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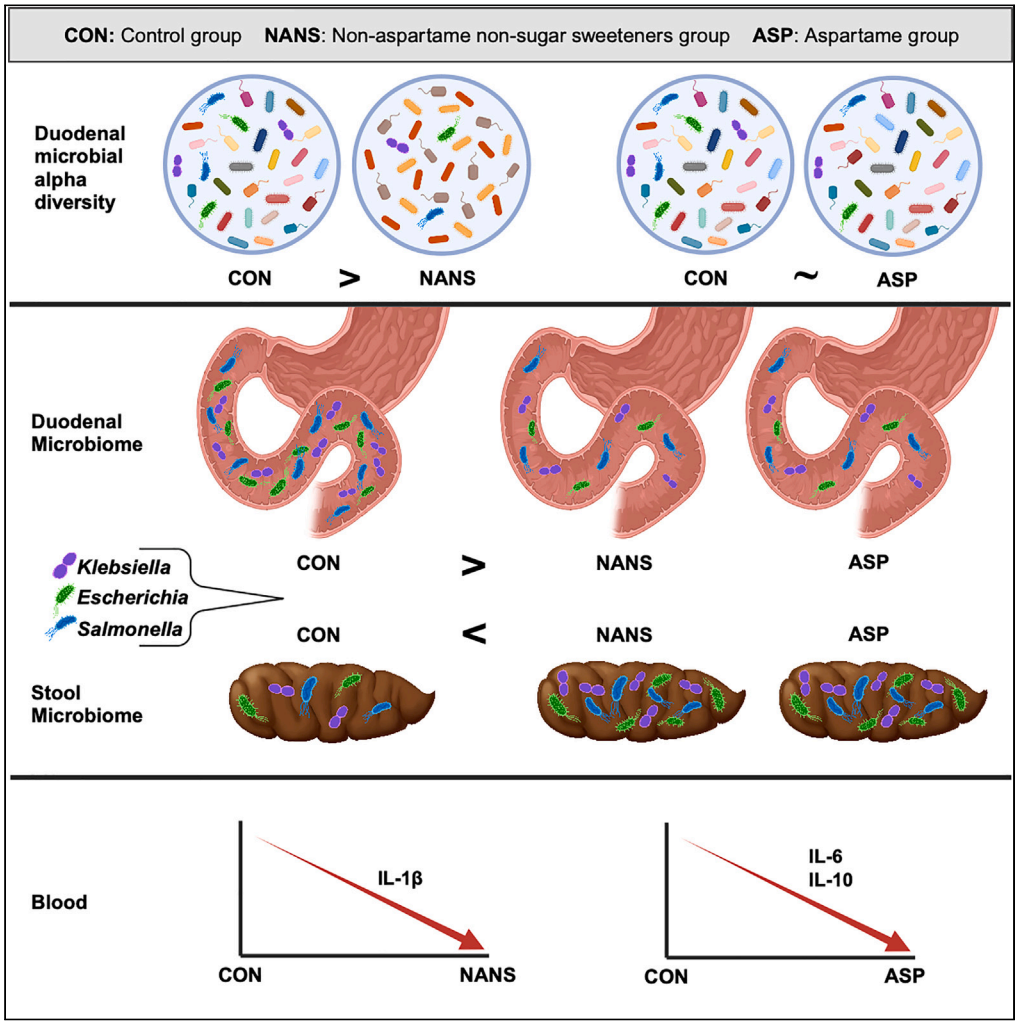

Ava Hosseini, Gillian M. Barlow, Gabriela Leite, ..., Ali Rezaie, Mark Pimentel, Ruchi Mathur

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Highlights

We examined non-aspartame non-sugar sweeteners (NANS) and aspartame (ASP) consumers

Duodenal alpha diversity is lower in NANS consumers vs. controls

Duodenal *Escherichia*, *Klebsiella*, and *Salmonella* are less abundant in NANS and ASP

Duodenal cylindrospermopsin biosynthesis is significantly enriched in ASP consumers

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Consuming artificial sweeteners may alter the structure and function of duodenal microbial communities

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SUMMARY

Studies using stool samples suggest that non-sugar sweetener (NSS) consumption affects gut microbiome composition. However, stool does not represent the entire gut. We analyzed the duodenal luminal microbiome in subjects consuming non-aspartame non-sugar sweeteners (NANS, N = 35), aspartame only (ASP, N = 9), and controls (CON, N = 55) and the stool microbiome in a subset (N = 40). Duodenal alpha diversity was decreased in NANS vs. CON. Duodenal relative abundance (RA) of *Escherichia, Klebsiella*, and *Salmonella* (all phylum Proteobacteria) was lower in both NANS and ASP vs. CON, whereas stool RA of *Escherichia, Klebsiella*, and *Salmonella* was increased in both NANS and ASP vs. CON. Predicted duodenal microbial metabolic pathways altered in NANS vs. CON included polysaccharides biosynthesis and D-galactose degradation, whereas cylindrospermopsin biosynthesis was significantly enriched in ASP vs. CON. These findings suggest that consuming non-sugar sweeteners may significantly alter microbiome composition and function in the metabolically active small bowel, with different alterations seen in stool.

INTRODUCTION

Artificial sweetener (AS) is a term used to identify sugar substitutes that are utilized to sweeten food and beverages. They include aspartame and sucralose, among others. AS and other low-calorie sweeteners such as the plant-based sweetener Stevia, are collectively termed non-sugar sweeteners (NSS). According to the US census data and Simmons National Consumer Survey, 141.18 million Americans used sugar substitutes in 2020.[1] Products containing NSS are most often used by people who are trying to reduce their sugar intake or manage their weight, as they are lower in calories than sugar.[2,3]

Recently, the use of NSS and their potential health effects have been called into question by the World Health Organization (WHO).[4] The WHO issued a new guideline advising against the use of NSS[5] based on evidence suggesting that NSS use do not help with weight control, and more importantly, they noted that NSS were linked to "potential undesirable effects from long-term use," including increased risks for type 2 diabetes (T2D) and cardiovascular disease.[5] In addition, the International Agency for Research on Cancer (IARC) recently classified aspartame specifically as possibly having carcinogenic effects,[6,7] based in part on findings that the consumption of >6 soft drink servings/week was positively associated with increased risk for hepatocellular carcinoma in humans.[8] In addition to these potential risks, there are also concerns as to how AS and NSS use may affect the gut microbiome[9,10] and whether the potential risks of NSS are linked to gut microbiome alterations.

