THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

MECHANISMS UNDERLYING THE EFFECTS OF EDIBLE MUSHROOMS ON GASTROINTESTINAL HEALTH

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A thesis
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ABSTRACT

White button (WB) mushrooms (*Agaricus bisporus*) have been shown to promote gastrointestinal health and protect the gastrointestinal tract from infection and injury. The protection provided by mushrooms is thought to be linked to the microbiota. The goal of this project was to look into the mechanism underlying this effect and to characterize the effect of mushrooms on the gut microbiota. One group of mice (n=7) was fed WB diet (1%) every day for 2 weeks. The control group (n=7) was fed the control (CTRL) diet.

^{1}H NMR analysis of the cecal contents showed higher levels of the metabolite succinate. When the same experiment was run in germ-free (GF) mice (mice that are free from microorganisms), succinate was not found in the cecum. Furthermore, microbial DNA was extracted from cecal contents and analyzed via qPCR, showing increased levels of the succinate-producing bacteria *Prevotella* in WB treated mice. This suggests that the succinate found in conventional (CV), WB-fed mice is a microbial product. Increased levels of succinate have been linked to improved glucose homeostasis via intestinal gluconeogenesis (IGN). The results from our experiment confirmed this, there were significantly higher levels of mRNA encoding for the IGN-related enzymes Glut2 and G6Pase in the jejunum of WB-fed mice. In addition to this, expression of two genes involved in energy homeostasis, *Sglt3* and *Ffar3*, was also found to be significantly elevated in WB-fed mice.

A potential mechanism of WB mushroom protection is through an increase in succinate-producing bacteria. This could contribute to improved glucose homeostasis through IGN, which is regulated via receptors like FFAR3 and SGLT3, which provide a connection between the gut and the nervous system.

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Stephanie Bora, Yang-Din Lin, and Lindsay Snyder have taught me how to become a better scientist (and a better baker). Shout out to my fellow undergrads for always being ready to commiserate with me: Elaine Santiago, Takara Richards, Val Pascoe, and Kevin Diehl. Additionally, I want to thank the members of the Patterson Lab for being welcoming of and patient with me. Thank you to Dr. Ming Tien, my honors adviser, and Dr. Andrew Patterson for their help as I wrote my thesis. Thanks also goes to the Office of Undergraduate Education for the Erickson Discovery Grant I received last summer to fund my research.

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Introduction

Humans are hosts to a plethora of microbes—on our skin, in our mouths, and perhaps most crucially, in our gastrointestinal tracts. In recent years, scientists have found that the gut microbiota has significant effects on human health, with some researchers even going so far as to call it an organ (1). The microbiota has roles in both immune function and metabolism and assists the body in fighting off pathogens (1). A lack of diversity in the microbial populations of the gut has been linked to higher incidence of obesity, cardiovascular disease, certain cancers (2), and type 2 diabetes (3).

The mechanism of the beneficial effects of a diverse microbiota has not been fully elucidated, especially since the gut microbiota is affected by things like geographical location, stress, age, and diet (2). One major way that the microbiota probably affects overall host health is through the production of short chain fatty acids, like butyrate, acetate, and propionate through microbial fermentation of dietary fiber. These short chain fatty acids are especially relevant in metabolism and energy homeostasis of the host (4).

Diet is the most controllable factor in gut microbiota composition, and it has been shown that dietetic supplementation can diversify gut flora populations (2). As a consequence, food and drug industries have been capitalizing on this by touting certain foods or supplements as "probiotic," claiming that they can help consumers maintain a healthy gut flora.

It can be difficult to distinguish which foods truly promote gastrointestinal health and which do not. However, the research that has been done on mushrooms indicates that they probably do have health benefits. There are many kinds of mushrooms and mushroom-related

treatments that are being studied. For example, there are extracts of medicinal mushrooms like *Agaricus blazei* (5) and *Lactarius deterrimus* (6) that have been linked to weight loss and diabetes control.

However, the reach of medicinal mushrooms is not as wide as that of edible mushrooms. According to the U.S. Department of Agriculture, in the United States, about 90% of mushrooms sold are *Agaricus bisporus*—this includes white button, crimini, and portabello mushrooms (7). As such, this is probably the most useful species of mushrooms to research. We chose to look specifically at white button (WB) mushrooms.

