The Impact of Diabetes on the Gut and Salivary IgA Microbiomes

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Running title: IgA-Biome of stool in saliva in those with diabetes
Abstract

Mucosal surfaces like those present in the lung, gut, and mouth interface with distinct external environments. These mucosal gateways are not only portals of entry for potential pathogens but homes to microbial communities that impact host health. Secretory immunoglobulin A (SIgA) is the single most abundant acquired immune component secreted into mucosal surfaces and via the process of immune exclusion, shapes the architecture of these microbiomes. Not all microorganisms at mucosal surfaces are targeted by SIgA, therefore a better understanding of the SIgA-coated fraction may identify the microbial constituents that stimulate host immune responses in the context of health and disease. Chronic diseases like type 2 diabetes are associated with altered microbial communities (dysbiosis) that in turn affect immune-mediated homeostasis. 16S rDNA sequencing of SIgA-coated/uncoated bacteria (IgA-Biome) was conducted on stool and saliva samples of normoglycemic participants and individuals with prediabetes or diabetes (n=8/group). These analyses demonstrated shifts in relative abundance in the IgA-Biome profiles between normoglycemic, prediabetic, or diabetic samples distinct from that of the overall microbiome. Differences in IgA-Biome alpha diversity were apparent for both stool and saliva, while overarching bacterial community differences (beta diversity) were also observed in saliva. These data suggest that IgA-Biome analyses can be used to identify novel microbial signatures associated with diabetes and support the need for further studies exploring these communities. Ultimately, an understanding of the IgA-Biome may promote development of novel strategies to restructure the microbiome as a means of preventing or treating diseases associated with dysbiosis at mucosal surfaces.
Introduction

Already a global epidemic, type 2 diabetes prevalence is expected to increase precipitously in coming decades (1). Worldwide, 382 million people were living with diabetes in 2013, but projections predict 600 million by 2035 (2). Type 2 diabetes increases the risk of chronic inflammation, immune dysregulation, stroke, neuropathy, other diabetes complications, and certain cancers (3-11). Evidence suggests that the composition of the gut microbiome can influence inflammation and host metabolism in a manner consistent with what is seen at the onset of type 2 diabetes, intimating a possible causal role of the gut microbiome in type 2 diabetes progression (12, 13). An understanding how the gut microbiome changes across time accompanying disease progression will provide insights into the etiology of type 2 diabetes and possibly identify novel diagnostic strategies and therapeutics. The challenge is how to identify among the hundred trillion (14) gut bacteria those species profiles most closely associated with health and disease. We addressed this challenge by analyzing changes to the immunoglobulin A (IgA) microbiome (IgA-Biome) of stool and saliva that accompany changes in glycemia as described previously.

Secretory immunoglobulin A (SIgA) is the single most abundant acquired immune component secreted into mucosal surfaces that shapes the architecture of mucosal microbiomes (15, 16). Although present at all mucosal surfaces (e.g., in the gut, lungs, mouth, eye), most SIgA is secreted into the gut (15), where it prevents direct interaction between the host intestinal mucosa and antigens derived from food, bacteria, or other sources (16). This process, known as immune exclusion (17-21), prevents antigen attachment through the agglutination of SIgA-bound targets that are subsequently
removed by peristaltic and mucociliary movements (22-24). SIgA also mediates immune inclusion, a process that involves linking commensal microbiota constituents to the gut mucosa via glycans present on the SIgA secretory component (16).

The present study examines the IgA-Biome of the stool and saliva of normoglycemic, prediabetic, and diabetic individuals as a means of identifying microbial SIgA signatures associated with these diabetes phenotypes. Remarkably, while altered serum IgA responses have been observed in those with type 2 diabetes (7, 8, 25, 26), little is known about SIgA in the gut in the context of diabetes or hyperglycemia. An understanding of the SIgA-coated repertoire will identify microbial constituents that stimulate the host’s immune response in the context of either health and disease. Using flow cytometry, we analyzed the IgA-Biome of stool and saliva using 16S rDNA sequencing. Characterization of the fecal and salivary IgA-Biome microbial signatures may yield diagnostic markers of diabetes and represent novel targets for the manipulation of the microbiome to reduce chronic inflammation and reverse or prevent diabetes progression.
Results

