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Celiac Disease and a Gluten-Free Diet Lead to Loss of Methanobrevibacter from the Gut Microbiome

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Abstract

Introduction: The importance of the human microbiome has become well known in recent years. The microbiome contains a diverse number of organisms including bacteria, archaea, fungi, and protozoa, which when imbalanced, can lead to a variety of dysbioses. Celiac disease (CD) is a condition where the immune system responds to gluten, a protein found in wheat, leading to chronic inflammation in the small intestine. Studies have found that microbial dysbiosis is often associated with patients who have CD, but few have looked at how gluten affects the microbiome in comparison to CD. This research sought to identify microbiome changes between people with CD (on a gluten-free diet), those on a gluten-free diet (without CD), and a control group (without CD and a gluten-free diet).

Methods: Twenty-nine eligible participants (screened via a survey) provided a single stool sample (12 CD, 8 gluten-free, and 9 control). The microbial DNA was extracted from the stool samples using the QIAamp PowerFecal DNA Kit (Qiagen) and the V3/V4 region of 16s RNA using the 600-cycleMiSeq kit (Illumina). Sequenced DNA was sent to the University of Utah for analysis. Changes in microbiome diversity were statistically analyzed using a Kruskal-Wallis analysis.

Results: No change in alpha or beta diversity was seen between any study groups. In addition, significance was not observed in common phyla normally affected by CD (Firmicutes, Bacteroides, and Actinobacteria). However, a statistically significant difference was seen in the archaeal genera *Methanobrevibacter*, which was found only in the control group ($p = 0.0212$).

Discussion: Previously reported changes in the microbiome of CD patients were not observed in this study. However, changes could be seen in the archaeal genius, Methanobrevibacter, which was found only control group at an abundance of 3.3%. Thus, when CD individuals were compared to healthy individuals with similar gluten-free diets there was little difference in gut microbial species suggesting that gluten-free diet may normalize CD-related microbiome changes.

Conclusion: The absence of Methanobrevibacter from CD and gluten-free groups requires additional analysis to understand what role *Methanobrevibacter* plays in the microbiome and how a gluten-free diet may affect that role.

Keywords: celiac disease; gluten; gut microbiome; gluten-free diet

Introduction

The gastrointestinal tract (GI) of the human body contains tens of trillions of microorganisms, classified as the intestinal microbiota [1]. Over the past decade, we have begun to understand the tremendous potential these microbes have to influence health and disease. The human gut microbiome is a collection of beneficial bacteria that play a variety of functions [1]. The microbiome aids the host in many areas including organ morphogenesis, metabolism, aging, pathogen protection, and maturation of the immune system [2,3]. Gut bacteria facilitate the digestion of soluble fiber, as well as producing vitamins such as vitamin K. This crucial symbiotic relationship between the host and the gut microbiome is important in programming the immune system, as well as teaching the difference between pathogens and commensal bacteria [3].

Celiac disease (CD) is an autoimmune disease that primarily affects the GI tract [4,5]. The disease targets the villi of the small intestines which help increase the absorption of digestive nutrients such as amino acids and monosaccharides [4,6]. In CD, the villi are affected by an inflammatory reaction that occurs upon the digestion of gluten [4]. Glutens are a group of storage proteins that are found in grains such as wheat, rye, and barley [5]. In normal digestion, long strands of these proteins are broken down by digestive enzymes [2]. These peptides can be broken down further and absorbed through the intestines [1,4]. For people with celiac disease, gluten cannot be broken down by the digestive enzymes, and the molecule triggers a T-cell inflammatory response [7,8]. Inflammation caused by gluten can lead to the villi of the small intestines becoming damaged or shortened [9]. Affected villi can lose the ability to absorb carbohydrates and fats, minerals,

vitamins such as A, D, E, and K, as well as other nutrients such as iron, folic acid, and calcium [4,9]. These can cause issues ranging from weight loss, to anemia, to even an increased occurrence of certain cancers such as lymphomas and carcinomas [4].