The gut microbiome plays significant roles in many aspects of human health and disease, including digestion and metabolism,[11,12] physiology,[13] and immune function,[14] and imbalances in gut microbial populations have been linked to health conditions ranging from gastrointestinal conditions such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS)[10] and metabolic conditions such as obesity and T2D[15,16] to immune-mediated inflammatory disease[9] and heart failure.[17] However, it is important to note that the majority of these links were identified using stool samples, which are not representative of the entire gastrointestinal tract.[18]

Within the gut, the small intestine plays key roles not only in digestion and nutrient absorption[16] but also in endocrine regulation,[19] innate immunity,[20] and host-microbial crosstalk.[21,22] Indeed, as recently noted by de Vos et al., one can live without a colon, but not without a small intestine.[21] Many small intestinal functions are mediated by differentiated cell types in the epithelium,[23–25] including columnar enterocytes, which absorb nutrients and secrete immunoglobulins; goblet cells, which secrete mucus that protects the gut epithelium[19]; and Paneth cells, which secrete antimicrobial peptides such as defensins.[26] There are also a wide variety of enteroendocrine cells,[23–25] including K-cells, which produce the incretin glucose-dependent insulinotropic polypeptide (GIP); I-cells, which produce cholecystokinin, stimulating bile release;

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Table 1. Subject demographics for duodenal analysis and fasting levels of circulating inflammatory markers

Group	NANS	ASP	CON	p-value NANS vs. CON	p-value ASP vs. CON
N	35	9	55	NS	NS
Female N (%)	19 (54%)	5 (55%)	31 (56%)	NS	NS
	Mean ± SD	Mean ± SD	Mean ± SD		
Age, years	59.50 ± 14.40	59.40 ± 17.60	60.30 ± 13.80	NS	NS
BMI, kg/m^2	26.90 ± 6.50	26.20 ± 6.50	25.90 ± 4.70	NS	NS
GM-CSF	4.72 ± 3.65	4.39 ± 1.26	6.43 ± 5.57	0.086	0.590
IFNγ	6.74 ± 13.47	1.31 ± 2.76	11.74 ± 42.63	0.993	0.671
IL-10	8.11 ± 6.88	3.75 ± 1.81	8.28 ± 9.13	0.718	**0.025**
IL-12P70	4.08 ± 1.66	3.70 ± 0.44	5.94 ± 7.38	0.299	0.532
IL-13	31.94 ± 114.99	3.20 ± 0.48	16.65 ± 41.03	0.952	0.137
IL-1β	2.29 ± 0.70	2.15 ± 0.32	2.67 ± 1.27	**0.042**	0.173
IL-2	0.97 ± 0.67	0.75 ± 0.56	1.88 ± 3.12	0.101	0.068
IL-4	64.28 ± 221.48	0.76 ± 0.77	68.66 ± 260.99	0.451	0.054
IL-5	4.62 ± 7.08	2.78 ± 0.53	16.46 ± 66.55	0.188	0.061
IL-6	19.72 ± 66.86	0.44 ± 1.19	18.17 ± 58.33	0.375	**0.025**
IL-8	14.35 ± 23.61	6.54 ± 1.72	14.52 ± 22.39	0.787	0.388
MCP1	716.89 ± 429.32	452.80 ± 230.84	632.02 ± 298.71	0.474	0.117
TNFα	16.41 ± 6.25	13.59 ± 7.29	19.07 ± 13.36	0.518	0.066

Significant values are indicated in bold font ($p < 0.05$).

enterochromaffin cells, which produce serotonin; D-cells, which produce somatostatin, regulating gastrointestinal hormones and gastric emptying[23–25]; M-cells, which produce motilin, regulating small intestinal motility and hunger signaling[27]; and L-cells, which secrete the incretin glucagon-like peptide 1 (GLP-1),[28] the peptide hormone GLP-2, which induces gut epithelial cell growth and proliferation and maintains mucosal integrity and gut barrier function,[29,30] and peptide YY (PYY), which regulates satiety.[16,19] The small intestine also differs from the large intestine in terms of pH, transit time, and biomass, although the use of improved techniques for sampling the small intestine[31] have revealed that its biomass is greater than previously understood.[18,32] Further, small intestinal microbial populations are significantly different from those of stool.[18] For all of these reasons, it is important to study the small bowel microbiome directly in evaluating its relationship to human disease.[33–39]

Recent studies have suggested that consuming NSS may affect the gut microbiome[40–43] and contribute to the development of glucose intolerance,[43] obesity,[44] IBD,[45] and non-alcoholic fatty liver disease (NAFLD),[46] although study results vary.[47] However, these studies only examined stool samples. In this study, we used samples from the REIMAGINE (Revealing the Entire Intestinal Microbiota and its Associations with the Genetic, Immunologic, and Neuroendocrine Ecosystem) study[31] to assess the potential effects of NSS consumption on the human duodenal luminal microbiome.

RESULTS

Subject demographics

This study utilized samples and data from the REIMAGINE study[31] at Cedars-Sinai. All individuals aged 18–85 years undergoing standard-of-care esophagogastroduodenoscopy (EGD) without colon prep are eligible for inclusion. All enrolled subjects provide duodenal luminal aspirates as well as fasting blood samples for analysis, and a subset also provide optional stool samples. A total of 650 REIMAGINE subjects had been recruited at the time of this study and had duodenal aspirates available for analysis. Of these, 101 subjects reported consumption of some type of non-sugar sweeteners (NSS). Subjects who had small intestinal bacterial overgrowth (SIBO), had type 2 diabetes (T2D), or were taking metformin were excluded due to potential confounding effects on the microbiome,[16,48,49] after which 44 subjects remained. These 44 NSS subjects were further subdivided into those who consumed non-aspartame non-sugar sweeteners (NANS, N = 35) and those who consumed aspartame only (ASP, N = 9). Aspartame was studied separately, as it is composed of amino acids (aspartic acid and phenylalanine), and we hypothesized that its effects on the microbiome might be different from the effects of other NSS. Control subjects (CON, N = 55) were matched to the NSS subjects for age, sex, and BMI, giving a total of 99 subjects in the analysis (see Table 1).

Circulating inflammatory markers are different between NANS, ASP, and CON groups

Fasting levels of a panel of 12 circulating inflammatory cytokines and chemokines were measured in serum samples. Levels of interleukin-1β (IL-1β) were found to be decreased in the NANS vs the CON group ($p = 0.042$, Table 1), and fasting levels of IL-10 ($p = 0.025$) and IL-6 ($p = 0.025$) were decreased in the ASP vs. the CON group (Table 1).

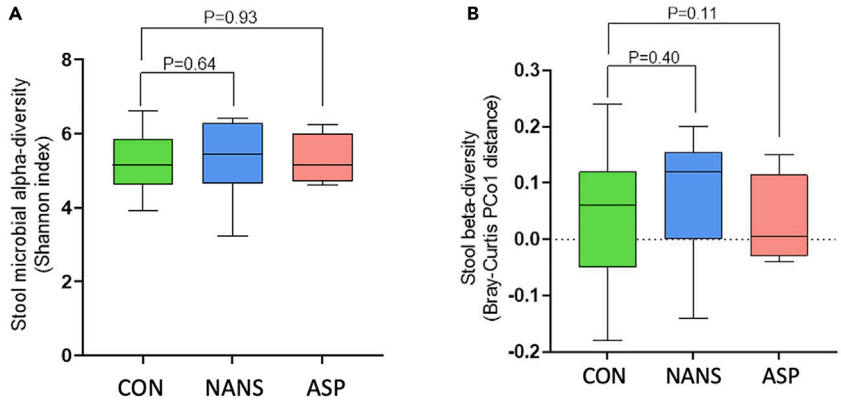

Figure 1. Stool microbial alpha and beta diversity in the CON, NANS, and ASP groups

(A) Alpha diversity, as determined using Shannon's index.