In the past, researchers from the Cantorna lab have found that adding WBM to the diets of mice has protective effects on mice subjected to gastrointestinal injury (8). The WB fed mice lost less weight and showed less colonic injury than their non-mushroom-fed counterparts (8,9). Others have shown that dietary supplementation of WB mushrooms assists in weight loss as a substitute for red meat in humans (10), boost the immune response to viral infection in mice (11), and promote antioxidant- and anti-inflammatory-related health benefits in adults predisposed to type 2 diabetes (12).

How exactly mushrooms provide health benefits is not known; however, it is suspected that it may be linked to the gut microbiota. WB mushroom feeding altered the microbial composition in the gut. Mice who consumed WB mushrooms had increased diversity of the gut microbiota, and also had lower levels of potentially pathogenic bacteria in the gut (9).

Materials and Methods

(Adapted from source 13)

Mice

For the conventional (CV) mice experiment, C57BL/6 wild type (WT) mice were used. They were originally from Jackson Laboratories (Bar Harbor, MN) and bred at the Pennsylvania State University (University Park, PA). The germ-free (GF) mice were from the Pennsylvania State University Gnotobiotic Facility. Animal experiments were performed using protocols approved by the Pennsylvania State University IACUC. Mice were male and 6 weeks old at the start of the experiments. CV mice were housed in the same animal room at the Pennsylvania State University. GF status was monitored continuously and confirmed for the mice at sacrifice.

Diets

The mice were all fed pellet diets from Teklad Diets (Madison, WI). The control diet was TD 89124, and the mushroom diet was the control (CTRL) diet with added *Agaricus bisporous* (WB) mushroom powder, at 1g/100g (1%). The mushrooms were a gift from Giorgio Foods (Temple, PA). The mushrooms were freeze-dried and ground into a fine powder at Penn State and sent to Teklad Diets (Madison, WI) to be incorporated into the control diet. Mice were fed the weight equivalent of 1 human serving of whole WB mushrooms which equals 75-100g fresh WB weight in a human diet (14). The WB and CTRL diets were sterilized through irradiation so that the same diets could be feed to GF and CV mice. The mice were randomly divided into two

groups. Half of the mice were fed CTRL diets and the other half of the mice were fed WB diets for 2 weeks.

Prevotella qPCR

Bacterial DNA from the cecal contents was extracted using the E.Z.N.A. ® stool DNA kit. qPCR used primers targeted at 16S ribosomal DNA for *Prevotella*. The samples from each mouse were run in duplicate and averaged. The results were normalized to 16S ribosomal (universal) DNA sequences and expressed as the relative difference compared to CTRL using the $\Delta\Delta C_T$ method (15).

Tissue RNA isolation and quantitative real-time PCR.

RNA was isolated from small intestine and colon tisses by TRIzol reagent according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA, then quantitative PCR (qPCR) reactions were performed, with the samples from each mouse being run in duplicate and averaged. qPCR conditions were 40 cycles of 95 °C for 20 s, 95 °C for 0.01 s, 60 °C for 20 s, 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. Primers used are listed in Table 1. Gene expression was normalized to beta-actin mRNA levels using $\Delta\Delta C_T$ method (15).

Statistics

Values are the means ± standard deviations (SD). Graphical illustrations and statistical analysis were performed using GraphPad Prism (v 7.0, GraphPad). Student's t-test with Mann-Whitney test analysis were performed and $p< 0.05$ were considered significant.

Table 1. Primers used for mRNA gene-targeted qPCR

Gene	**Abbreviation**	**Sequence (5'-3')**
Glucose-6-phosphatase	*G6pase*	CTGTGAGACCGGACCAGGA GACCATAACATAGTATACACCTGCTGC
Glucose transporter 2	*Glut2*	GTCCAGAAAGCCCCAGATACC GTGACATCCTCAGTTCCTCTTAG
Free fatty acid receptor 3	*Ffar3*	TGTCCAATACTCTGCATCTGTG AGGTCCGAAATGGTCAGGTT
Sodium glucose cotransporter 3a	*Sglt3a*	TGCTGAAGACGAACCGAAGCAC ACCAGCAGCAAGGCAAACGA
Sodium glucose cotransporter 3b	*Sglt3b*	TCGTACAGCGCTGCTTATGTGGT ACCGCAGTGCCACACTGTTTCT
Beta actin	*B-actin*	AGAGGGAAATCGTGCGTGAC CAATAGTGATGACCTGGCCGT

