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The Effect of a High-Fat Diet and Grape Powder on the Abundance of Sulfidogenic Bacteria in the Colonic Digesta of C57BL/6J Mice

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ABSTRACT

Recently scientists have made and interesting link with colon cancer, diet and *Bilophila wadsworthia*. For 16 weeks 50 C57BL/6J mice were fed four different high-fat diets and one low-fat diet each containing certain percentages of grape powder. At the end of the 16 weeks, colonic digesta samples were taken from the mice in order to analyze how much bacteria existed in their colon at the end of the 16 weeks. The *B. wadsworthia* abundance was higher in the low-fat diet than the 3% and 5% grape powder diets, suggesting other microbial targets may be appropriate for analysis.

INTRODUCTION

Sulfate reducing Bacteria (SRB) are microscopic organisms that acquire their energy by reducing sulfate to hydrogen sulfide. Through the process of the anaerobic respiration, which is the breaking down of organic material without the presence of oxygen, hydrogen sulfide is produced. This colorless and poisonous gas is what the bacteria produce in order to make energy and survive. Humans use oxygen as a terminal electron acceptor; SRB's, on the other hand, use sulfate. In addition to the SRB's, *Bilophila wadsworthia*, another closely related bacterium, produces hydrogen sulfide through taurine respiration.

Recently scientists have made an interesting link with sulfidogenic bacteria and colonic cancer. Evidence has shown that hydrogen sulfide gas produced by these bacteria may cause mutations in DNA. Scientists theorize that these bacteria are likely to be more abundant in hosts consuming a high fat diet. These fatty lipids cause the body to produce an excess of bile salts. Bile salts are steroid acids found in animals and most vertebrates (Hofmann, Hagey, Krasowski, 2010). Bacteria like *Bilophila wadsworthia* respire bile salts such as taurine and produce hydrogen sulfide. Suspecting these bacteria are related to colon cancer, scientists have started to run experiments to prove this connection.

Inflammation is one of the many symptoms of colon cancer. Although the results have been forth coming scientist cannot yet confirm that the bacteria are creating this inflammation until they have all the evidence they need to directly link the sulfidogenic bacterium with colorectal cancer. Previously, powder produced from California grapes was fed to mice on

high-fat and low-fat diets. This powder in turn lowered the inflammation in the colon of the mice that were fed the high-fat diet (Chuang, Shen , Chen , Xie , Jia , Chung , McIntosh, 2012). Our group theorizes mice on a high-fat are fed the grape powder then systemic inflammation in the colon will be lowered significantly. If this is true, then inflammation in the colon of low-fat fed mice should be even lower when fed the grape powder due to the low-fat diet. The low fat insures the body will not produce as many bile salts for the bacteria, *Bilophila wadswortha*, to respire and grow. Grape powder was fed to the mice in hope of restoring barrier functions in the colon to fight off the bacteria.

LITERATURE REVIEW

Obesity is a growing problem in the American culture. Approximately 69% percent of the American population is overweight; about 34.9% percent are obese (Ogden, Carroll, Kit, & Flegal, 2014). Being overweight and obese cause abnormal fat that accumulates and health problems. (WHO Media centre, 2014). Serious health problems that can develop as an effect of being overweight or obese are inflammatory bowel disease, type 2 diabetes, CVD (cardiovascular disease), hypertension, and colon cancer.

Recently scientists have made and interesting link with colon cancer, diet and *Bilophila wadsworthia*. *Bilophila wadsworthia* is a type of sulfidogenic bacterium that causes inflammation within the colon. The bacteria utilize the bile salt taurocholic acid, which is produced to help digest fat. In turn, these bacteria produce a poisonous gas, hydrogen sulfide, through taurine respiration. This poisonous gas infiltrates the colon and causes mutations within the cell wall; this is the cause of the inflammation.

These bacteria likely thrive in a high fat environment. This is where the diet portion becomes important. Professionals claim that consuming a western diet increases your chances of dying (Brooks, 2013). Consuming a fatty (western) diet causes the human digestive system to work harder, producing more bile salts. Bile salts are a mechanism your body uses in order to help digest fat (Hofmann, Hagey, Krasowski, 2010). Taurocholic acid is a certain type of bile salt that the bacteria use to live. These bacteria perform a process where they consume the taurine in the colon and chemically turn it into hydrogen sulfide. Once the colon contains increased levels of this bacterium, you can possibly be diagnosed with all of the diseases related to the inflammation of the colon; colonic cancer being the biggest threat on the human life.

