The salivary microbiome is altered in the presence of a high salivary glucose concentration

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Abstract

Background
Type II diabetes (T2D) has been associated with changes in oral bacterial diversity and frequency. It is not known whether these changes are part of the etiology of T2D, or one of its effects.

Methods
We measured the glucose concentration, bacterial counts, and relative frequencies of 42 bacterial species in whole saliva samples from 8,173 Kuwaiti adolescents (mean age 10.00 ± 0.67 years) using DNA probe analysis. In addition, clinical data related to obesity, dental caries, and gingivitis were collected. Data were compared between adolescents with high salivary glucose (HSG; glucose concentration ≥ 1.0 mg/dL, n = 175) and those with low salivary glucose (LSG; glucose concentration < 0.1 mg/dL n = 2,537).

Results
HSG was associated with dental caries and gingivitis in the study population. The overall salivary bacterial load in saliva decreased with increasing salivary glucose concentration. Under HSG conditions, the bacterial count for 35 (83%) of 42 species was significantly reduced, and relative bacterial frequencies in 27 species (64%) were altered, as compared with LSG conditions. These alterations were stronger predictors of high salivary glucose than measures of oral disease, obesity, sleep or fitness.

Conclusions
HSG was associated with a reduction in overall bacterial load and alterations to many relative bacterial frequencies in saliva when compared with LSG in samples from adolescents. We propose that hyperglycemia due to obesity and/or T2D results in HSG and subsequent
acidiﬁcation of the oral environment, leading to a generalized perturbation in the oral microbiome. This suggests a basis for the observation that hyperglycemia is associated with an increased risk of dental erosion, dental caries, and gingivitis. We conclude that HSG in adolescents may be predicted from salivary microbial diversity or frequency, and that the changes in the oral microbial composition seen in adolescents with developing metabolic disease may the consequence of hyperglycemia.

**Introduction**

Potentially outnumbering human cells 10:1, the millions of bacteria, archaea, microbial eukaryotes, and viruses that inhabit the human body, collectively known as the microbiome, display a vast biological and functional diversity. Over the past 10 years, it has become clear that deﬁning a healthy human microbial state at various sites (e.g., oral cavity, intestines, skin) is a critical step for discovering how variations in the microbiome may contribute to or cause a wide range of human diseases [1]. Indeed, it is still unclear whether the differences in the human microbiome that are seen in many disease states are a symptom of the disease or part of the underlying etiology [2]. The Human Microbiome Project, and other related research efforts in both industry and academia (including our own [3–5]), are now striving to understand and clarify the variations in microbial communities found in people in conditions of both health and disease [6].

The oral microbiome is one of the most diverse microbial communities in the body, with as many as 700 species of bacteria colonizing the hard surfaces of teeth and the soft tissues of the oral mucosa [7, 8]. Although there are substantial differences in the bacterial diversity of the oral microbiome between healthy people, the bacterial diversity of the oral microbiome remains relatively constant for any given person over time when that person remains in a state of health [9–11]. As with other body sites in which the human microbiome has been studied, long-term changes in bacterial diversity, frequency, and count in the oral cavity are observed in a number of chronic disease states. Any condition resulting in xerostomia [12], such as Sjögren’s disease and radiation therapy to the head [13], has the potential to alter salivary bacterial parameters. Other conditions such as kidney disease [14], some cancers [4, 15], cardiovascular diseases [16], and obesity [3, 17] have also been associated with changes in salivary bacterial parameters. Interestingly, type II diabetes mellitus (T2D) has been associated with clear changes in bacterial diversity and frequency in supragingival plaque in a few studies; however, an association between T2D and changes in salivary bacterial parameters is less clear [18, 19].

The prevalence of metabolic disease and T2D in adolescents appears to be increasing. The prevalence of T2D among U.S. children and adolescents in 2009 was 0.46/1000 [20], a 30% increase since 2001. Hyperglycemia is pathognomonic for diabetes. Values for fasting blood glucose greater than 100 mg/dL but less than 124 mg/dL are considered evidence of the prediabetic condition known as impaired fasting glucose [21]. Sustained values of fasting blood glucose greater than 125 mg/dL are considered diagnostic of diabetes. Such high levels of blood glucose are associated with high levels of salivary glucose, which has been promoted as a useful salivary biomarker for T2D [22]. To examine potential changes in the count and/or diversity of the bacterial species present in saliva in adolescents with high and normal concentrations of salivary glucose, we collected and analyzed whole saliva samples from 8,173 10-year old Kuwaiti adolescents. This population is of particular interest because Kuwaiti adults have one of the highest levels of T2D in the world [23], making this a high-risk adolescent population.
for T2D. Our previous work has indicated that, in this population, fasting salivary glucose concentrations of less than 1 mg/dL correlate with plasma concentrations of less than 100 mg/dL (normal, healthy range). Fasting salivary glucose concentrations above 1 mg/dL indicate plasma glucose concentrations of 100 mg/dL or higher (hyperglycemic) [24]. Therefore, we used the whole genomic DNA probe method commonly referred to as the “checkerboard assay” to analyze the salivary microbiota of these Kuwaiti adolescents, comparing differences in overall bacterial load, bacterial species counts, and the relative frequency of bacterial species between the group of samples with normal salivary glucose concentration and those with high salivary glucose concentration.

**Materials and methods**

**Study population**

The Kuwait study population and design have been previously described [25]. All of the participants enrolled were native Kuwaitis in 4th or 5th grade. A total of 8,317 adolescents participated in the study during 182 visits to 138 Kuwaiti schools made by study personnel between October 2, 2011 and May 15, 2012. The focus of this analysis is saliva samples from 8,173 adolescents in which salivary glucose concentration, bacterial counts, and relative bacterial frequencies were all measured. The study was approved by the Dasman Diabetes Institute Ethical Review Committee in Kuwait. Arabic language written informed consent was signed by parents/guardians and participant assent was signed on the day of their evaluation.

**Clinical examination**

All clinical data were captured on tablet computers (iPad®, Apple Corporation, Cupertino, CA, USA). Height in centimeters was measured using a stadiometer, and weight in kilograms was measured using a digital scale. Waist circumference was measured at the midpoint between the bottom of the rib cage and above the top of the iliac crest. Body Mass Index (BMI) was calculated by dividing body weight in kilograms by height in meters squared. Body weight categories were determined from BMI percentile using International Diabetes Federation values [26] as follows: underweight, <5th percentile; normal weight, 5th–84th percentile; overweight, 85th–94th percentile; obese >95th percentile. Systolic and diastolic blood pressure were measured using pediatric blood pressure cuffs. High blood pressure was defined as systolic ≥130 or diastolic ≥85 mmHg [26]. Fitness was measured by heart rate elevation following a standardized exercise [27]. Weekday sleep was self-reported.

Oral examinations were conducted by dentists assisted by trained nurses using portable dental chairs, halogen lights and intraoral mirrors [28]. No radiographic images were taken for this study, and no dental explorers were used. For each participant, the examiner recorded the number of primary teeth, the number of permanent teeth, the number of teeth with fillings, and the number of teeth with visible unfilled decay.

**Saliva collection**

Saliva was collected from all participants between 8:30 am and 9:30 am under fasting conditions [25]. Each participant rinsed with and swallowed 15 mL of water before saliva collection. Adolescents were given a dated, labeled, sterile, 15-mL plastic screw-top centrifuge tube (Product #430791, Corning Incorporated Life Sciences, Tewksbury, MA, USA). Whole saliva (approximately 3 mL) was collected while keeping the tube on ice by having the participant drool into the screw-top tube. A staff monitor recorded the start time of the saliva collection, verified that approximately 3 mL was collected from each participant, recorded the stop time
for each participant, and transferred the labeled tube to an ice bath for temporary storage. The salivary flow rate was computed by dividing the tarred weight of the saliva collection tube by the difference in the start and stop collection times.

