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Does Gut Flora Change in a Mouse Model of Autism Spectrum Disorders on a Ketogenic Diet?

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BY

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Abstract

The normal bacterial flora of an organism includes the non-disease causing bacteria that inhabit the human body under normal conditions. These bacteria are important for numerous reasons; for example, they excrete vitamins and prevent colonization by pathogens. Autism spectrum disorders (ASDs) are neurodevelopmental disorders that are characterized by social challenges, repetitive behaviors, and communication deficits. Comorbid conditions including gastrointestinal (GI) issues, depression, and anxiety are common. One popular way to attempt to alleviate the behavioral symptoms of ASDs is maintaining a ketogenic diet, which is seventy-five percent fat. Such a diet induces ketosis, a metabolic state when ketone bodies, not glucose, are used as the primary fuel. This study analyzes how the ketogenic diet affects the GI flora in a mouse model of ASDs and aims to determine if the benefits of a ketogenic diet are correlated with changes in the gut flora. In order to determine the types of bacteria present, the 16s rRNA gene was amplified from the fecal samples of mice in treatment groups. The amplified DNA was then digested with a restriction enzyme (*Hae*III) and Terminal Restriction Fragment Length Polymorphism (TRFLP) electropherograms were generated. Analyses of the electropherograms suggest there is no significant difference in number of species present or abundance of bacteria between any pre and post diet conditions, except for the normal mouse on a ketogenic diet. Additionally, there are 28 unidentified bacterial species that are common between two or more of the four experimental groups.

Introduction

Autism spectrum disorders (ASDs) are neurodevelopmental disorders that affect about 1

in 68 American children (Autism Spectrum Disorders, n.d.). They are defined by three main

symptoms: social challenges, repetitive behaviors, and communication deficits. For example, a

person on the autism spectrum could have limited verbal skills or have trouble maintaining eye

contact (Autism Spectrum Disorders, n.d.). People on the autism spectrum have a wide range of

cognitive impairments and varying levels of disability, causing treatments for ASDs to be unique

for each individual. They can include behavioral therapy and/or medications targeted to improve

a variety of "comorbid conditions," such as anxiety, depression, seizures, and gastrointestinal

(GI) issues ("How Is Autism Treated," n.d.). There seems to be a link between ASDs and the

ketogenic diet, and between ASDs and gut microbiota. This study investigates if there are

differences in the gut microbiome of a mouse model of ASDs on a normal diet compared to a

mouse model of ASDs on a ketogenic diet, and compares them to normal mice on a normal diet

and normal mice on a ketogenic diet.

In people with ASDs, GI disorders are among the most common, including chronic

diarrhea, constipation, and irritable bowel conditions. The Center for Disease Control (CDC)

found that children with ASDs are 3.5 times more likely to develop GI issues than their normally

developing peers (Schieve et al., 2012). The increased discomfort and pain from GI problems

can exacerbate other behavioral ASD symptoms (Autism and GI Disorders, n.d.). According to

surveys, as many as 40 percent of children with autism have been placed on special diets at one

point in their life in hopes of alleviating their behavioral symptoms. The ketogenic diet, in which

seventy-five percent of calories come from fat, induces ketosis, a metabolic state when ketone

bodies, not glucose, are used as the primary fuel. Such a diet is a popular choice for ASDs and has recorded benefits for treatment of epilepsy (Alpert, 2007).

For generations, an association between metabolism and brain function has been evident. Even in ancient times, epileptic seizures were treated by fasting. The ketogenic diet serves as an effective treatment for epilepsy because it mimics the conditions of starvation, without the lack of all nutrients, merely the lack of carbohydrates. Instead of getting fuel from carbohydrates like glucose, the body receives it from ketone bodies (Wilder, 1921). During limited carbohydrate intake, such as fasting or the ketogenic diet, the liver increases production of the ketone bodies, acetoacetate, acetone, and β-hydroxybutyrate, that get released to generate adenosine triphosphate (ATP) throughout the body (Aoki, 1981).

