitro* work with dendritic cells showed that 2, 3-butanediol has an anti-inflammatory affect when co-incubated at below toxic concentrations, but it was suggested that inhibition of the immune response in tissues such as the mucosa may ultimately be detrimental, contributing to chronic infection (Whiteson et al., 2017). There is no known health benefit

of 2, 3-butanediol in humans, whether it is synthesized by bacteria or formed in mammalian cells, especially in the liver after ethanol consumption. Our data showed that the predicted population proportion of 2, 3-butanediol producers relative to baseline increased in response to oral PG-inoculation then decreased during pregnancy by GD 18. This outcome is consistent with having a dysbiotic or "unhealthy" microbiome post PG-inoculation.

Riboflavin (Vitamin B2) is an essential precursor used to form the major coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), leading to the synthesis of vitamin B3 and B6 respectively, as well as other forms of Vitamin B and numerous flavoproteins. Riboflavin is synthesized by a wide variety of bacteria. Some bacterial species (e.g., most Firmicutes and Bacteroidetes) are high producers of riboflavin, secreting excess into their environment. Because riboflavin is essential for growth of all living cells, some bacterial species import riboflavin synthesized by other bacteria because they are either poor or non-producers. Humans cannot synthesize or store these water-soluble B vitamins and are dependent on diet and on biosynthesis by resident bacteria (Magnusdottir et al., 2015). Because bacterial non-producers compete with host cells for available riboflavin, understanding the mechanisms the host uses to preserve health in the face of microbial mechanisms used to survive in the host, in context of microbially produced essential metabolites like riboflavin, may provide insights into the etiology of observed microbiome shifts. For example, bacterially produced riboflavin is believed to play a direct role in immune function in the host though at least two mechanisms. Riboflavin is associated with reactive oxygen species generation in innate immune cells through priming of NADPH oxidase (Yoshii et al., 2019). Intermediates formed during microbial riboflavin biosynthesis also activate MAIT (mucosal-associated invariant T) cells through binding to the MR1 protein of MHC-I molecules on antigen presenting cells (Eckle et al., 2015; Yoshii et al., 2019). However, stimulation by commensal microorganisms are insufficient to fully elicit MAIT cell effector function (Berkson and Prlic, 2017). Thus, commensal bacterial mediated priming of MAIT cells is proposed to contribute to their immunological role in pathogen surveillance, which are then fully activated in the presence of pathogens at sufficient microbial load. Consequently, the presence of riboflavin biosynthesis intermediates has reportedly been used as a biomarker for microbial infection (Eckle et al., 2015).

Although we inoculated the oral cavity of rats with *P. gingivalis*, a robust and self-sufficient riboflavin producer, the consensus microbiome post-inoculation in animals with periodontal disease did not reflect overall increased community riboflavin production. Rather, we observed that the oral microbial population's predicted ability to synthesize riboflavin decreased by ∼50% in response to infection and then rebounded to surpass baseline levels during pregnancy by GD 18. With shallow understanding, this outcome would seem to contradict an expectation of high riboflavin production due to infection by *P. gingivalis*. However, presuming riboflavin production is generally beneficial in mammalian mucosa as reported in the literature for the GI tract, our data would be consistent with development of an "unhealthy" microbiome followed by recovery to a more beneficial microbial profile when viewed in terms of riboflavin production. This highlights how the consensus microbiome profile and its metabolome may better reflect important aspects of microbial health or dysbiosis that would not be revealed if only targeted characterization of a specific metabolic contribution of individual pathogens identified within a community was performed. Yet in this context, knowing the site-specific host response to individual pathogens increases our overall understanding of the disease process.

It is worth noting that the levels of MAIT cells found in different tissues sites differ, presumably impacting the local inflammatory response outcomes. Specifically, the abundance of MAIT cells in human apical periodontal tissues are reported to be similar to levels found in peripheral blood but markedly higher than levels found in gingival tissues (Davanian et al., 2019) and lower than levels found in the walls of the large intestine (epithelium and lamina propria) (Hama et al., 2019). Overall, microbial species that act as pathogens at sufficient microbial load, such as *P. gingivalis*, and that are also efficient riboflavin producers would presumably contribute to the localized chronic inflammatory response characteristic of periodontal disease. However, riboflavin producing pathogens may induce a more robust MAIT cell response in the intestines compared to the gingiva, or perhaps have a lower pathogen load threshold, due to the higher levels of resident MAIT cells. How increased or decreased riboflavin availability may further modulate the microbiome profile or disease development due to inherent differences in the environment, microbial load, and site-specific tissue characteristics of the gingiva are unknown and should be further investigated.

Collectively, our outcomes and current understanding shined a light on certain metabolic characteristics in the context of the observed microbiome profiles that we believe should be the target of future studies, including directly assessing select metabolic shifts of the oral microbial population in context of oral infection and pregnancy. However, we recognize that there are several limitations to this pilot study that may have negatively impacted our interpretations. A key limitation in interpreting microbiome data in general lies in the dependence on reference databases to create each microbiome profile. Because unmapped OTUs (**Figure S3**) are essentially discarded, metagenomic analyses can only "discover" and infer characteristics about the microbial community relative to what is already "known." In the future, when more reference genomes become available, these data can be reanalyzed to map the un-mapped reads. Similarly, in this study, we observed a large number of slash calls, thus limiting the level of the taxonomic hierarchy we could confidently compare between microbiomes. This is likely due to similarities of the particular 16S variable region sequence between closely related species. However, it is possible that with additional reference genomes, these slash calls might be differentiated.

When we designed this project, we opted to focus our pilot study on determining the longitudinal intra-animal shifts in the oral microbiome in response to *P. gingivalis* inoculation and pregnancy and did not assess the oral microbiome of sham-inoculated pregnant rats. It would be interesting to parse out the impact of pregnancy alone on the oral community in a future study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Wisconsin Animal Care and Use Committee, University of Wisconsin–Madison.

AUTHOR CONTRIBUTIONS

PP and her graduate student MW conducted all sample processing, sequencing, and sequencing data analysis. LR and her lab technician GP conducted all animal experiments, animal pathology assessments, and sample collection. PP, LR, AP-F, and MB are coinvestigators that together planned, developed, and sought funding for several collaborative projects that specifically targeted the role of oral pathogens in disease development in context of pregnancy. PP, LR, and MW are the primary writers of the manuscript with editorial contributions by the rest of the co-authors.

FUNDING

This work was funded by the Graduate Program Committee at Kirksville College of Osteopathic Medicine, A. T. Still University and the Eunice Kennedy Shriver National Institute of Child Health & Human Development of the National Institutes of Health under Award Number R15HD081439.

ACKNOWLEDGMENTS

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We would also like to acknowledge the assistance provided by Zachary Siress while he was being trained in our lab at A. T. Still University in performing various molecular techniques and Ms. Emily Director of UW Zoology Museum who assisted with defleshing of rat jaws. *P. gingivalis* strain A7UF was provided by Ann Progulske-Fox from the University of Florida.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00092/full#supplementary-material

REFERENCES

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., and Versalovic, J. (2014). The placenta harbors a unique microbiome. *Sci. Transl. Med.* 6:237ra265. doi: 10.1126/scitranslmed.3008599

Abusleme, L., Dupuy, A. K., Dutzan, N., Silva, N., Burleson, J. A., Strausbaugh, L. D., et al. (2013). The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J.* 7, 1016–1025. doi: 10.1038/ismej.2012.174

Anand, S., Kaur, H., and Mande, S. S. (2016). Comparative *in silico* analysis of butyrate production pathways in gut commensals and pathogens. *Front. Microbiol.* 7:1945. doi: 10.3389/fmicb.2016.01945

Barak, S., Oettinger-Barak, O., Machtei, E. E., Sprecher, H., and Ohel, G. (2007). Evidence of periopathogenic microorganisms in placentas of women with preeclampsia. *J. Periodontol.* 78, 670–676. doi: 10.1902/jop.2007.060362

Berkson, J. D., and Prlic, M. (2017). The MAIT conundrum - how human MAIT cells distinguish bacterial colonization from infection in mucosal barrier tissues. *Immunol. Lett.* 192, 7–11. doi: 10.1016/j.imlet.2017.09.013

Bolchakova, E., Schramm, A., Lackey, A., and Balachandran, P. (2015). *Assessment of Microbial Population Diversity in Polymicrobial Research Samples by 16S Single or Multi-V Region Sequencing. 115th ASM General Meeting.* Available online at: https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/Assessment-of-microbial-population-diversity16v.pdf (accessed May 30–June 2, 2015).

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chaparro, A., Blanlot, C., Ramirez, V., Sanz, A., Quintero, A., Inostroza, C., et al. (2013). *Porphyromonas gingivalis*, *Treponema denticola* and toll-like receptor 2 are associated with hypertensive disorders in placental tissue: a case-control study. *J. Periodontal. Res.* 48, 802–809. doi: 10.1111/jre.12074

Colwell, R. K., and Coddington, J. A. (1994). Estimating terrestrial biodiversity through extrapolation. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 345, 101–118. doi: 10.1098/rstb.1994.0091

Davanian, H., Gaiser, R. A., Silfverberg, M., Hugerth, L. W., Sobkowiak, M. J., Lu, L., et al. (2019). Mucosal-associated invariant T cells and oral microbiome in persistent apical periodontitis. *Int. J. Oral Sci.* 11:16. doi: 10.1038/s41368-019-0049-y

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., et al. (2010). The human oral microbiome. *J. Bacteriol.* 192, 5002–5017. doi: 10.1128/JB.00542-10

Eckle, S. B., Corbett, A. J., Keller, A. N., Chen, Z., Godfrey, D. I., Liu, L., et al. (2015). Recognition of vitamin B precursors and byproducts by mucosal associated invariant T cells. *J. Biol. Chem.* 290, 30204–30211. doi: 10.1074/jbc.R115.685990

Edgar, R. C. (2017). Accuracy of microbial community diversity estimated by closed- and open-reference OTUs. *PeerJ* 5:e3889. doi: 10.7717/peerj.3889

Griffen, A. L., Beall, C. J., Campbell, J. H., Firestone, N. D., Kumar, P. S., Yang, Z. K., et al. (2012). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. *ISME J.* 6, 1176–1185. doi: 10.1038/ismej.2011.191

Guncu, G. N., Tozum, T. F., and Caglayan, F. (2005). Effects of endogenous sex hormones on the periodontium–review of literature. *Aust. Dent. J.* 50, 138–145. doi: 10.1111/j.1834-7819.2005.tb00352.x

Hajishengallis, G. (2011). Immune evasion strategies of *Porphyromonas gingivalis*. *J. Oral Biosci.* 53, 233–240. doi: 10.1016/S1349-0079(11)80006-X

Hajishengallis, G. (2015). Periodontitis: from microbial immune subversion to systemic inflammation. *Nat. Rev. Immunol.* 15, 30–44. doi: 10.1038/nri3785

Hama, I., Tominaga, K., Yamagiwa, S., Setsu, T., Kimura, N., Kamimura, H., et al. (2019). Different distribution of mucosal-associated invariant T cells within the human cecum and colon. *Cent. Eur. J. Immunol.* 44, 75–83. doi: 10.5114/ceji.2019.84020

He, J., Li, Y., Cao, Y., Xue, J., and Zhou, X. (2015). The oral microbiome diversity and its relation to human diseases. *Folia Microbiol. (Praha).* 60, 69–80. doi: 10.1007/s12223-014-0342-2

Heller, D., Helmerhorst, E. J., Gower, A. C., Siqueira, W. L., Paster, B. J., and Oppenheim, F. G. (2016). Microbial diversity in the early *in vivo*-formed dental biofilm. *Appl. Environ. Microbiol.* 82, 1881–1888. doi: 10.1128/AEM.03984-15

Herrema, H., IJzerman, R. G., and Nieuwdorp, M. (2017). Emerging role of intestinal microbiota and microbial metabolites in metabolic control. *Diabetologia* 60, 613–617. doi: 10.1007/s00125-016-4192-0

Hughes, J. B., Hellmann, J. J., Ricketts, T. H., and Bohannan, B. J. (2001). Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl. Environ. Microbiol.* 67, 4399–4406. doi: 10.1128/AEM.67.10.4399-4406.2001

Kesavalu, L., Sathishkumar, S., Bakthavatchalu, V., Matthews, C., Dawson, D., Steffen, M., et al. (2007). Rat model of polymicrobial infection, immunity, and alveolar bone resorption in periodontal disease. *Infect. Immun.* 75, 1704–1712. doi: 10.1128/IAI.00733-06

Lai, C. H., Bloomquist, C., and Liljemark, W. F. (1990). Purification and characterization of an outer membrane protein adhesin from Haemophilus parainfluenzae HP-28. *Infect. Immun.* 58, 3833–3839. doi: 10.1128/IAI.58.12.3833-3839.1990

Legendre, P., and de Caceres, M. (2013). Beta diversity as the variance of community data: dissimilarity coefficients and partitioning. *Ecol. Lett.* 16, 951–963. doi: 10.1111/ele.12141

Lemos, L. N., Fulthorpe, R. R., Triplett, E. W., and Roesch, L. F. (2011). Rethinking microbial diversity analysis in the high throughput sequencing era. *J. Microbiol. Methods* 86, 42–51. doi: 10.1016/j.mimet.2011.03.014

Lin, W., Jiang, W., Hu, X., Gao, L., Ai, D., Pan, H., et al. (2018). Ecological shifts of supragingival microbiota in association with pregnancy. *Front. Cell Infect. Microbiol.* 8:24. doi: 10.3389/fcimb.2018.00024

Magnusdottir, S., Ravcheev, D., de Crecy-Lagard, V., and Thiele, I. (2015). Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. *Front. Genet.* 6:148. doi: 10.3389/fgene.2015.00148

Manrique, P., Freire, M. O., Chen, C., Zadeh, H. H., Young, M., and Suci, P. (2013). Perturbation of the indigenous rat oral microbiome by ciprofloxacin dosing. *Mol. Oral Microbiol.* 28, 404–414. doi: 10.1111/omi.12033

McCoy, C. O., and Matsen, F. A. (2013). Abundance-weighted phylogenetic diversity measures distinguish microbial community states and are robust to sampling depth. *PeerJ* 1:e157. doi: 10.7717/peerj.157

McLean, J. S., Fansler, S. J., Majors, P. D., McAteer, K., Allen, L. Z., Shirtliff, M. E., et al. (2012). Identifying low pH active and lactate-utilizing taxa within oral microbiome communities from healthy children using stable isotope probing techniques. *PLoS ONE* 7:e32219. doi: 10.1371/journal.pone.0032219

Olsen, I., Lambris, J. D., and Hajishengallis, G. (2017). *Porphyromonas gingivalis* disturbs host-commensal homeostasis by changing complement function. *J. Oral Microbiol.* 9:1340085. doi: 10.1080/20002297.2017.1340085

Paropkari, A. D., Leblebicioglu, B., Christian, L. M., and Kumar, P. S. (2016). Smoking, pregnancy and the subgingival microbiome. *Sci. Rep.* 6:30388. doi: 10.1038/srep30388

Phillips, P., Brown, M. B., Progulske-Fox, A., Wu, X. J., and Reyes, L. (2018). *Porphyromonas gingivalis* strain dependent inhibition of uterine spiral artery remodeling in the pregnant rat. *Biol. Reprod.* 95, 1045–1056. doi: 10.1093/biolre/ioy119