**Percent immunoglobulin-coated bacteria from stool and saliva.** Bacteria present in stool and saliva samples collected from normoglycemic individuals (n=8) or those with prediabetes (n=8) or diabetes (n=8) were incubated in the presence of fluorescently labeled anti-human IgA and/or anti-human IgM and sorted by flow cytometry. IgM coating was examined since this isotype can also be translocated into mucosal surfaces (14). In the stool samples examined, 11.1%, 10.63%, and 12.23% of bacteria from samples collected from normoglycemic, prediabetic, and diabetic individuals, respectively, were coated with IgA (Fig. 1A). The percent bacteria coated with both IgA and IgM or IgM alone ranged between 0.1%-1.35% (Table S1). In the saliva, 20.55%, 17.3%, and 15.68% of bacteria from samples collected from normoglycemic, prediabetic, and diabetic individuals, respectively, were IgA+/IgM- (Fig. 1B). Although the flow cytometer successfully detected IgM+ or IgA+/IgM+ bacteria, these organisms were not in sufficient abundance to consistently allow for successful DNA extraction and 16S sequencing; therefore, these sort types were not included in subsequent analyses. Scatter plots for respective stool and saliva sample samples used in subsequent analyses is shown in Fig. S1-S3 and the percent coated IgM+ or IgA+/IgM+ bacteria fractions described in Tables S1 and S2. In addition, Tables S1 and S2 describe the number of bacteria used for 16S rDNA sequencing for each stool and saliva sort type analyzed.

**Alpha and beta diversity.** Changes to both alpha and beta diversity have been linked to changing glycemia profiles resulting in diabetes (27-29). Alpha diversity analysis, which evaluates within sample diversity, did not identify differences in bacterial
richness (Observed OTU [operational taxonomic unit]) across stool IgA-Biome profiles (p=0.131; Fig. 2A). By comparison, differences in bacterial evenness (Shannon diversity index) were present (p=0.018) and largely driven by decreased diversity in the IgA fraction (IgA– vs. IgA+, p=0.003; IgA– vs. Presort, p=0.060). Similar patterns were observed in stratified analyses (i.e., by glycemia status), though reductions in evenness of the IgA– versus IgA+ stool microbiome only reached significance among those with diabetes in this limited dataset (p=0.049; Fig. 2B). These results suggest that while a similar number (OTU) of unique taxa are found in all stool IgA-Biome communities, the distribution (Shannon diversity index) of bacteria in the IgA– fraction is more skewed to favor one or more types of bacteria, that is, the IgA– fraction is less even than the presort or IgA+ fractions.

In contrast to stool, saliva samples demonstrated discernable differences in bacterial richness (p=0.008; Fig. 2A), with the collective IgA+/IgM– fraction less rich than that of the presort (p=0.013) and IgA+/IgM– microbiomes (p=0.050). These observations extended to stratified analysis but were not statistically significant within any diabetes phenotype (Fig. 2B). No difference in bacterial evenness was evident across salivary IgA-Biome profiles (p=0.203; Fig. 2A), indicating that while the salivary IgA+/IgM– bacterial population is comprised of fewer unique OTUs than the IgA+/IgM– and presort populations, the communities are similarly distributed within individuals. The only sort type that contained IgM-coated bacteria present at levels sufficient for 16S rDNA sequencing (Table S2) were IgA+/IgM+ bacteria sorted from saliva. However, alpha and beta diversity analysis as well as taxa abundance analysis did not identify significant differences between the IgA+/IgM+ and IgA+/IgM– populations (data not shown).
To investigate beta (between sample) diversity of the collective IgA-Biome communities, we visualized Bray-Curtis dissimilarity by principal coordinates analysis (PCoA). PCoA plots revealed distinct clustering according to IgA profile in saliva (p=0.001; Fig. 3B) but not stool (p=0.144; Fig. 3A). Notably, the salivary IgA+/IgM+ microbiome was markedly different from both the IgA+/IgM- and presort microbiomes (IgA+/IgM+ vs. IgA+/IgM-, p=0.001; IgA+/IgM+ vs. presort, p=0.001), while the latter two communities exhibited considerable overlap (p=0.578). Further analysis by diabetes status suggested these observations may be driven by nondiabetics (Fig. S4). We also examined inter-sample dissimilarity within each IgA-Biome signature by diabetes phenotype. IgA-coated communities in saliva were more similar to one another than uncoated populations independent of diabetes status (Fig. 3C). These data collectively reinforce the importance of analyzing these distinct microbial communities in the context of microbiome-disease association studies.