**Microbial assay**

Salivary microbiota were assayed using the whole genomic DNA probe method commonly referred to as the "checkerboard assay" [5]. By this method, numbers of bacteria are determined by comparison with linear regression of response from $10^5$ and $10^6$ standards for each probe. Assays were conducted using a 0.2 mL whole saliva sample obtained before centrifugation from each subject. Cell wall disruption was performed by boiling after adding 0.1 mL of 0.5 N NaOH and neutralizing by addition of 5 M ammonium acetate. It should be noted that dead cells with intact DNA can be measured by this technique but values obtained have acceptable association with those made by culture [29]. Samples were applied to the surface of a nylon membrane in a Minislot™ device (Immunetics, Cambridge, MA, USA) and evaluated by DNA probes to 42 species. Bacterial DNA were fixed to the membrane by ultraviolet exposure. Bacterial numbers were determined by image analysis of scanned samples (Typhoon™ Molecular Imager, GE Healthcare Life Sciences, Pittsburgh, PA, USA) using a covalently-bound fluorescent marker (AttoPhos®, Amersham Life Sciences, Arlington Heights, IL, USA). On each membrane, a mixture of DNA from each probe species at concentrations equivalent to $10^5$ and $10^6$ cells was applied to provide quantitation standards for each probe species. The concentrations of DNA probes used was adjusted to detect approximately $10^4$ bacteria (sensitivity) with 93.5% of cross-reactions exhibiting less than 5% of the homologous probe signal (specificity) [5]. The genus, species, and source of the bacteria used for DNA probes are provided in Table 1.

**Salivary glucose analysis**

Methods for measuring salivary glucose concentration have been previously described [24]. Briefly, saliva samples were weighed and then centrifuged to remove particulate debris, and a 30-μL aliquot of saliva supernatant was assayed. The assay used the glucose oxidase method with a fluorescent dye (Glucose Colorimetric/Fluorometric Assay Kit #K606-100, BioVision, Inc, Mountain View, California, USA) measured at Ex/Em = 535/590 nm and adapted to work on a Tecan Freedom EVO® 150 robotic processor with an 8-channel liquid handling arm (Tecan Group Ltd, Männedorf, Switzerland). Fluorescence was measured by a spectrophotometer (Infinite® 200 Pro, Tecan Group Ltd, Männedorf, Switzerland) using reverse 96-well plate reading mode. The 3 sigma detection limit of the glucose assay was 0.002 mg/dL. Standards of 0.12, 0.24, 0.48 and 0.96 mg/dL were assayed in triplicate on each run.

**Statistical and analytical**

Dental caries were evaluated as a percentage by counting the number of teeth (primary and permanent) with visible caries, or cavities plus fillings, and dividing by the total number of teeth. Gingivitis was evaluated as a percentage by counting the number of red sites around both deciduous and permanent teeth and dividing by the total number of sites in the mouth (four total sites/tooth). Differences in discrete measures were tested by chi-square analysis. Differences in continuous clinical variables (age, percent carious teeth, percent gingival redness, BMI, average sleep duration, and fitness level) were tested by two-sample t-test. Differences between parameters related to salivary bacterial composition were tested using the Kruskal-Wallis method.
Table 1. Bacterial species used to make oligonucleotide DNA probes. The mean bacterial count and mean bacterial percent were averaged over the study population of 8,173 adolescents.

| Bacterial Name                                      | Abbreviation     | Phylum                  | ATCC        | Numbers/ml x 10^5 | Percent   |
|-----------------------------------------------------|------------------|-------------------------|-------------|-------------------|-----------|
| Actinomyces gerencseriae                           | A. gerencseriae  | Actinobacteria          | 23860       | 1.95 ± 2.08       | 1.53 ± 0.90 |
| Actinomyces israelii                               | A. israelii      | Actinobacteria          | 12102       | 1.36 ± 1.39       | 1.03 ± 0.69 |
| Actinomyces naeslundii                             | A. naeslundii    | Actinobacteria          | 12104       | 1.92 ± 1.71       | 1.68 ± 1.04 |
| Actinomyces odontolyticus (serotype I)             | A. odontolyticus | Actinobacteria          | 17929       | 4.25 ± 4.89       | 3.24 ± 1.91 |
| Actinomyces viscosus                               | A. viscosus      | Actinobacteria          | 43146       | 2.10 ± 2.24       | 1.63 ± 1.05 |
| Aggregatibacter actinomycetemcomitans (serotypes a & b) | A. actinomycetemcomitans | Proteobacteria       | 43718 (Y4) & 29523 | 1.22 ± 1.09 | 1.06 ± 0.73 |
| Campylobacter gracilis                             | C. gracilis      | Proteobacteria          | 33236 (1084) | 0.26 ± 0.81       | 0.19 ± 0.29 |
| Campylobacter rectus                               | C. rectus        | Proteobacteria          | 33238 (371)  | 0.97 ± 1.84       | 0.80 ± 1.38 |
| Campylobacter showae                               | C. showae        | Proteobacteria          | 51146       | 1.45 ± 1.56       | 1.17 ± 0.78 |
| Capnocytophaga gingivalis                          | C. gingivalis    | Bacteroides             | 33624 (27)  | 1.69 ± 1.85       | 1.34 ± 0.89 |
| Capnocytophaga ochracea                            | C. ochracea      | Bacteroides             | (25)        | 1.52 ± 1.11       | 1.35 ± 0.68 |
| Capnocytophaga sputigena                           | C. sputigena     | Bacteroides             | 33612 (4)   | 2.51 ± 2.22       | 2.29 ± 1.72 |
| Eikenella corrodens                                | E. corrodens     | Proteobacteria          | 23834       | 12.11 ± 79.35     | 7.35 ± 5.73 |
| Fusobacterium nucleatum subsp. nucleatum           | F. nuc. nuc.     | Fusobacteria            | 25586       | 1.96 ± 8.92       | 1.40 ± 1.36 |
| Fusobacterium nucleatum subsp. polymorph           | F. nuc. polymorph| Fusobacteria            | 10953       | 1.55 ± 1.70       | 1.28 ± 0.70 |
| Fusobacterium nucleatum subsp. vincentii           | F. nuc.vinc.     | Fusobacteria            | 49256       | 1.62 ± 8.51       | 1.19 ± 2.18 |
| Fusobacterium periodonticum                        | F. periodonticum | Fusobacteria            | 33693       | 2.63 ± 4.38       | 2.02 ± 1.49 |
| Gemella morbillorum                                | G. morbillorum   | Firmicutes              | 27824       | 1.78 ± 1.63       | 1.50 ± 0.83 |
| Lachnoanaerobaculum saburreum                     | L. saburreum     | Firmicutes              | 33271       | 1.16 ± 0.84       | 1.03 ± 0.58 |
| Leptotrichia buccalis                             | L. buccalis      | Fusobacteria            | 14201       | 1.10 ± 2.73       | 1.02 ± 0.96 |
| Neisseria mucosa                                   | N. mucosa        | Proteobacteria          | 19696       | 13.99 ± 14.24     | 12.17 ± 6.42 |
| Parvimonas micra                                  | P. micra         | Firmicutes              | 33270       | 0.94 ± 0.76       | 0.91 ± 0.69 |
| Peptostreptococcaceae nodatum                     | P. nodatum       | Firmicutes              | 33099       | 0.45 ± 0.47       | 0.37 ± 0.38 |
| Porphyromonas gingivalis                          | P. gingivalis    | Bacteroidetes           | 33277       | 2.54 ± 3.01       | 2.35 ± 2.92 |
| Prevotella intermedia                             | P. intermedia    | Bacteroidetes           | 25611       | 2.04 ± 5.36       | 1.44 ± 1.07 |
| Prevotella melaninogena                            | P. melaninogena  | Bacteroidetes           | 25845       | 9.15 ± 8.86       | 7.27 ± 4.09 |
| Prevotella nigrescens                              | P. nigrescens    | Bacteroidetes           | 33563       | 3.89 ± 4.32       | 2.96 ± 1.72 |
| Propionibacterium acnes (serotypes I & II)         | P. acnes         | Actinobacteria          | 11827 & 11828 | 0.55 ± 0.60       | 0.41 ± 0.35 |
| Selenomonas noxia                                  | S. noxia         | Firmicutes              | 43541       | 2.06 ± 1.85       | 1.72 ± 0.89 |
| Streptococcus anginosus                            | S. anginosus     | Firmicutes              | 33397       | 1.17 ± 1.04       | 0.95 ± 0.53 |
| Streptococcus constellatus                        | S. constellatus  | Firmicutes              | 27823 (M32b) | 1.72 ± 1.78       | 1.47 ± 0.99 |
| Streptococcus gordonii                            | S. gordonii      | Firmicutes              | 10558       | 2.15 ± 2.10       | 1.70 ± 0.82 |
| Streptococcus intermedius                         | S. intermedius   | Firmicutes              | 27335       | 1.76 ± 1.62       | 1.47 ± 0.88 |
| Streptococcus mitis                               | S. mitis         | Firmicutes              | 49456       | 11.53 ± 11.42     | 10.0 ± 5.77 |
| Streptococcus mutans                              | S. mutans        | Firmicutes              | 25175       | 1.44 ± 1.57       | 1.26 ± 0.79 |
| Streptococcus oralis                              | S. oralis        | Firmicutes              | 35037       | 6.85 ± 7.14       | 5.58 ± 2.57 |
| Streptococcus salivarius                          | S. salivarius    | Firmicutes              | 27945       | 5.01 ± 7.56       | 3.72 ± 2.46 |
| Streptococcus sanguinis                           | S. sanguinis     | Firmicutes              | 10566       | 3.00 ± 2.68       | 2.55 ± 1.34 |
| Tannerella forsythia                              | T. forsythia     | Bacteroidetes           | 43037 (338) | 0.61 ± 0.55       | 0.48 ± 0.35 |
| Treponema denticola                               | T. denticola     | Spirochaetes            | (B1)        | 0.61 ± 0.67       | 0.51 ± 0.52 |
| Treponema socranskii                              | T. socranskii    | Spirochaetes            | (D40DR2) (S1) | 0.66 ± 0.95       | 0.57 ± 0.91 |