Results

The mice were fed the chow provided by the animal facility until they reached 6 weeks of age. At that point, the experimental feeding commenced. Mice were split into two groups of 6 mice each; one group received the CTRL diet, while the other received the WB diet (Fig. 1). After 2 weeks of feeding WB or CTRL diets, the mice were sacrificed (Fig. 1). Blood, liver, spleen, kidneys, small intestine, colon, small intestinal contents, cecal contents, urine, and feces were collected for analysis (Fig. 1). The tissues were used for qPCR to measure gene expression (Fig.1) The urine, feces, cecal contents, and serum were used for metabolomic analysis (Fig. 1). The experimental design was exactly the same for the germ-free (GF) (mice that are free from microorganisms) and CV mice (Fig. 1).

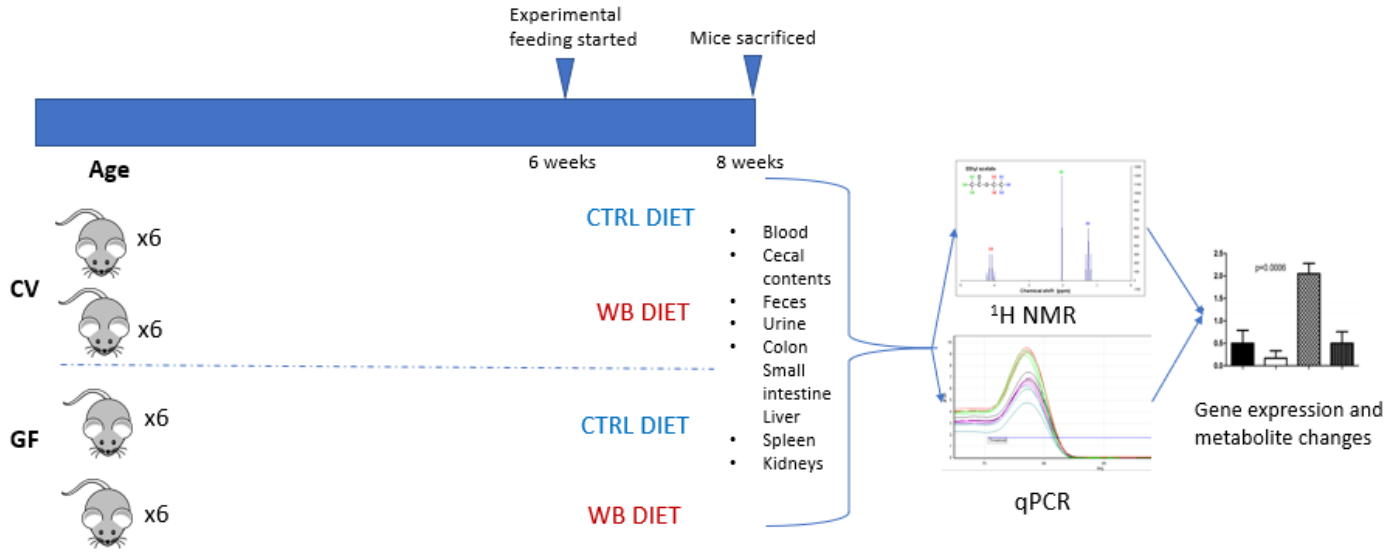

Figure 1. Experimental design

Metabolomic analysis was performed on the cecal contents to compare the effects of WB feeding to CTRL feeding, as well as to analyze the difference between GF and CV mice. This was done through ^{1}H NMR analysis, performed by Dr. Yuan Tian (YT).