IgA-Biome taxonomic composition. To identify bacterial taxa that discriminate the IgA coated versus uncoated sorting fractions, we used the biomarker discovery tool Linear Discriminant Analysis Effect Size (LEfSe) (30). Among stool samples, Ruminococcus_torques_group, Dorea, Pseudomonas, and Staphylococcus were preferentially abundant in IgA+ populations (all p<0.05), while no genera characterized the IgA− fractions at the standard cutoff α=0.05. Using the less strict α=0.10, Lachnospiraceae UCG 008, Ruminococcus_2, Brevibacterium, Enterobacter, and Erysipelatoclostridium further distinguished IgA-bound communities, with Escherichia_Shigella, Pannonibacter, and Butyricimonas differentiating IgA− sorted bacteria (Fig. 4A).
In saliva, *Prevotella_7, Haemophilus, Prevotella, Corynebacterium, Peptostreptococcus*, and *Eubacterium_nodatum_group* characterized the IgA⁺/IgM⁻ fractions, while *Selenomonas_3, Oribacterium, Capnocytophaga, Selenomonas, Campylobacter, Catonella, Butyrivibrio_2*, and *Fretibacterium* differentiated IgA⁻/IgM⁻ communities (all p<0.05) (Fig. 4B). Interestingly, *Dorea*, which characterized the IgA⁺ fraction in stool, was found in greater abundance in the IgA⁻/IgM⁻ population in saliva (p=0.050). This observation was not explained by differences in OTU content as the same *Dorea* OTU dominated both sites, highlighting the potential for niche-specific IgA responses.

We next sought to determine whether discriminant taxa identified by LEfSe varied according to diabetes phenotype using the IgA Coating Index (ICI) (31). The ICI measures enrichment of each genus in the IgA-coated versus the uncoated fraction, thereby accounting for inter-subject variation in taxonomic abundance variation readily apparent in both stool and saliva (Table S3). Of those stool bacteria differentiating IgA sorted fractions, none varied with diabetes status (Table S3). In contrast, ICI scores of the discriminant salivary bacteria *Oribacterium* (p=0.035) and *Selenomonas* (p=0.007), and to a lesser extent *Selenomonas_3* (p=0.068), increased with dysglycemia. Importantly, no significant differences in the abundance of these bacteria were evident among presort samples, which underscores the value of the IgA-Biome to inform previously unrecognized microbial relationships in the context of type 2 diabetes.
MATERIALS AND METHODS

Study population and sample collection. Stool and saliva samples were collected from age matched normoglycemic, prediabetic, and diabetic individuals (8 participants from each group) from Starr County, TX (Table 1). Subjects are participating in a larger study designed to identify changes in the nasal and gut microbiomes during the progression from normoglycemia to prediabetes to diabetes. This study was approved by the Institutional Review Board of the University of Texas Health Science Center and informed written consent was obtained from each participant. Following enrollment, saliva samples (approximately 2-3 ml) were collected at the field office and immediately frozen at -80°C. Approximately half of participants were able to provide a stool sample at time of enrollment. Stool was collected using a Feces Catcher (Abbexa, Houston, TX), and a portion of the sample was placed in a Covidien Precision™ Stool Collector and stored at -80°C. Participants unable to provide a stool sample at the field office collected samples in a similar fashion at home and contacted a Starr County Health Studies staff member when the sample was ready for pickup. Samples were stored at -20°C until they were transported to the field office where they were stored at -80°C. Samples were shipped to the Houston laboratories on dry ice and stored at -80°C until processed for flow cytometry.