(Continued)
The total number of bacteria in each sample was computed as the sum of the bacterial count for each of the 42 bacterial species probes used. Percentages of bacteria were calculated for each of the 42 species by dividing the bacterial count for each species by the sum of all bacteria counts measured in each sample. Bacterial numbers and percentages were computed as median values for 11 salivary glucose intervals which uniformly cover the range of values measured with LSG and HSG defined at the left and right extremes. These intervals (Fig 1) were 0\( \leq x < 0.1, 0.1 \leq x < 0.2 \ldots 0.9 \leq x < 1.0 \text{ and } x \geq 1.0.

We conducted subgroup analyses comparing results from the group (n = 2,537, 31%) of participants with low salivary glucose (LSG), which was defined as a salivary glucose concentration of less than 0.1 mg/dL, with those of the group of participants (n = 175, 2.1%) with high salivary glucose (HSG), which was defined as a salivary glucose concentration greater than or equal to 1.0 mg/dL. Differences in the total bacterial count per mL in samples between participants with LSG and those with HSG were tested for their ability to predict obesity by random forest analyses (Salford Systems, San Diego, CA, USA) that included dental caries, gingivitis, and BMI in the model, as well as by univariate receiver operating characteristic (ROC) analysis using linear support vector machines algorithms for multivariate analysis [30]. Statistically significant values were accepted when \( p < 0.001\), the Bonferroni adjusted \( p\)-value to obtain a familywise error rate of \( p < 0.05\) with 42 comparisons.

### Results

#### Patient characteristics

Saliva samples and clinical data were collected from 8,173 Kuwaiti adolescents aged 10.0 ± 0.67 years (Table 2). The mean saliva collection time for all participants was 8.1 ± 0.7 minutes. There were no significant age or BMI differences between sexes. The total study population contained significantly more girls (61.1%) than boys (38.9%), and a significantly higher percentage of boys were obese (38.2%) than were girls (31.0%). The average salivary flow rate was also significantly higher in boys (28.22 ± 17.06 mL/h) than girls (24.64 ± 14.36 mL/h). The mean salivary glucose concentration was significantly higher among boys (0.22 ± 0.28 mg/dL) than girls (0.18 ± 0.22 mg/dL). Boys had a larger percentage of carious or filled teeth and more untreated carious teeth than girls. Gingival redness was high in both boys and girls but was slightly higher among the girls.

#### Distribution of salivary glucose concentrations

Salivary glucose concentrations in study subjects were distributed as illustrated in Fig 1. HSG (salivary glucose concentration >0.1 mg/dL) was found in 2.1% of the population (n = 175).
LSG (salivary glucose concentration ≤0.1 mg/dL) was found in 31% of the population (n = 2,537).

Salivary glucose concentration differences and clinical characteristics

We compared clinical characteristics among the 2,712 participants who exhibited either LSG or HSG (Table 3). The analysis revealed striking differences in oral disease parameters between the LSG and HSG groups, but only relatively small differences in parameters related to metabolic disease. Among the oral tissue parameters, both dental caries and gingivitis were significantly increased with HSG. However, measures of obesity (BMI and waist circumference), fitness level, weekday sleep duration, and blood pressure were not significantly different between the groups. Among those adolescents in the HSG group, 57 (33%) were obese, 52
Salivary glucose (mg/dL) 0.19
Salivary flow rate (mL/h) 26.02
Age (y) 10.00

Obese (%) 2,789 (34.1%) 1,215 (38.2%) 1,574 (31.0%)

Gingival redness (%) 72.55
Carious teeth (% decayed) 5.43
Fitness (beats/min) 25.29
Sleep (hr) 8.82
Diastolic blood pressure (mmHg) 73.94
Systolic blood pressure (mmHg) 109.10
Age (y) 10.16

BMI (kg/m²) 20.89
Waist circumference (cm) 68.09
Systolic blood pressure (mmHg) 109.73

Carious teeth (%) 6.97
Fitness (beats/min) 25.29
Sleep (hr) 8.82
Diastolic blood pressure (mmHg) 73.94
Systolic blood pressure (mmHg) 109.10
Age (y) 10.16

Total number of bacteria (x 10⁵/mL) 124 ± 126

Salivary microbiome altered with high salivary glucose concentration

(30%) had high blood pressure, and 29 (17%) both had high blood pressure and were obese (Table 4). Of note, 95 adolescents with HSG (54%) were of normal weight and did not have high blood pressure, demonstrating that HSG was often present in the saliva of adolescents who did not exhibit clinical signs of metabolic disease. In the LSG group, 868 (34%) were obese, 589 (23%) had high blood pressure, and 346 (14%) both had high blood pressure and were obese.

Bacterial species counts in saliva samples were measured by hybridization with whole genomic probes (Table 1). Over 50% of the assayed bacteria were accounted for by seven species (N. mucosa, E. corrodens, S. mitis, P. melaninogenica, V. parvula, S. oralis and S. salivarius). Bacterial species present with the highest counts in the saliva samples included N. mucosa, E. corrodens and S. mitis, each present at mean concentrations of greater than 10⁵/mL. Bacteria with the lowest counts included P. nodatum and C. gracilis, each present at mean concentrations of less than 0.5 x 10⁵/mL.

**Table 2. Demographic and clinical variables for the total study population of 8,173 adolescents by sex.**

| Variable                      | Overall | Male | Female | P-value |
|-------------------------------|---------|------|--------|---------|
| N (%)                         | 8,173 (100%) | 3,181 (38.9%) | 5,068 (61.1%) | <0.001 |
| Obese (%)                     | 2,789 (34.1%) | 1,215 (38.2%) | 1,574 (31.0%) | <0.001 |
| Age (y)                       | 10.00 ± 0.67 | 9.99 ± 0.67 | 10.00 ± 0.67 | 0.5 |
| Salivary flow rate (mL/h)     | 26.02 ± 15.56 | 28.22 ± 17.06 | 24.64 ± 14.36 | <0.001 |
| Salivary glucose (mg/dL)      | 0.19 ± 0.24 | 0.22 ± 0.28 | 0.18 ± 0.22 | <0.001 |
| BMI (kg/m²)                   | 20.89 ± 5.21 | 20.92 ± 5.37 | 20.83 ± 5.12 | 0.5 |
| Carious or filled teeth (%)   | 10.92 ± 10.41 | 11.87 ± 10.82 | 10.43 ± 10.16 | <0.001 |
| Carious teeth (%)             | 6.97 ± 8.96 | 7.73 ± 9.35 | 6.58 ± 8.73 | <0.001 |
| Red gingival sites (%)        | 74.85 ± 21.16 | 73.47 ± 21.78 | 75.27 ± 21.07 | <0.001 |
| Total number of bacteria (x 10⁵/mL) | 124 ± 126 | 122 ± 166 | 126 ± 95 | 0.2 |