In the gut microbiota, there are three major phyla: *Firmicutes*, *Bacteroides*, and *Actinobacteria* [2]. In patients with clinically active celiac disease, studies report a dysbiosis of the microbiota with an increase in gram-negative bacilli, such as Bacteroides, and a decrease in gram-positive bacteria, such as Bifidobacterium and lactobacilli [2,3,6,10]. The only treatment for celiac disease is maintenance of a gluten-free diet (GFD) [10]. GFDs have been correlated with a decrease in the number of gram-negative bacteria [7,10]. However, it is not always correlated with an increase of the beneficial gram-positive bacteria, such as lactobacilli [2,7,8]. Studies have also shown that GFDs can lead to a reduction in the microbial diversity of the gut microbiome [6,7]. More research is needed to better understand the effect of a gluten-free diet on microbial diversity in the gut microbiome.

Diet is one of the leading environmental factors that affects the diversity of the gut microbiome. Studies have shown that both a gluten-free diet and celiac disease have been associated with changes in the gut microbiome [5,8,11]. However, many celiac disease studies have examined the microbiome of individuals not on GFD treatment. This study examined the microbiome of celiac individuals on a gluten-free diet compared to healthy individuals with or without a gluten-free diet to further identify microbiome changes associated with the disease compared to the diet.

Methods

Participant Eligibility and Enrollment

We compared the microbiome of three different groups of study participants: 1. those diagnosed with celiac disease and on a gluten-free diet, 2. those who do not have celiac disease and are on a gluten-free diet, and 3. those who do not have celiac disease and are not on a specific diet. Subjects were recruited from Utah Valley University, as well as the Orem and Provo area using flyers, emails, and personal recruitment. The study protocol was approved by the Institutional Review Board of Utah Valley University (IRB #01966). Written informed consent was obtained from all participants. In order to determine eligibility, participants were screened using a survey before being allowed to participate in the study (Supplementary Figure 1). Inclusion criteria was 18-65 years of age, not planning to or currently pregnant, and have no other GI or autoimmune disease other than celiac disease. Participants in the CD must have been diagnosed with celiacs disease and been on a gluten-free diet for at least three months to ensure any diet related changes in the microbiome had stabilized. Participants in the gluten-free group must have been on a gluten-free diet for at least three months. Participants were asked if they had taken or if they were currently on probiotics, as well as any antibiotics. Participants were excluded if they had been on an antibiotic within the past 3 months.

Fecal Collection

Eligible participants were given a collection kit consisting of instructions, a collection device, a storage container, and ice packs, which would allow for the subjects to self-collect a fecal sample. Upon receipt of samples, stool samples and their containers were placed in a -80 °C freezer for storage. Participants were sent a post survey after dropping off their fecal sample, which asked about their diet, eating habits, and demographics.

DNA Extraction and Processing of Samples

DNA was extracted from frozen stool samples using a QIAamp PowerFecal DNA (Qiagen) kit following manufacturer specifications. The V3-V4 region of the 16S rRNA gene was amplified and sequenced following the 16S Metagenomic Sequencing Library Preparation protocol for the Illumina MiSeq system (Illumina, San Diego, CA). Indexing was completed using the Nextera XT Index Kit v2 Set A (Illumina) and sequencing was performed by the High-Throughput Genomics Core at the University of Utah using the MiSeq platform with Reagent Kit v3 (600-cycle) (Illumina). Sequence data was analyzed using Nephele version 2.10.0. on the QIIME 2 pipeline (36,37) with default settings. Reads were clustered into Operational Taxonomic Units (OTU) using a 97% identity threshold and taxonomic assignment was made by comparison to Greengenes. After processing, unclassified OTU's were removed, and samples were rarified before analysis. All sequence data is accessible through the National Center for Biotechnology Information SRA Database SUB11675505.