Diabetes classifications. Participants were defined as normoglycemic, prediabetic, or diabetic based on their fasting glucose, 2-hour post load glucose levels, or percent glycated hemoglobin (%HbA1c) (Table 1). “Normoglycemic” classification required all 3 measures to be within normal range while an abnormal value on any one test was sufficient to classify a subject as “diabetic.”
Sample preparation for flow cytometry. Stool and saliva samples were prepared as described by Palm et al. with minor modifications (31). Stool samples were resuspended at a concentration of 0.1 g/ml in sterile filtered, cold phosphate buffered saline (PBS, pH 7.4). Stool samples were allowed to rehydrate for 30 min on ice, and saliva samples were allowed to thaw at room temperature. One mL of either stool or saliva were then added to Lysing Matrix D tubes (MP Biologicals, Santa Ana, CA) and homogenized for 7 sec using a BeadBeater (Biospec, Bartlesville, OK). Lysing Matrix D tubes were then centrifuged at 730 rpm for 15 min and 100 µl were collected for immunostaining. Each sample was washed 3X with 1 mL cold PBS/1% bovine serum albumin (BSA, Sigma, St. Louis, MO). Between washes, samples were centrifuged at 9,230 rpm. After the first wash, 20 µl were removed and frozen at -80ºC for use as the presort sample. After the last wash, samples were blocked in 25 µl of 20% mouse serum for 20 min on ice. Fluorophore-conjugated anti-IgA and anti-IgM antibodies were added to respective samples at the concentrations specified by the manufacturers (listed below) for 30 min on ice. Samples were washed 3 times in PBS/1%BSA. After the last wash, samples were resuspended in 1 mL PBS/1% BSA and transferred to a flow tube. All centrifugation steps were carried out in a cold room at 4ºC. Samples were maintained on ice until sorted.

Flow sorting of immunoglobulin-coated bacteria. Bacteria from stool and saliva samples were incubated with fluorescently labeled antibodies specific for either human IgA or IgM. Stool samples were sorted on two separate occasions, and saliva samples were sorted on a separate single day. Sorting was conducted using a BD SORP FACS Aria™ II (Special Order Research Product from Beckton Dickinson, San
Jose, CA), equipped with forward scatter photomultiplier tube (FSC-PMT) for improved small particle detection, at the Baylor College of Medicine Cytometry and Cell Sorting Core Facility where instruments go through daily QA/QC and follow NIH biosafety standards. The instrument was set up for small particle detection using both a forward scatter and side scatter, FSC and SSC respectively, thresholds to determine signal from background. Scatter voltage settings were determined using FSC height and SSC height parameters to adequately adjust signal over threshold values. FSC-PMT in both Area and Height was used to verify FSC and SSC threshold settings. Typical singlet gating discrimination was not performed to prevent excluding populations due to variations in scatters of bacteria. To reduce coincident events and sort error issues, the sample differential was maintained at the lowest setting and the sample threshold rate was decreased by diluting the sample as needed with phosphate buffered saline containing 1% bovine serum albumin. Background fluorescence was established using unlabeled specimens on all samples and examined in bivariate plots for the two channels of interest, FITC and PE and then PE and APC, setting gates on bias to account for a range of autofluorescence. Inter-channel compensation was done using single labeled samples of either conjugated antibody. Sorted samples were collected into chilled, pre-labeled epi tubes.