(B) Beta diversity, as determined using Bray-Curtis index. Data are presented as mean ± standard deviation.

Stool: Microbial alpha and beta diversity are not significantly altered in subjects consuming NSS

Analyses of stool samples were performed to determine whether findings in the REIMAGINE study population mirror those from other studies examining the effects of artificial sweeteners using stool samples.[43,47,50–52] Of the 99 subjects who had their small intestinal microbiome sequenced, 40 also had their stool microbiome sequenced (NANS N = 11, ASP N = 4, CON N = 25) (Table S1).

Stool microbial alpha diversity was not significantly different in both the NANS group when compared with the CON group (p = 0.64) and the ASP group when compared with the CON group (p = 0.93), as determined using Shannon index (Figure 1A). Stool microbial beta diversity, as determined using the Bray-Curtis index, was not significantly different in the NANS vs. CON group (p = 0.404) or in the ASP vs. CON group (p = 0.108, Figure 1B).

Stool: Microbial profiles in NANS and ASP subjects are significantly different from the CON group

Analysis of stool microbial profiles at the phylum level revealed lower relative abundance (RA) of Chloroflexi (log_2 fold change [FC] = −11.64, adj-P = 1.33E-7), Epsilonbacteraeota (FC = −7.29, adj-P = 8.11E-5), and Fusobacteria (FC = −6.79, adj-P = 8.11E-5) in the NANS vs. CON group. In contrast, the RA of phyla Lentisphaerae (FC = 3.77, p = 0.043, adj-P = 0.138) and Proteobacteria (FC = 1.41, p = 0.014, adj-P = 0.057) tended toward being higher in the NANS vs. CON group (Figure 2A, Table S2).

It is crucial to note that small sample size can significantly influence microbiome data and interpretation. The ASP group had 4 subjects, making it difficult to control for normal variations between subjects. Taking this into account, the RA of the archaeal phylum Euryarchaeota (FC = −17.24, adj-P = 4.76E-6) and the bacterial phyla Chloroflexi (FC = −11.36, adj-P = 9.64E-4) and Spirochaetes (FC = −15.93, adj-P = 2.06–5) were lower in the ASP group than in the CON group (Figure 2A). The RA of phylum Proteobacteria (FC = 2.33, adj-P = 1.78E-2) was higher in the ASP vs. CON group, similar to the findings for the NANS group.

Further analyses of the stool microbial profiles at the genus level showed higher RA of *Sanguibacteroides* (phylum Bacteroidetes) (FC = 15.14, adj-P = 1.09E-17), *Gibbsiella* (phylum Proteobacteria) (FC = 14.81, adj-P = 1.09E-17), *Faecalicoccus* (phylum Firmicutes) (FC = 12.31, adj-P = 6.11E-13), *Salmonella* (phylum Proteobacteria) (FC = 11.50, adj-P = 3.54E-11), *Klebsiella* (phylum Proteobacteria) (FC = 5.42, adj-P = 1.52E-3), and *Escherichia-Shigella* (phylum Proteobacteria) (FC = 3.32, adj-P = 4.02E-3) in the NANS vs. CON group (Figure 2B). In contrast, the NANS group had lower RA of *Arcobacter* (FC = −19.86, adj-P = 4.61E-17), *Succiniclasticum* (FC = −18.34, adj-P = 1.29E-14), *Methylobacterium* (phylum Proteobacteria) (FC = −16.68, adj-P = 2.75E-12), *Parvimonas* (phylum Firmicutes) (FC = −5.81, adj-P = 5.37E-3), and *Streptococcus* (phylum Firmicutes) (FC = −2.73, adj-P = 2.15E-2) when compared with the CON group.

The ASP group had higher RA of the genera *Providencia* (phylum Proteobacteria) (FC = 16.03, adj-P = 1.21E-10) and *Enorma* (phylum Actinobacteria) (FC = 13.55, adj-P = 2.03E-10) when compared with the CON group, and lower RA of *Butyrivibrio* (phylum Firmicutes) (FC = −19.75, adj-P = 4.20E-7) (Figure 2B). Similar to the findings for the NANS group, the RA of *Salmonella* (FC = 14.40, adj-P = 1.35E-8), *Klebsiella* (FC = 6.85, adj-P = 7.12E-3), and *Escherichia-Shigella* (FC = 4.37, adj-P = 1.02E-2) were higher in the ASP group vs. CON, and the RA of *Parvimonas* (FC = −19.72, adj-P = 4.20E-7), *Arcobacter* (FC = −19.69, adj-P = 4.20E-7), and *Streptococcus* (FC = −4.68, adj-P = 5.80E-3) were lower in the ASP group vs. CON.

Stool: Predicted microbial metabolic functions are different in the NANS vs. CON and ASP vs. CON groups

Analysis of predicted stool microbial metabolic functions indicated that functions that were significantly different in NANS vs. CON were similarly altered in ASP vs. CON. The top downregulated pathways in both NANS vs. CON and in ASP vs. CON were 8-oxo-(d)GTP detoxification II (NANS vs. CON FC = −8.05, adj-P = 7.84E-8; ASP vs. CON FC = −6.05, adj-P = 3.61E-2), 2,4-dichlorophenoxyacetate degradation (NANS vs. CON FC = −6.15, adj-P = 3.11E-3; ASP vs. CON FC = −14.38, adj-P = 8.25E-4), and 4-chloro-2-methylphenoxyacetate degradation (NANS vs.

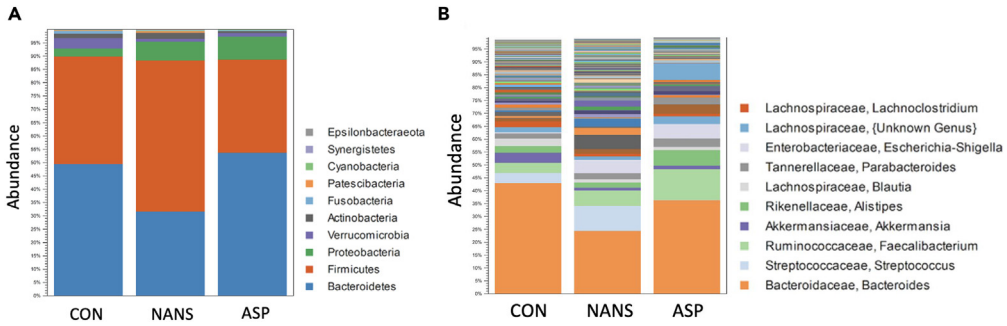

Figure 2. Stool microbial profiles in the CON, NANS, and ASP groups

(A) Phylum level.

