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Not Got Milk? The Effect on the Gut Microbiome of Removing Dairy from the Diet

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NOT GOT MILK? THE EFFECT ON THE GUT MICROBIOME OF REMOVING DAIRY FROM THE DIET

By
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ABSTRACT

Patrick Tyler Stovall: Not Got Milk? The Effect on the Gut Microbiome of Removing Dairy from the Diet
(Under the direction of Colin R. Jackson, Ph.D.)

The human gut contains a highly diverse set of bacteria that perform a wide range of duties that include much more than just nutrient acquisition. However, the composition of this community is subject to change, with diet, age and lifestyle playing roles in the development and maintenance of the gut microbiota. This study compared the bacterial composition of the human gut when consuming a normal diet versus a dairy-free diet. Samples were taken from a single subject during three periods: 1) control (normal) diet, 2) dairy-free diet, and 3) a return to normal diet. Gut bacterial communities were identified and compared using 16S rRNA gene sequencing. Relative to the total number of sequences within a sample, abundances were calculated for dominant bacterial groups starting at the phylum level and progressing to the smallest identifiable taxonomic group. Fluctuations were seen at taxonomic levels from class down to species. Non-metric multidimensional scaling ordination revealed that the samples from each dietary period were distinguishable from the other periods. Six significant operational taxonomic units (OTUs), from three phyla, were significantly related to dietary sample distributions. These OTUs consisted of two members of the Bacteroidetes (both genus *Bacteroides*), three from Firmicutes (genus *Megasphaera*, genus *Acidamniococcus*, and *Butyricicoccus pullicaecorum*), and one from Actinobacteria (*Collinsella aerofaciens*). This study shows that alterations to a diet can

cause changes of the relative abundances of bacteria in the human gut at multiple taxonomic levels, but that at the level of the entire community these shifts in gut microbiota can be reversed.

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Introduction

There are trillions of bacteria, representing more than 1,000 species, living on our skin and within our bodies, and it is estimated that bacterial cells out number human cells by ten to one (Ackerman 2012). These bacteria help keep us alive by performing important roles in metabolism and defense (Hopkins et al. 2001). Historically, it has been difficult to identify individual microbial species in such a diverse environment. However, each species of bacteria has a 16S rRNA gene sequence specific to it (Woese 1987), and modern approaches to microbial ecology rely on 16S rRNA gene sequencing to identify both the specific types of bacteria that are present and to estimate overall bacterial diversity (De Santis et al. 2006). The use of this 16S rRNA sequencing is critically important to the study of microbial diversity, as this technique removes the need to culture bacteria in order to identify them, an aspect that is particularly important in studies of the human gut microbiome, where many important bacteria, such as species of Bifidobacteria, may be anaerobic and difficult to culture using standard approaches (Turnbaugh et al. 2009). Indeed, over the last decade there have been a number of studies that have used 16S rRNA techniques to describe the microbiota in the human intestines (e.g. Garrity et al. 2004, Turnbaugh et al. 2009, Fujimura et al. 2010,).

Bacterial communities in the human gut are mainly composed of members of the Bacteroidetes and Firmicutes, which typically account for 17-60% and 35-80% of identified sequences, respectively (Shoaie et al. 2013). Bacteroidetes is a diverse phylum of bacteria consisting of four classes: Bacteroidia, Flavobacteria, Sphingobacteria, and

Cytophagia (Thomas et al. 2011). While all four classes are Gram negative, they are a mixture of physiological types, ranging from the strictly anaerobic Bacteroidia to the strictly aerobic Flavobacteria (Thomas et al. 2011). Given their obligate anaerobic metabolism, it is not surprising that Bacteroidia are the dominant class of Bacteroidetes in the human large intestine, and they are likely involved in the normal development of the gastrointestinal tract (Thomas et al. 2011), as well as activation of the T-cell mediated immune responses and limitation of gut colonization by potential pathogens (Mazmanian et al., 2008; Wen et al. 2008). Genome analysis of members of the Bacteroidetes shows that they have a large repertoire of genes involved in acquisition and metabolism of polysaccharides (Mahowald et al. 2009) so they are likely involved in polysaccharide degradation in the large intestine. Bacteroidetes are also thought to help maintain a healthy gut by producing butyrate, an end product of colonic fermentation that is thought to have antineoplastic properties (Kim and Milner, 2007), and in the transformation of toxic and/or mutagenic compounds (Smith et al, 2006).

The second major phylum, Firmicutes, has been found to be associated with obesity, likely because of their production of excess energy from consumed nutrients (Fujimura et al. 2010). A diet high in fat and sugar has been shown to result in a dominance of Mollicutes (a class within the Firmicutes), that was subsequently related to an increase in body fat and activity of metabolic pathways associated with the import and fermentation of simple sugars and host glycans (Fujimura et al. 2010). Changes in the microbial community such as this can not only have long-term negative effects on health, but can also be associated with the future growth of pathogenic microorganisms including species of Enterobacteriaceae species such as *Salmonella enterica* (Fujimura et al. 2010).

Members of Phylum Actinobacteria have also been identified as important gut bacteria, although at lower proportions than Bacteroidetes or Firmicutes. These organisms can still be important in immune response systems and development of the gut microbial community in the large intestine (Fujimura et al. 2010). Various Proteobacteria and even Euryarchaeota are also typically present in lower proportions, yet still play important roles in the large intestine (Shoaie et al. 2013).

With more and more studies on the human gut microbiome, researchers are identifying factors such as age and diet that are influential in gut bacterial composition. Aging causes structural changes in the microbiota, including changing the proportion of protective Bifidobacteria (members of the Actinobacteria; Garrity et al. 2004) that can have major effects on host physiology and metabolism, as well as on innate colonization resistance (Hopkins et al. 2001). The consequences of these age-related changes become exacerbated given that the elderly may be more prone to infection, and therefore undergo more frequent antibiotic therapy, which can further limit gut microbial diversity (Hopkins et al. 2001). As might be expected, a change in diet can result in changes in the relative abundances of major phyla of bacteria, which can then lead to diseases such as obesity. One study on humanized gnotobiotic mice analyzed bacterial community composition before and after a diet change. Mice that were switched to a high-fat, high-sugar Western diet showed increased levels of Bacilli and Erysipelotrichi (both Firmicutes) along the entire length of the gut, as well as lower proportions of Bacteroidetes in fecal samples relative to mice fed a low-fat, plant-polysaccharide rich diet (Turnbaugh et al. 2009). Changes in the ratio of Firmicutes to Bacteroidetes in lean subjects (3:1) compared to obese subjects (up to 35:1) have also been reported (Fujimura et al. 2010).