Prince, A. L., Ma, J., Kannan, P. S., Alvarez, M., Gisslen, T., Harris, R. A., et al. (2016). The placental membrane microbiome is altered among subjects with spontaneous preterm birth with and without chorioamnionitis. *Am. J. Obstet. Gynecol. 214,* 627.e1–627.e16. doi: 10.1016/j.ajog.2016.01.193

R Core Team (2013). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Reyes, L., Phillips, P., Wolfe, B., Golos, T. G., Walkenhorst, M., Progulske-Fox, A., et al. (2018). *Porphyromonas gingivalis* and adverse pregnancy outcome. *J. Oral Microbiol.* 10, 1374153. doi: 10.1080/20002297.2017.1374153

Rogers, G. B., Carroll, M. P., Hoffman, L. R., Walker, A. W., Fine, D. A., and Bruce, K. D. (2010). Comparing the microbiota of the cystic fibrosis lung and human gut. *Gut Microbes* 1, 85–93. doi: 10.4161/gmic.1.2.11350

Takahashi, N. (2015). Oral microbiome metabolism: from "who are they?" *to "what are they doing?". J. Dent. Res.* 94, 1628–1637. doi: 10.1177/0022034515606045

van Immerseel, F., Ducatelle, R., de Vos, M., Boon, N., van de Wiele, T., Verbeke, K., et al. (2010). Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. *J. Med. Microbiol. 59(Pt* 2), 141–143. doi: 10.1099/jmm.0.017541-0

Vanterpool, S. F., Been, J. V., Houben, M. L., Nikkels, P. G., de Krijger, R. R., Zimmermann, L. J., et al. (2016). *Porphyromonas gingivalis* within placental villous mesenchyme and umbilical cord stroma is associated with adverse pregnancy outcome. *PLoS ONE* 11:e0146157. doi: 10.1371/journal.pone.0146157

Verma, R. K., Rajapakse, S., Meka, A., Hamrick, C., Pola, S., Bhattacharyya, I., et al. (2010). *Porphyromonas gingivalis* and *Treponema denticola* mixed microbial infection in a rat model of periodontal disease. *Interdiscip. Perspect. Infect. Dis.* 2010:605125. doi: 10.1155/2010/605125

Whiteson, K., Agrawal, S., and Agrawal, A. (2017). Differential responses of human dendritic cells to metabolites from the oral/airway microbiome. *Clin. Exp. Immunol.* 188, 371–379. doi: 10.1111/cei.12943

Whitman, W.B. (2015). *Bergey's Manual of Systematics of Archaea and Bacteria*. Hoboken, NJ: Bergey's Manual Trust, and Wiley Online Library. doi: 10.1002/9781118960608

Wu, M., Chen, S. W., and Jiang, S. Y. (2015). Relationship between gingival inflammation and pregnancy. *Mediators Inflamm.* 2015:623427. doi: 10.1155/2015/623427

Yoshii, K., Hosomi, K., Sawane, K., and Kunisawa, J. (2019). Metabolism of dietary and microbial vitamin b family in the regulation of host immunity. *Front. Nutr.* 6:48. doi: 10.3389/fnut.2019.00048

Zenobia, C., and Hajishengallis, G. (2015). *Porphyromonas gingivalis* virulence factors involved in subversion of leukocytes and microbial dysbiosis. *Virulence* 6, 236–243. doi: 10.1080/21505594.2014.999567

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 10 June 2020
doi: 10.3389/fphys.2020.00528

Occlusional Modifications Reversibly Alter Aquaporin 5 Expression and Localization in Rat Salivary Glands

Eri Saito, Ippei Watari, Mariko Mizumachi-Kubono, Sumire Hsu-Hayashi and Takashi Ono*

Department of Orthodontic Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan

Background: Aquaporin 5 (AQP5) is a water channel–forming protein that plays a key role in saliva secretion. A decrease in masticatory function associated with the molar extraction adversely affects the submandibular salivary gland (SMG) in rats, inducing hypertrophic changes in the acinar cells and the expression of AQP5 in acinar cells or intercalated duct of the SMG. However, changes in AQP5 expression and localization in the SMG in association with occlusal modification have not been fully characterized.

Methods: We examined the influence of the decline and recovery of masticatory function on expression and localization of AQP5 in the rat SMG by inserting and removing an incisor bite plate (IBP). Thirty 5-week-old male Wistar rats were randomly divided into IBP (n = 12), recovery (REC) (n = 6), and control (CON) (n = 12) groups. Each rat in both the IBP and REC groups was fitted with the IBP on its maxillary incisors. Rats without the IBPs served as controls. All rats were fed powder diet and water *ad libitum*. Rats in the IBP and CON groups were sacrificed after 14 (n = 6) and 28 (n = 6) days after the IBP attachment. In the REC group, the IBP was detached on the 14th day and sacrificed on 28th day after the IBP attachment. *AQP5* mRNA expression was quantified by reverse transcription–polymerase chain reaction. Changes in the localization of AQP5 were tracked by immunohistochemical staining.

Results: Attachment of IBP resulted in a decrease in the expression of AQP5 in the IBP group. Changes in the localization of AQP5 were observed between 14 and 28 days in the IBP group. In contrast, changes in the expression and localization of AQP5 were not observed in the REC group.

Conclusion: Findings suggested that a loss of molar occlusion, due to the IBP attachment, altered AQP5 expression and localization in the rat SMG. However, removal of the bite plate allowed the recovery of both AQP5 expression and its normal localization in the SMGs.

Keywords: aquaporin 5, immunohistochemical staining, occlusion, submandibular gland, incisor biteplate

Abbreviations: APM, apical membrane; AQP5, aquaporin 5; BPM, basolateral membrane; H&E, hematoxylin and eosin; IBP, incisor bite plate; OD, optical density; PBS, phosphate-buffered saline.

OPEN ACCESS

Edited by:
Thimios Mitsiadis, University of Zurich, Switzerland

Reviewed by:
Victor E. Arana-Chavez, University of São Paulo, Brazil
Harald Osmundsen, University of Oslo, Norway

****Correspondence:***
Ippei Watari
ippeiwatari@gmail.com

Specialty section:
This article was submitted to Craniofacial Biology and Dental Research, a section of the journal Frontiers in Physiology

Received: *03 December 2019*
Accepted: *29 April 2020*
Published: *10 June 2020*

Citation:
Saito E, Watari I, Mizumachi-Kubono M, Hsu-Hayashi S and Ono T (2020) Occlusional Modifications Reversibly Alter Aquaporin 5 Expression and Localization in Rat Salivary Glands. Front. Physiol. 11:528. doi: 10.3389/fphys.2020.00528

INTRODUCTION

Saliva is important for the maintenance of the ambience and function of the oral cavity. Saliva has antibacterial, mucosal-protective, and bolus-forming activities. Thus, severe salivation disorders cause caries, oral infections, dysphagia, and aspiration pneumonia, resulting in a decrease in the quality of life (Hayashi et al., 2009; Vissink et al., 2010; Millsop et al., 2017). The function and morphology of the salivary glands are influenced by multiple factors, including systemic disease, drug side effects, radiation, and aging. Patients with Sjögren syndrome exhibit acinar atrophy and interstitial fibrosis (Daniels, 1984). Atrophy of the acinar cells, fat degeneration, and decreased saliva secretion have been observed in diabetic rats (Mahay et al., 2004). In rats, irradiation of the salivary glands causes atrophy of the acinar cells, dilation of the conduits, and a decrease in salivary flow (Marie et al., 2015). The number of acini decreases with age, with a concomitant increase in fatty and fibrous tissue (Liu et al., 2000).

Several reports have documented changes in the function and morphology of salivary glands in response to changes in the masticatory environment. When rats have been provided with an atypical hard bulk diet, an increase in the size of the parotid and submandibular glands, elevated amylase content of saliva, and changes in gene expression were observed. Conversely, there is a significant decrease in these indices when liquid or powder foods are provided during breeding (Johnson, 1982). Bulk diets stimulate increased saliva secretion, whereas liquid and powder diets decrease the volume of saliva secretion (Scott et al., 1990). Intriguingly, several reports have indicated that salivary gland morphology and function are restored when reintroducing solid foods. Salivary gland weight, neurotransmitter concentration, and saliva secretion recover nearly to the levels of rats fed with solid food following the change from liquid to solid diet. These indicate that the masticatory environment plays an important role in the maintenance of salivary gland function (Hall and Schneyer, 1964).

In rats, there are three major salivary glands: the parotid, submandibular, and sublingual glands. Additionally, a few small salivary glands are scattered on the oral mucosa and tongue. Protein and water secretions are performed in the salivary glands, with water constituting 99% of saliva. Therefore, secretion and transportation of water are considered to be essential for secretion of saliva. Salivation is controlled by the parasympathetic nervous system; acetylcholine acts on muscarinic receptors of the acinar cells, raising the intracellular calcium concentration and causing the secretion of water, mainly through a water channel called aquaporin (AQP).

Aquaporins, which are encoded by 13 genes in humans, are membrane proteins that function as a water channel. They are widely distributed throughout the body. Aquaporins 1, 5, and 8 are present in the cell membranes of the salivary glands (Baoxue et al., 2005). In particular, AQP5 is abundant in the salivary glands and has been reported to be involved in salivary function. Aquaporin 5 is located on the apical side of the acinar cells in the salivary gland, in the intercellular secretory capillary, and in the luminal cytoplasm of the interstitial conduit. A previous study has reported that expression of AQP5 decreases in the submaxillary glands in response to radiation (Li et al., 2006). Decreased AQP5 expression in the salivary glands has also been reported in Sjögren syndromic patients (Wang et al., 2009). Further, changes in the distribution of the acinar cells to the basal side (Steinfeld et al., 2001) and reduction in saliva volume have been observed in AQP5 knockout mice (Ma et al., 1999).

Our previous study suggested that maxillary molar extraction induced acinar cell hypertrophy and dispersed AQP5 expression in rat submandibular salivary gland (SMG) (Mizumachi-Kubono et al., 2012). However, little information is available regarding the relationship between impaired occlusion and salivary gland function. To date, it has been known that an occlusal hypofunction model, including molar extraction, causes functional deterioration of the masticatory muscles, periodontal tissue, and peripheral nerves in the oral cavity (Kaneko et al., 2001; Nakamura et al., 2013). Additional reports suggest that impaired oral function can be improved by restoring occlusal function. However, to our knowledge, similar studies on salivary gland hypofunction resulting from reduced occlusal function have not been reported. Furthermore, it is not known whether anatomical and functional changes in salivary glands are reversed following the recovery of occlusal function.

In this study, we aimed to evaluate the effect of decreased masticatory function and recovery of secretory function on salivary gland function in rodents. For this, we fashioned a masticatory function recovery model, by attaching a removable IBP over the rat maxillary incisors and a metal cap on the mandibular incisors, which prevent occlusal contact at the molars while the IBP was in position. Subsequently, we examined the expression and distribution of AQP5 in the salivary glands.

MATERIALS AND METHODS

Experimental Animals

Five-week-old male Wistar rats ($n = 30$) were randomly assigned to IBP ($n = 12$), recovery (REC; $n = 6$), and control (CON; $n = 12$) groups. All rats were fed a powdered diet (CE-2; CLEA, Tokyo, Japan) throughout the experimental period. In both the IBP and REC groups, an IBP and metal cap constructed from band material (0.180 × 0.005 inches; Rocky Mountain Morita Corp., Tokyo, Japan) were attached to the maxillary and mandibular incisors, respectively, using a light-curing composite resin (Clearfil Liner Bond II; Kuraray, Okayama, Japan) at the age of 5 weeks. The IBP was T-shaped with the maxillary incisors in the center, ensuring that the anterior teeth of the lower jaw made an early contact with the bite plate. The metal cap prevented any mandibular anterior tooth abrasion. Therefore, while the bite plate is being worn, there could be no occlusal molar contact. At 14 days, both the IBP and metal cap were removed from the rats in the REC group. Rats in both the CON and IBP groups were euthanized at 14 and 28 days. Rats in the REC group were euthanized at 28 days. Both SMGs were isolated immediately after sacrifice.

Wet Weight of SMG

The isolated submandibular glands were immediately weighed for wet rain on both the left and right sides. Then, the right side of the SMG was fixed with mild form, and the left side

frozen in RNA STAT-60 for reverse transcription–polymerase chain reaction (RT-PCR).

Preparation of the SMGs for Histological Analysis

The isolated SMGs were fixed immediately by overnight immersion in 4% paraformaldehyde (PBS; Mildform®; Wako Pure Chemical Industries, Osaka, Japan) at 4°C. After washing with PBS, the samples were then embedded in paraffin according to the standard protocol (Feng et al., 2014). Serial 5-μm-thick sections were cut, prepared for histological observation, and mounted on glass slides, as described below.

Hematoxylin-Eosin Staining and Morphological Evaluation

For morphometric analyses, sections were deparaffinized with xylene and rehydrated in a graded ethanol series. The sections were then stained with H&E and observed under light microscopy (Microphoto-FXA; Nikon, Tokyo, Japan) at ×400 magnification. Digital images were captured using a digital camera (DXM1200; Nikon). The area occupied by one acinus was measured using imaging software (ImageJ 1.44; National Institutes of Health, Bethesda, MD, United States). The number of acinar cells in each acinus was determined by counting the number of acinar nuclei. The area occupied by one acinar cell was calculated by dividing the acinar area by the number of acinar cells in that acinus. To obtain mean values, 10 records per sample for each gland were analyzed in randomly selected microscopic fields and measured using digital imaging fields. All samples were analyzed in a blind fashion.

Immunohistochemical Staining of AQP5 and Histological Evaluation

To evaluate AQP5 expression in the SMGs, we performed immunohistochemical staining using the three-step streptavidin–biotin–peroxidase method (VECTASTAIN ABC Staining Kit; Vector Laboratories, Burlingame, CA, United States). Briefly, sections were deparaffinized with xylene and rehydrated in a graded ethanol series. To activate the antigen, we used microwave treatment with unmasking solution (Antigen Unmasking Solution; Vector Laboratories). The solution was prewarmed for 10 min using three treatments of microwave irradiation 5 min each, followed by incubation at room temperature (RT) for 20 min. Endogenous peroxidase was blocked with peroxidase-blocking solution (Agilent Technologies, Santa Clara, CA, United States) for 10 min at RT. To prevent non-specific binding of antibodies, we incubated the sections with PBS containing 1% bovine serum albumin for 30 min at RT. Next, the sections were incubated for 1 h at RT with an anti-AQP5 primary antibody (#ab104751; Abcam, Cambridge, MA, United States) diluted 1:300 in PBS containing 0.3% Triton X-100 and 0.1% bovine serum albumin. The sections were then incubated with a diluted secondary anti-goat immunoglobulin G antibody (VECTASTAIN ABC Staining Kit; Vector Laboratories) for 30 min at RT.