(B) Genus level.

CON FC = −6.15, adj-P = 3.28E-3; ASP vs. CON FC = −14.38, adj-P = 8.25E-4). The top upregulated pathways in both the NANS vs. CON and the ASP vs. CON groups were phosphate acquisition (NANS vs. CON FC = 2.62, adj-P = 2.85E-4; ASP vs. CON FC = 2.99, adj-P = 1.03E-2), L-glucose degradation (NANS vs. CON FC = 2.56, adj-P = 8.49E-4; ASP vs. CON FC = 2.99, adj-P = 2.25E-2), and uracil degradation III (NANS vs. CON FC = 2.64, adj-P = 8.49E-4; ASP vs. CON FC = 3.04, adj-P = 2.39E-2, see Table S3).

Duodenal: Microbial alpha diversity, but not beta diversity, is significantly altered in the NANS group

Duodenal microbial alpha diversity was significantly lower in the NANS group (N = 35) when compared with the CON group (N = 55), as determined using Shannon index (p = 0.04) (Figure 3A). There were no significant differences in the duodenal microbial alpha diversity of the ASP group (N = 9) when compared with either the NANS or CON groups (p = 0.7) (Figure 3A). There were no significant differences in beta diversity in the NANS vs. CON (p = 0. 122) or ASP vs. CON (p = 0. 271) groups, as determined using the Bray-Curtis index (Figure 3B).

Duodenal: Microbial profiles in NANS and ASP subjects are significantly different from the CON group

The RA of the most predominant bacterial phylum in the duodenum, Firmicutes, was 1.25-fold higher in the NANS vs. CON group (adj-P = 8.36E-6), whereas the RA of phyla Proteobacteria was lower (FC = −1.40, adj-P = 0.01). The RA of Bacteroidetes (FC = −0.96, p = 0.04, adj-P = 0.1) and Fusobacteria (FC = −0.78, p = 0.04, adj-P = 0.1) also tended toward being lower in the NANS vs. CON group (Figure 4A, Table S4). In contrast, none of these phyla were significantly different in the ASP vs. CON group, but there was a trend toward lower Synergistetes RA (FC = −2.48, p = 0.04, adj-P = 0.16) and lower Spirochaetes RA (FC = −3.12, p = 0.01, adj-P = 0.10) (Figure 4A, Table S4).

At the genus level, RA of several bacterial features differed significantly in the NANS vs. CON group (adj. p < 0.05). These included several genera that were previously identified as part of the core duodenal microbiome, defined as the most widespread microbial components of the microbiome that are found across all subjects,[35] including 1.81-fold higher *Streptococcus* (phylum Firmicutes) RA (adj-P = 2.14E-7) and 1.14-fold lower *Fusobacterium* (phylum Fusobacteria) RA (adj-P = 0.04) in the NANS group (Figure 4B, Table S5). Additional non-core genera that had significantly lower RA in NANS vs. CON subjects included *Carnobacterium* (phylum Firmicutes) (FC = −10.02, adj-P = 0.00) and *Aeromonas* (phylum Proteobacteria) (FC = −10.14, adj-P = 0.00). Non-core genera that had significantly higher RA in NANS vs. CON subjects included *Paeniclostridium* (phylum Firmicutes) (FC = 8.78, adj-P = 0.00) and *Phocaeicola* (phylum Bacteroidetes) (FC = 6.50, adj-P = 8.2E-15).

The RA of several genera differed significantly in the ASP vs. CON group, including the core genera *Lactobacillus* (phylum Firmicutes) (FC = −5.79, adj-P = 1.17E-3) and *Fusobacterium* (phylum Fusobacteria) (FC = −1.98, adj-P = 0.045) (Figure 4B). Of the non-core genera, the RA of an unknown genus from family Neisseriaceae (phylum Proteobacteria) was 11.63-fold higher in the ASP vs. CON group (adj-P = 0.00), as was the RA of genera *Hydrogenophaga* (phylum Proteobacteria) (FC = 7.07, adj-P = 4.92E-14) and *Leifsonia* (phylum Actinobacteria) (FC = 6.88, adj-P = 1.15E-13). In contrast, the RA of genera *Roseomonas* (phylum Proteobacteria) (FC = −8.98, adj-P = 3.80E-05), *Lysinibacillus* (phylum Firmicutes) (FC = −8.35, adj-P = 5.84E-05), and *Aeromonas* (phylum Proteobacteria) (FC = −7.78, adj-P = 2.60E-4) were lower in the ASP vs. CON group (Figure 4B).

The RA of genera *Escherichia* (phylum Proteobacteria) and *Klebsiella* (phylum Proteobacteria), which are known disruptors in the duodenal microbiome,[32] were lower in both the NANS and ASP groups than in the CON group (NANS vs. CON: FC = −6.89, adj-P = 8.20E-15; FC = −7.03, adj-P = 2.13E-11, respectively; ASP vs. CON: FC = −7.81, adj-P = 1.04E-6; FC = −7.39, adj-P = 1.15E-4, respectively), as was the RA of genus *Salmonella* (NANS vs. CON: FC = −6.32, adj-P = 2.45E-8; ASP vs. CON: FC = −4.98, adj-P = 5.31E-3, Figure 4B).

Duodenal: Predicted microbial metabolic functions in NANS differ significantly from those in CON, with lesser differences in ASP

Analysis of predicted duodenal microbial metabolic functions found significant differences comparing the NANS with the CON group, with changes identified in a total of 386 pathways (Figure 5). The top downregulated pathways in the NANS group were xanthan biosynthesis

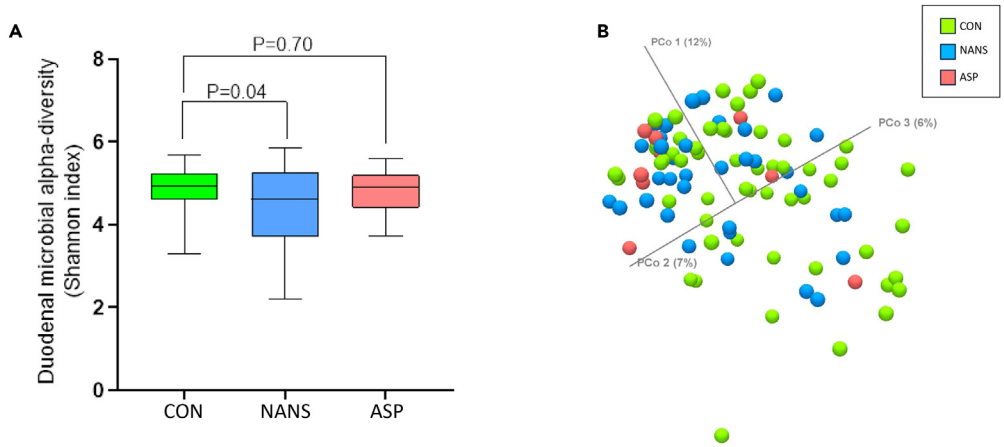

Figure 3. Duodenal microbial alpha and beta diversity in the CON, NANS, and ASP groups

(A) Alpha diversity, as determined using Shannon's index.