Shifts in gut community function because of dietary selective pressure can result in higher energy harvest from food, causing increased adipose tissue formation in the host (Fujimura et al. 2010). However, diet and environmental factors begin to affect the gut microbial community from the moment we are born. Infants born vaginally have higher levels of *Bifidobacterium* and *Bacteroides* whereas infants born via Cesarean section exhibited a gut microbial community dominated by *Staphylococcus*, *Streptococcus*, and *Clostridium difficile* (Fujimura et al. 2010). During infancy, exclusively formula-fed infants showed a greater abundance of *C. difficile* and *E. coli* while maintaining similar abundances of Bifidobacteria to exclusively breastfed infants. Conversely, exclusively breastfed infants showed greater gene expression in Bifidobacteria allowing them to metabolize a greater variety of complex oligosaccharides (Fujimura et al. 2010). From this information, it becomes obvious that maintaining a stable balance of bacteria in the human gut throughout life is essential to overall health.

In this study, I attempted to determine the effects of a major dietary change on the human gut microbiota. Specifically, I focused on the effects of eliminating dairy products from the diet on the composition of the microbiome of the human large intestine. Stool samples were obtained approximately every 5 days over a period of 10 weeks, covering a period on a regular diet followed by a dairy-free diet for 40 days, and the subsequent return to a regular diet. Such a dietary transition represents what a substantial proportion of the population might undergo when following an annual religious observance (Lent). Composition of the gut microbiome was characterized by next generation 16S rRNA gene sequencing, facilitating the analysis of this diverse community. My results suggest that eliminating dairy from one's diet can have an effect

upon the composition of the gut microbiome. While shifts were not seen in the Bacteroidetes or Firmicutes at the phyla level, changes in relative abundances could be detected at lower taxonomic levels. A shift in Proteobacteria could be seen at the phyla taxonomic level and further investigation exposed which class, family, genus, or species was responsible. Lastly, when examing the bacterial community as a whole, grouping could be seen among the separate sampling groups, suggesting that dietary change does play a role in structuring the gut microbiome.

Methods

Sample Collection

Stool samples (typically 0.1 g) were obtained from a single individual approximately every five days, beginning February 18 and ending April 30, 2014 with an additional sample taken on September 24. Five reference samples were taken between February 18 and March 7, before a dietary change. After March 7, the individual adjusted their diet and did not consume any further dairy products for 40 days. Eight samples were taken between March 16 and April 16, after eliminating dairy from the diet. After that time period, the individual resumed a normal diet, and a further four stool samples were collected from April 21 until April 30 and one on September 24. All samples were collected during regular defecation, using a sterile swab. Samples were immediately frozen (-20 °C) until all had been collected.

DNA Extraction and Sequencing

Frozen samples were thawed and DNA was extracted using a Mo Bio Power Fecal DNA Isolation kit, following the detailed protocol provided by the manufacturer (Mo Bio Laboratories, Carlsbad, CA). The presence of DNA in the end product was confirmed via agarose gel electrophoresis. The V4 region of the 16S rRNA gene was then amplified and sequenced using paired-end, barcoded Illumina next generation sequencing (Kozich et al. 2013). Sequence library preparation was performed at the UM campus, while the actual Illumina MiSeq sequencing run was conducted at the Molecular

and Genomics Core Facility at the University of Mississippi Medical Center (UMMC) in Jackson, MS. 16S rRNA gene sequence data was subsequently downloaded and assessed using the bioinformatics software package, mothur (Schloss et al. 2009) using the general procedures recommended by Schloss et al. (2011) and Kozich et al. (2013), and outlined below.

Briefly, raw FASTQ data derived from the sequencing process were downloaded and screened for length and potential base ambiguity. Sequences were then aligned against reference sequences in the SILVA database (Quast et al. 2013). Screening was performed to remove any remaining sequences that had runs of more than eight identical bases in a row, a potential indicator of sequencing error. Sequences differing by two bases or less were clustered together to remove potential amplification artifacts and chimeras were then checked for and eliminated using the incorporated UCHIME software (Edgar et al. 2011). Sequences were then classified using the Greengenes database, and contaminant sequences, such as those from chloroplasts, mitochondria, or Archaea, were removed from the dataset (Desantis et al. 2006). Final valid sequences were grouped into operational taxonomic units (OTUs) using a 97% sequence similarity criterion, as a surrogate for species to be used for diversity analysis and community comparisons.

Beta diversity was assessed by conducting Non-metric multidimensional scaling (NMDS) ordinations, followed by Spearman's rank correlations linking the presence of specific OTUs to NMDS axes scores.

Results

Stool samples were obtained and bacterial DNA extracted and analyzed according to the procedures mentioned previously. The presence of DNA was verified via gel electrophoresis (Figure 1), and all samples proved suitable for 16S rRNA gene sequencing. Sequencing was successful and yielded a total of 1,287,777 valid bacterial sequences, which classified into 535 operational taxonomic units (OTUs), across eighteen samples. Prior to any dietary change, Bacteroidetes and Firmicutes phyla dominated the dataset, accounting for 51.9% and 41.8% of the total sequences, respectively, while phyla Actinobacteria and Proteobacteria were also fairly prevalent and represented 4.0% and 2.2% of the total number of sequences, respectively (Table 1).

No significant changes were seen in overall percentages of the phylum Firmicutes following dietary change (Table 2, Figure 2a); however, a substantial decrease was seen in the order Lactobacillales (Table 2, Figure 2b). Bacteriodetes exhibited no major changes in their abundance (Table 2, Figure 3a) except for the genus *Parabacteroides* (Table 2, Figure 3b), which demonstrated an overall increase during dairy elimination (Table 2, Figure 4a). Gammaproteobacteria showed a clear surge in their relative abundance just after the diet alteration and then again when returning to a normal diet (Table 2, 3, Figure 4b). The initial spike seen in samples 7 and 8 can be attributed to a large increase in the proportion of *E. coli* (Table 2, Figure 5), and the broader group to which *E. coli* belongs, Enterobacteriaceae, was responsible for the second surge in relative abundance in sample 15 (Figure 5). Unfortunately, the genus and species that

correlated to the second surge were both listed as unclassified. Proportions of the genus *Sutterella*, a member of the Betaprotetobacteria, also showed an upward trend in relative abundance during the dietary change (Table 2, Figure 6).

Figure 1. PCR was preformed on the samples and DNA presence was confirmed via agarose gel electrophoresis. The samples were placed in the wells chronologically by date. Samples 1-9 were placed in the top wells and 10-18 were placed in the bottom wells.