After treatment with an avidin–biotin macromolecular complex (VECTASTAIN ABC Staining Kit; Vector Laboratories) for 30 min at room temperature and incubation with 3,3-diaminobenzidine for 1 min, the sections were washed in PBS, counterstained with hematoxylin, rinsed for 30 min under running tap water, and mounted with Mount-Quick "Aqueous" (Cosmo Bio Inc., Tokyo, Japan). We observed two sections per SMG sample and 20 fields of 200 × 200 pixels per section under ×400 magnification using light microscopy (DS-Ri1; Nikon). All procedures for image capture, identification, and processing were standardized before images were captured. The integral OD of each scanned image was computerized using a digital image analyzer in ImageJ 1.44 after the immunohistochemical stained area was extracted using Photoshop (Adobe Systems, San Jose, CA, United States), and the mean OD was obtained.

Quantitative Analysis of AQP5 mRNA (RT-PCR)

Submandibular salivary gland tissues for RNA preparation were quickly separated and frozen at −80°C. Total RNA was isolated using RNA STAT-60™ (Tel-Test Inc., Friendswood, TX, United States) reagent according to the instructions provided by the manufacturer. The cDNA was synthesized from 5.0 μg of total RNA using ReverTra Ace™ qPCR RT Master Mix (TOYOBO, Osaka, Japan) under the conditions suggested by the manufacturer. Quantitative RT-PCR was conducted with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) using KAPA SYBR® FAST qPCR Kits (KAPA BIOSYSTEMS, Cape Town, South Africa). The following oligonucleotide primers for rat β-actin, AQP5, were purchased from Thermo Fisher Scientific (Waltham, MA, United States): β-actin 5′-TCTGGTCGTACCACTGCATT-3′ (forward) and 5′-AGACGCAGATGCATGAG-3′ (reverse), AQP5 5′-CCCTGCTGTCATGAA-3′ (forward) and 5′-CAGTCCTCCTCCGGCTCATA-3′ (reverse). All reactions for quantitative RT-PCR were carried out under the following parameters: 95°C for 3 min and 35 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 15 s. Reverse transcriptase–PCR was performed three times for each primer, and the averaged results were used for statistical analysis. Relative mRNA expression was quantified using the $2^{-\Delta\Delta CT}$ method with β-actin as an internal control. The mRNA expression levels are expressed as the n-fold difference relative to the mRNA expression in control rats.

Statistical Analysis

All results are presented as means ± standard deviation. Data were analyzed after confirming whether the variance was equal in each group using the Barrett test; comparison between multiple groups was performed using the Tukey–Kramer test.

RESULTS

Body Weight

The body weight of the rats in the IBP and REC groups began to decrease on the second day after attaching the IBP. On the fourth day in the IBP group, mean weight decreased to 8.65 g and decreased to 1.59×10^2 g in the REC group. However, there were no significant differences in body weights between groups at 21 and 28 days (**Figure 1**).

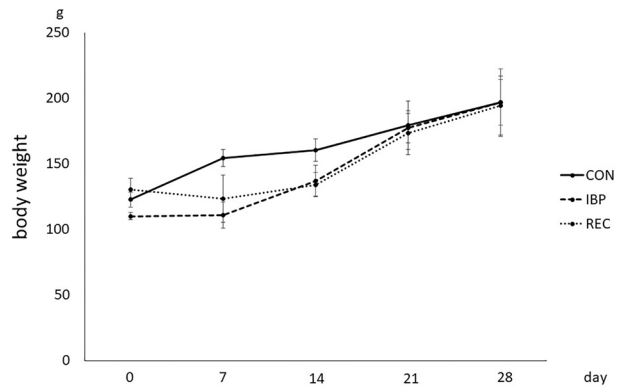


FIGURE 1 | Body weight of the rat in CON, IBP, and REC group. Changes in body weights during the experimental period in CON, IBP, and REC groups. Data are presented as means ± standard deviation (n = 6 each).

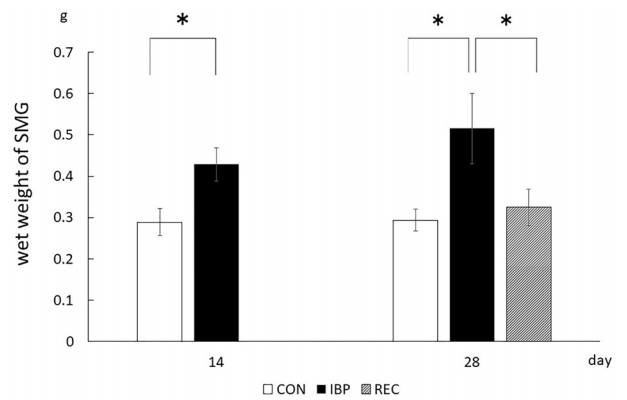


FIGURE 2 | Wet weights of submandibular glands. The weight of the left and right submandibular glands of each rat was measured, and the average value was taken. The weight in the REC group was not significantly different from that in the CON group, whereas it was significantly increased in the IBP group. *$p < 0.05$.

Wet Weight of SMG

The wet weight of SMGs in the CON group and IBP group was $2.89 \times 10^{-1} \pm 3.30 \times 10^{-2}$ g and $4.28 \times 10^{-1} \pm 3.96 \times 10^{-2}$ g, on day 14, respectively. On the 14th day after attachment of the IBP, the submandibular gland wet weight in the IBP group increased significantly compared to the CON group. The wet weight of salivary glands of CON, IBP, and REC group was $2.94 \times 10^{-1} \pm 2.65^{-2}$ g, $5.15 \times 10^{-1} \pm 8.49 \times 10^{-2}$ g, and $3.25 \times 10^{-1} \pm 4.37 \times 10^{-2}$ g, on day 28, respectively. On the 28th day, the IBP group's submandibular gland wet weight further increased compared to the CON group. Regarding the REC group, the wet weight was the same as that of the CON group on the 28th day (**Figure 2**).

Histological Findings of HE Staining

When observed at ×400 magnification with an optical microscope, acinar cells and ducts (intercalated, granulated, striated, and excretory) did not change with age in the CON group (**Figure 3**). In the IBP group, we observed marked hypertrophy of the acinar cells on 14th day after IBP attachment. This was accompanied by nuclear enlargement and thickening of myoepithelial cells. On the 28th day, the acinar cells shrank, but the nuclear enlargement and thickening of the myoepithelial cells persisted. In the REC group, a slight nuclear enlargement and thickening of myoepithelial cells were observed. However, the tissue exhibited normal properties when compared with the IBP group.

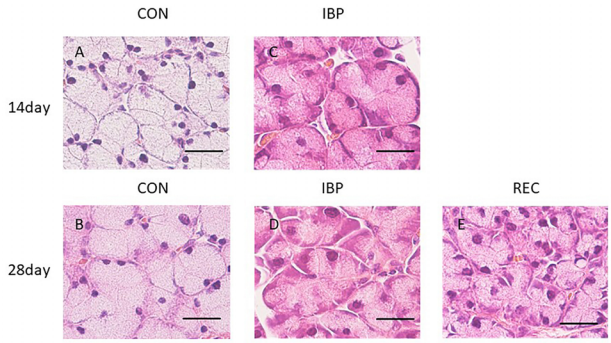


FIGURE 3 | Hematoxylin-eosin staining of SMG at ×400 magnification with an optical microscope. Morphological observation of HE staining of the SMG in the CON group **(A,B)**, IBP group **(C,D)**, and REC group **(E)**. The IBP group at 14 days **(C)** showed an enlargement of the acini. At 28 days, the size of the acini decreased **(D)** compared to the size at 14 days. **(E)** In the REC group at 14 days, the size of the acini was approximately the same as that of the CON group **(E)**. H&E, hematoxylin and eosin; CON, control group; IBP, incisor bite plate group; REC, recovery group. Bar = 25 μm, ×400 magnification.

Morphometric analysis showed that the cross-sectional area of acinar cells in the IBP group significantly ($p < 0.05$) increased compared with that of the CON group at 14 days. The acinar cell cross-sectional area significantly increased in the CON group between 14 and 28 days. Conversely, a significant decrease was observed in the IBP group over the same period. At 28 days, no significant differences in the cross-sectional area of acinar cells were observed among the control, IBP, and REC groups (**Figure 4**).

Expression and Distribution of AQP5 in SMG

The specificity of AQP5 immunohistochemical staining was demonstrated using the non-immune control, which did not exhibit non-specific staining. In normal salivary glands, AQP5 was localized to the APM of the acinar cells, intercellular canaliculi, and the luminal cytoplasm of the intercalated ducts. In the CON group, AQP5 was localized to the APM, intercellular canaliculi, and luminal side of the intercalated duct during all experimental periods, but not to the BPM, cytoplasm or nucleus of acinar cells, the granular conduit, the striated duct, or the excretory duct (**Figure 5**). In the IBP group, we observed faint staining and the changes of expression and localization in AQP5. The localization of AQP5 to the APM and intercellular canaliculi was rarely observed on days 14 and 28. On the other hand, in the REC group, AQP5 localization was confirmed in the APM

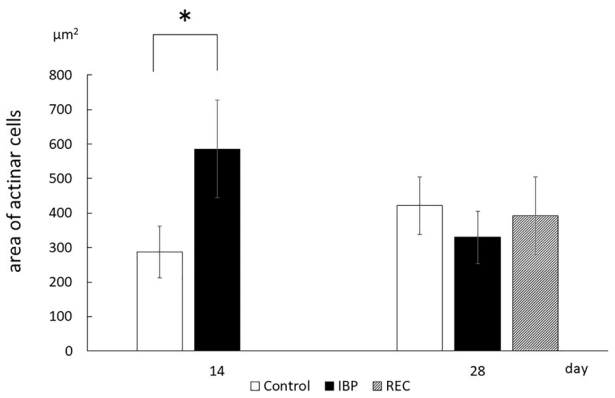


FIGURE 4 | The area of acinar cell. Morphometric measurements of acinar cells of the SMG. The area occupied by one acinar cell was calculated by dividing the acinar area by the number of acinar cells in that acinus. Data are presented as the mean ± standard deviation (n = 6 each, $^*p < 0.05$).

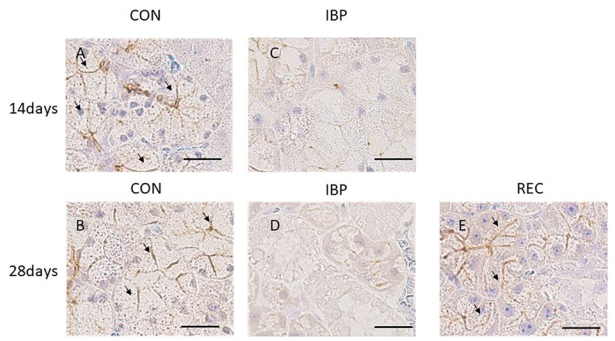


FIGURE 5 | Immunohistochemical images of AQP5 expression. **(A,B)** CON group; AQP5 was detected on the apical plasma membrane (APM) and in the intercellular secretory canaliculi of acinar cells and on the luminal side of the intercalated duct. **(C,D)** IBP group; immunostaining of AQP5 in the APM and intercellular secretory canaliculi of acinar cells; almost no staining was observed in the APM of the acinar cells. **(E)** REC group; AQP5 was detected at the same level as that of the CON group. AQP5, aquaporin 5; CON, control group; IBP, incisor bite plate group; REC, the recovery group.

of the acinar cell, intercellular canaliculi, and luminal side of the intercalated duct. Further, expression recovered to the same level as that of the CON group.

The respective OD values for the CON, IBP, and REC groups were $1.75 \times 10^4 \pm 2.89 \times 10^3$ and $4.33 \times 10^3 \pm 4.21 \times 10^3$ on the 14th day. On the 28th day, they were $1.26 \times 10^4 \pm 4.13 \times 10^3$, $3.86 \times 10^3 \pm 1.58 \times 10^2$, and $1.43 \times 10^4 \pm 6.21 \times 10^3$. A marked decrease in OD in the IBP group was observed on days 14 and 28 compared with the CON group. In contrast, there was no significant difference in OD values between the REC group and the CON group (**Figure 6**).

Quantitative Analysis of AQP5 mRNA (RT-PCR)

From the RT-PCR data, relative mRNA levels for AQP5 were $1.06 \pm 3.52 \times 10^{-1}$ in the CON group and $1.13 \pm 1.83 \times 10^{-1}$ in the IBP group. There were no significant differences observed between groups in the amount of AQP5 mRNA on the 14th day. On day 28, the levels were $1.01 \pm 1.87 \times 10^{-1}$ in the CON group, $6.57 \times 10^{-1} \pm 6.58 \times 10^{-2}$ in the IBP group, and $1.22 \pm 2.02 \times 10^{-1}$ in the REC group. There was significantly less AQP5 expression in the IBP group. On day 28, the AQP5 mRNA level in the IBP group was significantly decreased relative to the other two groups; however, there was no significant difference between the mRNA levels of the CON and REC groups (**Figure 7**).

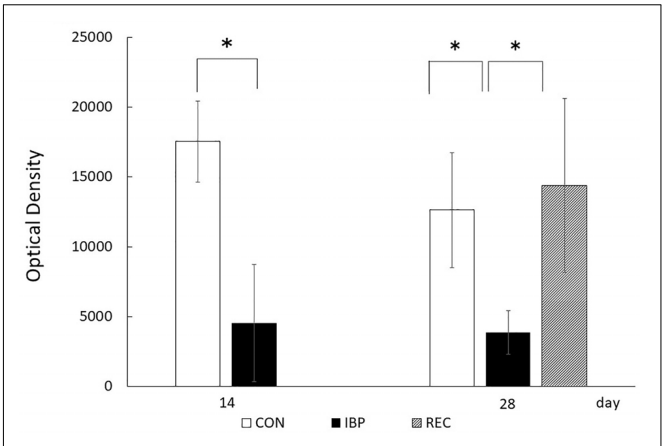


FIGURE 6 | Optical density (OD) of AQP5 immunohistochemical positive staining. The procedures for anti-AQP5 immunohistochemical image capturing, identification, and processing were standardized before images were captured. Concerning positive staining of AQP5, the integral OD of each scanned image was computerized using a digital image analyzer in ImageJ 1.44 after the immunohistochemical stained area was extracted using Photoshop (Adobe Systems), and the mean OD was obtained. Data are presented as means ± standard deviation (n = 6, each group; $^*p < 0.05$).

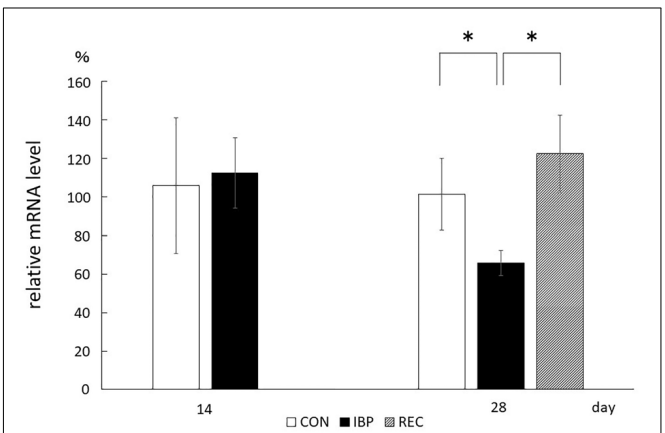


FIGURE 7 | Quantitative analysis of AQP5 mRNA (qPCR). Aquaporin 5 mRNA prepared from submandibular gland tissues was analyzed by RT-PCR using primers specific for AQP5. Aquaporin 5 mRNA levels normalized to β-actin. Data are means ± SE. $^*p < 0.05$.