(B) Beta diversity, as determined using the Bray-Curtis index. Data in (A) are presented as mean ± standard deviation.

(FC = −25.33, adj-P = 1.50E-03), acetan biosynthesis (FC = −24.03, adj-P = 2.10E-03), and sulfoacetaldehyde degradation III (FC = −18.31, adj-P = 1.55E-03), and the top upregulated pathways in the NANS group were 4,4-diapolycopenedioate biosynthesis (FC = 10.06, adj-P = 1.10E-04), cinnamoyl-CoAbiosynthesis (FC = 7.07, adj-P = 9.72E-03), and L-lysine degradation V (FC = 4.89, adj-P = 0.01) (see Table 2).

In contrast, predicted microbial metabolic functions in the ASP group were more similar to the CON group, and only one function, cylindrospermopsin biosynthesis, was significantly enriched in the ASP group (FC = 4.1, adj-P = 0.02) (see Table 2).

DISCUSSION

In this study, we explored the potential effects of consuming non-sugar sweeteners (NSS), including both non-aspartame non-sugar sweeteners (NANS) and aspartame (ASP), on the compositions of both the stool and duodenal microbiomes. Significant differences in both stool and duodenal microbial diversity and composition, as well as in levels of circulating inflammatory markers, were identified in NANS and ASP consumers compared with controls (CON). Further, alterations in predicted microbial metabolic pathways in both stool and the duodenum were also identified in both the NANS and the ASP groups when compared with the CON group. Interestingly, the microbial pathways predicted to be altered in the NANS and ASP groups were similar in stool but were strikingly different between these two groups in the duodenum. Given the crucial role played by small intestinal microbes in digestion, nutrient absorption, immune regulation, and endocrine functions, coupled with the substantial prevalence of NSS consumption among US adults (estimated at 41.4%), our findings have potential implications for metabolic and gastrointestinal health in a considerable proportion of the American adult population.[53]

NSS have become increasingly popular as sugar substitutes, are consumed in pre-packaged foods and beverages,[3] and considered safe by regulatory agencies such as the US Food and Drug Administration (FDA),[54] Health Canada,[55] and the European Food and Safety Authority (EFAS).[56] However, contrasting research has highlighted the growing concerns regarding their potential impacts on human health, including potential impacts on the gut microbiome.[57] The World Health Organization recently published a review exploring the health effects of NSS, including acesulfame K, advantame, cyclamate, neotame, saccharin, steviol glycosides, sucralose, and aspartame.[4] Through a systematic review and meta-analysis, they found higher intakes of NSS were associated with increases in BMI and risk of developing type 2 diabetes, all-cause mortality, cardiovascular events, any type of stroke, hypertension, and bladder cancer.[4] Additionally, the International Agency for Research on Cancer (IARC) recently described aspartame as "possibly carcinogenic to humans" based on "limited" evidence that aspartame may cause hepatocellular carcinoma in humans.[6,7] While the Joint Food and Agriculture Organization (FAO)/WHO Expert Committee on Food Additives (JECFA) concluded that there was "no sufficient reason" to alter the current acceptable daily intake for aspartame (40 mg/kg) body weight, they did also note that further studies and randomized controlled trials were needed.[7] These concerns illustrate that additional research on the effects of NSS, including aspartame, are warranted.

Many studies of the human intestinal microbiome to date have relied on stool samples. Therefore, while the focus of the REIMAGINE study and this paper is the small bowel microbiome, we included an analysis of stool samples provided by a subset of subjects in order to compare our data with previously published studies. We found no significant differences in stool microbial alpha diversity in either the NANS or ASP groups when compared with the CON group, which is consistent with the findings of Ahmad et al.[47] However, we did find significant changes in stool microbial composition at both the phylum level and genus level in the ASP and NANS groups when compared with the CON group. Specifically, we found the RA of *Klebsiella*, *Escherichia-Shigella*, and *Salmonella* (all anaerobes from family Enterobacteriaceae) were higher in both the NANS and the ASP group when compared with CON, which is consistent with the previous identification of a positive correlation between long-term AS consumption and Enterobacteriaceae in human subjects.[43] However, several other human studies did not find significant differences in stool microbial composition in subjects consuming aspartame,[50] various other NSS,[47,51,52] or artificially sweetened

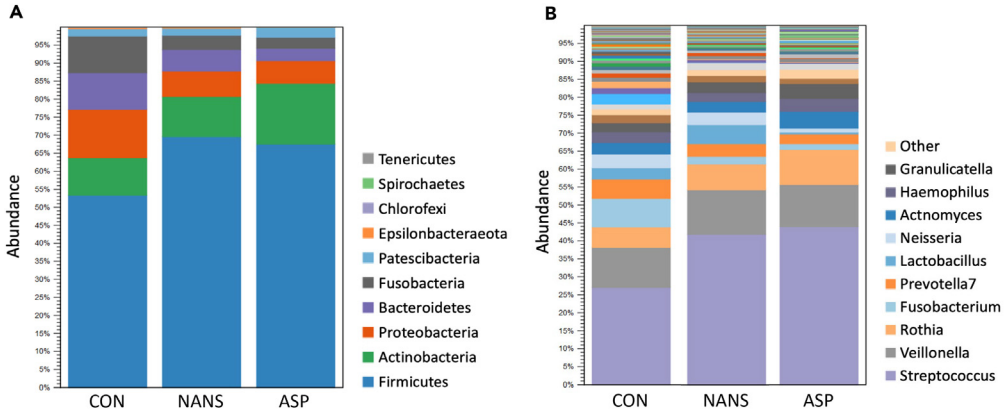

Figure 4. Small bowel microbial profiles in the CON, NANS, and ASP groups

(A) Phylum level.