Colon cancer is the second leading cancer-related death in the United States, taking about 50,310 deaths in the year 2014 (American Cancer Society, 2014). Colon cancer is a chronic inflammatory disease that resides within the colon and can be caused by obesity or being overweight.

The effect of a high-fat diet and grape powder on the abundance of sulifdogenic bacteria in the colonic digesta of C57BL/6J mice focuses on the abundance of *Bilophila wadsworhtia* that exist within the colon and whether it causes inflammation. For 16 weeks, 50 C57BL/6J mice were fed four different high-fat diets and one low-fat diet containing certain percentages of grape powder. At the end of the 16 weeks colonic digesta samples were taken from each of the mice in order to analyze how much bacteria existed in their colon at the end of the 16 weeks.

Past studies have shown that grape powder to be very effective in reducing inflammation in certain areas of the body, especially the colon. Although the grape powder has been shown to reduce inflammation scientist can't yet deem that the grape powder is the pure cause of the reduction. Scientists do not know what takes place between the cells and the grape powder once the grape powder is added to the colon (Scalbert, Johnson, Saltmarsh, 2005). This unknown factor has caused a ripple effect creating numerous continuous research.

"Research on the effects of dietary polyphenols on human health has developed considerably in the past 10 y. It strongly supports a role for polyphenols in the prevention of degenerative diseases, particularly cardiovascular diseases and cancers." (Scalbert, Johnson, Saltmarsh, 2005)

Despite their troubles scientists have been able to learn slightly more about the polyphenols. The process by which the polyphenols reduce the inflammation within the colon is much more complex.

Figure 1

Polyphenols, which are multi-phenolic organic compounds, derive naturally in plants like grapes. They exist as secondary metabolites, non-flavonoids and flavonoids, which have been studied for their anti-oxidant properties.
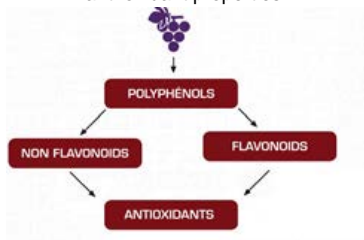
The figure above explains that polyphenols are chemically reacted and turned into antioxidants inside of the mice body (or human body). There are many different types of polyphenols- flavonoids, non-flavonoids and resveratrol (Tanner, et al. 2003). Flavonoids are polyphenols that exist in citrus-like fruits (Spencer, 2008). Resveratrol is a polyphenol that is found in plants and is produced if the plant is under attack by a pathogen, such as bacteria (Frémont, L. 2000).

Fruits and vegetables are absorbed within the body, and have polyphenolic compounds which have antioxidant effects. Once in the body these antioxidants protect against free radicals (unpaired electrons), which are created from oxidation. This has deemed antioxidants as reducing agents (Sies, 1997).

Need for further understanding of how the grape powder reduces inflammation is why scientists can't deem that the polyphenols in the grape powder are the sole reason for the reduction of inflammation inside of the *Bilophila wadsworthia* infiltrated colon (Scalbert, Johnson, Saltmarsh, 2005).

"As fruits and fruit extractions are abundant in bioactive components, such as polyphenols, they possess an immense potential to prevent the development of obesity-related inflammation and insulin resistance." (Overman, Mclntosh, et. al, 2009)

Scientists still hold on to the theory that polyphenols reduce inflammation and continue studies hoping to prove this theory. If scientists succeed within the next few years, it will open up doors for many people suffering from inflammatory diseases. Colon cancer could possibly be on the road to having a cure.

METHODOLOGY

2.1 Isolation of DNA from Colonic Digesta

Samples were collected from collaborators at UNCG. Using standard protocol for the isolation of DNA from colonic digesta, frozen mice fecal samples (180-220mg) were taken and put into separate centrifuge tubes. Buffer ASL (1.4 mL) was added to each sample and vortexed thoroughly to make a homogeneous solution. The suspension was heated for 5 minutes at a temperature of 70 degrees Celsius, then vortexed for 15 seconds before centrifuging (Eppendorf: Centrifuge 5415 R) the sample at full speed for a minute. Once the sample was centrifuged, 1.2mL of supernatant was pipetted into a 2 mL centrifuge tube. Half of a InhibitEX Tablet was placed into each sample and vortexed for 3 minutes. 1.5mL microcentrifuge tubes were prepared with 15uL of proteinase K and 200 uL of supernatant was added. After the right amount of superanatant has been pipetted into the 1.5 mL tubes 200 uL of Buffer AL was added and vortexed for 15s. Solution was incubated for 10 minutes at a temperature of 70 degrees Celsius. Next, 200 uL of ethanol was added to solution and mixed by vortexing. Solution was transferred into a spin column tube and centrifuged for a minute at full speed. Supernatant was discarded and process repeated three times, adding new Buffers: Buffer AW1, Buffer AW2 and Buffer AE, each time. When eluting the DNA only 100 uL of Buffer AE was used instead of 200uL.