DISCUSSION

Mastication is the critical importance of maintaining physiological function in stomatognathic system. The animal models of occlusal hypofunction, tooth extraction (Ejiri et al., 2006), feeding the soft/liquid diet (Mavropoulos et al., 2008), or resection of the masseter (Yonemitsu et al., 2007) are common; however, to avoid surgical stress and to allow recovery from occlusal hypofunction, we used IBP for making the occlusal modification in this study. We attached the IBP to the maxillary and mandibular incisors for creating occlusal hypofunction, preventing molar contacts during the entire experimental period. We set and detached the IBP non-invasively for allowing recovery. The body weights of the rats in the CON, IBP, and REC groups were measured during the experimental period. No significant differences were observed among the rats in the three groups at 0, 7, 14, 21, and 28 days with IBP attachment, so there was no apparent alteration of general metabolism.

After 14 days of IBP attachment, the wet weights of SMGs of the IBP group were significantly higher than those of the CON group. Histological observation showed that the hypertrophy of acinar cell area that increased the width of myoepithelial cells decreased significantly and enlargement of the nucleus of acinar cell observed in IBP group. Immunohistochemistry revealed that functional localization of AQP5 in acinar cells was not observed in the IBP group; however, the amount of AQP5 mRNA was different between the CON and IBP groups. These changes have also been observed in other hypofunctional animal models involving maxillary molar extraction (Mizumachi-Kubono et al., 2012) or repeated amputation of incisors (Wells et al., 1959; Takeda et al., 1986). The alterations in our experiment were smaller than those in these previous hypofunctional models were, but showed the same pattern. The reduction of afferent input from periodontal ligament of molars (Kaneko et al., 2001), alteration of autonomic activity affected by occlusal stimuli (Proctor and Carpenter, 2007), and the change of the jaw movement (Gavião et al., 2004) may influence SMG function in the IBP group. The SMG degeneration in this study is thought to be caused by reduction of water secreting function via AQP5 in acini due to impaired masticatory function.

On the other hand, at 28 days after IBP attachment, further histological degeneration of acinar cells, wet weight of SMG, and qualitative and quantitative reduction of AQP5 expression were observed in the IBP group relative to the CON group. Furthermore, normalization of functional localization of AQP5 and quantitative recovery of AQP5 mRNA were confirmed. These results showed that SMG degeneration induced by IBP attachment can be restored, especially the negative impact on AQP5, which plays a critical role in water transport in acini. This is the first report to indicate that salivary gland dysfunction associated with occlusal alteration can be restored. One possible reason for our observed restoration is the age of the rats. Functional maturation of SMG is known to continue to 10 weeks after birth (Jacoby and Leeson, 1959). In this experiment, we used younger rats with less-mature SMGs. Further studies are needed to examine the effects of aging.

In addition, the removal of IBP had improved the dryness of the mouth, which might have contributed to the histological recovery of SMG observed in the REC group. With IBPs on the incisors, it was difficult for the rats to close their mouths. The improvement of mouth dryness after IBP removal led to normalization of jaw movement, which might have helped in the recovery of SMG in REC group.

One limitation of our study is the uncertainty regarding the extent to which these results correspond to humans. Although there are some anatomical similarities between human and rat, differences do exist, such as the highly developed interstitial ducts in rat SMG. Another limitation is that, in our study, the functions other than water transportation in SMG were not investigated.

Our findings provide support for the hypothesis that the occlusal modification has an effect on salivary gland function. Clinical studies have shown that occlusal hypofunction causes a series of salivary disorders (Matsuda et al., 2009). This study supports these findings in terms of water transportation in salivary gland. The findings of our study also emphasize the importance of preventing malocclusion for maintaining physiologically normal saliva function.

CONCLUSION

The structure and water secretion function of the submandibular glands were significantly altered due to IBP-induced decrease in masticatory function. Removal of the bite plate allowed the recovery of both AQP5 expression and its normal localization in the SMGs, thus restoring the masticatory function.

DATA AVAILABILITY STATEMENT

The datasets generated during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

All experimental procedures were approved by the Institutional Animal Care and Use Committee (#0170353C2) and performed in accordance with the Animal Care Standards of Tokyo Medical and Dental University.

AUTHOR CONTRIBUTIONS

ES contributed to the design of the study, animal handling, data acquisition, data interpretation performed the statistical analysis, and drafted the manuscript. IW contributed to the design of the study, data interpretation, and participated in manuscript formatting. MM-K conceived the study. SH-H participated in the design of the study and data interpretation. TO participated in manuscript design and formatting. All authors read and approved the final manuscript.

FUNDING

This work was supported by Grants-in-Aid for Scientific Research in Japan (KAKEN) (grant numbers 26861774, 16K11781, and 20K10200).

ACKNOWLEDGMENTS

The authors acknowledge the facility and the scientific and technical assistance provided by Tokyo Medical and Dental University.

REFERENCES

Baoxue, Y., Yuanlin, S., Dan, Z., and Verkman, A. S. (2005). Phenotype analysis of aquaporin-8 null mice. *Am. J. Physiol. Cell Physiol.* 288, C1161–C1170. doi: 10.1152/ajpcell.00564.2004

Daniels, T. E. (1984). Labial salivary gland biopsy in Sjögren's syndrome. Assessment as a diagnostic criterion in 362 suspected cases. *Arthritis Rheum.* 27, 147–156. doi: 10.1002/art.1780270205

Ejiri, S., Toyooka, E., and Tanaka, M. (2006). Histological and histomorphometrical changes in rat alveolar bone following antagonistic tooth extraction and / or ovariectomy. *Arch. Oral Biol.* 51, 941–950. doi: 10.1016/j.archoralbio.2006.05.006

Feng, Z., Takahashi, R., Nakamura, T., Sato, D., Shirasawa, N., Nakayama, A., et al. (2014). Expression of microRNA-1, microRNA-133a, and Hand2 protein in cultured embryonic rat cardiomyocytes. *Vitr. Cell. Dev. Biol. Anim.* 50, 700–706. doi: 10.1007/s11626-014-9755-0

Gavião, M. B. D., Engelen, L., and Van Der Bilt, A. (2004). Chewing behavior and salivary secretion. *Eur. J. Oral Sci.* 112, 19–24. doi: 10.1111/j.0909-8836.2004.00105.x

Hall H. D., and Schneyer, C. A. (1964). Salivary gland atrophy in rat induced by liquid diet. *Proc. Soc. Exp. Biol. Med.* 117, 789–793. doi: 10.3181/00379727-117-29699

Hayashi, Y., Arakaki, R., and Ishimaru, N. (2009). Salivary gland and autoimmunity. *J. Med. Investig.* 56, 185–191. doi: 10.2152/jmi.56.185

Jacoby, F., and Leeson, C. R. (1959). The postnatal development of the rat submaxillary gland. *J. Anat.* 93, 201–216.

Johnson, D. A. (1982). Effect of a liquid diet on the protein composition of rat parotid saliva. *J. Nutr.* 112, 175–181. doi: 10.1093/jn/112.1.175

Kaneko, S., Ohashi, K., Soma, K., and Yanagishita, M. (2001). Occlusal hypofunction causes changes of proteoglycan content in the rat periodontal ligament. *J. Periodontal Res.* 36, 9–17. doi: 10.1034/j.1600-0765.2001.00607.x

Li, Z., Zhao, D., Gong, B., Xu, Y., Sun, H., Yang, B., et al. (2006). Decreased saliva secretion and down-regulation of AQP5 in submandibular gland in irradiated rats. *Radiat. Res.* 165, 678–687. doi: 10.1667/rr3569.1

Liu, P., Denny, P. A., and Denny, P. (2000). The effect of ageing on parenchymal cell populations in adult female mouse submandibular gland. *Arch. Oral Biol.* 45, 585–592. doi: 10.1016/S0003-9969(00)00014-5

Ma, T., Song, Y., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1999). Defective secretion of saliva in transgenic mice lacking aquaporin-5 water channels. *J. Biol. Chem.* 274, 20071–20074. doi: 10.1074/jbc.274.29.20071

Mahay, S., Adeghate, E., Lindley, M. Z., Rolph, C. E., and Singh, J. (2004). Streptozotocin-induced type 1 diabetes mellitus alters the morphology, secretory function and acyl lipid contents in the isolated rat parotid salivary gland. *Mol. Cell. Biochem.* 261, 175–181. doi: 10.1023/B:MCBI.0000028753.33225.68

Marie, A., Pedersen, L., and Pedersen, A. M. (2015). Xerostomia and Hypofunction of the salivary glands in cancer therapy Xerostomia and hypofunction of the salivary glands in cancer therapy. *Support Care Cancer* 11, 207–225. doi: 10.1007/s00520-002-0407-7

Matsuda, K. I., Ikebe, K., Ogawa, T., Kagawa, R., and Maeda, Y. (2009). Increase of salivary flow rate along with improved occlusal force after the replacement of complete dentures. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endodontol.* 108, 211–215. doi: 10.1016/j.tripleo.2009.03.020

Mavropoulos, A., Kiliaridis, S., and Anna, O. (2008). Do masticatory functional changes influence the mandibular morphology in adult rats. *Arch. Oral Biol.* 53, 1149–1154. doi: 10.1016/j.archoralbio.2008.07.004

Millsop, J. W., Wang, E. A., and Fazel, N. (2017). Etiology, evaluation, and management of xerostomia. *Clin. Dermatol.* 35, 468–476. doi: 10.1016/j.clindermatol.2017.06.010

Mizumachi-Kubono, M., Watari, I., Ishida, Y., and Ono, T. (2012). Unilateral maxillary molar extraction influences AQP5 expression and distribution in the rat submandibular salivary gland. *Arch. Oral Biol.* 57, 877–883. doi: 10.1016/j.archoralbio.2012.02.006

Nakamura, A., Zeredo, J. L., Utsumi, D., Fujishita, A., Koga, Y., and Yoshida, N. (2013). Influence of malocclusion on the development of masticatory function and mandibular growth. *Angle Orthod.* 83, 749–757. doi: 10.2319/083012-698.1

Proctor, G. B., and Carpenter, G. H. (2007). Regulation of salivary gland function by autonomic nerves. *Auton. Neurosci. Basic Clin.* 133, 3–18. doi: 10.1016/j.autneu.2006.10.006

Scott, J., Berry, M. R., Gunn, D. L., and Woods, K. (1990). The effects of a liquid diet on initial and sustained, stimulated parotid salivary secretion and on parotid structure in the rat. *Arch. Oral Biol.* 35, 509–514. doi: 10.1016/0003-9969(90)90080-T

Steinfeld, S., Cogan, E., King, L. S., Agre, P., Kiss, R., and Delporte, C. (2001). Abnormal distribution of aquaporin-5 water channel protein in salivary glands from Sjögren's syndrome patients. *Lab. Investig.* 81, 143–148. doi: 10.1038/labinvest.3780221

Takeda, Y., Hirose, H., and Enomoto, S. (1986). Enlargement of rat submandibular salivary gland induced by single amputation of lower incisor teeth: Histological, histometric and ultrastructural studies. *J. Oral Pathol. Med.* 15, 327–333. doi: 10.1111/j.1600-0714.1986.tb00634.x

Vissink, A., Mitchell, J. B., Baum, B. J., Limesand, K. H., Jensen, S. B., Fox, P. C., et al. (2010). Clinical management of salivary gland hypofunction and xerostomia in head-and-neck cancer patients: Successes and barriers. *Int. J. Radiat. Oncol. Biol. Phys.* 78, 983–991. doi: 10.1016/j.ijrobp.2010.06.052

Wang, D., Iwata, F., Muraguchi, M., Ooga, K., Ohmoto, Y., Takai, M., et al. (2009). Correlation between salivary secretion and salivary AQP5 levels in health and disease. *J. Med. Investig.* 56, 350–353. doi: 10.2152/jmi.56.350

Wells, H., Zackin, S. J., Goldhaber, P., and Munson, P. L. (1959). Increase in weight of the submandibular salivary glands of rats following periodic amputation of the erupted portion of the incisor teeth. *Am. J. Physiol.* 196, 827–830. doi: 10.1152/ajplegacy.1959.196.4.827

Yonemitsu, I., Muramoto, T., and Soma, K. (2007). The influence of masseter activity on rat mandibular growth. *Arch. Oral Biol.* 52, 487–493. doi: 10.1016/j.archoralbio.2006.10.019

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 16 September 2020
doi: 10.3389/fcimb.2020.530190

Bmal1 Regulates Coagulation Factor Biosynthesis in Mouse Liver in *Streptococcus oralis* Infection

Lili Chen[1,2*‡], *Shue Li*[1,2*], *Jiaming Nie*[1,2†], *Jiajia Zhao*[1,2†], *Shaoling Yu*[1,2], *Yaoxu Li*[1,2] and *Jinfeng Peng*[1,2]

[1] Department of Stomatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [2] Hubei Key Laboratory of Oral and Craniomaxillofacial Development and Regeneration, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Streptococcus oralis (S. oralis) has been recognized as a fatal pathogen to cause multiorgan failure by contributing to the formation of microthrombus. Coagulation and fibrinolysis systems have been found under the control of circadian clock genes. This study aimed to explore the correlation between *BMAL1* and coagulation factor biosynthesis in *S. oralis* infection. Mice were administered *S. oralis* to induce sepsis, and HepG2 cells were also infected by *S. oralis*. The expression of *BMAL1* of hepatocytes was downregulated in the *S. oralis* infection group, leading to the downregulation of coagulation factor VII (FVII) and the upregulation of the coagulation factor XII (FXII) *in vitro* and *in vivo*. Furthermore, we confirmed that the deficiency of *BAML1* contributed to the elevation of FVII and the decline in FXII by constructing BMAL1-deficiency (*Bmal1*$^{-/-}$) mice. The current result showed that BMAL1 regulates FVII directly. Thus, a novel insight into the coagulation abnormality in *S. oralis* infection was gained that may optimize the treatment of sepsis by rescuing the expression of BMAL1 in the liver.

