Distribution Statement

Distribution A: Public Release.

The views presented here are those of the author and are not to be construed as official or reflecting the views of the Uniformed Services University of the Health Sciences, the Department of Defense or the U.S. Government.

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
POSTGRADUATE DENTAL COLLEGE
ARMY POSTGRADUATE DENTAL SCHOOL
228 EAST HOSPITAL ROAD
FORT GORDON, GEORGIA 30905

THESIS APPROVAL PAGE FOR MASTER OF SCIENCE IN ORAL BIOLOGY

Title of Thesis: Influence of of Lactobacillus reuteri, Bifidobacterium animalis subsp. lactis, and prebiotic inulin on dysbiotic dental biofilm composition ex vivo

Name of Candidate: Aaron N. Colamarino
Master of Science Degree
October 17, 2022

THESIS/MANUSCRIPT APPROVED: DATE:

JOHNSON.THOMAS.MICHAEL.1073956591 Digitally signed by JOHNSON.THOMAS.MICHAEL.1073956591 Date: 2022.10.17 07:10:44 -04'00' October 17, 2022

Thomas M. Johnson
DEPARTMENT OF PERIODONTICS, ARMY POSTGRADUATE DENTAL SCHOOL
Committee Chairperson

STANCOVEN.BRIAN.WILLIAM.1239802733 Digitally signed by STANCOVEN.BRIAN.WILLIAM.1239802733 Date: 2022.10.17 09:54:55 -04'00' October 17, 2022

Brian W. Stancoven
DEPARTMENT OF PERIODONTICS, ARMY POSTGRADUATE DENTAL SCHOOL
Committee Member

LINCICUM.ADAM.RANDAL.1139359891 Digitally signed by LINCICUM.ADAM.RANDAL.1139359891 Date: 2022.10.17 08:11:35 -04'00' October 17, 2022

Adam R. Lincicum
DEPARTMENT OF PERIODONTICS, ARMY POSTGRADUATE DENTAL SCHOOL
Committee Member

DUTNER.JOSEPH.MICHAEL.1244007410 Digitally signed by DUTNER.JOSEPH.MICHAEL.1244007410 Date: 2022.10.25 07:36:10 -07'00' October 25, 2022

Joseph M. Dutner
DEPARTMENT OF ENDODONTICS, ARMY POSTGRADUATE DENTAL SCHOOL
Committee Member

Influence of *Lactobacillus reuteri*, *Bifidobacterium animalis* subsp. *lactis*, and prebiotic inulin on dysbiotic dental biofilm composition ex vivo

by

Aaron N. Colamarino, DDS

CPT, DC, USA

Thesis directed by:

Thomas M. Johnson, DMD, MS; COL, DC, USA
Professor, Department of Periodontics, Army Postgraduate Dental School

Thesis committee members:

Daniel M. Boudreaux, PhD; MAJ, MS, USA
Department of Clinical Investigation, Dwight David Eisenhower Army Medical Center

Joseph M. Dutner, DMD, MS; LTC, DC, USA
Associate Professor, Department of Endodontics, Army Postgraduate Dental School

Brian W. Stancoven, DMD, MS; LTC(P), DC, USA
Associate Professor, Department of Periodontics, Army Postgraduate Dental School

Adam R. Lincicum, DMD, MS; LTC(P), DC, USA
Assistant Professor, Department of Periodontics, Army Postgraduate Dental School

Thesis submitted to the Faculty of the
Army Postgraduate Dental School
Postgraduate Dental College
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Master of Science 2023

ABSTRACT

Background: Probiotic bacterial supplementation has shown promising results in the treatment of periodontitis and the maintenance of periodontal health. The purpose of this investigation was to evaluate the influence of *Lactobacillus reuteri* or *Bifidobacterium animalis* subsp. *lactis* supplementation with and without prebiotic inulin on biofilm composition using an ex vivo biofilm model.

Methods: Subgingival plaque specimens from three periodontitis-affected human donors were used to grow biofilms on hydroxyapatite disks in media supplemented with varying combinations of prebiotic inulin, *Lactobacillus reuteri,* and *Bifidobacterium animalis* subsp. *lactis*. Relative abundances of bacterial genera present in mature biofilms were evaluated using 16S rRNA next generation sequencing. Diversity metrics of microbial communities were evaluated using a next-generation microbiome bioinformatics platform.

Results: Inulin supplementation produced statistically significant dose-dependent increases in relative abundances of *Lactobacillus* and *Bifidobacterium* species ($p<0.001$) with concomitant decreases in relative abundances of *Streptococcus, Veillonella, Fusobacterium, Parvimonas,* and *Prevotella* species ($p<0.001$). Inoculation with *L. reuteri* or *B. animalis* subsp. *lactis* increased the relative abundance of only the supplemented probiotic genera ($p<0.05$). Supplemental inulin led to a statistically significant decrease in biofilm alpha diversity ($p<0.001$).

Conclusions: The described ex vivo model appears suitable for investigating effects of probiotic bacteria, prebiotic oligosaccharides, and combinations thereof on biofilm composition and complexity. Within the limitations imposed by this model, results from the present study

underscore the potential for prebiotic inulin to modify biofilm composition favorably. Additional research further elucidating biologic rationale and controlled clinical research defining therapeutic benefits is warranted.

KEY WORDS: Periodontitis, biofilms, dental plaque, probiotics, prebiotics, inulin

INTRODUCTION

For almost a half century, researchers have understood that the microbial cells colonizing the human body typically equal, or possibly far exceed, the somatic cell count.[1-3] Indeed, our microbial cohabitants are abundant and diverse. A healthy human may accommodate between 500 and 1000 bacterial species at any given time.[4] However, the mixture and relative abundance of bacterial species inhabiting various dermal and mucosal sites exhibit temporal variations, and individual patients may harbor profoundly different microbial collections.[1,3] For example, only about one third of the gut microbiota appears common to most humans, whereas the remaining two thirds comprises species that are specific to the individual.[3] Although some determinants of temporal and inter-individual microbiome variability are known, investigators do not fully understand the influence of these variations on health, wellness, and the onset/progression of disease.[1] Nevertheless, unfavorable changes in the microbiome—with associated immune, endocrine, and nervous system interactions—correlate with an array of human afflictions including inflammatory bowel disease, cancer, sinusitis, and periodontitis.[5-9]

Given the abundance and biodiversity of the human microbiome, it is unsurprising that manipulation of its composition—physically or pharmacologically—is an important strategy in the prevention and treatment of many diseases. In 1954, Kragen became the first author to report inoculation of the oral cavity with a beneficial bacterial species.[10] Lilly and Stillwell introduced the term "probiotics" in 1965,[11] and the World Health Organization subsequently established the widely accepted definition "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host."[12] In recent years, investigators have

utilized oral probiotics to treat or prevent dental caries, *Candida* infections, halitosis, and periodontal disease.[13,14] Dental practitioners have delivered these supplements as powders, suspensions, capsules, lozenges, and foods fortified with specific probiotic strains.[14]

New concepts in periodontitis etiopathogenesis may bolster interest in the therapeutic potential of oral probiotics. Periodontitis is an archetypical multifactorial disease process, whereby periodontal tissue destruction manifests through complex interactions between environmental and genetic factors.[8,9] Although genetic influences account for half of the variability associated with periodontitis,[15] behavioral and environmental factors emerging over the last two centuries appear responsible for a sudden surge in the prevalence of the disease.[9] For decades, investigators have associated the presence of *Porphyromonas gingivalis* and *Tannerella forsythia* in subgingival biofilms with increased risk of developing periodontitis, increased risk of progression to advanced disease, and decreased likelihood of successful treatment.[16] However, ample evidence now suggests that periodontitis results not from one or a few bacterial species but from true polymicrobial activity.[8] Accordingly, probiotics may benefit the host through a variety of mechanisms including competitive inhibition of pathogens, suppression of virulence factors, augmentation of the mucosal barrier function, development of the immune system, host immunomodulation, and synthesis of antimicrobial peptides.[17,18]

Researchers have characterized microbiological and clinical effects of numerous probiotic strains. Of these, *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis* have shown promising results in the treatment and prevention of periodontal disease with high safety margins.[18-30] *L. reuteri* is an indigenous microorganism of the human gastrointestinal

tract known to modulate cytokine levels, suppress inflammation, and produce reuterin, an antimicrobial protein.[30, 31] In randomized controlled trials, *L. reuteri* supplementation has reduced the presence of specific periodontal pathogens, decreased levels of cytokines and other inflammatory markers, and improved periodontitis treatment outcome measures such as probing depth, clinical attachment level, plaque index, gingival index, and bleeding on probing.[18, 19-22] *B. animalis* subsp. *lactis* is also considered a normal resident of the human microbiome, exhibiting a symbiotic relationship with the host through antimicrobial and immunomodulatory properties.[23] In an in vitro study, probiotics of the *Bifidobacterium* genus increased IL-10 levels and inhibited IL-1b and TNF-α effects.[24] In randomized controlled trials and rodent models, *B. animalis* subsp. *lactis* supplementation has decreased pro-inflammatory and increased anti-inflammatory cytokine levels, reduced the relative abundances of orange- and red-complex bacteria, and produced clinical benefits comparable to those described for *L. reuteri*.[13, 23-27]

A technical challenge limiting the clinical application of oral probiotics for therapeutic and preventative purposes is the inability to establish beneficial species as prominent members of the host microbiota without consistent inoculation. Although *L. reuteri* and *B. animals* subsp. *lactis* are capable of surviving in oral biofilms,[25] most researchers agree that colonization of these species is transient without sustained/repeated intake.[21, 28, 29] Efforts to overcome this obstacle have led to studies involving prebiotics—non-digestible oligosaccharides that promote proliferation of beneficial commensal species.[32] Three criteria have been proposed for classifying a carbohydrate as a prebiotic: 1) resistance to hydrolysis and absorption in the upper gastrointestinal tract, 2) fermentation by selective intestinal bacteria, and 3) enrichment of

beneficial bacterial species within the intestinal microbiota.[33] Only inulin and fructo-oligosaccharides, which together comprise the β2-1 fructans, have satisfied all three criteria; other oligosaccharides are considered candidate prebiotics.[33] Prebiotics have shown encouraging results in the stimulation of indigenous gastrointestinal bacteria, leading to a shift in the microbiota to a symbiotic state with multiple health benefits.[32, 33] In addition to positively influencing intestinal health, prebiotic supplementation has been used to selectively promote beneficial bacterial species within the oral microbiota.[34-36] Slomka and colleagues reported that supplementation with N-acetyl-D-mannosamine resulted in biofilm composition consisting of 97% beneficial species.[34] Clinical studies have demonstrated the effectiveness of prebiotics such as inulin to selectively enrich oral biofilms in species of the *Lactobacillus* and *Bifidobacterium* genera.[35, 37]

Although prior reports have demonstrated oral health benefits of *L. reuteri* and *B. animalis* subsp*. lactis,* the preferred probiotic species, the most effective vehicle of administration, and the optimal regimen remain unestablished. Moreover, prior research has not adequately characterized the value of combining probiotic and prebiotic supplementation. The current study aimed to evaluate the influence of *L. reuteri* or *B. animals* subsp. *lactis,* with and without prebiotic inulin, on microbial diversity and relative abundances of various bacterial species within dental biofilm cultures ex vivo.

MATERIALS AND METHODS

Ethical guidelines

This protocol utilized de-identified dental biofilm and saliva specimens and did not involve contact with patients or patient records. The Dwight David Eisenhower Army Medical Center Human Research Protections Office determined this research to be exempt from IRB review requirements (protocol #20-11301/931759), and the protocol was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. Approval was granted to use de-identified specimens from the Fort Gordon Dental Health Activity Saliva and Dental Plaque Repository, which was reviewed and approved by the Naval Medical Center Portsmouth Institutional Review Board (protocol #20-10581/941839). Patients providing biofilm and saliva specimens for the repository provided written consent.

Inclusion and exclusion criteria

Three pairs of matched biofilm and saliva specimens were requested from the repository described above. The repository manager provided plaque and saliva from donors meeting the following criteria:

a) Periodontal diagnosis of Stage III or IV periodontitis
b) Radiographic alveolar bone loss and probing depth ≥ 6 mm at site of specimen collection
c) Age ≥ 18 years
d) Systemically healthy
e) Nonsmoker

f) No surgical or non-surgical periodontal therapy in the last 12 months

g) No antibiotic use in the last 12 months

Dental biofilm and saliva collection

Investigators included in the repository protocol collected dental biofilm specimens by inserting sterile coarse endodontic paper points[*] to the depth of the periodontal pocket of the first molar displaying the most severe periodontitis (Figure 1C). The paper point was moved laterally within the sulcus for 10 seconds and immediately placed into 1 mL of Amies transport medium[†] at 4°C. Unstimulated saliva was collected from the same donors and diluted ten-fold in reduced Ringers solution[‡]. Diluted saliva specimens were centrifuged at 1200 g × 10 min to remove large particulate matter then filter-sterilized through a membrane with 0.2-µm pores, divided into 2-mL aliquots, and stored at -20 °C.

Culture conditions

This study utilized a modification of the ex vivo biofilm model described by Velsko and Shaddox.[38] Hydroxyapatite (HA) disks,[§] 9.5 x 2 mm, were prepared for biofilm development by coating the disks in diluted sterile saliva for two hours at room temperature. The saliva was then removed and the disks were washed with 3 mL reduced Ringers solution. One washed disk was placed in each well of a 24-well plate,[‖] and 1 mL reduced sterile tryptic soy broth supplemented with 5 µg/mL hemin[¶] and 1 µg/mL menadione[#] (TSB-hm) was then gently added to each well. Gentle sonication of the biofilm specimens in a water bath for 30 seconds dispersed the plaque, and each well received 10 µL of a biofilm specimen from one of the three donors.

Broth cultures of *L. reuteri* (Strain designation 11284)[**] and *B. animalis* subsp*. lactis* (Strain designation IDCC 4301)[**] were grown to an optical density of 0.5 at 600 nm. These cultures were used to create 1/10 and 1/100 dilutions in reduced Ringer's solution. TSB-hm media was prepared and supplemented with inulin[††] to create final inulin concentrations of 0, 5, and 20 mg/mL.

A unique probiotic (*L. reuteri* or *B. animalis* subsp*. lactis*; control, undiluted, 1/10 dilution, or 1/100 dilution) and prebiotic (0, 5, or 20 mg/mL) combination was added to each well, producing a total of 21 experimental conditions (Figure 1A). Each well received 10 µL of the indicated probiotic culture and 1 mL of TSB-hm media with or without inulin supplementation. Well plates were then placed in an anaerobic incubator[‡‡] at 37°C-75% N_2/10% CO_2/10% H_2 for static growth.

Every 48 hours all media was gently removed from each well and 1 mL of fresh media with or without inulin was added. On the eighth day, new saliva-coated and washed HA disks were added to fresh wells. The HA disk with mature biofilm (eighth day of growth) from each of the 21 wells for each of the three donors was removed from the well and added to 1 mL reduced Ringer's solution. The disks were sonicated in an ice water bath for 30 seconds, vortexed briefly to disperse any remaining deposits, and 50 µL of the suspension was used to inoculate the appropriate well for the next generation. This procedure was repeated for 10 total generations (80 days of bacterial growth) for each of the three biofilm specimens. The residual biofilm suspensions not used to inoculate the next generation were used for DNA extraction (Figure 1B).

Viable cell count estimations

The biofilm and probiotic suspensions used to inoculate the first generation for each biofilm specimen were plated to estimate viable cell counts. Tryptic soy agar with hemin and menadione, *Lactobacillus* MRS agar,[§§] and *Bifidobacterium* selective agar[‖‖] were used to plate serial dilutions of the biofilm specimens, *L. reuteri* cultures, and *B. animalis* subsp. *lactis* cultures, respectively. The plates were incubated at 37°C-75% N_2/10% CO_2/10% H_2 and read after 72 hours of growth.

Differential species abundance determination (human oral microbiome identification)

The residual biofilm suspensions from generations 1, 2, 3, 4, 6, 8, and 10 (days 8, 16, 24, 32, 48, 64, and 80, respectively) were centrifuged at 10,000 rpm for 2 min to pellet cells, and the supernatant discarded. The pelleted cells were stored at -20°C for DNA extraction. DNA was extracted from the biofilm pellets of interest and submitted to a commercial lab[¶¶] for human oral microbiome analyses via 16S rRNA sequencing.[##] This system is capable of identifying over 600 genera. Of these, 19 genera have been associated with periodontitis.[39, 40] The influence of probiotic inoculation and prebiotic supplementation on the relative abundance of 19 periodontitis-associated genera was recorded, and seven genera were selected for the statistical analyses described below.

Relative abundance analyses

Genus-level relative abundance analyses were completed at the donor and study population levels. Hierarchical multiple regression models were conducted using Statistical

Package for the Social Sciences[***] to determine if the addition of inulin or probiotic strains influenced the relative abundance of seven selected genera over the ten generations observed. The full models consisted of growth time, concentration of *B. animalis* or *L. reuteri* inoculation, and concentration of supplemental inulin. Independent samples t-tests were used to compare mean relative abundance values in experimental versus control cultures for each genera of interest. Statistical significance was assessed at an alpha level of 0.05.

Community diversity analyses

All community diversity analyses were completed at the study population level using an open-source next-generation microbiome bioinformatics platform.[†††, 41] Intra-group diversity (alpha diversity) was assessed using the metrics observed operational taxonomic units (OTUs) and Faith's phylogenetic diversity (FPD) on tables rarefied to a depth of 35,300 reads/sample. Rarefications were performed and alpha diversity metrics calculated 10 times and then averaged. Differences in alpha diversity between experimental groups were evaluated using the Kruskal–Wallis *H* test. Statistical significance was assessed at an alpha level of 0.05. Inter-group diversity (beta diversity) was assessed using weighted UniFrac distance. Principal coordinates analysis was performed on beta diversity metrics, and the results were plotted on three axes.

RESULTS

At eight days of growth, biofilms of adequate mass for DNA extraction/purification and establishment of subsequent generations were present in all inoculated wells. Extraction and purification of DNA from all samples yielded sufficient quantity and quality for microbial identification and relative abundance analysis via 16S rRNA sequencing.

Figure 2 presents study population-level changes in relative abundance values for the 19 periodontitis-associated genera over ten generations of ex vivo biofilm growth. As expected, relative abundance values remained stable in cultures receiving no probiotic or prebiotic supplementation. The relative abundance of *Lactobacillus* and *Bifidobacterium* species increased and putative periodontal pathogens decreased in cultures receiving inulin supplementation versus cultures not receiving inulin.

In relative abundance analysis (Figure 3), addition of either probiotic strain alone (no inulin supplementation) produced no statistically significant change for any genera of interest. Among cultures receiving only inulin supplementation (no probiotic inoculation), each genera of interest exhibited statistically significant changes in mean relative abundance values, with *Streptococcus, Veillonella, Fusobacterium, Parvimonas,* and *Prevotella* species decreasing in relative abundance and native *Bifidobacterium* and *Lactobacillus* species increasing in relative abundance. In cultures inoculated with either probiotic strain, inulin supplementation produced statistically significant changes in mean relative abundance for each genera of interest similar to the effects noted in cultures receiving inulin alone. For some genera, the effect of inulin supplementation appeared dose dependent.

Donor-level and study population-level results of the hierarchical multiple regression analyses are presented in Tables 1 and 2, respectively. At the donor-level and study population-level, inulin supplementation led to statistically significant increases in mean relative abundance values of *Lactobacillus* and *Bifidobacterium* species ($p<0.001$). Furthermore, inulin supplementation resulted in statistically significant ($p<0.001$) decreases in *Streptococcus, Veillonella, Fusobacterium, Parvimonas,* and *Prevotella* species. Inoculation with *L. reuteri* led to a statistically significant increase in *Lactobacillus* species only ($p<0.05$); likewise, inoculation with *B. animalis* subsp. *lactis* produced a statistically significant increase in *Bifidobacterium* species only ($p<0.05$). Inoculation with either *L. reuteri* or *B. animalis* subsp. *lactis* produced no statistically significant change in the relative abundances of the pathogenic genera evaluated.

Figure 4 presents the study population-level diversity analyses completed in QIIME 2. In alpha analyses by observed OTUs and FPD, inulin supplementation at 20 mg/ml with or without *L. reuteri* or *B. animalis* subsp. *lactis* inoculation led to statistically significant reduction in alpha diversity ($p<0.001$). Beta diversity across experimental groups also appears altered with a distinct change in community profile between control conditions and biofilms receiving inulin supplementation at 20 mg/ml with or without *L. reuteri* or *B. animalis* subsp. *lactis* inoculation; control conditions clustered separately from inulin treated biofilms.

DISCUSSION

The purpose of this investigation was to assess the influence of two probiotic bacterial strains, alone and in combination with a prebiotic, on biodiversity within human dental biofilm cultures ex vivo. Outcome measures recorded in this investigation included two distinct diversity indices. Alpha diversity indices reflect the richness (number of unique taxa) and evenness (similarity in relative abundance values of the taxa present) within a microbial community, whereas beta diversity scores permit comparison of the overall dissimilarity in community structure across samples.[8] Other dimensions of microbial ecology include relative abundance (percentage of the total microbes in a community represented by a particular microbe), abundance (absolute quantity of a microbe within a sample), and prevalence (mere presence of a particular microbe within a sample).[8] In addition, the abundance ratio of two taxa can provide practical insight. For example, in gut microbial ecology, the *Bacteroidetes*:*Firmicutes* ratio has been proposed as a relevant biomarker.[5]

Probiotic and prebiotic applications in the prevention and treatment of inflammatory disease represent areas of intense research focus in recent years.[12-14, 17-37, 42] Within the gut, prebiotics/metabolites stimulate mucin production due to intraluminal pH reduction, disfavor colonization of some pathogens by acidifying the local environment, bind specific G protein coupled receptors on immune cell surfaces, and modulate gene expression in epithelial cells.[33] Researchers have associated prebiotic supplementation with positive effects on both mucosal and systemic immune function.[33] Likewise, in the oral cavity probiotic supplementation may favorably alter the microbiome and dampen the ensuing host immune response,[17-30, 35, 36] and

supplemental prebiotics may enhance the clinical benefit predominantly by enriching the microbiota in beneficial bacterial strains.[32-35, 37]

To our knowledge, no prior study has evaluated the influence of prebiotic inulin on dental plaque specimens from periodontitis patients ex vivo. Under the described conditions, initial supplementation with probiotic *L. reuteri* or *B. animalis* subsp. *lactis* produced no significant alteration of the biofilm composition in terms of pathogen abundance or overall diversity. In contrast, inulin supplementation alone led to dose-dependent increases in health-associated genera and decreases in pathogenic genera. Inulin supplementation also significantly decreased the alpha diversity of the microbiota, as assessed by observed OTUs and FPD. Abusleme and colleagues compared microbial diversity under conditions of health versus periodontitis using 16S rRNA sequencing and reported higher diversity and greater biomass in the periodontitis cohort.[40] Thus, our observation of reduced microbial diversity among cultures receiving inulin may imply a shift toward a healthy microbiota.

Caution is prudent in the interpretation of our results. Cultures evaluated in this study derived from only three human donors. Moreover, although investigators have made substantial progress in understanding oral microbial profiles differentiating health and disease,[9, 10, 40] alpha diversity per se has not been validated as a reliable marker. Indeed, considering the unique microbial ecosystems across the various body regions, it appears necessary to interpret the significance of microbial diversity in context. Reduction in biodiversity is not universally favorable. It is accepted that community stability and high species diversity in the gut are attributes of a healthy microbiota, with exogenous variables

such as exercise, diet, and probiotic/prebiotic supplementation influencing the structure of gut communities.[42-44] Likewise, complex microbiota have been observed in maxillary sinuses of patients with and without chronic sinusitis; however, microbial communities in inflamed sinuses typically exhibit reduced diversity.[7] In contrast, molecular analysis of microbiota associated with bacterial vaginosis suggests dramatic increases in bacterial abundance and diversity compared with healthy controls.[45] Intraorally, reports identifying the biodiversity of microbiota at dental caries sites are conflicted.[46] Although some studies have found increased microbial complexity at caries-affected sites, low pH can select for acid-tolerant species, leading to a less diverse and more extreme microflora.[46] Biofilms at the dentogingival interface are among the most complex in the human body.[8,9,40] Interpreting observed biodiversity in such communities remains an area of investigation in periodontics. Eubiosis—a functional balance within the microbial ecosystem—has been characterized by diversity of species, ability to withstand perturbation (resistance), ability to return to baseline after removal of a stressful stimulus (resilience), and stability between the microbial community and the host.[9] Nevertheless, at least one study has reported higher diversity in periodontitis compared with control samples.[40]

Inulin supplementation purportedly produces beneficial effects by selecting for health-associated inulin-fermenting species. [32-35, 37] Degradation of inulin—a long, water-soluble polymer—produces smaller fructans, which neighboring species may metabolize.[47] In research involving the gut microbiota of humans and feed animals, this interspecies cross-feeding has been found to promote gastrointestinal health.[48, 49] It is possible that observations in the

present ex vivo oral biofilm study reflect a combination of direct promotion of inulin-fermenting bacteria and cross feeding of secondary consumers in similar and distant genera.

Results of the present investigation are consistent with findings from previous studies reporting prebiotic effects on oral microbiome composition. In an in vitro biofilm model consisting of only 14 oral bacterial species, Slomka and colleagues reported that three prebiotic substrates successfully increased the beneficial proportion of genera to > 95%.[34] While this study did not evaluate inulin as a prebiotic, the authors did assess similar long-chain, water-soluble polysaccharides that exhibit degradation comparable to that of inulin.[34] In a randomized controlled trial, Mousquer and colleagues used a combination of inulin and *Lactobaciullus salivarius* for the treatment of halitosis.[50] This combination resulted in a significant decrease in oral malodor compared to placebo suggesting a modification of the microbiome, although microbiological analysis was limited in this study.[50] No prior study has evaluated the effect of inulin in combination with a probiotic strain in the treatment of periodontitis or maintenance of periodontal health.

The ex vivo model utilized in the present study appears appropriate for evaluating prebiotics, probiotics, and prebiotic/probiotic combinations. The composition of control cultures remained consistent over ten generations of biofilm growth in the present study, as expected. For many genera of interest, the effect induced by each experimental condition versus control was evident by the earliest time point evaluated. Having validated our methods, future studies involving larger sample sizes and more powerful statistical analyses are necessary to confirm observations in this initial investigation. One limitation of the described model is

that no data were generated prior to the eighth day of growth in the first biofilm generation. Another potential limitation of this study is in the use of relative abundance values. While this quantifier permits comparisons in biofilm composition, it provides no information on absolute CFU counts for the genera of interest. In this study, large increases in relative abundances of *Lactobacillus* and *Bifidobacterium* species were observed in the presence of inulin. However, the actual counts of the pathogenic genera may have decreased, remained stable, or even increased.

Based on our findings, prebiotics appear to be promising adjuncts in the prevention and treatment of periodontal disease through the modulation of the subgingival biofilm. Although in vitro culture methods allow for sustained contact between the biofilm and supplemented media, in vivo applications of inulin to subgingival biofilms with prolonged contact time will be a challenging therapeutic obstacle. Additional study further elucidating the underlying biologic rationale and optimal mechanism of administration through controlled clinical research are warranted.

CONCLUSIONS

In conclusion, we have demonstrated a dose-dependent decrease in the relative abundance values of select periodontal pathogens accompanied by a dose-dependent increase in the probiotic genera *Lactobacillus* and *Bifidobacterium* in response to continued inulin supplementation. These results suggest that prebiotic supplementation may represent a viable strategy for promotion of periodontal health through favorable modification of the oral microbiome.

AUTHOR CONTRIBUTIONS

Conception and design of the study: AC, JA, DB, TJ. Acquisition, analysis, or interpretation of data: AC, DB, JD. Drafting the work or revising it critically for important intellectual content: AC, TJ, DB, BS, AL, JA. Final approval of the version to be published: all authors.

ACKNOWLEDGEMENTS

The authors report no conflicts of interest related to this report. The views expressed in this manuscript are those of the authors and do not necessarily reflect the official policy of the Department of Defense, Department of Army, U.S. Army Medical Department, or Uniformed Services University of the Health Sciences.

[*]Dentsply; York, PA

[†]Puritan; Guilford, Maine

[‡]Millipore; Burlington, MA

[§]Clarkson Chromatography; South Williamsport, PA

[‖]Neogen; Lansing, MI

[¶]Thermo Scientific; Waltham, MA

[#]Sigma; St. Louis, MO

[**]ATCC; Manassas, Virgina

[††]Thermo Scientific; Waltham, MA

[‡‡]Bactron 600-2; MRC Laboratory Instruments; London, UK

[§§]BD Difco; Fischer Scientific; Waltham, MA

[|||]BIFIDO; Anaerobe Systems; Morgan Hill, CA

[¶¶]CD Genomics, Shirley, NY

[##]MiSeq System, Illumina, San Diego, CA

[***]IBM SPSS for Windows, v.27, SPSS Inc., Chicago, IL

[†††]Quantitative Insights Into Microbial Ecology-2, QIIME 2

REFERENCES

1. Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch SV, Knight R. Current understanding of the human microbiome. *Nat Med* 2018;24:392-400.

2. Sender R, Fuchs S, Milo R. Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans. *Cell* 2016;164:337-340.

3. Qin J, Li R, Raes J, Arumugam M, et al. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010;464:59–65.

4. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature* 2007;449:804-810.

5. Bamola VD, Ghosh A, Kapardar RK, et al. Gut microbial diversity in health and disease: experience of healthy Indian subjects, and colon carcinoma and inflammatory bowel disease patients. *Microb Ecol Health Dis* 2017;28:1322447. doi: 10.1080/16512235.2017.1322447.

6. Kostic AD, Chun E, Robertson L, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 2013;14:207-215.

7. Lee JT, Frank DN, Ramakrishnan V. Microbiome of the paranasal sinuses: update and literature review. *Am J Rhinol Allergy* 2016;30:3-16.

8. Scannapieco FA, Dongari-Bagtzoglou A. Dysbiosis revisited: Understanding the role of the oral microbiome in the pathogenesis of gingivitis and periodontitis: A critical assessment. *J Periodontol* 2021;92:1071-1078.

9. Kumar PS. Microbial dysbiosis: The root cause of periodontal disease. *J Periodontol* 2021;92:1079-1087.

10. Kragen H. The treatment of inflammatory affections of the oral mucosa with a lactic acid bacterial culture preparation. *Zahnarztl Welt* 1954;9:306-308.

11. Lilly DM, Stillwell RH. Probiotics: growth-promoting factors produced by microorganisms. *Science* 1965;147:747-748.

12. Reid G, Gadir AA, Dhir R. Probiotics: reiterating what they are and what they are not. *Front Microbiol* 2019;10:424.

13. Matsubara VH, Bandara HM, Ishikawa KH, Mayer MP, Samaranayake LP. The role of probiotic bacteria in managing periodontal disease: a systematic review. *Expert Rev Anti Infect Ther* 2016;14:643-655.

14. Kuru BE, Laleman I, Yalnizoglu T, Kuru L, Teughels W. The influence of a Bifidobacterium animalis probiotic on gingival health: a randomized controlled clinical trial. *J Periodontol* 2017;88:1115-1123.

15. Michalowicz BS, Diehl SR, Gunsolley JC, et al. Evidence of a substantial genetic basis for risk of adult periodontitis. *J Periodontol* 2000;71:1699-1707.

16. Ezzo PJ, Cutler CW. Microorganisms as risk indicators for periodontal disease. *Periodontol 2000* 2003;32:24-35.

17. Teughels W, Loozen G, Quirynen M. Do probiotics offer opportunities to manipulate the periodontal oral microbiota? *J Clin Periodontol* 2011;38 Suppl 11:159-177.

18. Ince G, Gursoy H, Ipci SD, Cakar G, Emekli-Alturfan E, Yilmaz S. Clinical and biochemical evaluation of lozenges containing Lactobacillus reuteri as an adjunct to non-surgical periodontal therapy in chronic periodontitis. *J Periodontol* 2015;86:746-754.

19. Teughels W, Durukan A, Ozcelik O, Pauwels M, Quirynen M, Haytac MC. Clinical and microbiological effects of Lactobacillus reuteri probiotics in the treatment of chronic periodontitis: a randomized placebo-controlled study. *J Clin Periodontol* 2013;40:1025-1035.

20. Romani Vestman N, Chen T, Lif Holgerson P, Ohman C, Johansson I. Oral microbiota shift after 12-week supplementation with Lactobacillus reuteri DSM 17938 and PTA 5289; a randomized control trial. *PLoS One* 2015;10:e0125812.

21. Tekce M, Ince G, Gursoy H, et al. Clinical and microbiological effects of probiotic lozenges in the treatment of chronic periodontitis: a 1-year follow-up study. *J Clin Periodontol* 2015;42:363-372.

22. Schlagenhauf U, Rehder J, Gelbrich G, Jockel-Schneider Y. Consumption of Lactobacillus reuteri-containing lozenges improves periodontal health in navy sailors at sea: A randomized controlled trial. *J Periodontol* 2020;91:1328-1338.

23. Ricoldi MST, Furlaneto FAC, Oliveira LFF, et al. Effects of the probiotic Bifidobacterium animalis subsp. lactis on the non-surgical treatment of periodontitis. A histomorphometric, microtomographic and immunohistochemical study in rats. *PLoS One* 2017;12:e0179946.

24. Oliveira LF, Salvador SL, Silva PH, et al. Benefits of Bifidobacterium animalis subsp. lactis probiotic in experimental periodontitis. *J Periodontol* 2017;88:197-208.

25. Toiviainen A, Jalasvuori H, Lahti E, et al. Impact of orally administered lozenges with Lactobacillus rhamnosus GG and Bifidobacterium animalis subsp. lactis BB-12 on the number of salivary mutans streptococci, amount of plaque, gingival inflammation and the oral microbiome in healthy adults. *Clin Oral Investig* 2015;19:77-83.

26. Invernici MM, Salvador SL, Silva PHF, et al. Effects of Bifidobacterium probiotic on the treatment of chronic periodontitis: A randomized clinical trial. *J Clin Periodontol* 2018;45:1198-1210.

27. Invernici MM, Furlaneto FAC, Salvador SL, et al. Bifidobacterium animalis subsp lactis HN019 presents antimicrobial potential against periodontopathogens and modulates the immunological response of oral mucosa in periodontitis patients. *PLoS One* 2020;15:e0238425.

28. Allaker RP, Stephen AS. Use of probiotics and oral health. *Curr Oral Health Rep* 2017;4:309-318.

29. Dassi E, Ferretti P, Covello G, et al. The short-term impact of probiotic consumption on the oral cavity microbiome. *Sci Rep* 2018;8:10476.

30. Armitage GC. A brief history of periodontics in the United States of America: Pioneers and thought-leaders of the past, and current challenges. *Periodontol 2000* 2020;82:12-25.

31. Ma D, Forsythe P, Bienenstock J. Live Lactobacillus rhamnosus [corrected] is essential for the inhibitory effect on tumor necrosis factor alpha-induced interleukin-8 expression. *Infect Immun* 2004;72:5308-5314.

32. Roberfroid M. Prebiotics: the concept revisited. *J Nutr* 2007;137:830S-837S.

33. Lomax AR, Calder PC. Prebiotics, immune function, infection and inflammation: a review of the evidence. *Br J Nutr* 2008;101:633-658.

34. Slomka V, Herrero ER, Boon N, et al. Oral prebiotics and the influence of environmental conditions in vitro. *J Periodontol* 2018;89:708-717.

35. Slomka V, Hernandez-Sanabria E, Herrero ER, et al. Nutritional stimulation of commensal oral bacteria suppresses pathogens: the prebiotic concept. *J Clin Periodontol* 2017;44:344-352.

36. Nguyen T, Brody H, Radaic A, Kapila Y. Probiotics for periodontal health-Current molecular findings. *Periodontol 2000* 2021;87:254-267.

37. Kolida S, Tuohy K, Gibson GR. Prebiotic effects of inulin and oligofructose. *Br J Nutr* 2002;87 Suppl 2:S193-197.

38. Velsko IM, Shaddox LM. Consistent and reproducible long-term in vitro growth of health and disease-associated oral subgingival biofilms. *BMC Microbiol* 2018;18:70.

39. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;25:134-144.

40. Abusleme L, Dupuy AK, Dutzan N, et al. The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *ISME J* 2013;7:1016-1025.

41. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019;37:852-857.

42. Mengheri E. Health, probiotics, and inflammation. *J Clin Gastroenterol* 2008;42 Suppl 3 Pt 2:S177-178.

43. Campbell SC, Wisniewski PJ. Exercise is a novel promoter of intestinal health and microbial diversity. *Exerc Sport Sci Rev* 2017;45:41-47.

44. Bamola VD, Ghosh A, Kapardar RK, et al. Gut microbial diversity in health and disease: experience of healthy Indian subjects, and colon carcinoma and inflammatory bowel disease patients. *Microb Ecol Health Dis* 2017;28:1322447. doi: 10.1080/16512235.2017.1322447.

45. Ling Z, Kong J, Liu F, Zhu H, Chen X, Wang Y, Li L, Nelson KE, Xia Y, Xiang C. Molecular analysis of the diversity of vaginal microbiota associated with bacterial vaginosis. *BMC Genom* 2010;11:1-6.

46. Marsh PD. Microbiology of dental plaque biofilms and their role in oral health and caries. *Dent Clin N Am* 2010;54:441-454.

47. Rakoff-Nahoum S, Foster KR, Comstock LE. The evolution of cooperation within the gut microbiota. *Nature* 2016;533:255-259.

48. Vandeputte D, Falony G, Vieira-Silva S, et al. Prebiotic inulin-type fructans induce specific changes in the human gut microbiota. *Gut* 2017;66:1968-1974.

49. Zhu Y, Liu J, Lopez JM, Mills DA. Inulin fermentation by Lactobacilli and Bifidobacteria from dairy calves. *Appl Environ Microbiol* 2020;87.

50. Mousquer CR, Della Bona A, Milani DC, et al. Are Lactobacillus salivarius G60 and inulin more efficacious to treat patients with oral halitosis and tongue coating than the probiotic alone and placebo? A randomized clinical trial. *J Periodontol* 2020;91:775-783.

FIGURES

A

Condition	Inulin mg/mL	Probiotic dilutions	
		L. reuteri (OD_{600} 0.5)	*B. animalis* (OD_{600} 0.5)
1	0	--	--
2	5	--	--
3	20	--	--
4	0	1/100	--
5	5	1/100	--
6	20	1/100	--
7	0	1/10	--
8	5	1/10	--
9	20	1/10	--
10	0	1/1	--
11	5	1/1	--
12	20	1/1	--
13	0	--	1/100
14	5	--	1/100
15	20	--	1/100
16	0	--	1/10
17	5	--	1/10
18	20	--	1/10
19	0	--	1/1
20	5	--	1/1
21	20	--	1/1

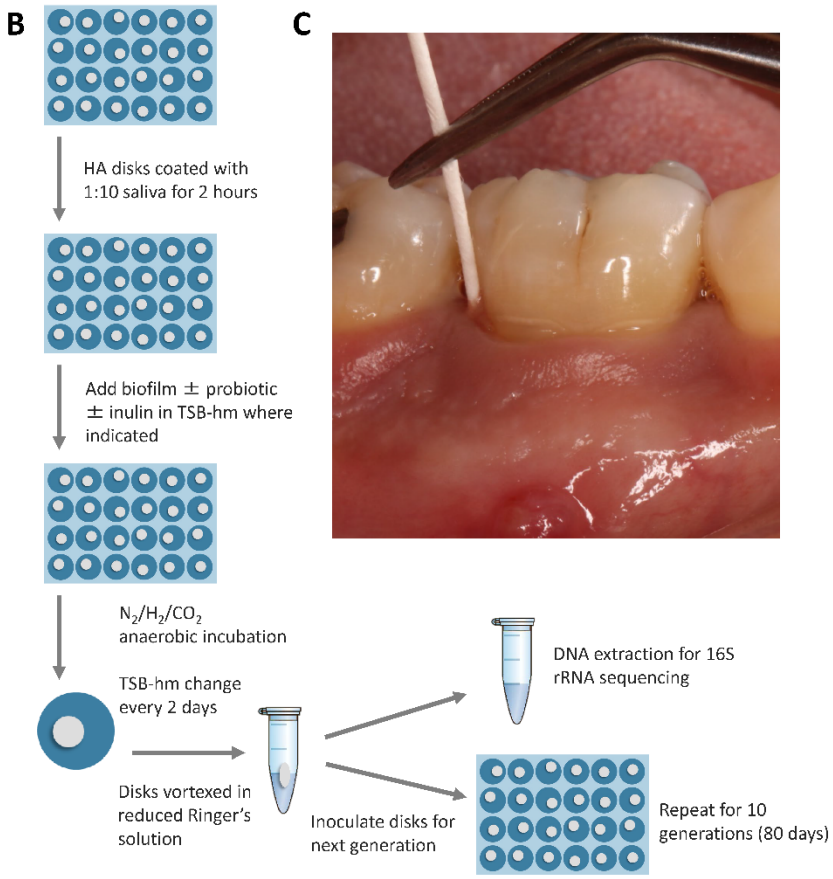


FIGURE 1. Experimental methods. **A)** Experimental conditions applied to each donor biofilm. **B)** Biofilm development schematic utilizing 24-well plates containing hydroxyapatite disks. All ex vivo biofilm growth completed in anaerobic chamber at 37°C-75% N_2/10% CO_2/10% H_2. **C)** Demonstration of the biofilm collection technique. Dental biofilm and saliva were requested from a repository of de-identified specimens. Donors had received stage III or IV periodontitis diagnoses, and the investigator collected biofilm using a sterile paper point at the first molar site exhibiting greatest bone/attachment loss.

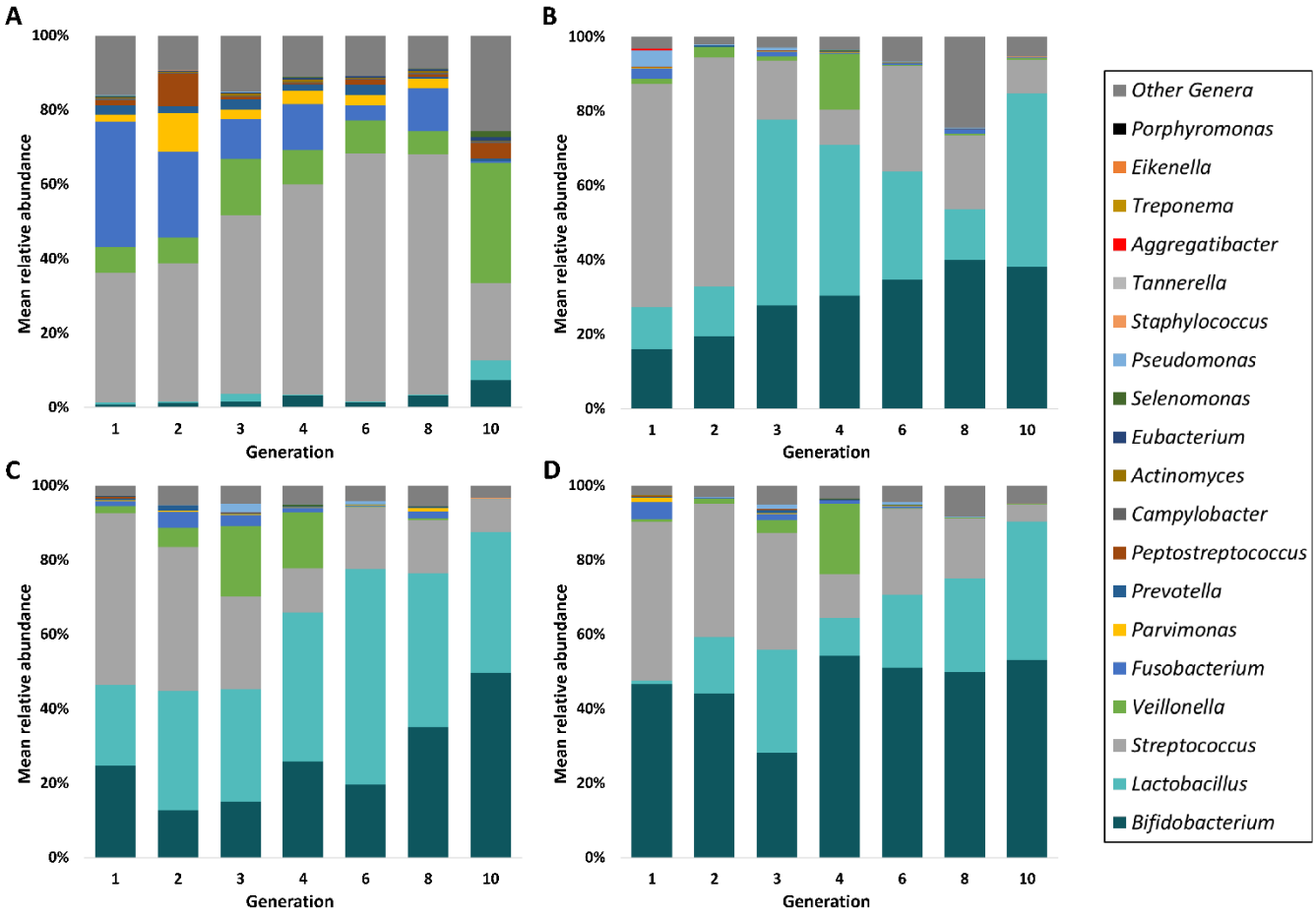


Figure 2. Mean relative abundance of 19 genera at each generation of ex vivo growth. **A**) Control biofilms not inoculated with probiotic or supplemented with inulin showed minimal change in abundance of pathogenic species or naturally occurring probiotic species. **B**) Cultures supplemented with 20 mg/ml inulin exhibited increased relative abundance of native *Lactobacillus* and *Bifidobacterium* species as well as decreased relative abundance of pathogenic genera. **C**) *L. reuteri* inoculation plus 20 mg/ml supplemental inulin led to a greater increase in *Lactobacillus* species compared with inulin supplementation alone. **D**) *B. animalis* inoculation plus 20 mg/ml supplemental inulin led to a greater increase in *Bifidobacterium* species compared to inulin supplementation alone.

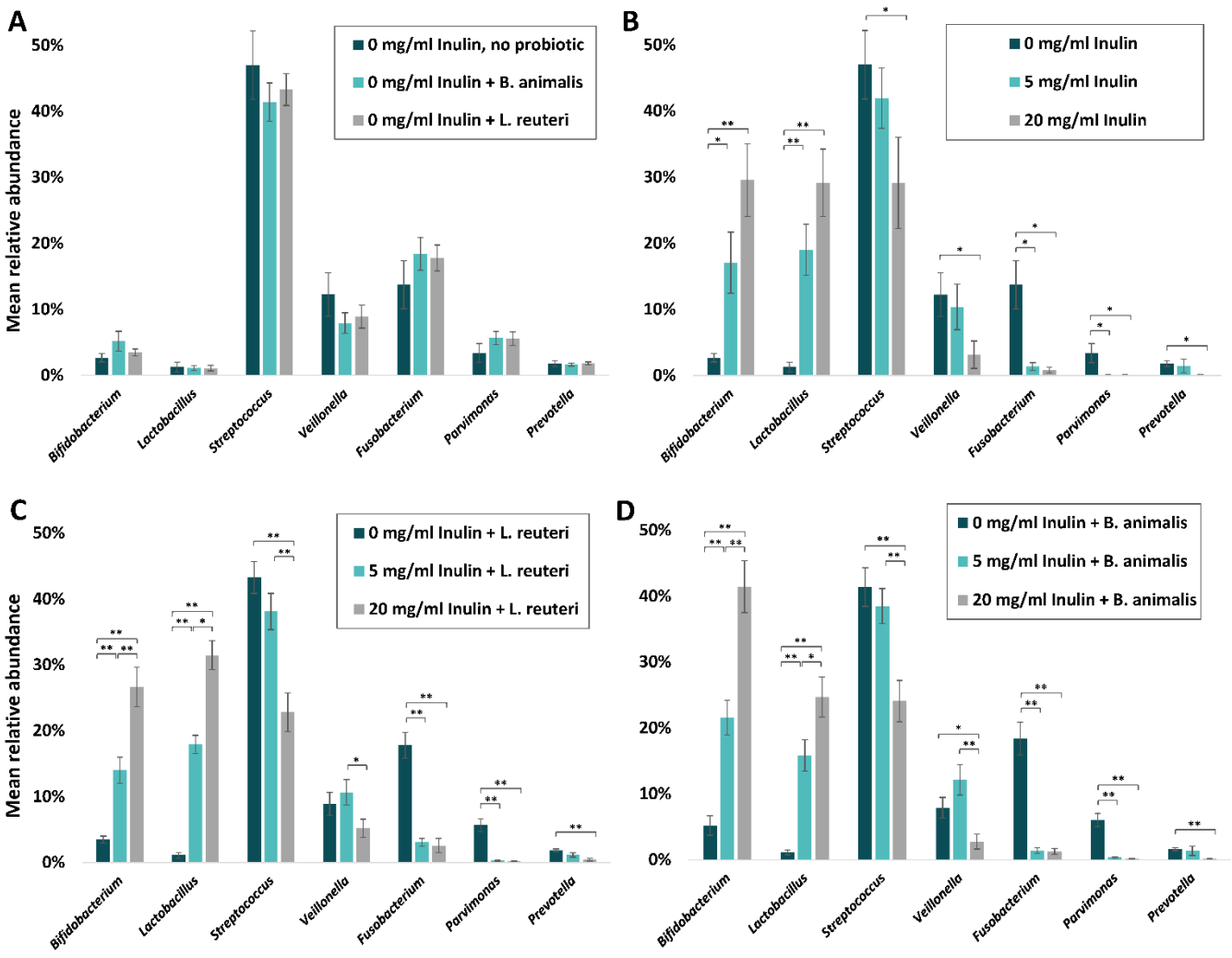
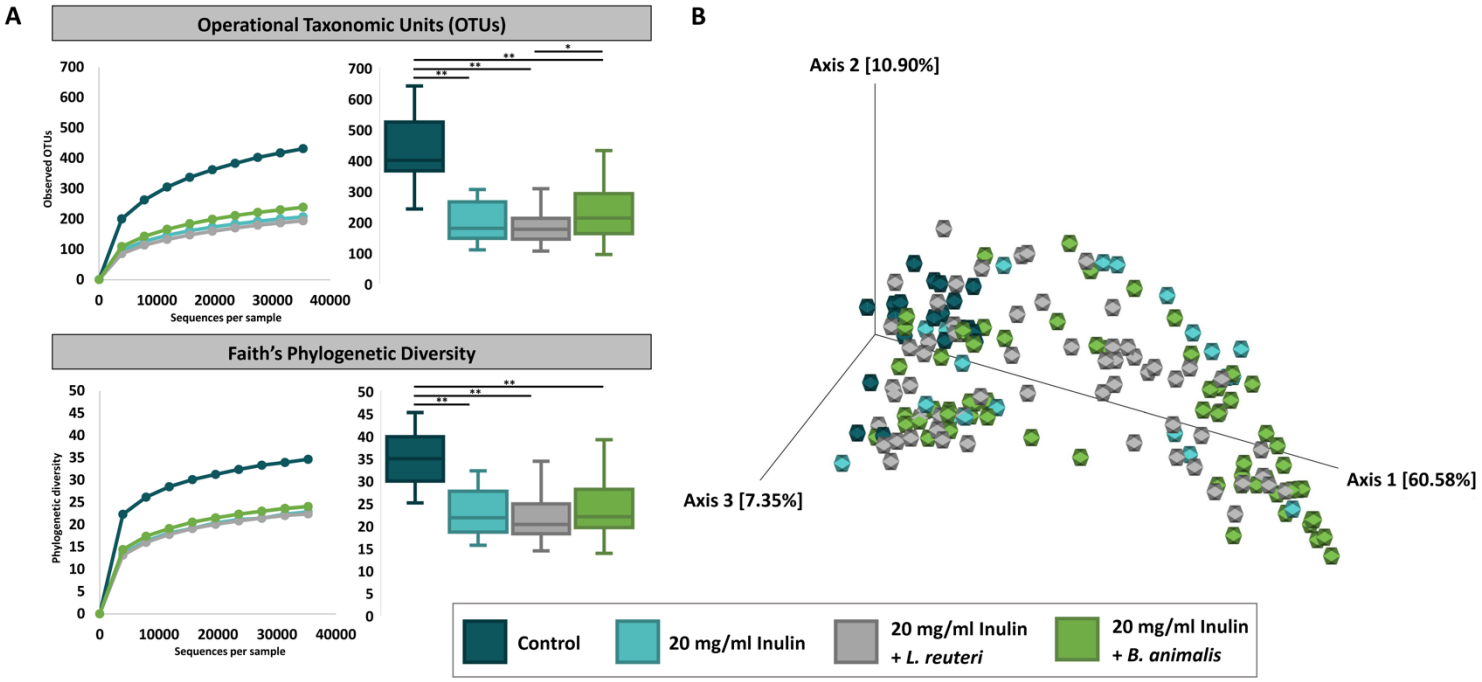


Figure 3. Mean relative abundance values across 10 generations of ex vivo growth. $^{**}p<0.001$, $^{*}p<0.05$. **A**) Varying initial probiotic inoculation with no prebiotic inulin produced no statistically significant changes in relative abundance of any evaluated genera. **B**) Prebiotic inulin supplementation with no initial probiotic inoculation led to significant increases in relative abundance of native *Bifidobacterium* and *Lactobacillus* species as well as significant decreases in pathogenic genera. **C**) *L. reuteri* inoculation plus inulin supplementation and inulin supplementation alone led to led to significant increases in relative abundance of native

Bifidobacterium and *Lactobacillus* species as well as significant decreases in pathogenic genera. **D**) *B. animalis* inoculation plus inulin supplementation and inulin supplementation alone led to significant increases in relative abundance of native *Bifidobacterium* and *Lactobacillus* species as well as significant decreases in pathogenic genera.



** p<0.001, * p<0.05

Figure 4. Ex vivo plaque biofilm diversity analysis. **A**) Alpha diversity. All cultures receiving prebiotic inulin exhibited reduction in alpha diversity, as assessed by recorded operational taxonomic unit counts (top) and Faith's phylogenetic diversity (bottom). **B**) Beta diversity. In weighted UniFrac distance analysis, control and inulin-treated cultures clustered separately.

TABLES

Genus	Variables added to model	Donor 1 Coef.	Donor 1 95% CI Lower Bound	Donor 1 95% CI Upper Bound	Donor 1 R^2	Donor 2 Coef.	Donor 2 95% CI Lower Bound	Donor 2 95% CI Upper Bound	Donor 2 R^2	Donor 3 Coef.	Donor 3 95% CI Lower Bound	Donor 3 95% CI Upper Bound	Donor 3 R^2
Bifidobacterium	Conc. of *B. animalis*	0.093	0.047	0.140	0.108	***0.087**	0.003	0.172	0.035	***0.090**	0.003	0.177	0.147
	Conc. of *L. reuteri*	-0.002	-0.049	0.044	0.108	-0.084	-0.169	0.000	0.046	-0.020	-0.107	0.067	0.148
	Conc. of Inulin	***0.002**	0.001	0.004	0.147	****0.024**	0.021	0.027	0.602	****0.015**	0.011	0.018	0.432
Lactobacillus	Conc. of *B. animalis*	-0.003	-0.107	0.101	0.133	-0.042	-0.103	0.019	0.028	***-0.073**	-0.132	-0.014	0.041
	Conc. of *L. reuteri*	***0.111**	0.007	0.215	0.133	0.056	-0.005	0.117	0.020	0.023	-0.036	0.081	0.010
	Conc. of Inulin	****0.018**	0.014	0.022	0.426	****0.012**	0.009	0.014	0.410	****0.008**	0.006	0.011	0.287
Streptococcus	Conc. of *B. animalis*	-0.083	-0.175	0.009	0.020	-0.087	-0.176	0.002	0.037	-0.010	-0.119	0.098	0.239
	Conc. of *L. reuteri*	-0.034	-0.127	0.058	0.024	-0.022	-0.112	0.067	0.038	-0.072	-0.180	0.036	0.247
	Conc. of Inulin	-0.002	-0.006	0.002	0.032	****-0.020**	-0.024	-0.017	0.498	***-0.006**	-0.010	-0.002	0.284
Veillonella	Conc. of *B. animalis*	-0.008	-0.083	0.067	0.055	***0.073**	0.021	0.125	0.129	-0.006	-0.068	0.055	0.034
	Conc. of *L. reuteri*	-0.039	-0.114	0.036	0.062	0.012	-0.041	0.064	0.130	0.048	-0.014	0.109	0.049
	Conc. of Inulin	-0.001	-0.004	0.002	0.067	***-0.004**	-0.006	-0.002	0.218	***-0.004**	-0.006	-0.001	0.111
Fusobacterium	Conc. of *B. animalis*	0.000	-0.076	0.076	0.094	0.004	-0.034	0.042	0.080	-0.015	-0.050	0.019	0.057
	Conc. of *L. reuteri*	-0.035	-0.111	0.042	0.098	***0.043**	0.004	0.081	0.105	0.240	-0.011	0.058	0.067
	Conc. of Inulin	****-0.010**	-0.013	-0.007	0.301	****-0.004**	-0.006	-0.003	0.256	****-0.004**	-0.006	-0.003	0.265
Parvimonas	Conc. of *B. animalis*	***0.022**	0.001	0.043	0.032	-0.016	-0.043	0.012	0.044	0.009	-0.009	0.027	0.029
	Conc. of *L. reuteri*	-0.010	-0.031	0.012	0.037	0.000	-0.028	0.027	0.044	0.016	-0.002	0.035	0.048
	Conc. of Inulin	****-0.002**	-0.003	-0.001	0.148	****-0.003**	-0.004	-0.002	0.210	****-0.001**	-0.002	-0.001	0.137
Prevotella	Conc. of *B. animalis*	-0.002	-0.007	0.004	0.088	0.003	-0.003	0.009	0.013	0.001	-0.020	0.022	0.061
	Conc. of *L. reuteri*	0.000	-0.005	0.006	0.088	0.003	-0.003	0.009	0.021	0.002	-0.019	0.023	0.061
	Conc. of Inulin	****-0.001**	-0.001	0.000	0.271	****-0.003**	-0.001	0.000	0.121	****-0.001**	-0.002	0.000	0.100

Table 1. Donor-level hierarchical multiple regression analysis. Effect of probiotic and prebiotic supplementation on relative abundance predictive values. *$p < 0.05$, **$p < 0.001$. Coef. values represent the predicted change in genus relative abundance per unit change in variable added to model. Concentrations (Conc.) of *B. animalis* and *L. reuteri* calculated as dilutions of initial broth culture with OD of 0.5. Concentration (Conc.) of inulin calculated as mg/ml.

Genus	Variables added to model	Combined Coef.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound	R^2
Bifidobacterium	Conc. of *B. animalis*	***0.090**	0.035	0.146	0.027
	Conc. of *L. reuteri*	-0.035	-0.091	0.020	0.030
	Conc. of Inulin	****0.014**	0.011	0.016	0.277
Lactobacillus	Conc. of *B. animalis*	-0.039	-0.091	0.013	0.042
	Conc. of *L. reuteri*	***0.063**	0.011	0.115	0.038
	Conc. of Inulin	****0.013**	0.011	0.015	0.279
Streptococcus	Conc. of *B. animalis*	-0.060	-0.122	0.001	0.050
	Conc. of *L. reuteri*	-0.043	-0.104	0.018	0.052
	Conc. of Inulin	****-0.009**	-0.012	-0.007	0.162
Veillonella	Conc. of *B. animalis*	0.020	-0.018	0.058	0.005
	Conc. of *L. reuteri*	0.007	-0.031	0.045	0.005
	Conc. of Inulin	****-0.003**	-0.005	-0.002	0.041
Fusobacterium	Conc. of *B. animalis*	-0.004	-0.037	0.030	0.016
	Conc. of *L. reuteri*	0.011	-0.023	0.044	0.017
	Conc. of Inulin	****-0.006**	-0.007	-0.005	0.163
Parvimonas	Conc. of *B. animalis*	0.005	-0.008	0.019	0.002
	Conc. of *L. reuteri*	0.002	-0.011	0.016	0.002
	Conc. of Inulin	****-0.002**	-0.003	-0.002	0.122
Prevotella	Conc. of *B. animalis*	0.001	-0.007	0.008	0.026
	Conc. of *L. reuteri*	0.002	-0.006	0.009	0.027
	Conc. of Inulin	****-0.001**	-0.001	0.000	0.073

Table 2. Study population-level hierarchical multiple regression analysis. Overall effect of probiotic and prebiotic supplementation on relative abundance predictive values. *$p < 0.05$, **$p < 0.001$**.** Coef. values represent the predicted change in genus relative abundance per unit change in variable added to model. Concentrations (Conc.) of *B. animalis* and *L. reuteri* calculated as dilutions of initial broth culture with OD of 0.5. Concentration (Conc.) of inulin calculated as mg/ml.