Studies have found that in humans, the ketogenic diet is correlated to the functions of the central nervous system (CNS). It can decrease anxiety and even improve symptoms of ASDs (Brinkworth et al., 2009). One pilot study evaluated children with ASDs who maintained a ketogenic diet by using the Childhood Autism Rating Scale. Significant improvement was reported for 11% of children, average improvement for 44%, and mild improvement for 44%. Interestingly, the most improvements were seen in children exhibiting mild autistic behavior (Evangeliou et al., 2003).

Many studies use the inbred BTBR T+ tf/j (BTBR) strain of mice as a model for ASDs because they have been characterized as having an autism-like phenotype. BTBR mice share the same core traits as ASDs, and even some minor traits, such as selective food preference (McFarlane et al., 2007). BTBR mice are widely accepted as a mouse model of ASDs and were used in a study by Ruskin et al. (2015) which confirmed that the BTBR mice have low sociability, increased repetitive behavior, and reduced communication compared to other strains

exhibiting normal behaviors. The study analyzed how these symptoms were effected by a ketogenic diet, and the results showed the ketogenic diet improved the behavioral symptoms significantly. This study by Ruskin et al. (2015) was groundbreaking because it was the first time a diet showed significant behavioral benefits in a mouse model of ASDs.

Modifying diet can affect neurological disorders, whether ASDs or epilepsy, due to the gut-brain axis (GBA), a term commonly used to describe the bidirectional communication system that exists between the CNS and the gut (Grenham et al., 2011). One study by Sudo et al. (2004) used germ-free (GF) mice to examine how the intestinal microbiome influences the CNS, showing that colonization of the gut by normal flora must occur to ensure normal development of the hypothalamic-pituitary-adrenal (HPA) axis, which is responsible for regulating many processes, ranging from digestion to stress. The same study revealed a decrease in brain derived neurotrophic factor (BDNF), a neurotrophin related to neuronal growth and survival, in mice whose normal flora were never established fully (Sudo et al., 2004).

Some studies have examined the opposing direction of influence- how the brain impacts the gut microbiome. Kiliaan et al. (1998) confirmed that stress induces permeability of the gut, allowing certain bacteria to enter, in turn activating an immune response which then changes the composition of the intestinal microbiome. Another study on rhesus monkeys looked at maternal separation, an early life stressor. The fecal bacteria were analyzed to measure gut microbiota, and by day 3 post separation there was a significant decrease in *Lactobacilli* (Bailey and Coe, 1999). A study by O'Mahony et al. (2009) analyzed the long term effects of maternal separation in rats and found a significantly altered gut microbiome when compared to the non-separated control group.

Numerous mechanisms of action have been proposed to explain how the GBA works, including both neural and humoral routes. One study identified the vagus nerve, a major part of the parasympathetic nervous system, which unconsciously controls the digestive tract, as the key communication pathway between the gut and the CNS (Bravo et al., 2011). Bravo et al. (2011) found that mice whose vagus nerve was surgically cut did not show the same neurochemical or behavioral effects as control mice when subjected to a variety of stressors. Other mechanisms could be related to modulation of systemic tryptophan levels (Grenham et al., 2011).

Many studies examine the bacterial flora of the gut to learn about the CNS and the GBA. The normal bacterial flora of an organism includes the non-disease causing bacteria that inhabit the human body under normal conditions. These bacteria are important for numerous reasons, including metabolic and protective functions. *Escherichia coli* excrete metabolites such as vitamin K, which is necessary for blood clotting, but not made by the human body (Davis, 1996). The normal flora also prevents colonization by pathogens by occupying space in the gastrointestinal tract, making it unavailable to disease causing pathogens (Davis, 1996). Development of the innate immune system, which helps initially distinguish potential pathogens from harmless microbes, is influenced by ligands of the normal flora, including lipopolysaccharide and lipoteichoic acid (Grenham et al., 2011).