Keywords: *S. oralis*, bmal1, FVII, FXII, coagulation factor biosynthesis

OPEN ACCESS

Edited by:
Lorenzo Lo Muzio, University of Foggia, Italy

Reviewed by:
Kristina Marie Feye, University of Arkansas, United States
Kun Zhang, Virginia Commonwealth University, United States

***Correspondence:**
Lili Chen
chenlili1030@hust.edu.cn
Shue Li
lishue737@hotmail.com

†*These authors share first authorship*

‡*Lead Contact*

Specialty section:
This article was submitted to Microbiome in Health and Disease, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *28 January 2020*
Accepted: *18 August 2020*
Published: *16 September 2020*

Citation:
Chen L, Li S, Nie J, Zhao J, Yu S, Li Y and Peng J (2020) Bmal1 Regulates Coagulation Factor Biosynthesis in Mouse Liver in Streptococcus oralis Infection. Front. Cell. Infect. Microbiol. 10:530190. doi: 10.3389/fcimb.2020.530190

INTRODUCTION

Streptococcus oralis (*S. oralis*) is an oral biofilm-deriving opportunistic pathogen (Li et al., 2004) that has been found in endocarditis (Turnier et al., 2009) and cerebral abscess (Thiagarajan et al., 2016). Reportedly, in neutropenic patients, *S. oralis* induces sepsis (Penack et al., 2014; Shelburne et al., 2014) and inflicts death (Cornely and Schirmacher, 2001). Thus, the mechanisms of *S. oralis*-oriented abnormal coagulation to reduce the mortality need to be deduced. The overwhelming host response can progress to disseminated intravascular coagulation (DIC) by upregulating the activation of the coagulation cascade (Fourrier et al., 1992). It is characterized by excessive activated coagulation systems and suppressed fibrinolytic systems and is known to contribute to the formation of microthrombus, which leads to multiorgan failure (Vervloet et al., 1998). In this process, several coagulation factors patriciate in both classic clotting pathways (Amaral et al., 2004). In addition, most of the coagulation factors in coagulation cascade are generated by hepatocytes. Blood coagulation factor VII (FVII) and factor XII (FXII) are the key enzymes in the extrinsic and intrinsic coagulation cascades (Furie and Furie, 1988). When interacted with tissue factor (TF), FVII is converted to the active state (FVIIa), forms TF–FVIIa complex, and initiates the downstream extrinsic clotting cascade (Bogdanov et al., 2003). In the case of the intrinsic pathway of coagulation, FXII initiates the activation of the blood coagulation zymogen FXI and leads to the formation of factor IXa (Schmaier, 2008). Thus, further exploration of FVII and FXII may provide an in-depth insight into the aberrant coagulation in sepsis.

The circadian clock genes in mammals are endogenous oscillators, containing interlocked and negative feedback loops (Takahashi et al., 2008; Duong et al., 2011). These loops are basic helix-loop-helix-PER-ARNT-SIM (PAS) transcriptional activators *BMAL1* and *CLOCK*, *NPAS2* in addition to Period (*Per1* and *Per2*), and cryptochrome (*Cry1* and *Cry2*) genes (Koike et al., 2012). Among these genes, *BMAL1* is a non-redundant and an essential component of circadian clock genes for maintaining normal physiology and behaviors (Bunger et al., 2000). Additionally, deficiency of *BMAL1* in mice causes an imbalance in the coagulation mechanism and has potential for blood thrombin generation (Ninivaggi et al., 2014; Hemmeryckx et al., 2019), while other circadian clock genes such as *CLOCK* (Ohkura et al., 2006; Cheng et al., 2011), *Per2* (Oishi et al., 2009), and *Cry1/2* (Ohkura et al., 2006) participate in hemostasis via regulation of fibrinolytic systems rather than coagulation systems. Nevertheless, the mechanisms underlying the functional role of *BMAL1* in affecting coagulation in bacterial infection, such as *S. oralis*, remain largely obscure.

In this study, we demonstrated that *S. oralis* downregulates the expression of *BMAL1* and then disrupts the biosynthesis of coagulation factors. As a transcription factor, *BMAL1* regulates the coagulation genes through the E-box elements. For the first time, this study demonstrated the correlation between *S. oralis* and *BMAL1*. The formation of microthrombus in hematosepsis is related to the balance of abnormal coagulation factors from hepatocyte due to the decline in *BMAL1*. Therefore, recovering *BMAL1* expression might be a promising strategy to inhibit abnormal coagulation for reducing the formation of microthrombus and lowering the mortality.

MATERIALS AND METHODS

Cell Culture and *S. oralis* Infection

HepG2 cells were purchased from ATCC (HB-8065, USA) and cultured in Dulbecco's minimum essential media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 at 37 °C. At 70–80% confluency, the cells were trypsinized and challenged by *S. oralis* with a multiplicity of infection (MOI)=50 for 24 h.

Animals

C57BL/6J mice were bought from the Beijing HFK Bioscience Co. Ltd (Beijing, China) and grouped randomly into either LD12:12 or jet-lagged. Homozygous BMAL1-deficiency ($Bmal1^{-/-}$) mice in the C57BL/6J background were produced by breeding heterozygous BMAL1-deficiency mating pairs ($Bmal1^{+/-}$), which was a kind gift from Dr. Ying Xu (Soochow University, Jiangsu, China). T BMAL1-deficiency was confirmed by Western blot as described (Bunger et al., 2000).

RNA Isolation and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted on ice using TRIzol (Vazyme, China), according to the manufacturer's protocols. cDNA synthesis was performed with reverse transcription reagent (Vazyme). Real-time RT-PCR was performed using SYBR Green PCR protocol on an ABI 7300 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Relative mRNA expression of the target genes was normalized against that of *GAPDH* using the $2^{-\Delta\Delta Ct}$ method, and presented as mean±SD of replicates. The primers used for amplification are listed in **Supplementary Table 1**.

Western Blot Analysis

Cells were lysed on ice by modified RIPA buffer (P0013B; Beyotime, Shanghai, China) to obtain total protein extract that was quantitated by BCA protein Assay kit (P0010; Beyotime, Shanghai, China), and resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes that were then blocked in 5% milk diluted with TBST for 1 h at room temperature, probed with primary antibodies, such as BMAL1 (Abcam, 1:1000), CLOCK (Abcam, 1:1000), PER2 (Abcam, 1:1000), CRY2 (Abcam, 1:1000), GAPDH (Santa Cruz Biotechnology Inc., 1:10000), FVII (Santa Cruz Biotechnology Inc., 1:1000), and FXII (Santa Cruz Biotechnology Inc., 1:500), at 4°C overnight. Then, the membranes were incubated with a secondary goat anti-rabbit antibody (1:2000) on the following day for 1 h at room temperature. ECL enhanced chemiluminescence substrate kit (Millipore) was used for imaging and quantitation of the immunoreactive bands by the Image J software.

Immunohistochemistry (IHC)

Liver tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight and sliced into 5-μm-thick paraffin-embedded sections. After deparaffinization and rehydration, antigen retrieval was performed by boiling the sections in citrate antigen retrieval solution (P0081; Beyotime) for 15 min. Next, the endogenous peroxidase activity of the samples was blocked by 3% H_2O_2 for 15 min, followed by pre-blocking with 5% BSA (A1933; Sigma–Aldrich) for 1 h at room temperature. The levels of proteins were investigated in liver tissues using primary antibodies against BMAL1 (Abcam, 1:800), FVII (Santa Cruz Biotechnology Inc., 1:400), and FXII (Santa Cruz Biotechnology Inc., 1:400) using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), followed by the DAB Substrate kit (Vector Laboratories). All protocols were followed according to the manufacturer's instructions.

Immunofluorescence (IF)

Cells were plated at a density of 1×10^5 cells/well in 24-well plates and then grown on glass coverslips for 12 h and infected with *S. oralis*, followed by staining with 5 mM SYTO® Green-Fluorescent Nucleic Acid Stains (Thermo Fisher, USA) for 30 min. After gentle washing for three times with PBS, the cells were fixed for 10 min in 4% paraformaldehyde (wt/vol). The cells were pre-blocked with 5% BSA (A1933; Sigma–Aldrich), labeled with TRITC Phalloidin (Yeasen, 1 μg/mL), and incubated with anti-rabbit Alexa Fluor 594 (Invitrogen) secondary antibodies (1:300) for 1 h at room temperature. The cell nuclei were stained with by DAPI dihydrochloride staining

solution. Images were collected using a confocal microscope (SP2-AOBS Leica Microsystems).

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of mouse plasma FVII (Cusabio, China) and mouse plasma FXII (Cusabio) were measured using ELISA kits according to the manufacturer's instructions. HepG2 cells were seeded overnight in 1% FBS-DMEM in six-well plates (6×10^6/well) and infected with *S. oralis* in 100 U/mL penicillin and 100 μg/mL streptomycin for 24 h (MOI=50). Then, cell-free-culture supernatants were collected and analyzed for FVII or FXII content by a specific ELISA (), according to the manufacturer's recommendations. The cells without infection are similar to infected cells. Briefly, the standard protein was solubilized and diluted to 20, 10, 5, 2.5, 1.25, 0.625, and 0.312 ng/mL for constructing a standard curve. The diluted samples were added to the reaction plate and incubated at 37°C for 2 h. The wells were washed five times, and the biotin primary antibody was added, and the reaction was incubated at 37°C for 1 h. Subsequently, horseradish peroxidase (HRP)-avidin was added and incubated at 37°C for 1 h, followed by the addition of a substrate chromogenic solution that was incubated at 37°C for 30 min. Finally, the reaction terminator and OD_{450} were measured.

Fluorescence *in situ* Hybridization (FISH)

The following probe was used for FISH: *S. oralis* (probe sequences targeted 16S ribosomal-RNA (rRNA) genes; 6FAM-CTCCTACGGGAGGCAGCAGTAGGGA-BHQ-3; fluorescence emission maximum at 900 nM). As mentioned above, the liver sample was fixed in 4% paraformaldehyde for 2 h at room temperature and stored in 96% ethanol. Then, the paraffin-embedded sections (5-μm-thick) were incubated in hybridization buffer (100 mM Tris-HCl pH 7.2, 0.9 M NaCl, 0.1% SDS) containing 8 ng/mL of the *S. oralis* probe overnight at 37°C. Slides were washed 3 times for 15 min in prewarmed (37°C) hybridization buffer, following the labeling of the cell nucleus by DAPI. Images were collected using a confocal microscope (SP2-AOBS Leica Microsystems).

In vivo Imaging of Animals

For *in vivo* imaging, mice (LD12:12 and jet-lagged) were injected 10^9 fluorescence-labeled bacteria through the tail vein for 6 h prior to imaging, as described previously. Subsequently, the animals were euthanized with sodium pentobarbital, the hairs were wiped off, and the organs were removed and placed on plates. The fluorescence was detected at 490 nm (*in-vivo* FX PRO BRUKER). The images were analyzed using Bruker software. All procedures were performed using a standard protocol.

Coagulation Array

A volume of 1.8 mL heart blood was collected in a blood collection tube containing 0.2 mL of 3.2% sodium citrate anticoagulant (blood volume <1.8 mL was prepared according to the ratio of blood to anticoagulant 1:9) and mixed gently. The plasma was separated at 3,000 rpm for 15 min. The prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen time, and fibrinogen concentration assays were conducted using a specific kit (Hanlisisw, China). All assays were carried out with an automatic coagulator (BE, Germany).

Bacterial Strains and Culture Conditions

The strain used for research: *S. oralis* from ATCC. Brain Heart Infusion (BHI) medium (Becton, Dickinson and Company, USA) was used for bacterial growth under aerobic conditions for 24–48 h at 37°C in order to reach the exponential growth phase. Subsequently, 10-fold serial dilutions of each bacterium were performed in PBS to achieve concentrations of $\sim 1 \times 10^8$ CFU/mL. Specific primers were designed for *S. oralis* (forward primer: CAACGATACATAGCCGACCTGAG; reverse primer: TCCATTGCCGAAGATTCC).

Chromatin Immunoprecipitation Assay (ChIP)

Chromatin immunoprecipitation assays were conducted using chromatin immunoprecipitation kit (Millipore), according to the manufacturer's protocol. Briefly, HEPG2 cells were fixed in 1% formaldehyde, cross-linked with glycine, and lysed. Cell lysates were sonicated into fragments and precleared with Protein A/G Agarose beads for 1 h. After centrifugation (1,000 rpm × 1 min at 4°C), 10% of the supernatants were stored as input, and the remaining supernatants were divided into two parts, one for detection by BMAL1 antibody and the remaining for IgG reactivity (negative control). The DNA was purified in a final volume of 50 mL and subjected to PCR. Specific primers were designed for FVII promoter (forward primer: GACCGTCTACCCCAGTGTTT; reverse primer: CGGCAGTCCACGTCATTTC).

Luciferase Reporter Assay

Bmal1-overexpression and *Bmal1*-knockout HepG2 cells were constructed and plated at a density of 5×10^5 cells/well in 12-well plates. These cells were transfected with the wild-type FVII promoter, its deletion mutant, its mutation with mutated BMAL1-binding site, and empty vector (PGL3). Furthermore, firefly luciferase reporter vectors (100 ng) and Renilla luciferase reporter vectors pRL-SV40 (10 ng; Promega, USA) were transfected into cells using Lipofectamine™ 3,000 transfection kit (Thermo Fisher, USA) for 48 h. Subsequently, firefly and Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The data were normalized by the firefly/Renilla ratio.

Transformation of *S. oralis*

Briefly, mid-logarithmic phase cultures of *S. oralis* were diluted 1:20 in complete medium (Brain Heart Infusion, Becton, Dickinson, and Co.) containing 1 mM calcium chloride, 0.2% BSA (Sigma Aldrich Ltd, Dorset, UK) and 100 ng/mL competence-stimulating peptide 1 (CSP1; Mimotopes, Clayton, Victoria, Australia). The transformation reactions were incubated for 90 s at 42 °C and placed on ice for 2 min. Then, the sensory bacteria were transferred to 6-well plate with ampicillin for 16 h. The primers were designed for *GFP*

(forward primer: GACCGTCTACCCCAGTGTTT; reverse primer: CGGCAGTCCACGTCATTTC).

Statistical Analysis

All the statistical analyses were performed with GraphPad Prism Software and based on normally distributed datasets with equal variance (Bartlett's test). The investigators were not blinded during the experiments or outcome assessment. The data points were presented as mean ± SD values unless otherwise stated. Data were inferred as statistically significant if *P*-values were <0.05. Significance between two groups was determined by independent Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used with Tukey's multiple comparisons *post-hoc* test unless stated otherwise.