Statistical Analyses

Statistical analyses were completed with GraphPad Prism software (V8, GraphPad Software Inc., La Jolla, CA). Demographic data was analyzed with two-tailed p-values calculated through Fisher's exact test and chi-squared test. Alpha diversity and relative abundance comparisons between the celiac, gluten-free, and control arms were analyzed with the nonparametric Kruskal-Wallis, using Dunn's test for multiple comparisons. Gut microbiome dissimilarities between groups were visualized by Principal Coordinate Analysis (PCoA), using Bray-Curtis dissimilarities.

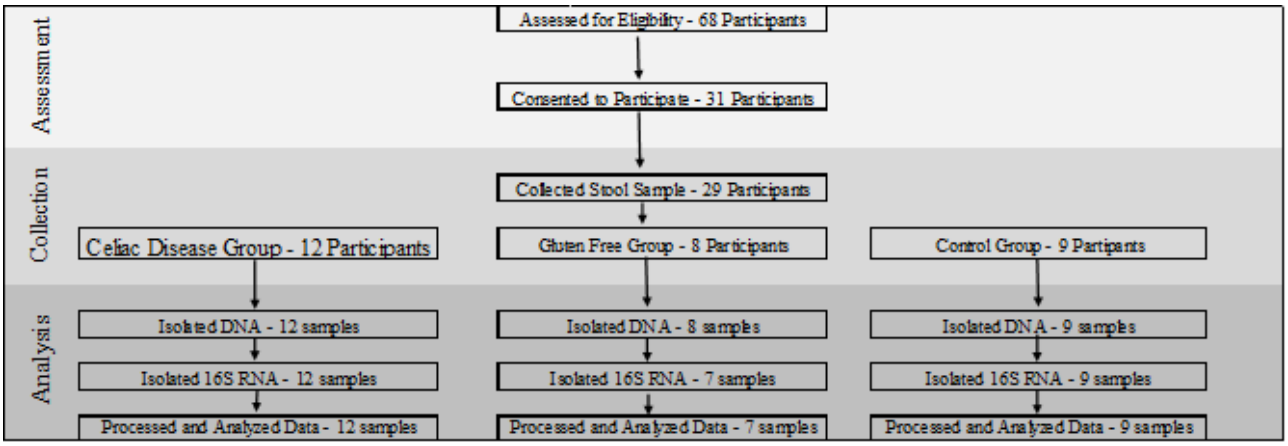

Figure 1. CONSORT flowchart. In total, 68 participants completed the enrollment survey. Of those, 31 met eligibility requirements and consented to participate. Twenty-nine participants returned a collected stool sample and were organized into study groups based on the enrollment survey. There were 12 placed in the CD group, 8 in the gluten-free group, and 9 in the control group. One sample from a participant in the gluten-free group was not able to be used due to the sample not having enough isolated DNA. All other participants provided fecal samples that were able to be fully processed. Figure made using Excel.

Results

Study Population

Study demographics include 28 total participants, with 12 allocated to the celiac disease group, 7 to gluten-free group, and 9 to control group (Table 1). Participant ages ranged from 18 to 54 years old. The mean age group was 25-34 with the other age groups getting roughly equal representation. All of the participants declared Caucasian as their race. The largest gender represented was female accounting for approximately 80% of the survey population. Three total participants reported taking probiotics during the last month.

Table 1. Participant Demographics

	CELIAC DISEASE GROUP	GLUTEN FREE GROUP	CONTROL GROUP
PARTICIPANTS (N=29)	43%	25%	32%
AGE (YEARS)			
18-24	16%	29%	22%
25-34	16%	29%	56%
35-44	33%	14%	11%
45+	25%	0%	0%
NO RESPONSE	8%	29%	11%
GENDER			
FEMALE	83%	86%	56%
MALE	17%	14%	44%
NO RESPONSE	0%	0%	0%
ETHNICITY			
CAUCASIAN	92%	71%	89%
OTHER	0%	0%	0%
NO RESPONSE	8%	29%	11%
PROBIOTICS			
Yes	8%	29%	0%
No	92%	71%	100%

Diversity of GI Microbiota

The health of a gut microbiome can be correlated to the diversity and microbial richness it contains. To this end, the alpha diversity of the hierarchal clustering of OTUs was analyzed using Shannon index and OTU Abundance (Figure 2). Results indicated no statistical difference between study arms. Beta diversity quantifies dissimilarities between study groups as another measure of microbial richness (Figure 3). There were no significant diversity dissimilarities between study groups as evidenced by the lack of clustering by study arm.

