

EFFECTS OF SUN DRIED RAISINS ON GUT MICROBIOTA COMPOSITION IN
HEALTHY ADULTS

By

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For my beloved grandmother

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
BMI	Body Mass Index
CRC	Colorectal Carcinoma
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IL	Interleukin. A glycoprotein, a marker of immune response. IL-1 produced by macrophages, B cells, monocytes and dendritic cells, IL-10 produced by monocytes, T-helper cells type-2, CD8+ T cells, mast cells, macrophages and B cells.
NCBI	National Center for Biotechnology Information
OTU	Operational Taxonomic Unit. Part of a unique sequence that represents a certain taxonomic group or organism.
PCNA	Proliferative Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
QIIME	Quantitative Insight Into Microbial Ecology. An Open source microbiome pipeline
SCFA	Short Chain Fatty Acid. A fatty acid with equal or less than six carbon atoms. A secondary metabolite of microbial fermentation of fibers.

Abstract of Thesis Presented to the Graduate School
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Emerging evidence suggests a potential role of gut microbiome in the etiopathogenesis of various diseases. Thus, the maintenance and targeted modification of gut microbiota composition has potential for improving various health parameters. Raisins are rich in phytochemicals that may affect gut microbiota composition. As the association between gut microbiota and raisins has not been investigated in humans the objective of this study is to determine how adding raisins to the diet affects gut microbiota of healthy adults.

A 14 day feeding study was conducted with thirteen healthy volunteers between the ages of 18 and 59 years. Participants consumed three servings (one ounce each) of sun dried raisins daily. Fecal samples were collected prior to raisin consumption (baseline) and after addition of raisins to the diet (on day 7 and 14). The composition of fecal microbiota was characterized by sequencing 16S rDNA; sequences were then subjected to quality control and clustered into Operational Taxonomic Units (OTU) at 95% and 98% similarity levels.

Overall microbiota diversity was not significantly affected by adding raisins to the diet. An increase of specific OTUs matching *Faecalibacterium prausnitzii* and

Bacteroidetes sp. along with a decrease of OTUs closest to *Klebsiella sp.*, *Prevotella sp.* and *Bifidobacterium spp.* correlated with the addition of raisins to the diet. These OTU level changes indicate beneficial changes and a reduced risk for potential pathogen effects such as *Klebsiella sp.*

To better establish benefits of increased raisin intake future studies should target quantifiable health endpoints correlate gut microbiota with improved immune function.

CHAPTER 1 LITERATURE REVIEW

The Human Gut Microbiota

The human gut harbors a diverse and dynamic ecosystem of mostly symbiotic microorganisms, frequently referred to as gut microbiota (1-3). Gut microbiota composition and activities have been proposed to correlate with various aspects of human health (1, 4). Mutualistic gut microbiota functions contribute to digestion, absorption, excretion as well as immune protection. Specific contributions of the gut microbiota include the degradation of insoluble fibers into short chain fatty acids (SCFA), the de-novo synthesis of biomolecules such as vitamins (B12, K) and linoleic acids, biotransformation of phytochemicals and energy conversion (Butyrate, a SCFA is the main energy source of colonocytes), induction of gut motility to a beneficial speed and immune homeostasis (1, 5-8). Gut microbiota in a healthy adult is diverse and fairly stable providing protection from enteric pathogens (8). Dietary intake can influence the microbial milieu and affect the physiology in the human gut (9, 10). Previous microbiome studies suggest that an increased intake of fruits and other plant-based foods correlates with beneficial changes in gut microbiota composition (11-15). However, most of these studies struggle to establish such correlations between fruits and the gut microbiome. This chapter will review potential health benefits of raisins, with an emphasis on microbiota-mediated effects as discussed in previous research studies. This chapter will also provide information and rationale behind the methodologies used in this study.

Intrinsic and extrinsic factors shape the gut microbiota composition. Each individual harbors a unique microbiome that is shaped by maternal inoculation during

birth and then adapts to the person's genetics, age and lifestyle habits such as diet and exercise (16).

Bacteria are more abundant in the human gut when than other microorganisms such as eukaryotes and viruses. The bacterial cell count outnumbers the host cell count by a ratio of ten to one (1). Many of these bacteria are obligatory anaerobes contributing to host immune function (7, 17). Many of the microorganisms in the gut cannot be grown by conventional culture methods due to complex growth requirements. The advancement of high throughput sequencing has allowed characterization of such complex microbial communities and studies of their associations with human health (18).

Human Microbiome Characterization

The gut microbiome is the collection of microbial genomes in the gut. A recent review of current microbiome research reports that a healthy human adult gut has more than 35,000 total bacterial species and over ten million total bacterial genes (19). Large scale microbiome projects such as "European Metagenomics of the Human Intestinal Tract" (MetaHIT) and "US Human Microbiome Project" (HMP) have provided insight into host microbiome signatures as disease diagnostic markers (2, 6, 20).

Fecal microbiota DNA extraction, the first step in non-culture-based gut microbiome characterization. Microbial DNA extracted from fecal samples are representative of the distal gut microbiota. However, the details of preservation and extraction protocol can potentially have a decisive influence on the yield and quality of DNA (21, 22). The current study uses QIAamp® DNA stool mini kit to extract DNA, because several studies have shown effectiveness of the protocol in obtaining a representative gut microbiome sample (23-25). Adding a bead beating step further

increases the DNA extraction efficiency (23, 24). This study uses RNAlater solution to preserve fecal samples to improve stability of DNA and to prevent degradation. A recent study has demonstrated RNAlater to be more effective in terms of both the DNA stability and cost (26). The same study recommends -80 °C as the fecal sample storage temperature to prevent any DNA degradation (26).

The 16S rDNA microbiome characterization approach. Characterization of gut microbiome is challenging because of its diversity and taxonomic complexity. Bacteria dominate the gut microbiome, thus 16S rDNA amplicon sequencing can capture almost all microorganisms in the gut that evade conventional culture methods. Selected combinations of the hyper-variable regions (V1-V9) of 16S rDNA in bacterial genome are amplified using PCR and clustered into Operational Taxonomic Units (OTUs) (27). This study uses the Illumina MiSeq sequencing platform which is an efficient sequencing platform for multiple samples of 16S rDNA (28). The 16S rDNA approach is efficient for analyzing complex microbial communities (27).

Identification and classification of sequenced bacterial DNA. Raw sequence data obtained after Illumina sequencing must be clustered and assigned into biologically meaningful Operational Taxonomic Units (OTUs) to identify the bacterial composition in the fecal samples (29). OTUs are sequence reads that are similar to each other at a specified similarity level (95% or 98%). QIIME (Quantitative Insights Into Microbial Ecology) is one of the widely used software packages to perform pre-processing of raw sequence reads (quality filtering and removal of chimeras), clustering sequences based on a similarity threshold, OTU picking, taxonomic assignment and downstream core diversity analyses (21, 29). The current study uses the UPARSE algorithm with

USEARCH greedy clustering approach to generate OTUs. The OTU clusters are then matched with greengenes reference database using a 16S rDNA specific microbiome pipeline to assign phylogeny (27, 29-32). Many studies have identified that the clustering and OTU generation approaches used in the current study are efficient in human gut microbiome research (21, 29, 33). This study uses 95% and 98% similarity levels to cluster sequences. This study expects to identify bacterial composition by clustering sample sequences with less stringent and more stringent phylogenetic relatedness.

Downstream OTU analysis. Apart from OTU prevalence analysis, changes in diversity and richness measures overtime can help to determine gut microbiome stability or indicate dysbiosis. The Alpha is OTU diversity within each sample and the beta diversity is OTU diversity between samples (34). The core diversity analyses in QIIME can produce a Chao1 rarefaction curve, which visualizes the alpha diversity of each sample based on rare OTUs and sequencing depth. Other diversity indices for alpha diversity are Shannon index and Simpson's index (34, 35). QIIME use UniFrac distance to calculate the beta diversity; UniFrac distance is based on both the phylogenetic distance as well as the sample composition dissimilarity (weighted UniFrac distance is a quantitative measure of beta diversity where abundance of sequences are also included, and un-weighted UniFrac distance is a qualitative measure where abundance is not considered) (32).

Targeted 16S rDNA-based gut microbiota studies need standardized, cost-effective and efficient DNA preservation, extraction, upstream and downstream sequence processing and analyzing methods to allow cross comparison between

research studies (22, 33). Also to use gut microbial changes as prognostic or disease diagnostic biomarkers, the standardized methods must be sensitive, reliable and accurate (22, 33). Direct changes in gut microbiota composition by modified dietary habits have the potential to improve health (36). Currently the extent of the association between diet and human gut microbiota and its correlation to health is not fully understood.

Dietary Influences on Gut Microbiota

There has been renewed interest among gut microbiome researchers to understand how dietary manipulations can impact gut microbiota and human health (8, 36, 37). Among factors that can influence the gut microbiota, diet is easily manipulated to allow beneficial changes in the gut microbiota composition. Even though the human gut microbiota is adapted to long term dietary habits, alterations to the diet can change the microbiota composition (9, 38, 39). A diet abundant with fruits, vegetables and fibers is recognized as one of the most important preventive factors, only second to tobacco cessation in cancer prevention strategies (11, 40-42). Increasing the dietary intake of fruits, vegetables and fibers can be suggested as an effective means of improving the daily diet.

Latest dietary guidelines for healthy Americans, recommend an average daily intake of two to two and a half cup equivalents of fruits per 2,000 calorie diet (43-45). According to the dietary evaluations from 2007-2010 National Health and Nutrition Examination Survey (NHANES), the daily intake of fruits by adults is well below the recommended intake (43, 46). Taking into account the benefit of consuming dried fruits with regard to fulfilling the dietary requirements of fruits, raisins can contribute to the daily intake of fibers and nourishing with micronutrients (43, 47, 48). Therefore

expanding the knowledge on dried fruits such as raisins would have a significant impact on nutrition and public health research.