The first set of stool samples (n=4 per glycemia group) were incubated with either mouse fluorescein isothiocyanate (FITC)-conjugated anti-human IgM (SA1-19598, Thermofisher, Waltham, MA) or phycoerythrin (PE)-conjugated mouse anti-human IgA (130-093-128, Miltenyi Biotec Inc., Auburn, CA). Accordingly, bacteria were sorted as either IgA+, IgA−, IgM+, or IgM−. Only 2/4 IgM+ sorts contained sufficient
bacteria to the successful extraction of DNA at a concentration suitable for 16S rRNA sequencing. Therefore, these samples were not included in subsequent analyses. The second set of stool samples (n=4 per group) was conducted after the PE-anti-human IgA (130-093-128) used in the first experiment was no longer available for purchase. Instead, a recombinant PE conjugated anti-human IgA (130-116-878, Miltenyi) was paired with an allophycocyanin (APC)-conjugated anti-human IgM (130-093-076, Miltenyi) in the same sample tube; accordingly, these stool bacteria were sorted as either IgA+/IgM-, IgA+/IgM+ or IgA-/IgM+. Comparisons of percent IgA-positive bacteria following incubation with the two different anti-IgA antibodies did not identify significant differences in percent IgA-coating, that is, the percent IgA+ or IgA+/IgM+ coated bacteria were similar (Fig. S5). For analysis purposes, sequence data obtained from IgA+ (first set of sorted stool samples) and from IgA+/IgM+ (second set of samples) were combined into a single ‘IgA+’ group.

Saliva samples were analyzed using the PE-conjugated anti-IgA (130-116-878, Miltenyi)/APC-conjugated anti-human IgM (130-093-076, Miltenyi) antibody pair. In contrast to stool sample analyses, all saliva samples were incubated simultaneously with anti-IgA and anti-IgM, enabling dual label identification.

Most stool and saliva IgA-Biome analyses were conducted on half a million sorted bacteria although some sorts contained fewer bacteria (Table S1 and S2). IgM+ bacteria in stool (n=12) or IgA+/IgM+ or IgA-/IgM+ bacteria in stool (n=12) and saliva (n=24) were present in numbers insufficient to sort (Fig. S1-S3 and Table S1 and S2). Only IgA+/IgM+ bacteria sorted from saliva samples were suitable for 16S rDNA sequencing (rDNAseq) (Table S2).
Sorted samples along with sheath fluid controls were transported on ice and centrifuged at 4°C at 10,000 rpm for 15 min. The majority of the supernatant was aspirated, leaving approximately 100 µl and then frozen at -80°C until processed for 16S sequencing (31).

**16S RNA gene sequencing.** Genomic DNA was extracted with the MagAttract PowerSoil DNA kit (Qiagen) following methods adapted from the NIH Human Microbiome Project (32). The V4 region of the 16S rDNA gene was amplified by PCR using barcoded primers (515F,806R) and sequenced on the MiSeq platform (Illumina) via the 2x250 bp paired-end protocol, targeting at least 10,000 reads per sample.

Demultiplexed read pairs were merged using USEARCH v7.0.1090 (33) with the following parameters: merged length ≥252 bp, minimum 50 bp overlap with zero mismatches, and truncation quality >5. Merged reads were further filtered against a maximum expected error rate of 0.05, and PhiX sequences were removed. Reads were clustered into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using UPARSE (34) and underwent chimera removal via USEARCH (33). OTUs were mapped against an optimized version of SILVA Database v132 (35) containing only sequences from the v4 region of the 16S rDNA gene. To reduce the influence of potential contamination from the sorting process, OTUs identified in at least two of three sheath fluid samples were removed from the dataset if their average relative abundance among presort samples was <50% of the sheath samples. This resulted in the removal of six OTUs representing *Acinetobacter*, *Bradyrhizobium*, *Methylobacterium*, *Pseudomonas*, *Tyzzerella*, and a Microbacteriaceae family member. Following removal of putative contaminants, data were rarefied to 1,319 reads per sample (Fig. S6).
Statistical analysis. Agile Toolkit for Incisive Microbial Analyses 2 (ATIMA2), a stand-alone tool for microbial data exploration, was used as an integrated solution for analyzing and visualizing microbiome data (atima2.jplab.net). ATIMA2 uses PERMANOVA to evaluate differences in overarching community composition (beta diversity), while comparisons of community dispersion were determined via the Mann-Whitney U test. Alpha diversity analyses were performed in STATA 16 using repeated measures ANOVA and the paired t-test as appropriate. Differentially abundant taxa by IgA-bound status were determined via LEfSe (36) using parameters $\alpha=0.10$ and minimum LDA=2.0. LEfSe analysis was limited to taxa identified in at least 10% of samples. Finally, ICI was calculated as relative abundance (IgA$^+$) /relative abundance (IgA$^-$) (31). When taxa were detected in the IgA$^+$ fraction but not the IgA$^-$ fraction, a pseudoabundance of 0.0008 (the limit of detection) was applied to the denominator. Samples in which taxa were not detected in either fraction were excluded from analysis. Differences in ICI were tested using the Kruskal-Wallis test. Unless otherwise indicated, results are considered significant at $p<0.05$. 
Recent efforts highlight the promise of the gut and oral microbiome as targets in the development of novel prognostic, diagnostic, and therapeutic interventions. These microorganisms represent the bulk of the human microbiota (37) and as residents of mucosal microenvironments they also face the additional selection pressures imposed by that of the host’s adaptive and innate immune systems (15). In the present study, we examined the stool and salivary microbiomes in the context of the host immune response by characterizing the IgA-Biome, which profiles bacteria based on their S IgA-bound status. Specifically, we investigated differences in the IgA-Biome in type 2 diabetes, a chronic inflammatory disease with known alterations to both the intestinal and oral microbiota (13, 37-41).