The gut is inhabited by about 10^{14} microorganisms, which is more than 10 times the number of human cells in our entire body (Davis, 1996). During the postnatal period, after leaving the sterile uterine environment, facultative bacteria begin colonization of the gut. Next, the gram positive bacteria colonize via breast feeding and then gram negative bacteria alter the flora when the baby expands types of foods in its diet (Davis, 1996). Eventually, the normal

balance of flora is established, creating a stable, adult-like signature, which can be altered by infection, disease, diet, and antibiotics (Grenham et al., 2011).

Bacterial flora that is normal in one area of the body can be pathogenic in a different location (Davis, 1996). For example, species of *Streptococcus* can be commensal in an oral habitat, but become pathogenic in artificial joints after arriving via the bloodstream. Similarly, during birth through the vaginal canal, neonates can uptake *Streptococcus* that can travel to the respiratory tract and result in bacterial meningitis.

Knowledge of the normal flora increased substantially when germ-free (GF) animals became available for studies. GF animals are obtained by surgical delivery and then raised in a sterile environment, which hinders the acquisition of normal flora (Davis, 1996). These GF animals serve as comparison for animals that are exposed to normal flora during their lifetime. Studies using GF animals helped bridge the gap in the knowledge of the intestinal microbiome, eventually enabling scientists to determine that the gut contains a high abundance of the phyla *Bacteroidetes* and *Firmicutes,* and a lower abundance of *Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia* (Eckburg et al., 2005). Also, following meals, the gut flora is highest, while after digestion, it is frequently undetectable (Davis, 1996).

Many diseases have shown an association with the intestinal microbiome. Often, instances of stress and disease are bidirectionally linked to abnormal gut function and flora, while a healthy status is related to normal gut physiology and flora (Grenham et al., 2011). A study conducted by Backhed et al. (2007) analyzed a potential microbial basis for obesity, in addition to the many other factors contributing to the disease. Researchers took GF mice and colonized their GI tract with fecal flora. The resulting weight gain supported evidence of the

connection between the microbiome and disease, which can be applied to other diseases where the gut flora is known to be abnormal.

Not only have patients with ASDs shown increased GI problems, they have exhibited differences in the gut microbiota. Increased amounts of *Clostridium* were seen in the guts of patients with ASDs (Song et al., 2004). One study found that due to the presence of increased toxin producing bacteria, ASDs could worsen (Parracho et al., 2005). The same study by Parracho et al. (2005) observed *Sutterella* and *Clostridium bolteae* in ASD patients with GI issues, but not in control populations with similar GI problems. Another study discovered lower levels of *Bifidobacterium* and higher levels of *Lactobacillus* in children with ASDs (Finegold et al., 2010). Also, in patients with ASDs, increased gut permeability has been correlated with progression of the disease most likely due to an escape of microbes and cytokines into the blood stream (Turner, 2009).

Sandler et al. (2000) found that the antibiotic vancomycin was effective at normalizing the intestinal microbiome and improving symptoms. The study did not recommend their protocol as a treatment for ASDs, but it did support a relationship between the intestinal microbiota and CNS. Martin et al. (2008) saw a significant increase in *Bifidobacterium* and *Bacteroides distasonis* and a significant decrease in *E. coli* and *Clostridium perfringens* when patients were provided prebiotics. Antibiotics, prebiotics, and probiotics are promising potential treatments for gut microbiome related diseases, however more research needs to be completed (Grenham et al., 2011). Fecal transplants are another potential treatment option because they help re-establish the normal gut flora. They have been successful in laboratory animals and in certain human cases, but still need more research to be fully understood (Petrof and Khoruts, 2014).