Many of the existing research studies focus on the chemical aspect of fruits and its effect on the gut chemistry and physiology. In order to understand the overall influence of fruits on human gut, more studies should focus on investigating the effects of specific fruits on the gut microbiome and identify fruits that promote beneficial changes. Dietary interventions and epidemiologic studies have documented various effects of fruits on the gut microbiota (8, 49). However, interpreting results based on dietary interventions must be dwelt with caution by considering any confounding effects that may be attributed to environmental, lifestyle and individual host factors to ensure reliability of the results.

Having a beneficial phytochemical profile and the ability of the phytochemicals to influence gut microbiota must be considered when selecting a fruit to test influence on gut microbiota. Commonly cultivated, easily accessible and popular fruits with medicinal values could be prioritized to expand the knowledge about specific fruits and its influence on the human gut microbiome.

Raisins and the Gut Microbiota Composition

Raisins are dried grapes (*Vitis vinifera*) a popular dietary constituent used since 120 to 900 BC in European and Mediterranean regions (50, 51). Previous studies have demonstrated various effects of grapes and grape products (raisins, pomace, wine and seeds) on the gut microbiota (14, 52-56). However, little is known about the effects of raisins on human gut microbiota.

Grapes and raisins contain relatively high concentrations of beneficial phytochemicals (47, 57, 58). These phytochemicals include simple sugars (glucose,

fructose), fibers (30% soluble fibers such as fructo-oligosaccharides and inulin, and insoluble fibers), tartaric acid, and phenolic compounds (flavonols such as quercetin and kaempferol, phenolic acids such as caftaric and coutaric acids, and proanthocyanidins) (57, 59). Several studies have shown that the production process of raisins (sun drying and processing) increases the concentration of flavonol, phenolic acid, fructo-oligosaccharides (FOS), tartaric acid and fibers not only by condensation (17, 60, 61).

Phytochemicals present in raisins has a potential to positively influence the gut microbiota (41, 62, 63). Many in vitro and animal model studies have demonstrated effects of grape phytochemicals on gut microbiota to be beneficial for human health (17, 48, 57, 58, 62-65). However, human studies are needed to confirm or strengthen these assumptions based on in vitro and animal studies.

Anti-Inflammatory and Pathogen-Resistant Properties of Raisins

Raisins could potentially influence the epithelial cell integrity. Gut epithelial integrity is essential for absorption of nutrients, providing a barrier for pathogens and reduce gut inflammation (1, 8). Yang *et al* has shown that grape seeds contribute to better cell integrity in mouse models that mimic inflammatory bowel disease (66). Many phytochemicals present in grape seeds can also be found in raisins. Fibers, polyphenolics and SCFA are some of these compounds that are known to exert beneficial effects on gut epithelial integrity (48, 57).

Evidence suggests raisins could potentially increase anti-pathogenic byproducts of microbiota. Gut microbiota can contribute to both active pathogen inhibition (via bacterial metabolites such as SCFA, bacteriocins and peroxides) and by passive inhibition (via bio-film barrier formation and competing for resources) (8, 58).

Polyphenolic compounds and also fibers contribute to anti-microbial activity (1, 67). Several gut microbiome studies have reported inhibitory effect of grapes and grape derivatives on bacterial pathogens such as *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* and fungal pathogens such as *Penicillium expansum*, *Candida albicans* and *Aspergillus niger* have been observed (52-54, 68, 69).

Phytochemicals in raisins are suggestive of their ability to potentially reduce chronic inflammation. Many studies report disrupted fermentation process as a predictor of inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), and also pathogen invasion (8, 41, 66, 70, 71). Phytochemical compounds of raisins could potentially contribute to anti-inflammatory activity by modulating the gut microbiota. Spiller *et al* has demonstrated increased SCFA with raisin consumption, indicating improved fermentation process. Di Lorenzo *et al* has demonstrated the anti-inflammatory activity of raisins by suppression of pro-inflammatory molecules using an in vitro study on healthy human gastrointestinal epithelial cells (17).

Anti-Cancer Properties

While some gut microbiota are associated with carcinogenesis by prolonged infection exerting pro-inflammatory effects, others may contribute to cancer prevention. The phytochemicals that are present in grapes and grape products have shown to be protective against cancers (41). The Yang *et al* study on mice has demonstrated that grape seeds reduce proliferative cell nuclear antigen (PCNA), indicating a reduction of hyper-proliferation in epithelial cells (66). The Kountouri *et al* study on colorectal cancer (CRC) cell line (HT29) has demonstrated an exposure time-dependent antioxidant and anti-inflammatory activity of raisins (42). Increased production of butyrate (a SCFA) is associated with reduced risk of ulcerative colitis and CRC (14). Raisins have ample

amounts of fibers and tartaric acids that could potentially be converted to SCFA by microbial activity (59). Antioxidants (flavonoids and phenolic acids) present in grapes and raisins are also correlated with reduced risk of CRC (17, 41). Camire *et al* demonstrated the bile acid binding ability of raisins (60). Dietary fibers reduce the concentration of bile acids in the colon. Excess fecal bile acids (FBA) are an indicator of increased CRC risk (64). Raisin consumption has shown to decrease FBA in healthy adults (64).

Fibers, tartaric acid and SCFA increase bowel movement by reducing the transit time, and subsequent moderate increase of fecal weight contributes to dilution and effective elimination of carcinogens and prevents constipation (59, 60, 64, 72). Raisins seem to exert protective effects against colorectal carcinogenesis and some seem to be associated with gut microbiota activity.

Overall Benefits of Raisins and Possible Means of Introducing Raisins to American Diet

Raisins are beneficial to health in many aspects, thus they are better alternative for commonly used high fat, high sugar snack food. Raisins have a low to moderate glycemic index, and a low insulinemic index than other snack food (72, 73). Raisins have more fiber content than any other commonly used snack food, and also the FOS content of raisins is greater than that of grapes (57, 60). Raisins also seem to correlate with improved total serum cholesterol level (reduced LDL, and triglycerides) and weight loss by regulating appetite (72).

Raisins are stable, have a greater shelf life and lesser tendency for spoilage. Few studies have focused on sun dried raisins as a healthier and natural alternative to replace commercial carbohydrate snacks. Rietschier *et al* has claimed that sun dried

raisins appear to be a cost effective source of energy for people doing moderate to high intense exercise in comparison to sports jelly beans or sports chew (58, 74). It is the best and the easiest way to acquire adequate amounts of fibers, micronutrients and beneficial phytochemicals that have the potential to promote immune function and enhanced gut comfort (14, 59, 63, 75). Potential long term effects may include the reduced risk of colorectal cancer and prevention of chronic inflammation.

Strengths and Limitations of Raisins and Gut Microbiome Studies

The Mandalari *et al* study is the first to describe the correlation between sun dried raisins and the human gut microbiota using a gastrointestinal digestion model (14). Authors claims that “sun dried raisins exhibit immense potential in their capacity to promote the colonization and proliferation of beneficial bacteria in the human gastrointestinal tract” (14). Authors documented a significant increase in *Bifidobacterium sp.*, Proteobacteria, Actinobacteria, *Roseburia sp.* and decreased in overall Bacteroidetes, *Faecalibacterium prausnitzii*, and Ruminococcacea. Although the authors make inferences based on fecal samples provided by one healthy individual, artificially digested raisins, and a short incubation period (24 hours), they comment on the need for a long term human intervention (14).

Spiller *et al* describe the effects of sun dried raisins on healthy adults (59, 64). Authors measured the amount of SCFA transit time, fecal weight and bile acid binding as markers of gut microbiota activity (59, 60, 64). Although raisins seem to correlate with gut health via modification of microbiota activities, their effects on microbiota are not established. In vitro studies cannot fully replicate the real life changes occurring in the human gut influenced by internal and external host factors. Therefore finding direct effect of raisins on human gut microbiota can contribute to future research.

CHAPTER 2 INTRODUCTION

The interplay of human gut microbiome, diet and health has received much attention in the past decade (76). There are many means through which gut microbiota can influence good health; fermentation of dietary fiber to short chain fatty acids is one such important mechanism (6, 36, 76). Recent research efforts have focused on investigating the effect of dietary patterns, specific foods and individual dietary compounds on the gut microbiome (10, 36). The current study investigates the effect of sun-dried raisins on gut microbiota composition. The study utilizes a targeted 16S rDNA sequencing approach, a recognized method of microbiota characterization (21), to determine the effects of adding raisins to diet on gut microbiota for a two-week period. Several studies have investigated the influence of either whole raisins or isolated phytochemicals from raisins (42, 57, 59, 64), on the amounts of microbial metabolites produced or the gut microbiota composition using in vitro cell culture or gastric digestion models (14, 59, 60, 64). However, human feeding studies are needed to translate these observations.

Maintaining a diverse gut microbiome is associated with an improved immune function (20, 21). The effect of raisins on the overall gut microbiota diversity has not been documented in earlier studies. Thus this study measures the alpha and beta diversity to identify the effects of raisins on gut microbiota diversity. Previous studies suggest potential effect of raisins on specific bacterial signatures, thus this study also measures the relative abundance of bacterial signatures before and after raisin consumption (10, 19, 58, 76).

Raisins have been shown to reduce the risk of many chronic diseases (38, 58). Investigating the effects of raisins on gut microbiota can potentially determine the means of chronic disease prevention. This research will contribute to the understanding of the benefits of raisin-mediated influence on gut microbiota.

Research Question

How can raisin consumption change the human gut microbiota in healthy adults?

Study Hypothesis

Sun dried raisins modify the human gut microbiota towards a beneficial composition.

Study Objectives

To evaluate the effect of sun dried raisins on the gut microbiome composition in healthy adults.