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**Table 3. Clinical characteristics (mean ± standard deviation) of adolescents with LSG and HSG.**

| Clinical characteristics       | LSG (n = 2537) | HSG (n = 175) | % difference | Hypothesis testing (p, t) |
|-------------------------------|----------------|---------------|--------------|--------------------------|
| Carious teeth (% decayed)     | 5.43 ± 7.71 | 8.35 ± 8.55 | 53.9 | <0.001 , -4.40 |
| Gingival redness (% red)      | 72.55 ± 19.94 | 78.58 ± 19.45 | 8.3 | <0.001 , -3.96 |
| Total number of bacteria (x 10⁵/mL) | 123.97 ± 86.64 | 67.61 ± 52.39 | -45.5 | <0.001 , 13.05 |
| Saliva flow rate (ml/h)       | 25.83 ± 15.61 | 27.93 ± 16.79 | 8.2 | 0.1, -1.61 |
| BMI (kg/m²)                   | 21.12 ± 5.22 | 20.65 ± 5.45 | -2.2 | 0.3, 1.10 |
| Waist circumference (cm)      | 68.09 ± 21.56 | 67.06 ± 12.41 | -1.5 | 0.3, 1.00 |
| Fitness (beats/min)           | 25.29 ± 19.17 | 25.61 ± 22.01 | 1.3 | 0.9, -0.19 |
| Sleep (hr)                    | 8.82 ± 1.60 | 9.09 ± 1.67 | 3 | 0.04, -2.06 |
| Diastolic blood pressure (mmHg) | 73.94 ± 13.25 | 75.00 ± 14.10 | 1.4 | 0.3, -0.97 |
| Systolic blood pressure (mmHg) | 109.10 ± 16.61 | 109.73 ± 18.36 | 0.6 | 0.7, -0.44 |
| Age (y)                       | 10.16 ± 0.66 | 9.89 ± 0.70 | -2.6 | <0.001 , 4.89 |

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Salivary glucose concentration. The only bacterial species to increase in count (p < 0.001, NS) with increasing salivary glucose concentration was *P. micra* (Fig 2G).

The median bacterial count under HSG and LSG conditions for each species are listed in Table 5. About 88% of the species (n = 37) were found to have statistically significant (p < 0.001) changes in count between the LSG and HSG conditions. The order of reduction was in the direction of the reported aciduric strength of the bacterial species, with *Prevotella* spp. being most sensitive and *S. mutans* among the most resistant.

Salivary bacterial load predicts salivary glucose concentration

Using a random forest analysis and a multivariate ROC analysis, the overall bacterial load was found to accurately predict HSG (Table 5). The area under the curve was 0.949 by random forest analysis and 0.935 by multivariate ROC analysis. When clinical measures (sex, BMI, fitness level, sleep duration, percentage of carious teeth, and gingival redness) were included, they were given a random forest importance of less than or equal to 13.2 in prediction of HSG. In the random forest analysis, the most predictive individual bacterial species was *A. actinomycetemcomitans*, for which a decrease in bacterial count predicted HSG (AUC = [0.79]). In the multivariate ROC analysis, the most predictive individual bacterial species was *P. melaninogenica*, for which a decrease in bacterial count predicted HSG (AUC = 0.83).

Relative bacterial species frequency is altered with high salivary glucose

We next determined if the relative frequency of each bacterial species to the overall mean salivary bacterial count differed between HSG and LSG conditions. For this analysis, we considered the bacterial count for each species under conditions of LSG as representative of the normal, healthy state. We then computed the percent difference in bacterial count for each species between the LSG and HSG conditions (Table 6). A significant change in percentage was seen for 26 (62%) of the bacterial species under conditions of HSG. Of these, 15 (36%) species decreased in percentage, while 11 (27%) bacterial species increased in percentage. The remaining 16 (38%) did not significantly change in percentage between the LSG and HSG conditions. The *Prevotella* spp exhibited the largest percentage reduction in the HSG condition, while the percentage of *S. mutans* did not significantly change between conditions. *P. micra* exhibited both the highest univariate AUC (0.82) and was also the most important species in the random forest analysis in prediction of HSG.

Phylum counts and relative frequency are altered with high salivary glucose

We also analyzed phylum counts and found that all decreased under HSG conditions when compared with counts seen under LSG conditions (Table 7). The phylum *Bacteroidetes* decreased to the greatest extent. Relative phyla frequencies under conditions of LSG and HSG

| Body weight & salivary glucose category | Normal BP | High BP | Total |
|----------------------------------------|-----------|---------|-------|
| Normal weight-HSG                       | 95 (54%)  | 23 (13%)| 118 (67%)|
| Obese-HSG                              | 28 (16%)  | 29 (17%)| 57 (33%) |
| Total-HSG                              | 123 (70%) | 52 (30%)| 175 (100%)|
| Normal weight-LSG                       | 1426 (56%)| 243 (10%)| 1669 (66%)|
| Obese-LSG                              | 522 (21%) | 346 (14%)| 868 (34%) |
| Total-LSG                              | 1948 (77%)| 589 (23%)| 2537 (100%)|

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are listed in Table 8. The phylum Bacteroidetes decreased to the greatest extent (-5.7%) under HSG conditions, whereas the phylum Firmicutes increased by the greatest extent (4.3%) under HSG conditions.

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Fig 2. Bacterial count (median number x 10⁵/mL) in saliva with increasing salivary glucose concentration. The total bacterial load (sum of the 42 species measured) is shown in (A). The mean bacterial count of the 42 bacterial species evaluated are shown in (B) through (H).

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Table 5. Median bacterial counts (number/mL x 10^{-5}) in LSG and HSG conditions, sorted by univariate area under the curve (AUC). Percent difference was computed as [100 x (HSG-LSG)/LSG] between the HSG group and the LSG group. Negative values represent a reduction in bacterial count. The random forest ROC area under the curve was 0.935. Source data is listed in S1 Table.

| Bacteria                  | Random Forest Importance | Univariate AUC | Low salivary glucose (LSG) | High salivary glucose (HSG) | % difference |
|---------------------------|--------------------------|----------------|---------------------------|-----------------------------|--------------|
| *P. melanogenica*         | 81.3                     | 0.83           | 8.25                      | 1.78                        | -78.5        |
| *P. nigrescens*           | 79.5                     | 0.81           | 3.41                      | 1.12                        | -67.1        |
| *P. intermedia*           | 90.7                     | 0.81           | 1.66                      | 0.37                        | -77.5        |
| *A. actinomycetemcomitans*| 100.0                    | 0.79           | 1.29                      | 0.45                        | -64.6        |
| *P. acnes*                | 34.9                     | 0.78           | 0.44                      | 0.08                        | -82.5        |
| *A. odontolyticus*        | 24.6                     | 0.77           | 3.15                      | 1.19                        | -62.2        |
| *A. gerencseriae*         | 38.3                     | 0.76           | 1.68                      | 0.68                        | -59.5        |
| *G. morbillorum*          | 43.1                     | 0.75           | 1.77                      | 0.77                        | -56.2        |
| *S. salivarius*           | 12.7                     | 0.75           | 3.51                      | 1.62                        | -53.8        |
| *A. viscosus*             | 29.1                     | 0.74           | 1.67                      | 0.81                        | -51.7        |
| *E. corrodens*            | 11.8                     | 0.74           | 6.30                      | 2.57                        | -59.2        |
| *A. israelii*             | 31.0                     | 0.74           | 1.18                      | 0.42                        | -64.0        |
| *V. parvula*              | 16.4                     | 0.73           | 6.56                      | 2.73                        | -58.4        |
| *N. mucosa*               | 12.8                     | 0.73           | 11.21                     | 5.57                        | -50.3        |
| *F. periodonticum*        | 15.2                     | 0.73           | 1.86                      | 1.14                        | -38.9        |
| *S. mutans*               | 22.4                     | 0.73           | 1.43                      | 0.85                        | -40.5        |
| *C. gingivalis*           | 5.4                      | 0.72           | 1.35                      | 0.81                        | -39.9        |
| *S. oralis*               | 17.2                     | 0.72           | 5.84                      | 2.63                        | -55.0        |
| *A. naeslundii*           | 23.4                     | 0.71           | 1.58                      | 1.01                        | -35.9        |
| *S. anginosus*            | 22.9                     | 0.71           | 1.14                      | 0.46                        | -59.8        |
| *F. nucleomorphus*        | 27.0                     | 0.71           | 1.44                      | 0.80                        | -44.7        |
| *S. mitis*                | 10.5                     | 0.71           | 9.38                      | 4.78                        | -49.0        |
| *T. forsythia*            | 6.4                      | 0.70           | 0.57                      | 0.21                        | -63.1        |
| *S. noxia*                | 9.7                      | 0.70           | 1.76                      | 1.09                        | -38.2        |
| *P. gingivalis*           | 59.7                     | 0.69           | 1.88                      | 0.88                        | -53.1        |
| *C. ochracea*             | 21.4                     | 0.69           | 1.42                      | 0.96                        | -32.4        |
| *C. showae*               | 14.0                     | 0.69           | 1.13                      | 0.57                        | -49.9        |
| *S. gordonii*             | 13.3                     | 0.69           | 1.76                      | 0.89                        | -49.5        |
| *F. nucleomorphus*        | 26.1                     | 0.68           | 1.40                      | 0.98                        | -29.8        |
| *C. gracilis*             | 8.6                      | 0.68           | 0.18                      | 0.06                        | -66.5        |
| *P. nodatum*              | 9.6                      | 0.64           | 0.35                      | 0.20                        | -43.0        |
| *L. saburreum*            | 16.1                     | 0.62           | 1.18                      | 0.99                        | -16.3        |
| *S. constellatus*         | 24.7                     | 0.61           | 1.31                      | 1.02                        | -22.2        |
| *S. intermedius*         | 27.4                     | 0.61           | 1.29                      | 0.89                        | -30.9        |
| *S. sanguinis*            | 11.5                     | 0.59           | 2.07                      | 1.56                        | -24.5        |
| *F. nucleomorphus*        | 26.1                     | 0.59           | 1.01                      | 0.79                        | -22.2        |
| *C. rectus*               | 30.9                     | 0.58           | 0.31                      | 0.19                        | -39.6        |
| *T. denticola*            | 9.6                      | 0.57           | 0.39                      | 0.27                        | -30.7        |
| *C. sputigena*            | 23.4                     | 0.56           | 1.68                      | 1.28                        | -23.9        |
| *P. micra*                | 51.3                     | 0.55           | 0.75                      | 0.92                        | 22.8         |
| *T. socrauski*            | 35.3                     | 0.54           | 0.25                      | 0.20                        | -21.2        |
| *L. buccalis*             | 22.3                     | 0.52           | 1.01                      | 0.95                        | -6.0         |