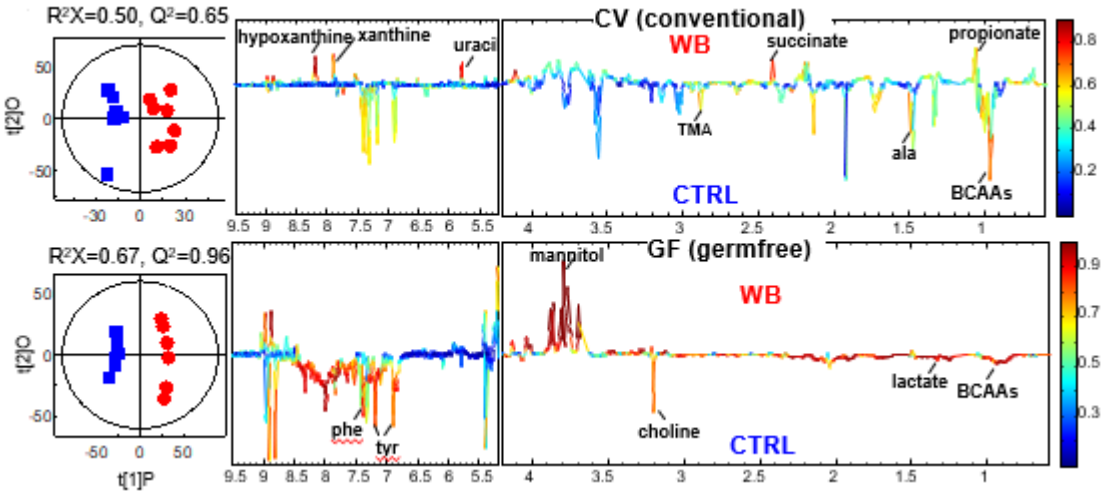

Figure 2. Coefficient plots of ^{1}H NMR spectra of CV and GF cecal contents after 2 weeks feeding. YT extracted metabolites from the cecal contents of both the CV and GF mice and analyzed the types and levels of metabolites present in the cecal contents. n=6-7. Graph courtesy of YT.

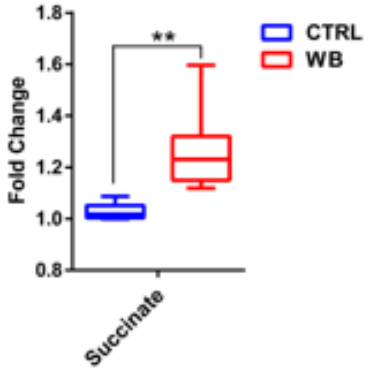

Figure 3. WB-fed mice have significantly higher levels of succinate in the cecum after 2 weeks of feeding. Using the ^{1}H NMR data, YT isolated and analyzed the succinate levels in the CV mice. p<0.01, n=6-7 Graph courtesy of YT.

The ^{1}H NMR results show, among other things, an increase in levels of the short chain fatty acid propionate, as well as the metabolite succinate in WB-fed CV mice compared to the

CTRL fed CV mice (Fig. 2, Fig. 3). The absence of propionate and succinate in the WB-fed GF mice suggests that these metabolites were microbially produced. (Fig. 2) The analysis also showed mannitol in WB-fed GF mouse cecal contents but not in WB fed CV cecal contents (Fig. 2). Mannitol is found in WB mushrooms; we hypothesize that the gut microbiota of the CV mice fermented the mannitol in the WB mushrooms to produce the succinate and propionate.

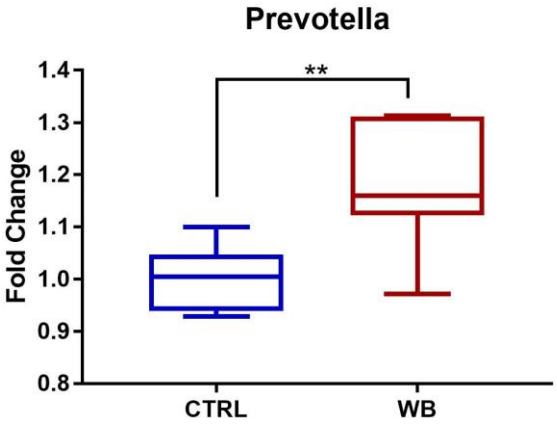

Figure 4. WB-fed mice have higher levels of bacteria of the genus *Prevotella* present in the cecum, normalized to a universal bacterial control. Using real-time PCR, we analyzed the levels of *Prevotella* present in both CTRL and WB-fed CV mice. p=0.008, n=6-7.