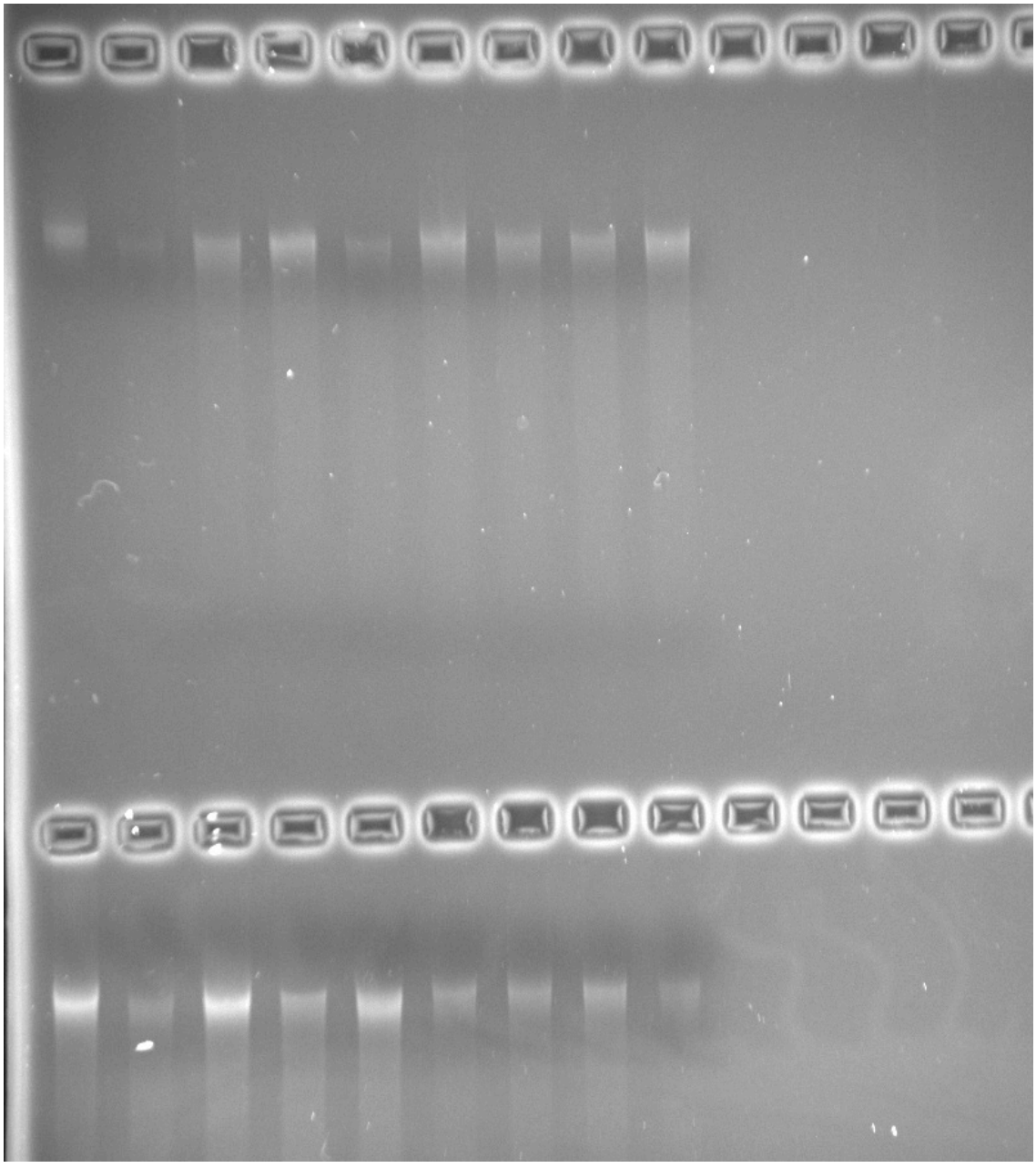


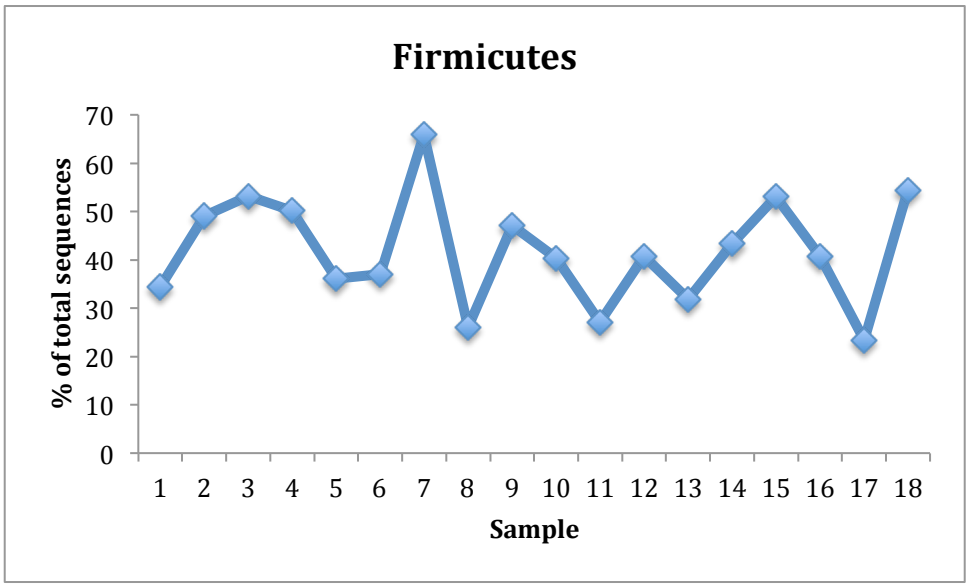
Table 1. Bacterial community composition of fecal samples obtained from a human subject on a regular diet, prior to switching to dairy free. Samples are listed by date (month/day 2014). Major bacterial taxonomic groups are listed on the left with their taxonomic level listed below, with nested taxa being part of those above them. Numbers represent the number of sequences obtained that classified to that taxon, with corresponding percentages of the total from that sample below them.

taxon	total	02/18	02/25	03/03	03/06	03/07
Bacteria	1287777	67489	66758	77153	65440	57430
Actinobacteria	51891	1252	2729	5639	9623	1202
phylum	4.03%	1.86%	4.09%	7.31%	14.70%	2.10%
Bacteroidetes	668574	42078	30617	29888	22110	35204
phylum	51.92%	62.35%	45.86%	38.74%	33.79%	61.30%
Bacteroides	588503	39866	22587	26312	19049	32866
genus	45.70%	59.07%	33.83%	34.10%	29.11%	57.23%
Parabacteroides	23063	631	2645	1643	866	779
genus	1.79%	0.93%	3.96%	2.13%	1.32%	1.36%
Firmicutes	538556	23224	32768	40993	32809	20758
phylum	41.82%	34.41%	49.08%	53.13%	50.14%	36.14%
Clostridia	532046	22985	32298	40507	32434	20530
class	41.32%	34.06%	48.38%	52.50%	49.56%	35.75%
Lactobacillales	1403	25	169	56	187	48
order	0.11%	0.037%	0.25%	0.073%	0.29%	0.084%
Streptococcaceae	1281	22	132	49	181	45
family	0.099%	0.033%	0.20%	0.064%	0.28%	0.078%
Proteobacteria	28064	895	632	615	888	244
phylum	2.18%	1.33%	0.95%	0.80%	1.36%	0.42%
Betaproteobac.	16064	506	459	526	746	159
class	1.25%	0.75%	0.69%	0.68%	1.14%	0.28%
Sutterella	16021	500	455	523	746	158
genus	1.24%	0.74%	0.68%	0.68%	1.14%	0.28%
Gammaproteobac.	9941	263	95	28	51	62
class	0.77%	0.39%	0.14%	0.036%	0.078%	0.11%
Enterobacteriaceae	8670	190	71	27	50	49
family	0.67%	0.28%	0.11%	0.035%	0.076%	0.085%
E. coli	6604	189	68	22	48	48
species	0.51%	0.28%	0.10%	0.029%	0.073%	0.084%