The mucosal immune system functions beyond simply protecting the host from infection. It also effectively serves as a ‘bouncer,’ selecting the residents of the intestinal microbiota as a means of maintaining gut homeostasis (16, 42, 43). To do this, it must decide between an active inflammatory response driven by either pathogen- (e.g., lipopolysaccharide, peptidoglycan) (44, 45) or self-derived danger signals (e.g., S100 proteins, heat shock proteins) (44-46) or a non-inflammatory maintenance response governed by safety signals delivered by commensal bacteria and their by-products, healthy epithelial cells, and dietary components (42). Part of this decision-making process is established in early life, paradoxically through interactions between the developing immune system and the gut microbiota. This process is essential to both the development of CD4+ and CD8+ T-cells as well as to the maturation of B-cells into IgA-secreting plasma cells (43).
Unlike traditional gut and oral microbiome sequencing approaches, the IgA-Biome offers a different perspective of the microbial communities colonizing mucosal surfaces. Specifically, it provides an understanding of host immune awareness to the myriad targets present in these ecological niches. Although bacteria found in stool are frequently used as a surrogate for the mucosal microbiome, data demonstrate these two populations are distinct (47, 48). SlgA primarily targets microbes that come in contact with host tissues, therefore the SlgA-coated fraction of the IgA-Biome is likely to be more similar to the mucosal microbiota than the microbiota colonizing the lumen of the intestine (49, 50). We hypothesized therefore that IgA-Biome profiles may be a better indicator of the mucosal-associated microbiota since these organisms will, by definition, have come in contact with the host’s immune system and are thus more likely to have been in closer proximity to mucosal surfaces (16). The specificity of SlgA in the gut and at other mucosal sites (e.g., the oral mucosa, lungs, eyes) dictates and shapes microbial community architecture, topography, invasiveness profiles, and the immunometabolome via antibody-mediated immune exclusion and inclusion (16, 49). The IgA-Biome signature therefore provides a snapshot of the organisms that have the attention of the host’s immune system or, in the context of disease, organisms that should have the host’s attention but for unknown reasons do not. Therefore, further characterizing IgA-Biome at the species level will identify the microbial constituents that stimulate and interact with the host immune system to promote type 2 diabetes (31, 51-54).

In addition to the gut IgA-Biome, we examined IgA microbial signatures in saliva. The salivary microbiome represents a composite of various oral cavity niches (55) and
has been linked to both oral and systemic disease, including type 2 diabetes (37, 39, 56-58). Unlike the gut, the salivary microbiome appears more resistant to environmental stressors such as antibiotics (59). Zaura et al. hypothesized that this resiliency arose as a consequence of overcoming daily disruptions in the form of oral hygiene procedures as well as significant fluctuations in temperature and oxygen that do not occur in the gut (59).