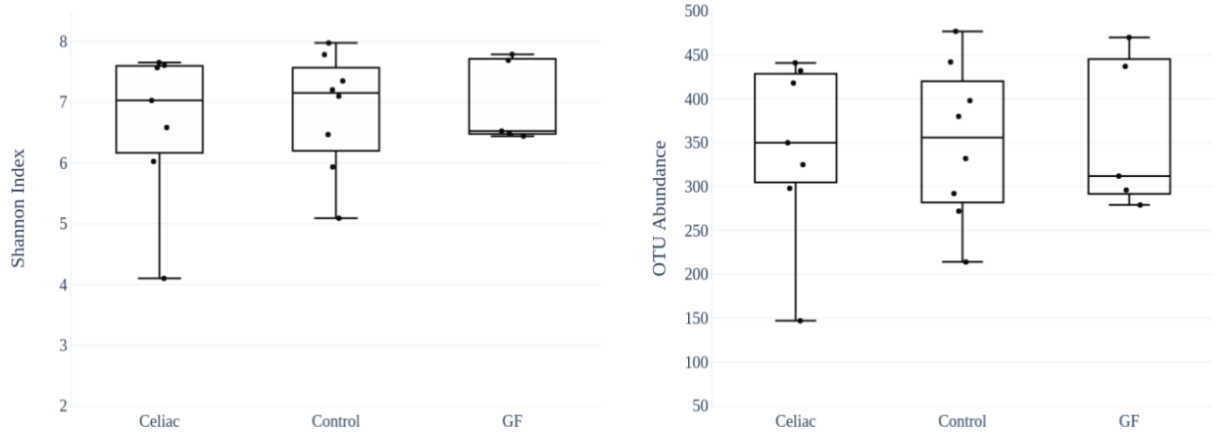

Figure 2. Alpha diversity. Species richness was measured using the Shannon Index (left panel) and OTU abundance (right panel). No significant diversity difference in individual sample diversity between groups is seen. Figure made using Excel.

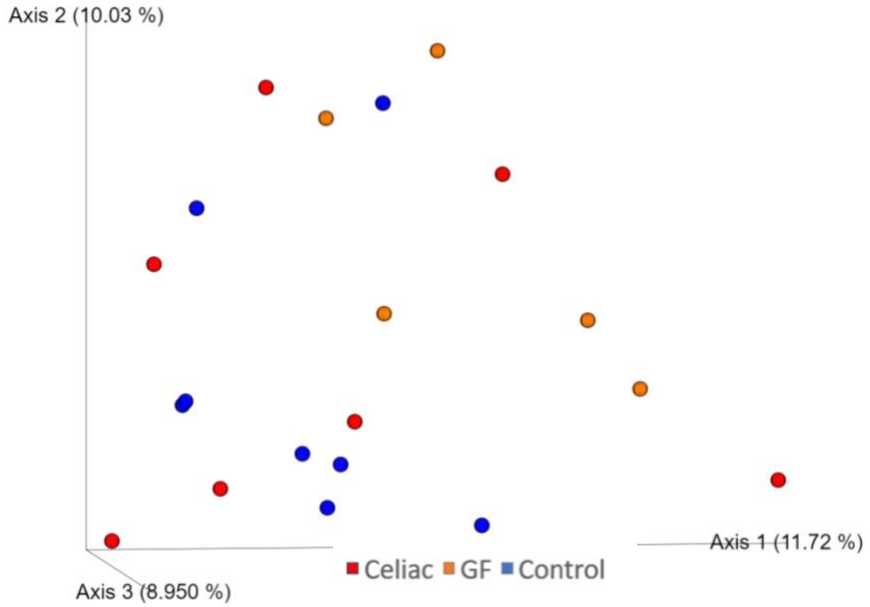

Figure 3. Principal coordinate analysis of Brays-Curtis beta diversity comparing celiac (red), gluten-free diet (orange), and control (blue) groups. No clustering is seen from the different treatments. Figure made using Excel.