Cecal succinate was significantly higher in CV mice fed WB mushrooms for 2 weeks (Fig. 3). *Prevotella* is a genus of bacteria that produces succinate (16). Bacterial DNA was extracted from the cecal contents and was analyzed in two ways. YT used 16S RNA gene sequencing to do global analysis of the microbial populations in the gut; among her findings, she found elevated levels of *Prevotella* in the cecums of WB-fed CV mice (13). In addition, qPCR for *Prevotella* showed higher levels of the bacteria *Prevotella* present in the cecums of WB-fed CV mice (Fig. 4). Both methods confirm higher levels of *Prevotella* present in the cecums of WB-fed CV mice and suggest that *Prevotella* produced the succinate observed in WB-fed CV mice.

It has been shown that microbially produced succinate can improve glucose homeostasis via intestinal gluconeogenesis (IGN) (16). Gluconeogenesis is the biological process of producing glucose from non-carbohydrate sources. While gluconeogenesis largely occurs in the liver of mammals, IGN also contributes to glucose control. Glucose-6-phosphatase (G6Pase) is an enzyme that hydrolyzes glucose-6-phosphate, which then allows glucose to be transported out of the cell. Glucose transporter 2 (Glut2) is a protein that then transports glucose out of the cell. These two enzymes are integral players in the IGN pathway (16).

Certain microbially produced metabolites are taken in by epithelial cells in the gut and converted to glucose-6-phosphate, the substrate of G6Pase that is converted into glucose (16). The glucose is transported into the bloodstream by Glut2 (16).

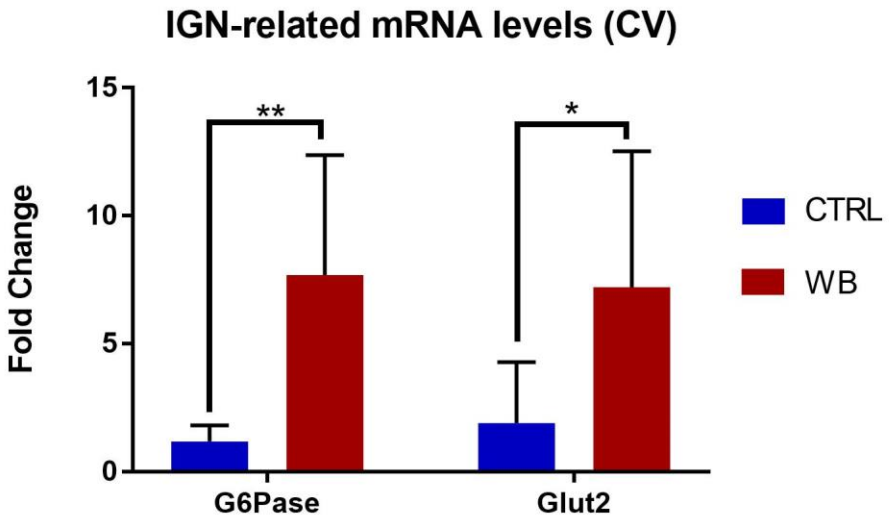

Figure 5. Real-time PCR analysis of the expression of intestinal gluconeogenesis related genes showed significantly higher levels of mRNA encoding for *G6Pase* (p=0.007) and *Glut2* (p=0.05) in the jejunum with WB feeding, normalized to beta-actin. n=6-7.

Feeding CV mice with WB mushrooms significantly increased expression of *G6Pase* and *Glut2* compared to CTRL fed mice (Fig. 5). The higher levels of these enzymes suggest that there is increased IGN occurring in the small intestine and therefore higher levels of glucose

circulating in the bloodstream. These higher levels of glucose are linked to lower glucose production in the liver, which in turn is linked to improved glucose and energy homeostasis (16).

We hypothesize that these changes came about as a direct result of microbially produced succinate. To ensure that the change was indeed linked to the microbiota, we also had to check expression of these two genes in the GF mice.