**Clinical measures**

| Age (y) | 13.2 | 0.62 | 10.1 | 9.7 | -4.0 |

(Continued)
Discussion

In the current study of samples of whole saliva from 8,173 Kuwaiti adolescents, we conducted a checkerboard assay to analyze the salivary microbiota, comparing differences in overall bacterial load and the count and relative frequency of 42 different bacterial species between the group of samples with LSG and the group with HSG. We found that over 50% of the assayed bacteria overall were accounted for by seven species (N. mucosa, E. corrodens, S. mitis, P. melaninogenica, V. parvula, S. oralis, and S. salivarius). The overall mean salivary bacterial count decreased with increasing salivary glucose concentration. Indeed, overall salivary counts more accurately predicted HSG in this cohort than did clinical measures, including sex, BMI, fitness level, sleep duration, percentage of decayed teeth, and gingival redness. When considering the individual bacterial species, 88% of the 42 species exhibited a statistically significant difference in count between LSG and HSG conditions, and 62% exhibited a statistically difference in relative frequency between LSG and HSG conditions. For those species that displayed a reduced bacterial count and/or frequency under HSG conditions, the magnitude of the difference reflected the growth sensitivity of each bacterial species to an acidic environment.

Since glucose is a well-known energy source for many oral bacteria, it is no surprise that alterations in the salivary glucose concentration would affect the salivary microbiome. However, we did not expect overall bacterial counts to decrease with increasing concentrations of glucose. This is particularly true given that many of the species most affected by increases in salivary glucose concentration, including P. nigrescens, P. intermedia, and P. melaninogenica, are asaccharolytic. Therefore, we propose the following hypothesis to account for the observed changes (Fig 3).

The central theme of our hypothesis is that hyperglycemia changes the oral microbial environment by salivary acidification. The first step in this process (Fig 3, Point 1) is the increase in salivary glucose concentration that occurs with hyperglycemia in blood. Glucose transporters have been found in both the acinar and ductal cells of rodent salivary glands [31]. The positioning of the GLUT1, GLUT4, and SGLT1 transporters suggests that glucose may be transported from blood to saliva into the collecting duct to sustain ductal cellular metabolism. Meta-analyses of controlled studies demonstrate elevated salivary glucose concentrations in patients with both type 1 and type 2 diabetes [22]. We have previously shown that the salivary glucose concentration can be related to the blood glucose concentration via a threshold model [24]. When the blood glucose concentration exceeds 84.8 mg/dL, glucose begins to appear in saliva. When the blood glucose concentration reaches hyperglycemic levels (≥100 mg/dL), the salivary concentration becomes ≥1 mg/dL. This threshold effect is hypothesized to occur.

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**Table 5. (Continued)**

| Bacteria                  | Random Forest Importance | Univariate AUC | Low salivary glucose (LSG) | High salivary glucose (HSG) | % difference |
|---------------------------|--------------------------|----------------|-----------------------------|------------------------------|--------------|
| % of teeth with untreated decay** | 0.8                      | 0.61           | 3.8                         | 7.1                          | 86.8         |
| % of red gingival sites**  | 0.0                      | 0.59           | 74.2                        | 83.3                         | 12.3         |
| Weekday sleep*            | 0.0                      | 0.55           | 9                           | 9                            | 0.0          |
| BMI                       | 0.0                      | 0.54           | 19.9                        | 18.9                         | -3.0         |
| Fitness*                  | 0.0                      | 0.50           | 24.5                        | 24                           | -1.0         |

*p<0.05  
**p<0.001

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Table 6. Median salivary bacterial percentages under conditions of LSG and HSG sorted by univariate AUC. Difference was computed as \([\text{HSG-LSG}]\), such that negative values represent a reduction in percentage as salivary glucose increases. The random forest ROC area under the curve = 0.968. Source data is listed in S2 Table.

| Bacteria % decrease with high glucose | Random Forest Importance | Univariate AUC | Low Salivary Glucose (LSG) | High Salivary Glucose (HSG) | Difference |
|--------------------------------------|--------------------------|----------------|----------------------------|-----------------------------|------------|
| **P. melanogenica**                  | 53.8                     | 0.80           | 7.51                       | 3.71                        | -3.80      |
| **P. nigrescens**                    | 78.0                     | 0.76           | 3.22                       | 1.91                        | -1.31      |
| **P. intermedia**                    | 68.5                     | 0.76           | 1.55                       | 0.75                        | -0.80      |
| **P. acnes**                         | 24.7                     | 0.74           | 0.41                       | 0.16                        | -0.25      |
| **A. actinomyctemcomitans**          | 52.0                     | 0.68           | 1.19                       | 0.79                        | -0.40      |
| **A. gerencseriae**                  | 74.3                     | 0.67           | 1.58                       | 1.20                        | -0.39      |
| **A. israelii**                      | 16.6                     | 0.65           | 1.07                       | 0.74                        | -0.32      |
| **A. odontolyticus**                 | 6.6                      | 0.64           | 2.99                       | 2.44                        | -0.55      |
| **A. viscosus**                      | 14.6                     | 0.61           | 1.61                       | 1.30                        | -0.31      |
| **T. forsythia**                     | 8.6                      | 0.60           | 0.49                       | 0.32                        | -0.17      |
| **S. salivarius**                    | 8.5                      | 0.60           | 3.40                       | 3.01                        | -0.39      |
| **G. morbillorum**                   | 18.3                     | 0.60           | 1.63                       | 1.41                        | -0.22      |
| **C. gracilis**                      | 4.5                      | 0.58           | 0.16                       | 0.12                        | -0.04      |
| **S. anginosus**                     | 16.9                     | 0.58           | 0.99                       | 0.85                        | -0.14      |
| **E. corrodens**                     | 6.5                      | 0.57           | 6.14                       | 5.30                        | -0.84      |
| **P. gingivalis**                    | 31.5                     | 0.57           | 1.63                       | 1.19                        | -0.44      |
| **C. gingivalis**                    | 2.9                      | 0.56           | 1.28                       | 1.13                        | -0.15      |
| **C. showae**                        | 10.1                     | 0.55           | 1.02                       | 0.91                        | -0.11      |
| **F. periodonticum**                 | 6.3                      | 0.55           | 1.88                       | 1.78                        | -0.11      |
| **V. parvula**                       | 8.5                      | 0.54           | 6.00                       | 5.56                        | -0.43      |
| **S. oralis**                        | 8.0                      | 0.53           | 5.37                       | 5.33                        | -0.04      |
| **F. nuc. polymorph.**               | 10.6                     | 0.51           | 1.39                       | 1.37                        | -0.01      |
| **C. rectus**                        | 29.7                     | 0.51           | 0.28                       | 0.27                        | -0.01      |
| **N. mucosa**                        | 9.0                      | 0.50           | 11.30                      | 10.83                       | -0.47      |