(B) Genus level. The top 10 most abundant genera in the duodenal microbiome are indicated on the right in (B).

beverages.[58] In contrast, most studies using rodent models have found that NSS consumption does affect gut microbiome composition, although specific findings are highly dependent on both dose and route of administration.[59] Interestingly, Rodriguez-Palacios et al. identified an association between sucralose consumption and increased relative abundance of Proteobacteria and decreased relative abundance of Chloroflexi in stool samples,[45] which is consistent with our findings of increased Proteobacteria relative abundance and decreased Chloroflexi relative abundance in the NANS group when compared with controls, and Abou-Donia et al. found that rats fed sucralose exhibited decreases in anaerobic bacteria and lactobacilli,[60] which is also consistent with our findings. Further, Bian et al. found that mice consuming sucralose also exhibited decreases in *Streptococcus* in stool, again consistent with our findings.[61]

With respect to the small bowel, we found significant differences in duodenal microbial composition in NSS consumers compared with controls, including higher relative abundance of phylum Firmicutes and genus *Streptococcus* in the NANS group, and lower relative abundance of genus *Fusobacteria* in both the NANS and ASP groups. Unexpectedly, the duodenal RA of genera *Escherichia* (phylum Proteobacteria) and *Klebsiella* (phylum Proteobacteria) were lower in both the NANS and ASP groups than in CON, which differs from our findings in stool samples. *Escherichia* and *Klebsiella* are known disruptors in the duodenal microbiome[32] and both are associated with GI conditions such as inflammatory bowel disease.[62,63] Interestingly, NSS such as aspartame have been shown to have antimicrobial activity against common periodontal pathogens,[64] and saccharin specifically has previously been shown to inhibit *E. coli* growth *in vitro*.[42] Further, a meta-analysis by Tepler et al. found that AS consumption was linked to a 19% decreased risk of developing luminal gastrointestinal cancer.[65] Taken together, these and our findings may suggest a beneficial effect of NSS consumption with respect to small bowel microbial populations.

The small bowel microbiome also contributes to innate immunity[20] and host-microbial crosstalk.[21,22] We found that circulating levels of IL-1β were significantly decreased in NANS vs. CON subjects. IL-1β is a pro-inflammatory cytokine that plays a crucial role in the immune response, promoting inflammation as part of the host defense mechanism.[66] Reducing IL-1βactivity has emerged as an effective treatment option in autoinflammatory diseases such as atherosclerosis, heart failure, recurrent pericarditis, and rheumatoid arthritis.[67–69] Additionally, decreases were identified in IL-6 and IL-10 in the ASP group when compared with the CON group. IL-6 is a pro-inflammatory cytokine that plays central roles in both innate and adaptive immunity[70] and has been shown to be elevated in diseases such as IBD, colorectal cancer, and rheumatoid arthritis.[71–73] However, IL-6 also plays key roles in intestinal epithelial regeneration and repair, so much so that the safety of IL-6 blockade therapy has been questioned.[74] In contrast, IL-10 is an anti-inflammatory cytokine that protects against uncontrolled immune responses by regulating inflammation, and decreased levels of IL-10 have been demonstrated in diseases such as psoriasis and chronic obstructive pulmonary disease.[75,76] The decreases in IL-10 and IL-6 levels identified here may suggest that alterations in pro-inflammatory and anti-inflammatory immune responses play a role in mediating the effects of NSS on the host.

Further, analysis of predicted microbial pathways revealed significant differences in the ASP and NANS groups when compared with the CON group. While similar changes in predicted stool microbial metabolic pathways were identified in both the ASP and NANS groups, significantly more duodenal microbial metabolic pathways were predicted to be altered in the NANS group than in the ASP group, again illustrating that changes in small intestinal microbial populations may have greater implications for microbial functions. The duodenal microbial pathways predicted to be downregulated in the NANS group include pathways for the biosynthesis of the polysaccharides xanthan and acetan, as well as the D-galactose degradation pathway, which may be indicative of shifts in the metabolism of sugars in subjects consuming NSS. These pathways were not altered in the ASP group, possibly reflecting a lesser effect of aspartame on metabolism of sugars. Pathways related to lysine metabolism were also altered in the NANS group, which is consistent with findings in humans consuming AS.[43] Regarding the ASP group, it was interesting that only one pathway was significantly altered in the ASP group vs. CON, the cylindrospermopsin biosynthesis pathway. Cylindrospermopsin is recognized for its harmful effects on the liver and the nervous system, and it is classed as a potential cancer-causing agent.[77] Cylindrospermopsin can hinder the synthesis of the antioxidant glutathione as well as

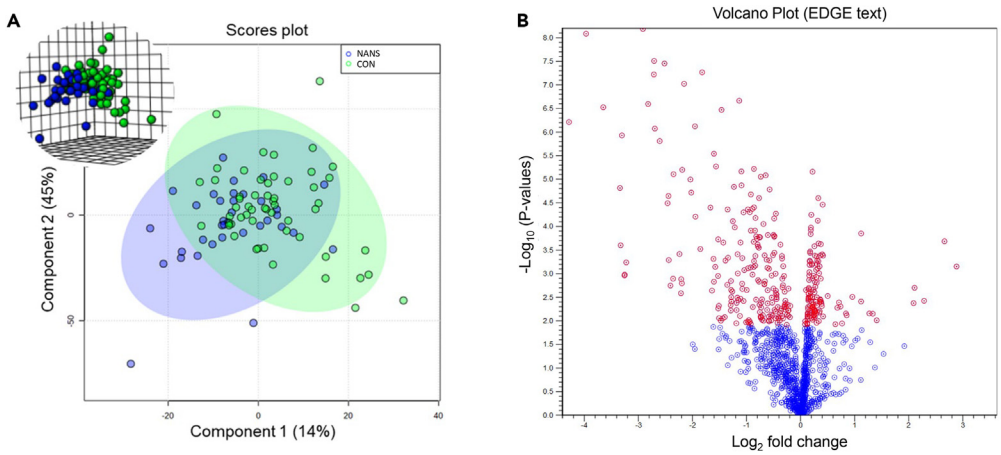

Figure 5. Differences in MetaCyc predicted duodenal microbial metabolic functions in the CON (green) and NANS (blue) groups

(A) Dimensionality reduction analysis plot of all microbial metabolic functions detected in the duodenum.

(B) Volcano plot of differences in microbial metabolic functions between the NANS and CON groups.

several proteins and has inhibitory effects on cytochrome P450 enzymes, found primarily in liver cells.[77] This finding is particularly interesting given recent concerns regarding aspartame and risks for hepatocellular cancer,[6–8] as well as the known effects of cylindrospermopsin in inducing stress in human liver cells.[78]

Limitations of the study

First, subjects underwent upper endoscopy for a variety of reasons, including evaluation of intestinal complaints and screening for familial and other risk factors. Consequently, the study population may not be fully representative of normal, healthy individuals. Additionally, after applying all of the filters for potential confounders, the duodenal sample size for the ASP group was small. The stool sample sizes were

Table 2. Top duodenal microbial metabolic functions predicted to be enriched or depleted in the NANS vs. CON groups and the ASP vs. CON groups