Comparison of GI Microbiota

The composition and number of organisms present in the microbiome can affect how the microbiome interacts with, and influences. the host. Examining phyla level data indicated no significant difference in phyla identified between study groups (Figure 3). While all the traditional phyla found in the gut microbiome were observed, the levels correlate with what is traditionally seen in the literature [9,12]. Key groups such as *Ruminococcus*, *Furmicutes*, *Prevotella*, *Proteobacteria*, and *Blautia* were

all observed (Figure 4,5). A healthy microbiome normally has a higher quantity of good bacteria such as *Lactobacillus* and *Bifidobacterium* and low quantities of bad bacteria such as *Escherichia* and *Closterium* [2,9]. These groups were all present in our study, but the counts did not differ significantly across our study groups (celiac disease, gluten-free, and control).

The genus *Methanobrevibacter* was the only genera found to be significantly different. *Methanobrevibacter* is a genus of archaea that was only found in the control group with an abundance of 3.3% (1263 OTUs) (Figure 6). This was significant with a p-value of 0.0212.

Discussion

The purpose of this study was to examine the microbiome changes in those with celiac disease when compared to those on a gluten-free diet. To that end, 28 healthy adults were recruited to participate in the study and were assigned to groups corresponding to whether they had celiac disease (and a gluten-free diet), a gluten-free diet (with no celiac disease), and a control group consisting of participants who had neither celiac disease nor a gluten-free diet. Participants produced fecal samples, which went through a rigorous process of DNA extraction after which the V3-V4 region was amplified and sequenced with the help of Illumina. From this data, the taxonomic data to the genus level as well as microbial abundance was established.

We observed statistical significance in one genus that was found in significantly different numbers in the various groups. *Methanobrevibacter* was only found in the control group ($p = 0.0212$) with an abundance of 3.3% (1263 OTUs) *Methanobrevibacter* is an archaeon from the from the phylum Euryarchaeota, which is one of the two phyla of archaea and is noted for containing members that are methanogenic and are found in the intestines of several organisms including humans.[11,13] One noted member of this genera is *Methanobrevibacter smithii*, which is the predominate archaeon in the microbiota of the human gut microbiome [14,15]. This archaeon plays a role in the digestion of complex polysaccharides by consuming the final products of bacterial fermentation and in the process converting carbon dioxide to methane [16]. Archaea in the *Methanobrevibacter* genera are strictly anaerobic and are thought to play a role in obesity [15]. Due to the reliance of this archaea on complex polysaccharides, which can be found in foods that have gluten such as wheat, barley, and rye, it is plausible that an individual with a gluten-free diet may be lacking enough complex polysaccharides to allow the archaea to survive. This could explain why we saw no *Methanobrevibacter* in the celiac disease and gluten-free group.

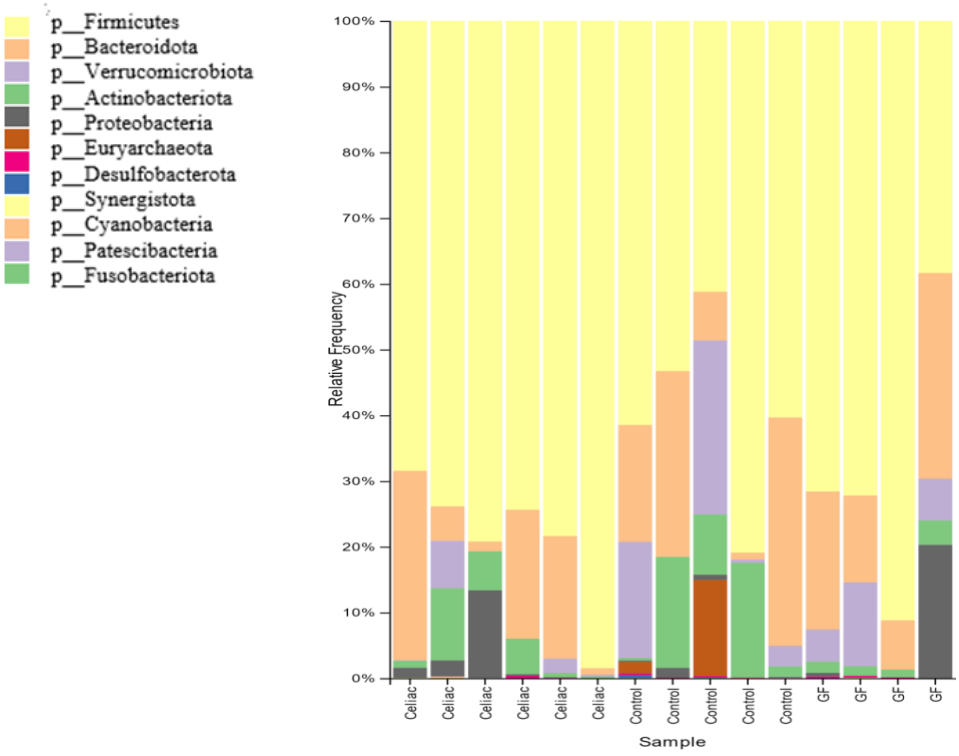

Figure 4. Phyla level diversity. Identified phyla in each sample are shown. Samples are organized based on their corresponding study group of Celiac disease (Celiac), healthy with no diet control (Control), and healthy on a gluten-free diet (GF). Figure made using Excel.