Table 2. Bacterial community composition of fecal samples obtained from a human subject on an altered diet, after switching to dairy free. Samples are listed by date (month/day 2014). Major bacterial taxonomic groups are listed on the left with their taxonomic level listed below, with nested taxa being part of those above them. Numbers represent the number of sequences obtained that classified to that taxon, with corresponding percentages of the total from that sample below them.

taxon	total	03/16	03/21	03/26	03/31	04/02	04/07	04/11	04/16
Bacteria	1287777	89153	86589	84998	81396	84242	91019	50579	66439
Actinobacteria	51891	2008	2779	1419	4526	2794	1268	2463	1931
phylum	4.03%	2.25%	3.21%	1.67%	5.56%	3.32%	1.39%	4.87%	2.91%
Bacteroidetes	668574	51233	23672	57923	36668	45043	62984	26697	40556
phylum	51.92%	57.47%	27.34%	68.15%	45.05%	53.47%	69.20%	52.78%	61.04%
Bacteroides	588503	45599	20211	54296	29126	38390	55728	22224	36599
genus	45.70%	51.15%	23.34%	63.88%	35.78%	45.57%	61.23%	43.94%	55.09%
Parabacteroides	23063	1596	897	562	1625	1299	1303	723	683
genus	1.79%	1.79%	1.04%	0.66%	2.00%	1.54%	1.43%	1.43%	1.03%
Firmicutes	538556	33033	57058	22105	38354	34008	24625	20579	21142
phylum	41.82%	37.05%	65.90%	26.01%	47.12%	40.37%	27.05%	40.69%	31.82%
Clostridia	532046	32574	56380	21614	37859	33589	24341	20372	20978
class	41.32%	36.54%	65.11%	25.43%	46.51%	39.87%	26.74%	40.28%	31.57%
Lactobacillales	1403	20	24	39	74	25	33	7	60
order	0.11%	0.022%	0.028%	0.046%	0.091%	0.030%	0.036%	0.014%	0.090%
Streptococcaceae	1281	15	18	35	73	24	10	6	58
family	0.099%	0.017%	0.021%	0.041%	0.090%	0.028%	0.011%	0.012%	0.087%
Proteobacteria	28064	2856	2881	3361	1834	2390	2120	834	2759
phylum	2.18%	3.20%	3.33%	3.95%	2.25%	2.84%	2.33%	1.65%	4.15%
Betaproteobacteria	16064	2308	488	907	1286	1585	1876	436	2289
class	1.25%	2.59%	0.56%	1.07%	1.58%	1.88%	2.06%	0.86%	3.45%
Sutterella	16021	2307	486	891	1284	1583	1876	436	2286
genus	1.24%	2.59%	0.56%	1.05%	1.58%	1.88%	2.06%	0.86%	3.44%
Gammaproteobacteria	9941	94	2338	2370	508	607	68	294	283
class	0.77%	0.11%	2.70%	2.79%	0.62%	0.72%	0.075%	0.58%	0.43%
Enterobacteriaceae	8670	32	2247	2357	367	597	53	291	268
family	0.67%	0.036%	2.60%	2.77%	0.45%	0.71%	0.058%	0.58%	0.40%
E. coli	6604	27	2220	2345	361	570	51	282	36
species	0.51%	0.030%	2.56%	2.76%	0.44%	0.68%	0.056%	0.56%	0.054%

(a)



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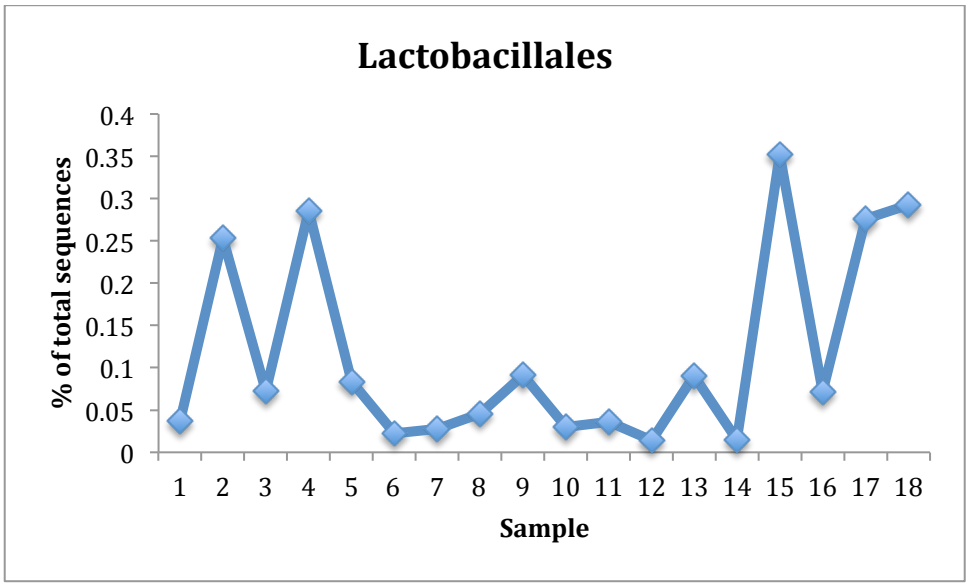
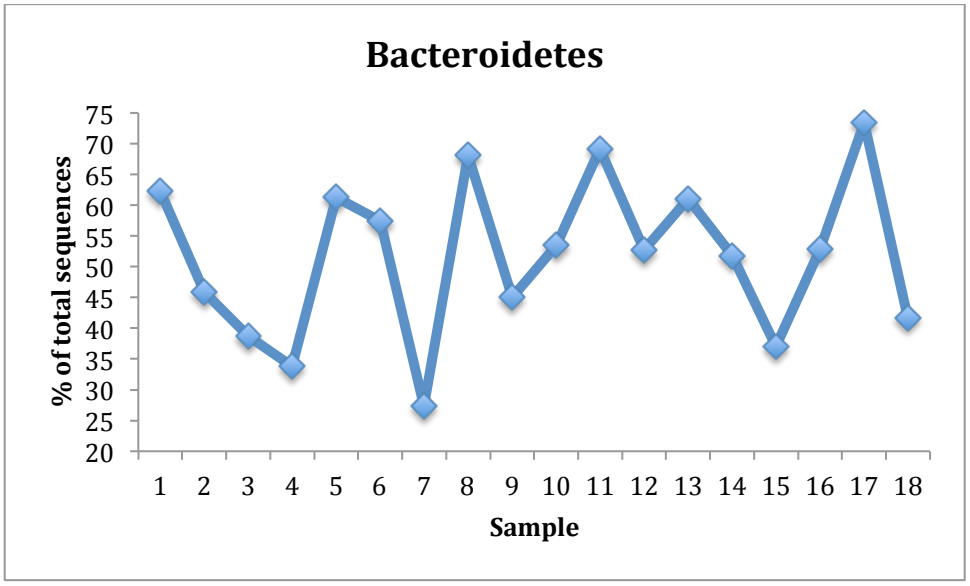


Figure 2. Changes in the relative abundance of phylum Firmicutes (a) and the order Lactobacillales within that phylum (b) in bacterial communities in stool samples taken from an individual on a regular diet (samples 1-5), after switching to a dairy free diet (samples 6-13), and after resuming a regular diet (samples 14-18) over a period of two months.