| Bacteria % increase with high glucose | Random Forest Importance | Univariate AUC | Low Salivary Glucose (LSG) | High Salivary Glucose (HSG) | Difference |
|--------------------------------------|--------------------------|----------------|----------------------------|-----------------------------|------------|
| **P. micra**                         | 100.0                    | 0.82           | 0.73                       | 1.51                        | 0.78       |
| **L. buccalis**                      | 39.0                     | 0.74           | 0.91                       | 1.54                        | 0.63       |
| **C. sputigena**                     | 58.9                     | 0.73           | 1.63                       | 2.52                        | 0.89       |
| **S. sanguinis**                     | 37.9                     | 0.71           | 1.96                       | 3.05                        | 1.09       |
| **S. constellatus**                  | 55.8                     | 0.64           | 1.20                       | 1.64                        | 0.44       |
| **F. nuc. vinc.**                    | 29.8                     | 0.63           | 0.86                       | 1.15                        | 0.28       |
| **S. intermedius**                   | 41.4                     | 0.61           | 1.20                       | 1.48                        | 0.28       |
| **L. saburreum**                     | 8.2                      | 0.60           | 1.04                       | 1.20                        | 0.15       |
| **T. denticola**                     | 17.9                     | 0.59           | 0.36                       | 0.52                        | 0.16       |
| **T. socranski**                     | 30.1                     | 0.59           | 0.23                       | 0.34                        | 0.11       |
| **C. ochracea**                      | 6.6                      | 0.58           | 1.34                       | 1.55                        | 0.21       |
| **F. nuc. nuc.**                     | 7.8                      | 0.57           | 1.31                       | 1.42                        | 0.10       |
| **S. noxia**                         | 14.7                     | 0.56           | 1.67                       | 1.89                        | 0.22       |
| **S. mitis**                         | 7.7                      | 0.53           | 8.61                       | 9.27                        | 0.66       |
| **S. gordonii**                      | 5.9                      | 0.52           | 1.63                       | 1.72                        | 0.10       |
| **P. nodatum**                       | 5.6                      | 0.51           | 0.33                       | 0.37                        | 0.04       |
| **S. mutans**                        | 18.7                     | 0.51           | 1.33                       | 1.40                        | 0.07       |
| **A. naeslundii**                    | 3.5                      | 0.51           | 1.49                       | 1.61                        | 0.12       |

(Continued)
when glucose transport exceeds ductal cell uptake in conditions of hyperglycemia, such as in T2D. When glucose appears at a high concentration in saliva, one would expect an increased level of bacterial synthesis of acidic metabolites (Fig 3, Point 2), given that between 39.9% and 44.2% of salivary bacteria are acidogenic Firmicutes (Table 8). Indeed, the presence of dietary carbohydrates is well recognized as a stimulus for lowered salivary pH due to incomplete carbohydrate metabolism by oral acidogenic bacteria [32]. Further, the rapid lowering of pH in dental biofilms following exposure to glucose rinses (the “Stephan curve”) has been known for over 70 years [33]. Many investigators have reported that the salivary pH is lower in adults and adolescents with diabetes than it is in healthy adults and adolescents [34–36]. Reported differences are of sufficient magnitude to be measured by pH paper [37, 38], and to have been proposed as a simple screening measure for detection of metabolic syndrome or diabetes [39]. Therefore, according to our model, blood hyperglycemia in patients with T2D will lead to elevated levels of salivary glucose concentration, an increased synthesis of acidic metabolites by oral acidogenic bacteria, and salivary acidification (Fig 3, Point 3).

Acidification is proposed to interfere with bacterial reproduction (Fig 3, Point 4), altering the relative bacterial species frequency and count of the oral microbiome. This proposal is consistent with the reduction of oral bacterial growth under conditions of lowered pH that has been reported in many studies [40–43]. Studies of acid resistance in oral bacteria [40, 41] indicate that S. mutans is more acid resistant than S. salivarius, and A. viscosus, which is also seen in Table 5. About 35% to 75% of S. oralis and Actinomyces populations are reported to be killed within 1 hour at pH 4.2–4.4 [42]. Other studies comparing oral bacterial growth on blood agar versus acid agar at pH 5 [43] found that the phyla Bacteroidetes, Fusobacteria, and

### Table 6. (Continued)

| Clinical measures                        | Median Bacteria Percentage |
|-----------------------------------------|----------------------------|
| Age (y)**                                | 13.4                       |
| % of teeth with untreated decay**       | 1.2                        |
| % of red gingival sites**               | 0.1                        |
| Weekday sleep*                          | 0.7                        |
| BMI                                     | 2.2                        |
| Fitness                                 | 0.0                        |

*p<0.05  **p<0.001

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| Phylum                  | Random forest Importance | Univariate AUC | Low salivary glucose (LSG) | High salivary glucose (HSG) | % Difference | Hypothesis testing (p, \( \chi^2 \)) |
|-------------------------|--------------------------|----------------|---------------------------|---------------------------|--------------|-----------------------------------|
| Bacteroides             | 66.6                     | 0.79           | 21.8                      | 8.6                       | -60.8        | <0.0001, 170                     |
| Actinobacteria          | 100.0                    | 0.77           | 10.3                      | 4.2                       | -59.0        | <0.0001, 135                     |
| Proteobacteria          | 20.0                     | 0.71           | 20.8                      | 10.4                      | -50.1        | <0.0001, 104                     |
| Firmicutes              | 37.0                     | 0.69           | 42.5                      | 22.6                      | -46.8        | <0.0001, 89                      |
| Fusobacteria            | 20.7                     | 0.66           | 6.9                       | 4.9                       | -28.6        | <0.0001, 52                      |
| Spirochaetes            | 49.0                     | 0.53           | 0.7                       | 0.6                       | -7.7         | 0.3, 1.3                         |

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Proteobacteria were not significantly enhanced on pH 5 agar. Our study indicates that, of all the phyla tested, Bacteroidetes is most sensitive to increased salivary glucose concentration, though the species isolated were non-saccharolytic.

It is known that salivary acidification is a major factor in the development of both dental caries [44] and gingivitis [45, 46] because it alters the oral microbiome to favor caries-associated bacterial species, such as Bifidobacterium dentium, Bifidobacterium longum, and S. mutans [40]. An elevated salivary glucose concentration also appears, in our study, to increase the risk of dental erosion, dental caries, and gingivitis (Fig 3, Point 5). We observed that adolescents with HSG had almost twice the percentage of carious teeth than did adolescents with LSG (Table 3). We also observed that adolescents with HSG had a significantly increased

**Table 8. Median phyla frequencies under conditions of LSG and HSG.** Data are sorted by difference (HSG-LSG), such that negative values represent a decrease in percentage under HSG conditions. The random forest ROC area under the curve for phyla = 0.884. Source data is listed in S2 Table.

| Phyla                  | Median Bacteria % | Random forest Importance | Univariate AUC | Low salivary glucose (LSG) | High salivary glucose (HSG) | Difference | Hypothesis testing (p,χ²) |
|------------------------|-------------------|--------------------------|----------------|---------------------------|----------------------------|------------|--------------------------|
| Bacteroidetes          | 100.0             | 1.77                     | 20.3           | 14.6                      | -5.7                       | <0.001, 117 |                          |
| Actinobacteria         | 45.5              | 0.69                     | 9.6            | 7.6                       | -2.0                       | <0.001, 60 |                          |
| Proteobacteria         | 21.4              | 0.51                     | 20.7           | 20.4                      | -0.3                       | 0.3, 0.6   |                          |
| Phyla % increasing with high glucose |     |                          |                |                           |                            |            |                          |
| Firmicutes             | 36.2              | 0.64                     | 39.9           | 44.2                      | 4.3                        | <0.001, 23 |                          |
| Spirochaetes           | 70.8              | 0.62                     | 0.6            | 1.0                       | 0.4                        | <0.001, 37 |                          |
| Fusobacteria           | 45.1              | 0.60                     | 6.7            | 8.1                       | 1.4                        | <0.001, 32 |                          |

Proteobacteria were not significantly enhanced on pH 5 agar. Our study indicates that, of all the phyla tested, Bacteroidetes is most sensitive to increased salivary glucose concentration, though the species isolated were non-saccharolytic.