RESULT

S. oralis Reduced BMAL1 Expression and Disrupted the Biosynthesis of Coagulation Factors *in vitro*

α-hemolytic *Streptococcus*, such as *S. oralis*, exhibits strong pathopoiesia either by generating various enzymes and exotoxin indirectly or invading into a host directly. In order to elucidate how *S. oralis* interacts with hepatocytes, nucleic acid dye-treated bacteria had infected cells in MOI=10,000 for 30 min, which showed that *S. oralis* directly invaded into the cells (**Figure 1A**). Owning to the lack of elements initiating coagulation, the clotting factors were not consumed, and hence, it was found that FVII slightly decreased, and FXII increased significantly

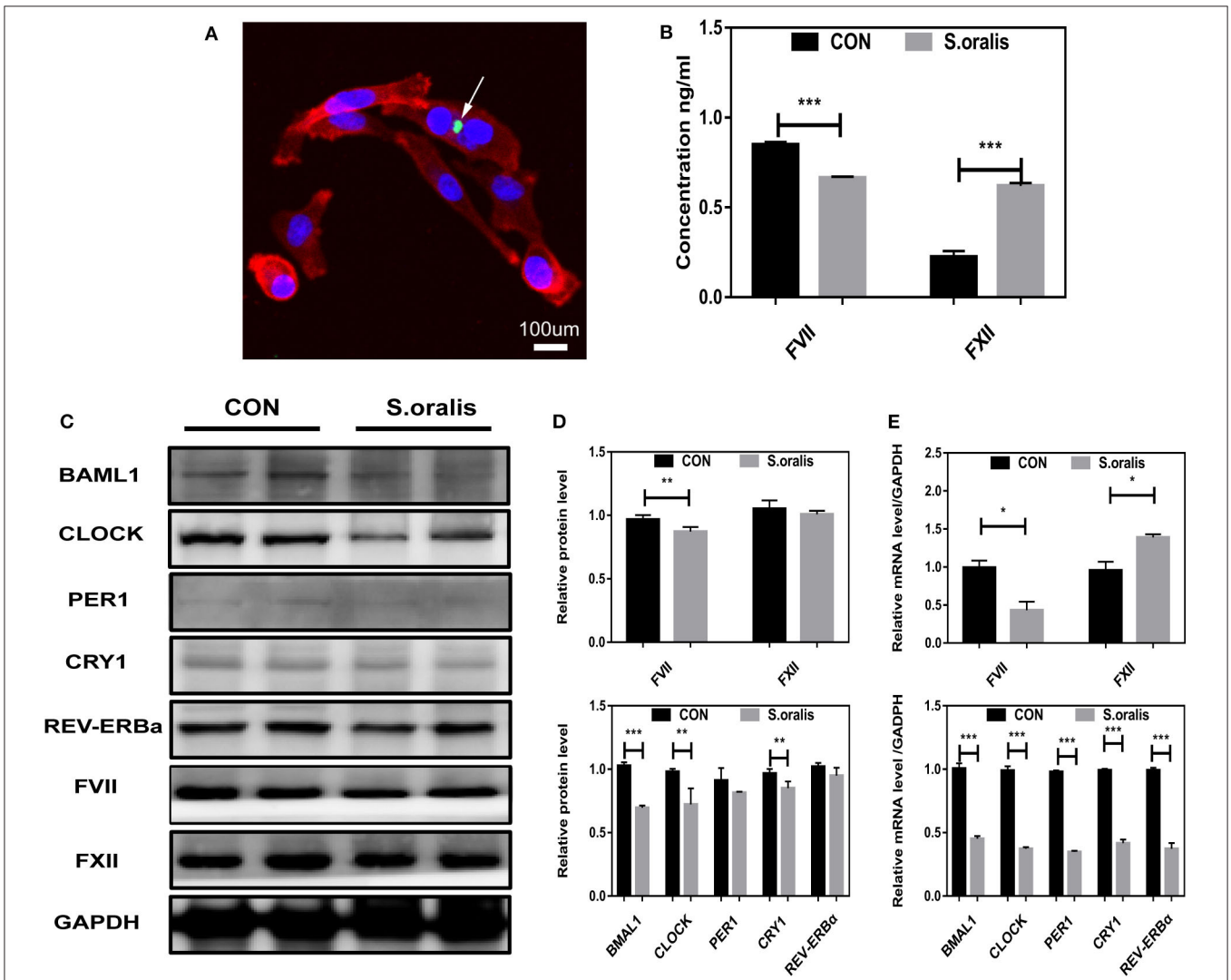


FIGURE 1 | *S. oralis* reduced BMAL1 expression and disrupted the biosynthesis of coagulation factors *in vitro*. **(A)** Confocal images for HepG2 cells infected with *S. oralis* for 30 min, stained for DAPI (blue), F-actin (red), and *S. oralis* (green). The white arrow represents bacteria invading into cells. Scale bars, 100 μm. **(B)** Relative supernatant concentration of coagulation factors VII and XII in HepG2 cells infected with *S. oralis* for 48 h as compared to the uninfected group (CON). **(C,D)** Western blot analysis of circadian clock genes and coagulation factors, and the relative values of densitometry analysis. **(E)** qRT-PCR analysis of circadian clock genes and coagulation factors. The experiments were performed three times independently. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

in the cellular supernatant of infected HepG2 (**Figure 1B**). To explore the correlation between *S. oralis* infection and circadian clock genes in hepatocytes, cells were treated with *S. oralis*, and the total protein and mRNA were extracted. Consequently, *BMAL1* expression was significantly decreased as compared to the other circadian clock genes, and it was found that the coagulation factor FVII decreased slightly, while the FXII did not alter (**Figures 1C,D**). In another cohort of cells with the same treatment, the expression of *BMAL1* and other circadian clock genes was lower in *S. oralis* infection group, and obvious upregulation of FVII and mild downregulation of FXII was detected at the transcriptional level (**Figure 1E**).

S. oralis Reduced BMAL1 Expression and Disrupted the Biosynthesis of Coagulation Factors *in vivo*

Correspondingly, to explore whether *S. oralis* modified the circadian clock genes and coagulation factor levels *in vivo*, another series of studies were carried out. The mice were injected with *S. oralis* into the tail vein. After the mice were sacrificed, the whole blood was collected for a routine examination, and it was found that the neutrophils increased significantly. In addition, the total DNA of the liver and spleen was extracted and amplified to detect the bacteria in these organs using *S. oralis*-specific primers (**Figure 2A**). To further consolidate the presence of bacteria in the liver, FISH was conducted, which showed green EGFP-labeled-bacterium in the liver, and many of them had invaded into the hepatocytes (**Figure 2B**). This phenomenon might suggest that *S. oralis* plays a functional role both directly and indirectly. The remaining of the whole blood was collected for coagulation arrays and ELISA. It was observed that the level of both plasma coagulation factors, FVII and FXII, was downregulated (**Figure 2C**). Additionally, it was found that PT, APTT, and fibrinogen reaction time were prolonged and plasma fibrinogen was declined (**Figure 2D**). In sepsis, bacteria can activate both intrinsic and extrinsic coagulation by the cytokine response. The serial sections of liver, spleen, kidney and lung stained with hematoxylin and eosin (H&E) indicated inflammation after infection (**Figure 2E**). Moreover, most of the coagulation factors, including FVII and FXII were generated in the liver. To investigate the correlation between *S. oralis* infection and coagulation factors, serial sections of the liver tissues were stained with H&E, and histochemistry was conducted (**Figure 2F**). *S. oralis* infection declined the expression of FVII expression, while that of FXII was elevated (**Figure 2G**). In addition, the livers of mice were collected and the total proteins were extracted. The results showed that BMAL1 expression was significantly decreased as compared to that of the other genes; also, an obvious decline was detected in FVII and a mild elevation of FXII as compared to the uninfected group (**Figures 2H,I**). On the other hand, *BAML1* level was also decreased, while the mRNA expression of *REV-ERBa* and *CRY1* was elevated, *FVII* was significantly downregulated, and *FXII* was markedly upregulated (**Figure 2J**). Therefore, we confirmed that *S. oralis* infection declined the level of FVII and BMAL1 while that of FXII was elevated at both protein and transcriptional levels *in vivo*.

Downregulation of FVII and Upregulation of FXII in *Bmal1*$^{-/-}$ Mice

To confirm the role of BMAL1 in coagulation factor biosynthesis, we constructed BMAL1-deficiency (*Bmal1*$^{-/-}$) mice, and the genotypes were verified by Western blot (**Figure 2D**). Interestingly, we found that BMAL1 had two-aspect functions in the coagulation process. The blood coagulation arrays showed that PT was slightly deduced while APTT was significantly prolonged with a huge reduction in these fibrinogen reaction time owning to elevated plasma fibrinogen in *Bmal1*$^{-/-}$ mice (**Figure 3A**). FVII and FXII were the key enzymes that initiated the extrinsic and intrinsic coagulation cascade, respectively. Therefore, the serial sections of the liver tissues were stained with H&E, and histochemistry was performed (**Figure 3B**). The result showed that FVII approximately decreased to a third, and FXII increased nearly four-fold in *Bmal1*$^{-/-}$ mice (**Figure 3C**). To further verify the correlation between BMAL1 and the biosynthesis of coagulation factors, the total protein of the liver tissues was extracted. This phenomenon was similar to that FVII decreased significantly while FXII increased by three-fold in *Bmal1*$^{-/-}$ mice (**Figures 3D,E**). In addition, that the expression of the coagulation factor was altered at the mRNA level, which was synchronous with the changes in the protein level, indicating that BMAL1 was likely to act as a transcription factor to regulate the biosynthesis of coagulation factors (**Figure 3F**). Although coagulation factors FVII and FXII played major roles in coagulation cascades, other coagulation factors, such as vWF, also played a significant role. Therefore, we analyzed the expression of other coagulation factors (**Figure 3G**). To investigate the effect of BAML1 on the biosynthesis of coagulation factors *in vitro*, *Bmal1*-overexpression or *BAML1*-knockout was used (**Figure 3H**), which showed that FVII expression increased mildly and FXII significantly decreased in *Bmal1*-overexpressing cells. However, a contrast result was observed in *BAML1*-knockout cells (**Figure 3I**). It was also confirmed that the deficiency of BMAL1 negatively regulated FVII expression but positively regulated FXII.

BMAL1 Regulates Coagulation Factors VII in a Direct Way

Firstly, we found that *FVII* mRNA level showed 12-h circadian changes and reached nadir around ZT8 and ZT20, but FXII did not exhibit circadian changes; several circadian clock genes in the liver showed obvious rhythmicity, and *BMAL1* mRNA expression was synchronous with FVII expression by hitting valleys around ZT8 and ZT20 (**Figure 4A**). Furthermore, the correlation between circadian clock genes and FVII showed that the expression of *BMAL1* was strongly associated with that of *FVII* (**Figure 4B**). To further verify the rhythmicity of FVII expression, we measured the plasma coagulation factor at different time points. Strikingly, FVII expression reached nadir around ZT8 and ZT20, while that of FXII lacked circadian changes (**Figure 4C**). Typically, BMAL1 acts as a transcription

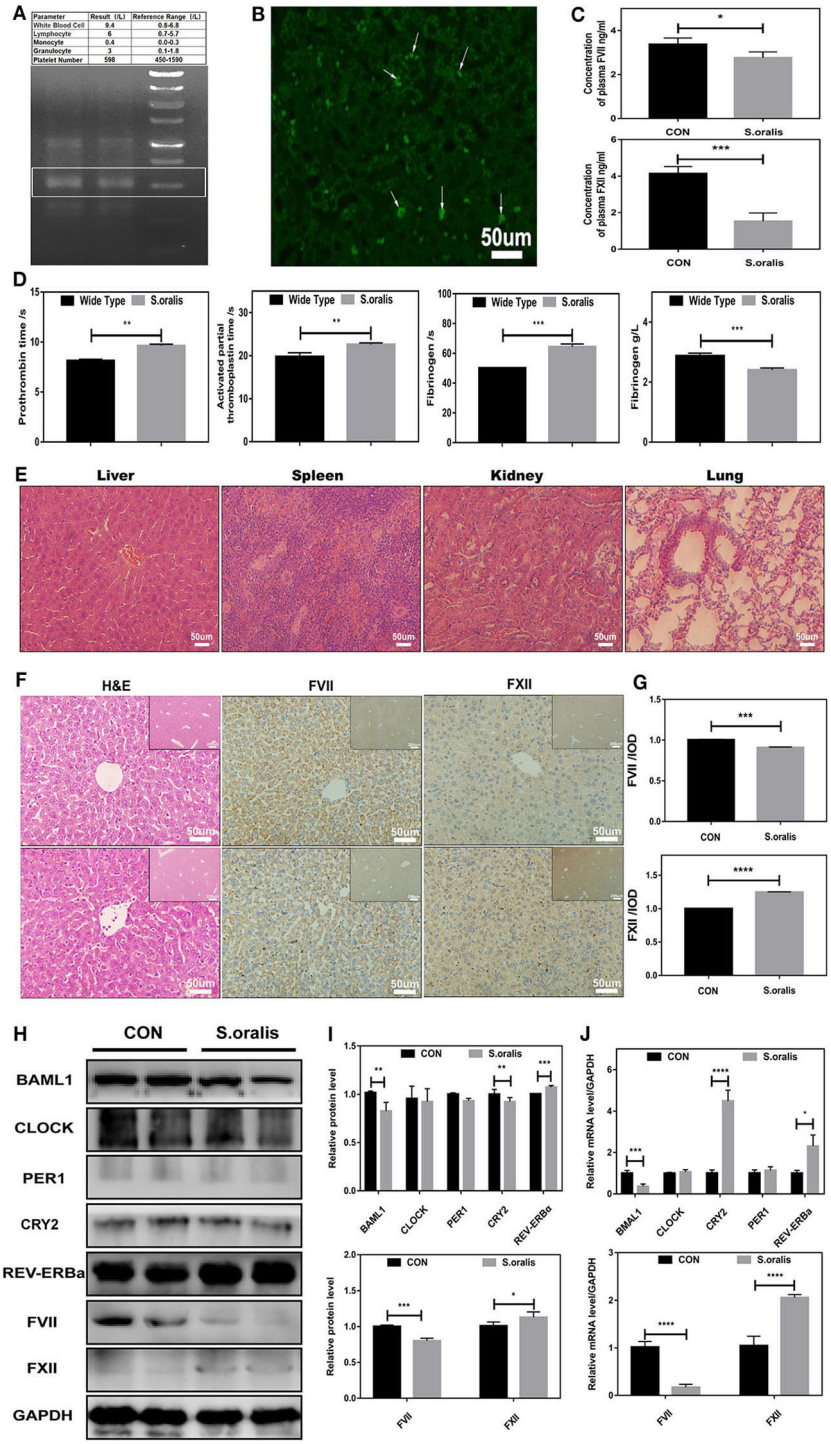


FIGURE 2 | *S. oralis* reduced BMAL1 expression and disrupted the biosynthesis of coagulation factors *in vivo*. Every mouse was infected by *S. oralis* in about 1×10^8 CFU (colony-forming units) for 24 h. **(A)** The upper part for the blood routine test of the infected mouse, the lower part for DNA extraction of infected mouse livers. Special primers were designed for *S. oralis*. **(B)** FISH for the detection of *S. oralis* in infected mouse livers. Special primers were designed for *S. oralis*. Scale bars, 50 mm. **(C)** Plasma concentration of coagulation factors VII and XII in the infected group as compared to the control group. **(D)** Routine coagulation array for mice in infected and uninfected groups. **(E)** H&E staining for liver, spleen, kidney, and lung of infected mice. **(F,G)** IHC staining for coagulation factors VII and XII of the liver and the relative values of IHC optical density. **(H,I)** Western blot analysis of circadian clock genes and coagulation factors, and the relative values of densitometry analysis of hepatocytes. **(J)** qRT-PCR analysis of circadian clock genes and coagulation factors. The experiments were performed three times independently. $^*P < 0.05$; $^{**}P < 0.01$ $^{***}P < 0.005$; $^{****}P < 0.001$.