Previous studies have presented two major concepts: that the ketogenic diet improves ASD symptoms via the GBA, and that ASDs are associated with changes in the gut microbiome that affect disease progression via the GBA. This exploratory study combines these two ideas in order to examine the effects that the ketogenic diet has on the gut microbiota in BTBR mice. More specifically, this study aims to determine if there is a difference in the number of bacterial species present or the overall abundance of bacteria in the gut on and off a ketogenic diet. This study also explores the possibility of improved ASD symptoms being attributed to changes in gut microbiota. Additionally, the bacterial population similarities between treatment groups (Normal Mouse, BTBR Mouse, Normal Diet, and Ketogenic Diet) are analyzed to identify potentially significant species that should be investigated further via DNA sequencing.

After the most of the data had been collected for this study, Klein et al. published a paper exploring how the ketogenic diet affects the gut microbiome in BTBR mice (2016). They kept the mice on a normal diet or placed them on a ketogenic diet at 5 weeks of age. Two weeks later, feces were collected and the mice were sacrificed. DNA was extracted from the samples, quantitative PCR was conducted to amplify the 16s rRNA gene, and microbial profiling was completed to identify the bacterial species present and their abundances. Klein et al. found that the ketogenic diet significantly reduced total microbe numbers and that diet, rather than mouse type, was the main driver of microbial changes. Because this study was published, the results of our study can be compared directly to the study by Klein et al.

Our experimental parameters relied on previously successful and widely accepted techniques for exploring individual bacterial species in a population. The 16s rRNA gene is highly conserved in different bacterial species, but has a number of hyper-variable regions that can enable identification of specific species via sequencing (Kolbert and Persing, 1999). In this

study, DNA was isolated from fecal samples, the 16s rRNA gene was amplified using PCR, and Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis was conducted to determine the flora present in the intestinal microbiota. TRFLP analysis has been successfully used to identify flora extracted from feces, further supporting the experimental design of this study (Hayashi et al., 2005).
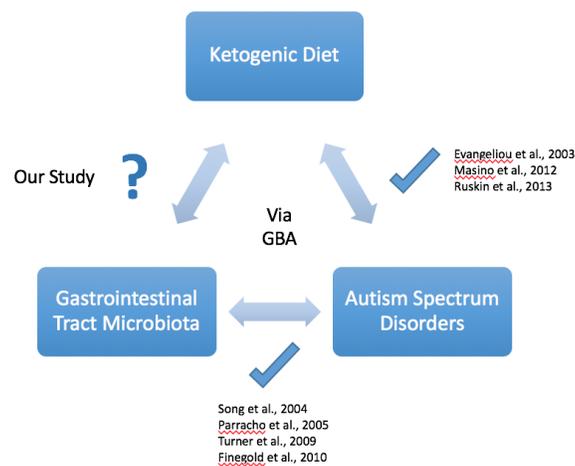

Figure 1. Pictorial representation of the study goals

Materials and Methods

Obtaining Fecal Samples

Fecal samples were obtained from 5-week old normal mice on a normal diet, normal mice on a ketogenic diet, BTBR mice on a normal diet, and BTBR mice on a ketogenic diet (collected from Dr. Masino's lab, n = 6, 9, 16, 13 respectively). When mice were removed from the diet at 8 weeks of age, another fecal sample was taken (n = 6, 8, 16, 12). All samples were coded to prevent bias and stored in the -80°C freezer until DNA was isolated.

DNA Isolation

DNA was isolated from 88 samples using the MO BIO PowerFecal® DNA Isolation Kit. The Trinity College Biology Department's NanoDrop was then used to quantify the amount of DNA.

Polymerase Chain Reaction (PCR) with labeled primers

To amplify the 16s rRNA gene, PCR was performed on each fecal sample. Two fluorescently labeled (FAM, HEX) universal primers that have been identified to amplify the 16s rRNA gene across bacterial species were used (Kuramae et al., 2010; Baker et al., 2003). The forward primer was 27F-FAM [5'-(6FAM)AGAGTTTGATCCTGGCTCAG-3'] and the reverse primer was 926R-HEX [5'-(HEX)CCGTCAATTCMTTTRAGTTT-3']. Samples were placed in the thermocycler with settings for 95°C initial denaturation, 50°C primer annealing, and 72°C extension for 35 cycles. The results were checked on a 0.8% agarose gel.