It is known that salivary acidification is a major factor in the development of both dental caries [44] and gingivitis [45, 46] because it alters the oral microbiome to favor caries-associated bacterial species, such as Bifidobacterium dentium, Bifidobacterium longum, and S. mutans [40]. An elevated salivary glucose concentration also appears, in our study, to increase the risk of dental erosion, dental caries, and gingivitis (Fig 3, Point 5). We observed that adolescents with HSG had almost twice the percentage of carious teeth than did adolescents with LSG (Table 3). We also observed that adolescents with HSG had a significantly increased
percentage of gingival redness compared with adolescents with LSG. This is in line with data from a study in which increased dental erosion was reported in patients with hyperglycemia and renal disease [47]. Consumption of sugar has long been associated with dental decay, even though consumed sugars are rapidly cleared from the oral cavity, with a halftime of about 2.2 minutes [32]. We propose that it is underlying hyperglycemia that is the real cause of dental decay, because patients with hyperglycemia can have persistent salivary glucose concentrations exceeding 1 mg/dL.

Both gingivitis and dental caries have been related to the development of obesity [32, 48] and T2D [49, 50]. Those investigating the role of bacteria in the gastrointestinal tract as a cause of obesity (“infectobesity”) have generally sought identification of specific pathogenic bacteria. These observations, though somewhat variable, have reported that obesity is associated with high proportions of *Firmicutes* and reduced proportions of *Bacteroidetes* [51] in the oral microbiome. This is similar to what we have found (Table 8). Identification of specific pathogenic bacteria, however, has eluded scientific enquiry. Our study suggests that differences in gut bacterial composition between people who are obese and those of normal weight may be largely the consequence of hyperglycemia, rather than the cause (Fig 3, Point 6). Also, since all gastric bacteria are introduced through the oral cavity, it is possible that a lowered salivary pH due to hyperglycemia may act as a filter to inhibit replenishment of gastric *Bacteroidetes*, while more facilely transmitting gastric *Firmicutes* species.

**Conclusions**

There are reports of major changes in the bacterial frequency and/or bacterial species counts in saliva from obese individuals compared with samples from those of healthy weight. T2D has been associated with changes in bacterial diversity and frequency in supragingival plaque, but an association between T2D and changes in salivary bacterial parameters has been less clear. In considering the role of oral bacteria in obesity and the development of T2D, one is hard pressed to determine whether the observed changes to the oral microbiome are the cause of these conditions, or one of the effects. In the current study, we have confirmed that the overall salivary bacterial load, as well as the bacterial counts and relative frequencies of various bacterial species, are significantly altered in adolescents with HSG. Our current study supports the idea that increased concentrations of salivary glucose may be the root cause of these perturbations in the oral microbiome, as has been suggested by others as well [52]. One cannot rule out the possibility that a bacterial species, such as that of *P. micra*, or a bacterial species we did not measure by the DNA probe method, could have a direct effect on the development of obesity and T2D. However, our data support a model in which hyperglycemia associated with obesity and T2D results in an increased salivary glucose concentration, which appears to reduce the salivary pH. This, in turn, reduces the overall bacterial count of the oral microbiome and alters the relative bacterial frequencies to favor aciduric bacterial species. This sequence of events suggests a basis for the observation that hyperglycemia is associated with an increased risk of dental erosion, dental caries, and gingivitis. Further studies will elucidate the utility of analyzing salivary bacterial load and species frequency as predictors for hyperglycemia and/or T2D.

**Supporting information**

S1 Table. The number of bacteria in each sample (x 10^5).
(XLSX)

S2 Table. The percentage of bacteria in each sample.
(XLSX)
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References

1. McDonald D, Birmingham A, Knight R: Context and the human microbiome. Microbiome 2015, 3:52.
2. Ursell LK, Clemente JC, Rideout JR, Gevers D, Caporaso JG, Knight R: The interpersonal and intraper-sonal diversity of human-associated microbiota in key body sites. The Journal of allergy and clinical immunology 2012, 129(5):1204–1208. doi: 10.1016/j.jaci.2012.03.010 PMID: 22541361
3. Goodson JM, Groppo D, Halem S, Carpino E: Is obesity an oral bacterial disease? J Dent Res 2009, 88 (6):519–523. doi: 10.1177/0022034509338353 PMID: 19587155
4.  Mager DL, Haftajee AD, Devlin PM, Norris CM, Posner MR, Goodson JM: The salivary microbiota as a diagnostic indicator of oral cancer: A descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. J TranslMed 2005, 3(1):27.

5.  Socransky SS, Haftajee AD, Smith C, Martin L, Haftajee JA, Uzel NG, et al.: Use of checkerboard DNA-DNA hybridization to study complex microbial ecosystems. Oral Microbiol Immunol 2004, 19(6):352–362. doi: 10.1111/j.1399-302x.2004.00168.x PMID: 15491460

6.  Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, et al: The NIH Human Microbiome Project. Genome research 2009, 19(12):2317–2323. doi: 10.1101/gr.006651.109 PMID: 19819907

7.  Duran-Pinedo AE, Frias-Lopez J: Beyond microbial community composition: functional activities of the oral microbiome in health and disease. Microbes and infection 2015, 17(7):505–516. doi: 10.1016j.micinf.2015.03.014 PMID: 2582077

8.  Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, et al: The NIH Human Microbiome Project. Genome research 2009, 19(12):2317–2323. doi: 10.1101/gr.006651.109 PMID: 19819907

9.  Belstrom D, Holmstrup P, Bardow A, Kokars A, Fiehn NE, Paster BJ: Temporal Stability of the Salivary Microbiota in Oral Health. PLoS One 2016, 11(1):e0147472. doi: 10.1371/journal.pone.0147472 PMID: 26799067

10.  Caporaso JG, Lauber CL, Costello EK, Berg-Lyons D, Gonzalez A, Stombaugh J, et al: Moving pictures of the human microbiome. Genome biology 2011, 12(5):R50. doi: 10.1186/gb-2011-12-5-r50 PMID: 21624126

11.  Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JJ, Knight R: Bacterial community variation in human body habitats across space and time. Science (New York, NY) 2009, 326(5960):1694–1697.

12.  Guobis Z, Kareiviene V, Baseviciene N, Paipaliene P, Niedzelskiene I, Sabalys G, et al: Microflora of the oral cavity in patients with xerostomia. Medicina (Kaunas) 2011, 47(12):646–651.

13.  Almstahl A, Wikstrom M, Fagerberg-Mohlin B: Microflora in oral ecosystems and salivary secretion rates—A 3-year follow-up after radiation therapy to the head and neck region. Arch Oral Biol 2015, 60(9):1187–1195. doi: 10.1016/j.archoralbio.2015.04.004 PMID: 26058004

14.  Ertugrul F, Elbek-Cubukcu C, Sabah E, Mir S: The oral health status of children undergoing hemodialysis treatment. Turk J Pediatr 2003, 45(2):108–113. PMID: 12921296

15.  Whitmore SE, Lamont RJ: Oral bacteria and cancer. PLoS pathogens 2014, 10(3):e1003933. doi: 10.1371/journal.ppat.1003933

16.  Schafer CA, Schafer JJ, Yakob M, Lima P, Camargo P, Wong DT: Saliva diagnostics: utilizing oral fluids to determine health status. Monogr Oral Sci 2014, 24:88–98. doi: 10.1159/000358791 PMID: 24862597

17.  Shillitoe E, Weinstock R, Kim T, Simon H, Planer J, Noonan S, et al: The oral microflora in obesity and type-2 diabetes. Journal of oral microbiology 2012, 4.

18.  Hintao J, Teanpaisan R, Chongsuvivatwong V, Ratarasan C, Dahlen G: The microbiological profiles of saliva, supragingival and subgingival plaque and dental caries in adults with and without type 2 diabetes mellitus. Oral Microbiol Immunol 2007, 22(3):175–181. doi: 10.1111/j.1399-302X.2007.00341.x PMID: 1748443

19.  Kampoo K, Teanpaisan R, Ledder RG, McBain AJ: Oral bacterial communities in individuals with type 2 diabetes who live in southern Thailand. Applied and environmental microbiology 2014, 80(2):662–671. doi: 10.1128/AEM.02821-13 PMID: 24242241

20.  Dabelea D, Mayer-Davis EJ, Saydah S, Imperatore G, Linder B, Divers J, et al: Prevalence of type 1 and type 2 diabetes among children and adolescents from 2001 to 2009. Jama 2014, 311(17):1778–1786. doi: 10.1001/jama.2014.3201 PMID: 24794371

21.  Hagman E, Reinehr T, Kowalski J, Ekbom A, Marcus C, Holl RW: Impaired fasting glucose prevalence in two nationwide cohorts of obese children and adolescents. Int J Obes (Lond) 2014, 38(1):40–45.