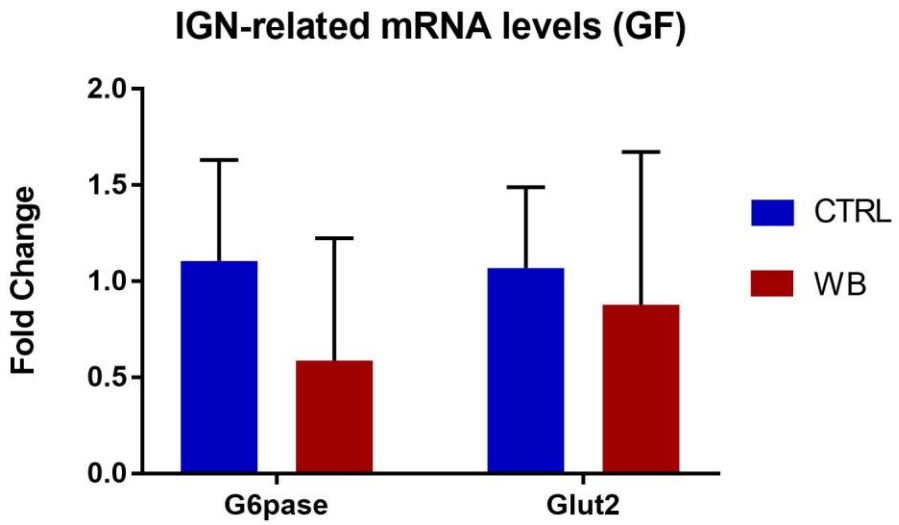

Figure 6. Real-time PCR analysis of the expression of intestinal gluconeogenesis related genes showed no significant difference in *Glut2* and *G6Pase* expression in GF mice after 2 wks WB feeding. n=5-6.

In GF mice, there were no significant changes in *G6Pase* and *Glut2* expression with WB feeding (Fig. 6). Because there was no difference in expression between the two groups in GF mice, that suggests that the WB mushrooms interact with the gut microbiota to produce succinate, which in turn increases IGN, which has beneficial effects on metabolic health.

However, IGN is not the only intestinal factor that is linked to glucose/energy homeostasis. In addition, it has been shown that intestinal gluconeogenesis is linked to the gut-brain neural circuit (17). Two intestinal proteins involved in energy homeostasis are sodium glucose cotransporter 3 (SGLT3) and free fatty acid receptor 3 (FFAR3). SGLT3 is a glucose

sensor. In humans, there is only one *SGLT3* gene, but in mice, there are two genes that encode for two different (but similarly functioning) proteins: *Sglt3a* and *Sglt3b* (18). FFAR3 is a receptor that activates a neural circuit regulating intestinal gluconeogenesis (17).

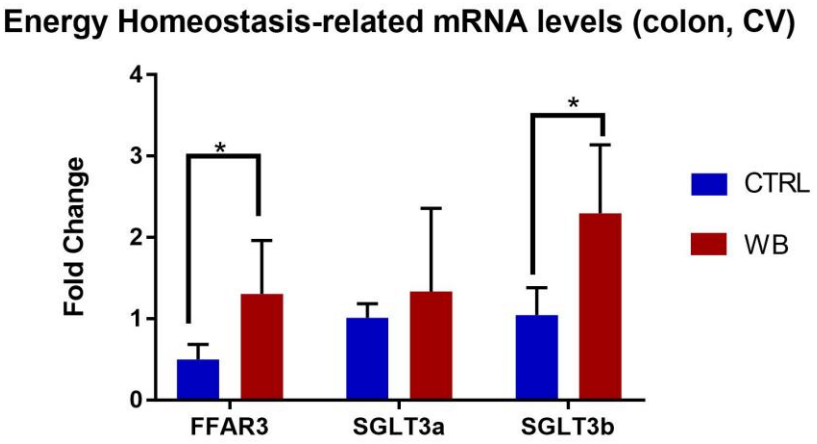

Figure 7. Real-time PCR analysis of the genes related to energy homeostasis showed significantly higher levels of both *Ffar3* (p=0.02) and *Sglt3b* (p=0.02) in the colon with 2 wks WB feeding. n=6-7.