Restriction Digests

All 88 amplified samples were digested with the restriction enzyme *Hae*III at 37°C for two to three hours. A 1.2% agarose gel was used to confirm digestion of the samples.

Purification

Restriction digest fragments were purified based on the procedure outlined in the QIAquick® Spin Handbook titled "QIAquick PCR Purification Kit Protocol Using a Microcentrifuge." A 1.2% agarose gel was run to check purification.

Terminal Restriction Fragment Length Polymorphism (TRFLP) Analysis

Purified fragments from each restriction digest were diluted using highly deionized formaldehyde (HDF) to two concentrations. The high concentration was 7 µl sample to 3 µl of HDF and the low concentration was 5 µl sample to 5 µl HDF. A plate of samples was sent to

Yale University where each sample underwent fragment analysis. GeneMarker® was used to

analyze the electropherograms and extract peak heights to determine overall abundance of

bacteria, number of peaks per sample to determine the number of species, and peak sizes.

Determination of Common, Unidentified Species

Sample peak sizes were compared to determine which species were common between

two or more of the experimental groups.

Statistical Analyses

T-tests were conducted to compare the means of the samples from 5 week old mice and

the samples from 8 week old mice. More specifically, P-values calculated in Microsoft Excel

determined the statistical significance (95% confidence) between the number of species present

at 5 weeks of age and 8 weeks of age, and between the overall abundance of bacteria at 5 weeks

of age and 8 weeks of age. Two-way Analysis of Variance (ANOVA) was used to compare the

means of the overall abundance of bacteria in the four experimental conditions in the samples

from 8 week old mice.

Results

Similarities Between the Four Experimental Groups

There were twenty-eight unidentified bacterial species (A-BB) that were common

between two or more of the experimental groups, based on the comparison of sample peak sizes

(Table 1). There were ten bacterial species found in all four of the sample groups; nine species

found in only the normal mouse and normal diet groups; five species found in the BTBR mouse

sample and both diet samples; one species found in the BTBR mouse strain and the ketogenic

diet samples; one species found in both the normal and BTBR mouse strain and the normal diet;

and one species found in the BTBR strain and normal diet sample groups (Figure 1).

Table 1. Associated peak size ranges for the 28 unidentified bacterial species (A-BB) that were

common between two or more of the experimental groups.