Alpha diversity analyses of the stool IgA-Biome identified similarities in richness (OTUs); however, the presort and IgA⁺ stool fractions were more even (Shannon diversity index) compared to the IgA⁻ fraction. By contrast, salivary IgA-Biome analyses revealed significant differences in richness between the IgA⁺/IgM⁺ compartment compared to the presort and the IgA⁺/IgM⁻ microbiome. When parallel analyses were conducted comparing IgA-Biome profiles across diabetes phenotypes, statistical differences in richness were not evident although a trend for increased richness in the SlgA-coated compartment for both stool and saliva (independent of diabetes status) was observed. Shannon diversity analyses by diabetes status of the stool IgA-Biome showed a similar trend, that is, the SlgA-coated microbiota was more diverse (and statistically different in the those with diabetes) compared to the uncoated fraction. Interestingly, the opposite trend was observed for the salivary IgA-Biome in that the SlgA-coated compartment was less diverse than the uncoated microbiota although these differences were not statistically significant.

Beta diversity analyses of the IgA-Biome of stool and saliva identified significant differences in the salivary but not the stool microbiome using both PCoA and pairwise comparisons of Bray-Curtis dissimilarity values. Salivary IgA-Biome analysis

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demonstrated a lower IgA+/IgM- inter-sample dissimilarity (i.e., Bray-Curtis dissimilarity closer to 0.0) indicating that the salivary IgA+/IgM- microbial communities, independent of diabetes status, were more similar to each other than those of the IgA-/IgM- microbiome, highlighting the importance of analyzing these distinct microbial communities in the context of mucosal microbiome studies.

Human studies of the gut microbiome and diabetes have largely focused on differences between individuals of varied glycemic status. *Bifidobacterium*, the most consistently identified taxa, showed an inverse relationship with hyperglycemia (60). Other bacteria negatively associated with prediabetes or diabetes are *Roseburia*, *Faecalibacterium*, and other Clostridiales members (61-64). Bacteria with frequent positive associations with type 2 diabetes include *Ruminococcus*, *Lactobacillus*, and various members of the Proteobacteria phylum (61-65). These relationships are not surprising given that Proteobacteria may result in loss of gut barrier function, and hyperglycemia is itself associated with intestinal permeability (66). Of these taxa, we identified *Escherichia_Shigella* and *Pseudomonas* (Proteobacteria) to be associated with the IgA+ and IgA- organisms, respectively, in addition to members of the Order Clostridia (*Dorea* and *Ruminococcus*).

Similar analysis of the salivary IgA-Biome identified increased levels of *Rothia* and *Prevotella* in the SlgA-coated fraction. These genera were previously found to be associated with diabetes (67-70), while *Prevotella* has additionally been linked to poor oral health and increased body mass index (55, 71). *Alloprevotella*, also preferentially bound to SlgA, has by contrast been inversely associated with diabetes (58, 72). Taxa found to be differentially coated with SlgA included *Capnocytophaga*, *Leptotrichia*, and
Tannerella—three genera previously linked to diabetes or impaired fasting glucose (67, 68, 72–76). Selenomonas and Oribacterium were also preferentially found among uncoated bacteria; however, the ICI for these taxa varied by diabetes status. Specifically, prediabetes and diabetics exhibited higher scores compared to nondiabetics, indicating higher propensity for these bacteria to be found in the IgA-coated fraction among those with dysglycemia. The relative abundance of these bacteria in presort samples did not vary with diabetes phenotype, demonstrating the potential for IgA-coating profiles to identify taxa linked to changes in glycemia.

Identifying changes to the gut IgA-Biome that can be linked to transitions across glycemic profiles and to changes in IgA-Biome profiles at other mucosal sites i.e., the oral IgA-Biome, may help identify subtle fluctuations associated with diabetes phenotypes and potentially identify IgA-Biome signatures that are shared between mucosal microenvironments. The presence of Dorea, for example, a genus associated with gut microbiota changes in those with diabetes was identified in this study at both anatomic sites and in both the IgA-coated (stool) and -uncoated (saliva) compartments (62, 77). Dorea (and potentially other genera with similar profiles) may represent candidate taxa that can be used to track changes to the SlgA response at different anatomic sites in the context of changing glycemia profiles.