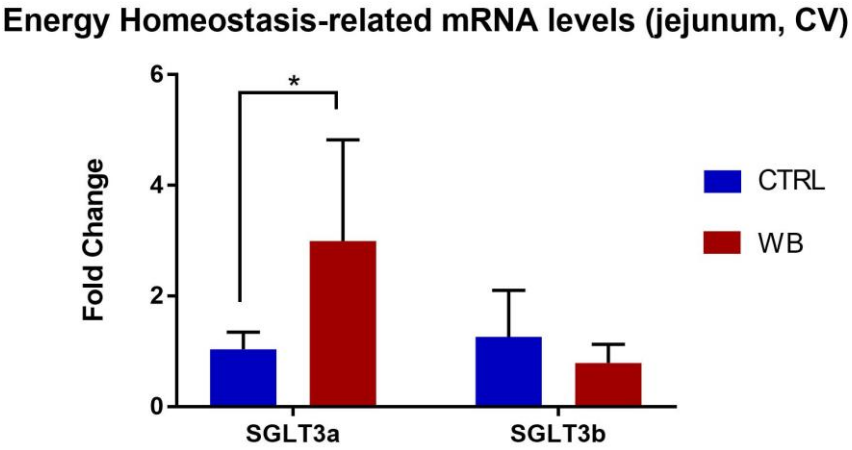

Figure 8. Real-time PCR analysis of the genes related to energy homeostasis showed significantly higher levels of *Sglt3a* (p=0.03) in the jejunum with 2 wks WB feeding. n=6-7

In the WB-fed CV mice, there were significantly higher levels of *Ffar3* and *Sglt3b* present in the colon (Fig. 7) as well as significantly higher levels of *Sglt3a* in the jejunum (Fig. 8). The increased expression of these genes indicate that there is increased regulation of energy

homeostasis. It has been shown that increased levels of these proteins these genes encode for send signals to the brain to curb the appetite (17); coupled with the increased IGN, the data suggests that WB mushroom feeding helps with glucose homeostasis overall.

Again, because the hypothesis was that these changes are all linked to the microbiota, it was important to look at the expression of these enzymes in GF mice. There were no significant changes in expression of *Ffar3*, *Sglt3a*, and *Sglt3b* among the GF mice with WB feeding (Fig. 9). Because there was no significant difference in GF mice, this suggests that the changes observed were related to the gut microbiota.

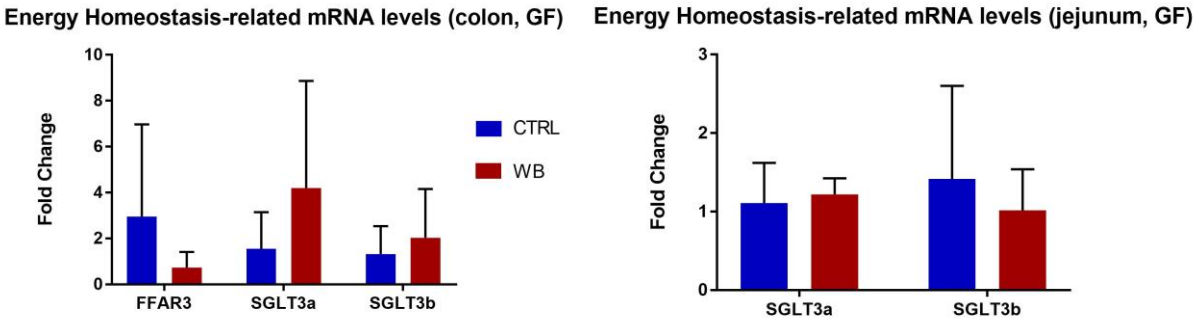

Figure 9. Real-time PCR analysis of the genes related to energy homeostasis show that there is no significant difference in *Ffar3, Sglt3a,* or *Sglt3b* expression in GF mice after 2 wks WB feeding. n=5-6.

Discussion

In the past, the Cantorna lab has conducted research on mushrooms that found that WB mushrooms have protective effects on mice subjected to gastrointestinal injury; WB-fed mice lose less weight and show greater protection from colonic injury than their control diet counterparts (8). Furthermore, it was found that WB feeding also affects the microbial composition of the gut (9). Our hypothesis was that it was these changes in the gut microbiota that caused the improvement in the mice's gastrointestinal health. As such, YT was interested in exploring the metabolic and microbial changes that occur with WB feeding. Past experiments conducted by the Cantorna lab involved mice with compromised gastrointestinal tracts—either through bacterial infection or chemical injury (8,9). However, the mice in the experiment outlined here were healthy.

YT investigated many different aspects in this experiment: metabolite levels, microbial composition in the gut, glucose and glycogen levels in the liver, ergothionine levels, glucose tolerance, and of course, the gene expression mentioned in the results section. My role in the experiment was to look at expression of genes (via quantification of mRNAs) in the small intestine and colon, as well as targeted bacterial analysis. As such, I helped YT with mRNA extraction from tissue, cDNA synthesis from that RNA, and then subsequent qPCR. I also ran the qPCR for the targeted bacterial analysis of *Prevotella*.