MetaCyc functions	Pathway description	Fold change	Adj-P-value
NANS vs. CON			
4,4-diapolycopenedioate biosynthesis	Biosynthesis of a precursor to the red pigment, diapolycopene	10.06	1.10E-04
Cinnamoyl-CoAbiosynthesis	Biosynthesis of cinnamoyl-CoA, a key intermediate in lignin biosynthesis and secondary metabolism	7.07	9.72E-03
L-lysine degradation V	Degradation of lysine via the saccharopine pathway	4.89	0.01
Alpha-diglucosyldiacylglycerol biosynthesis	Biosynthesis of the membrane lipid α-diglucosyldiacylglycerol, which is found in chloroplasts and some bacteria	3.33	2.37E-04
UDP-α-D-galacturonate biosynthesis II (from D-galacturonate)	Biosynthesis of UDP-α-D-galacturonate, a key intermediate in pectin biosynthesis	2.58	0.02
(S,S)-butanediol biosynthesis	Fermentative biosynthesis of (S,S)-butanediol, a potential biofuel and platform chemical	1.85	0.01
Xanthan biosynthesis	Biosynthesis of xanthan, a high-molecular-weight extracellular polysaccharide produced by bacteria such as *Xanthomonas campestris*	−25.33	1.50E-03
Acetan biosynthesis	Biosynthesis of acetan, a low-molecular-weight extracellular polysaccharide produced by bacteria such as *Acetobacter xylinum*	−24.03	2.10E-03
Sulfoacetaldehyde degradation III	Degradation of sulfoacetaldehyde, a toxic intermediate of taurine catabolism	−18.31	1.55E-03
D-galactose degradation II	Degradation of D-galactose via the Leloir pathway	−15.72	4.17E-04
L-lysine degradation IV	Degradation of lysine via the aminoadipate pathway	−14.65	2.46E-03
ASP vs. CON			
Cylindrospermopsin biosynthesis	Cylindrospermopsin is a toxic secondary metabolite produced by certain species of cyanobacteria	4.1	0.02

also limited, which can make it difficult to control for normal variations between subjects. However, stool data were primarily included to allow comparisons with prior published analyses. Our duodenal aspirate samples reflect the luminal microbiome, not the mucosa-associated microbiota. In addition, our samples are collected at a single time point, which limits our ability to establish causal relationships. Lastly, we do not have data regarding the absolute volume and frequency of artificial sweetener consumption among the subjects.

Conclusions

This study provides novel insights into the potential impacts of NSS on the gut microbiome, including the metabolically relevant duodenal microbiome. Our findings suggest that NSS affect the stool and small bowel microbiomes differently and that there are also differences between the effects of NANS and ASP. Future research efforts will aim to elucidate the mechanisms underlying these effects and explore potential interventions to mitigate adverse outcomes resulting from artificial sweetener consumption.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, R.M. and M.P.; resources, A.H. and MR; investigation, G.L., G.B., G.P., W.M., and S.W.; formal analysis, G.L., J.W., A.R., and M.P.; project administration, G.B., M.P., and R.M.; writing—original draft, A.H., G.L., G.B., and R.M.; writing—review & editing, G.L., G.B., A.R., M.P., and R.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human Duodenal Aspirates	REIMAGINE Study Subjects	https://www.cedars-sinai.org/programs/digestive-liver-diseases/clinical/gi-motility/clinical-trials/small-intestinal-sampling-study-reimagine.html Leite et al. 2019[31]
Human Blood Samples	REIMAGINE Study Subjects	As above
Human Stool Samples	REIMAGINE Study Subjects	As above
Chemicals, peptides, and recombinant proteins		
Sputolysin (Dithiothreitol (DTT))	EMD Millipore Corp	CAS 578517
Allprotect Tissue Reagent	Qiagen	Cat. No. 76405
PhiX Control v3	Illumina	FC-110-3001
Critical commercial assays		
Human Cytokine/Chemokine Magnetic Bead Panel	EMD Millipore Corp	HCYTOMAG-60K
MagAttract PowerMicrobiome DNA/RNA EP Kit	Qiagen	27500-4-EP
MagAttract PowerSoil DNA EP Kit	Qiagen	27100-4-EP
Qubit 1X dsDNA, High Sensitivity Assay kits	Invitrogen	Q33231
MiSeq Reagent Kit v3 (600 cycles)	Illumina	MS-102-3003
Deposited data		
16S rRNA gene sequencing data	This paper	National Center for Biotechnology Information (NCBI) BioProject Repository https://www.ncbi.nlm.nih.gov/bioproject) under BioProject: PRJNA1010208
Oligonucleotides		
16S Amplicon PCR Forward Primer TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	Leite et al., 2019[31]	N/A
16S Amplicon PCR Reverse Primer 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC	Leite et al., 2019[31]	N/A
Software and algorithms		
CLC Genomic Workbench v.20.0.3	Qiagen	https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-clc-genomics-workbench/
CLC Microbial Genomics Module v.2.5	Qiagen	https://digitalinsights.qiagen.com/plugins/clc-microbial-genomics-module/
MetaCyc Pathway Database	MetaCyc	https://metacyc.org/
GraphPad Prism 7.02	GraphPad Software, La Jolla, CA, USA	https://www.graphpad.com/scientific-software/prism/
IBM SPSS Statistics Version 28	IBM	https://www.ibm.com/products/spss-statistics
Other		
OMNIgene GUT tubes	DNA Genotek	MP-018

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr Ruchi Mathur (ruchi.mathur@cshs.org).

Materials availability

This study did not generate new unique reagents.

DATA AND CODE AVAILABILITY

- The 16S rRNA gene sequencing datasets generated during this study have been deposited at the National Center for Biotechnology Information (NCBI) BioProject Repository and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Potential study subjects were identified from the REIMAGINE study.[31] Based on responses given in the diet portion of their questionnaire, subjects were divided into 3 groups: those who consume non-aspartame, non-sugar artificial sweeteners (NANS), which included Stevia, sucralose and 'other', those who consume aspartame only (ASP), and controls who do not consume non-sugar artificial sweeteners (CON). Any subject with type 2 diabetes (T2D) was excluded from the study cohort, as were subjects who were taking metformin for additional indications, as both T2D[16] and metformin[48] can affect gut microbial composition. Subjects with small intestinal bacterial overgrowth (SIBO) were also excluded, due to the confounding effects of the small bowel disturbances in SIBO.[49] The groups were then matched by age ($\pm$5 years), body mass index (BMI, ($\pm$3 kg/m^2), and sex. The final compositions of the groups were as follows:

(1) NANS: N=35, number of females (F)=16, age= 59.50 $\pm$ 14.40 years
(2) ASP: N=9, F=5, age= 59.40 $\pm$ 17.60 years
(3) CON: N=55, F=31, age=60.30 $\pm$ 13.80 years

The study subjects reported races and ethnicities as follows (of note, some subjects selected more than one race):

(1) NANS: Caucasian N=32, African American N=2, Asian N=0, American Indian or Alaska Native N=2, Native Hawaiian or other Pacific Islander N=0, Unknown N=0. Hispanic N=2, Non-Hispanic N=26, Unknown N=7.
(2) ASP: Caucasian N=9, African American N=0, Asian N=0, American Indian or Alaska Native N=0, Native Hawaiian or other Pacific Islander N=0, Unknown N=0. Hispanic N=2, Non-Hispanic N=7, Unknown N=0.
(3) CON: Caucasian N=46, African American N=3, Asian N=1, American Indian or Alaska Native N=1, Native Hawaiian or other Pacific Islander N=1, Unknown N=3. Hispanic N=3, Non-Hispanic N=48, Unknown N=4.