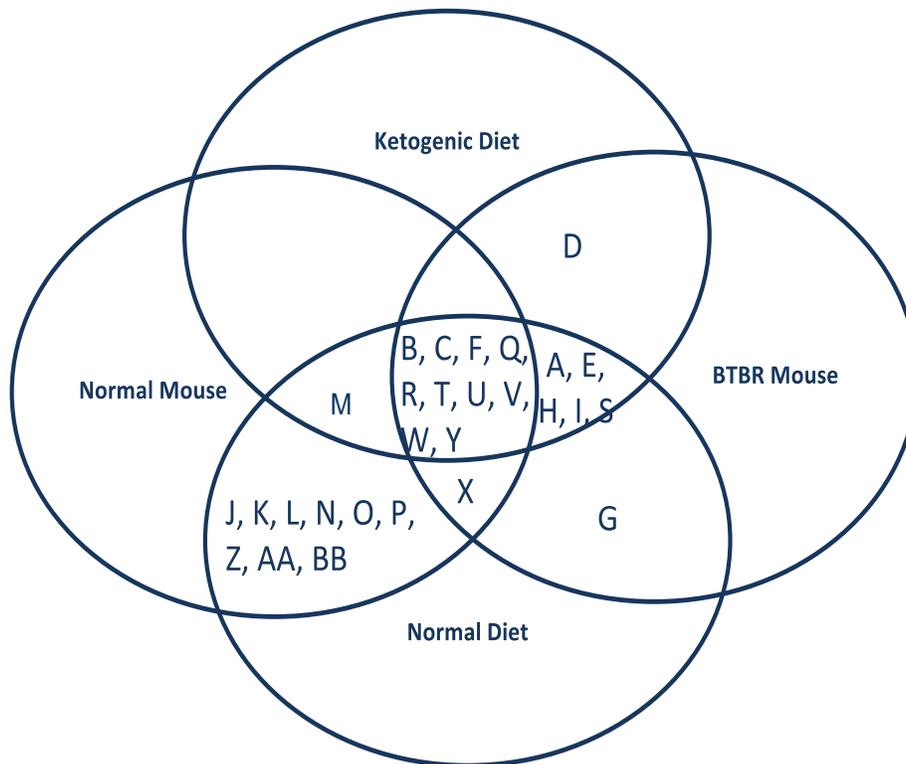| Species	Peak Size	Species	Peak Size
A	81.80 ± 0.90	O	248.95 ± 0.35
B	177.75 ± 0.45	P	253.15 ± 1.25
C	203.15 ± 0.65	Q	251.50 ± 0.30
D	204.30 ± 0.50	R	255.15 ± 0.55
E	209.20 ± 0.80	S	258.50 ± 0.70
F	211.45 ± 0.85	T	260.50 ± 0.90
G	213.60 ± 0.30	U	263.60 ± 1.10
H	215.60 ± 0.30	V	262.80 ± 0.70
I	217.80 ± 0.30	W	265.25 ± 0.75
J	220.65 ± 0.85	X	265.65 ± 0.25
K	225.15 ± 0.65	Y	272.35 ± 0.55
L	226.80 ± 1.00	Z	283.65 ± 0.55
M	237.40 ± 0.30	AA	287.95 ± 0.75
N	245.70 ± 0.20	BB	304.15 ± 0.35

Figure 1. Pictorial representation of the 28 unidentified bacterial species (A-BB) and their

respective experimental groups. Each species is distinguished by a unique peak size.

Number of Bacterial Species Present

A significant increase was seen between the number of bacterial species found in a normal mouse before and after a ketogenic diet (P=0.003; Figure 2). No significant difference was seen between the number of bacterial species pre and post diet in the normal mouse on a normal diet (P=0.224), BTBR mouse on a normal diet (P=0.199), and BTBR mouse on a ketogenic diet (P=0.113; Figure 2).
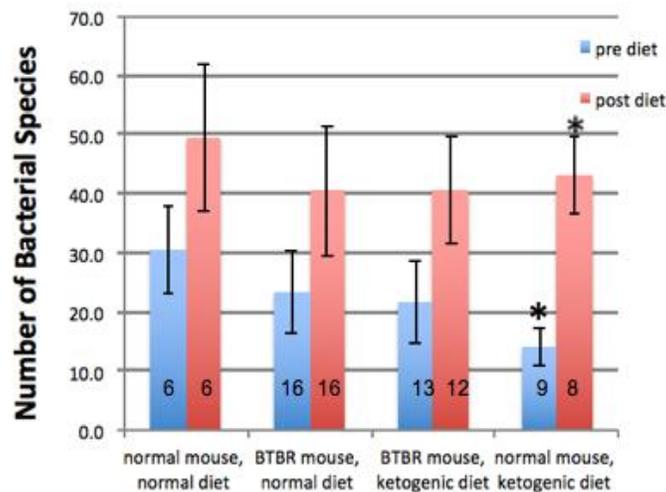

Figure 2. Number of bacterial species present in the four experimental groups (normal mouse on a normal diet, BTBR mouse on a normal diet, BTBR mouse on a ketogenic diet, and normal mouse on a ketogenic diet) before and after a ketogenic or normal diet. Blue bars represent samples taken before mice were put on a ketogenic diet or kept on a normal diet. Red bars represent samples taken three weeks later. Respective n values are shown on each bar. Error bars represent standard error of the mean.