The salivary IgA-Biome may provide a different avenue for the early detection of chronic disease states and serve as a potential alternative to the gut microbiome since immune responses originating in the gut have implications at distant mucosal surfaces. For example, it was demonstrated that gut dysbiosis negatively impacted immune responses in the lung in response to a Mycobacterium tuberculosis infection (78). Due
to the low number of participants in each diabetes phenotype examined in this study, IgA-Biome data should be interpreted with caution. However, even in this limited sample size, differences between the IgA-Biome and the presort microbiome were observed. Future studies will employ whole genome sequencing to identify organisms at the species level associated with changes in glycemia status in both the gut and saliva. These analyses will allow us to also test the hypothesis that changes in the gut IgA-Biome that accompany worsening glycemia are reflected by changes to the salivary IgA-Biome. These analyses would allow for the identification of salivary microbiome constituents that could serve as indicators of changes to the gut IgA-Biome.

Characterizing the IgA-Biome provides an awareness of taxa targeted by the host immune response and, equally important, those taxa not targeted in the context of specific disease states. Such studies can only further our understanding of disease etiology and may additionally promote development of new modalities to restructure the microbiome as a means of preventing or treating diseases associated with dysbiosis at mucosal surfaces. Continued research of these unique signatures is greatly warranted.

Acknowledgements

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Table 1. Participant Characteristics by Diabetes Status

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1Values in ‘grey’ indicate a measure that qualifies individuals as prediabetic or diabetic based on the indicated cutoff values. Some individuals have more than one measure that qualifies them as prediabetic or diabetic. Any measure of fasting glucose, 2-hour post-load glucose, or %HbA1c can be used to classify individuals as having prediabetes or diabetes.

2A 2-hour-post-load glucose is not conducted on individuals with a fasting plasma glucose ≥160 mg/dL.
Figures

FIG 1

A

%IgA-Coated Bacteria-Stool

B

%IgA-Coated Bacteria-Saliva

Normoglycemic, Prediabetes, Diabetes
FIG 3

A
Stool
Weighted Bray-Curtis PCoA
P-Value: 0.144, R-Squared: 0.0381

B
Saliva
Weighted Bray-Curtis PCoA
P-Value: 0.001, R-Squared: 0.0857

C

Stool

Saliva

Bray-Curtis Distance

Nondiabetic  Prediabetic  Diabetic

*
REFERENCES


Figure Legends

FIG 1 Percent IgA-coated bacteria in stool (A) and saliva (B) according to diabetes status. No significant differences in percent coating were observed within groups. Each dot represents a study participant. Horizontal bars represent the mean ± standard error.

FIG 2 Alpha diversity of the stool and salivary IgA-Biomes. A) Box plots depict sample richness (Observed OTU) and evenness (Shannon Diversity Index) among collective presort and sorted samples in both stool and saliva. B) Same as in (A) except box plots indicate differences in diversity according to IgA-bound status stratified by diabetes phenotype. Only relationships with p < or = 0.05 by repeated measures ANOVA are indicated.

FIG 3 Beta diversity analyses of stool and salivary IgA-Biomes. Differences in beta diversity by IgA-bound status were not evident in stool (A) but were observed in saliva (B). Beta diversity was measured by weighted Bray-Curtis distance and visualized by principal coordinates analysis. Statistical significance was determined using PERMANOVA. (C) Box plots depict weighted Bray-Curtis dissimilarities by IgA-Biome profile in stool (top) and saliva (bottom) according to diabetes phenotype. Significant differences were observed between IgA-coated and uncoated bacteria in saliva only. *p<0.01, Kruskal-Wallis.

FIG 4 LEfSE analysis identified bacterial biomarkers associated with IgA coated and uncoated genera in (A) stool and (B) saliva. Analyses were conducted using parameters α<0.10 and linear discriminant analysis (LDA) threshold ≥2.0.