(a)



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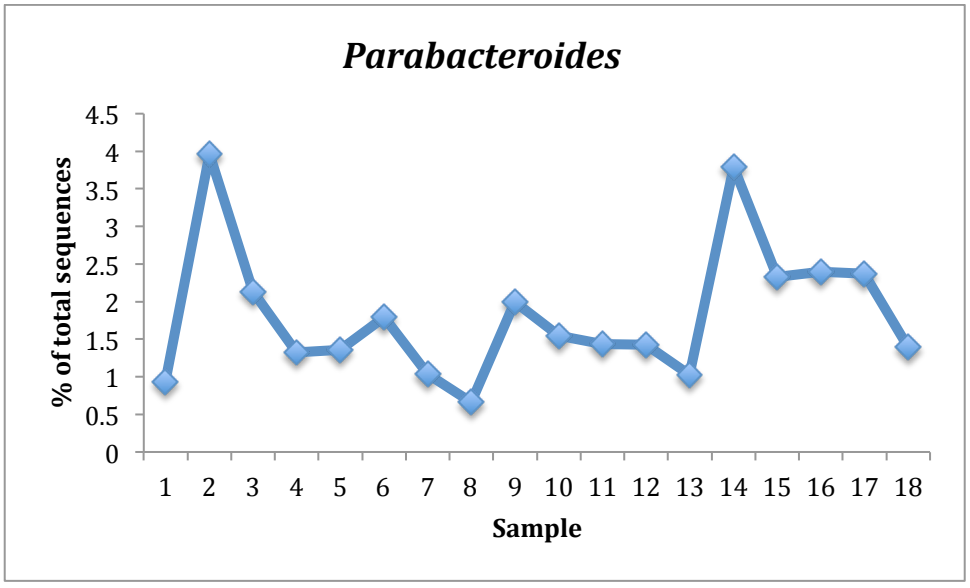
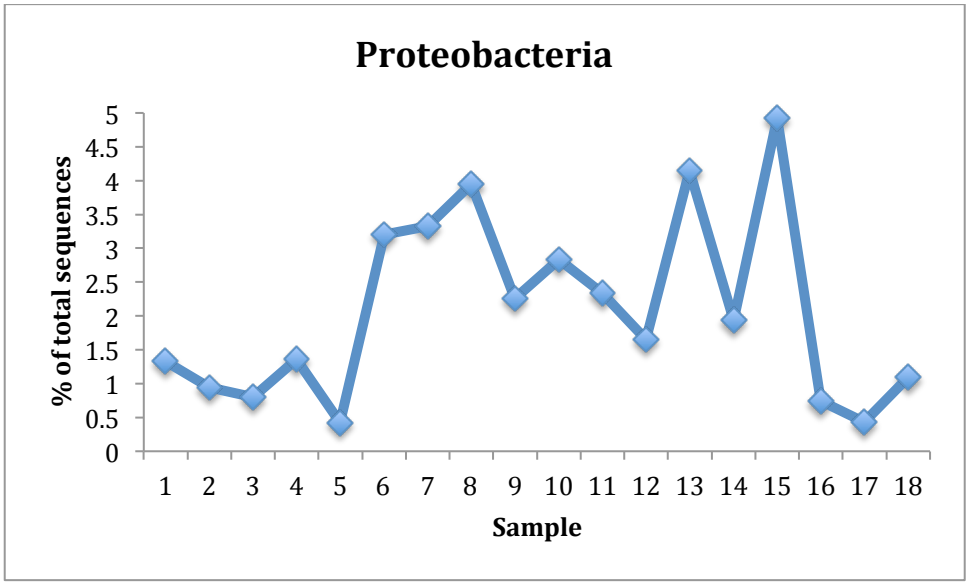


Figure 3. Changes in the relative abundance of phylum Bacteroidetes (a) and the genus Parabacteroides within that phylum (b) in bacterial communities in stool samples taken from an individual on a regular diet (samples 1-5), after switching to a dairy free diet (samples 6-13), and after resuming a regular diet (samples 14-18) over a period of two months.

(a)



(b)

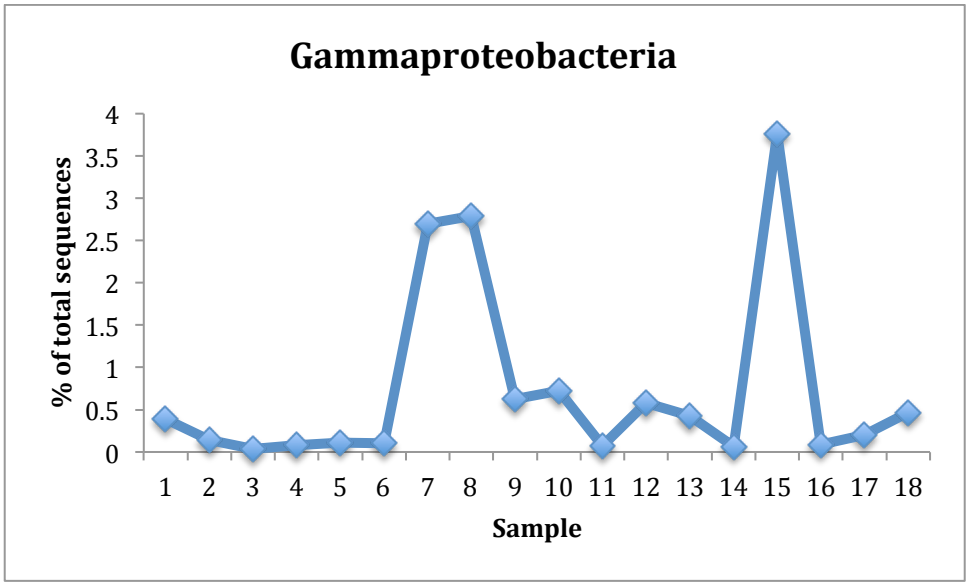
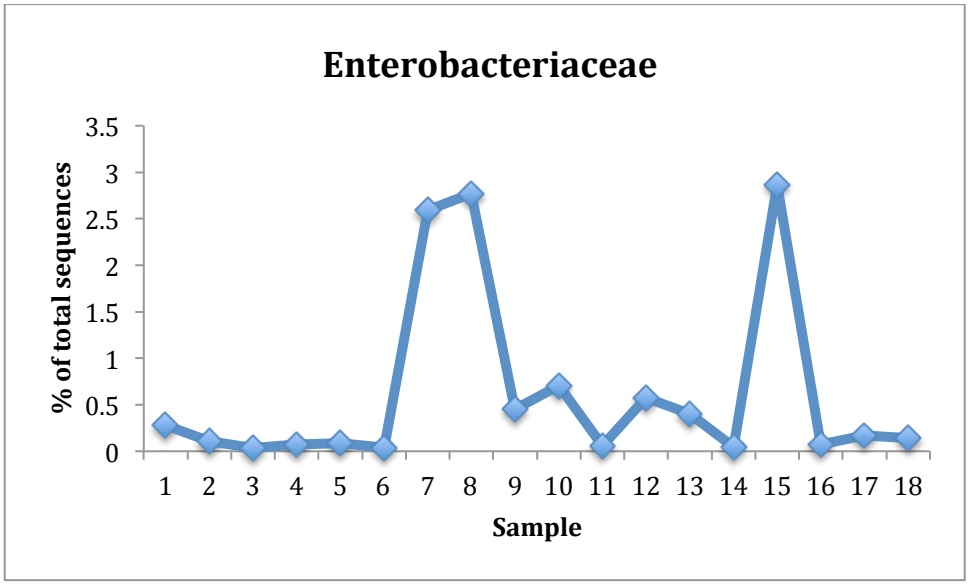


Figure 4. Changes in the relative abundance of phylum Proteobacteria (a) and the class Gammaproteobacteria within that phylum (b) in bacterial communities in stool samples taken from an individual on a regular diet (samples 1-5), after switching to a dairy free diet (samples 6-13), and after resuming a regular diet (samples 14-18) over a period of two months.

Table 3. Bacterial community composition of fecal samples obtained from a human subject after returning to a normal diet after being dairy-free. Samples are listed by date (month/day 2014). Major bacterial taxonomic groups are listed on the left with their taxonomic level listed below, with nested taxa being part of those above them. Numbers represent the number of sequences obtained that classified to that taxon, with corresponding percentages of the total from that sample below them.

taxon	total	04/21	04/22	04/25	04/30	09/24
Bacteria	1287777	61152	62748	69997	62358	62837
Actinobacteria	51891	1727	3075	3975	1733	1748
phylum	4.03%	2.82%	4.90%	5.68%	2.78%	2.78%
Bacteroidetes	668574	31696	23235	37005	45769	26196
phylum	51.92%	51.83%	37.03%	52.87%	73.40%	41.69%
Bacteroides	588503	27246	20900	31818	42213	23473
genus	45.70%	44.55%	33.31%	45.46%	67.69%	37.36%
Parabacteroides	23063	2320	1463	1677	1478	873
genus	1.79%	3.79%	2.33%	2.40%	2.37%	1.39%
Firmicutes	538556	26529	33328	28493	14562	34188
phylum	41.82%	43.38%	53.11%	40.71%	23.35%	54.41%
Clostridia	532046	26360	32948	28343	14287	33647
class	41.32%	43.11%	52.51%	40.49%	22.91%	53.55%
Lactobacillales	1403	9	221	50	172	184
order	0.11%	0.015%	0.35%	0.071%	0.28%	0.29%
Streptococcaceae	1281	8	213	50	165	177
family	0.099%	0.013%	0.34%	0.071%	0.26%	0.28%
Proteobacteria	28064	1185	3092	523	267	688
phylum	2.18%	1.94%	4.93%	0.75%	0.43%	1.09%
Betaproteobac.	16064	894	723	412	126	338
class	1.25%	1.46%	1.15%	0.59%	0.20%	0.54%
Sutterella	16021	894	723	410	126	337
genus	1.24%	1.46%	1.15%	0.59%	0.20%	0.54%
Gammaproteobac.	9941	42	2362	59	126	291
class	0.77%	0.069%	3.76%	0.084%	0.20%	0.46%
Enterobacteriaceae	8670	29	1800	51	104	87
family	0.67%	0.05%	2.87%	0.07%	0.17%	0.14%
E. coli	6604	25	85	42	98	87
species	0.51%	0.041%	0.14%	0.60%	0.16%	0.14%

(a)



(b)

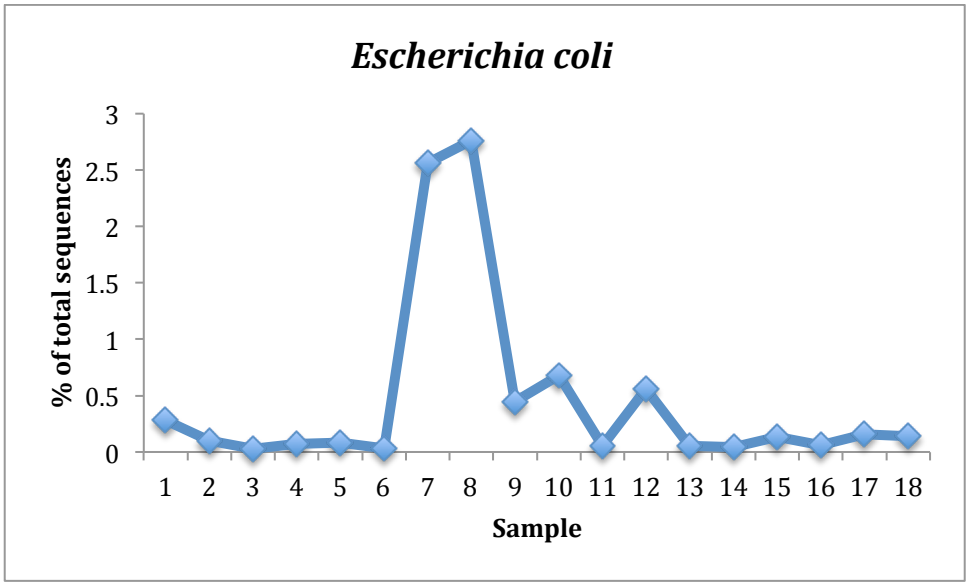
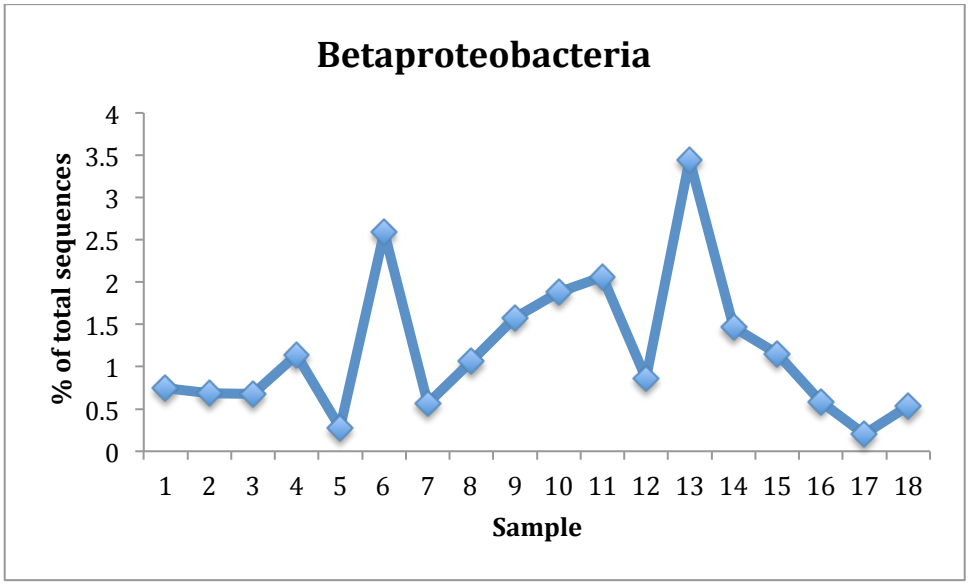