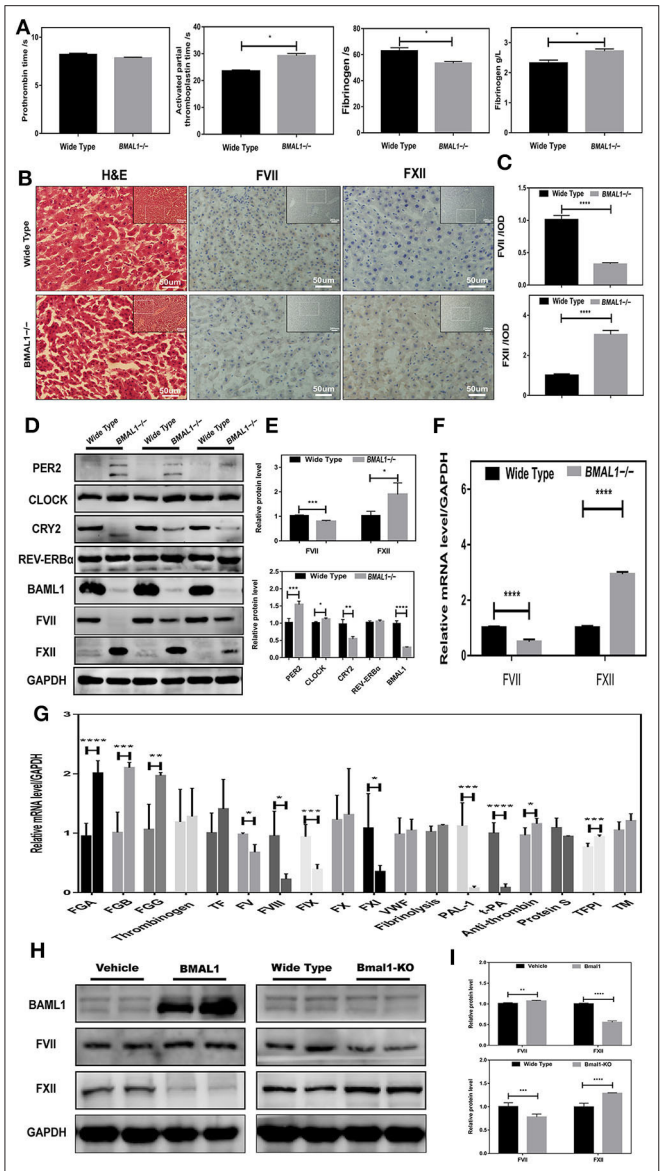


FIGURE 3 | Disrupted coagulation factors VII and XII in Bmal1$^{-/-}$ mice. **(A)** Routine coagulation array for whole blood in *Bmal1*$^{-/-}$ vs. WT mice (3/group). **(B,C)** H&E and IHC staining for coagulation factors VII and XII in slices of livers, and the relative values of IHC optical density. **(D–G)** Western blot analysis and qRT-PCR analysis of circadian clock genes and coagulation factors in mouse liver. **(H,I)** Western blot analysis of coagulation factors VII and XII in *Bmal1*-overexpression and Bmal1-knockout HepG2 cells. The experiments were performed three times independently. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$.

factor that forms a heterodimer with CLOCK to activate the expression of the downstream genes, such as E-box-controlled genes PER1 and CRY1 (Duong et al., 2011). Therefore, it could be deduced that BMAL1 regulated *FVII* through transcription factor binding sites (**Figures 4D,E**).

Low activity of *FVII* promoter could be found in cells transfected with both the *FVII* promoter mutants with *Bmal1* binding site and the *FVII* promoter deletion mutant. To confirm the role of BMAL1, firefly luciferase reporter vectors were transfected into *Bmal1*-overexpressing and *Bmal1*-knockout cells. Next, we found that the activity of *FVII* promoter was enhanced in *Bmal1*-overexpressing cells, while it was declined in *Bmal1*-knockout cells. Together, these results suggested that BMAL1 directly regulates the expression of *FVII* in a positive way (**Figure 4F**).

Circadian Rhythm Disruption-Driven Coagulation Prevents Extravasation of *S. oralis*

Since BMAL1 plays a key role in circadian rhythm, investigating the circadian rhythm disruption-driven coagulation is crucial for unveiling the role of BMAL1 in coagulation. To further explore the roles of circadian clock gene *BMAL1* in coagulation, a jet-lagged mouse model was established to observe the impact of circadian rhythm disruption on the impairment of the liver ossification process. All mice were maintained under 12/12-h light/dark cycles with the light on from 8:00 a.m. (Zeitgeber time 0, ZT0) to 8:00 p.m. (ZT12) and fed antibiotic-free food and water *ad libitum*. In the experiment, C57BL/6J mice were placed on a previously described jet-lagged schedule with 8-h light advanced every 2–3 days for 12 weeks, which mimicked the circadian disturbance that humans undergo during shift work (Zhao et al., 2017). PT was mildly shortened, while APTT was significantly prolonged, and the fibrinogen reaction time was shortened owing to elevated plasma fibrinogen in jet-lagged mice (**Figure 5A**). To eliminate the impact of body weight and liver weight, measurement experiments were essential, which seemed to have no impact on the coagulation (**Figures 5B,C**). However, jet-lag seemed to induce a slight decline in BT, suggesting that circadian rhythm disruption-driven downregulation of BMAL1 might be conducive to thrombus formation (**Figure 5D**). In order to elucidate whether circadian rhythm disruption-driven coagulation prevents extravasation of *S. oralis*, EGFP-labeled *S. oralis* was constructed and confirmed by PCR amplification (**Figure 5E**). We found that the number of bacteria invaded into the liver was less than that in the LD12:12 group due to disruption-driven coagulation, which prevents the invasion of EGFP-labeled *S. oralis*. Intriguingly, a similar phenomenon was observed in the lung and spleen, and that the number of bacteria invaded into the kidneys was large in quantity in both the jet-lagged and LD12:12 groups because of abundant blood vessels (**Figures 5F,G**).

DISCUSSION

To gain new insight into the mechanism underlying the influence of the circadian gene *BMAL1* on coagulation factors in bacterial infectious liver disease, we performed the experiment described above and showed that the level of BMAL1 in hepatocytes was downregulated in the *S. oralis* infection group, leading to the downregulation of coagulation factor VII and the upregulation of coagulation factor XII. Similar results were found *in vitro*. *Bmal1* overexpression significantly increased the coagulation factor VII

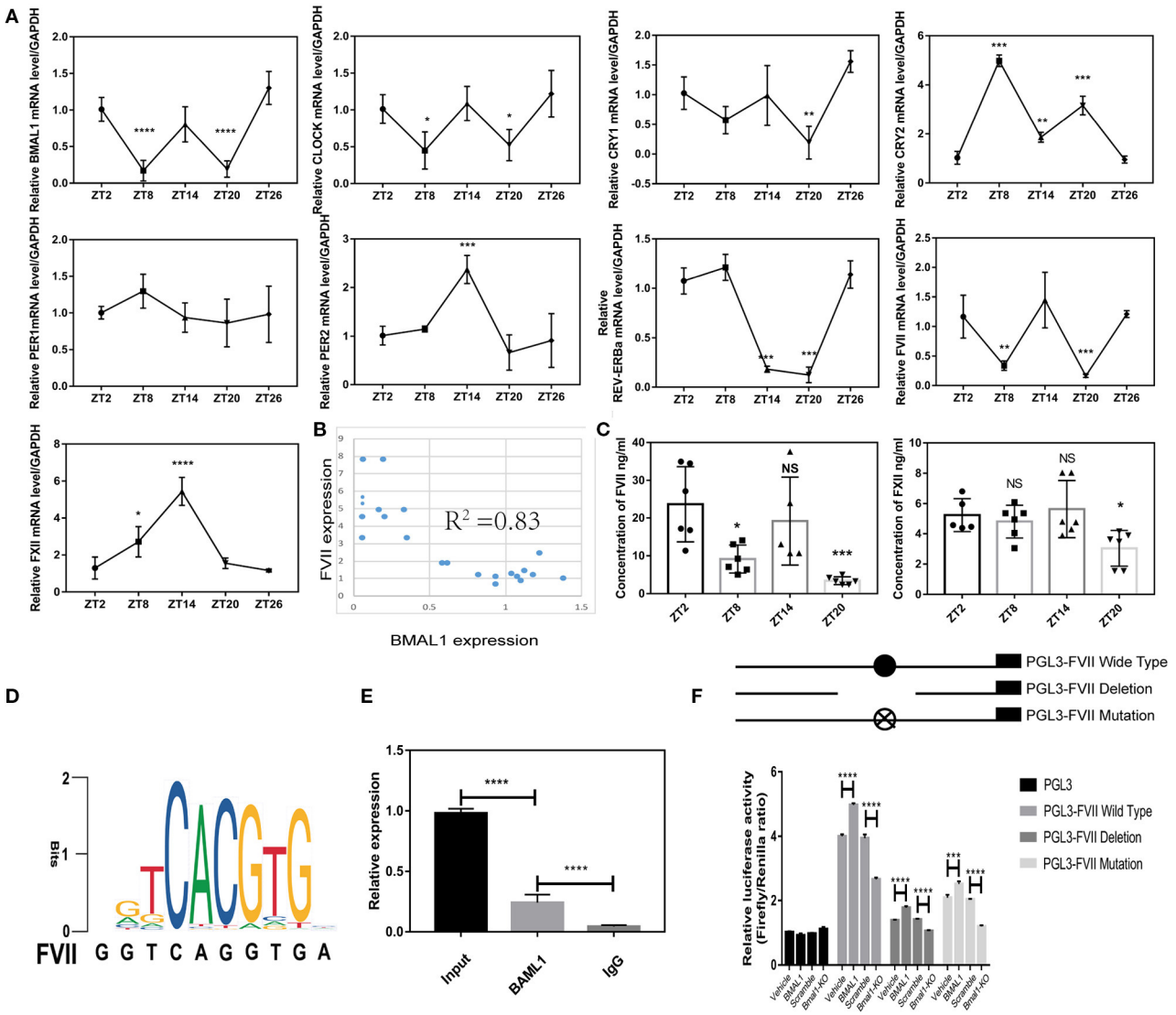


FIGURE 4 | BMAL1 regulates coagulation factors VII in a direct way. **(A)** Relative expression of circadian clock genes and coagulation factors at different time points (4 mice/each point). **(B)** Correlation analysis between BMAL1 and FVII. **(C)** Plasma concentration of coagulation factors VII and XII at each time point. **(D)** Motif sequence signature of BMAL1 in the promoter region of coagulation factor FVII. **(E)** ChIP assays were performed using an antibody against BMAL1, and IgG as negative controls. Specific primers were designed for FVII promoter. **(F)** Luciferase reporter assays were performed to measure the activities of WT FVII promoter (PGL3- FVII WT), its deletion mutant (PGL3- FVII deletion), its mutant (PGL3- FVII mutation) with mutated BMAL1-binding site and empty vector (PGL3) in *Bmal1*-overexpression and *Bmal1*-knockout HepG2 cells. The experiments were performed three times independently. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$.

while it decreased the level of coagulation factor XII in HEPG2 cell line. According to these findings, circadian gene *BMAL1* plays a critical role in coagulation by regulating the biogenesis of clotting factors in *S. oralis* infection.

Reportedly, the gut homeostatic microbiome is necessary for normal oscillations in both intestine and liver, which is effectuated via circadian genes, and participating in diurnal fluctuations of host physiology, disease susceptibility, lipid absorption, and sleep rhythms (Miyazaki et al., 2014; Thaiss et al., 2016; Kuang et al., 2019). The courses of bacterial invasion into the host include bacterial adsorption on the body surface, invading tissues or cells, growing and breeding, producing toxins, spreading, and resisting a series of defensive functions of the host. Colonized *S. oralis* derived from blood bacteria was found in the liver and was also found to be invading into hepatocytes (**Figures 1A**, **2A**). Herein, we observed that the downregulation of BMAL1 contributed to the secretion of FXII without the upregulation of FXII protein level *in vitro* (**Figure 1B**). Consistent with the current findings, some studies have shown that the deficiency of BMAL1 upregulates the secretion of many biochemical parameters by restraining their repressors, such as CXCL5 (Gibbs et al., 2014) and interleukin-1β

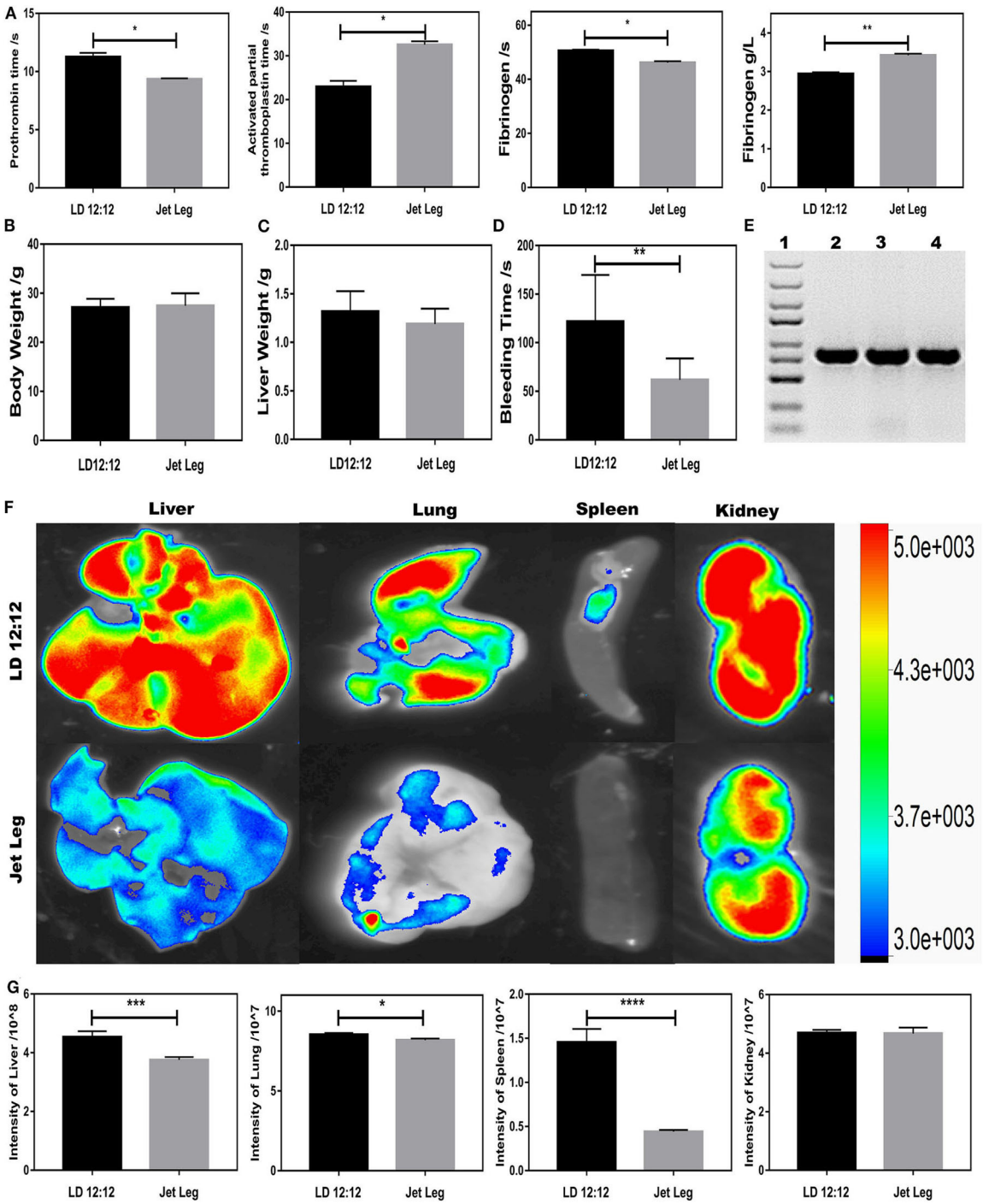


FIGURE 5 | Circadian rhythm disruption-driven coagulation prevents extravasation of *S. oralis*. **(A)** Routine coagulation array (4 mice/group) for whole blood in LD12:12 vs. jet-lag mice at ZT12. **(B,C)** Bodyweight and liver weight of mice in LD12:12 vs. jet-lag at ZT12. **(D)** Tail bleeding time of mice. **(E)** DNA extraction of EGFP-labeled *S. oralis*. Specific primers for GFP. **(F,G)** Imaging of mice in LD12:12 vs. jet-lag mice at ZT2 *in vivo* (4 mice/group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$.