Overall Abundance of Bacteria

A significant increase was seen between the overall abundance of bacteria found in the normal mouse before and after a ketogenic diet (P=0.003; Figure 3). No significant difference was seen between the overall abundance of bacteria pre and post diet in the normal mouse on a

normal diet (P=0.334), BTBR mouse on a normal diet (P=0.632), and BTBR mouse on a
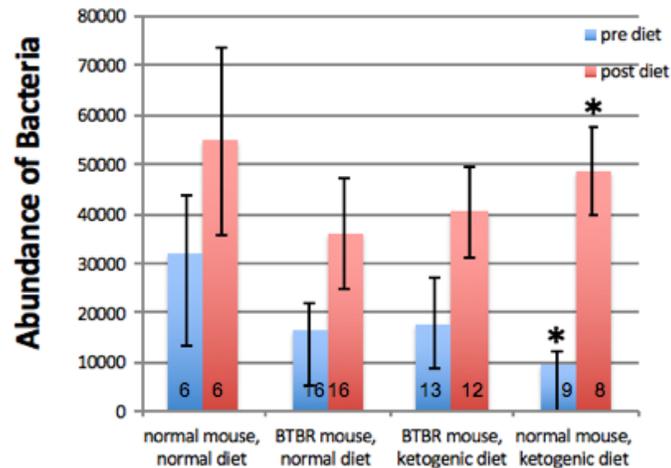
ketogenic diet (P=0.097; Figure 3).

Figure 3. Overall abundance of bacteria present in the four experimental groups (normal mouse

on a normal diet, BTBR mouse on a normal diet, BTBR mouse on a ketogenic diet, and normal

mouse on a ketogenic diet) before and after a ketogenic or normal diet. Blue bars represent

samples taken before mice were put on a ketogenic diet or kept on a normal diet. Red bars

represent samples taken three weeks later. Respective n values are shown on each bar. Error bars

represent standard error of the mean.

Two-Way ANOVA for Comparison to Klein et al. 2016

The two-way ANOVA showed no significant difference between the ketogenic diet and

the normal diet (P=0.8243) and between the normal mouse and the BTBR mouse (P=0.3031). No

significant relationship was seen between diet and mouse type, either (P=0.7104).

Discussion

Similarities Between the Four Experimental Groups

There were twenty-eight unidentified bacterial species (A-BB) that were common

between two or more of the experimental groups, based on the comparison of sample peak sizes

(Table 1; Figure 1). By looking at which unidentified species are present in which groups, species that are potentially significant for diagnosis or treatment can be identified.

For example, species M, A, E, H, I, and S are all potential biomarkers of ASDs. Species M is only found in the normal mouse, regardless of diet. Its absence could be a biomarker of ASDs because species M is not found in the BTBR mouse. Conversely, species A, E, H, I, and S can also be biomarkers of ASDs because they are only present in the BTBR mouse.

Species D, G, and X provide insight about potential therapies of ASDs. Species D is only present in the BTBR mouse on the ketogenic diet. The presence of species D could help explain the improvements in behavioral symptoms seen in mouse models of ASDs on the ketogenic diet (Ruskin et al., 2015). It is possible that supplementing species D into a normal diet via a probiotic could improve the behavioral symptoms of ASDs. Both species G and species X are eliminated by the ketogenic diet. Species G is only found in the BTBR mouse on the normal diet. Species X is present in both mouse types on the normal diet, but not on the ketogenic diet. Species G might have harmful effects on the BTBR mouse because improvements in behavioral symptoms are seen in the mice lacking species G, the BTBR mice on the ketogenic diet. Eliminating these species could be another target for ASDs treatment. Future studies should sequence the significant, unidentified species in order to identify and learn more about them.