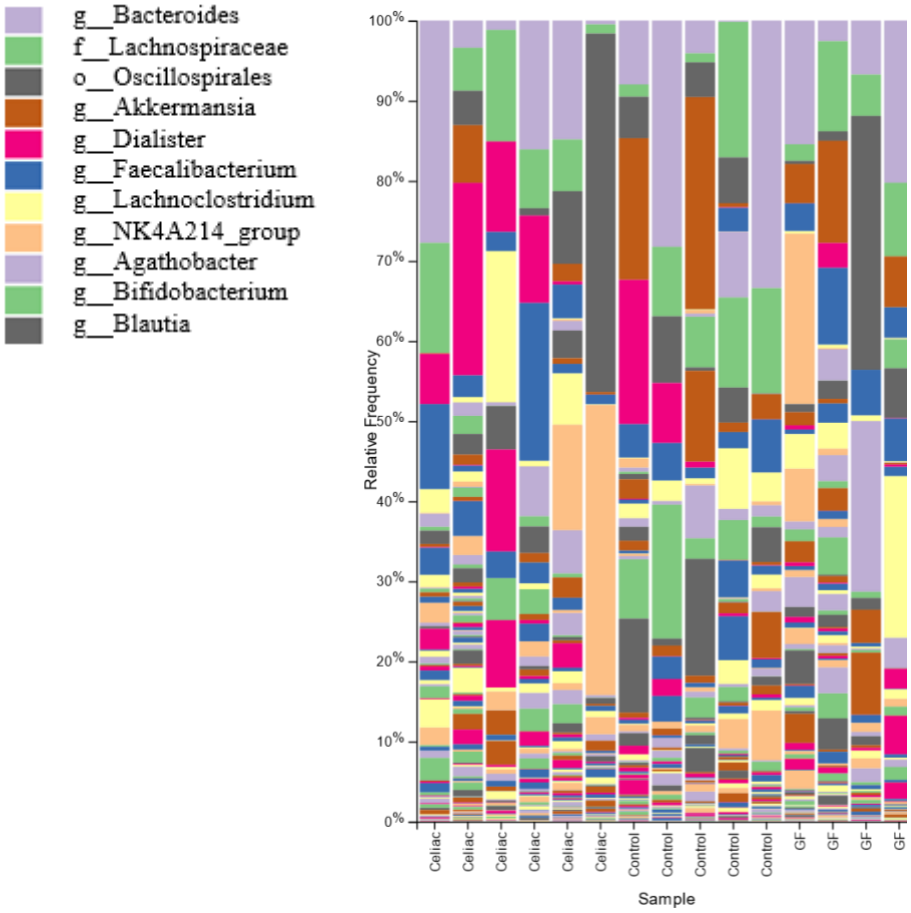

Figure 5. The genus level diversity observed in our study along with their corresponding group. Colors were used to indicate the associated genera. Figure made using Excel.

The identification of archaea through microbiome analysis has several limitations. While archaea and bacteria share mainly similarities, such as similar cell walls structures and lack of membrane-bound organelles, archaea also resemble eukaryotes [17,18]. One of those similarities is the ribosome [17-19]. The primary sequence of archaeal rRNA and r-proteins are closer to those found in eukaryotes than to bacteria [17]. Furthermore, while the V3/V4 region of rRNA is best for the identification of most bacteria, it is not necessarily the best for archaea [19]. Depending on the family or genus in question other regions such as the V1/V2 can be more effective [19]. The larger variance in the effectiveness of the 16S hypervariable regions, in combination with the differences in ribosome structure, is one of the reasons that microbiome processing can be so difficult in complex microbial communities composed of both bacteria and archaea. This information could explain why *Methanobrevibacter* was only found in a couple of people in the control group. While the *Methanobrevibacter* was found to be significant its isolation in combination with the fact that it was found in a small number of individuals makes it hard to come to a definitive conclusion regarding its presence and effect on the microbiome.

In a healthy gut microbiome, *Firmicutes* and *Bacteroidetes* account for approximately 90% of microbes in the gut microbiota followed by other groups such as *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia* [12]. Celiac patients generally see a reduction in beneficial species (*Lactobacillus* and *Bifidobacterium*) and an increase in pathogenic species (*E. coli* and *Bacteroides*) [9,12]. Our study saw no significate change in groups such as *Firmicutes* and *Bacteroides* in celiac participants when compared to the gluten-free and control groups. This is similar to changes in *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, where no significant changes were detected. One possible explanation for the lack of visible significance in the normally seen groups (as well as others) could come from the smaller sample size. The small sample size, N = 28, as well the small sample size in each induvial group could all have played a role in the lack of significance. Another plausible explanation could come

from the nature of our study population. Our study population was noted to be largely female (around 80%), completely Caucasian, with a mean age of 25-34. It has been noted that the microbiome is different between sexes, which could have influenced results [1,2]. The microbiome also changes as we age, which could have affected results since so many of our study population was in the same age group [1,2].