Figure 5. Changes in the relative abundance of the family Enterobacteriaceae (a) and species *E. coli* (b) within that family in bacterial communities in stool samples taken from an individual on a regular diet (samples 1-5), after switching to a dairy free diet (samples 6-13), and after resuming a regular diet (samples 14-18) over a period of two months.

(a)



(b)

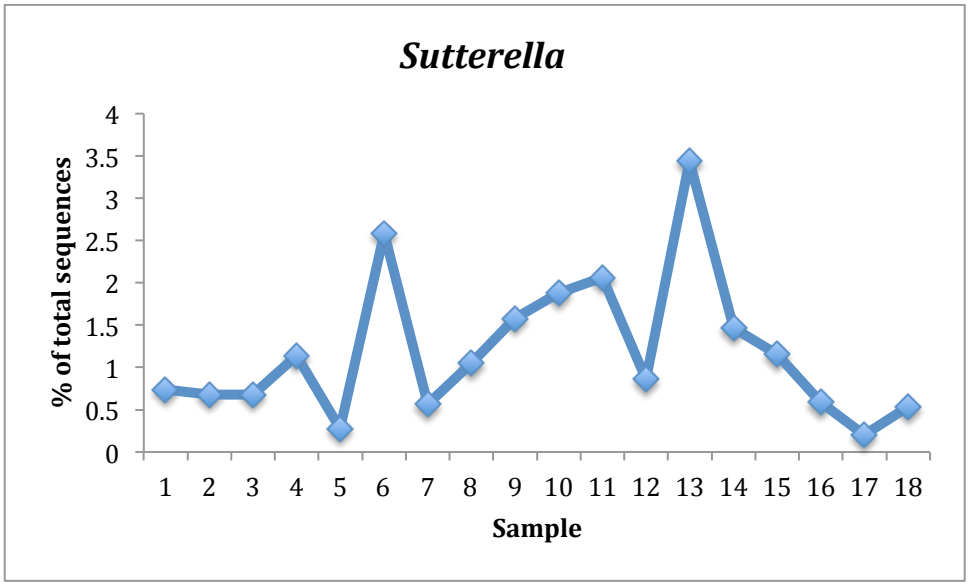


Figure 6. Changes in the relative abundance of class Betaproteobacteria (a) and genus Sutterella (b) within that class in bacterial communities in stool samples taken from an individual on a regular diet (samples 1-5), after switching to a dairy free diet (samples 6-13), and after resuming a regular diet (samples 14-18) over a period of two months.

While there was overlap in the spatial arrangement of community types, NMDS ordinations generally separated samples into three types: control, dairy-free diet, and a return to normal diet (Figure 7). Control (pre-dietary change) samples showed a high degree of variation but did tend to separate from the samples taken after switching to a dairy free diet. Those samples tended to shift to negative scores on the first NMDS axis (Figure 7). After returning to a normal diet, the gut microbiome appeared to show signs of reverting back to its original composition, with points on the NMDS ordination shifting back towards positive scores on the first axis (Figure 7). However, the first sample taken after returning to a normal diet (sample 14 in Figure 7) remained in the negative scores of the NMDS axis, suggesting that the microbiome requires a certain amount of time to shift after dietary change.

OTUs that were significantly related to sample distributions are labeled in Figure 7 and their relevant data is listed in Table 4. These OTUs can be linked to specific bacteria and serve to express which bacteria may have driven the scattering of data points. OTU0003 (a member of genus *Bacteroides* within the phylum Bacteroidetes, species unclassified) drove the samples toward the negative scores on the first NMDS axis, the direction in which samples shifted following the adoption of a dairy-free diet (Figure 7). OTU0001 (a member of the *Bacteroides*, species unclassified) drove the samples towards positive values on the second NMDS axis. OTU0004, OTU0013, and OTU0014 were clustered together on the positive end of the first NMDS axis. OTU0004 (a member of genus *Megasphaera*) and OTU0014 (a member of genus *Acidamniococcus*) both of which belong to the family Veilonellaceae, a member of phylum Firmicutes, drove samples towards positive scores on first NMDS axis, the direction in which

samples shift following the return of normal diet. OTU0013 (identified as *Collinsella aerofaciens*, a member of the Actinobacteria) also drove the samples toward positive scores on the first NMDS axis. Lastly, OTU0028 (identified as *Butyricicoccus pullicaecorum*, a member of phylum Firmicutes) drove the samples towards negative scores on the second NMDS axis.

Figure 7. NMDS ordination of bacterial communities in the human large intestine on a regular, control diet (blue diamonds), on a non-dairy diet (red squares), and after reverting to a regular diet (green triangles). Numbers near each symbol indicate sample order, and OTU drivers are represented by purples X indicating the direction that those OTUs pull the community.

Table 4. Operational taxonomic units (OTUs) responsible for driving the NMDS ordination in Fig. 7. OTUs are listed in descending order of their length, which reflect the overall influence of that OTU on community comparisons. The p-values are the probability that a grouping of samples occurred by chance. OTUs were identified as follows: OTU0003 is a member of genus *Bacteroides* within the phylum Bacteroidetes, species unclassified; OTU0001 is a member of the *Bacteroides*, species unclassified; OTU0004 is a member of genus *Megasphaera* and OTU0014 is a member of genus *Acidamniococcus*, both of which belong to the family Veilonellaceae, a member of phylum Firmicutes; OTU0013 is *Collinsella aerofaciens*, a member of the Actinobacteria; OTU0028 is *Butyricicoccus pullicaecorum*, a member of phylum Firmicutes.