Number of Bacterial Species Present and Overall Abundance of Bacteria

No significant difference was seen between the number of bacterial species present pre and post diet in the normal mouse on a normal diet (P=0.224), BTBR mouse on a normal diet (P=0.199), and BTBR mouse on a ketogenic diet (P=0.113; Figure 2). No significant difference was seen between the overall abundance of bacteria pre and post diet in the normal mouse on a normal diet (P=0.334), BTBR mouse on a normal diet (P=0.632), and BTBR mouse on a

ketogenic diet (P=0.097; Figure 3). However, a general trend of increase is seen when comparing the before diet data to the after diet data, which can be attributed to the establishment and increase of normal flora as the mice age.

Both the significant differences seen between the number of bacterial species found and between the overall abundance of bacteria found in a normal mouse on a ketogenic diet pre and post diet are questionable. The number of bacterial species found in a normal mouse that has not yet been put on a normal diet is expected to be similar to the the number of bacterial species found in a normal mouse that has not yet been put on a ketogenic diet. Similarly, the overall abundance of bacteria found in a normal mouse that has not yet been put on a normal diet is expected to be similar to the overall abundance of bacteria found in a normal mouse that has not yet been put on a ketogenic diet. Up to that point, both groups had the same treatment, therefore, it is expected they would have similar numbers of bacterial species present and similar overall abundances of bacteria (Figure 2, Figure 3).

It is plausible that if the normal mouse on a ketogenic diet had more species present (like the normal mouse on a normal diet) that there would no longer be a statistically significant difference between the pre and post diet groups. It is also possible that if the normal mouse on a normal diet had fewer species present (like the normal mouse on the ketogenic diet) that there would be a significant difference between the pre and post diet groups for the normal mouse on the ketogenic diet. If both normal mouse groups had a significant difference between pre and post diet conditions, one conclusion could be that the normal mouse might not have established their baseline level of the number of bacterial species in their guts at the pre diet stage. This same concept can be applied to the overall abundance of bacteria results, as well.

Future studies should increase the sample sizes to validate the results from this study. Additionally, other studies could put the mice on either the ketogenic diet or normal diet when the mice are older, in order to control for the possibility that normal mice might not have established their baseline level of the number of bacterial species and/or overall abundance of bacteria in their guts at the pre diet stage.

Comparison to Klein et al. 2016

In our study, a two-way ANOVA was used to obtain information on the overall abundance of bacteria data that could be compared to the study by Klein et al. (2016). This two-way ANOVA showed no significant difference between the ketogenic diet and the normal diet (P=0.8243) and between the normal mouse and the BTBR mouse (P=0.3031). No significant interaction was seen between diet and mouse type, either (P=0.7104). However, Klein et al. (2016) found that the ketogenic diet significantly reduced total microbe numbers and that diet, rather than mouse type, was the main driver of microbial changes.

Despite having similar experimental set ups, our study and Klein et al.'s study had different results. Our study had slightly larger sample sizes, our mice were on the diet for three weeks, and we used qualitative PCR. Klein et al.'s study had their mice on the diet for two weeks and used quantitative PCR. However, none of these differences can fully explain the discrepancies in the results. The major difference between the studies and one possible explanation for the differences is that we used TRFLP analysis and Klein et al. used microbial profiling, which is more sensitive. Microbial profiling uses next-generation sequencing and compares its information to a known database, while TRFLP merely identifies a peaks from unknown bacteria. Future studies should repeat this experiment using the more accurate method of microbial profiling in order to validate the results seen in our study and Klein et al.'s.

Conclusion

There were twenty-eight unidentified bacterial species (A-BB) found to be common between two or more experimental groups. Also, there was a significant difference (P=0.003) in the number of bacterial species and in the overall abundance of bacteria in the normal mouse before and after a ketogenic diet. Lastly, Klein et al. (2016) found diet to be the driver of microbial changes, while our study could not identify a source of microbial changes.

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