Gender identities are not available as these were not recorded in the REIMAGINE study questionnaire at the time of study inception. The REIMAGINE study is approved by the Institutional Review Board at Cedars-Sinai Medical Center (IRB #35192). All subjects gave informed written consent before participating in the study.

Stool subanalysis

While the primary focus of the REIMAGINE study is the small bowel microbiome, all participants who are willing are asked to contribute stool samples for analysis. Of the 99 subjects in this study, 40 had also provided stool samples:

(1) NANS: N=11, F=8, age=59.6 $\pm$ 13.8 years
(2) ASP: N=4, F=3, age=60.2 $\pm$ 12.0 years
(3) CON: N=25, F=16, age=62.2 $\pm$ 13.6 years

METHOD DETAILS

Study subjects

Subjects in this study were recruited for the REIMAGINE study.[31] Individuals aged between 18-85 years old undergoing standard of care upper endoscopy (esophagogastroduodenoscopy [EGD]) without colon preparation are eligible for participation.[31] Prior to EGD, REIMAGINE subjects are asked to complete a questionnaire that includes self-documented family and medical history. All medical information provided by subjects is verified against medical records. All data were de-identified prior to analysis.

Blood collection and analysis

After completing the study questionnaire, fasting blood samples were collected. Circulating cytokine and chemokine levels were analyzed on a Luminex FlexMap 3D (Luminex Corporation, Austin, TX, USA) using a bead-based multiplex panel that included: granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFNγ), interleukin (IL)-10, IL-12P70, IL-13, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, and tumor necrosis factor alpha (TNFα) (EMD Millipore Corp., Billerica, MA, USA, cat. #HCYTOMAG-60K).

Duodenal luminal aspirates collection and processing

Samples of duodenal luminal fluid were obtained during the EGD, using a sterile double-lumen aspiration catheter (Hobbs Medical, Inc.).[31] Aspirates were collected via a sterile inner catheter that was pushed through a sterile bone wax cap only after the second portion of the duodenum was reached, in order to reduce contamination from the mouth, esophagus and stomach. Due to the high viscosity of aspirates, an equal volume of sterile 1x dithiothreitol (DTT) was added to each duodenal aspirate (~1mL) and the samples were vortexed until fully liquified (~30 s).[31] 100 µl of each sample was then serially diluted with 900 µL sterile 1x PBS and plated on MacConkey agar (Becton Dickinson, Franklin Lakes, NJ, EUA), and on blood agar (Becton Dickinson). Plates were incubated at 37°C for 16-18 hours under aerobic (MacConkey) or anaerobic (blood agar) conditions. Colony forming units (CFU) were then counted electronically using a Scan 500 (Interscience, Paris, France).

The remainder of each sample (the portion not used for microbial culture) was centrifuged at maximum speed (>13000 RPM) for 5 minutes. The supernatant was removed, and 1 mL of sterile Allprotect reagent (QIAGEN, Hilden, Germany) was added to the microbial pellet. The pellets under Allprotect were then stored at −80°C prior to DNA isolation for sequencing.[31]

Stool collection

Stool samples were self-collected at home, within days of the upper endoscopy procedure, placed in OMNIgene GUT tubes (DNA Genotek, Ottawa, ON, Canada) at room temperature and returned promptly to the laboratory for analysis.

DNA isolations

On the day of DNA isolation, duodenal aspirate microbial pellets under Allprotect were thawed on ice and 1x DTT was added in a 1:1 ratio and the mixture was vortexed.[31] Microbial DNAs were isolated from duodenal aspirates using the MagAttract PowerMicrobiome DNA/RNA EP Kit (Qiagen) on a KingFisher Duo (Thermo Fisher Scientific, Waltham, MA, USA).[31] Stool DNAs were isolated using MagAttract PowerSoil DNA EP Kits (Qiagen) and purified using a KingFisher Duo automated system (ThermoFisher Scientific, Waltham, MA). Isolated DNAs were quantified using Qubit 1X dsDNA, High Sensitivity Assay kits (Invitrogen by Thermo Fisher Scientific) on a Qubit 4 Fluorometer (Invitrogen, Carlsbad, CA, USA).

Library preparation and 16S rRNA sequencing and analysis

The V3 and V4 regions of the 16S rRNA gene were amplified using custom primers that had been modified to include adapters for Illumina sequencing as follows:

16S Amplicon PCR Forward Primer: 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG.

16S Amplicon PCR Reverse Primer: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

The purity and concentration of the amplicon library was checked using an Agilent 2100 Bioanalyzer System. The library was then sequenced on a MiSeq System Illumina, San Diego, California) via paired-end sequencing with 2x301 cycles of amplification, using the MiSeq Reagent Kit v3 (600 cycles) and 5% to 10% PhiX (Illumina).

QUANTIFICATION AND STATISTICAL ANALYSIS

The closed source software programs CLC Genomic Workbench v.20.0.3 and CLC Microbial Genomics Module v.2.5 (Qiagen) were used to group similar DNA sequences into Operational Taxonomic Units (OTUs) and identify the taxonomy of each OTU by comparing it to the SILVA database v.132. Samples with low sequencing depth (<5,000 sequences per sample) were removed from the analysis. CLC Microbial Genomics Module Package v.2.5 (Qiagen) tools were used to calculate different measures of microbial diversity (alpha diversity) and the differences in microbial communities (beta-diversity) between groups. Data are presented as mean ± standard deviation.

To identify which OTUs were present in different amounts between groups, the OTU table was rarefied to the minimal number of reads found in any sample and a statistical model (Negative Binomial GLM) was used to estimate the fold change (FC) of an OTU between groups, again using the CLC Microbial Genomics Module Packages v.2.5 (Qiagen). The significance of these changes was determined using the Wald test and adjusting for multiple comparisons using the False Discovery Rate (FDR). A P-value <0.05 was considered to be statistically significant.

Analyses of predicted microbial pathways and functions was conducted using the Functional Analysis tool from CLC Microbial Genomics Module Package v.2.5 (Qiagen). Functional profiles were determined based on the EC database and Clusters of Orthologous Genes (COGs) terms provided by the CLC package, while pathway identification was performed using the MetaCyc Pathway Database (2022-05), also provided by the CLC package (Qiagen). Randomization analyses were carried out with 1000 replicates, with inclusion of super-pathways.

Additional statistical analyses such as Two-tailed Spearman r correlations and Mann-Whitney tests were performed using normalized OTU tables and software such as GraphPad Prism 7.02 (GraphPad Software, La Jolla, CA, USA) and IBM SPSS Statistics Version 28 software.