First, YT's ^{1}H NMR analysis showed significantly higher levels of succinate in the cecal contents from mice fed WB diet. When the same experiment was run in GF mice, succinate was not found in the cecum. Furthermore, microbial DNA was extracted from cecal contents and analyzed via qPCR, showing increased levels of the succinate-producing bacteria *Prevotella* in WB treated mice. This suggests that the succinate found in conventional, WB-fed mice is a

microbial product. Increased levels of succinate have been linked to improved glucose homeostasis via intestinal gluconeogenesis (16). The results from our experiment confirmed this; in the small intestine of WB-fed mice, there were significantly higher levels of expression of *Glut2* and *G6Pase*. These are genes that are important in intestinal gluconeogenesis and thus, glucose homeostasis.

Glucose homeostasis refers to the tight regulation of blood sugar within the body. Usually, this process is discussed in terms of hormonal control by insulin and glucagon. However, glucose homeostasis is also linked to release of glucose through the intestines, which is related to a gut-brain neural circuit. To that end, we also looked at two enzymes involved in this circuit, which help with energy homeostasis. SGLT3 is a glucose sensor, and FFAR3 is a receptor engaged in a neural circuit that regulates intestinal gluconeogenesis (17). The elevated levels of these enzymes in the small intestine and colon in WB-fed mice support the hypothesis that dietary WB mushroom supplementation can improve glucose homeostasis.

Thus, a potential mechanism of WB mushroom protection is through an increase in succinate-producing bacteria. This could contribute to improved glucose homeostasis through increased intestinal gluconeogenesis (as shown by the increase in the expression of *G6Pase* and *Glut2*), which is regulated via enzymes like FFAR3 and SGLT3, which provide a connection between the gut and the nervous system.

This has potential implications for diabetes and obesity research. However, this was a short-term experiment—the mice were only fed for 2 weeks. Future experiments should look at longer-term feeding to see what the results are. In addition, dosage is another aspect that ought to be investigated. These mice were fed a 1% (by weight) WB mushroom supplemented diet every day. How much mushroom consumption would that be for humans, and would someone looking

to lose weight or control his/her diabetes be willing to consume that amount of mushrooms every day? In addition, future experiments could investigate what exactly it is about the mushrooms that causes these changes. Is it the additional dietary fiber? Is it a certain compound contained within the mushroom? These are all potential future directions.

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Curriculum Vitae

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        - Resume writing and formatting
        - Mock parent-teacher conferences
        - Ordering at a restaurant

EXTRACURRICULARS AND COMMUNITY SERVICE:

Assistant Coach, State College High School Science Olympiad team *Fall 2015-Present*

- Coaching high school students in the areas of forensic science, chemistry, microbiology, geology, and environmental science; this includes teaching and testing in order to prepare for regional and state competitions

Volunteer, Mount Nittany Medical Center *June 2012-December 2017*

- 401 hours of service completed
- Worked in the Physical Therapy and Emergency Departments
- Trained new volunteers

Spanish Translator, United Way of Utah County *January 2016-January 2017*

- Volunteer position
- On-call translator of flyers and other miscellaneous documents for South Franklin Community Center in Provo, UT

Staff Writer, *Unscooped Bagel* *March 2016-July 2016*

- Biweekly blogger for the literary magazine *Unscooped Bagel*
- Writer of two columns: one on poetry, the other on pop song parodies
- The editors had trouble sustaining the magazine and blog financially, which is why the time period was so short

HONORS/SCHOLARSHIPS:

Erickson Discovery Grant Recipient *Summer 2017*

- A $3,500 grant given annually to approximately 60 students at Penn State to fund summer research

Schreyer Honors College Academic Excellence Scholarship *Fall 2014-Present*
Dean's List, The Pennsylvania State University *Fall 2014-Present*
National Merit Scholar *Fall 2014-Present*
Scholarship, National Spanish Exam *Fall 2014*

PUBLICATIONS/LITERARY PURSUITS:

National Gold Medal (Personal Essay), Scholastic Art & Writing Awards *April 2014*
"Fire and Ice Revisited," *Unscooped Bagel* *February 2016*

- Poem published on website and in Februrary 2016 eMagazine.