CHAPTER 3 MATERIALS AND METHODOLOGY

Study Population

A total of 18 eligible volunteers were recruited from the area surrounding the University of Florida, Gainesville to participate in a two-week feeding study. Volunteers were recruited by advertising with flyers. Potential participants were screened for eligibility. They were asked to provide information on dietary habits and medical history. Participants with an age between of 18 and 75 years, in general good health and having regular bowel movements (at least three times a week) were included in the study. Exclusion criteria of the study were having underlying gastrointestinal disorders such as ulcers, Irritable bowel syndrome and chronic constipation, having diarrhea during the past month (at least three soft or watery stools within 24 hour period), experiencing a change in body weight of more than 10% in the past three months, having a colonoscopy screening within the last two months and using medication that affects bowel function or microbiota such as antibiotics and laxatives during the past month. After the written informed consent, participants were asked to complete a demographic questionnaire at baseline, a compensation form, a fecal sample collection log and a gastrointestinal health questionnaire during each visit. Participants were given monetary compensation for each fecal sample collected.

Each participant was assigned to consume three servings (one ounce per serving) of commercial “Sun Maid Natural California Raisins” per day for a continuous 14 days. Participants were considered as their own controls. Thirteen participants completed the feeding study successfully. Those participants who completed the study were included in the microbiota analysis.

Funding Source and Project Approval

The gut microbiota human participant study protocol has been approved by Institutional Review Board, University of Florida (IRB-01) with the approval number IRB201500607. The current study is funded by Sun-Maid growers of California.

Product Information

This study used “Sun-Maid” commercial sun dried raisins, with no added sugar. A packet of 28.3g (one ounce) “Sun-Maid” raisins contains 20g of natural sugars (fructose), 2g of fibers, 1g of protein, 5mg of sodium and micronutrients like iron (47, 77) (Figure 3-1).

Study Design and Fecal Sample Collection

For microbiota analysis, a single fecal sample was collected from each of the 13 participants at three time points: day 0 (prior to raisin consumption; baseline), day 7 and day 14 (study design: Figure 3-2). Participants were asked to provide the first bowel movement of the day and to fill a short questionnaire using gastrointestinal symptom response scale (zero to six; zero having no discomfort at all and six having very severe discomfort). The gastrointestinal health questionnaire included questions about stomach ache, acid reflux, hunger pain, rumbling in the stomach, bloating, being bothered by burping, passing gas or flatus, constipation, diarrhea, loose stools, urgent need for bowel movement and feeling of incomplete bowel emptying. It also included a section for physical activity (predominantly sedentary, occasionally active, moderately active and vigorously active) (Appendix C). For Table 4-1, predominantly sedentary and occasionally active was considered as low activity level and the latter two categories was re-classified as high activity group. The Demographic questionnaire included age, gender, race, height and weight. Participants were asked to collect fecal samples into

SIGMA stool collection kit, transfer to sterile plastic collection tubes with RNAlater solution, and transported within six hours for immediate storage at -80 °C.

DNA Extraction and PCR Amplification

Bacterial genomic DNA was isolated from fecal samples using QIAamp DNA Stool mini kit with an initial bead beating step (appendix B). DNA samples were quantified and then amplified using bar-coded Illumina primers targeting the V1 and V2 region of the bacterial 16S rDNA. Primers used in this study can be found in Appendix A (Figure A-1). PCR products were purified using the Axygen magnetic separation kit to prepare DNA libraries.

DNA Sequencing and Clustering into Operational Taxonomic Units (OTUs)

DNA library was sequenced on Illumina MiSeq platform (Appendix A, Figure A-2). Sequences of low quality (expected error >0.50 quality filter and chimera) or with a length less than 290 nucleotides were removed from the analysis. Using a modified UPARSE pipeline, the sequences were clustered at similarity levels of 95% and 98% using the UPARSE algorithm. The representative sequences from each OTU are annotated through the Greengenes 16S reference database using a Bayesian RDP classifier. Sequences were then binned into OTUs using the USEARCH algorithm resulting in a completed OTU table with OTUs as rows and samples as columns.

Statistical Analysis

Microbiome diversity analysis. Shannon-Weaver and Simpson index and species richness were calculated in Microsoft Excel to measure alpha diversity. Chao1 rarefaction curve was generated using the Quantitative Insights into Microbial Ecology (QIIME) software package (31). UniFrac distances and principle component analysis

plots were generated using the QIIME software package. The mean UniFrac distance calculations were conducted using Microsoft Excel.

Relative abundance measures of OTUs. Percent relative abundance of bacterial signatures grouped by phyla and genera were calculated. OTUs annotated as un-classified or classified only up to the kingdom level by the UPARSE algorithm were manually re-aligned using the BLAST tool in NCBI (78). If re-aligned sequences matched to sequences belonging to phages or vectors, and/or if sequence similarity score and query coverage is less than 95%, then these OTUs were excluded from the analysis.

The significance of differences in the proportion of participants showing the presence/absence of specific OTUs was calculated using z-test. Heat maps were generated to include OTUs that reached significance (79). The significance in mean counts of OTUs was calculated by t-test.



Figure 3-1. Sun dried raisins used in the study. A) nutritional facts label of 1 oz package of raisins, and B) the six pack raisins provided for participants, each small package contains one ounce of raisins. Photo Courtesy ((14) and the author).

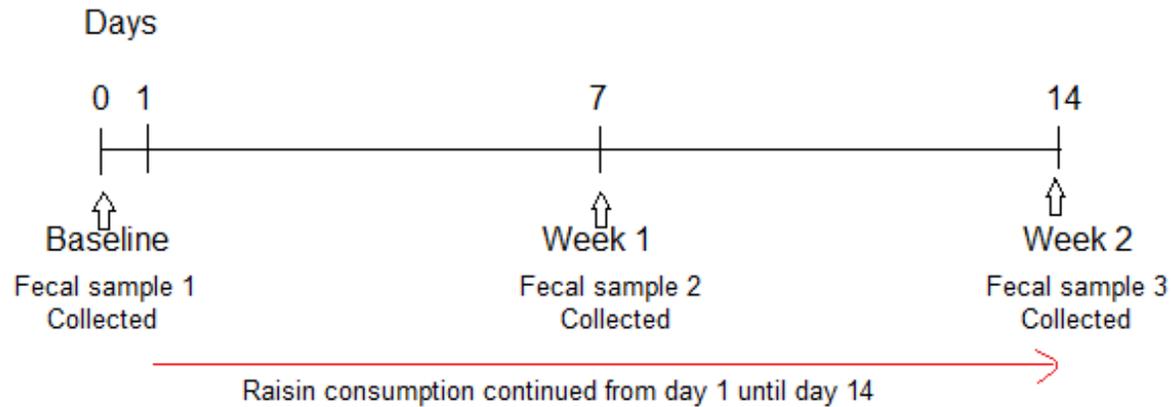


Figure 3-2. Study design. Red arrow represents the raisin feeding period (day one to day 14). Fecal samples collected before raisin intake (day 0/baseline) and one week after addition of raisins (day 7/ week 1) and two weeks of raisin intake (day 14/ week 2). After informed consent the monetary compensation forms and demographic questionnaire were given at baseline. Gastrointestinal health questionnaire was given at baseline, week one and week two.

CHAPTER 4 RESULTS

Participant Characteristics

Demographic characteristics of the participants are shown in Table 4-1. Participants did not report any discomfort (overall health, stomach comfort and fecal movement) during the raisin feeding period. However, most participants disliked consuming three one-ounce servings of raisins daily.

Output of 16S rDNA Sequencing

Sequencing using Illumina MiSeq platform generated a total of 5,533,527 sequence reads from 39 fecal samples (samples from 13 participants each at baseline, week one and week two). After removal of low quality and short length sequences, a total of 4,477,275 sequences were retained, with an average of 106,475 sequences per sample and an average sequence length of 322.25 nucleotides. The sequences binned using UPARSE algorithm generated 1,238 and 2,168 unique OTUs at the 95% and 98% similarity levels respectively.

Microbiome Diversity and Richness

Alpha diversity measured using Shannon-Weaver index, Simpson index and OTU richness did not show statistically significant difference with raisin intake (data not shown). Also, the Chao-1 rarefaction curve did not differ by time point (Figure 4-1). The principle coordinate analysis plots based on the UniFrac distances show that overall microbiota composition of fecal samples were grouped by participants and not by time points (Figure 4-2 and Appendix A, Figure A-1).

Relative Abundance of Gut Bacteria

In the phylum level analysis, OTUs matching Bacteroidetes and Firmicutes were dominant across all study samples. OTUs matching phylum Actinobacteria and Proteobacteria were observed to a lesser extent. Relative proportions of bacterial phyla were not significantly affected by raisin consumption (Figure 4-3, Figure 4-4 and Appendix A, Figure A-2). Phylum distribution at 98% similarity level was similar to that of 95%, thus the data plots are not shown for 98%. Even though the effect in raisin consumption on the Firmicutes to Bacteroidetes ratio across time points was not significant (with an average of 2.33 for baseline, 1.56 for week one and 1.60 for week two), relative proportion of the Bacteroidetes seem to increase and the proportion of Firmicutes seem to decrease with raisin consumption (Figure 4-3 and Appendix A, Figure A-2). Genus level effect by raisins was also not statistically significant.

At 95% similarity level, heat maps show 16 OTUs that were significantly affected by the first week of raisin consumption, 11 OTUs significantly changed by the second week, compared to only 4 OTUs that show significant change when comparing the two feeding periods (week one to week two, Figure 4-5). At 98% similarity level, heat maps show 28 OTUs and 19 OTUs significantly affected by the first and second week of raisin consumption respectively, compared to only 13 OTUs changed between the feeding periods (Figure 4-6). OTU matching *Klebsiella sp.* presented in Figure 4-5 was used to generate another heat map with that has an enhanced OTU color separation range (0 to 100) for clear visualization. (Appendix A, Figure A-3)

Table 4-1. Demographic characteristics of study participants

Characteristics		Range or Percentage (N)
Age		18 to 59 years (13)
Gender		
	Female	62% (8)
	Male	38% (5)
BMI		
	Normal	46% (6)
	Overweight	39% (5)
	Obese	15% (2)
Exercise		
	Low	46% (6)
	High	54% (7)
Race		
	White	46% (6)
	Black	8% (1)
	Other	46% (6)

Exercise categories: predominantly sedentary and occasionally active categorized as low activity level and the latter two categories re-classified as high activity group. Body Mass Index (BMI) categories: BMI between 18.5 to 24.9kgm⁻² as normal, 25.0 to 29.9kgm⁻² as overweight and 30kgm⁻² and above as obese.