OTU	Size	axis1	p-value	axis2	p-value	length
Otu0003	212975	-0.919505	0	-0.347781	0.15159	0.983077
Otu0004	122254	0.927761	0	-0.031992	0.895059	0.928312
Otu0001	185594	-0.308566	0.203285	0.80805	0.00005	0.864961
Otu0013	30901	0.826625	0.000023	-0.060888	0.801779	0.828865
Otu0014	37590	0.822497	0.000028	0.013416	0.955887	0.822607
Otu0028	4347	0.24871	0.305147	-0.737874	0.000473	0.778662

Discussion

This study examined the effects of eliminating dairy from the diet on the human gut microbiome. A total of eighteen samples were taking during three periods: a control period on a regular diet, a dairy-free period, and finally a return to normal diet. Over each time period 16S rRNA sequencing was used to determine the bacterial taxa from each sample, and suggested that changes in the gut bacterial community did occur. Other studies have found that diet affects gut microbe composition (Muegge et al. 2011, Wu et al. 2011, David et al. 2014), and this can have consequences on human health (Walker et al. 2011, Claesson et al. 2012). It was expected that samples taken during the period on a dairy-free diet would differ from those of the control and return to normal diet periods, and to some extent this was the case. Fluctuations in the relative abundances of different bacterial populations were also expected, although it was uncertain which bacteria, and at what taxonomic levels the fluctuations would be seen.

Sequences classified as members of the phyla Bacteroidetes and Firmicutes were dominant within the samples, followed by sequences identified as Proteobacteria. Bacteroidetes are commonly found in the human gut, and may be important in the development of the gastro-intestinal tract as well as interacting with the immune system and limiting gut colonization by pathogens (Thomas et al. 2011). This phylum is known for its symbiotic activity in the degradation of biopolymers and polysaccharides in the large intestine (Mahowald et al. 2009, Thomas et al. 2011). Members of the Firmicutes are also common with the gut, and some groups have been linked to obesity because of

their ability to efficiently produce excess energy from food (Fujimura et al. 2010). Higher numbers of *Lactobacillus* (a genus within the Firmicutes) have been reported in obese individuals compared to lean and anorexic subjects (Armougom et al. 2009). The relative abundance of Firmicutes is dependent upon dietary selective pressures and a higher ratio of Firmicutes to Bacteroidetes tends to be correlated with obesity (Fujimura et al. 2010), although it may be that a decrease in the numbers of Bacteroidetes, rather than an actual increase in Firmicutes, is responsible for the increased ratio of Firmicutes to Bacteroidetes in obese subjects (Armougom et al. 2009).

Lactobacillales, the order within Firmicutes that contains *Lactobacillus*, decreased in abundance during the dairy-free period and returned to control levels when dairy was reintroduced to the diet. Lactobacillales falls within the lactic acid bacteria, a group that is microaerophilic and ferment hexose sugars, such as lactose, into lactic acid (Makarova et al. 2006). Thus, with a reduction of the intake of dairy products, of which the main sugar is lactose, a corresponding decrease in these bacteria would be expected. Given that higher numbers of *Lactobacillus* can correlate with obesity (Armougom et al. 2009), eliminating dairy from the diet would therefore seem to be beneficial for obese individuals, as it would not only potentially reduce caloric intake, but also potentially reduce the proportions of a group of obese-associated gut bacteria. A reduction in the abundance of *Parabacteroides*, a genus of Bacteroidetes, was also observed during the dairy-free period of the experiment. No other studies on dietary change have reported a reduction in *Parabacteroides*, although a recent study suggests that host genotype can influence the numbers of this genus of bacteria, among others (Kashyap et al. 2011). The reduction in this genus during the dairy free period could simply be a result of lowered

intake of food, including polysaccharides that are likely required by *Parabacteroides*, because of the stringent restrictions upon the subject's diet.

The representation of *Escherichia coli* in the gut community increased temporarily immediately following the start of the dairy-free diet. No reason for this change in relation to dairy intake could be found from other studies. However, a study on early onset atopic eczema found that in infants, IgE concentration correlated directly with *E.coli* in highly sensitized groups, indicating that *E. coli* and other bacteria are associated with atopic sensitization (Kirjavainen et al. 2002). Another study found that relative numbers of *E. coli* increase following inflammation in the gut, characterized by a decrease in obligate anaerobic bacteria, because of a growth advantage conferred by the nitrate by-products of inflammation (Winter et al., 2013). Thus the proportion of *E. coli* in the gut can clearly be influenced by other factors, although the mechanism by which they showed short-term fluctuations immediately after dietary change are unclear. It could be that the varying levels of *E. coli* are simply related to the highly variable diet of a college-aged male. Another member of the Proteobacteria, *Sutterella*, demonstrated a general increase in its proportions in the gut during dairy elimination. While no prior studies could be found that linked dairy consumption to a fluctuation of *Sutterella*, it has been associated with autistic children who also suffer from gastrointestinal (GI) dysfunction, which may be correlated to the severity of the autism (Williams et al. 2012). *Sutterella* was found to be a major component of the gut microbiota in subjects with autism and GI dysfunction, while absent from subjects with just GI dysfunction (Williams et al. 2012). *Sutterella* 16S rRNA gene sequences have also been detected in fecal and intestinal biopsy samples from subjects with Crohn's disease and ulcerative

colitis (Williams et al. 2012).

Non-metric multidimensional scaling (NMDS) was used to compare the samples of each period to the other periods and revealed background variability in the gut community. Beginning with the control samples, the data points are slightly scattered, likely reflecting the unregulated and highly varied diet typical of a college-age male. Moving to the dairy-free diet samples, there is a clear and distinct shift towards the negative scores on the first NMDS axis. These findings are supported by other studies that demonstrate diet having an effect on gut microbiome composition (David et al. 2014) and that a drastically different diet can influence gut microbiome composition in as little as twenty-four hours (Wu et al. 2011). After returning to a normal diet, the first sample in this period appears to group with the dairy-free samples; however, subsequent samples do not group with the dairy-free samples, and are more similar to the control samples. That first sample after returning to a normal diet was taken on the second day after reintroducing dairy, suggesting that the gut microbiota required >48 hours for dairy to have an effect on overall composition. This timeframe is not as fast as the results from a controlled-feeding study that showed detectable differences within twenty-four hours (Wu et al. 2011).

This study supports the original hypothesis, as well as confirming the results of other studies, that diet has a direct and significant impact on the composition of the human gut microbiota. Relative abundances of bacteria at many taxonomic levels showed both positive and negative fluctuations. While the specific reasons for a majority of these changes in abundances are outside the scope of this study, further work could be done to determine what specific changes caused these fluctuations. The findings of this

study have implications that can affect the everyday lives of ordinary people, because the length of time of eliminating dairy (40 days) was typical of what many people may follow during an annual religious observance (Lent). Not everyone undergoing such temporary dietary change realizes that diet can have such as significant influence on their gut microbiome, even more so than ones genetic history or genotype (Zhang et al. 2010). Simple lifestyle choices can have serious negative or positive effects on our health, and since gut composition relies so heavily on diet, I guess you can say that we really are what we eat.

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