22.  Mascarenhas P, Fateia B, Barahona I: Effect of diabetes mellitus type 2 on salivary glucose—a systematic review and meta-analysis of observational studies. PloS One 2014, 9(7):e101706. doi: 10.1371/journal.pone.0101706 PMID: 25025218

23.  IDF Diabetes Atlas, 5th edition Brussels, Belgium [http://www.idf.org/diabetesatlas]

24.  Hartman ML, Goodson JM, Barake R, Aismadi O, Al-Mutawa S, Ariga J, Soparkar P, Behbehani J, Behbehani K, Welty F: Salivary glucose concentration exhibits threshold kinetics in normal-weight, overweight, and obese children. Diabetes, metabolic syndrome and obesity: targets and therapy 2015, 8:9–15.

25.  Goodson JM, Kantarci A, Hartman ML, Denis GV, Stephens D, Hasturk H, et al: Metabolic disease risk in children by salivary biomarker analysis. PloS One 2014, 9(6):e98799. doi: 10.1371/journal.pone.0098799 PMID: 24915044
26. Zimmet P, Alberti KG, Kaufman F, Tajima N, Silink M, Ansarian S, et al: The metabolic syndrome in children and adolescents—an IDF consensus report. Pediatric diabetes 2007, 8(5):299–306. doi: 10.1111/j.1399-5448.2007.00271.x PMID: 17850473

27. Suriano K, Curran J, Byrne SM, Jones TW, Davis EA: Fatness, fitness, and increased cardiovascular risk in young children. The Journal of Pediatrics 2010, 157(4):552–558. doi: 10.1016/j.peds.2010.04.042 PMID: 20542285

28. Goodson JM, Tavares M, Wang X, Niederman R, Cugini M, Hasturk H, et al: Obesity and Dental Decay: Inference on the Role of Dietary Sugar. PLoS One 2013, 8(10):e74461. doi: 10.1371/journal.pone.0074461 PMID: 24130667

29. Maiden MF, Tanner A, McArdle S, Najpauer K, Goodson JM: Tetracycline fiber therapy monitored by DNA probe and cultural methods. J Periodontal Res 1991, 26(5):452–459. PMID: 1832458

30. Xia J, Broadhurst DI, Wilson M, Wishart DS: Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics 2013, 9(2):280–299. doi: 10.1007/s11306-012-0482-9 PMID: 23543913

31. Cetik S, Hupkens E, Malaisse WJ, Sener A, Popescu IR: Expression and localization of glucose transporters in rodent submandibular salivary glands. Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology 2014, 33(4):1149–1161.

32. Douglas CW, Naylor K, Phansopa C, Frey AM, Farmilo T, Stafford GP: Physiological adaptations of key oral bacteria. Adv Microb Physiol 2014, 65:257–335. doi: 10.1016/bbs.ampbs.2014.08.005 PMID: 25476768

33. Stephan RM, Miller BF: A quantitative method for evaluating physical and chemical agents which modify production of acids in bacterial plaques on human teeth. J Dental Research 1943, 22:45–51.

34. Prathibha KM, Johnson P, Ganesh M, Subhashini AS: Evaluation of Salivary Profile among Adult Type 2 Diabetes Mellitus Patients in South India. Journal of clinical and diagnostic research: JCDR 2013, 7(8):1592–1595. doi: 10.7860/JCDR/2013/5749.3232 PMID: 24086848

35. Tremblay M, Brisson D, Gaudet D: Association between salivary pH and metabolic syndrome in women: a cross-sectional study. BMC Oral Health 2012, 12:40. doi: 10.1186/1472-6831-12-40 PMID: 22958748

36. Lopez ME, Colloca ME, Paez RG, Schallmacher JN, Koss MA, Chervonagura A: Salivary characteristics of diabetic children. Braz Dent J 2003, 14(1):26–31. PMID: 12656461

37. Koc Ozturk L, Ulucan K, Akyuz S, Furuncuglu H, Bayer H, Yarat A: The investigation of genetic polymorphisms in the carbonic anhydrase VI gene exon 2 and salivary parameters in type 2 diabetic patients and healthy adults. Mol Biol Rep 2012, 39(5):5677–5682. doi: 10.1007/s11033-011-1374-1 PMID: 22198626

38. Rai K, Hegde AM, Kamath A, Shetty D: Dental caries and salivary alterations in Type I Diabetes. The Journal of clinical pediatric dentistry 2011, 36(2):181–184. PMID: 22524081

39. Aitken-Saavedra J, Rojas-Alca yaga G, Maturana-Ramirez A, Escobar-Alvarez A, Cortes-Coloma A, Reyes-Rojas M, et al: Salivary gland dysfunction markers in type 2 diabetes mellitus patients. J Clin Exp Dent 2015, 7(4):e501–505. doi: 10.4317/jced.52329 PMID: 26535097

40. Takahashi N, Takahashi N, Beighton D: Resistance to acidic environments of caries-associated bacteria: Bifidobacterium dentium and Bifidobacterium longum. Caries research 2010, 44(5):431–437. doi: 10.1159/000318582 PMID: 20814202

41. Niederman R, Buyle-Bodin Y, Lu BY, Robinson P, Naleway C: Short-chain carboxylic acid concentration in human gingival crevicular fluid. J Dent Res 1997, 76(4):501–505. doi: 10.1177/0022034510379602 PMID: 26535097

42. Horiuuchi M, Washio J, Mayanagi H, Takahashi N: Transient acid-impairment of growth ability of oral Streptococcus, Actinomyces, and Lactobacillus: a possible ecological determinant in dental plaque. Oral Microbiol Immunol 2009, 24(4):319–324. doi: 10.1111/j.1399-302X.2009.00517.x PMID: 19572895

43. Tanner AC: Anaerobic culture to detect periodontal and caries pathogens. J Oral Biosci 2015, 57(1):18–26. doi: 10.1016/j.job.2014.08.001 PMID: 25678835

44. Takahashi N, Nyvad B: The role of bacteria in the caries process: ecological perspectives. J Dent Res 2011, 90(3):294–303. doi: 10.1177/0022034510379602 PMID: 20924061

45. Niederman R, Buyle-Bodin Y, Lu BY, Robinson P, Nailew C: Short-chain carboxylic acid concentration in human gingival crevicular fluid. J Dent Res 1997, 76(1):575–579. PMID: 9042080

46. Niederman R, Zhang J, Kashket S: Short-chain carboxylic-acid-stimulated, PMN-mediated gingival inflammation. Crit Rev Oral Biol Med 1997, 8(3):269–290.

47. Eltas A, Tozoglu U, Keles M, Canakci V: Assessment of oral health in peritoneal dialysis patients with and without diabetes mellitus. Perit Dial Int 2012, 32(1):81–85. doi: 10.3747/pdi.2010.00113 PMID: 21632442
48. Carneiro VL, Fraiz FC, Ferreira Fde M, Pintarelli TP, Oliveira AC, Boguszewski MC: The influence of glycemic control on the oral health of children and adolescents with diabetes mellitus type 1. Arch Endocrinol Metab 2015, 59(6):535–540. doi: 10.1590/2359-3997000000117 PMID: 26677088

49. Kim EK, Lee SG, Choi YH, Won KC, Moon JS, Merchant AT, et al: Association between diabetes-related factors and clinical periodontal parameters in type-2 diabetes mellitus. BMC Oral Health 2013, 13:64. doi: 10.1186/1472-6831-13-64 PMID: 24195646

50. Haseeb M, Khawaja KI, Ataullah K, Munir MB, Fatima A: Periodontal disease in type 2 diabetes mellitus. Journal of the College of Physicians and Surgeons—Pakistan: JCPSP 2012, 22(8):514–518. PMID: 22868018

51. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI: Obesity alters gut microbial ecology. Proc Natl Acad Sci USA 2005, 102(31):11070–11075.

52. Choromanska K, Choromanska B, Dabrowska E, Baczek W, Mysliwiec P, Dadan J, Zalewska A: Saliva of obese patients—is it different? Postepy Hig Med Dosw (Online) 2015, 69(0):1190–1195.