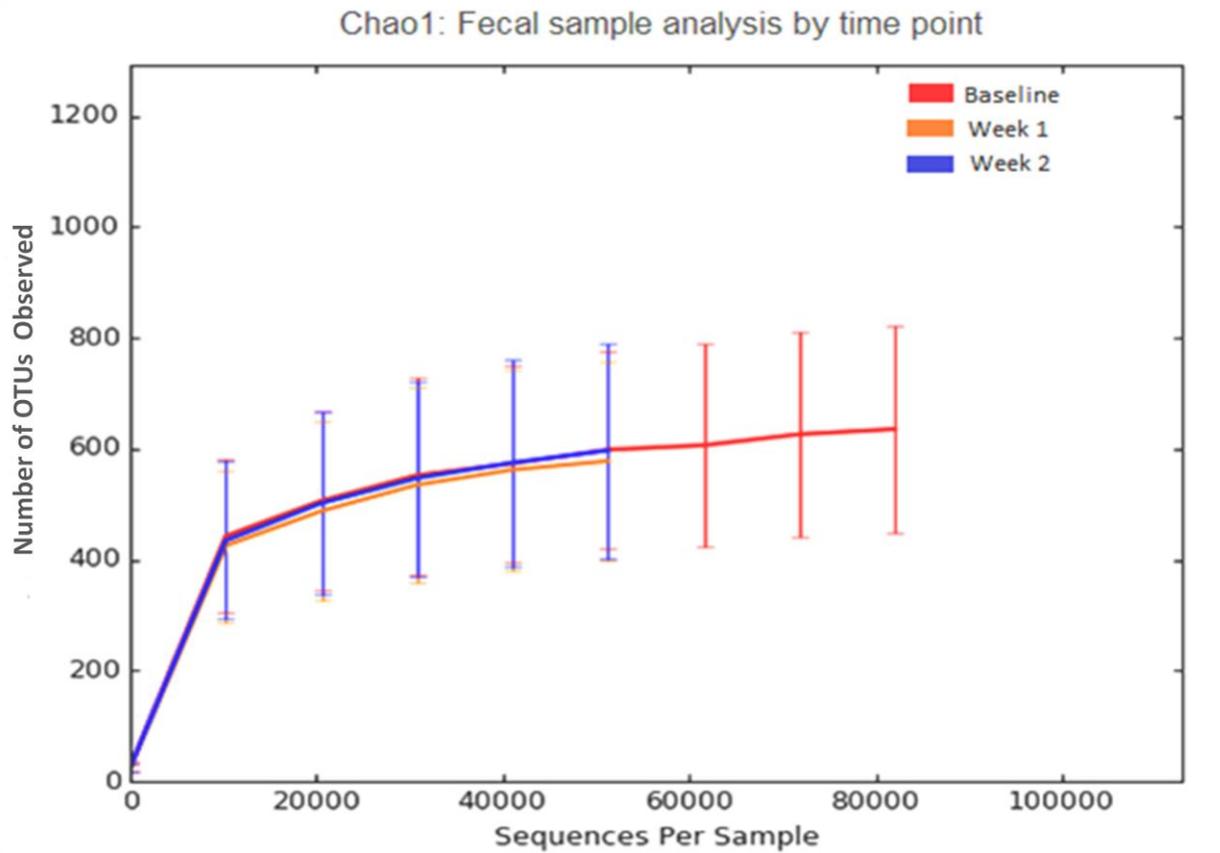


Figure 4-1. Chao1 rarefaction diversity (α -diversity). Chao diversity was calculated from sequence distribution at baseline, week 1 (one week after raisin starting consumption) and week 2 (two weeks after raisin consumption).

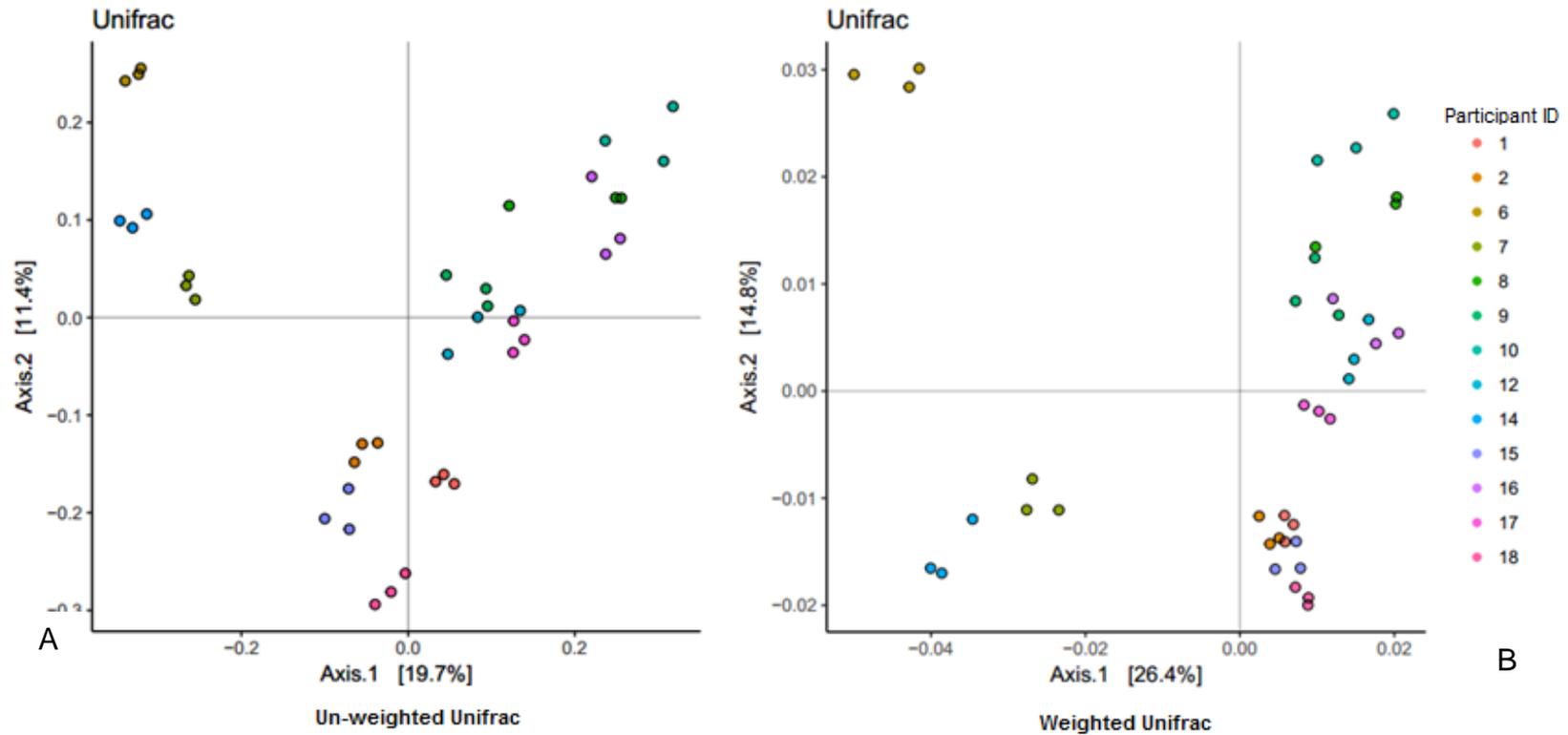


Figure 4-2. UniFrac beta diversity analysis by study participant. The principle coordinate plots based on un-weighted (A) and weighted (B) UniFrac presents microbiota composition in stool samples measured at baseline, week 1 (one week after starting raisin consumption) and week 2 (two weeks after raisin consumption). The samples are 16S rDNA MiSeq Illumina sequences obtained from each study participant given with a participant ID 1,2,6,7,8,9,10,12,14,15,16,17 and 18. Each individual has three samples from three time points and each individual was assigned a different color coded circle to visualize intra individual variation before and after the feeding period.