(Luyts et al., 2014). Furthermore, the interaction between *Bmal1* of hepatocytes and bacterial infection revealed that the expression of BMAL1 descended at the transcriptional level both *in vitro* and *in vivo* in the microorganism. This finding is in line with the results of the previous studies (Lou et al., 2017; Li et al., 2018; Coiffard et al., 2019). With the infection of *Mycobacterium tuberculosis*, the level of BMAL1 was significantly decreased in both the lung and the spleen (Lou et al., 2017). Conversely, some

studies showed a certain promoting effect of BMAL1 on gastric epithelial cells with the infection of *Helicobacter pylori* (Li et al., 2018), which could be attributed to the various roles of BMAL1 in different diseases or the various types of bacteria executing infections in different conditions. The reciprocal role between bacteria and BMAL1 has been reported. The magnitude of response to the pathogenic bacterial challenge under the control of Bmal1 has been established (Bhardwaj et al., 2011; Wang et al., 2011; Gibbs et al., 2012, 2014). However, the mutual role of *Bmal1* and microbiota needs to be investigated in the future.

Circadian rhythm is a regular recurrence of biological processes or activities with an ∼24-h cycle. The circadian changes have been detected in human coagulation factors, such as antithrombin, FVII, and FVIII (Kapiotis et al., 1997; Undar et al., 1999; Iversen et al., 2002). In the current study, not only mouse plasma FVII but also FVII in mouse hepatocyte exhibit rhythmicity (**Figure 4A**). BMAL1 plays a core and elemental role (Bunger et al., 2000). However, other components of circadian clock genes, such as *CLOCK* and *CRY1/2* (Ohkura et al., 2006; Cheng et al., 2011), were largely relevant to the fibrinolytic system rather than the coagulation system. The properties of diurnal rhythm were entirely lost for FVII and antithrombin. Nonetheless, additional evidence on the regulation of coagulation factors needs to be unveiled in *Bmal1*$^{-/-}$ mice. This study showed that FVII level was tremendously declined in *Bmal1*$^{-/-}$ mice (**Figures 3C,E**). Interestingly, FXII level was markedly elevated in *Bmal1*$^{-/-}$ mice (**Figures 3C,E**). Thus, our findings may be a significant breakthrough that BMAL1 plays a two-sided role in regulating the coagulation process. In the endogenous coagulation pathway, decreased FXI and FXI might explain the prolonged APPT despite the elevated level of FXII in *Bmal1*$^{-/-}$ mice. In the exogenous coagulation pathway, deficiency of *BAML1* might explain the unchanged PT by affecting the activation of coagulation factors despite deduced FVII in *Bmal1*$^{-/-}$ mice (**Figure 3G**). This displays that the regulation of FVII is complicated and sophisticated. BMAL1 is crucial for the regulation of FVII expression. Low expression of BMAL1 could still be found in *Bmal1*-knockout cells (**Figure 3G**). These phenomena might explain the low activity of FVII promoter, which could be found in *Bmal1*-knockout cells (**Figure 4F**). In the future, it might be essential to study the correlation between BMAL1 and coagulation.

The elevated FXII level ascribes the potentially elevated generation of endogenous thrombin larger in BMAL1-deficient mice (Hemmeryckx et al., 2019). Additionally, the level of FGA (Fibrinogen α chain), FGB (Fibrinogen β chain), and FGG (Fibrinogen γ chain) was elevated in *Bmal1*$^{-/-}$ mice in accordance with the elevated plasma fibrinogen in the blood coagulation arrays, which contributed to the formation of the crypto-fibrin network (**Figure 3G**; Ninivaggi et al., 2014). Although circadian rhythm disruption-driven coagulation prevents the extravasation of *S. oralis*, more macrothrombusus is induced than in the control group, which leads to multiple organ failure on account of deduced BMAL1 expression.

In summary, our results demonstrated that the downregulation of BMAL1 by *S. oralis* infection disrupts these clotting factors in hepatocytes. Therefore, we identified the coagulation factors VII and XII that were largely regulated in *Bmal1*$^{-/-}$ mice. Interestingly, this study on circadian clock genes *BMAL1* might provide a novel view for the generation of microthrombus in hematosepsis induced by *S. oralis*.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee of Ethics of Wuhan Union Hospital and the Institutional Research Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LC and SL designed the study and critically revised the manuscript. JN performed the experiments, analyzed the data, and drafted the manuscript. JZ participated in the implementation of the study. SY, YL, and JP collected the data. All authors read and approved the final manuscript.

FUNDING

This work was financially supported by grants from the National Science Foundation for Distinguished Young Scholars of China (31725011 to LC) and the National Natural Science Fund (81700946 to JZ).

ACKNOWLEDGMENTS

We thank Prof. Ying Xu for the heterozygous BMAL1-deficient mating pairs (*Bmal1*$^{wt/-}$).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.530190/full#supplementary-material

REFERENCES

Amaral, A., Opal, S. M., and Vincent, J.-L. (2004). Coagulation in sepsis. *Intensive Care Med.* 30, 1032–1040. doi: 10.1007/s00134-004-2291-8

Bhardwaj, V., Meier, S., Petersen, L. N., Ingle, R. A., and Roden, L. C. (2011). Defence responses of *Arabidopsis thaliana* to infection by *Pseudomonas syringae* are regulated by the circadian clock. *PLoS ONE* 6:e26968. doi: 10.1371/journal.pone.0026968

Bogdanov, V. Y., Balasubramanian, V., Hathcock, J., Vele, O., Lieb, M., and Nemerson, Y. (2003). Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat. Med.* 9, 458–462. doi: 10.1038/nm841

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., et al. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 103, 1009–1017. doi: 10.1016/S0092-8674(00)00205-1

Cheng, S., Jiang, Z., Zou, Y., Chen, C., Wang, Y., Liu, Y., et al. (2011). Downregulation of Clock in circulatory system leads to an enhancement of fibrinolysis in mice. *Exp. Biol. Med.* 236, 1078–1084. doi: 10.1258/ebm.2011.010322

Coiffard, B., Diallo, A. B., Culver, A., Mezouar, S., Hammad, E., Vigne, C., et al. (2019). Circadian rhythm disruption and sepsis in severe trauma patients: *Shock* 52, 29–36. doi: 10.1097/SHK.0000000000001241

Cornely, O. A., and Schirmacher, P. (2001). Clinical picture: bacterial translocation in neutropenic sepsis. *Lancet.* 358:1842. doi: 10.1016/S0140-6736(01)06884-2

Duong, H. A., Robles, M. S., Knutti, D., and Weitz, C. J. (2011). A molecular mechanism for circadian clock negative feedback. *Science* 332, 1436–1439. doi: 10.1126/science.1196766

Fourrier, F., Chopin, C., Goudemand, J., Hendrycx, S., Caron, C., Rime, A., et al. (1992). Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared patterns of antithrombin III, protein C, and protein S deficiencies. *Chest* 101, 816–823. doi: 10.1378/chest.101.3.816

Furie, B., and Furie, B. C. (1988). The molecular basis of blood coagulation. *Cell* 53, 505–518. doi: 10.1016/0092-8674(88)90567-3

Gibbs, J., Ince, L., Matthews, L., Mei, J., Bell, T., Yang, N., et al. (2014). An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action. *Nat. Med.* 20, 919–926. doi: 10.1038/nm.3599

Gibbs, J. E., Blaikley, J., Beesley, S., Matthews, L., Simpson, K. D., Boyce, S. H., et al. (2012). The nuclear receptor REV-ERBα mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. *Proc. Natl. Acad. Sci. U.S.A.* 109, 582–587. doi: 10.1073/pnas.1106750109

Hemmeryckx, B., Frederix, L., and Lijnen, H. R. (2019). Deficiency of Bmal1 disrupts the diurnal rhythm of haemostasis. *Exp. Gerontol.* 118, 1–8. doi: 10.1016/j.exger.2018.12.017

Iversen, P. O., Groot, P. D., Hjeltnes, N., Andersen, T. O., Mowinckel, M. C., Sandset, P. M., et al. (2002). Impaired circadian variations of haemostatic and fibrinolytic parameters in tetraplegia. *Br. J. Haematol.* 119, 1011–1016. doi: 10.1046/j.1365-2141.2002.03953.x

Kapiotis, S., Jilma, B., Quehenberger, P., Ruzicka, K., Handler, S., and Speiser, W. (1997). Morning hypercoagulability and hypofibrinolysis. Diurnal variations in circulating activated factor VII, prothrombin fragment F1+2, and plasmin-plasmin inhibitor complex. *Circulation* 96, 19–21. doi: 10.1161/01.CIR.96.1.19

Koike, N., Yoo, S.-H., Huang, H.-C., Kumar, V., Lee, C., Kim, T.-K., et al. (2012). Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. *Science* 338, 349–354. doi: 10.1126/science.1226339

Kuang, Z., Wang, Y., Li, Y., Ye, C., Ruhn, K. A., Behrendt, C. L., et al. (2019). The intestinal microbiota programs diurnal rhythms in host metabolism through histone deacetylase 3. *Science* 65, 1428–1434. doi: 10.1101/580613

Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., et al. (2004). Identification of early microbial colonizers in human dental biofilm. *J. Appl. Microbiol.* 97, 1311–1318. doi: 10.1111/j.1365-2672.2004.02420.x

Li, T., Shao, W., Li, S., Ma, L., Zheng, L., Shang, W., et al. (2018). H. pylori infection induced BMAL1 expression and rhythm disorder aggravate gastric inflammation. *EBioMedicine* 39, 301–314. doi: 10.1016/j.ebiom.2018.11.043

Lou, J., Wang, Y., Zhang, Z., and Qiu, W. (2017). Activation of MMPs in macrophages by *Mycobacterium tuberculosis* via the miR-223-BMAL1 signaling pathway. *J. Cell. Biochem.* 118, 4804–4812. doi: 10.1002/jcb.26150

Luyts, K., Smulders, S., Napierska, D., van kerckhoven, S., Poels, K., Scheers, H., et al. (2014). Pulmonary and hemostatic toxicity of multi-walled carbon nanotubes and zinc oxide nanoparticles after pulmonary exposure in Bmal1 knockout mice. *Part Fibre Toxicol.* 11:16. doi: 10.1186/s12989-014-0061-5

Miyazaki, K., Itoh, N., Yamamoto, S., Higo-Yamamoto, S., Nakakita, Y., Kaneda, H., et al. (2014). Dietary heat-killed Lactobacillus brevis SBC8803 promotes voluntary wheel-running and affects sleep rhythms in mice. *Life Sci.* 111, 47–52. doi: 10.1016/j.lfs.2014.07.009

Ninivaggi, M., Kelchtermans, H., Kuijpers, M. J., Hemmeryckx, B., Heemskerk, J. W. M., Lindhout, T., et al. (2014). Whole blood thrombin generation in Bmal1-deficient mice. *Thromb. Haemost.* 112, 271–275. doi: 10.1160/TH13-11-0910

Ohkura, N., Oishi, K., Fukushima, N., Kasamatsu, M., Atsumi, G.-I., Ishida, N., et al. (2006). Circadian clock molecules CLOCK and CRYs modulate fibrinolytic activity by regulating the *PAI-1* gene expression. *J. Thromb. Haemost.* 4, 2478–2485. doi: 10.1111/j.1538-7836.2006.02210.x

Oishi, K., Miyazaki, K., Uchida, D., Ohkura, N., Wakabayashi, M., Doi, R., et al. (2009). PERIOD2 is a circadian negative regulator of PAI-1 gene expression in mice. *J. Mol. Cell. Cardiol.* 46, 545–552. doi: 10.1016/j.yjmcc.2009.01.001

Penack, O., Becker, C., Buchheidt, D., Christopeit, M., Kiehl, M., von Lilienfeld-Toal, M., et al. (2014). Management of sepsis in neutropenic patients: 2014 updated guidelines from the infectious diseases working party of the German society of hematology and medical oncology (AGIHO). *Ann. Hematol.* 93, 1083–1095. doi: 10.1007/s00277-014-2086-0

Schmaier, A. H. (2008). The elusive physiologic role of Factor XII. *J. Clin. Invest.* 118, 3006–3009. doi: 10.1172/JCI36617

Shelburne, S. A., Sahasrabhojane, P., Saldana, M., Yao, H., Su, X., Horstmann, N., et al. (2014). *Streptococcus mitis* strains causing severe clinical disease in cancer patients. *Emerg. Infect. Dis.* 20, 762–771. doi: 10.3201/eid2005.130953

Takahashi, J. S., Hong, H.-K., Ko, C. H., and McDearmon, E. L. (2008). The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat. Rev. Genet.* 9, 764–775. doi: 10.1038/nrg2430

Thaiss, C. A., Levy, M., Korem, T., Dohnalov,á, L., Shapiro, H., Jaitin, D. A., et al. (2016). Microbiota diurnal rhythmicity programs host transcriptome oscillations. *Cell* 167, 1495–1510.e12. doi: 10.1016/j.cell.2016.11.003

Thiagarajan, S., Krishnamurthy, S., Raghavan, R., Mahadevan, S., Madhugiri, V. S., and Sistla, S. (2016). *Streptococcus oralis* cerebral abscess following monkey bite in a 2-month-old infant. *Paediatr. Int. Child Health* 36, 160–162. doi: 10.1179/2046905515Y.0000000020

Turnier, L., Nausheen, S., and Cunha, B. A. (2009). Fatal Streptococcus viridans (S. oralis) aortic prosthetic valve endocarditis (PVE) with paravalvular abscesses related to steroids. *Heart Lung* 38, 167–171. doi: 10.1016/j.hrtlng.2007.12.008

Undar, L., Ertugrul, C., Altunba,ş, H., and Akça, S. (1999). Circadian variations in natural coagulation inhibitors protein C, protein S and antithrombin in healthy men: a possible association with interleukin-6. *Thromb. Haemost.* 81, 571–575. doi: 10.1055/s-0037-1614526

Vervloet, M. G., Thijs, L. G., and Hack, C. E. (1998). Derangements of coagulation and fibrinolysis in critically III patients with sepsis and septic shock. *Semin. Thromb. Hemost.* 24, 33–44. doi: 10.1055/s-2007-995821

Wang, W., Barnaby, J. Y., Tada, Y., Li, H., Tör, M., Caldelari, D., et al. (2011). Timing of plant immune responses by a central circadian regulator. *Nature* 470, 110–114. doi: 10.1038/nature09766

Zhao, J., Zhou, X., Tang, Q., Yu, R., Yu, S., Long, Y., et al. (2017). BMAL1 deficiency contributes to mandibular dysplasia by upregulating MMP3. *Stem Cell Rep.* 10, 180–195. doi: 10.1016/j.stemcr.2017.11.017

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership