Characterizing Changes in Oral Microbiota with Cardiometabolic Risk Factors

by

Ahmed Ibrahim Warsi

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Project Advisor: Dr. Jo. Max Goodson

I have reviewed this thesis. It represents work done by the author under my guidance/supervision.

Primary Mentor: Dr. Tsute Chen

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Thesis Committee Members:

1. Student: Dr. Ahmed Ibrahim Warsi
2. Primary Mentor: Dr. Tsute Chen
3. Co-Primary Mentor: Dr. J. Max Goodson
4. External Content Advisor: Dr. Bruce Paster
5. MMSCI Program Representative: Dr. Enid Martinez
6. Program Director: Dr. Ajay Singh
7. Program Co-Director: Dr. Finnian Mc Causland

Overview

Cardiometabolic risk factors (CMR) such as obesity and hypertension in adolescents are independent risk predictors for the development of cardiovascular disease in adulthood (1,2). Recent evidence has demonstrated the dysbiosis of oral and gut microbiota with CMR (3–5). While longitudinal studies examining the association between oral dysbiosis with the evolution of CMR in children are lacking. I aim to examine changes in oral microbiota with the development of obesity (Project-I), and hypertension (Project-II), among adolescents in Kuwait Healthy Lifestyle Study Cohort (KHLS).

The Human Microbiome Project indicated that the oral and gut microbiome overlap in nearly 45% of the population (6). The premise that oral and gut have an anatomical connection and that ingesting oral bacteria could be a source for colonization of gut microbiota is plausible (7,8). In inflammatory systemic diseases, increased numbers of oral bacteria have been observed in the intestine (9–11). Moreover, it has been reported that an increased abundance of pathogenic, gram-negative, oral Bacteroidetes, such as *Porphyromonas gingivalis* can alter the gut microbial ecosystem (12). Numerous studies have indicated that obesity-associated gut microbiome has increased capacity for energy harvest,(13–15) by digesting otherwise indigestible complex polysaccharides, the gut microbiota is now recognized as an additional contributing factor towards the pathophysiology of obesity (13,16). However, it is still not clear where did the gut microbiota come from? And how could oral microbiota influence the gut microbiome in the development of obesity (8)? To bridge the gap, we compared the oral microbiota in healthy, overweight, and obese phenotypes. To place the oral dysbiosis in context with the

pathophysiology of obesity, I compared the study results with existing studies on oral and gut dysbiosis and obesity development.

Project-I: The KHLS cohort study was envisioned following the observation published in 2009 (17), suggesting that obesity could be the result of oral bacterial infection. The KHLS investigators reasoned that by the selection of children from a population with high levels of adult obesity, we would be able to determine if obesity could be predicted. In this longitudinal study, I analyzed data from 67 adolescents, sampled at visits 1 and 2, two-years apart. In this cohort, 28 healthy, 33 overweight, and 6 obese children at baseline were followed for two-years. At visit-2, 19 healthy, 10 overweight, and 38 obese subjects were identified. In summary, 47% of our baseline subjects became obese by visit 2. These remarkable changes in host-phenotypes concerning weight gain, allowed me to characterize changes in oral microbiota with changes in host phenotypes – using both cross-sectional and longitudinal analyses. I also observed drastic changes in systemic inflammatory and metabolic factors, which could also influence changes in oral microbiota (18). To compare oral microbial changes across healthy, overweight, and obese phenotypes; and to investigate the possibility of effect-modification with inflammatory and metabolic factors, I constructed three sub-cohorts comparing: (i) healthy-vs-overweight subjects, (ii) overweight-vs-obese subjects, and (iii) healthy-vs-obese subjects.

Project-II: From the analysis, I learned that Kuwait has one of the highest prevalence of adolescent hypertension (nearly 40% within KHLS cohort), as compared to 5.5% reported for the USA (19). This raises concerns about the involvement of multiple pathways responsible for a higher prevalence of cardio-metabolic risk factors amongst the KHLS

cohort. Besides other known cardiometabolic risk factors that have already been reported (20), I assessed the role of the "Enterosalivary-Nitrate-Nitrite-NO pathway" with pediatric hypertension (21,22). The second project was aimed at assessing whether the changes in nitrate and nitrite-reducing oral bacteria, could influence changes in blood pressure amongst the KHLS cohort. The enterosalivary nitrate-nitrite-nitric oxide pathway is an alternative route of nitric oxide generation, potentially linking the oral microbiome to blood pressure regulation (21–25). I hypothesized that the reduction of nitrate and nitrite-reducing oral bacteria would be associated with an increase in blood pressure and higher odds of having hypertension.

In both projects, I aim to assess whether oral dysbiosis is associated with the development of cardiometabolic risk factors. If that is the case, there is a possibility of linking oral-systemic connection through microbial pathways. If oral dysbiosis precedes changes in host phenotype (onset of obesity and hypertension), there could be a potential causal-route which has been un-explored. But if the oral dysbiosis occurs as a manifestation of changes in host phenotype, it could indicate a potential effect-pathway. In either case, the study of oral dysbiosis is imperative to anticipate the onset of periodontal diseases. In my thesis work, I aim to characterize changes in oral microbiome with obesity and hypertension, using longitudinal observational study design, to understand the depth of oral-systemic connection.

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PROJECT - I

Changes in Oral Microbiota with the Development of Adolescent Obesity, and Effect-Modification with Metabolic and Inflammatory factors

I. Warsi[1,3], J.M. Goodson[2,3], P. Gajare[3], A.P.V. Colombo[4], P. Shi[3], M.L. Hartman[3], T. Chen[2,3]

[1.] Masters' in Medical Sciences and Clinical Investigation, Harvard Medical School, Boston, MA, USA

[2.] Department of Oral Medicine, Infection, and Immunology, Harvard School of Dental Medicine, Boston, MA, USA

[3.] The Forsyth Institute, Cambridge, MA, USA

[4.] Department of Medical Microbiology, Institute of Microbiology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.

Corresponding Author:

Ibrahim Warsi, BDS, Cert. Implant,
MMSCI, Harvard Medical School
Research Fellow | The Forsyth Institute
Email: ahmed_warsi@hms.harvard.edu
Contact number: +1 (832) 458-5039
Address: 614A, Shawmut Ave, Unit-4,
Boston, MA, 02118, USA.

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ABSTRACT

Dysbiosis of oral and gut microbiota has been implicated with obesity. However, oral microbiota changes with the development of adolescent obesity have not been studied. Next-generation-sequencing of the 16S-rRNA V3-V4 region was used to characterize salivary microbial changes with the development of adolescent obesity, at two-time points, two-years apart. We investigated whether the compositional changes of oral microbiota are associated with changes in host phenotypes – healthy, overweight, and obese. We also investigated, whether changes in oral microbiota observed with host-phenotypes are confounded by changes in the visit, inflammatory factors (gingivitis, and salivary C-reactive-protein), and salivary metabolic markers (insulin, high-density-lipo-protein-cholesterol, and glucose levels). A higher *Firmicutes-to-Bacteroidetes* phylum-ratio, a hallmark feature of gut-microbiota of obese subjects, was also observed in the oral microbiota of overweight subjects. Interestingly the ratio decreased in obese subjects when compared to overweight ones. In obese subjects, we observed a higher proportion of pro-inflammatory *Prevotella sp.*, and mucin-degrading oral bacteria (i.e., members of the *Lachnospiracae* family). This is consistent with the higher expression of salivary C-reactive-protein that we observed and is indicative of systemic inflammation. Furthermore, we demonstrated that metabolic factors such as high insulin, high glucose, and low high-density-lipoprotein-cholesterol are phenotype-specific effect modifiers for oral microbial dysbiosis in adolescent obesity.

Keywords: Microbiota, Saliva, Pediatric obesity, Overweight, Dysbiosis, Host Microbial Interactions

INTRODUCTION:

The worldwide prevalence of childhood obesity has risen dramatically from 4% in 1975 to over 18% in 2016 (WHO 2018). Of the adolescents developing obesity, 80% will become obese as adults (1).

Obesity is a complex disease with many environmental-influences, amongst which the human gut-microbiome has been identified as an important modulating-factor. Numerous studies on gut-microbiota have demonstrated that changes in gut-microbiome are associated with obesity. Gut microbial dysbiosis has been attributed to triggering host inflammatory and metabolic responses, which contributes to the development of obesity (2–4). It has been observed that obese individuals exhibit a decrease in both oral/gut microbiome diversity and richness, relative to healthy-counterparts (5,6); while an increase in Firmicutes-to-Bacteroidetes (F-to-B) ratio has been attributed as a marker for gut-microbial dysbiosis in obese phenotype (4). Accumulating evidence suggests that gut-microbiota affect nutrient acquisition, increased energy-harvest, and contributes to a myriad of host-metabolic pathways (6). Evidence from mouse-model studies has demonstrated the causal-relationship between diet, the composition of the gut microbiota, and host-energy equilibrium (7). Transplantation of the gut-microbiome from obese-to-lean donors resulted in increased-adiposity (8). This mechanism has attributed gut-microbiota as increasing energy-harvest and shifting the energy equilibrium which results in weight gain (9).

The oral cavity is the gateway to the gut, and a source for passage of microbial entry into the gut, mediated by the ingestion of saliva (10–12). Little is known, however, about the

influence of oral bacteria on obesity. Recently, three studies have characterized the oral microbiome with rapid-weight-gain in infants**,** and microbial changes with adulthood-obesity (5,13,14). Longitudinal studies describing oral microbiome changes with weight gain and development of obesity, particularly in adolescents are lacking. In a prospective study design, we examined the oral microbiota of 67 adolescents, sampled at two-time points, two-years apart. Here, we investigated whether the changes in the composition of oral microbiota are associated with changes in host phenotypes – healthy, overweight, and obese. We also investigated whether oral microbial changes observed with host-phenotypes are confounded by changes in visit-2 (two-years apart), inflammatory factors (gingivitis, and salivary C-reactive-protein), or salivary metabolic markers (insulin, high-density lipoprotein cholesterol – HDLC, and glucose levels).

METHODS:

Study Design:

Subjects of this study are a subset of the Kuwait-Healthy-Lifestyle-Study (KHLS). This prospective study investigated changes in oral microbiota, with changes in host phenotypes - healthy, overweight, and obese. We sampled 80 adolescent subjects at visit-1 and 2, two-years apart. Each subject had two observations, making a total of 160 observations. During the sequence quality-filtration process (DADA2)(25,26), thirteen unique observations were deleted, hence we eliminated their corresponding observation, leaving 134 observations (of 67 subjects) for analysis of both visits 1 & 2. Refer to the CONSORT flow chart (Figure-1).

To compare oral microbiota changes in overweight phenotype (relative to healthy), we pooled in subjects who remained healthy at both visits (H_{v1}/H_{v2}=19), healthy individuals who became overweight (H_{v1}/Ov_{v2}=3), and overweight individuals who maintained their phenotype at visit-2 (Ov_{v1}/Ov_{v2}=7); to create a cohort of healthy-vs-overweight subjects (Cohort-I; N=29 subjects, Figure-1).

Similarly, for comparison of overweight and obese phenotypes, we pooled together a cohort of subjects who remained overweight at both visits (Ov_{v1}/Ov_{v2}=7), overweight individuals who became obese (Ov_{v1}/Ob_{v2}=26), and obese individuals who maintained their phenotype at visit-2 (Ob_{v1}/Ob_{v2}=6); to create a cohort of overweight-vs-obese subjects (Cohort-II; N=39 subjects, Figure-1).

Further, for comparison of healthy and obese phenotypes, we pooled together a cohort of subjects who remained healthy at both visits (H_{v1}/H_{v2}=19), healthy individuals who became obese (H_{v1}/Ob_{v2}=6), and obese individuals who maintained their phenotype at visit-2 (Ob_{v1}/Ob_{v2}=6); to create a cohort of healthy-vs-obese subjects (Cohort-III; N=31 subjects, Figure-1).

The rationale for constructing three cohorts is to evaluate whether changes in oral microbiota are specific to host-phenotypes and/or associated with phenotype-dependent effect modifiers such as inflammatory and metabolic factors evaluated in this study.

Saliva collection and processing:

An unstimulated (3 ml) whole saliva sample was obtained by passive-drooling between 8:30-9:30 am, under fasting conditions, at visit-1 and 2(15,27). We added a stabilization reagent (150uL RNAlater, Thermofisher Scientific) to 200uL of the whole saliva to protect

the nucleotides. To characterize the oral microbiome, whole saliva samples collected from the subjects of both study visits were sequenced based on 16s-rRNA NGS, on Illumina-Miseq platform, using primers for hypervariable-region (V3-V4). Also, multiplex-assays were used to measure salivary CRP, insulin, adiponectin, leptin, glucose, high-density lipoprotein (HDLC). The laboratory assay-protocols have been published earlier (15,28–30). Note that in this manuscript when we refer to metabolic or inflammatory markers, we are strictly referring to their saliva measurements and not plasma values.

Study Parameters:

For assessment of demographic traits and general health, we recorded age, gender, waist circumference (cm), height (cm), weight (kg), BMI (kg/m^2), systolic and diastolic blood pressure (SBP, DBP - mmHg), heart rate (HR in bpm), and fitness scores. For quantification of the systemic inflammatory pathway, we measured salivary CRP (levels high if $\geq$219 pg/mL)(29,30). For the ascertainment of the local inflammatory effect, we recorded the percentage of red/inflamed gingival sites (gingival erythema) as a marker for gingivitis. Generalized gingivitis (binary variable) was defined as $\geq$50% gingival erythematic sites. In addition, for assessment of metabolic markers we measured salivary insulin levels (high if $\geq$128 pg/mL), HDLC levels (low if <0.6 mg/dL), and salivary glucose concentration (high if levels $\geq$0.12mg/dL, cutoff obtained from the KHLS population and average median of 160 observations)(15,28–30). Moreover, metabolic measures of adiposity were also quantified using salivary adiponectin, and leptin levels.

Oral clinical parameters such as the number of deciduous and permanent teeth, and teeth with decay or filled, and gingivitis was recorded(28,31). Obesity cutoffs were obtained

from the original KHLS population (28). Body-mass-index (BMI) computed from weight and height (kg/M^2) was used to define weight categories: healthy ($<85^{th}$ percentile), overweight ($\geq$85%-95% percentile), and obese ($\geq$95% percentile).

Statistical Analysis:

Visit-1 and 2 study variables (shown in table-1) were recorded as continuous variables. Due to the non-normal distribution of the data, the Kruskal-Wallis test was used to compare medians (and interquartile ranges) of study variables amongst healthy, overweight, and obese subjects. While the chi-square test was used to compare frequencies for categorical variables. The diversity in microbial profile amongst our study cohorts was assessed using alpha-diversity by observed OTUs, Shannon, and Simpson diversity indices (32,33). Similarities in microbial profiles were evaluated using the Bray-Curtis similarity metric(33). (Refer to Supplement Methods and Results; Supplement-Figures 1, and 2)

Linear Discriminant Analysis effect-size (LEFSE) analysis was aimed at identifying predictive microbial markers (at phylum and genus taxa-level)(33). It employs a Kruskal-Wallis rank-sum test to detect significant differential-abundance with respect to healthy, overweight, or obese phenotype, followed by Linear Discriminant Analysis (LDA) to evaluate the effect-size of differential abundance features(33,34). We used the false-discovery-rate (FDR) method to adjust for multiple testing(35). Moreover, the longitudinal model,(36) was used to identify association and direction of change in microbial phyla with respect to change in the visit, host phenotypes, and systemic inflammatory/metabolic effect-modifiers. Refer to supplement methods and results.

RESULTS:

Study population and demographic characteristics:

We compared study demographics, systemic inflammatory, and metabolic factors, and oral clinical parameters across healthy, overweight, and obese subjects at both visits 1 and 2 (as shown in Table-1). Briefly, there was no significant difference between age, gender, and governate (state) distribution. Phenotypic traits associated with obesity, such as waist circumference, height, weight, systolic/diastolic blood pressure, and heart rate significantly differed across the three groups in both visits. The frequency of subjects with high insulin, high CRP, and low HDLC levels was higher in visit-2 and significantly associated with obesity. While, the frequency of subjects with high glucose, and gingivitis largely differed between the two study visits, and have previously been reported to affect the composition of oral microbiota(15). Hence, these variables were assessed for the possibility of effect-modification (for changes in oral microbiota), and subsequently adjusted in the longitudinal analysis (Supplementary Tables 1-3). Oral clinical parameters (except gingivitis) did not differ with either change in the visit or host phenotype.

Overview of Taxonomy changes at Phylum-level with host-phenotypes and effect modifiers:

Figure 2a. demonstrates that higher proportions of *Actinobacteria* were significantly associated with obese subjects having high insulin, and individuals with gingivitis. Whereas, lower proportions of *Actinobacteria* were significantly associated with obese and overweight subjects at visit-2, and obese subjects with low HDLC (a metabolic marker for adiposity). **Figure 2b.** demonstrates that higher proportions of *Bacteroidetes* were associated with subjects in visit-2, overweight subjects with high glucose or gingivitis, and

obese subjects with low HDLC or gingivitis. Besides, higher, and lower proportions of *Bacteroidetes*, were associated with obese subjects at visit-2, and overweight subjects at visit-2, respectively. Whereas, lower proportions of *Bacteroidetes* were associated with obese subjects with high insulin. This also demonstrates that both low-HDLC and high-insulin are strong effect-modifiers and belong to different systemic pathways that counter influence the proportion of *Bacteroidetes* in obese subjects. **Figure 2c.** demonstrates that higher proportions of *Fusobacteria* were associated with high insulin, whereas, lower proportions were associated with high CRP (active systemic inflammation), and overweight and obese subjects at visit-2. **Figure 2d.** demonstrates that higher proportions of *Firmicutes* were associated with overweight subjects with low-HDLC, whereas, lower proportions were associated with obese subjects at visit-2. **Figure 2e.** demonstrates that higher proportions of *Firmicutes-to-Bacteroidetes* (F-to-B-ratio) were associated with overweight subjects at visit-2, and subjects with high salivary glucose. Whereas lower proportions of F-to-B-ratio were associated with overweight subjects (with high glucose or gingivitis), and obese subjects (with gingivitis or within visit-2). The data indicate that gingivitis is a strong effect-modifier, i.e., the presence of gingivitis is either overweight or obese subjects demonstrated a reduction in F-to-B-ratio, irrespective of phenotype. Another observation is that both glucose, and gingivitis demonstrated a contrasting effect modification with the composition of F-to-B-ratio. Note that changes attributed solely with the effect of visit-2, could be attributed to time-varying changes within the two-year interval with the onset of puberty, along with the increase in age, blood pressure, waist circumference, BMI, changes in dietary patterns, tooth eruption, and other unmeasured/unknown confounders.

Change in members of Actinobacteria:

The abundance of phylum *Actinobacteria* predominated in subjects at visit-1, relative to visit-2. In exception, the abundance of phylum *Actinobacteria,* however, was lower in obese subjects (relative to overweight subjects) with low HDLC (estimate decrease=-3.79, p=0.04; Supplement-Table-2/Fig-2a). At follow-up visit-2, we identified that the abundance of *Actinobacteria* was lower in overweight subjects relative to healthy subjects (estimate decrease=-6.59, p<0.001, Supplement-Table-1/Fig-2a), and in obese subjects relative to both overweight (estimate decrease=-6.86, p<0.001, Supplement-Table-2/Fig-2a), and healthy subjects (estimate decrease=-6.54, p<0.001, Supplement-Table-3). In summary, we observed that the relative abundance of *Actinobacteria* demonstrated a dose-response relationship across the developmental stages of obesity (i.e., Healthy > Overweight > Obese; Fig-2a)

Within phenotype comparison at baseline (visit-1), identified that in obese subjects (relative to overweight subjects), the abundance of phylum *Actinobacteria* was higher in subjects with gingivitis (estimate increase=0.65, p<0.001) or high insulin (estimate increase=1.30, p=0.01; Supplement-Table-2/Fig-1). Similarly, in obese subjects at baseline (relative to healthy subjects), a higher proportion of *Actinobacteria* was associated with an increase in salivary insulin (estimate increase=1.03, p=0.02; Supplement-Table-3). In summary, these changes in phylum *Actinobacteria* can be attributed to the influence of visit-dependent effect modifiers - gingivitis, and high insulin.

At the genus-level, *Rothia* demonstrated a marked change (LDA~2.8), attributed to the effect of the visit. However, within phenotype comparison at visit-1 demonstrated a higher

abundance of *Rothia* in healthy (relative to overweight; Table-3) and in overweight (relative to obese; Table-4) subjects.

Change in members of Bacteroidetes:

In comparing healthy-vs-overweight subjects, both healthy and overweight individuals at visit-2 demonstrated a relatively higher abundance of *Bacteroidetes*, which was more pronounced amongst healthy relative to overweight individuals (5510.6 vs 4545.8; LDA=3.0; Table-2). This change in the proportion of *Bacteroidetes* amongst overweight individuals was attributed to the combined effect of both visit-2 and overweight phenotype (estimate decrease=-0.38, p=0.02; Supplement-Table-1/ Fig-2b).

In obese subjects, relative to both healthy and overweight subjects, the proportion of *Bacteroidetes* was significantly higher in visit-2 compared to baseline (Table-2). The proportion of *Bacteroidetes* was relatively higher in obese subjects (relative to overweight subjects) having low HDLC (estimate increase=0.86, p=0.04), or gingivitis (estimate increase=0.79, p=0.02), but relatively lower in obese subjects having high insulin (estimate decrease=-0.47, p=0.04) (Supplement-Table-2/ Fig-2b). In summary, we found that the proportion of *Bacteroidetes* was relatively higher in obese subjects, having gingivitis, or low HDLC supplements the abundance of *Bacteroidetes*; while obese subjects with high insulin, negatively influenced the abundance of *Bacteroidetes* (Fig-2b)

At the genus-level, we identified that *Prevotella* demonstrated a higher abundance in obese subjects, relative to healthy (LDA=3.0; Table-5) and overweight (LDA=2.9; Table-4) subjects.

Change in members of Firmicutes:

The LEFSE analysis did not demonstrate a significant change in *Firmicutes* at the phylum-level but demonstrated significant changes at the genus-level. On the other hand, the longitudinal analysis demonstrated that the proportion of Firmicutes was higher in obese subjects, relative to healthy (estimate increase=0.16, p=0.04; Supplement-Table-3), and overweight (estimate increase=0.16, p=0.02; Supplement-Table-2) subjects at baseline. In exception, the proportion of Firmicutes was higher in overweight subjects with low HDLC-levels, relative to healthy subjects (estimate increase=0.29, p=0.05; Fig-2c). In summary, a higher abundance of phylum Firmicutes was attributed to obese subjects and overweight subjects with low HDLC (Supplement-Tables 1-3; Fig-2c).

At the genus-level, however, we observed a generalized decrease in median abundance at visit-2 (relative to visit-1). We identified at baseline, that genera within the *Lachnospiraceae* family, were significantly higher in obese subjects, relative to healthy and overweight subjects: *Lachnoaerobaculum, Oribacterium, Stomatobaculum, Lachnospiraceae, Catonella,* and *Butyrivibrio* (Table 4 and 5). The expression of most members of the *Lachnospiraceae* family was relatively lower with visit-2, except *Ruminococcaceae (G2).*

The trend in the change of Firmicutes-to-Bacteroidetes (F-to-B) ratio:

At visit-2, the F-to-B ratio was significantly higher in overweight subjects (relative to healthy subjects) and lower in obese subjects (relative to healthy and overweight subjects) (Supplement-Tables 1-3/Fig-2e).

The change in the F-to-B ratio was strongly associated with the change in the visit, which can be attributed to the significant effect of glucose and gingivitis that changed between the two study visits. In particular, we observed that the F-to-B ratio significantly increased

with high salivary glucose or gingivitis but decreased in overweight individuals having high glucose or gingivitis (Fig-2e).

DISCUSSION:

In this study, of oral bacterial perturbation related to the development of obesity, there are five major findings: (i) change in F-to-B ratio, (ii) a higher proportion of *Prevotella sp.*, (iii) a higher proportion of mucin-degrading oral bacteria, (iv) reduced salivary-glucose effects, and (v) reduction in nitrate-reducing oral bacteria.

(i). Change in F-to-B ratio: One of the principal findings in our study was that a relatively higher oral F-to-B ratio was observed in overweight subjects, relative to healthy and obese subjects. When we compared the overweight-to-obese transition, however, the oral F-to-B ratio decreased in obese subjects. Furthermore, these transitions in oral microbiota were modulated by the presence of metabolic and inflammatory mediators. This differs from the observations reported from the gut microbiome.

The gut-microbiome of obese subjects, exhibited a higher abundance of phylum Firmicutes, and a lower abundance of Bacteroidetes (i.e., an increase in F-to-B ratio) is associated with increased energy-harvest, leads to weight gain, adiposity, and associated with the development of obesity, compared to a healthy-state (7,8). In our study of oral bacteria, when we compared overweight-to-obese transition (cohort-II), we observed that 26 of 33 overweight subjects at visit-1, became obese at visit-2. These overweight subjects exhibited a higher F-to-B ratio, which significantly lowered with the development of obesity. We postulate that the oral-bacteria within overweight-subjects could translocate into the gut microbiota, and might play a role in converting complex otherwise indigestible-

polysaccharides into monosaccharides and short-chain-fatty-acids (SCFA's), which serves as a substrate for lipogenesis in hepatocytes and adipocytes (4,12,16) (11,17).

(ii) A higher proportion of Prevotella: In obese subjects, at visit-2 we observed a relatively higher proportion of *Prevotella sp.,* compared to healthy and overweight subjects. These *Bacteroidetes* members are opportunistic, anaerobic, gram-negative bacteria, and contain pathogenic lipo-poly-saccharide capsule (LPS). Higher proportions of LPS-containing *Prevotella sp*. were observed in parallel with a significant increase in CRP levels in obese subjects, which is indicative of active systemic inflammation (Tables 1, 4, and 5). It has been suggested that the bacterial-mediated-LPS, binds to human immune pattern-recognition-receptors such as the toll-like-receptor (TLR's), which initiates systemic-inflammation (18). Besides, increased nutritional fatty-acids (SCFA) have also shown to activate TLR and induce inflammation (19).

(iii) A higher proportion of mucin-degrading oral bacteria: In obese subjects (relative to healthy and overweight subjects at baseline), we observed a higher proportion of mucin-degrading bacteria (esp. the Lachnospiraceae family – genera *Lachnoaerobaculum, Lachnospiraceae (G-2), Stomatobaculum, Oribacterium, Ruminococcaceae[G-2], Catonella,* and *Butyrivibrio*), which is attributed to gut-barrier dysfunction (20–22). Therefore, under increased gut-permeability (i.e., gut-barrier-dysfunction), increased bacterial-LPS and/or SCFA's in the gut could leak into the systemic circulation, giving rise to a condition known as metabolic-endotoxemia, which leads to the development of both insulin resistance and the onset of obesity (3,18).

(iv) Salivary glucose effects: In obese subjects, the proportion of *Bacteroidetes* (esp. *Prevotella* spp.) was relatively lower with high salivary glucose concentration. This phenomenon was observed particularly in visit-1 subjects who were healthy or overweight and expressed early signs of salivary hyperglycemia (high glucose concentration) before the development of obese status by visit-2. With the development of obesity, in visit-2, the frequency of hyperglycemia was reduced, and consequently, higher proportions of *Bacteroidetes* was observed. The data also indicate that hyperglycemia was one of the earliest metabolic markers towards the development of obesity. Consistent with our findings, it has been reported that higher salivary glucose concentration is associated with a reduction in acid-sensitive *Bacteroidetes* species such as *Prevotella melaninogenica* (15). These observations indicate that the salivary glucose concentration is an effect-modifier, influencing the concentration of *Bacteroidetes*.

(v) Reduction in nitrate-reducing oral bacteria: Consistent with the development of obesity (at visit-2) and the onset of pediatric hypertension (median SBP/DBP $\geq$ 130/90 mmHg), we observed a relatively lower proportion of nitrate-reducing oral bacteria (phylum *Actinobacteria* - esp. *Rothia* and *Actinomyces spp.*). It has been reported that commensal nitrate-reducing oral bacteria play a key role in the enterosalivary nitrate-nitrite-NO pathway which has a homeostatic effect in the maintenance of normal blood pressure (23,24). Does that mean the reduction in nitrate-reducing oral bacteria (NRB) could lead to the onset of hypertension (potential causal pathway), or, obese subjects with hypertension could influence lower proportions of oral-NRB (potential effect pathway). This hypothesis needs to be investigated in future studies.

Besides phenotype, the microbial changes observed with changes in the visit cannot be overlooked. Because it highlights the impact of changes in host inflammatory or metabolic pathways associated with obesity which have demonstrated a profound impact on oral microbial perturbation. Also, it is evident that a significant difference in metabolic and inflammatory factors across the healthy, overweight, and obese phenotypes were observed only at visit-2, and not at visit-1 (Table-1). Although we did not measure visit-related changes in growth hormones or the effect of puberty, adjusting for the variable – 'visit-2' in longitudinal analysis, we took into account, the afore-mentioned time-varying changes, observed with puberty, or development of obesity. We also believe that these microbial changes between visits 1 and 2, could be attributed to the original nature of the study design. The original sub-cohort was designed to study changes in oral microbiome concerning obese and non-obese, based on waist circumference. We enrolled non-obese subjects at visit-1, half of whom became obese at visit-2, while the other half remained non-obese at visit-2, two-years later. This study design, however, did not allow us to investigate microbial changes with the developmental stages of childhood obesity. To compare microbial changes across the developmental stages of obesity (i.e., by comparing healthy, overweight, and obese phenotypes), we used BMI instead of waist circumference.

This is the first longitudinal study that characterized changes in oral microbiota with the development of adolescent obesity, and obesity-associated inflammatory and metabolic pathways. This is a hypothesis-generating study, that highlights the role of effect-modifiers with changes in host phenotype and related oral microbial perturbation. In this study, the sample size was reduced by 28%, because of loss-to-follow-up, and poor quality of microbial sequencing. This limited our ability to estimate small effect sizes of changes in

the oral microbiome at the genus and species level. Although, we adjusted for multiple comparisons using the FDR approach in our cross-sectional analysis, which addressed our study's primary objective. But we did not adjust for multiple comparisons in our longitudinal analysis. Future researchers investigating the role of individual effect modifiers reported in our study can refer to the effect size for sample-size estimation. The microbial changes observed in this study concerning host phenotypes, gingivitis, and systemic changes in inflammatory or metabolic factors, need to be validated in longitudinal study design; with a larger sample size, longer follow-up, and multiple time-points to ensure temporality in data.

CONCLUSIONS:

In summary, the oral microbiome composition was found to significantly differ in healthy, overweight, and obese phenotypes. We found that the overweight phenotype, which is considered as a high-risk for obesity, demonstrated an increase in oral Firmicutes-to-Bacteroidetes ratio – a feature of obese gut microbiota. We also observed that metabolic markers associated with the development of obesity strongly influenced oral microbial perturbations. Particularly, in transitions from healthy to overweight, and overweight to obese phenotypes, we identified high insulin, low HDLC, and high salivary glucose as effect-modifiers. Higher proportions of genus *Prevotella* and family *Lachnospiracae* suggests that the oral bacteria may potentially degrade gastric mucus. This degradation may increase the risk of gut mucosal permeability, which might contribute to the leakage of LPS into the circulation and increase systemic inflammation. Considering the evidence presented, we

postulate that the oral microbiota of overweight subjects may translocate to the gut and might set the stage for childhood obesity.

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AUTHOR CONTRIBUTION:

All authors gave their final approval and agree to be accountable for all aspects of the work. Individual author contributions are listed below.

Author 1: Contributed to design, data acquisition and interpretation, performed all statistical and bioinformatics analyses, drafted and critically revised the manuscript

Author 2: Principal Investigator - contributed to conception, design, data acquisition and interpretation, drafted and critically revised the manuscript.

Author 3: data acquisition, bioinformatics analysis, and interpretation, and critically revised the manuscript

Author 4: analysis, and interpretation, and critically revised the manuscript

Author 5: performed all statistical analyses, and critically revised the manuscript

Author 6: Contributed to design, data acquisition and critically revised the manuscript

Author 7: Senior Author – supervised the project, data acquisition, and interpretation, performed bioinformatics analyses, and critically revised the manuscript

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FIGURE LEGEND

Figure-1: CONSORT flowchart describes our study population and construction of the three cohorts, to compare changes in oral microbiota, amongst Healthy-vs-Overweight, Overweight-vs-Obese, and Healthy-vs-Obese subjects. Healthy (n=28), Overweight (n=33), and Obese (n=06) adolescents at baseline were followed for two years. At visit 2, 19 healthy individuals remained healthy (H_{v1}/H_{v2}), while 3 became overweight ($H_{v1}/Over_{v2}$), and 6 became obese (H_{v1}/Obe_{v2}). On the other hand, 07 Overweight remained overweight ($Over_{v1}/Over_{v2}$), while 26 overweight subjects became obese ($Over_{v1}/Obe_{v2}$). Besides, 6 obese subjects remained obese (Obe_{v1}/Obe_{v2}). The rationale for constructing three cohorts is to compare longitudinal changes in oral microbiota across healthy, overweight, and obese phenotypes. Also, separately comparing these individual cohorts allow us to identify phenotype-dependent effect modifiers (for changes in oral microbiota).

Figure-2: Forest plots summarize key significant results from longitudinal analysis (Supplementary Tables 1-3). The estimates and 95% confidence intervals are plotted, which represents compositional changes in each phylum concerning changes in host phenotypes, visit-2, inflammatory factors (gingival inflammation/gingivitis, and high-CRP), and salivary metabolic markers (low HDLC concentration, high glucose, and high insulin concentration). Estimates>0 represent a relatively higher proportion of respective phylum abundance, whereas, estimates<0 represent a relatively lower proportion of phylum abundance.

Figure 1: CONSORT Flow Chart

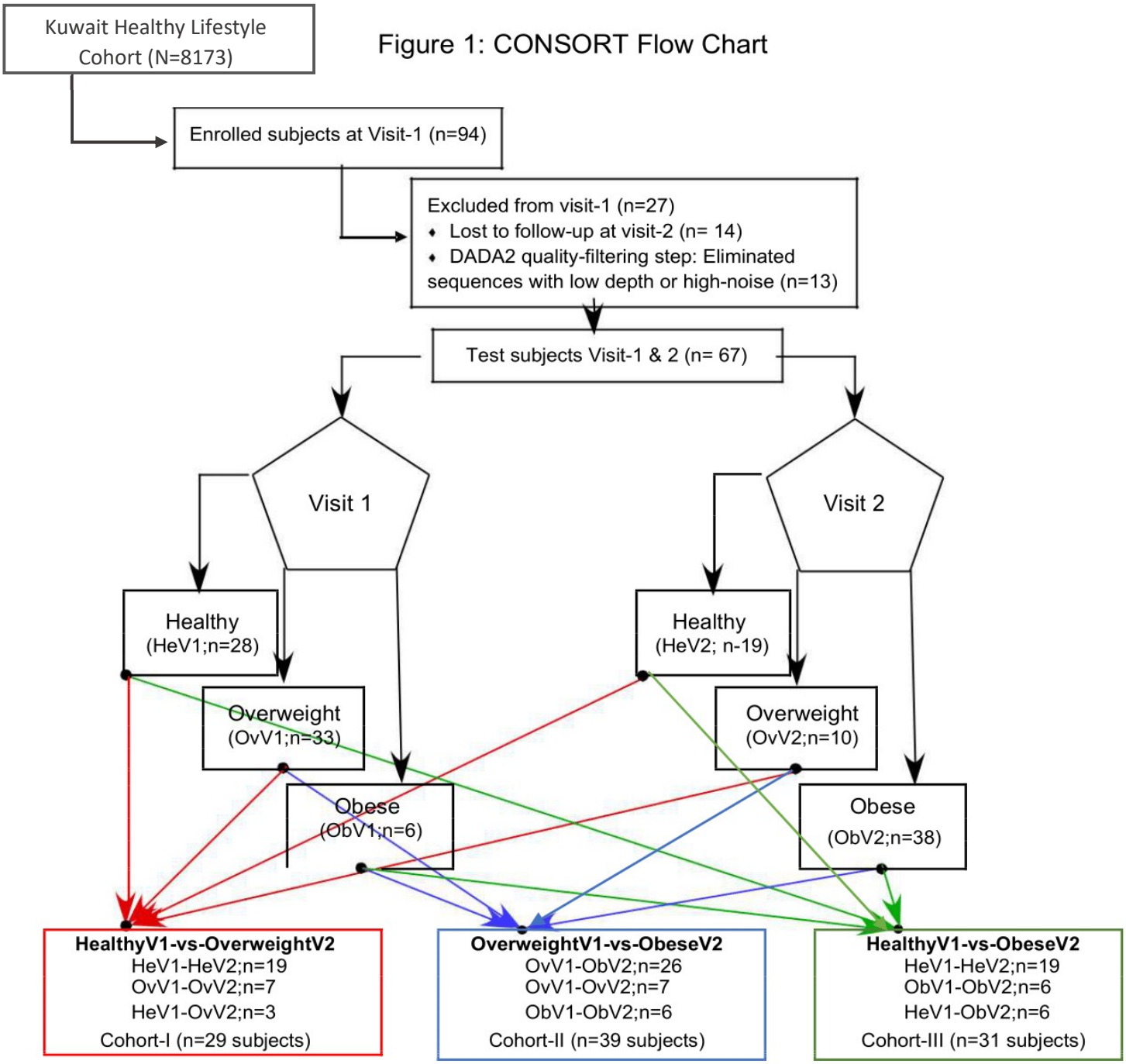
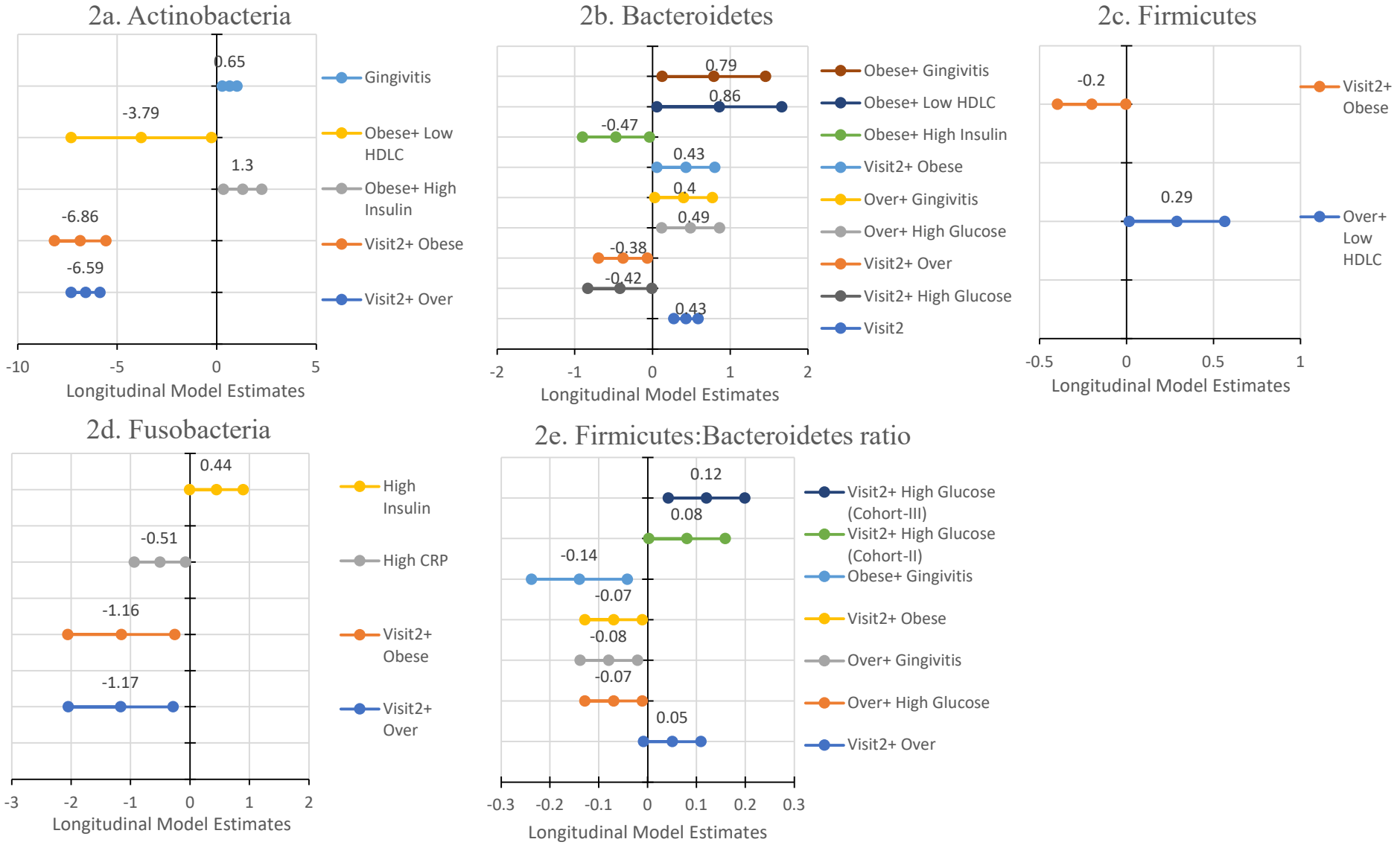


Figure-2: Forest plots summarize key significant results from longitudinal analysis (Supplementary Tables 1-3). The estimates and 95% confidence intervals are plotted, which represents compositional changes in each phylum concerning changes in host phenotypes, visit-2, inflammatory factors (gingival inflammation/gingivitis, and high-CRP), and salivary metabolic markers (low HDLC concentration, high glucose, and high insulin). Estimates>0 represent a relatively higher proportion of respective phylum abundance, and vice-versa.



TABLES

Table-1: Comparing population characteristics at baseline (visit-1) and after two years of follow-up (at visit-2)

	Visit-1 (N=67)				*Visit-2 (N=67)*			
Study Characteristics	**Healthy (n=28)**	**Overweight (n=33)**	**Obese (n=6)**	**p-value**	**Healthy (n=19)**	**Overweight (n=10)**	**Obese (n=38)**	**p-value**
Demographic characteristics of the study population								
Age (years)	9.7 (9.2,10.3)	9.8 (9.4,10.2)	9.7 (9.6,9.9)	0.93	11.9 (11.5,12.5)	12.0 (11.5,12.4)	11.8 (11.3,12.3)	0.67
Male Gender	16 (57.1%)	12 (36.4%)	02 (33.3%)	0.22	9 (47.4%)	4 (40.0%)	17 (44.7%)	0.93
Governates				0.83				0.92
Al-Ahmadi	5 (17.9%)	7 (21.2%)	1 (16.7%)		4 (21.1%)	1 (10.0%)	8 (21.1%)	
A- Asimah (Capital)	5 (17.9%)	8 (24.2%)	1 (16.7%)		5 (26.3%)	3 (30.0%)	6 (15.8%)	
Al-Farwaniyah	5 (17.9%)	5 (15.2%)	0		4 (21.1%)	1 (10.0%)	5 (13.2%)	
Al-Jara	8 (28.6%)	10 (30.3%)	4 (66.7%)		4 (21.1%)	4 (40.0%)	14 (36.8%)	
Hawalli	1 (3.6%)	1 (3.0%)	0		1 (5.3%)	0	1 (2.6%)	
Al-Kabeer	4 (14.3%)	2 (6.1%)	0		1 (5.3%)	1 (10.0%)	4 (10.5%)	
Fitness Score (bpm)	14.0 (4.0,31.6)	34.0 (9.0,47.5)	29.3 (20.8,57.6)	0.08	23.0 (13.8,28.5)	30.8 (20.0,37.3)	23.3 (14.1,34.1)	0.53
Waist (cm)	60.9 (58.4,63.5)	68.6 (66.0,71.1)	73.7 (71.8,73.7)	<0.01	67.0 (64.0,69.5)	75.5 (72.0,79.0)	89.0 (86.3,92.0)	<0.01
Height (cm)	132.5 (130.8,137.3)	139.0 (134.0,144.0)	139.0 (134.3,140.8)	0.019	145.0 (140.0,152.5)	156.5 (145.0, 160.0)	152.0 (147.3, 156.0)	0.01
Weight (kg)	30.5 (30.5,43.5)	40.0 (37.0,43.0)	48.0 (45.0,48.8)	<0.01	38.0 (34.2,42.1)	53.7 (47.8,57.3)	59.4 (57.6,63.0)	<0.01
BMI (kg/m2)	16.5 (15.7, 17.3)	20.6 (19.8, 21.2)	24.3 (23.1,25.1)	<0.01	17.6 (17.1, 18.3)	21.8 (21.4,22.5)	26.1 (24.7, 26.9)	<0.01
SBP (mmHg)	100.5 (98,114.0)	116.0 (107,125.0)	116.5 (103,128.5)	<0.01	103.0 (96.5,107.0)	121.5 (118.0,130.5)	131.0 (124.0,136.0)	<0.01
DBP (mmHg)	67.0 (63.8,72.3)	79.0 (68.0,87.0)	85.5 (84.0,87.8)	<0.01	69 (66.0,81.0)	78 (68.5,94.3)	90 (78.5,100.5)	<0.01
HR (bpm)	88.0 (78.5,100.3)	97.0 (84.0,101.0)	86.5 (81.3,96.3)	0.45	88 (74.5,94.5)	83 (65.25,89.25)	94 (81.0,102.0)	0.05
Assessment of Metabolic and Inflammatory Factors								
Saliva HDLC	1.0(0.7,1.5)	0.8 (0.7,1.3)	0.8(0.7, 0.9)	0.59	0.5 (0.2, 1.5)	0.5 (0.3, 0.8)	0.3 (0.2, 0.4)	0.11
Low HDLC	0	03 (9.1%)	0	-	12 (63.2%)	7 (70.0%)	35 (92.10%)	0.02
Saliva Glucose	0.1 (0.1, 0.2)	0.15 (0.13, 0.19)	0.17 (0.12, 0.17)	0.96	0.04 (0.02, 0.08)	0.01 (0, 0.10)	0.05 (0.001, 0.11)	0.60
High Glucose	21 (75.0%)	26 (78.8%)	04 (66.7%)	0.69	2 (10.5%)	3 (30.0%)	7 (18.4%)	0.40
Saliva Insulin	67.9 (1.6, 180.6)	121.5 (22.3, 218.8)	94.8 (82.3, 103.7)	0.31	72.7 (28.1, 106.9)	160.1 (103.1, 229.7)	177.8 (85.7, 244.4)	<0.01
High Insulin	11 (39.3%)	16 (48.5%)	01 (16.7%)	0.33	2 (10.5%)	7 (70.0%)	26 (68.4%)	<0.01
Saliva CRP	175.1 (42.4, 596.7)	144.4 (52.5, 334.4)	133.9 (14.4, 287.8)	0.61	55.3 (25.2, 142.4)	57.3 (42.0, 62.4)	458.3 (184.1, 895.7)	<0.01

High CRP	12 (42.9%)	12 (36.4%)	03 (50.0%)	0.77	3 (15.8%)	2 (20.0%)	26 (68.4%)	<0.01
Saliva Adiponectin	13801.6 (7277.4, 19204.1	10136.9 (5738.2, 20297.2)	14020.6 (10624.3, 17263.2)	0.33	9680.6 (5437.2, 15274.1)	8487.5 (4054.8, 23540.4)	7010.8 (3814.9, 11892.0)	0.28
Saliva Leptin	0.5 (0.5,3.3)	0.5 (0.5,6.2)	0.5 (0.5,3.2)	0.36	2.9 (0.5,4.4)	3.5 (0.5,5.2)	2.9 (0.5, 5.0)	0.81
Oral Clinical Parameters								
Saliva Flow rate	29.3 (15.8,40.2)	30.4 (20.3,35.4)	22.5 (13.3,39.7)	0.87	16.74 (11.5,27.6)	22.3 (16.6,40.1)	28.3 (17.5,36.3)	0.16
No .Decay/ filled teeth	10.8 (4.6,18.2)	8.7 (0,20.8)	8.7 (8.4,9.0)	0.95	11.5 (8.7, 18.7)	18.33 (8.81,28.6)	15.1 (1.8, 21.1)	0.54
No. Deciduous teeth	8.0 (4.0, 11.0)	7.0 (3.0, 10.0)	6.5 (5, 9.5)	0.59	1 (0, 4)	0 (0, 1.8)	0 (0, 1)	0.25
No. Permanent teeth	13.5 (12.0, 18.5)	16.0 (14.0, 20.0)	16.5 (14.3, 18.8)	0.2	22.0 (20.0, 26.0)	25.5 (23.3, 27.8)	26.0 (24.0, 27.8)	0.11
Eruption Rate (%)	36.2 (11.5, 78.3)	56.3 (28.6, 85.0)	59.4 (33.1, 73.3)	0.43	95.2 (79.4, 100)	100 (93.4, 100)	100 (96, 100)	0.22
% Gingival redness	75.6 (48.2, 94.9)	84.9 (61.7, 94.4)	86.6 (78.1, 97.8)	0.52	38.5 (30.6, 46.0)	37.3 (31.3, 44.9)	46.1 (35.7, 59.6)	0.28
Gingivitis	20 (71.4%)	27 (81.8%)	05 (83.3%)	0.69	4 (21.1%)	2 (20.0%)	14 (36.8%)	0.36

Due to the non-normal distribution of the data, the Kruskal Wallis test was used to assess differences in continuous variables concerning host phenotypes. The results are reported median (and interquartile range) with p-values. SBP and DBP – systolic and diastolic blood pressure (mmHg); HR- heart rate (beats per minute or bpm); Salivary High-density lipoprotein (HDLC) $\leq$0.6 mg/dL was categorized as low HDLC; Salivary glucose concentration $\geq$0.12mg/dL was defined as high-salivary-glucose (based on median cutoff). Insulin $\geq$128 pg/mL was categorized as high-insulin; C-Reactive Protein- CRP $\geq$219 pg/mL was categorized as high-CRP; Salivary flow rate was measured in mL/hr. Percent of gingival red sites were recorded (% gingival redness), an individual having $\geq$50% gingival sites was categorized into a binary variable (gingivitis). Permanent teeth eruption rate was calculated based on the following equation: [1-(ratio of deciduous-to-permanent number of teeth)]*100%; where a hundred percent denotes eruption of permanent dentition is completed, and the deciduous teeth are no longer retained in the dentition.

Table-2: Using Linear Discriminant Analysis effect size (LEFSE) to compare phylum-level changes across the three cohorts: healthy vs overweight, overweight vs obese, and healthy vs obese.

Comparing Healthy vs. Overweight (Cohort-I)			Visit-1		Visit-2		LDA
	P-values	FDR	Healthy	Over	Healthy	Over	score
Actinobacteria	2.63E-10	1.58E-09	**1959.2**	1901.1	28.7	63.7	2.99
Bacteroidetes	3.74E-03	1.12E-02	3497.8	4230.3	**5510.6**	4545.8	3.00
Fusobacteria	1.34E-01	2.68E-01	832.1	565.9	535.7	541.5	2.17
Proteobacteria	2.33E-01	3.50E-01	2505.5	2213.4	2983.8	3775.1	2.89
Saccharibacteria (TM7)	6.66E-01	8.00E-01	55.3	45.7	58.8	46.5	0.89
Firmicutes	9.37E-01	9.37E-01	6277.1	6170.6	6009.4	6154.4	2.13
Comparing Overweight vs. Obese (Cohort-II)			Visit-1		Visit-2		
	P-values	FDR	Over	Obese	Over	Obese	LDA score
Actinobacteria	6.36E-12	3.82E-11	**1584.1**	1407.0	8.0	137.7	2.90
Bacteroidetes	2.13E-04	6.38E-04	2947.3	2847.8	3350.0	**4434.7**	2.90
Proteobacteria	5.02E-02	1.00E-01	1936.5	1421.3	3241.9	2037.4	2.96
Fusobacteria	9.84E-02	1.48E-01	496.2	661.2	386.6	428.8	2.14
Firmicutes	4.20E-01	4.22E-01	5155.9	5800.8	5138.9	5078.5	2.56
Saccharibacteria (TM7)	4.22E-01	4.22E-01	34.0	15.8	28.7	36.8	1.06
Comparing Healthy vs. Obese (Cohort-III)			Visit-1		Visit-2		
	P-values	FDR	Healthy	Obese	Healthy	Obese	LDA score
Actinobacteria	3.43E-11	2.06E-10	**1863.8**	1777.0	27.263	8.0	2.97
Bacteroidetes	8.68E-04	2.60E-03	3676.9	3558.0	5510.5	**5695.3**	3.03
Fusobacteria	1.57E-03	3.15E-03	**961.8**	824.3	542.8	366.5	2.48
Firmicutes	2.09E-01	2.29E-01	6250.8	7219.3	5979.6	5873.7	2.83
Saccharibacteria (TM7)	2.21E-01	2.29E-01	60.1	19.5	56.3	43.0	1.33
Proteobacteria	2.29E-01	2.29E-01	2313.6	1728.8	3010.5	3140.5	2.85

LEFSE analysis compares median inter-phyla abundance concerning obesity phenotypes in both visits and uses the Kruskal-Wallis rank-sum test to detect differences in the medians across the four groups compared in each analysis. Significance is reported as a p-value, which has been adjusted using the Benjamini-Hochberg false discovery rate (FDR). P-value <0.05 is considered significant. Linear Discriminant Analysis (LDA) is used to evaluate the effect size of differential abundance features across the four groups of comparison. LEFSE analysis has been computed using MicrobiomeAnalyst. Values in **BOLD** represent the highest and significant medians amongst the groups of comparison.

Table-3: Linear Discriminant Analysis effect size (LEFSE) to compare genus-level changes in Healthy vs Overweight subjects, at baseline and visit-2 (Cohort-I).

Healthy vs. Overweight (Cohort-I)			Visit-1		Visit-2		LDA
Genus	Phylum	FDR (p-value)	Health	Over	Health	Over	
Increase in Overweight (v2)							
Aggregatibacter	Proteobacteria	4.29E-02	137.2	52.0	131.9	**276.9**	2.05
Streptobacillus	Fusobacteria	2.79E-03	19.1	5.4	7.9	50.1	1.37
Lautropia	Proteobacteria	1.58E-04	21.0	21.8	12.2	40.7	1.18
Increase in Overweight (v1)							
Actinomyces	Actinobacteria	1.81E-09	171.5	**284.4**	2.0	2.0	2.15
Solobacterium	Firmicutes	1.81E-09	88.1	111.1	1.0	1.0	1.75
Atopobium	Actinobacteria	4.80E-07	120.2	125.3	21.7	56.7	1.72
Lachnoanaerobaculum	Firmicutes	1.40E-03	88.6	91.0	31.0	42.7	1.49
Megasphaera	Firmicutes	8.58E-04	41.4	71.1	33.2	27.0	1.36
Selenomonas	Firmicutes	3.72E-06	14.6	21.6	1.0	1.0	1.05
Mogibacterium	Firmicutes	2.52E-06	9.7	14.7	1.9	2.8	0.87
Peptococcus	Firmicutes	1.37E-04	10.3	10.9	1.0	1.0	0.77
Increase in Healthy (v2)							
Butyrivibrio	Firmicutes	2.24E-03	6.9	5.0	10.4	1.0	0.76
Increase in Healthy (v1)							
Rothia	Actinobacteria	1.81E-09	**1620.5**	1480.4	3.0	3.0	2.91
Leptotrichia	Fusobacteria	2.19E-04	**462.6**	218.1	95.4	203.9	2.27
Peptostreptococcus	Firmicutes	3.72E-06	85.4	70.3	33.2	1.0	1.64
Abiotrophia	Firmicutes	9.64E-06	72.9	38.7	41.2	1.0	1.57
Corynebacterium	Actinobacteria	2.01E-06	47.1	11.0	2.0	2.0	1.37
Campylobacter	Proteobacteria	4.45E-02	87.3	45.4	44.2	61.6	1.35
Lachnospiraceae (G-2)	Firmicutes	6.08E-05	41.9	22.3	1.0	11.5	1.33
Stomatobaculum	Firmicutes	1.73E-07	41.5	36.9	2.0	2.0	1.32
Ruminococcaceae (G-1)	Firmicutes	4.80E-07	30.9	18.0	9.0	1.0	1.20
Catonella	Firmicutes	1.25E-03	19.2	18.3	6.6	1.0	1.00
Ruminococcaceae (G-2)	Firmicutes	3.87E-03	11.6	6.3	7.3	10.5	0.56

LEFSE analysis compares median inter-genus abundance amongst healthy and overweight phenotype in both visits and uses a Kruskal-Wallis rank-sum test to detect differences in the medians across the four groups: Healthy (visit-1), Overweight

(visit-1), Healthy (visit-2), and Overweight (visit-2). P-values have been adjusted for multiple-comparison using Benjamini-Hochberg false discovery rate (FDR). P-value <0.05 is considered significant. Linear Discriminant Analysis (LDA) is used to evaluate the effect size of differential abundance features across the four groups of comparison. LEFSE analysis has been computed using MicrobiomeAnalyst. Values in **BOLD** represent the highest and significant medians of respective genera amongst the four groups of comparison.

Table-4: Linear Discriminant Analysis effect size (LEFSE) to compare genus-level changes in Overweight vs. Obese subjects, at baseline and visit-2 (Cohort-II).

Overweight vs. Obese (Cohort-II)			Visit-1		Visit-2		LDA
Genus	Phylum	FDR	Over	Obese	Over	Obese	
Increase in Obese (v2)							
Prevotella	Bacteroidetes	5.44E-03	2442.60	2257.30	2354.10	**3842.80**	2.90
Megasphaera	Firmicutes	5.96E-05	37.03	24.67	8.71	40.88	1.23
Increase in Obese (v1)							
Leptotrichia	Fusobacteria	5.85E-09	229.12	**355.00**	4.00	101.56	2.25
Peptostreptococcus	Firmicutes	2.41E-10	71.36	140.83	1.00	10.66	1.85
Lachnoanaerobaculum	Firmicutes	1.92E-05	65.97	**122.83**	21.29	26.97	1.71
Oribacterium	Firmicutes	4.51E-02	72.85	**125.67**	97.14	48.19	1.60
Abiotrophia	Firmicutes	3.00E-09	55.58	67.83	1.00	23.66	1.54
Solobacterium	Firmicutes	8.28E-10	69.15	**70.50**	1.00	24.56	1.55
Aggregatibacter	Proteobacteria	1.16E-05	69.88	109.17	39.57	60.75	1.55
Stomatobaculum	Firmicutes	5.17E-11	28.73	**37.83**	2.00	2.97	1.28
Lachnospiraceae (G-2)	Firmicutes	4.65E-07	24.85	**33.67**	1.00	5.63	1.24
Catonella	Firmicutes	1.15E-07	10.06	**18.50**	1.00	1.31	0.99
Selenomonas	Firmicutes	2.41E-10	14.94	16.83	1.00	1.00	0.95
Butyrivibrio	Firmicutes	4.65E-07	11.91	14.17	1.00	5.56	0.88
Peptococcus	Firmicutes	9.86E-06	7.15	7.50	1.00	1.00	0.63
Mogibacterium	Firmicutes	2.17E-06	8.36	8.83	3.43	3.88	0.57
Increase in Overweight (v1)							
Rothia	Actinobacteria	8.91E-11	**1337.30**	1238.20	3.00	130.41	2.82
Corynebacterium	Actinobacteria	2.41E-10	24.30	17.33	2.00	2.00	1.08
Ruminococcaceae (G-1)	Firmicutes	1.83E-04	11.91	10.83	1.00	9.50	0.81
Increase in Overweight (v2)							
Streptobacillus	Fusobacteria	1.54E-02	13.27	40.83	63.29	24.50	1.42
Bergeyella	Bacteroidetes	7.09E-04	26.18	15.00	53.86	18.13	1.31
Lautropia	Proteobacteria	6.60E-04	15.58	17.33	33.57	20.69	1.00
Ruminococcaceae (G-1)	Firmicutes	1.16E-05	9.00	7.17	12.29	3.28	0.74

LEFSE analysis compares inter-genera median abundance amongst overweight and obese phenotype in both visits and uses a Kruskal-Wallis rank-sum test to detect differences in the medians across the four groups: Overweight (visit-1), Obese (visit-1), Overweight (visit-2), and Obese (visit-2). P-values have been adjusted for multiple-comparison using Benjamini-Hochberg false discovery rate (FDR). P-value <0.05 is considered significant. Linear Discriminant Analysis (LDA) is used to evaluate the effect size of differential abundance features across the four groups of comparison. LEFSE analysis has been computed using MicrobiomeAnalyst. Values in **BOLD** represent the highest and significant medians of respective genera amongst the four groups of comparison.

Table-5: Linear Discriminant Analysis effect size (LEFSE) to compare genus-level changes in Healthy vs. Obese subjects, at baseline and visit-2 **(Cohort-III).**

Healthy vs. Obese (Cohort III)			Visit-1		Visit-2		LDA
Genus	Phylum	FDR	Healthy	Obese	Healthy	Obese	
Inc in Obese (v2)							
Prevotella	Bacteroidetes	8.56E-02	3038.0	2822.8	4611.5	**4870.0**	**3.01**
Megasphaera	Firmicutes	1.74E-03	57.6	30.7	35.0	73.2	1.35
Ruminococcaceae (G-2)	Firmicutes	1.84E-03	12.2	9.3	7.8	13.3	0.58
Inc in Obese (v1)							
Rothia	Actinobacteria	4.67E-10	1529.1	**1564.5**	3.0	3.0	**2.89**
Peptostreptococcus	Firmicutes	5.74E-07	79.8	176.0	34.3	1.0	1.95
Lachnoanaerobaculum	Firmicutes	2.25E-06	91.5	**158.2**	34.2	8.4	1.88
Abiotrophia	Firmicutes	1.68E-05	71.8	81.3	42.4	13.6	1.54
Stomatobaculum	Firmicutes	3.07E-09	48.8	**49.8**	2.0	2.0	1.40
Lachnospiraceae (G-2)	Firmicutes	4.70E-07	41.1	**47.3**	1.0	3.3	1.38
Streptobacillus	Fusobacteria	3.31E-03	15.8	48.0	7.8	42.1	1.32
Selenomonas	Firmicutes	4.98E-06	15.9	22.3	1.0	1.0	1.07
Mogibacterium	Firmicutes	1.90E-08	9.8	12.2	1.2	1.6	0.81
Butyrivibrio	Firmicutes	1.72E-03	8.5	**17.0**	11.1	9.8	0.72
Inc in Healthy (v1)							
Leptotrichia	Fusobacteria	7.30E-07	**565.8**	443.7	96.5	15.6	2.44
Actinomyces	Actinobacteria	4.67E-10	178.9	125.3	2.0	2.0	1.95
Atopobium	Actinobacteria	4.88E-09	127.0	64.7	20.3	1.0	1.81
Solobacterium	Firmicutes	2.75E-09	104.7	96.0	1.0	9.0	1.72
Corynebacterium	Actinobacteria	3.35E-07	28.7	22.5	2.0	2.0	1.16
Ruminococcaceae (G-1)	Firmicutes	2.08E-06	27.7	15.7	9.0	10.7	1.01
Catonella	Firmicutes	9.02E-04	19.6	19.0	6.7	4.2	0.94
Peptococcus	Firmicutes	4.90E-06	12.5	9.2	1.0	1.0	0.83
Lautropia	Proteobacteria	5.25E-06	20.2	19.8	12.2	11.2	0.74
Inc in Healthy (v2)							
Aggregatibacter	Proteobacteria	5.85E-03	114.8	135.0	136.6	52.3	1.64

LEFSE analysis compares inter-genera median abundance amongst healthy and obese phenotype in both visits and uses a Kruskal-Wallis rank-sum test to detect differences in the medians across the four groups: Healthy (visit-1), Obese (visit-1), Healthy (visit-2), and Obese (visit-2). P-values have been adjusted for multiple-comparison using Benjamini-Hochberg false discovery rate (FDR). P-value <0.05 is considered significant. Linear Discriminant Analysis (LDA) is used to evaluate the effect size of differential abundance features across the four groups of comparison. LEFSE analysis has been computed using MicrobiomeAnalyst. Values in **BOLD** represent the highest and significant medians of respective genera amongst the groups of comparison.

SUPPLEMENT

OF

PROJECT-I

SUPPLEMENT METHODS:

Ethics and Funding:

The study population and KHLS study design have been previously described, while the study conformed to the STROBE guidelines (28,31). The study was approved by both the Dasman Diabetes Institute Ethical Committee in Kuwait, and The Forsyth Institute (USA). Informed Consent (in Arabic) was taken from parents/guardians before enrollment and assent were taken from children before the time of their examination.

Bioinformatics:

Raw 16S-rRNA paired-end sequence reads were merged, de-multiplexed, denoised, and quality-filtered with the DADA2 algorithms in the QIIME2 package (25,26)**.** During our quality-control step, we eliminated 13 unique samples that had poor sequencing depth, their corresponding observations from either visit were also deleted; and left with 134 samples for analysis (Supplement Figure-1). We BLASTN searched against four 16s-rRNA reference sequence databases: Human microbial taxa (HMT) RefSeq V15.1, human oral microbial database (eHOMD) RefSeq Extended V1.11, GreenGeneGold V1, and NCBI 16s-rRNA reference library (37). Reads matched with single species at a matching criterion of >= 98% were pooled in the OTU (operational-taxonomy-unit) table (38). As part of the quality-control step, reads matched with multiple species, and un-matched reads were assessed for the possibility of chimera, using

USEARCH. Chimeric-reads, short-reads, singletons, and read matches with <98% coverage were removed. Later, the non-chimeric reads matched with multiple species, and non-chimeric, unmatched reads were incorporated in the OTU table. The latter were clustered into OTU's, with closest species assigned – could be potential novel species. High-quality amplicon-sequence-variants (ASVs – much better than conventional de-novo OTUs) were generated and subjected to taxonomy assignment. Using MicrobiomeAnalyst, samples were rarefied to even sequencing depth based on the sample having the lowest sequencing depth, later for variance stabilization, relative log-transformation was employed.

Alpha and Beta Diversity analysis:

For community-profiling, we performed alpha-diversity analysis (32) using three different diversity metrics- Observed OTU's, Shannon, and Simpson. We investigated whether oral-microbial diversity and richness are influenced concerning the change in visit and changes in host phenotype (healthy, overweight, or obese) (Supplementary Figure 1). To assess similarities in microbial profiles, the beta-diversity analysis was done using the Bray-Curtis similarity metric. Non-metric multidimensional scaling (NMDS) plot was used to visualize similarities in microbial profiles (see also Supplementary Figure 2).

Longitudinal Analysis:

In investigating the influence of overweight and obese phenotype in the transition from healthy, or overweight status amongst the two visits, we also adjusted for the effect of unmeasured confounding introduced by visit-2 (which accounts for the increase in age by two-years, the effect of growth and puberty related changes, changes in dietary patterns, and differential increase in BMI, waist circumference, and blood pressure). Other changes that were attributed to change in between the two study visits, were adjusted for effect modifiers. Referring to Table-1, variables that changed drastically with the visit, and significantly differed concerning host phenotype were termed as effect modifiers and adjusted in the longitudinal model to investigate whether the changes in microbial profile as evident by visit, were introduced due to effect modifiers). Effect modifiers include gingivitis, and systemic effect of inflammatory/metabolic factors including high CRP, high insulin, low HDLC, and high salivary glucose concentration.

The longitudinal model was used to investigate associations of change in the oral microbiome (at phylum-level) with phenotype, visit, gingivitis, or systemic inflammatory/metabolic markers. Given that there is a low sample size within each cohort, the effect size for estimation of systemic markers is very small, we were underpowered to study the role of effect-modifiers using longitudinal analysis at the genus-level. Moreover, it could have raised the risk of getting a false positive association. Therefore, in doing longitudinal analysis at phylum-level we cautiously cross-referenced each significant finding with results from the established microbial biomarker discovery tool (LEFSE analysis).

The interpretation of the beta estimates from a longitudinal model.1 (see also Supplementary Table-1) shown below is as follows. β_0 (model intercept) refers to healthy at baseline, β_1 refers to estimate the change in Y amongst overweight subjects relative to healthy subjects; β_2 refers to estimate the change in Y at visit-2, compared to visit-1; β_3 refers to estimate the change in Y in overweight subjects (relative to healthy subjects) at visit-2.

For example, the interpretation of a longitudinal model with CRP as an effect modifier (shown below – Model.2) is as follows. β_0 (model intercept) refers to healthy at baseline, β_1 refers to estimate the change in Y amongst overweight subjects relative to healthy subjects; β_2 refers to estimate the change in Y at visit-2, compared to visit-1; β_3 refers to estimate the change in Y with high-CRP (relative to normal CRP); β_4 refers to estimate the change in Y in overweight subjects (relative to healthy subjects) at visit-2; β_5 refers to estimate change in Y in overweight (relative to healthy) subjects with high-CRP; β_6 refers to estimate the change in Y in visit-2 (relative to visit-1), with high CRP. Key significant results (including estimates and 95% CI) from the longitudinal analysis (Supplementary Tables 1-3) have been presented in the forest plots (Manuscript Figure-2).

Model.1: $Y = (\beta\_0)+(\beta\_1*\text{Overweight})+(\beta\_2*\text{Visit})+(\beta\_3*\text{Overweight*Visit})+e$

Model.2: $Y = (\beta\_0)+(\beta\_1*\text{Overweight})+(\beta\_2*\text{Visit})+(\beta\_3*\text{ CRP})+(\beta\_4*\text{Overweight*Visit})+(\beta\_5*\text{Overweight* CRP})+(\beta\_6*\text{Visit* CRP})+e$

SUPPLEMENT RESULTS AND DISCUSSION:

Numerous studies have characterized the gut-microbiota in obese and non-obese subjects, while emphasis has been laid on how obese-phenotype influences microbial-dysbiosis. A comprehensive characterization of the oral microbiome in parallel with the course of obesity onset has not been explored. One of the inherent limitations, however, is that we only sampled at two-time points. Therefore, we could not study the longitudinal development of obesity in the same individuals at three different stages of the development of obesity: healthy, overweight, and obese states. To address oral microbial changes in the three phenotypes involved in the development of obesity, given the inherent limitations in our study design, we created three cohorts to compare health-vs-overweight, overweight-vs-obese, and healthy-vs-obese. Assuming, that the healthy-vs-overweight cohort (I) if followed overtime until the development of obesity, will follow similar changes as observed in the overweight-vs-obese cohort (II). Under this assumption, it allowed us to identify oral microbial markers associated with healthy, overweight, and obese phenotypes. Besides, it also enabled us to identify changes in systemic pathways specific to the three cohorts and adjust for these effect modifiers in our longitudinal analysis.

Alpha Diversity comparison with changes in host-phenotype: We observed a marked reduction in alpha diversity from baseline to visit-2. Besides, the obese phenotype demonstrated a decrease in median alpha-diversity compared to their healthy and overweight counterpart (Refer to Supplement Figure 1). The diversity decreases from visit-1 to visit-2, irrespective of phenotype. But the median diversity was relatively lower in overweight phenotype compared to

healthy phenotype (Supplement Fig. 1a). Besides, the median diversity increased in obese phenotype relative to overweight, in both study visits (Supplement Fig. 1b). But the median diversity in obese phenotype was lower, compared to healthy phenotype, as observed in both study visits (Supplement Fig. 1c).

Beta Diversity comparison with changes in host-phenotype: We compared beta diversity amongst healthy-vs-overweight (Supplement Fig. 2a), overweight-vs-obese (Supplement Fig. 2b), and healthy-vs-obese cohorts (Supplement Fig. 2c). In general, we observed a clear separation between microbial profiles from visit-1 and 2. We also observed that within visit-1, healthy and overweight, overweight and obese, and healthy and obese phenotypes demonstrated significant separation between their profiles (as shown by the interaction between green and pink ellipsoids).

Identification of systemic effect-modifiers:

In obese subjects, the compositional changes in oral-Actinobacteria differed drastically in the presence of high insulin, and low HDLC. A higher relative abundance was observed in obese subjects with high salivary insulin levels, and low relative abundance was observed in obese subjects with low salivary HDLC concentration (Fig. 2a). Similarly, the presence of gingivitis, low HDLC, and high insulin levels, served as an effect modifier for modulating the relative abundance of oral-Bacteroidetes in obese subjects (Fig. 2b). These observations indicate that gingivitis and metabolic factors (such as high insulin, and low HDLC) are phenotype-specific, effect-modifiers, and play a key role in shaping the oral microbiota with the development of adolescent obesity.

Changes attributed with the effect of visit: The oral microbial changes associated with the effect of visit-2, could be attributed to time-varying changes within the two-year interval with the onset of puberty, along with an increase in age, blood pressure, waist circumference, BMI, changes in dietary patterns, tooth eruption, and other unmeasured/unknown confounders. To illustrate, with alpha diversity analysis, we observed a loss of microbial diversity in visit-2, relative to baseline. Given that there was a strong influence of visits on microbial changes between the two study visits, we treated the variable, 'visit' as an effect modifier, and adjusted in the longitudinal model. The LEFSE analysis allowed us to identify microbial predictors specific to host-phenotype on a cross-sectional basis. But, with LEFSE we could not account for time-varying changes or changes introduced due to systemic inflammatory and metabolic factors, hence we used longitudinal analysis to adjust for the above-mentioned effect modifiers. This approach allowed us to identify the association between the oral microbial predictors with the development of overweight and obese phenotypes when adjusted for the effect of visit-related effect modifiers. In summary, we identified that inflammatory factors such as high salivary CRP and presence of gingivitis; and metabolic factors such as the high salivary concentration of glucose, insulin, and low HDLC levels demonstrated evidence of effect modification specific to each phenotype. Henceforth, we treated the cumulative effect of visit-2, as an effect modifier, and adjusted in the longitudinal analysis. In our analysis, we compared two statistical approaches to identify microbial predictors concerning the host phenotype – LEFSE, and Longitudinal analysis.

SUPPLEMENT TABLES

Supplement -Table 1: Longitudinal Model showing changes in Oral Microbial Phyla with the effect of overweight status, visit, systemic inflammatory/ metabolic changes, and gingivitis amongst **HEALTHY vs. OVERWEIGHT- (OVER) adolescents** (Cohort-I: N=29 subjects/ 58 samples).

LEGEND: [1]The microbial profiles (at phylum-level) have been log-transformed and treated as the outcome variable (Y) in the Longitudinal model (**The model equation** is shown below). The changes in microbial profile have been documented concerning healthy/overweight status, baseline/visit-2 status, combined effect of being overweight and visit-2 (interaction term), along with the binary effect of changes in confounding variables (X): high CRP, high Insulin, low HDLC, high Glucose, presence of Gingivitis (Models 2-6). To estimate and adjust for the effect of other confounding variables (Variable X), our model included: the individual effect of X (representing healthy individuals at baseline), the effect of X in Overweight (relative to healthy subjects), and the effect of X at visit-2 (relative to visit-1) as shown in the longitudinal model equation below. Significant p-values$\leq$0.05 are in bold. The significant p-value for the interaction term indicates that the confounding variable for interaction is an effect-modifier.

Model.1: $Y = (\beta\_0)+(\beta\_1*\text{Overweight})+(\beta\_2*\text{Visit2})+(\beta\_3*\text{Overweight*Visit2})+e$
Model equation: $Y = (\beta\_0)+(\beta\_1*\text{ Overweight})+(\beta\_2*\text{Visit2})+(\beta\_3*\text{ X})+(\beta\_4*\text{ Overweight*Visit2})+(\beta\_5*\text{Overweight*X})+(\beta\_6*\text{Visi2t* X})+e$
Model.2: $Y = (\beta\_0)+(\beta\_1*\text{Overweight})+(\beta\_2*\text{Visit2})+(\beta\_3*\text{ CRP})+(\beta\_4*\text{Overweight*Visit2})+(\beta\_5*\text{Overweight* CRP})+(\beta\_6*\text{Visit2* CRP})+e$
Model.3: $Y = (\beta\_0)+(\beta\_1*\text{Overweight})+(\beta\_2*\text{Visit2})+(\beta\_3*\text{ Insulin})+(\beta\_4*\text{Overweight*Visit2})+(\beta\_5*\text{Overweight* Insulin})+(\beta\_6*\text{Visit2* Insulin})+e$
Model.4: $Y = (\beta\_0)+(\beta\_1*\text{Overweight})+(\beta\_2*\text{Visit2})+(\beta\_3*\text{ Glucose})+(\beta\_4*\text{Overweight*Visit2})+(\beta\_5*\text{Overweight* Glucose})+(\beta\_6*\text{Visit2* Glucose})+e$
Model.5: $Y = (\beta\_0)+(\beta\_1*\text{Overweight})+(\beta\_2*\text{Visit2})+(\beta\_3*\text{ low HDLC})+(\beta\_4*\text{Overweight*Visit2})+(\beta\_5*\text{Overweight* low HDLC})+(\beta\_6*\text{Visit2* low HDLC})+e$
Model.6: $Y = (\beta\_0)+(\beta\_1*\text{Overweight})+(\beta\_2*\text{Visit2})+(\beta\_3*\text{ Gingivitis})+(\beta\_4*\text{Overweight*Visit2})+(\beta\_5*\text{Overweight* Gingivitis})+(\beta\_6*\text{Visit2* Gingivitis})+e$

S-Table – 1: **Longitudinal Analysis comparing changes in the bacterial phylum, in comparison to Healthy vs. Overweight phenotype**

Effect of Overweight and 2-year FU (Visit-2)				Effect of systemic Inflammatory & Metabolic factors												Gingivitis (Gg)		
	Model.1			Model.2: CRP			Model.3: Insulin (I)			Model.4: Glucose (G)			Model.5: HDLC (H)			Model.6: Gingivitis (Gg)		
Phyla[1]	Over	Visit2	Over *Visit 2	CRP	Over * CRP	Visit2 * CRP	I	Over*I	Visit2 *I	G	Over* G	Visit2 * G	Low H	Over * Low H	Visit2* Low H	Gg	Over * Gg	Visit2* Gg
Actinobacteria																		
estimate	-0.05	**-6.59**	0.40	0.10	-0.13	-0.68	0.22	-0.12	0.53	0.30	-0.18	0.19	0.66	-0.11	-0.17	0.10	0.38	-0.34
(SE)	(0.19)	(0.37)	(0.63)	(0.19)	(0.47)	(0.74)	(0.19)	(0.38)	(0.71)	(0.22)	(0.39)	(0.74)	(1.29)	(1.19)	(1.10)	(0.22)	(0.41)	(0.67)
p-value	0.81	**<0.01**	0.52	0.59	0.78	0.36	0.26	0.76	0.46	0.18	0.65	0.87	0.61	0.93	0.88	0.66	0.36	0.61
Bacteroidetes																		
estimate	**0.26**	**0.43**	**-0.38**	0.02	0.44	0.10	-0.001	-0.15	-0.06	**-0.36**	**0.49**	-0.34	-0.04	-0.29	-0.19	**-0.34**	0.40	0.30
(SE)	(0.13)	(0.08)	(0.16)	(0.11)	(0.27)	(0.20)	(0.12)	(0.19)	(0.23)	(0.13)	(0.19)	(0.20)	(0.38)	(0.26)	(0.35)	(0.12)	(0.19)	(0.19)
p-value	**0.05**	**<0.01**	**0.02**	0.85	0.11	0.62	0.99	0.42	0.81	**0.01**	**0.01**	0.10	0.91	0.27	0.60	**0.01**	**0.04**	0.11
Firmicutes																		
estimate	-0.01	-0.05	0.04	-0.01	0.02	0.06	0.02	0.10	0.03	0.002	-0.06	0.20	-0.39	**0.29**	0.38	0.12	-0.19	-0.13
(SE)	(0.07)	(0.05)	(0.09)	(0.07)	(0.15)	(0.12)	(0.07)	(0.11)	(0.12)	(0.08)	(0.12)	(0.12)	(0.22)	(0.14)	(0.20)	(0.07)	(0.11)	(0.11)
p-value	0.86	0.28	0.66	0.88	0.91	0.63	0.75	0.37	0.80	0.98	0.62	0.10	0.08	**0.05**	0.07	0.10	0.10	0.26
Fusobacteria																		
estimate	-0.27	-1.17	0.52	-0.09	0.83	-0.61	-0.004	-0.61	-0.32	-0.17	0.83	0.08	-0.18	0.21	-0.14	-0.004	-0.35	0.28
(SE)	(0.29)	(0.45)	(0.76)	(0.27)	(0.79)	(0.97)	(0.28)	(0.53)	(1.09)	0.33	0.58	(1.02)	1.83	(1.71)	(1.56)	(0.33)	(0.61)	(0.97)
p-value	0.36	**0.01**	0.50	0.74	0.30	0.53	0.99	0.25	0.77	0.61	0.16	0.94	0.92	0.90	0.93	0.99	0.58	0.77
Proteobacteria																		
estimate	-0.05	0.34	0.30	0.27	-1.31	0.03	0.85	-0.51	-0.45	-0.17	-0.28	0.46	0.76	-0.43	-0.10	0.11	-0.67	0.32
(SE)	(0.62)	(0.28)	(0.56)	(0.51)	(0.77)	0.66	(0.52)	(0.56)	(0.72)	(0.62)	(0.66)	(0.68)	(1.59)	0.61	(1.56)	(0.59)	(0.63)	(0.70)
p-value	0.93	0.23	0.59	0.59	0.09	0.97	0.11	0.37	0.53	0.78	0.67	0.50	0.64	0.49	0.95	0.86	0.29	0.66
Saccharibacteria-TM7																		
estimate	0.18	0.16	-0.98	0.45	0.86	-0.20	-0.41	-0.63	0.03	-0.48	0.15	0.59	0.53	0.14	-0.09	0.17	-0.11	0.52
(SE)	(0.55)	(0.44)	(0.80)	(0.53)	(1.21)	(1.01)	(0.53)	(0.90)	(1.08)	(0.63)	(0.99)	(1.06)	(2.00)	(1.48)	(1.82)	(0.62)	(1.00)	(0.99)
p-value	0.75	0.71	0.23	0.40	0.48	0.84	0.45	0.49	0.98	0.45	0.88	0.58	0.79	0.93	0.96	0.78	0.91	0.60
Firmicutes-to-Bacteroidetes ratio																		
estimate	-0.03	**-0.06**	**0.05**	-0.01	-0.03	-0.01	-0.001	0.03	0.01	**0.05**	**-0.07**	**0.08**	-0.04	0.07	0.06	**0.06**	**-0.08**	-0.05
(SE)	(0.02)	(0.02)	(0.03)	(0.02)	(0.04)	(0.04)	(0.02)	(0.03)	(0.04)	(0.02)	(0.03)	(0.03)	(0.07)	(0.04)	(0.06)	(0.02)	(0.03)	(0.03)
p-value	0.16	**<0.01**	0.06	0.73	0.43	0.73	0.98	0.42	0.72	**0.04**	**0.03**	**0.02**	0.60	0.11	0.32	**0.01**	**0.02**	0.15

Supplement-Table 2: Longitudinal Model showing changes in Oral Microbial Phyla with the effect of obesity (OBE) status, visit, systemic inflammatory/ metabolic changes, and gingivitis amongst **OVERWEIGHT- (OVER) vs. OBESE (OBE) adolescents** (Cohort-II: N=39 subjects/ 78 samples)

LEGEND: The microbial profiles (at phylum-level) have been log-transformed and treated as the outcome variable (Y) in the Longitudinal linear mixed effect model (**The model equation** is shown below). The changes in microbial profile have been documented concerning overweight/obese status, baseline/visit-2 status, combined effect of obesity and visit-2, along with the binary effect of changes in confounding variables (X): high CRP, high Insulin, low HDLC, high Glucose, presence of Gingivitis (Models 2-6). For the effect of other confounding variables (Variable X) we estimated and adjusted for the individual effect of X (for obese subjects at baseline), the interaction of variable-X and Obesity (represented as X*Obesity), and interaction of X with visit-2 (X*Visit) as shown in the longitudinal model equation below. In the longitudinal model, the variables are reference coded in a binary format, in case of obesity variable: '0' is treated as a reference which refers to overweight, while '1' means obese. Significant p-values≤0.05 are in bold. The significant p-value for the interaction term indicates that the confounding variable for interaction is an effect-modifier.

Model.1: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{Obese*Visit})+e$
Model equation: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{X})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese*X})+(\beta\_6 * \text{Visit* X})+e$
Model.2: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{CRP})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese*CRP})+(\beta\_6 * \text{Visit* CRP})+e$
Model.3: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{Insulin})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese* Insulin})+(\beta\_6 * \text{Visit* Insulin})+e$
Model.4: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{Glucose})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese* Glucose})+(\beta\_6 * \text{Visit* Glucose})+e$
Model.5: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{low HDLC})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese* low HDLC})+(\beta\_6 * \text{Visit* low HDLC})+e$
Model.6: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{Gingivitis})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese* Gingivitis})+(\beta\_6 * \text{Visit* Gingivitis})+e$

S-Table – 2: **Longitudinal Analysis comparing changes in the bacterial phylum, in comparison to Overweight vs. Obese phenotype**

Effect of Obesity and Visit-2 (two-year FU)				Effect of systemic Inflammatory & Metabolic pathways												Gingivitis (Gg)		
	Model.1			Model.2: CRP			Model.3: Insulin (I)			Model.4: HDLC (H)			Model.5: Glucose (G)			Model.6: Gingivitis (Gg)		
Phyla	OBE	Visit2	OBE* Visit2	CRP	OBE* CRP	Visit2 * CRP	I	OBE* I	Visit2 *I	Low H	OBE* Low H	Visit2 * Low H	G	OBE* G	Visit2 * G	Gg	OBE* Gg	Visit2* Gg
Actinobacteria																		
estimate	-0.22	-6.86	0.81	-0.09	-0.53	0.81	-0.02	1.30	-0.41	-0.13	-3.79	0.22	0.21	0.18	1.04	0.65	-0.82	0.35
(SE)	(0.21)	(0.66)	(0.75)	(0.17)	(0.41)	(0.72)	(0.16)	(0.49)	(0.72)	(0.29)	(1.80)	(1.59)	(0.20)	(0.44)	(0.73)	(0.19)	(0.51)	(0.82)
p-value	0.31	**<0.01**	0.29	0.59	0.20	0.27	0.89	**0.01**	0.57	0.65	**0.04**	0.89	0.29	0.68	0.16	**<0.01**	0.11	0.67
Bacteroidetes																		
estimate	-0.08	0.05	0.43	0.01	-2e-3	-0.14	-0.02	-0.47	0.19	0.08	0.86	-0.54	-0.10	0.16	-0.42	-0.21	0.79	-0.55
(SE)	(0.13)	(0.14)	(0.19)	(0.11)	(0.23)	(0.23)	(0.10)	(0.22)	(0.23)	(0.18)	(0.41)	(0.40)	0.12	(0.21)	(0.21)	(0.13)	(0.34)	(0.33)
p-value	0.54	0.71	**0.03**	0.92	0.99	0.54	0.81	**0.04**	0.42	0.67	**0.04**	0.18	0.43	0.44	**0.04**	0.11	**0.02**	0.11
Firmicutes																		
estimate	0.16	0.002	-0.19	0.04	0.09	-0.17	0.02	-0.12	0.24	-0.04	-0.12	0.33	0.01	-0.16	0.17	-0.02	-0.29	0.28
(SE)	(0.07)	(0.09)	(0.11)	(0.06)	(0.12)	(0.13)	(0.05)	(0.13)	(0.14)	(0.09)	(0.27)	(0.25)	(0.06)	(0.12)	(0.13)	(0.07)	(0.18)	(0.18)
p-value	**0.02**	0.98	0.09	0.44	0.44	0.21	0.65	0.35	0.09	0.66	0.68	0.20	0.87	0.22	0.19	0.71	0.11	0.13
Fusobacteria																		
estimate	0.49	-0.77	-0.55	-0.09	0.02	-1.00	-0.26	0.15	0.65	-0.43	0.28	-1.02	-0.41	0.48	0.32	0.12	-0.22	0.44
(SE)	(0.31)	(0.79)	(0.91)	(0.25)	(0.61)	(0.89)	(0.24)	(0.73)	(0.94)	(0.42)	(2.58)	(2.29)	(0.29)	(0.63)	(0.95)	(0.32)	(0.84)	(1.11)
p-value	0.11	0.33	0.54	0.72	0.97	0.26	0.29	0.83	0.49	0.30	0.91	0.66	0.16	0.44	0.74	0.71	0.79	0.69
Proteobacteria																		
estimate	-0.12	0.64	-0.60	0.02	0.01	0.09	0.17	-0.40	0.16	0.26	-0.59	-0.35	0.04	-0.11	0.41	-0.08	0.41	0.05
(SE)	(0.22)	(0.24)	(0.32)	(0.19)	(0.40)	(0.41)	(0.17)	(0.39)	(0.45)	(0.29)	(0.79)	(0.73)	(0.20)	(0.38)	(0.38)	(0.22)	(0.56)	(0.56)
p-value	0.59	**0.01**	0.07	0.90	0.98	0.82	0.31	0.32	0.72	0.37	0.45	0.63	0.86	0.78	0.28	0.72	0.47	0.93
Saccharibacteria-TM7																		
estimate	-0.49	-1.09	1.50	0.08	-0.66	0.56	-0.17	0.78	-0.93	0.20	-0.96	1.45	-0.26	-0.73	0.01	-0.69	1.48	0.08
(SE)	(0.51)	(0.61)	(0.81)	(0.43)	(0.94)	(0.98)	(0.41)	(0.98)	(1.03)	(0.71)	(1.97)	(1.84)	(0.48)	(0.90)	(0.91)	(0.52)	(1.37)	(1.40)
p-value	0.34	0.08	0.07	0.85	0.48	0.57	0.67	0.43	0.37	0.77	0.63	0.44	0.59	0.42	0.99	0.19	0.29	0.96
Firmicutes-to-Bacteroidetes																		
estimate	0.03	-0.01	**-0.07**	0.01	0.004	-3e-3	0.01	0.05	-2e-3	-0.01	-0.13	0.11	0.02	0.04	**0.08**	0.02	**-0.14**	0.10
(SE)	(0.02)	(0.02)	(0.03)	(0.02)	(0.04)	(0.04)	(0.02)	(0.04)	(0.04)	(0.03)	(0.08)	(0.07)	(0.02)	(0.04)	(0.04)	(0.02)	(0.05)	(0.05)
p-value	0.19	0.73	**0.03**	0.72	0.91	0.99	0.74	0.16	0.97	0.62	0.09	0.14	0.35	0.24	**0.03**	0.26	**0.01**	**0.06**

Supplement-Table 3: Longitudinal Model showing changes in Oral Microbial Phyla with the effect of obesity (OB) status, visit, systemic inflammatory/ metabolic changes, and gingivitis amongst **HEALTHY vs. OBESE (OBE) adolescents** (Cohort-III: N=31 subjects/ 62 samples)

LEGEND: HDLC cannot be adjusted in this model, in this population we are comparing healthy individuals who either remained healthy or became obese; essentially all visit-1 healthy/obese subjects had normal salivary HDLC levels (>=0.6mg/dL). The microbial profiles (at phylum-level) have been log-transformed and treated as the outcome variable (Y) in the Longitudinal linear mixed effect model (the equation is shown below). The changes in microbial profile have been documented concerning healthy and obese status, baseline/visit-2 status, the combined effect of obesity, and visit-2, along with the binary effect (low/high) of changes in confounding variables (X): CRP, Insulin, Glucose, Gingivitis. For the effect of other confounding variables (Variable X), we estimated and adjusted for the individual effect of X (for overweight subjects at baseline), the interaction of X: Obesity, and interaction of X with visit-2 as shown in the longitudinal model equation below. Significant p-values≤0.05 are in bold. The significant p-value for the interaction term indicates that the confounding variable for interaction is an effect-modifier.

Model.1: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{Obese*Visit})+e$
Model equation: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{X})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese*X})+(\beta\_6 * \text{Visit*X})+e$
Model.2: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{CRP})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese*CRP})+(\beta\_6 * \text{Visit*CRP})+e$
Model.3: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{Insulin})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese*Insulin})+(\beta\_6 * \text{Visit*Insulin})+e$
Model.4: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{Glucose})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese*Glucose})+(\beta\_6 * \text{Visit*Glucose})+e$
Model.5: $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{Gingivitis})+(\beta\_4 * \text{Obese*Visit})+(\beta\_5 * \text{Obese*Gingivitis})+(\beta\_6 * \text{Visit*Gingivitis})+e$

S-Table – 3: **Longitudinal Analysis comparing changes in the bacterial phylum, in comparison to Healthy vs. Obese phenotype**

Effect of Obesity and Visit-2 (two-year FU)				Effect of Systemic Inflammatory & Metabolic pathways									Gingivitis		
	Model.1			Model.2: CRP			Model.3: Insulin (I)			Model.4: Glucose (G)			Model.5: Gingivitis (Gg)		
Phyla	OBE	Visit2	OBE * Visit2	CRP	OBE* CRP	Visit2 *CRP	Insulin	OBE* Insulin	Visit2 * Insulin	Glucose	OBE* Glucose	Visit2* Glucose	Gg	OBE* Gg	Visit2* Gg
Actinobacteria															
estimate	-0.20	**-6.54**	-0.08	0.08	-0.58	-0.02	0.13	1.03	-0.88	0.25	0.08	-0.56	-0.05	0.02	-0.14
(SE)	(0.21)	(0.25)	(0.42)	(0.18)	(0.38)	(0.5)	(0.17)	(0.44)	(0.53)	(0.20)	(0.41)	(0.58)	(0.20)	(0.46)	(0.47)
p-value	0.34	**<0.01**	0.85	0.65	0.13	0.97	0.44	0.02	0.10	0.22	0.85	0.34	0.81	0.96	0.77
Bacteroidetes															
estimate	-0.08	**0.39**	0.06	-0.04	0.01	0.11	0.08	-0.37	-0.16	0.22	0.11	**-0.43**	-0.04	0.27	-0.25
(SE)	(0.13)	(0.09)	(0.18)	(0.11)	(0.21)	(0.22)	(0.11)	(0.23)	(0.25)	(0.13)	(0.23)	(0.22)	(0.12)	(0.23)	(0.22)
p-value	0.55	**<0.01**	0.73	0.71	0.98	0.63	0.46	0.11	0.53	0.09	0.64	0.06	0.73	0.26	0.25
Firmicutes															
estimate	0.16	-0.05	**-0.20**	0.05	0.07	-0.17	-0.01	0.002	0.09	-0.05	-0.14	0.25	0.002	**-0.25**	0.05
(SE)	(0.08)	(0.06)	(0.10)	(0.07)	(0.12)	(0.13)	(0.07)	(0.14)	(0.15)	(0.07)	(0.14)	(0.14)	(0.07)	(0.13)	(0.12)
p-value	0.04	0.40	0.06	0.44	0.58	0.18	0.91	0.99	0.54	0.48	0.32	0.09	0.98	0.07	0.66
Fusobacteria															
estimate	-0.04	-1.16	-0.74	**-0.51**	0.21	1.55	0.44	-0.67	0.77	0.29	0.14	0.07	0.28	-0.22	1.23
(SE)	(0.27)	(0.46)	(0.76)	(0.22)	(0.48)	(0.91)	(0.23)	(0.63)	(1.01)	(0.26)	(0.56)	(1.11)	(0.25)	(0.64)	(0.85)
p-value	0.88	0.01	0.34	**0.03**	0.67	0.10	0.06	0.29	0.45	0.28	0.80	0.95	0.27	0.74	0.15
Proteobacteria															
estimate	0.52	0.46	-0.39	0.45	-0.35	-0.06	0.24	-0.68	0.33	-0.52	0.27	0.92	-0.27	0.35	0.74
(SE)	(0.60)	(0.26)	(0.58)	(0.46)	(0.59)	(0.63)	(0.49)	(0.64)	(0.74)	(0.54)	(0.75)	(0.67)	(0.52)	(0.60)	(0.67)
p-value	0.39	0.09	0.50	0.32	0.55	0.93	0.62	0.29	0.66	0.34	0.73	0.17	0.60	0.56	0.28
Saccharibacteria-TM7															
estimate	-1.03	0.02	0.29	0.35	0.70	-1.65	-0.38	-1.16	0.67	-0.02	-0.63	-0.50	0.27	0.45	0.05
(SE)	(0.45)	(0.33)	(0.59)	(0.35)	(0.63)	(0.75)	(0.37)	(0.79)	(0.88)	(0.43)	(0.86)	(0.86)	(0.40)	(0.82)	(0.78)
p-value	0.03	0.95	0.63	0.32	0.28	0.03	0.31	0.15	0.45	0.96	0.46	0.56	0.51	0.59	0.95
Firmicutes-to-Bacteroidetes ratio															
estimate	0.03	**-0.05**	-0.03	0.01	0.01	-0.04	-0.02	0.06	0.03	0.02	-0.04	**0.12**	0.003	-0.06	0.04
(SE)	(0.02)	(0.02)	(0.03)	(0.02)	(0.04)	(0.03)	(0.02)	(0.04)	(0.04)	(0.02)	(0.04)	(0.04)	(0.02)	(0.04)	(0.04)
p-value	0.28	**<0.01**	0.37	0.56	0.71	0.27	0.41	0.19	0.48	0.39	0.33	**<0.01**	0.87	0.17	0.27

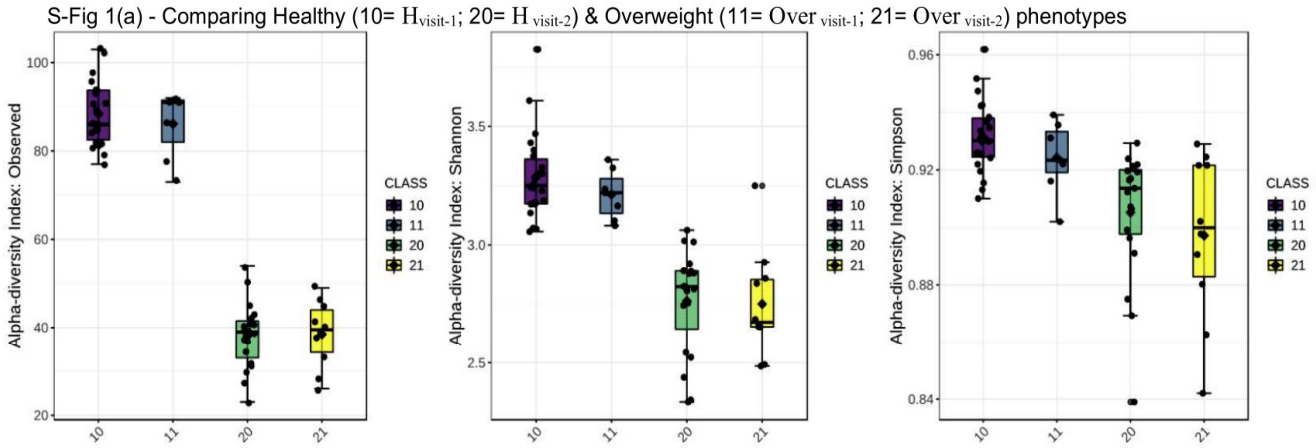
Supplement -Table 4: Using Longitudinal Model to compare three cohorts to identify changes in oral microbial genera with the individual effect of overweight (Over) or obesity (OBE) status, visit, and the combined effect of visit and overweight (Visit*Over), and visit and obese (Visit*OBE).

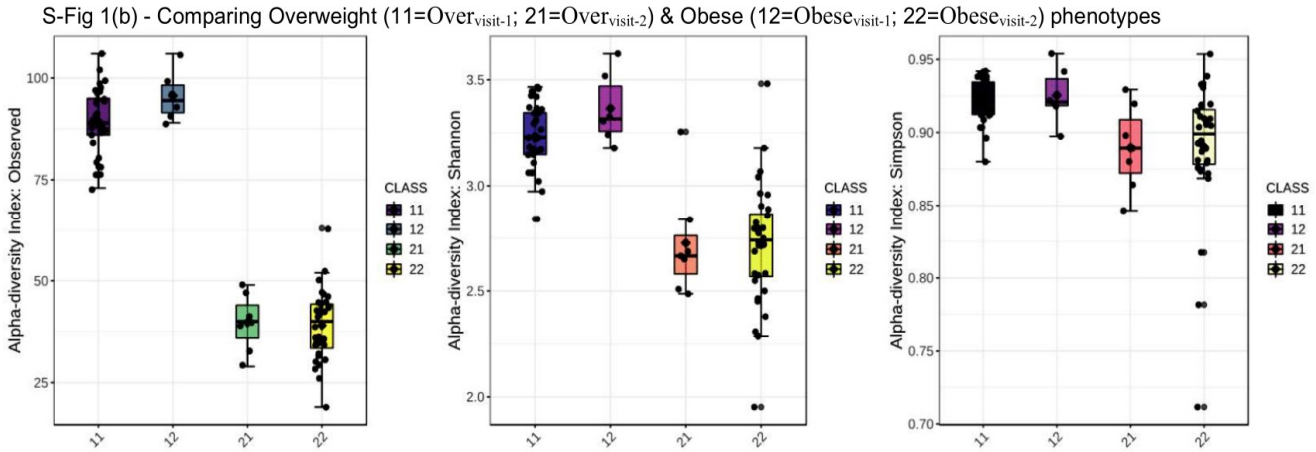
S- Table 4	Healthy & Overweight- OVER (n=29/ 58 samples)						Overweight vs. Obese - OBE (n=39/ 78 samples)						Healthy & Obese- OBE (n=31/ 62 samples)					
Genus	Over	p	Visit2	p	Visit2* Over	p	OBE	p	Visit2	p	Visit2* OBE	p	OBE	p	Visit2	p	Visit2* OBE	p
Abiotrophia	-2.2E-03	0.16	-1.8E-03	0.14	-6.3E-04	0.77	1.3E-03	0.36	-3.4E-03	0.16	-6.7E-04	0.82	1.0E-03	0.55	-1.8E-03	0.16	-3.0E-03	0.20
Actinomyces	-2.2E-03	0.16	-1.8E-03	0.14	-6.3E-04	0.77	1.3E-03	0.36	-3.4E-03	0.16	-6.7E-04	0.82	1.0E-03	0.55	-1.8E-03	0.16	-3.0E-03	0.20
Aggregatibacter	-3.6E-03	0.35	1.0E-03	0.87	8.7E-03	0.40	4.2E-03	0.08	-3.1E-04	0.94	-4.7E-03	0.32	1.7E-03	0.61	5.9E-04	0.83	-6.3E-03	0.19
Alloprevotella	-8.4E-03	0.15	1.1E-02	0.19	1.7E-02	0.26	6.5E-03	0.26	3.4E-02	**<0.01**	-3.3E-02	**0.01**	6.6E-03	0.39	1.1E-02	0.11	-8.1E-03	0.50
Atopobium	-2.9E-05	0.99	-7.0E-03	**0.02**	2.4E-03	0.65	-1.7E-03	0.44	-6.3E-03	**<0.01**	2.2E-03	0.34	-4.7E-03	0.26	-7.4E-03	**<0.0**	3.3E-03	0.46
Bergeyella	-9.7E-04	0.25	-6.6E-04	0.45	2.7E-03	0.09	-7.0E-04	0.41	3.1E-03	**0.03**	-3.1E-03	0.07	-3.3E-04	0.58	-3.5E-04	0.69	2.0E-03	0.19
Butyrivibrio	-9.7E-04	0.25	-6.6E-04	0.45	2.7E-03	0.09	2.2E-04	0.73	-6.0E-04	0.23	-1.8E-04	0.82	4.7E-04	0.12	1.3E-04	0.79	-3.7E-04	0.65
Campylobacter	-2.2E-03	**0.03**	-2.7E-03	**<0.0**	3.6E-03	**<0.01**	2.7E-03	**<0.01**	1.1E-03	0.58	-2.1E-03	0.35	1.0E-03	0.47	-2.5E-03	**0.03**	1.4E-03	0.50
Catonella	-2.5E-04	0.67	-9.3E-04	**0.01**	-7.4E-05	0.91	1.2E-03	**<0.01**	-6.5E-04	**<0.01**	-1.2E-03	**<0.0**	3.4E-04	0.57	-9.1E-04	**0.01**	-4.3E-04	0.54
Corynebacterium	-2.5E-04	0.67	-9.3E-04	**0.01**	-7.4E-05	0.91	1.2E-03	**<0.01**	-6.5E-04	**<0.01**	-1.2E-03	**<0.0**	3.4E-04	0.57	-9.1E-04	**0.01**	-4.3E-04	0.54
Fusobacterium	-2.7E-03	0.77	4.8E-03	0.49	-4.7E-03	0.70	4.4E-04	0.96	3.8E-03	0.61	3.2E-04	0.98	-5.4E-03	0.54	4.4E-03	0.54	-3.7E-03	0.77
Gemella	-8.7E-03	0.28	1.2E-02	0.06	1.6E-02	0.16	4.5E-03	0.51	2.7E-02	**0.01**	-1.6E-02	0.17	7.8E-03	0.34	1.2E-02	**0.03**	-6.8E-03	0.49
Granulicatella	-4.1E-03	0.66	3.6E-03	0.65	-2.4E-03	0.87	9.6E-03	0.17	1.5E-02	0.15	-1.6E-02	0.20	1.8E-02	0.07	7.2E-03	0.36	1.7E-03	0.90
Haemophilus	-4.5E-03	0.77	3.1E-02	**0.04**	-1.8E-03	0.94	-1.8E-02	0.16	5.6E-02	**<0.01**	-3.7E-02	0.07	-1.0E-02	0.52	3.0E-02	**0.01**	-3.7E-03	0.86
Kingella	-2.4E-04	0.30	1.1E-04	0.71	5.6E-04	0.28	-1.2E-04	0.65	4.2E-04	0.35	1.1E-04	0.84	3.0E-05	0.89	3.4E-04	0.46	1.0E-03	0.18
Lachnoanaerobaculum	2.6E-04	0.81	-3.8E-03	**<0.0**	5.5E-04	0.75	5.2E-03	**<0.01**	-3.9E-03	**<0.01**	-4.6E-03	**0.04**	3.8E-03	**0.05**	-4.1E-03	**<0.0**	-5.5E-03	**0.02**
Lachnospiraceae(G-2)	-1.5E-03	0.24	-2.7E-03	**<0.0**	2.1E-03	0.15	9.5E-04	0.50	-2.0E-03	**0.01**	-6.0E-04	0.69	2.1E-04	0.88	-2.7E-03	**<0.0**	-7.6E-05	0.96
Lautropia	-1.1E-04	0.89	-6.6E-04	0.49	2.0E-03	0.23	1.6E-04	0.73	1.5E-03	0.38	-1.1E-03	0.56	-1.3E-05	0.99	-5.5E-04	0.47	7.9E-05	0.95
Leptotrichia	-1.3E-02	0.30	-2.5E-02	**<0.0**	2.2E-02	0.15	1.3E-02	0.16	-1.7E-02	**0.06**	-6.2E-03	0.63	-9.2E-03	0.55	-3.2E-02	**<0.0**	3.0E-03	0.84
Megasphaera	2.6E-03	**0.03**	-6.1E-04	0.55	-2.1E-03	0.25	-1.5E-03	0.27	-3.4E-03	0.20	5.2E-03	0.10	-2.2E-03	0.10	-7.7E-04	0.61	2.9E-03	0.25
Mogibacterium	8.2E-05	0.74	-5.3E-04	**<0.0**	1.2E-04	0.64	2.7E-04	0.29	-2.2E-04	0.55	-3.0E-04	0.52	1.9E-04	0.44	-5.7E-04	**<0.0**	-1.7E-04	0.51
Moraxella	-6.6E-03	0.17	2.4E-03	0.54	6.3E-03	0.36	-1.0E-03	0.60	1.1E-02	**0.01**	-1.0E-02	**0.03**	-1.0E-03	0.74	2.0E-03	0.35	2.3E-05	0.99
Neisseria	-2.5E-02	0.30	1.1E-02	0.44	2.9E-02	0.24	-3.2E-02	0.19	4.7E-02	0.09	-1.1E-02	0.77	-1.1E-02	0.58	1.5E-02	0.37	4.7E-02	0.10
Oribacterium	1.7E-03	0.32	-3.3E-04	0.81	-4.8E-04	0.85	5.2E-03	**0.07**	1.5E-03	0.46	-8.7E-03	**0.01**	4.2E-03	0.20	-6.6E-04	0.68	-7.4E-03	**0.03**

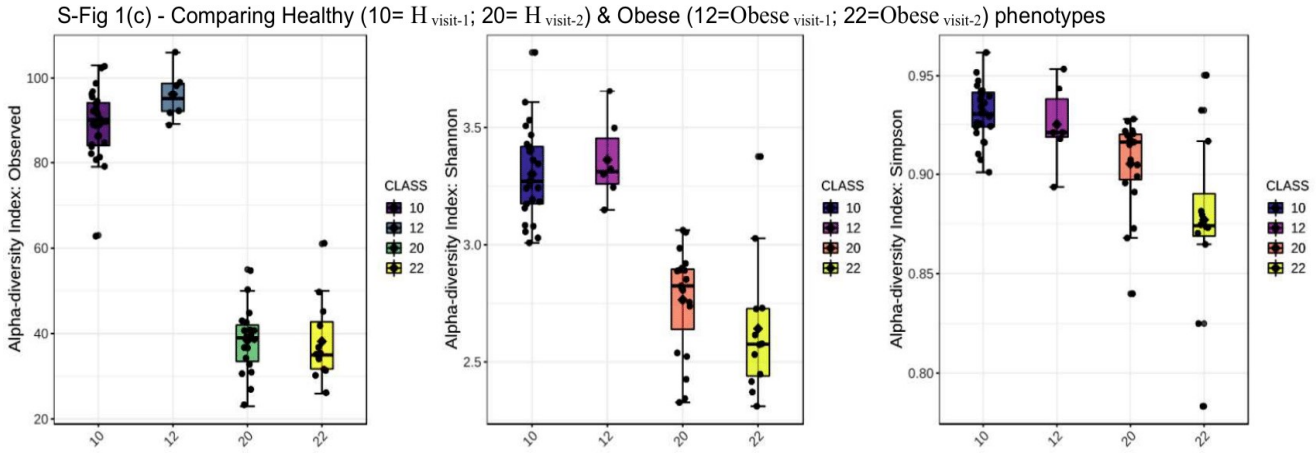
Peptococcus	1.7E-03	0.32	-3.3E-04	0.81	-4.8E-04	0.85	5.2E-03	**0.07**	1.5E-03	0.46	-8.7E-03	**0.01**	4.2E-03	0.20	-6.6E-04	0.68	-7.4E-03	**0.03**
Peptostreptococcus	-1.0E-03	0.64	-3.3E-03	**0.01**	-1.1E-03	0.66	6.9E-03	**0.04**	-5.4E-03	**<0.01**	-6.2E-03	0.09	7.1E-03	**0.04**	-3.1E-03	0.06	-9.1E-03	**0.01**
Porphyromonas	-9.8E-03	0.20	3.6E-03	0.49	6.6E-03	0.47	8.4E-04	0.90	1.3E-02	0.12	-1.6E-02	0.16	1.7E-03	0.83	4.2E-03	0.45	-3.3E-03	0.74
Prevotella	9.1E-02	**0.01**	1.1E-01	**<0.0**	-1.1E-01	**0.03**	-3.1E-02	0.34	-4.6E-02	0.32	2.0E-01	**<0.0**	-3.6E-02	0.21	1.1E-01	**<0.0**	2.1E-02	0.67
Rothia	-7.9E-2	0.6	-1.1E-1	**0.01**	-7.9E-3	0.70	-6.5E-3	0.80	-1.1E-01	**<0.01**	1.7E-02	0.57	-3.6E-02	0.21	1.1E-01	**<0.0**	2.1E-02	0.67
Ruminococcaceae (G1)	-8.0E-04	0.50	-1.6E-03	**0.03**	3.8E-04	0.78	-3.6E-05	0.93	-8.9E-04	0.13	7.9E-04	0.28	-1.1E-03	0.35	-1.5E-03	**0.03**	1.3E-03	0.32
Ruminococcaceae (G2)	-3.8E-04	0.16	-2.8E-04	0.43	6.6E-04	0.29	-1.9E-04	0.53	1.7E-04	0.66	-5.2E-04	0.30	-1.5E-04	0.62	-3.2E-04	0.29	2.7E-04	0.59
Saccharibacteria TM7	-6.7E-06	1.00	3.2E-04	0.74	-2.1E-04	0.91	-1.9E-03	0.25	-3.8E-04	0.74	2.2E-03	0.26	-2.9E-03	0.09	5.3E-05	0.95	1.9E-03	0.27
Selenomonas	-6.7E-06	1.00	3.2E-04	0.74	-2.1E-04	0.91	-1.9E-03	0.25	-3.8E-04	0.74	2.2E-03	0.26	-2.9E-03	0.09	5.3E-05	0.95	1.9E-03	0.27
Solobacterium	-6.7E-06	1.00	3.2E-04	0.74	-2.1E-04	0.91	-1.8E-04	0.93	-5.8E-03	**0.03**	1.9E-03	0.57	-5.9E-04	0.77	-6.7E-03	**<0.0**	1.2E-03	0.56
Stomatobaculum	-6.7E-06	1.00	3.2E-04	0.74	-2.1E-04	0.91	1.2E-03	0.22	-2.2E-03	**<0.01**	-1.1E-03	0.26	-5.9E-04	0.77	-6.7E-03	**<0.0**	1.2E-03	0.56
Streptobacillus	-8.8E-04	0.31	-8.6E-04	0.47	3.8E-03	0.07	9.2E-04	0.20	5.2E-03	**<0.01**	-5.5E-03	**<0.0**	1.8E-04	0.85	-1.8E-04	0.82	3.1E-04	0.80
Streptococcus	-6.1E-03	0.83	-2.8E-02	0.16	5.5E-02	0.14	3.7E-02	0.17	3.6E-02	0.21	-8.2E-02	**0.04**	5.0E-02	0.12	-2.3E-02	0.21	-2.8E-02	0.44
Veillonella	2.1E-02	0.13	1.2E-02	0.23	-3.0E-02	0.08	-6.5E-03	0.49	-3.8E-02	**0.01**	5.2E-02	**0.01**	-2.8E-02	**0.02**	1.3E-02	0.11	-1.0E-02	0.48

Each microbial profile (at genus-level) have been normalized by rank-sum, and log-transformed and treated as the outcome variable (Y) in the Longitudinal linear mixed effect model (**Model.1** equation shown below). The estimated mean change (and p-values) in microbial profile have been documented concerning overweight or obese status, baseline/visit-2 status, the combined effect of obesity, and visit-2, amongst the three study cohorts. The significant p-value for the interaction term indicates that the confounding variable for interaction is an effect-modifier. **Model.1:** $Y = (\beta\_0)+(\beta\_1 * \text{Obese})+(\beta\_2 * \text{Visit})+(\beta\_3 * \text{Obese*Visit})+e$

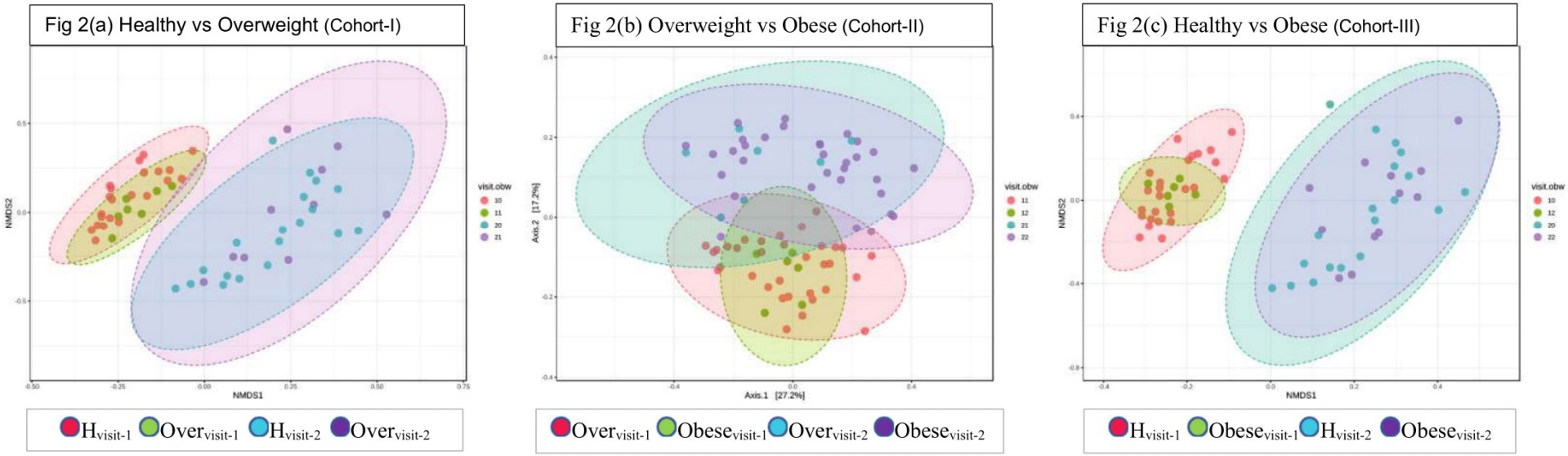
Supplement Figure 1: Demonstrating Alpha Diversity plots (using Observed, Shannon, and Simpson metrics) to compare changes in the diversity of oral microbiota amongst healthy-vs-overweight (Cohort-I; Fig-1a), overweight-vs-obese (Cohort-II; Fig-1b), and healthy-vs-obese (Cohort-III; Fig-1c) phenotypes.







Supplement Figure 2: NMDS (non-metric multidimensional scaling) plots compares Beta Diversity amongst the three study cohorts: (i) Healthy-vs-Overweight, (ii) Overweight-vs-Obese, and (iii) Healthy-vs-Obese. Within each cohort, the comparing groups are coded as 10 (healthy at visit-1), 20 (healthy at visit-2), 11 (overweight at visit-1), 21 (overweight at visit-2), 12 (obese at visit-1), and 22 (obese at visit-2). The separation of microbial profiles is based on the Bray-Curtis dissimilarity metric.



PROJECT - II

Reduction of Nitrate and Nitrite-reducing Oral Microbiota is Associated with Pediatric Hypertension

Ibrahim Warsi,[1,3*] J. Max Goodson,[2,3*] Hatice Hasturk,[2,3] Prasad Gajare,[3] Tsute Chen,[2,3]

*Both I. Warsi, and J.M. Goodson are co-first authors.

[1.] Masters' in Medical Sciences and Clinical Investigation, Harvard Medical School, Boston, MA, USA

[2.] Department of Oral Medicine, Infection, and Immunology, Harvard School of Dental Medicine, Boston, MA, USA

[3.] The Forsyth Institute, Cambridge, MA, USA

Corresponding Author:

Ibrahim Warsi, BDS, Cert. Implant,
MMSCI, Harvard Medical School
Research Fellow | The Forsyth Institute
Email: ahmed_warsi@hms.harvard.edu
Contact number: +1 (832) 458-5039
Address: 614A, Shawmut Ave, Unit-4,
Boston, MA, 02118, USA.

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ABSTRACT:

Nitric-oxide has a strong vasodilatory-effect, contributing to homeostasis of normal blood pressure. Nitrate and nitrite reducing oral bacteria play a key role in the production of nitric oxide, through the enterosalivary nitrate-nitrite-nitric-oxide pathway. In this study, we primarily investigated, the cross-sectional association between reduction in nitrate and nitrate-reducing oral bacteria, and changes in blood pressure. We also investigated whether changes in oral bacteria amongst normotensive individuals at baseline, could predict hypertension (>120/80 mmHg), two-years later. Ten-to-eleven-year-old children were enrolled in a longitudinal Kuwait-Healthy-Lifestyle study (n=6209). Systolic and diastolic blood pressure were recorded at both visits 1 and 2, two-years apart. Whole saliva sample from visit-1, were examined for fifteen nitrate and nitrite-reducing bacterial numbers using the DNA-DNA-hybridization method. Adjusted linear and logistic regression analyses were used to investigate the association and prediction aims of our study. We observed that a reduction in major ***nitrate-reducing***, *Actinomyces sp*. (*odontolyticus*, *israelii*, and *viscosus*), was associated with an increase of 3.14mmHg systolic, and 5.95mmHg diastolic blood pressure, and a higher-odds of hypertension (OR=0.87, 95%CI=0.79,0.96). Whereas, a reduction in ***nitrite-reducing,*** *Streptococcus sp.* (*mutans* and *salivarius*), was associated with an increase of 5.67mmHg systolic blood pressure, and a higher-odds of hypertension (OR=0.82, 95%CI=0.71,0.96). Further, a reduction in ***nitrite-reducing***, *Prevotella melaninogenica,* amongst normotensive children at baseline, was associated with a 19% risk (AOR=0.81; 95%CI=0.66,1.00) of having hypertension, at visit-2. Our results suggest that dysbiosis of oral microflora is linked to hypertension. A reduction in nitrate and nitrite-

reducing oral bacteria were significantly associated with higher odds of pediatric hypertension.

Keywords: Pediatrics, Hypertension, Blood Pressure, Nitrates, Nitrites, Nitric Oxide, Bacteria, Nitrite Reductases, oral microbiome, epidemiology

INTRODUCTION:

Hypertension affects 1.39 billion adults worldwide (in 2010), with a global prevalence of 31.1% (1). In the US, 75 million American adults (29%) suffer from high blood pressure (BP), which is about one in three adults (1). High blood pressure during childhood is an independent risk factor for the onset of hypertension and cardiovascular events later in life (2,3). In the USA, the prevalence of hypertension (in 2013-2016) in children between the age of 8 to 12 years is 5.5% (4). Hypertension is the most common risk factor for cardiovascular morbidity and mortality. Nitric oxide (NO) is a key regulator of cardiovascular function, and evidence indicates that oxidative stress and subsequent NO deficiency is associated with the development of hypertension (5,6). There is a growing interest in treatment modalities that increase NO production, which might have important implications in preventing and treating cardiovascular disease (7).

The commensal bacteria in the oral cavity play an essential role in the enterosalivary nitrate-nitrite-NO pathway in humans. Interestingly, it is only bacteria and not human cells that can reduce nitrate to nitrite (8). Dietary and endogenous inorganic nitrate is reduced to nitrites by facultative-anaerobic oral bacteria on the dorsal surface of the tongue (5,9). Nitrites may be further reduced to NO by either nitrate-reducing oral/gut bacteria or

through a stomach acid-catalyzed reduction process (10,11). Nitrite and NO mediates several physiological effects, including vasodilation, reduces blood pressure, inhibits platelet adhesion, modulation of mitochondrial function, and prevents endothelial dysfunction from ischemia-reperfusion injury (12,13). The reduction of oral commensal bacteria with chlorhexidine mouthwash demonstrated a 2-3.5 mmHg increase in systolic and diastolic blood pressure (14). In a meta-analysis of clinical trials, the intake of nitrate from solution or beetroot juice was associated with a reduction of 4.2–4.5 mmHg in systolic BP (15).

Although there is compelling evidence supporting the role of the oral microbiome in the enterosalivary nitrate-nitrite-NO pathway, large-scale epidemiological studies on the association between nitrate and nitrite-reducing oral bacteria (NRB) and hypertension are lacking (16). Previous studies have focused on specific age groups (adults and older women), but there are no studies on a pediatric population (16,17). In a prospective study, we enrolled 10 to 11-year-old, Kuwaiti adolescents (n=6209), examined for hypertension at baseline, and visit-2, two-years apart. We examined whole saliva samples (at baseline) for fifteen NRB-species using the checkerboard DNA-DNA hybridization method (18). We aim to investigate the relationship between NRB and pediatric hypertension using two approaches – association and prediction. We investigated primarily (cross-sectional) association between changes in oral-NRB, with effects on blood pressure changes. We also investigated whether changes in oral-NRB of normotensive children at baseline, could predict hypertension onset, two-years later (visit-2).

METHODS:

Study Population:

In a longitudinal study design, we sampled Kuwaiti school-aged children at two visits, two-years apart. At baseline (visit-1), we enrolled 8,173 Kuwaiti school-going children in their fourth and fifth grades (10-year-old) in the Kuwait Healthy Lifestyle Study (KHLS), during 182 visits, from 138 Kuwaiti schools. The focus of this analysis is to measure blood pressure and record whole salivary bacterial counts and relative bacterial frequencies (19). In a follow-up (visit-2), two years later, we recorded blood pressure measurements and study characteristics of 6,209 adolescents from the same group in their sixth and seventh grades. One purpose of the follow-up visit was to assess whether the salivary bacterial composition of visit-1 could predict disease in the second visit. Both the Dasman Diabetes Institute (Kuwait) and The Forsyth Institute (USA) ethical review committees reviewed and approved the research proposal. Parents (or guardians) read and signed informed consent before the conduct of the study, and participant children read and signed an assent on the day of their evaluation.

Clinical Examination:

During the clinical examination, we recorded socio-demographic details (such as age, gender, state/ethnicity background). We captured all data on tablet computers (19). Body Mass Index (BMI) was calculated by dividing body weight in kilograms by height in meters squared. Systolic (SBP)/ Diastolic (DBP) blood pressure were measured using pediatric blood pressure cuffs.

We divided blood pressure readings into four categories: normotension (SBP/DBP: < 120/< 80 mmHg), elevated blood pressure (120-129/<80)mmHg, stage-I hypertension

(130-139/80-89 mmHg), and stage-II hypertension (≥140/90 mmHg) (20). We defined binary hypertension as SBP ≥120 or DBP ≥80 mmHg. Mean arterial blood pressure (MAP) was calculated using the following formula: 1/3(SBP)+2/3(DBP) (21). We measured fitness by the heart rate elevation following a standardized exercise (22). We conducted oral examination using portable dental chairs, halogen lights, and intraoral mirrors for assessment of the number of inflamed gingival sites and the number of teeth with dental decay. Besides, we measured salivary glucose concentration (Glucose Colorimetric/Fluorometric Assay (Kit #K606-100, BioVision, Inc, Mountain View, California, USA) (19).

Saliva Collection and Storage:

Whole saliva was collected by expectoration from 6209 children between 8:30–9:30 am, under fasting conditions. Each participant rinsed with and swallowed 15mL of water before saliva collection. Approximately 3mL whole saliva was collected by a passive drooling method in a 15-mL plastic screw-top centrifuge tube (Product #430791, Corning Incorporated Life Sciences, Tewksbury, MA, USA). Saliva was maintained ice until transported to the Dasman Diabetes Institute, where they were placed in a 96-vial rack (Thermo Scientific Latch Rack) and frozen at −80°C. Racks were air-transferred from Kuwait under temperature-monitored dry ice (Biocair, Boston MA) to the Forsyth Institute and maintained at −80°C until assay (average time to assay = 0.88±0.06 y) (19).

Microbial Assay:

We measured whole saliva microbiota using the DNA-DNA-hybridization or whole genomic-probe method, commonly referred to as the checkerboard assay (18). By this method, we determined the number of bacteria by linear regression of 10^5 and 10^6

standards for each bacterium. We conducted assays on a 0.2 mL aliquot of the whole saliva sample from each subject. For cell-wall disruption, 0.1mL of 0.5N NaOH neutralized by 5M ammonium acetate was added to the sample and then boiled. This procedure allows us to extract intact DNA. Values obtained by this method have an acceptable association with culture-based methods (23,24). Samples were then applied to the surface of a nylon membrane in a Minislot™ device (Immunetics, Cambridge, MA, USA) and evaluated by DNA probes to 42 species (19). Bacterial DNA was fixed to the membrane by ultraviolet exposure. We determined bacterial numbers by image analysis of scanned samples (Typhoon™ Molecular Imager, GE Healthcare Life Sciences, Pittsburgh, PA, USA). We detected bound bacterial DNA by using a covalently bound fluorescent marker (AttoPhos®, Amersham Life Sciences, Arlington Heights, IL, USA). We adjusted the concentrations of DNA probes used to detect approximately 10^4 bacteria (sensitivity), with 93.5% of cross-reactions exhibiting less than 5% of the homologous probe signal (specificity) (18).

Statistical Methods:

We calculated the total number of oral nitrate-reducing bacteria (NRB) in each sample as a sum of bacterial counts for each of the forty-two bacterial species probes used. To quantify the relative proportion of each of the forty-two species present, we divided the bacterial count for each species by the sum of bacterial counts in each sample. We identified fifteen of forty-two species as nitrate or nitrate-reducing species. Square root transformation was performed for each bacterium to obtain a normal distribution. We adjusted for covariate using Propensity score (PS) method (25). We first determined the variables to include in the propensity score model based on findings from the literature. Using logistic regression, we modeled the confounders age, sex, BMI, and percentage of inflamed gingival sites, on

the indicator outcome variable (i.e., hypertension), and estimated the propensity score for each subject. Throughout in our analysis of linear and logistic regression, we adjusted for propensity score as a confounder. Further, multiple linear and logistic regression models were compared with periodic covariate adjustments (using propensity score), to ensure that the estimated odds of hypertension, and estimated changes in systolic and diastolic blood pressure, concerning changes in oral-NRB are valid, and not confounded by other potential confounders (Sensitivity Analysis, refer to Supplement Table 3). The results of linear and logistic regression analysis are presented as adjusted estimates (E mmHg), and adjusted odds ratios (AOR), along with 95% confidence intervals (CI). To account for multiple comparison, a p-value ≤ 0.003 was considered significant based on Bonferroni correction.

We divided our statistical methods into four parts. **First,** using logistic regression analysis, we assessed whether changes in the microbiota (continuous exposure) are associated with hypertension (defined as SBP/DBP $\geq$ 120/80mmHg; binary outcome) when adjusted for confounding via propensity score method. **Second**, using linear regression while adjusting for the propensity score, we estimated whether changes in oral microbiota (continuous exposure) are associated with an increase in SBP, DBP, and MAP (continuous outcome). **Third**, we investigated whether the changes in oral microbiota (continuous outcome) are associated with the advancing stages of hypertension (categorical exposure). The categories for hypertension stages include normotension (reference), elevated blood pressure, stage-I, and stage-II hypertension. **Fourth**, we attempted to identify oral

microbial markers amongst normotensive children at visit-1, which could predict hypertension at visit-2, two-years later.

RESULTS:

Table 1 shows the baseline characteristics of the study population, stratified based on normal blood pressure (normotensive), and high blood pressure (hypertensive) groups. In Table-2, we identify that a reduction in most oral-NRB is associated with higher odds of hypertension. These include *Streptococcus mutans* (AOR=0.62, 95% CI=0.44,0.87), *Selenomonas noxia* (AOR=0.64, 95% CI=0.46,0.90), and *Actinomyces* species including *A. israelii* (AOR=0.62, 95%CI=0.44,0.87), *A. odontolyticus* (AOR=0.77, 95%CI=0.61,0.96), *A. viscosus* (AOR=0.72, 95%CI=0.53,0.97). The only significant exception was an increase in *Eikenella corrodens*, associated with higher odds of hypertension (AOR=1.25, 95%CI=1.08,1.45; Table-2). Similarly, an increase in *E. corrodens* was directly associated with an 8.33mmHg increase in DBP (95%CI=2.33,14.34), and 6.44mmHg increase in MAP (95%CI=1.61,11.27) (Table 3).

Using linear regression, we estimated the changes in SBP and DBP with the changes in abundance of oral-NRB, adjusted for confounding using the propensity score method (Table-3). We found that with a one-unit increase in the abundance of *Streptococcus mutans*, an estimated decrease of 12.61mmHg SBP was observed (95%CI=22.55,-2.61). Although not significant, *Streptococcus salivarius* demonstrated a reduction of 7.46mmHg in DBP (95%CI=-15.44,0.51). In other words, a reduction in *S*

.mutans, and *S. salivarius* was associated with an increase in 12.61mmHg SBP, and 7.46mmHg in DBP, respectively.

Besides, a one-unit increase in the abundance of *Actinomyces viscosus* produced an estimated decrease of 9.62mmHg SBP (95%CI=-19.29,0.06; Table-3). Although not significant, *Actinomyces odontolyticus* (6.73mmHg reduction, 95%CI=-13.82,0.36; p=0.06), and *Selenomonas noxia* (9.97 mmHg reduction, 95%CI=-20.55,0.61; p=0.06) also exhibited a similar trend. In other words, a reduction in *S. noxia, A. viscosus,* and *A. odontolyticus* were associated with an increase in 9.97 mmHg, 9.62 mmHg, 6.73 mmHg SBP, respectively.

Similarly, when assessed whether an increase in oral NRB is associated with a reduction in DBP (Table-3). We found that an increase in three *Actinomyces* species significantly reduced DBP. These include *A. odontolyticus* (a 15.03mmHg reduction, 95%CI=-24.20,-5.85), *A. israelii* (a 14.57mmHg reduction, 95%CI=-28.34,-0.80), and *A. viscosus*, (a 12.99mmHg reduction, 95%CI=-25.51,-0.46). In other words, a reduction in *Actinomyces* species – *A. odontolyticus*, *A. israelii*, and *A. viscosus*, were significantly associated with an increase in 15.03mmHg, 14.57mmHg, and 12.99mmHg DBP, respectively.

At the genus-level (Table-3), we found that a one-unit reduction in the *Actinomyces* genus was significantly associated with a 3.14mmHg increase in SBP, 5.95mmHg increase in DBP, and 5.01mmHg increase in MAP. Similarly, a one-unit reduction in the *Streptococcus* genus was significantly associated with a 5.67mmHg increase in SBP and a 5.82mmHg increase in MAP. Only the *Actinomyces* genus maintained a significant association with DBP and MAP, even after Bonferroni correction (p-value$\leq$0.003).

We found that changes in the abundance of oral-NRB follow a pattern when compared across the advancing stages of hypertension (Figure-1/ Supplement Table-1). We observed

a significant reduction in oral-NRB (esp. *S. mutans, S. noxia, P. acnes, A. israelii,* and *A. naeslundii)* when compared across subjects with advancing stages of hypertension. *E.corrodens*, exhibited a unique exception in that it was related to a significant increase in stage I and II hypertension, relative to elevated blood pressure.

In our secondary analysis (Table-4), we aimed to assess whether changes in oral microbiota of normotensive children (at baseline), could predict the onset of hypertension at visit-2, two-years apart. We found that a reduction in *P. melaninogenica* was associated with 19% risk (AOR=0.81; 95%CI=0.66,1.00) of having hypertension (SBP/DBP>120/80 mmHg), while a 22% higher risk (AOR=0.78; 95%CI=0.64,0.96) of having stage-I and II hypertension (when predicting SBP/DBP≥130/80 mmHg). Further, a reduction in nitrite-reducing bacteria, *P. melaninogenica,* was also associated with a 4.59mmHg increase in DBP (Supplementary Table 2). Similarly, a reduction in nitrate-reducing, *S. noxia,* amongst normotensive individuals at baseline, was associated with 39% risk (AOR=0.61; 95%CI=0.39,0.95) of having stage-I and II hypertension (SBP/DBP≥130/80 mmHg) at visit-2, two-years later.

In a sensitivity analysis, using logistic regression, we assessed the relationship between the composition of forty-two oral bacteria and hypertension prevalence (Supplement Fig.1). In addition to *Eikenella corrodens*, an increase in the abundance of *Porphyromonas gingivalis* (AOR=1.34, 95%CI=1.14,1.58), and *Treponema socranskii* (AOR=1.54, 95%CI=1.17,2.03) was associated with significantly higher odds of hypertension.

DISCUSSION:

Our study identified three major findings. First, the reduction in nitrate-reducing bacteria is associated with higher odds of hypertension prevalence, and an increase in blood pressure. Second, a dose-response reduction in the abundance of a few oral-NRB was associated with increasing severity of hypertension. Third, the oral microbial changes in normotensive individuals can predict the onset of hypertension, two-years later.

In the present study, we have shown that of the fifteen nitrate or nitrite reducing oral bacteria (NRB) examined, reduction of five species was associated with an increase in mean systolic (SBP), diastolic (DBP), and arterial blood pressure (MAP). Of note, the five species belonged to two genera – *Actinomyces,* and *Streptococcus*. At the genus-level, we identified a reduction in *Actinomyces* genera was associated with a significant mean increase of 3.14mmHg SBP, 5.95mmHg DBP, and 5.0mmHg MAP (Table-3). Whereas, a reduction in *Streptococcus* genera demonstrated a mean increase of 5.67mmHg SBP and 5.82mmHg MAP. Mean arterial blood pressure (MAP) is a function of cardiac output and the amount of resistance provided by blood vessels (21). MAP is a strong predictor of cardiovascular disease risk (21). A study indicated that by inhibiting endogenous NO production via eNOS-pathway, was associated with a 10% increase in MAP, and a 46% increase in total peripheral resistance (41). Our results indicate that a reduction in *Actinomyces,* and *Streptococcus* genera, is associated with a ~5mmHg increase in MAP. Other investigators have demonstrated a similar triad relationship with nitrate supplementation, an increase in *Actinomyces*, and a decrease in MAP (9).

At species-level, we observed that a reduction in nitrite-producing (or nitrate-reducing) bacteria (26,27), i.e., *Actinomyces species (A.israelii, A.odontolyticus, A.viscosus)* was associated with a significant increase in DBP, whereas, a reduction in NO-producing (or nitrate-reducing) bacteria (28–30), i.e., *Streptococcus mutans* was associated with a significant increase in SBP (Table-3). The association of reduction in both *Actinomyces* and *Streptococcus* species could mean that the compositional changes in either nitrate or nitrite-reducers, could affect blood pressure homeostasis, even though by two different pathways. We also observed this phenomenon when investigating changes in abundance of oral-NRB, with advancing stages of hypertension (Figure-1/ Supplementary Table-1). To illustrate, we observed a marked reduction of *Streptococcal sp.* in stage-I hypertension, relative to elevated BP, and stage-II hypertension (Figure-1). Whereas, *Actinomyces sp.* demonstrated a marked reduction in stage-II hypertension, compared to other groups. The species belonging to their respective genera, also followed a similar gradient, as illustrated in Figure-1. *Streptococcus sp.* forms a major population on the tongue microflora (~40%), followed by *Veillonella*, and *Actinomyces* species (26,27). Other investigators have reported that *Actinomyces odontolyticus* is a major contributor to nitrite-production, and amongst the second most common tongue isolate. The phenomenon of reduction in *Actinomyces*, together with an increase in DBP and MAP, which was significant even after Bonferroni correction, cannot be overlooked. Other investigators have reported similar, but smaller effects (16,31).

Nitric oxide (NO) is an important signaling molecule, with a strong vasodilatory-effect, and is responsible for the maintenance of normal blood pressure. The human body can produce endogenous NO through endothelial-NO-synthase (eNOS) dependent conversion of L-arginine to NO (32). Other investigators have reported a decrease in the amount of bioavailable NO through an eNOS-mediated pathway, which occurs by endothelial dysfunction under certain conditions like hypertension, diabetes, hypercholesterolemia, atherosclerosis, and aging (33,34). The commensal oral microbiota supplements host NO requirements, by reducing dietary nitrates to nitrites. Nitrite is then converted non-enzymatically to NO in the stomach and absorbed into the systemic circulation. Twenty-five percent of un-reduced nitrates are actively taken-up by salivary glands and converted into nitrites by the action of nitrate-reducing oral bacteria through a process referred to as the enterosalivary-nitrate-nitrite-NO pathway (35). Numerous clinical trials have demonstrated that the disruption of the enterosalivary pathway by use of mouthwash, has resulted in an increase in blood pressure by around ~3mmHg (36). Most human studies have investigated the effects of nitrate consumption on changes in blood pressure, amongst normotensive subjects. Evidence from ten dietary nitrate-consumption studies have indicated a significant reduction of 3 to 10 mmHg SBP, and between 3 to 8 mmHg DBP (37). Two studies also indicated a positive association between nitrate consumption and improvement in vascular flow-mediated dilatation (37–39). Similarly, our results report an increase of 3 to 5 mmHg in SBP and DBP, with a reduction in two major nitrate/nitrite-reducing genera (*Actinomyces* and *Streptococcus*), which concurs with previously reported ranges. This magnitude of change in blood pressure, concerning changes in the abundance of oral-NRB, is clinically meaningful. It has been reported that a reduction of up to 5mmHg

blood pressure is estimated to reduce the risk of stroke by 34%, and ischemic heart disease by 21% (40).

Our findings reflect that the reduction of oral NRB starts at earlier developmental stages of hypertension (i.e., during elevated blood pressure stage), which is even before the onset of vascular endothelial dysfunction – feature attributed to stage-I and II hypertension (42,43). Also, we reported that a reduction in nitrate-reducing (i.e., *S. noxia*) (27), and nitrite-reducing (i.e., *P. melaninogenica*) oral bacteria (26), amongst normotensive individuals at baseline, demonstrated a 39%, and 22% (respectively), higher risk of having hypertension, two-years later. These two important findings pose two major questions to address in future longitudinal studies.

First, what caused the reduction of oral-NRB? It could be to the result of poor oral hygiene practices that precipitate systemic changes in the host-inflammatory pathway. These factors, however, tend to favor the growth of opportunistic bacteria such as *P*. *gingivalis* and *E*. *corrodens* (36,44). In addition to the nitrate-reduction pathway, the role of inflammatory-pathway in the homeostasis of blood pressure cannot be ruled out (45,46). Numerous studies have implicated a positive relationship between periodontal pathogen - *P. gingivalis* and hypertension via the inflammatory route (7,45–47). *E. corrodens* is a gram-negative, facultative-anaerobic bacteria, which tends to induce inflammatory responses in periodontal tissues of adults and promote the development of chronic periodontitis (48). In the present study of children, gingivitis was the principle periodontal disease, and early evidence of an increase in *E*. *corrodens* and *P. gingivalis*, amongst hypertensive children,

could lay a framework for later development of adult periodontitis. In this scenario, commensal oral microbiota undergoes an ecological shift from aerobic, gram-positive bacteria (in healthy periodontium), being replaced by pathogenic, anaerobic, gram-negative bacteria in periodontal disease (49). We observed a similar phenomenon, i.e., reduction of growth-inhibiting species (*Actinomyces sp.*, and *Streptococcus sp.)* for periodontal pathogens (such as *P.gingivalis*), was consistent with an increase in the abundance of *P. gingivalis* – an opportunistic, periodontal pathogen, implicated with hypertension prevalence (50). In summary, we attribute an increase in *E. corrodens* and a reduction in oral-NRB, due to poor oral hygiene awareness and practices amongst the Kuwaiti school-going population (51).

Second, since we did not measure salivary or plasma nitrate/nitrite/NO concentrations, we do not know whether the reduction in oral NRB could lower the bioavailability of NO, leading to elevated blood pressure? Or, could the dysfunction in the eNOS pathway (i.e., endothelial dysfunction), responsible for changes in blood pressure, amongst individuals at high-risk for developing hypertension. Future longitudinal studies should take both pathways into account during the assessment of the relationship between oral-NRB and hypertension-onset.

Besides, our study has some limitations. First, we used hypertension definition based on cut-offs for children aged >13 years, rather than percentile-based hypertension. We justified it by using a consistent definition of hypertension for visit-1, where children were 10-11 years, and for visit-2, where children were 12-13 years old. When the analysis was repeated with a percentile-based definition for hypertension, we obtained similar results

(see also Supplementary Table 4). Second, we did not measure changes in oral microbiota, in our follow-up at visit 2. Hence, our analysis is limited to prediction and not causal inference. Third, we did not adjust for dietary factors, which might influence changes in oral microbiota. Fourth, we did not measure changes in saliva or plasma nitrate or nitrite levels, which could be useful to establish an accurate relationship between changes in oral-NRB and enterosalivary-nitrate-nitrite circuit. Fifth, our results are generalizable to pediatric hypertension but might be restricted to the Kuwaiti population. Given that the prevalence of pediatric hypertension in the USA was around 5.5%, compared to nearly 40% for the KHLS cohort (4). This raises concerns about the involvement of multiple pathways responsible for a higher prevalence of cardio-metabolic risk factors amongst the KHLS cohort (52). In particular, environmental factors such as pollutants emitted from oil and gas extraction could also be associated with a higher prevalence of hypertension and early development of cardiovascular disease amongst Kuwait children (53,54). Sixth, we did not assess oral hygiene awareness and practices. Although regular use of antibacterial mouthwashes has been shown to significantly raise blood pressure in adults (55), no evidence of this effect in children has been reported. In a national survey, good oral hygiene awareness and practices amongst Kuwaiti school-going children were only 3.9%, which nearly eliminates the possibility of widespread use of oral antimicrobial products (51). Finally, we based our analysis on the DNA-DNA-hybridization (or checkerboard) method, which only examined 15 of the known 28 oral-NRB, and relative to 16s rRNA sequencing method, it lacks specificity at species-level (56). We tried to overcome that limitation by also comparing genus-level changes with hypertension. The results of the checkerboard method are, however, comparable with culture-based methods (~85%) (23,24).

Assessment of oral bacterial profiles using the checkerboard method is relatively inexpensive and useful for large, population-based, epidemiological studies (18,24,47,57). In our case, it was well-suited with our study objective, the semi-quantitative estimation of bacteria counts (abundance), rather than relative-abundance, which is possible through our checkerboard approach, compared to 16s-rRNA-sequencing approach (56,58).

This population-based, longitudinal cohort study, is the first to addresses two important research questions, i.e., whether a reduction in nitrate/nitrite-reducing oral bacteria is associated with pediatric hypertension; and whether changes in the composition of oral-NRB could predict the onset of pediatric hypertension. Until now, only two cross-sectional studies, have investigated a link between the composition of oral NRB and hypertension in adults (16), and older women (17), while studies on pediatric hypertension are lacking. These studies have used modern sequencing methodologies like 16s-rRNA methods, but their sample population was relatively small, and specific to certain groups. Moreover, these studies have not explored the association concerning nitrite-reducing oral bacteria. Besides, these studies examined subgingival oral microbiota, which does not reflect the actual abundance of nitrate and nitrite reducing oral bacteria, residing on the surface of the tongue (26,36). Our research aims to address these gaps in knowledge. In a human life cycle, early phases of hypertension start developing in the early-adolescent phase and is an independent risk factor for hypertension, and cardiovascular events in adulthood (3). Our study indicated a potential for microbiological assessment amongst the pediatric population could predict adulthood hypertension. We believe, that if the relationship between the reduction of oral-NRB and hypertension onset proves to be causal, in the

future, the manipulation of the oral microbiota at early stages, might also improve cardiovascular health and hence prevent hypertension-onset in adulthood.

PERSPECTIVES:

We provide epidemiological evidence of an association between reduction of nitrate and nitrite reducing oral bacteria (NRB) and higher odds of hypertension. Reduction in the abundance of nitrate-reducing, *Actinomyces sp.* and nitrite-reducing, *Streptococcus sp.* were associated with an increase in systolic, diastolic, and mean arterial blood pressure. The abundance of these species also decreased in a dose-response manner across the advancing stages of hypertension. Our results support the hypothesis, that the reduction of oral-NRB in normotensive children could predict the onset of pediatric hypertension. Our findings also indicate that changes in oral-NRB are preceded by hypertension onset. Future longitudinal cohort studies are, however, needed to validate our findings concerning the enterosalivary-nitrate-nitrite-NO circuit. Treatment modalities aimed at manipulating the oral microbiota, increasing the bioavailability of NO production, could have better implications in the management of hypertension and ultimately preventing CVD onset in adulthood.

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NOVELTY AND SIGNIFICANCE

What Is New?

- This is the first, large-scale, epidemiological study, to examine the relationship between nitrate, and nitrite-reducing oral microbiota, with hypertension prevalence, and onset.
- A dose-response reduction in the abundance of oral nitrate and nitrite reducing bacteria is associated with advancing stages of hypertension.
- Reduction of certain nitrate and nitrite-reducing oral bacteria amongst normotensive children can predict the onset of pediatric hypertension.

What is Relevant?

- In this epidemiological study, we demonstrated that a reduction in oral nitrate and nitrite reducing oral bacteria is associated with a 3 to 5 mmHg higher systolic and diastolic blood pressure.
- This may translate to clinical management by a 5mmHg reduction in blood pressure, which is estimated to reduce the risk of stroke by 34%, and ischemic heart disease by 21%.

Summary?

- Reduction in nitrate and nitrite-reducing oral bacteria is associated with pediatric hypertension.

MANUSCRIPT TABLES and FIGURES

Table 1: Baseline Characteristics

Study characteristics	Normotension	Hypertension	P-value
N	3752 (60.43%)	2457 (39.57%)	
Age (years)	9.96 (0.65)	10.04 (0.65)	<0.001
Male sex	1351 (36.0%)	924 (37.6%)	0.21
Kuwait Governates (or States)			<0.001
Al-Ahmadi	861 (22.95%)	442 (17.99%)	
Al-Farwaniyah	563 (15.01%)	389 (15.83%)	
Hawali	340 (9.06%)	261 (10.62%)	
Al-Jara	793 (21.14%)	617 (25.11%)	
Al-Asimah (capital)	940 (25.05%)	604 (24.58%)	
Mubarak Al-Kabeer	255 (6.80%)	144 (5.86%)	
Waist circumference (cm)	65.37 (17.31)	73.52 (25.12)	<0.001
BMI (Kg/m^2)	18.57 [16.32, 21.70]	22.45 [18.51, 26.40]	<0.001
DBP (mmHg)	66.45 (8.55)	85.23 (10.91)	<0.001
SBP (mmHg)	100.10 (10.82)	123.17 (12.64)	<0.001
MAP (mmHg)	77.67 (8.11)	97.87 (9.13)	<0.001
Heart rate (bpm)	90.84 (12.84)	94.27 (14.61)	<0.001
Saliva flow rate (mL/hr)	22.78 [15.28, 32.40]	23.25 [15.64, 32.88]	0.164
Fitness score (bpm)	21.5 [4.00, 37.00]	26 [8.00, 42.00]	<0.001
Sleep (weekend sleep hours)	8.85 (1.62)	8.79 (1.63)	0.198
Decayed or Filled (%)	8.7 [4.00, 17.39]	8.33 [0.00, 16.67]	<0.001
Gingival inflammation (%)	77.78 [59.72, 92.75]	78.26 [61.11, 93.06]	0.522
Salivary glucose (mg/dL)	0.15 [0.09, 0.19]	0.15 [0.09, 0.19]	0.787

Table 1 demonstrates distribution of study characteristics, stratified based on Normotension (SBP/DBP < 120/80 mmHg) and Hypertension (SBP/DBP $\geq$ 120/80 mmHg). T-test was used to assess differences in means (and standard deviations) for the following variables: Age, Waist circumference, DBP (Diastolic blood pressure), SBP (Systolic blood pressure), MAP (Mean Arterial blood pressure), Heart rate, and Sleep. Mann-Whitney test was used to assess differences in medians and inter-quartile ranges for the following variables: BMI (Body mass index), Saliva flow rate, Fitness score (heart rate elevation following a standard exercise [Suriano K, 2010]), Decay or Filled (percentage of decayed or filled teeth), Gingival inflammation (percentage of inflamed gingival sites), and Salivary glucose (salivary glucose concentration). A Chi-square test was used to assess differences in frequency (and percentages) for sex and governates. P-value is considered significant at <0.05.

Table 2: Logistic Regression analysis demonstrating the association between binary hypertension and oral nitrate and nitrite-reducing bacteria (NRB).

Association between Hypertension and oral-NRB					
Microbial Predictors	**Phylum**	**Ref.#**	**AOR**	**95% CI**	**p-value**
Nitrate-reducing species					
Actinomyces israelii	Actinobacteria	27	0.62	0.44 – 0.87	**0.005**
Actinomyces naeslundii	Actinobacteria	27	0.82	0.60 – 1.13	0.229
Actinomyces odontolyticus	Actinobacteria	26, 27, 35	0.77	0.61 – 0.96	**0.021**
Actinomyces viscosus	Actinobacteria	25, 26	0.72	0.53 – 0.97	**0.033**
Capnocytophaga sputigena	Bacteroidetes	27	1.01	0.80 – 1.29	0.920
Eikenella corrodens	Proteobacteria	27	1.25	1.08 – 1.45	**0.003 ***
Fusobacterium nucleatum polymorph	Fusobacteria	26	0.83	0.58 – 1.19	0.311
Propionibacterium acnes	Actinobacteria	27	0.69	0.46 – 1.05	0.084
Selenomonas noxia	Firmicutes	27	0.64	0.46 – 0.90	**0.009**
Nitrite-reducing species					
Prevotella melaninogenica	Bacteroidetes	26	0.90	0.77 – 1.04	0.160
Streptococcus mitis	Firmicutes	28, 30	1.08	0.93 – 1.24	0.313
Streptococcus mutans	Firmicutes	28, 29	0.66	0.48 – 0.90	**0.009**
Streptococcus salivarius	Firmicutes	28, 30	0.85	0.70 – 1.04	0.110
Nitrate & Nitrite – reducing species					
Neisseria mucosa	Proteobacteria	26, 36	1.01	0.89 – 1.15	0.848
Veillonella parvula	Firmicutes	26	0.90	0.74 – 1.09	0.266
Nitrate & Nitrite – reducing genera					
Actinomyces[1]	Actinobacteria	26	0.87	0.79 – 0.96	**0.005**
Streptococcus[2]	Firmicutes	28, 29, 30	0.82	0.71 – 0.96	**0.013**

#Literature references have been cited, on information about oral bacterial nitrate or nitrite reduction capability. Significant p-values (<0.05) are listed in boldface type. *Represents significant after Bonferroni correction (p-value≤0.003). [1]*Actinomyces* genera (nitrate-reducers) represents a cumulative additive score from four species: *A.israelii, A.naeslundii, A.odontolyticus, A.viscosus.* Whereas, [2]*Streptococcus* genera (nitrite-reducers) represents a cumulative additive score from two species: *S. mutans, and S. salivarius.* The results are presented as adjusted odds ratios (AOR), with 95% confidence intervals (CI), and p-values. The adjustment was done with age, sex, BMI, and percent gingival inflammation using the propensity score method. AOR<1 represent a reduction in bacterial numbers is associated with hypertension, while AOR>1 represents an increase in bacterial numbers is associated with hypertension.

Table 3: Estimated blood pressure change by linear regression analysis. Values represent mean estimated changes in systolic and diastolic blood pressure (the outcome) with changes in the composition of oral nitrate or nitrite reducing bacteria – NRB (the exposure).

Association between Oral-NRB & Systolic, Diastolic, and Mean Arterial Blood Pressure									
Microbial Predictors	**Systolic Blood Pressure**			**Diastolic Blood Pressure**			**Mean Arterial Pressure**		
	E (mmHg)	95% CI	p	E (mmHg)	95% CI	p	E (mmHg)	95% CI	p
Nitrate-reducing species									
A. israelii	-8.59	-19.23 – 2.04	0.113	-14.57	-28.34 – -0.80	**0.038**	-12.58	-23.65 – -1.50	**0.026**
A. naeslundii	-3.84	-13.72 – 6.05	0.447	-9.66	-22.46 – 3.13	0.139	-7.72	-18.01 – 2.57	0.141
A. odontolyticus	-6.73	-13.82 – 0.36	0.063	-15.03	-24.20 – -5.85	**0.001***	-12.26	-19.64 – -4.88	**0.001***
A. viscosus	-9.62	-19.29 – 0.06	**0.051**	-12.99	-25.51 – -0.46	**0.042**	-11.87	-21.94 – -1.79	**0.021**
C. sputigena	5.29	-2.22 – 12.81	0.167	2.63	-7.11 – 12.36	0.597	3.52	-4.31 – 11.34	0.379
E. corrodens	2.65	-1.99 – 7.29	0.262	8.33	2.33 – 14.34	**0.007**	6.44	1.61 – 11.27	**0.009**
F. nucpolymorph	5.31	-5.87 – 16.49	0.352	7.48	-7.00 – 21.96	0.311	6.76	-4.89 – 18.41	0.255
P. acnes	1.76	-11.27 – 14.80	0.791	-15.58	-32.45 – 1.29	0.07	-9.80	-23.37 – 3.77	0.157
S. noxia	-9.98	-20.56 – 0.60	0.065	-11.3	-25.00 – 2.40	0.106	-10.86	-21.87 – 0.16	**0.053**
Nitrite-reducing species									
P. melaninogenica	-1.78	-6.47 – 2.91	0.457	-4.66	-10.74 – 1.41	0.132	-3.70	-8.59 – 1.18	0.137
S. mitis	1.99	-2.52 – 6.50	0.386	2.36	-3.48 – 8.20	0.429	2.24	-2.46 – 6.93	0.350
S. mutans	-12.61	-22.55 – -2.67	**0.013**	-6.17	-19.05 – 6.70	0.347	-8.32	-18.68 – 2.04	0.115
S. salivarius	-4.61	-10.78 – 1.55	0.142	-7.46	-15.44 – 0.51	0.067	-6.51	-12.93 – -0.10	**0.047**
Nitrate & Nitrite reducing species									
N. mucosa	1.56	-2.54 – 5.65	0.456	0.14	-5.16 – 5.44	0.959	0.61	-3.65 – 4.88	0.779

V. parvula	-4.08	-10.13 – 1.96	0.185	-4.36	-12.19 – 3.46	0.274	-4.27	-10.56 – 2.02	0.183
Nitrate or Nitrite-reducing genera									
Actinomyces[1]	-3.14	-6.13 – -0.16	**0.039**	-5.95	-9.81 – -2.08	**0.003***	-5.01	-8.12 – -1.90	**0.002***
Streptococcus[2]	-5.67	-10.44 – -0.90	**0.020**	-5.90	-12.07 – 0.28	0.061	-5.82	-10.79 – -0.85	**0.022**

[1]Actinomyces genera (nitrate-reducers) represents a cumulative additive score from four species: *A.israelii, A.naeslundii, A.odontolyticus, A.viscosus.* Whereas, [2]Streptococcus genera (nitrite-reducers) represents a cumulative additive score from two species: S. *mutans,* and S. *salivarius.* P-values <0.05 are considered significant (in boldface) and are shown up to three decimal places, to address the strength of the association with (adjusted) linear regression estimates (E in mmHg), and 95% confidence intervals (CI). The estimates are adjusted for baseline confounding (age, gender, BMI, percent gingival inflammation) using the propensity score method. *Represents significant after Bonferroni correction (p-value≤0.003)

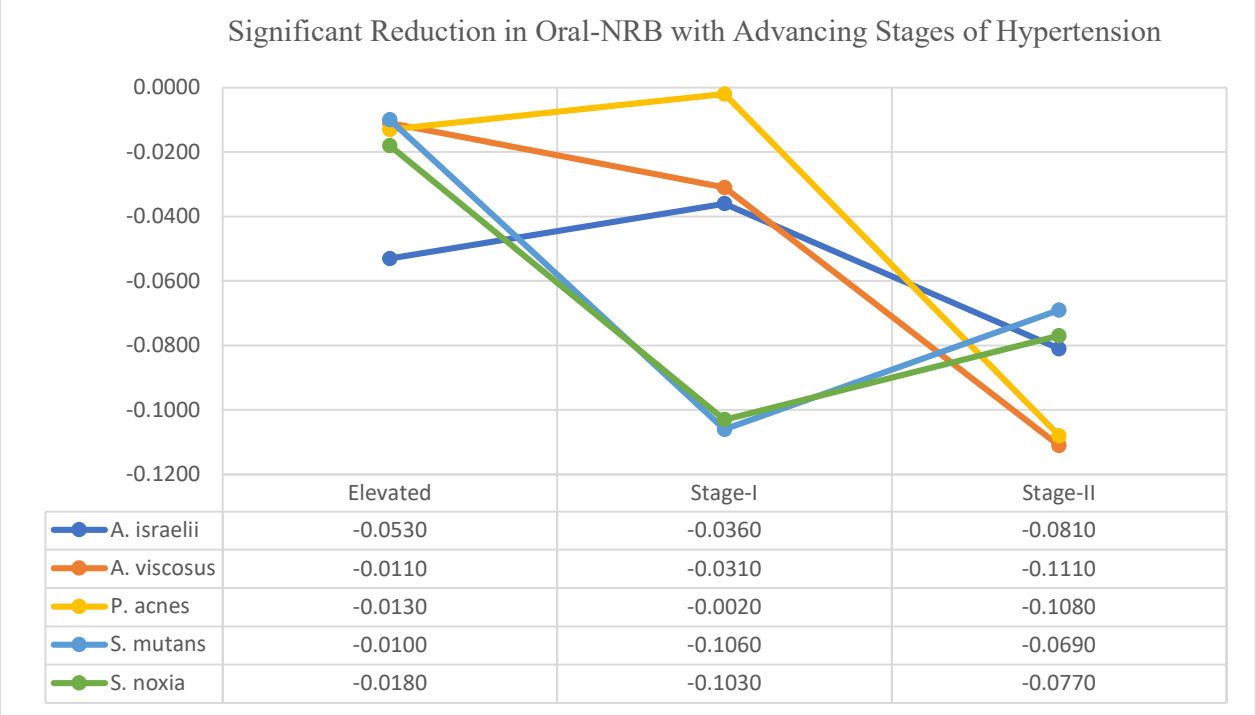
Table 4: **Secondary Analysis (Prediction)**: Significant changes amongst 42 oral microbiota, predicting hypertension at visit-2, in a cohort of normotensive subjects at baseline (n=3749), half of whom developed hypertension at visit-2 (n=1834, 48.9%), compared to healthy controls at visit-2 (n=1915; 51.1%).

Changes in Oral Bacteria Predicting Hypertension onset				
Predicting Hypertension (SBP/DBP > 120/80 mmHg)				
Predictors	**AOR**	**95% CI**	**p**	**Role of Oral Bacteria**
P. melaninogenica	0.81	0.66 – 1.00	0.045	Orange-complex/ Nitrite-reducers
C. gracilis	0.67	0.51 – 0.87	0.003	Light-orange-complex (Periodontopathogen)
Predicting Stage I & II Hypertension (SBP/DBP > 130/80 mmHg)				
Predictors	**AOR**	**95% CI**	**p**	
F. periodonticum	1.50	1.04 – 2.15	0.029	Orange-complex (Periodontopathogen)
T. forsythia	0.47	0.26 – 0.86	0.013	Red-complex (Periodontopathogen)
S. noxia	0.61	0.39 – 0.95	0.030	Other-complex/ Healthy commensal/ Nitrate-reducers
P. melaninogenica	0.78	0.64 – 0.96	0.018	Orange-complex/ Nitrite-reducers
C. gracilis	0.73	0.56 – 0.95	0.018	Light-orange-complex (Periodontopathogen)

The results are presented as adjusted odds ratios (AOR), with 95% confidence intervals (CI), and p-values. The adjustment was done with baseline confounding: age, sex, BMI, and percent gingival inflammation (using the propensity score method). AOR<1 represent a reduction in bacterial numbers is associated with hypertension, while AOR>1 represents an increase in bacterial numbers is associated with hypertension.

MANUSCRIPT FIGURE

Figure 1: Linear Regression estimates are plotted to compare changes in the relative-mean abundance of oral-NRB, concerning advancing stages of hypertension - Normotension (taken as a reference - SBP/DBP< 120/80) mmHg, Elevated blood pressure (SBP/DBP= 120-129/<80) mmHg, Stage-I hypertension (SBP/DBP= 130-139/ 80-89) mmHg, and Stage-II hypertension (SBP/DBP ≥140/90) mmHg. The results are a diagrammatic representation of Supplementary Table-1.



	Elevated	Stage-I	Stage-II
A. israelii	-0.0530	-0.0360	-0.0810
A. viscosus	-0.0110	-0.0310	-0.1110
P. acnes	-0.0130	-0.0020	-0.1080
S. mutans	-0.0100	-0.1060	-0.0690
S. noxia	-0.0180	-0.1030	-0.0770

The figure shows the association between reduction in the abundance of oral nitrate and nitrite-reducing bacteria (NRB), with increasing severity of hypertension stages. The reduction estimates are obtained from the linear regression table shown in Supplementary Table-1**.** The estimates represent the significant changes in the relative abundance of bacteria, concerning stages of hypertension. All estimates shown here, have been multiplied by a hundred percent for easy interpretation in real numbers, instead of exponents (shown in supplement Table-1).

SUPPLEMENT
OF
PROJECT-II

SUPPLEMENT TABLES AND FIGURE

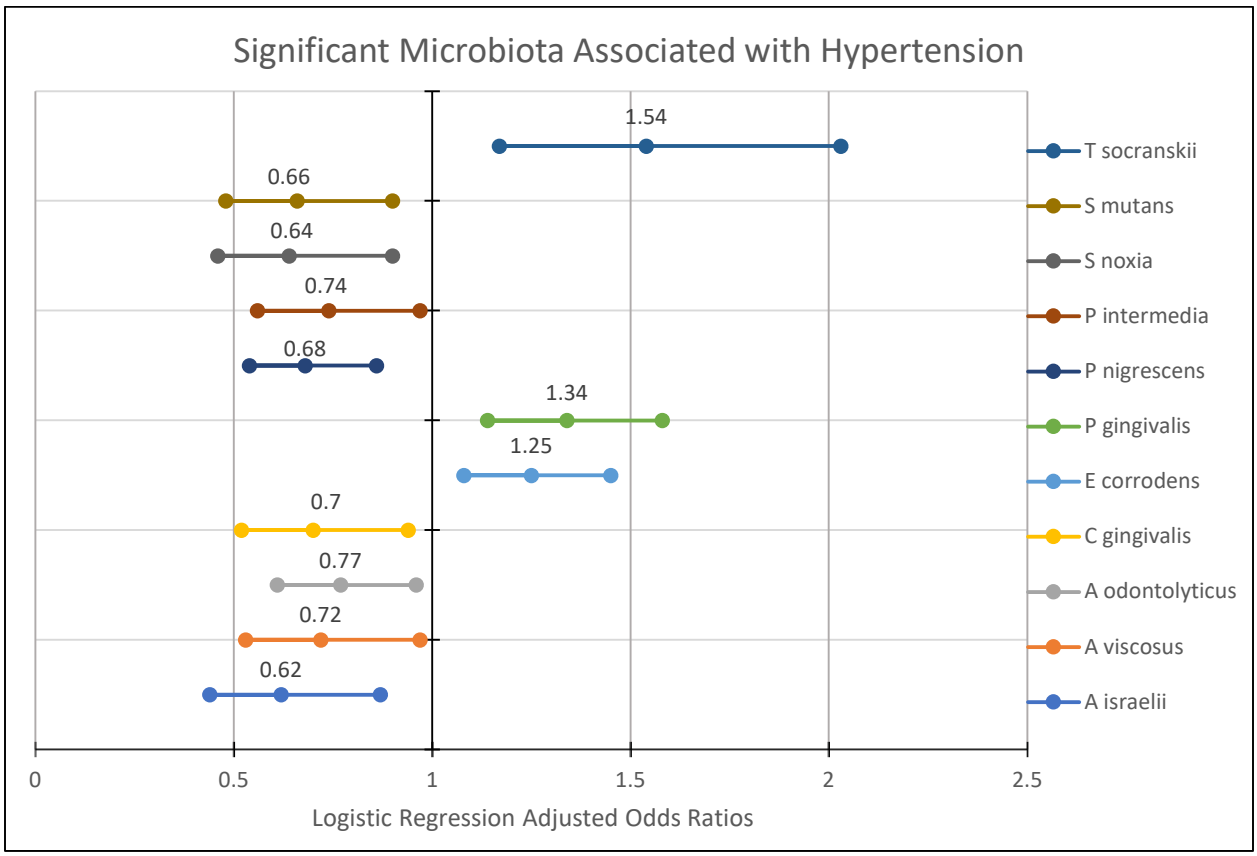
Supplementary Table-1: Linear regression analysis demonstrating the **association** between the abundance of oral-nitrate and nitrite reducing bacteria - NRB (the outcome) with advancing stages of hypertension (the exposure).

Oral - NRB	Advancing Stages of Hypertension								
	Elevated (n=795)			Stage-I (n=1389)			Stage-II (n=1046)		
	Estimates	95% CI	p-value	Estimates	95% CI	p-value	Estimates	95% CI	p-value
A. israelii	-5.30E-04	-0.00138, 0.00031	2.18E-01	-3.60E-04	-0.00105, 0.00033	3.02E-01	-8.10E-04	-0.00160, -0.00003	4.10E-02
A. naeslundii	-1.70E-04	-0.00108, 0.00073	7.10E-01	-5.80E-04	-0.00132, 0.00016	1.22E-01	0.00E+00	-0.00083, 0.00084	9.92E-01
A. odontolyticus	9.10E-04	-0.00030, 0.00213	1.41E-01	-8.10E-04	-0.00180, 0.00019	1.11E-01	-9.90E-04	-0.00212, 0.00013	8.30E-02
A. viscosus	-1.10E-04	-0.00106, 0.00085	8.27E-01	-3.10E-04	-0.00109, 0.00047	4.39E-01	-1.11E-03	-0.00199, -0.00023	1.40E-02
C. sputigena	-8.70E-04	-0.00216, 0.00042	1.87E-01	7.60E-04	-0.00029, 0.00181	1.56E-01	8.20E-04	-0.00038, 0.00201	1.80E-01
E. corrodens	-3.90E-04	-0.00305, 0.00227	7.75E-01	2.77E-03	0.00060, 0.00494	1.20E-02	5.85E-03	0.00339, 0.00832	<0.001
F. nucpolymorph	-4.90E-04	-0.00132, 0.00034	2.48E-01	5.70E-04	-0.00010, 0.00125	9.70E-02	-6.30E-04	-0.00140, 0.00014	1.07E-01
N. mucosa	-7.90E-04	-0.00327, 0.00169	5.32E-01	-6.00E-05	-0.00037, 0.00090	9.55E-01	7.00E-05	-0.00066, 0.00079	2.24E-01
P. acnes	-1.30E-04	-0.00081, 0.00056	7.19E-01	-2.00E-05	-0.00208, 0.00196	9.40E-01	1.42E-03	-0.00087, -0.00371	1.00E-03
P. melaninogenica	-1.60E-04	-0.00208, 0.00175	8.69E-01	-8.00E-04	-0.00058, 0.00053	3.16E-01	-1.08E-03	-0.00171, -0.00045	6.04E-01
S. mitis	-4.70E-04	-0.00296, 0.00202	7.11E-01	1.86E-03	-0.00236, 0.00076	7.30E-02	-4.70E-04	-0.00224, 0.00130	5.97E-01
S. mutans	-1.00E-04	-0.00100, 0.00081	8.34E-01	-1.06E-03	-0.00017, 0.00389	5.00E-03	6.20E-04	-0.00168, 0.00293	1.06E-01
S. noxia	-1.80E-04	-0.00098, 0.00062	6.52E-01	-1.03E-03	-0.00179, -0.00032	2.00E-03	-6.90E-04	-0.00152, 0.00015	4.10E-02
S. salivarius	1.16E-03	-0.00030, 0.00263	1.20E-01	-2.50E-04	-0.00168, -0.00037	6.85E-01	-7.70E-04	-0.00151, -0.00003	3.86E-01
V. parvula	9.90E-04	-0.00049, 0.00248	1.88E-01	-1.16E-03	-0.00144, 0.00095	6.00E-02	-6.00E-04	-0.00196, 0.00076	2.16E-01

The results of linear regression analysis are presented as estimates, with corresponding p-values, and 95% confidence intervals (CI). P-values <0.05 are considered significant (in boldface). Baseline confounders have been adjusted using the propensity score method. The estimates represent changes in the

abundance of oral-NRB (outcome), across the four advancing stages of hypertension: Normotension - reference category (SBP <120, DBP <80), Elevated blood pressure (SBP= 120-129, DBP <80), Stage-I hypertension (SBP= 130-139, DBP= 80-89), Stage-II hypertension (SBP ≥140, DBP ≥90).

Supplement Figure-1: Forest plot of logistic regression (**sensitivity analysis**) showing an association between significant oral microbiota of the 42 microbes (measured by the DNA-DNA-checkerboard-hybridization method) and prevalence of hypertension (SBP/DBP >120/80 mmHg).



The plot represents the odds of having hypertension, with changes in the abundance of oral bacterial species. The adjusted odds ratios (AOR's) have been labeled, while the upper and lower 95% confidence intervals are shown as right and left extensions from the estimated AOR's, respectively. The AOR>1 represents a direct, positive association between an increase in abundance of oral bacteria linked with increased odds of hypertension prevalence; whereas, an AOR<1 represents a decrease in the abundance of oral bacteria is associated with hypertension prevalence.

In a sensitivity analysis, we assessed the relationship between the composition of the 42 oral bacteria and hypertension (**Supplement Fig.1).** In a logistic regression analysis, we identified 11 oral bacteria that significantly changed in composition with hypertension. Increase in the abundance of *Treponema socranskii* (AOR=1.54, 95% CI=1.17,2.03), *Porphyromonas gingivalis* (AOR=1.34, 95% CI=1.14,1.58), and *Eikenella corrodens* (AOR=1.25, 95% CI=1.08,1.45) were associated with higher odds of hypertension prevalence. While, a decrease in the abundance of *Actinomyces israelii* (AOR=0.62, 95%CI=0.44,0.87), *Selenomonas noxia* (AOR=0.64, 95% CI=0.46,0.90), *Streptococcus*

mutans (AOR=0.66, 95%CI=0.48,0.90), *Prevotella nigrescens* (AOR=0.68, 95%CI=0.54,0.86), *Campylobacter gingivalis* (AOR=0.70, 95%CI=0.52,0.94), *Actinomyces viscosus* (AOR=0.72, 95% CI=0.53,0.97), *Prevotella intermedia* (AOR=0.74, 95% CI=0.56,0.97), and *Actinomyces odontolyticus* (AOR=0.77, 95% CI=0.61,0.96) were associated with prevalence of hypertension.

Supplement Table 2 (Prediction- Secondary Analysis): Linear and Logistic Regression results are presented in the table. Demonstrating whether changes in oral-NRB could predict changes in SBP, DBP, and the onset of hypertension during follow-up of the KHLS cohort (n=6209), at visit-2, two-years later.

Microbial Predictors	**SBP – Visit-2**			**DBP – Visit-2**			**Hypertension - Visit-2**		
	E	**95% CI**	**p**	**E**	**95% CI**	**p**	**AOR**	**95% CI**	**p**
P. melaninogenica	-1.67	-6.72 – 3.39	0.519	**-4.59**	-8.94 – -0.25	**0.038**	**0.90**	0.78 – 1.05	0.185
A. israelii	1.42	-10.04 – 12.89	0.808	-2.13	-11.98 – 7.72	0.672	1.07	0.76 – 1.50	0.718
A. naeslundii	-4.90	-15.56 – 5.75	0.367	-2.16	-11.32 – 7.00	0.644	1.02	0.74 – 1.40	0.898
A. odontolyticus	-2.55	-10.20 – 5.09	0.513	-1.84	-8.41 – 4.73	0.583	0.97	0.77 – 1.22	0.788
A. viscosus	-2.75	-13.18 – 7.67	0.605	-2.26	-11.22 – 6.70	0.621	0.84	0.62 – 1.15	0.276
C. sputigena	5.02	-3.09 – 13.12	0.225	4.69	-2.27 – 11.65	0.186	1.06	0.84 – 1.36	0.611
E. corrodens	2.12	-2.88 – 7.12	0.406	-0.72	-5.02 – 3.58	0.743	1.00	0.86 – 1.16	0.968
F. nucpolymorph	-2.53	-14.59 – 9.53	0.681	-1.61	-11.98 – 8.75	0.76	0.96	0.67 – 1.38	0.831
N. mucosa	-0.46	-4.88 – 3.95	0.837	-0.58	-4.38 – 3.21	0.764	0.96	0.84 – 1.10	0.557
P. acnes	-3.60	-17.65 – 10.46	0.616	-8.82	-20.90 – 3.25	0.152	0.76	0.50 – 1.16	0.209
S. mitis	-0.59	-5.45 – 4.28	0.814	-0.6	-4.78 – 3.58	0.778	0.92	0.79 – 1.06	0.242
S. mutans	6.04	-4.68 – 16.77	0.269	5.83	-3.38 – 15.05	0.215	1.23	0.89 – 1.69	0.21
S. noxia	-0.07	-11.48 – 11.34	0.99	-3.94	-13.74 – 5.87	0.431	0.83	0.59 – 1.17	0.289
S. salivarius	1.09	-5.56 – 7.73	0.748	-1.01	-6.72 – 4.70	0.728	0.98	0.80 – 1.20	0.843
V. parvula	-0.73	-7.25 – 5.78	0.826	0.13	-5.47 – 5.73	0.964	1.10	0.91 – 1.34	0.325

Changes in SBP and DBP (at visit-2), due to changes in oral-NRB (at baseline, visit-1) has been estimated using linear regression analysis, while adjusting for baseline confounding (age, sex, BMI, and percent gingival inflammation) using propensity score method. While adjusted odds (AOR) of having hypertension in the KHLS cohort at visit-2 were estimated using logistic regression analysis while adjusting for baseline confounding using propensity score method.

Supplement Table-2 demonstrates the results of linear and logistic regression analysis. This analysis was aimed at predicting hypertension and changes in systolic and diastolic blood pressure, at visit-2, concerning changes in the abundance of oral-NRB. We found that a reduction in *Prevotella melaninogenica* was associated with an increase of 4.59mmHg DBP (95% CI=-8.94,-0.25), and a 10% higher risk of having hypertension (AOR=0.90, 95%CI=0.78,1.05), at visit-2.

Supplement Table 3 (a) and (b): Internal Validity - Multiple linear and logistic regression models were compared with periodic adjustments, to ensure that the estimated odds of hypertension, and estimated changes in systolic and diastolic blood pressure (E in mmHg), concerning changes in Actinomyces (table 3a), and Streptococcus (table 3b), are valid, and not confounded by other potential confounders.

Table 3a.	**Hypertension**			**Systolic Blood Pressure**			**Diastolic Blood Pressure**		
Actinomyces	AOR	95% CI	p-value	E (mmHg)	95% CI	p-value	E (mmHg)	95% CI	p-value
Model.1	0.87	0.79 – 0.96	**0.005**	-3.14	-6.13 – -0.16	**0.039**	-5.95	-9.81 – -2.08	**0.003**
Model.2	0.87	0.79 – 0.96	**0.006**	-3.12	-6.10 – -0.13	**0.041**	-5.93	-9.79 – -2.06	**0.003**
Model.3	0.87	0.79 – 0.96	**0.006**	-3.15	-6.14 – -0.15	**0.039**	-5.83	-9.71 – -1.96	**0.003**
Model.4	0.88	0.80 – 0.96	**0.006**	-3.10	-6.09 – -0.10	**0.043**	-5.80	-9.68 – -1.92	**0.003**
Table 3b.	**Hypertension**			**Systolic Blood Pressure**			**Diastolic Blood Pressure**		
Streptococcus	AOR	95% CI	p-value	E (mmHg)	95% CI	p-value	E (mmHg)	95% CI	p-value
Model.1	0.82	0.71 – 0.96	**0.013**	-5.67	-10.44 – -0.90	**0.020**	-5.90	-12.07 – 0.28	0.061
Model.2	0.81	0.70 – 0.95	**0.008**	-6.27	-11.04 – -1.51	**0.010**	-6.30	-12.48 – -0.12	**0.046**
Model.3	0.82	0.70 – 0.95	**0.010**	-6.08	-10.85 – -1.30	**0.013**	-6.07	-12.26 – 0.11	0.054
Model.4	0.82	0.70 – 0.95	**0.010**	-6.05	-10.82 – -1.27	**0.013**	-6.05	-12.24 – 0.13	0.055

Model.1 adjusted for age, sex, BMI, percent gingival redness; **Model.2** adjusted for Model.1 + Salivary glucose concentration; **Model.3** adjusted for Model.2 + Percentage of Decay/Filled teeth; **Model.4** adjusted for Model.3 + Salivary flow rate.
All periodic adjustments were calculated using the propensity score method. HTN (hypertension); SBP (systolic blood pressure); DBP (diastolic blood pressure); AOR (logistic regression - adjusted odds ratios); E (linear regression estimates showing changes in blood pressure in mmHg, when adjusted across models 1 to 4); 95% CI (95% upper and lower confidence intervals); p-value≤0.05 is considered significant.

We also assessed the internal validity of our study as shown in **Supplement Table-3**. Here, we validated whether the changes in blood pressure (SBP and DBP), and odds of having hypertension, concerning changes in the abundance of Actinomyces (Table-3a), and Streptococcus genera (Table-3b) are consistent when periodically adjusted with other study variables. We obtained consistently robust estimates, and odds ratios, when we additionally adjusted for salivary glucose concentration, percentage of decay and filled teeth, and salivary flow rate.

Supplement Table 4: Sensitivity Analysis to assess odds of prevalent (percentile-based) hypertension, concerning changes in the abundance of nitrate, and nitrite-reducing oral bacteria (NRB).

Percentile-based Hypertension			
Predictors	*AOR*	*95% CI*	*p-value*
Nitrate-reducing species			
A. israelii	0.63	0.47 – 0.85	**0.002***
A. naeslundii	0.82	0.62 – 1.09	0.173
A. odontolyticus	0.73	0.60 – 0.89	**0.002***
A. viscosus	0.76	0.58 – 1.00	**0.047**
C. sputigena	1.18	0.95 – 1.45	0.128
E. corrodens	1.19	1.05 – 1.36	**0.007**
F. nucpolymorph	0.94	0.69 – 1.29	0.713
P. acnes	0.56	0.39 – 0.81	**0.002***
S. noxia	0.73	0.54 – 0.98	**0.034**
Nitrite-reducing species			
P. melaninogenica	0.9	0.79 – 1.03	0.115
S. mitis	1.04	0.91 – 1.18	0.572
S. mutans	0.79	0.60 – 1.04	0.093
S. salivarius	0.84	0.71 – 1.00	**0.050**
Nitrate, and Nitrite-reducing species			
N. mucosa	1.03	0.92 – 1.16	0.567
V. parvula	0.95	0.80 – 1.13	0.556
Nitrate & Nitrite – reducing genera			
Actinomyces[1]	0.87	0.80 – 0.95	**0.001***
Streptococcus[2]	0.85	0.75 – 0.98	**0.020**

The table presents the results of logistic regression analysis, demonstrating an association between binary hypertension (outcome) and oral-NRB (microbial predictors). Here hypertension was defined as ≥90th percentile to <95th percentile. Accordingly, the prevalence of percentile-based hypertension was 23.79%. Significant p-values (<0.05) are listed in boldface type. *Represents significant after Bonferroni correction (p-value≤0.003). [1]*Actinomyces* genera (nitrate-reducers) represents a cumulative additive score from four species: *A.israelii, A.naeslundii, A.odontolyticus, A.viscosus.* Whereas, [2]*Streptococcus* genera (nitrite-reducers) represents a cumulative additive score from two species: *S. mutans, and S. salivarius.* The results are presented as adjusted odds ratios (AOR), with 95% confidence intervals (CI), and p-values. The adjustment was done with age, sex, BMI, and percent gingival inflammation using the propensity score method. AOR<1 represent a reduction in bacterial numbers is associated with hypertension, while AOR>1 represents an increase in bacterial numbers is associated with hypertension.

Summary of Project-I

Given that Kuwait exhibits a very high prevalence of adolescent obesity, it allowed us to register new cases of obesity within a two-year time frame. Hence, we were able to conduct this study that has compared longitudinal changes in oral microbiota across healthy, overweight, and obese phenotypes within the adolescent population, at two-time points. Further, we were able to ascertain changes in host inflammatory and metabolic pathways, using surrogate salivary biomarkers. While we observed that oral microbiota is sensitive to changes in host phenotype, we also observed phenotype-dependent, phylum-specific, effect-modification of oral microbiota with high insulin, high salivary glucose concentration, low salivary HDLC levels, and gingivitis.

One of the principal microbial findings in our study was that a relatively higher oral *Firmicutes-to-Bacteroidetes (F-to-B)* ratio was observed in overweight subjects, relative to healthy and obese subjects. A higher *F-to-B*-ratio is attributed to the gut microbiota of obese subjects. Increased abundance of *Firmicutes* tends to digest otherwise indigestible polysaccharides – which play a key role in the pathogenesis of obesity. Besides, higher proportions of pro-inflammatory, Lipopolysaccharide (LPS) containing *Prevotella sp*., were observed in parallel with, a significant increase in CRP levels, in obese subjects, which is indicative of active systemic inflammation. It has been suggested that the bacterial-mediated-LPS, binds to human immune pattern-recognition-receptors such as the toll-like-receptor (TLR's), which initiates systemic-inflammation. Based on our findings, we hypothesize that oral bacteria may translocate to the gut and might set the stage for childhood obesity.

Summary of Project-II

In this longitudinal KHLS study, we enrolled 10-11-year-old, 8,173 Kuwaiti school-going children, during 182 visits, from 138 Kuwaiti schools, across all six Kuwaiti states (governates). At visit-2, two-years apart, we followed-up on 6,209 children. From our analysis, we learned that Kuwait has one of the highest prevalence of adolescent hypertension (nearly 40% within KHLS cohort), compared to 5.5% reported for the USA. This raises concerns about the involvement of multiple pathways responsible for a higher prevalence of cardio-metabolic risk factors amongst the KHLS cohort. We assessed the role of the "Enterosalivary-Nitrate-Nitrite-NO pathway" with pediatric hypertension.

We identified that a reduction in major *nitrate-reducing*, *Actinomyces sp*. (*A. odontolyticus, A. israelii*, and *A. viscosus*) was associated with a 3.14mmHg increase in systolic blood pressure, 5.95mmHg increase in diastolic blood pressure, and 5.01mmHg increase in mean arterial blood pressure. Whereas, a reduction in *nitrite-reducing, Streptococcus sp.* (*mutans* and *salivarius*) was associated with a 5.67mmHg increase in systolic blood pressure and a 5.82mmHg increase in mean arterial blood pressure. Besides, we found that a reduction in *nitrite-reducing, P. melaninogenica,* was associated with 19% risk of having hypertension (SBP/DBP>120/80 mmHg), while a 22% higher risk of having stage-I and II hypertension (when predicting SBP/DBP$\geq$130/80 mmHg), at visit-2.

Discussion and Perspectives – Project-I

The oral cavity is the gateway to the gut and serves as a microbial reservoir that constantly replenishes the gut flora. While numerous studies have implicated gut microbiota as a potential route towards the pathophysiology of obesity; little is known, however, about the influence of oral bacteria on obesity. This is the first longitudinal study, characterizing changes in oral microbiota with the development of adolescent obesity. While most data come from studies of the gut microbiome, perhaps one of the shortcomings of this study is that I did not characterize the gut microbiota. To understand these results with the development of obesity, I rely upon studies from other investigators from a different population. Further, I could not analyze the data from 28% of the originally sampled population, because of two main reasons – (i) poor sequencing quality, and (ii) loss to follow-up at the second visit. Given the limitations, I utilized the opportunity to generate a hypothesis for future investigators. I addressed the study aims broadly using cross-sectional, and longitudinal analysis.

While future studies are needed to validate these preliminary findings, I hypothesized that the translocation of LPS-containing, pathogenic, oral *Bacteroidetes* (esp. *Prevotella sp*.) to the gut, could be responsible for triggering metabolic endotoxemia. A sub-clinical, persistent, low-grade inflammation because of increased, circulating endotoxins – LPS, which plays a key role in the pathophysiology of obesity. To investigate changes in oral and gut microbiota with the development of obesity, future longitudinal studies should account for the measurement of salivary and plasma LPS levels.

Further, changes in oral microbiota could also reflect a tendency towards the development of obesity and related cardiometabolic risk factors. If the relationship proves to be causal, early screening, and manipulation of oral and gut microbiota, might also prevent the onset of obesity. On the other hand, the oral microbial assessment of individuals at high-risk for the development of obesity could also help alleviate the burden of oral diseases, resulting as a consequence of oral dysbiosis.

Discussion and Perspectives – Project-II

This is the first attempt to investigate a potential relationship between changes in nitrate and nitrite reducing oral bacteria, with the prevalence of pediatric hypertension, and onset. One of the limitations of this study is the use of DNA-DNA-hybridization (or checkerboard method). This might raise questions on the validity of the microbial assessment. While this approach to identify oral bacteria was groundbreaking in the early twenty-first century and helped shaped core concepts on periodontal microbiology, it was replaced by a high-throughput 16s-rRNA next-generation sequencing approach. The checkerboard lacks specificity at species-level, compared to sequencing methods. I justified the use of the checkerboard method, by examining for a selected group of oral bacteria that possess either nitrate, or nitrate-reducing capability, and the diagnostic accuracy has been correlated well with culture-based identification methods. On the other hand, the checkerboard approach was inexpensive and allowed the investigators to conduct a large-scale microbiological assessment of bacterial counts, which has added to the study's advantage.

While future longitudinal studies are needed to validate the study findings. I believe, that if the relationship between the reduction of nitrate/nitrite-reducing oral bacteria, and hypertension onset proves to be causal, in the future, the microbiological assessment amongst the pediatric population could predict adulthood hypertension. Manipulation of the oral microbiota at early stages, might also improve cardiovascular health and hence prevent hypertension-onset in adulthood. This may translate to clinical management by a 5mmHg reduction in blood pressure, which is estimated to reduce the risk of stroke by 34%, and ischemic heart disease by 21%.

Since numerous probiotic trials for periodontitis have shown promising results, there is a potential for developing targeted probiotics to replenish the oral microbiota involved in the enterosalivary-nitrate-nitrite-NO pathway. From this epidemiological study, and previous nutrition-based clinical trials, it seems that the enterosalivary route has a potential in the homeostasis of blood pressure. However, the results of future prospective studies and clinical trials, seem to dictate our approach in implementing oral probiotic therapy for the management of blood pressure.