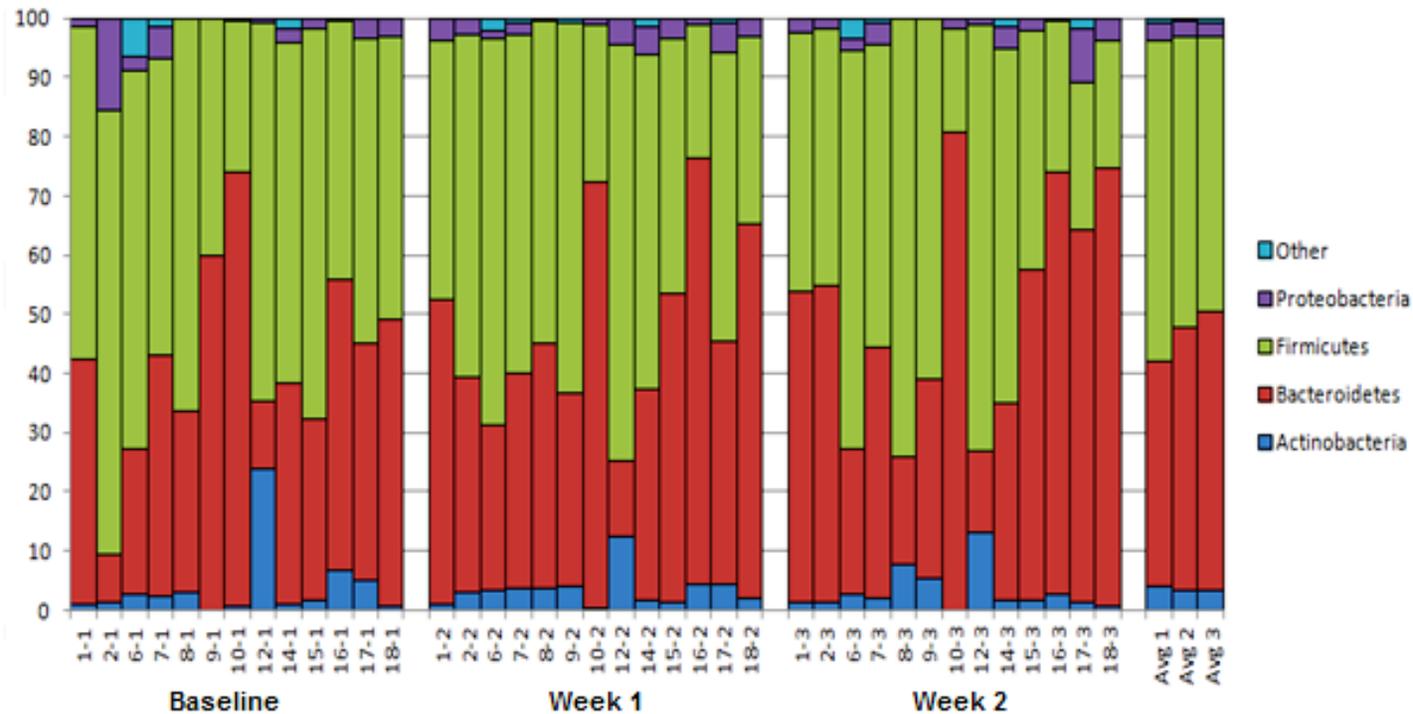


Figure 4-3. Proportion of bacterial OTUs at 95% similarity level grouped by phyla and time points. The gut microbiota variation based on 16S rDNA abundance at phylum level. Bacteria grouped into four most dominant phyla (Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria) and other group belongs to those OTUs that had sequence abundance average of less than 1.0% at each time point. Cyanobacteria, Fusobacteria, Lentisphaerae, Synergistetes, Tenericutes, TM7 and Verrucomicrobia were combined into other phyla group. Percent relative abundance of baseline, week 1 (one week after raisin consumption) and week 2 (two weeks after raisin consumption) samples are shown. Each column shows the bacterial composition of one fecal sample based on the OTU clustering with greengenes reference database. Bacterial OTUs classified only up to kingdom were not included in this analysis (these sequences had a query coverage and similarity of less than 95% when aligned with BLAST or they belonged to phage DNA used in the sequencing process. All unclassified bacterial sequences were also excluded from the figure as they were also identified as phages from BLAST analysis).

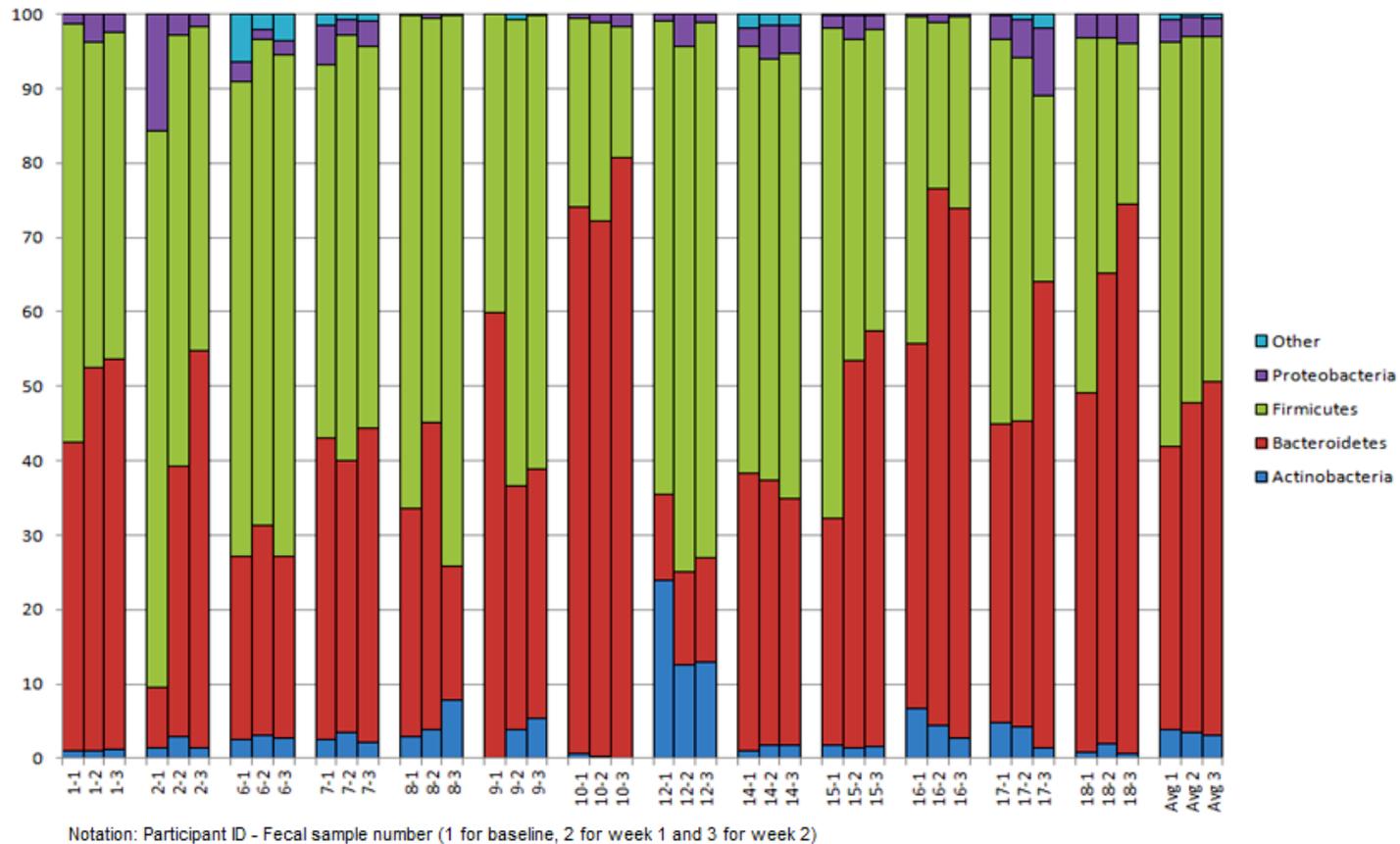


Figure 4-4. Proportion of bacterial OTUs at 95% similarity level grouped by phyla and by participants. Illumina MiSeq sequencing of 16S rDNA for microbial OTUs abundance grouped into dominant phyla (Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria) and other. OTUs that had sequence abundance average of less than 1.0% at each time point were grouped as other phyla. This group includes Cyanobacteria, Fusobacteria, Lentisphaerae, Synergistetes, Tenericutes, TM7 and Verrucomicrobia. Samples from baseline, week1 (one week after raisin consumption) and week 2 (two weeks after raisin consumption) were grouped by participant ID (1,2,6,7,8,9,10,12,14,15,16,17 and 18). Each column shows the bacterial composition of one fecal sample based on the OTU clustering with greengenes reference database.

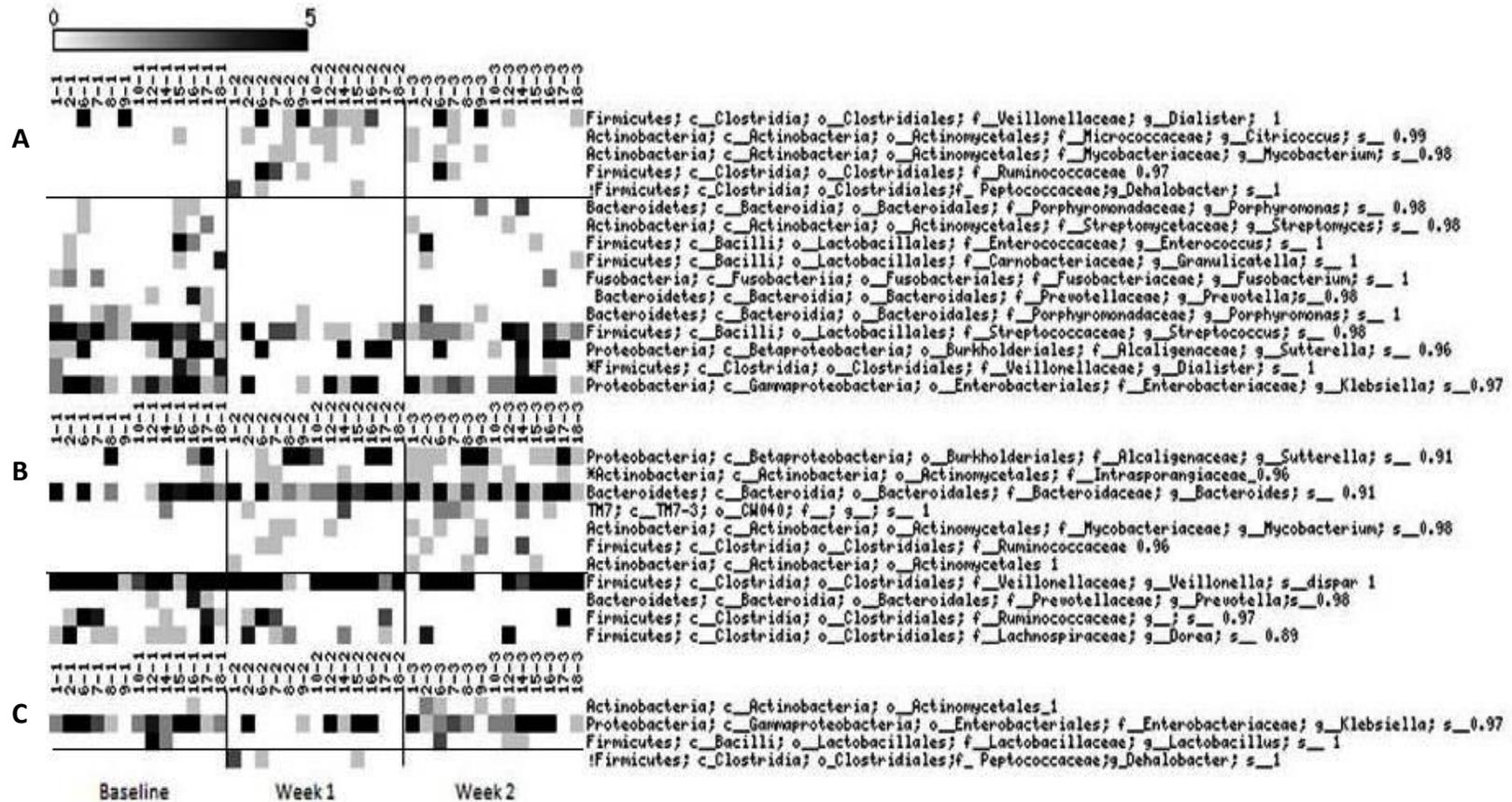


Figure 4-5. Bacterial OTUs affected by sun dried raisin consumption at 95% similarity level. Left column separator: baseline, middle column separator: week 1 (one week after raisin consumption) and right column separator: week 2 (two weeks after raisin consumption). Each column represents one fecal sample (three samples from each individual with the participant ID no: 1,2,6,7,8,9,10,12,14,16,17,18) and each row shows one OTU with its taxonomic classification of the closest match to the greengenes microbiota reference database. All samples are significant by z-test. Other notations: (*) significant by two sample t-test and (!) annotated using BLAST tool.

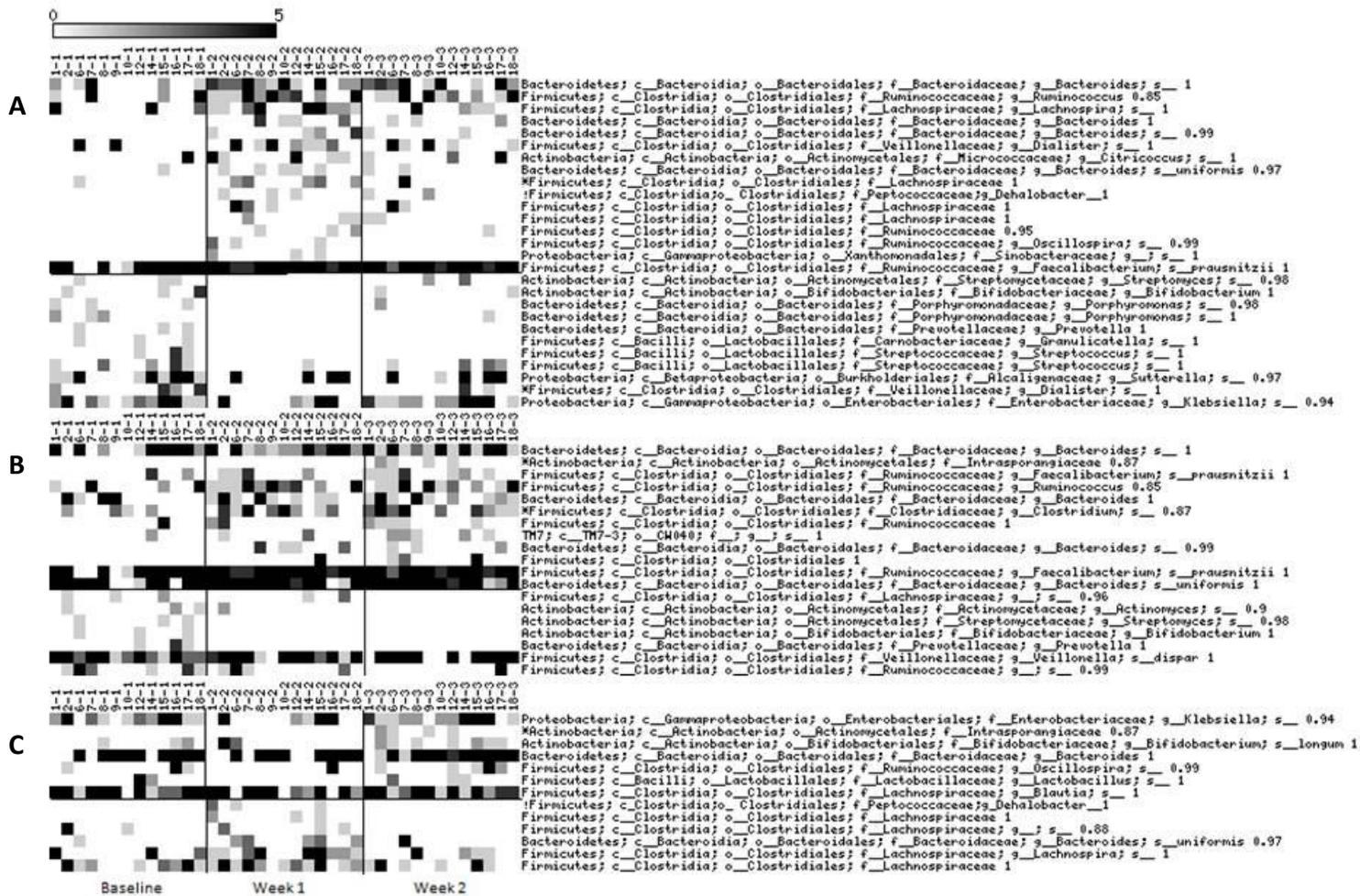


Figure 4-6. Bacterial OTUs affected by sun dried raisin consumption at 98% similarity level. Columns show fecal samples and rows show annotated OTUs. All samples are significant by z-test.

CHAPTER 5 DISCUSSION AND CONCLUSION

Increasing the dietary intake of raisins has been suggested to have beneficial effects on human gut microbiota and improved health (14, 58-60, 64). However, the association between raisin intake and gut microbiota has not been investigated among humans. Therefore, this study evaluated the effect of raisins on the overall gut microbiome composition and specific bacterial signatures by including thirteen healthy participants for a two- week study.

Effect of raisins on overall gut microbial diversity has not been investigated before. Raisin intake did not significantly change both the alpha diversity (within time points) and beta diversity (between time points) (Figure 4-1, Figure 4-2 and appendix A, Figure A-1). Although raisin intake seemed to increase the relative abundance of Bacteroidetes and decrease Firmicutes, phylum level OTU abundance did not differ significantly with raisin consumption (Figure 4-3, Figure 4-4 and Appendix A, Figure A-2). Inter-individual variation could possibly interfere with the net effect of raisins observed in this study due to differences in age, BMI and possible dietary habits (Figure 4-2, Table 4-1). Many dietary interventions with fruits have documented similar observations as to overall gut microbiota composition (13, 36, 38). In fact, polyphenolic compounds in the grape extract are found to be associated with reshaping gut microbiota towards a homeostatic balance (55, 57). Thus these observations may suggest a possible contribution of raisins in gut microbial homeostasis in healthy adults.

Although the overall microbiome composition seems to remain at homeostatic balance, some specific bacterial signatures significantly correlate with raisin consumption (Figure 4-5, Figure 4-6). This study's observations suggest that raisins

exert complex and selective effects on different groups of gut microbiota, possibly due to the complex nutrient and polyphenolic profile of raisins. Among many OTUs evaluated, abundance of several OTUs with known health correlations were influenced with raisin intake, including *Faecalibacterium prausnitzii*, *Klebsiella sp.*, *Prevotella sp.*, certain *Bacteroidetes spp.*, *Bifidobacterium spp.*, Ruminococcaceae and *Lactobacillus sp.* (Figure 4-5, Figure 4-6). These observations regarding OTUs must be interpreted carefully because without replicating the study, accuracy or the reliability of the observations cannot be confirmed.

In contrast to Mandalari *et al.*, who suggested reduced abundance of *Faecalibacterium prausnitzii* in human fecal samples after incubation with digested sun dried raisin (human gastrointestinal model) (14), this study observed an increase of OTUs matching *F. prausnitzii* in week one and week two fecal samples obtained after raisin intake (Figure 4-5). Decrease of *F. prausnitzii* in the gut correlates with chronic inflammation, colon polyps and many other diseases (80, 81). Increase of *F. prausnitzii* has been reported with increased intake of Inulin, a common phytochemical present in raisins (82). Higher concentration of Inulin or a combination of phytochemicals might have increased the OTUs matching *F. prausnitzii* (57, 82). Future studies can investigate the effect of specific phytochemicals present in raisins on human gut microbiota.

Bacterial signatures matching *Klebsiella sp.* show a significant decrease in the number of OTUs, one week after addition of raisins to the diet. This may suggest that increased intake of raisins correlate with reduced risk of enteric inflammation associated with subclinical infection (Figure 4-5, Figure 4-6 and Appendix A, Figure A-3). However,

the number of OTU seems to increase slightly by the end of the week two. Non-compliance with the study protocol or adaptation of bacteria to raisins could be two possible explanations. Two studies have suggested inverse association of potential enteric pathogens with sun dried raisin, and the combination of red wine and grape juice extracts, by using human gastrointestinal models (14). However, the potential pathogen resistant effect of raisins is consistent with many of the in vitro and in vivo grape seed studies (52, 54, 68). The presence of certain polyphenolics such as flavonoids and proanthocyanidine, gallate esters, and fibers in grape seeds induces the anti-pathogenic activity (65, 69). Pathogen resistant compounds similar to that of grape seeds may also be present in raisins (65, 69).

This study observed a decrease of OTUs closest to *Prevotella sp.* with the addition of raisins to the diet. Previous studies have shown an increase in *Prevotella sp.* with increased intake of fibers and other plant derived food compared to low fiber, high fat, western diet (63, 83). Although many studies correlate *Prevotella sp.* with improved gut health (84), others state that some species such as *Prevotella copri* could indicate chronic inflammation, rheumatoid arthritis and cardiovascular disease (83, 85, 86).

Four OTUs that are similar to *Bacteroidetes sp.* including *B. uniformis* showed an increase with the addition of raisins to the diet. Previous studies have demonstrated that grape derived products have increased *B. uniformis* (65, 68). Increased number of *B. uniformis* is associated with stimulation of the adaptive immune function, ameliorating metabolic dysfunction as well as reducing the weight gain in obese animal models (87, 88). *B. uniformis* may also contribute to the pathogen resistant properties of raisins by

converting quercetin (flavonol) in raisins to Aglycon; Aglycon has detrimental effects on the growth of *Staphylococcus aureus* and *Helicobacter pylori* (65).