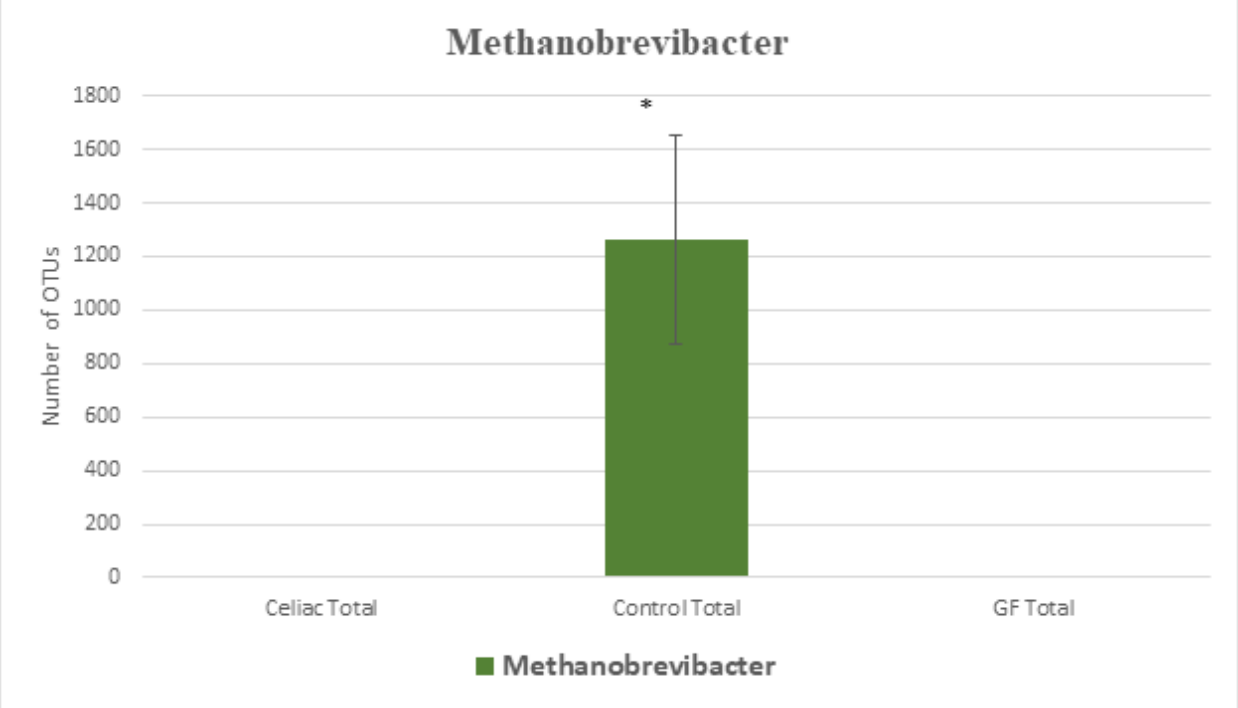

Figure 6. Counts of *Methanobrevibacter* identified in each study group. No OTUs aligning to *Methanobrevibacter* were found in the celiac or GF groups. The * indicates data that was significant (p-value ≤ to 0.05). Figure made using Excel.

While we do not report some of the celiac-related microbiome changes seen in other studies [2,5,7,9], we did identify *Methanobrevibacter* as a genus that may be lost by celiac patients once they start a gluten-free diet. The presence of this genus in healthy individuals not on a restricted diet presents an interesting opportunity to learn more about the effect gluten has on the microbiome and how that can be related to those with celiac disease.

Conclusions

The human gut microbiome and its connection to celiac disease and gluten remain a topic in need of future research. This study contributes to the work already done on celiac disease and gluten by adding new insights into the effect diet many have on Methanobrevibacter in the gut microbiome. The role *Methanobrevibacter* plays in healthy populations is an area that should be explored. A longitudinal study, with a much larger sample size, looking at how *Methanobrevibacter* affects the microbiome and perhaps the role (if any) its loss to the microbiome plays in celiac disease symptoms, has merit, and should be considered.

List of Abbreviations Used

GI: gastrointestinal tract
GFD: gluten-free diet
CD: celiac disease
OTU: operational taxonomic units
PCoA: principal coordinate analysis

Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

This study was approved by the Utah Valley University IRB #01966. All participants were provided written informed consent documents which they signed after receiving oral explanations of the research and participant expectations. All participants were given opportunity to ask questions during the consent process.

Authors' Contributions

CR: collected and analyzed data, generated figures, drafted the manuscript and gave final approval of the version to be published.

VH: organized study, consented participants, collected and analyzed data and gave final approval of the version to be published.
RH: made contributions to the study design, wrote IRB application, organized the study, and consented participants and gave final approval of the version to be published.
MAGS: made contributions to the study design, analyzed and interpreted data, critically revised the manuscript, and gave final approval of the version to be published.

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