A significant decrease in OTUs representing *Bifidobacterium sp.* with the addition of raisins into the diet was observed (Figure 4-6). In addition, one OTU matching *Bifidobacterium longum* show a significant increase after two weeks of raisin consumption compared to week one (Figure 4-6). Mandalari *et al* claim that raisins increase *Bifidobacterium sp* (14). Previous studies have documented that *Bifidobacterium spp.* are subjected to selective growth with grape polyphenols based on sensitivity/resistance to anti-bacterial grape polyphenols (52, 68). *Bifidobacterium spp.* are also SCFA producers and are associated with beneficial health outcomes and are recommended by the World Health Organization (WHO) as a probiotic supplement (82, 89). Reduction of certain *Bifidobacterium sp.* could reduce dietary fermentation and correlate with dysbiosis (89). Increase in *B. longum* with grape inulin and fructo-oligosaccharide has been documented (48, 82). Future studies could target *Bifidobacterium spp.* to identify species specific effects with raisin consumption.

Raisin consumption has shown diverse effects on bacterial signatures matching Ruminococcaceae. A significant increase in two OTUs matching Ruminococcaceae was observed with raisin consumption while another OTU matching Ruminococcaceae show a decrease only at week two (Figure 4-6). An OTU matching *Lactobacillus sp.* shows a significant increase in week two compared to week one (Figure 4-6). Previous studies suggest that the effect on *Lactobacillus sp.* can vary with different grape products (52, 90). Both *Lactobacillus sp.* and Ruminococcaceae benefit gut environment by pathogen inhibition, providing essential nutrients and SCFA and immune modulation (8, 52).

This is the first human feeding study to evaluate the influence of sun dried raisins on gut microbiota. Mandalari *et al* was the first to evaluate short term effects of raisins on gut microbiota (less than one hour to 24 hours) using a gut stimulator mimicking the human gut (14). However, their choice of comparison group, duration of the study, use of in vitro model and different analytical methods restrict inferences regarding the effect of raisins on human gut microbiota and also limit direct comparison with this study (14).

Although study findings are consistent with the hypothesis that raisin intake contributes to beneficial changes in the human gut microbiota composition, further research needs to be conducted with controlled inter-individual parameters such as age, gender, race, BMI and other dietary habits or a representative sample from the US population with more study participants. Because there were no previous human studies on raisins and gut microbiota interaction, the effect size could not be determined to calculate an ideal sample size. However, many small scale dietary interventions with fruits have found significant observations in gut microbiota indicating that the sample size used in this study is sufficient to observe a significant change (82, 84, 91).

The number of OTUs significantly affected by raisin intake is greater in week one compared to week two. Raisins seem to exert a stronger effect during week one or it could be that the participants did not fully meet the required raisin intake even though the reported compliance to the study protocol was high. Participants' dislike of continuous consumption of raisins suggests the need for reduced number of servings (three) or feeding frequency (less than daily). Incorporating raisins to other food products such as yogurt or meals such as salads could be suggested to improve compliance.

To provide support to observed changes in microbiota due to raisin intake, future studies could incorporate measures of SCFA and bile acid concentration, fecal weight, Bristol stool measurements and a food log or conduct a metabolomics analysis of fecal microbiota (60, 64). A prospective epidemiologic study could determine potential long term effects of raisins on gut microbiota and health in the general population. Carefully controlled dietary interventions could identify specific contributions of raisins to human health.

Conclusion. Adding sun dried raisins to the diet seems to induce beneficial changes in several bacterial OTUs including the increase of OTUs matching *Faecalibacterium prausnitzii*, and *Bacteroidetes sp.* and the decrease of a bacterial OTU matching enteric pathogen *Klebsiella sp.*

APPENDIX A
SUPPLEMENTARY FIGURES OF RESULTS SECTION

Table A-1. Primer combination for 16S rRNA gene PCR amplification

Primer	Primer sequence
27F Forward Primer	5' AATGATACGGCGACCACCGAGATCTACAC TATGGTAATT CC AGMGTTYGATYMTGGCTCAG 3' (60)
338R Reverse Primer	3' CAAGCAGAAGACGGCATACGAGAT TCCCTTGTCTCC AGTCAGTCAG AA GCTGCCTCCCGTAGGAGT 5' (66)

27F_Forward primer: 5' Illumina adapter, 2, Forward primer pad, 3, Forward primer linker, 4, Forward primer; 338R_Reverse primer: Reverse complement of 3' Illumina adapter, 2, Golay barcode, 3, Reverse primer pad, 4, Reverse primer linker, 5, Reverse primer, *Primers contain a linker sequence that bind to the flow cell. Specific bar-code to identify different samples, adapters provide sticky ends to attach to the flow cell. Length of the PCR product is 250 base pairs.

Table A-2. Primer combination for 16S rRNA gene sequencing on Illumina MiSeq

MiSeq custom sequence primers	Primer sequence
Read 1	5' GAGATCTACAC TATGGTAATT CC AGMGTTYGATYMTGGCTCAG 3' (43)
Read 2	3' AGTCAGTCAG AA GCTGCCTCCCGTAGGAGT 5' (30)
Index	3' ACTCCTACGGGAGGCAGC TT CTGACTGACT 5' (30)

Index sequence allows 96 different samples to be sequenced on one flow cell, thus increasing the throughput. Read 1 and Read 2 allow accurate sequence alignment, detect insertions or deletions and PCR duplicates.

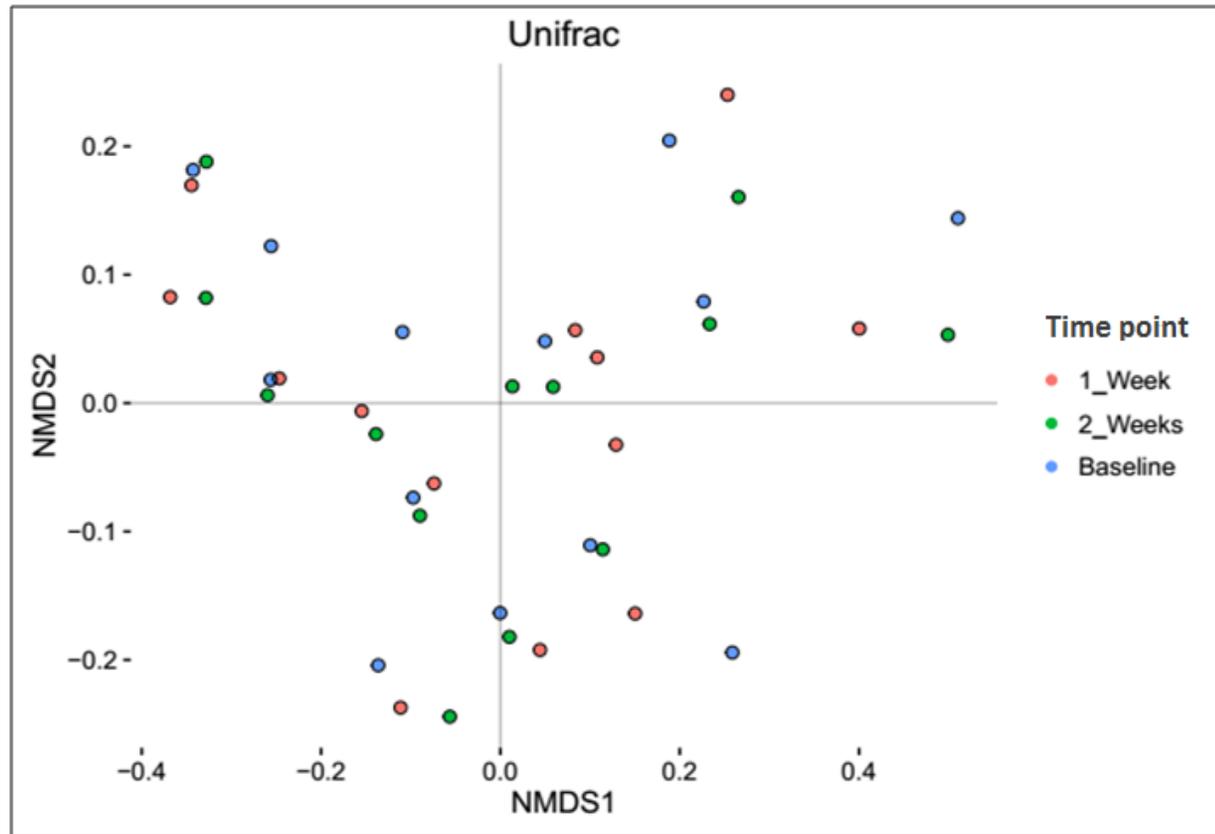


Figure A-1. UniFrac beta diversity analysis by feeding time points. Un-weighted UniFrac analysis based on 16S rDNA MiSeq sequences from stool samples (circles), measured before, during and after the raisin feeding period (baseline/day0, week1/day 7 and week2/day14 respectively) All the samples from each time point were assigned a different color code (Baseline: blue, week 1: red and week 2: green) to visualize the microbial diversity variation at each time point. A single circle represents a single sample from one of thirteen healthy volunteers (total sample size 39).

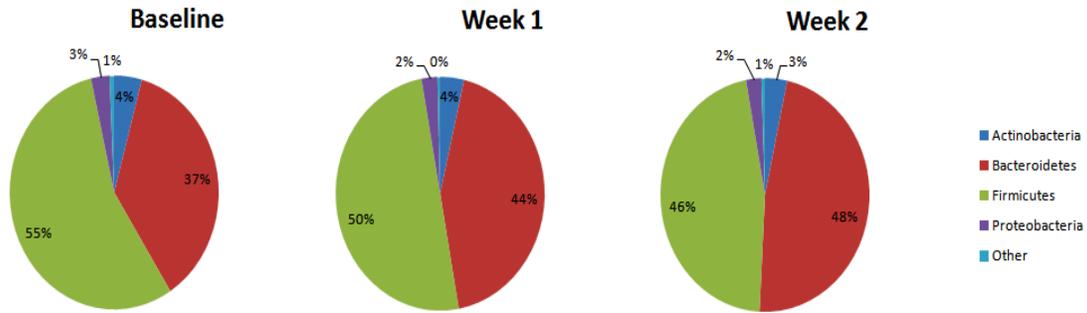


Figure A-2. Changes in the proportion of operational taxonomic subunits grouped into four most abundant bacterial phyla. Based on 16S rDNA similarity, individual sequence reads were matched to the closest reference sequence in greengenes database at 95% similarity level. OTUs were grouped into phylum level at each time point. Stool samples of 13 participants were analyzed at baseline (before raisin consumption), week 1 (during raisin consumption at day 7) and week 2 (after raisin consumption at day 14). Phyla with less than 1% prevalence at all three time points were grouped as other phyla

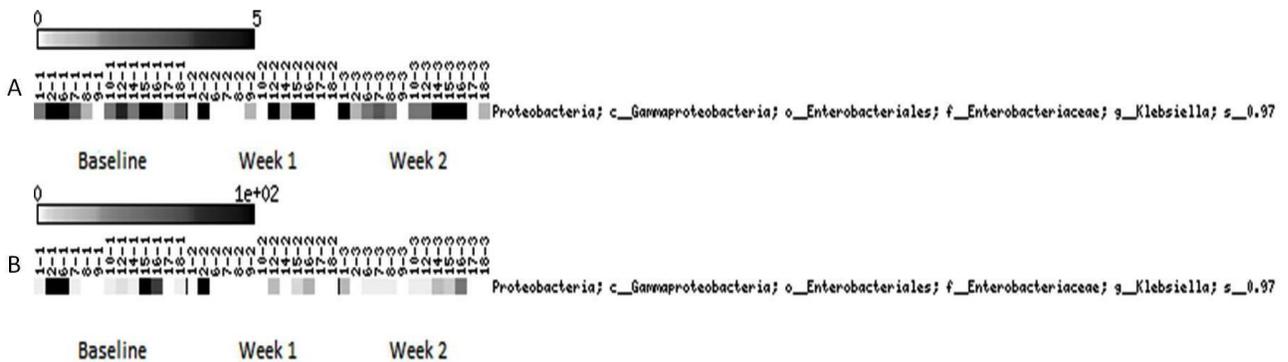


Figure A-3. Heat-map depicting the variations in an OTU closest to *Klebsiella spp.* affected by raisin consumption at 95% similarity level. The OTU matching *Klebsiella spp.* taken from figure 4-8 with an OTU count color separation range of 0 to 5, B: same OTU with a enhanced color separation range of 0 to 100 as indicated in the top left color intensity bar.

APPENDIX B DNA EXTRACTION PROTOCOL

Pre-processing of fecal samples. Fecal samples were collected and stored at -80°C in RNAlater solution. Stock samples were thawed once, homogenized and half a pea (200-300 mg) sized solid stool or 300-500 µL loose stool were measure into separate 2mL micro centrifuge tubes (stored in 4°C or used immediately without storage). Three glass beads were added to the stool sample tubes along with 1 mL 0.05 M phosphate buffer and vortexed until the stool was thoroughly homogenized. Thereafter the samples were centrifuged at maximum speed (Table centrifuge >10.000 rpm) and pellets were saved. Washing of the samples were carried out again using 1 mL 0.05 M phosphate buffer similar to previous step.

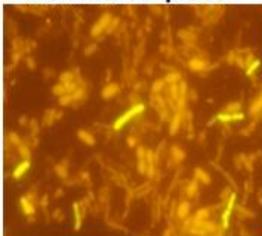
DNA extraction. DNA isolation was conducted using QIAamp stool mini DNA kit (25). Few changes to the original protocol have been made. After heating the fecal sample and ASL buffer suspension, 0.3 g of zirconia beads were added to each tube and bead beating was performed for 3 min. In this protocol after incubation with InhibitEX tablet and centrifugation, 1.2 mL of the supernatant was used for the next step. Volumes of chemical solutions used in this study slightly differ from the original protocol: 25µL Proteinase K, 400µL AL buffer and a 400 µL of 200proof ethanol. After applying the lysate and completing the washing steps as in the original protocol, pre-heated 100 µL of AE buffer (heated at 65°C for 5-10 minute) was transferred to the QIAamp spin column with a new, labeled 1.5 mL micro centrifuge tube. Tubes were incubated at room temperature for 5 minute and then centrifuged at full speed for 1minute to elute DNA.

APPENDIX C

GASTROINTESTINAL HEALTH QUESTIONNAIRE SAMPLE

<p>Weekly Questionnaire (Raisins Study)</p> <p>Subject number _____ Date: _____</p> <p>1. Gastrointestinal Symptoms Response Scale</p> <p>(0) no discomfort at all (4) moderately severe discomfort</p> <p>(1) slight discomfort (5) severe discomfort</p> <p>(2) mild discomfort (6) very severe discomfort</p> <p>(3) moderate discomfort</p> <p>Questions: Answer each question using the response scale above.</p> <p>_____ Have you been bothered by stomach ache or pain during the past week? <i>Stomach ache refers to all kinds of aches or pains in your stomach or belly.</i></p> <p>_____ Have you been bothered by heartburn during the past week? <i>By heartburn we mean a burning pain or discomfort behind the breastbone in your chest.</i></p> <p>_____ Have you been bothered by acid reflux during the past week? <i>By acid reflux we mean regurgitation or flow of sour or bitter fluid into your mouth.</i></p> <p>_____ Have you been bothered by hunger pains in the stomach or belly during the past week? <i>This hollow feeling in the stomach is associated with the need to eat between meals.</i></p> <p>_____ Have you been bothered by nausea during the past week? <i>By nausea we mean a feeling of wanting to vomit.</i></p> <p>_____ Have you been bothered by rumbling in your stomach or belly during the past week? <i>Rumbling refers to vibrations or noises in the stomach.</i></p> <p>_____ Has your stomach felt bloated during the past week? <i>Feeling bloated refers to swelling in the stomach or belly.</i></p> <p>_____ Have you been bothered by burping during the past week? <i>Burping refers to bringing up air or gas through the mouth.</i></p> <p>_____ Have you been bothered by passing gas or flatus during the past week? <i>Passing gas refers to the release of air or gas from the bowels.</i></p> <p>_____ Have you been bothered by constipation during the past week?</p>	<p><i>Constipation refers to a reduced ability to empty the bowels.</i></p> <p>_____ Have you been bothered by diarrhea during the past week? <i>Dianhea refers to frequent loose or watery stools.</i></p> <p>_____ Have you been bothered by loose stools during the past week? <i>If your stools have been alternately hard and loose, this question only refers to the extent you have been bothered by the stools being loose.</i></p> <p>_____ Have you been bothered by hard stools during the past week? <i>If your stools have been alternately hard and loose, this question only refers to the extent you have been bothered by stools being hard.</i></p> <p>_____ Have you been bothered by an urgent need to have a bowel movement during the past week? <i>This urgent need to open your bowels makes you rush to the toilet.</i></p> <p>_____ When going to the toilet during the past week, have you had the feeling of not completely emptying your bowels? <i>The feeling that after finishing a bowel movement, there is still some stool that needs to be passed.</i></p> <p>2. Physical Activity</p> <p>How would you classify your physical activity for the past week (select one response)?</p> <p>_____ Predominantly sedentary – not participating in physical activity on a regular basis (less than once a week)</p> <p>_____ Occasionally active – participating in physical activity on an intermittent basis (once or twice a week)</p> <p>_____ Moderately active – accumulating 30 minutes of moderate-intensity physical activity (equivalent to brisk walking, recreational swimming, or bicycling on some hills) on most if not all days of the week</p> <p>_____ Vigorously active – participating in vigorous physical activity (running, swimming laps, bicycling up steep terrain) 3 to 5 days a week</p>
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APPENDIX D
PARTICIPANT RECRUITMENT POSTER AND SAMPLE COLLECTION FORM

<p>Study ID: IRB201500607 Date Approved: 10/27/2015 Expiration Date: 10/22/2018</p> <p style="text-align: right;"></p> <p style="text-align: center;">Attention: Study Volunteers Needed!</p> <p style="text-align: center;">"Effects of adding raisins to the American diet on fecal microbiota composition"</p> <p style="text-align: center;"></p> <p style="text-align: center;">This study will investigate the effects of adding raisins to your diet on bacteria in the gut and associated markers of health.</p> <p style="text-align: center;">Eligible subjects will be asked to provide information on dietary habits and medical history. Subjects will add raisins to their diet and collect stool samples. Subjects will be compensated for their efforts.</p> <p style="text-align: center;">If interested, please contact Dr. Volker Mai at vmai@ufl.edu or (352) 273-9398. Please include your name and also your phone number in your message and you will be contacted.</p>	<p style="text-align: center;">Raisins Study</p> <p style="text-align: center;">Stool Collection Instructions</p> <ol style="list-style-type: none">1. Please provide first bowel movement of the day on your specified collection days, if possible.2. If possible, please notify one of the contacts below prior to providing a stool sample so that we may plan for collection.3. Please drop off stool sample immediately after collection, before 2pm. If you have no transportation, please contact us to pick up the stool sample collection. Please contact Sheldon Waugh (239-404-8668), or Dr. Maria Ukhanova (352-273-9401).4. Please place stool sample in provided bag along with the provided ice pack until the sample is in our possession. <p>Please provide your first stool sample of the day on one of the days during the date periods indicated below (Note: the below time periods include weekdays only. Please notify us if it is not possible to provide sample on one of the specified dates.):</p> <p>Collection Date 1: _____</p> <p>Start ingesting Raisins: _____</p> <p>Collection Date 2: _____</p> <p>Collection Date 3: _____</p>
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BIOGRAPHICAL SKETCH

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