2.2 Concentrations of Isolated DNA from Colonic Digesta

For the most accurate results, a pre-clean of DNA was necessary. The nano (NanoDrop Spectrophotometer ND-1000) drop application was opened on the computer and distilled water was applied onto the measurement application area. Once the nano drop was initialized the arm was lifted and distilled water was wiped off using a Kim Wipe. 1.5uL of Buffer AE was measured and applied to loading area. Clicking on blank started the nano drop blank test. Once the nano drop completed the second step, the arm was lifted and

cleaned using a Kim Wipe. This completed the cleaning portion of the process. The sample was vortexed for a few seconds and 1.5 uL of sample was measured out. The measured amount was applied to the loading area, the arm was pushed down, and the computer started to measure. The computer supplied the concentration in ng/uL. Once the concentrations were recorded two formulas ($C_1V_1=C_2V_2$; where C_1 is the desired concentration of 5ng, V_1 is desired volume, and C_2 is the concentration of sample) are used into order to find 1) how much of your sample needed to be used 2) how much water needed to be added to the sample.

2.3 Dilutions

The two formulas used to find the amounts of sample and water in *Concentrations of Isolated DNA from Colonic Digesta* were needed in this step of the procedure. Using the numbers from the formula separate amounts were put into separate wells in a 96 well plate.

2.4 Prepare Standards

Specific Target: Cloned plasmids were prepared that contain target of interest using TOPO TA cloning kit. Clean PCR product was produced using Zymo research DNA clean and concentrator. Target length was validated through electrophoresis. Target sequences were validated through BLAST results. Product was brought to concentration of 5ng/µL. Serial dilutions of plasmids were performed to 10^-10.

2.5 Prepare qPCR Master Mix

Number of samples + number of standards + number of no template controls were calculated, than multiplied by 3 to run in triplicate. The following were mixed in a reservoir per well:

    a. 4µL of SYBR green
    b. 0.1µL of Forward Primer
    c. 0.1µL of Reverse Primer
    d. 2.8µL of H_2O

A 1 mL pipette was used to slowly mix the reagents together, being sure not to create any bubbles. 7µL of master mix was loaded into all wells that were utilized.

2.6 Prepare DNA

The Nanodrop procedure was used to confirm the DNA concentrations of all samples. Each sample was diluted to a standard concentration of 5ng/µL in a 96 well plate. This was done by using the equation $C_1V_1=C_2V_2$, (where C_1 is the desired concentration of 5ng, V_1 is desired volume, and C_2 is the concentration of sample). V_2 was calculated to give the volume of sample necessary to your sample to 5ng/µL. The sample volume to calculate the amount of DNA-ase free water to be added subtracted the desired volume. 1µL of sample DNA was added per well.

2.7 Prepare 384-well plate

The work area was cleaned with 70/30 bleach/ethanol. A 384-well plate was placed on a piece of clean foil to prevent contaminates from obscuring optical area. 7µL of Master Mix and 1µL of DNA was added into all wells that were be utilized. Plate layout below was followed. No template controls that had 7µL of Master Mix and 1µL of H_2O. Once completed, plate was covered with an optical clear adhesive film. Plate was wrapped immediately in foil, (SYBR green is light sensitive). Plate was stored at -20^{0}C until ready to run.

2.8 Run plates using 7900HT Fast Real-Time PCR System

SDS Automation Que was opened and then the SDS 2.4 program was opened as an administrator. A new document was started and the barcode of the first plate was scanned. The plate was centrifuged for 1 minute at 3000 RPM. Program "PattyW" was selected and copied to plate document. All cells were selected in documents and indicated that they should all run PattyW. The sample volume was changed to 8µL. The plate was saved as: SampleNameLocationTypeTarget-date (ex: GrapeMouseIlleumMucosaDSV-72214) and then sent to Que. The plate was stored under foil and the steps were repeated for additional plates. Once all plates were ready, stacked in order, and placed into the input column the program was run.

2.9 Collecting the Analysis

Once the program was finished, plates were discarded. Files were opened and Analysis->Analyze was clicked. Once analyzed was clicked File->Export->Gaskins->Export. Files were saved in a txt. file so they could be opened in notebook. Once file was exported and moved to a thumb drive to be opened on a personal computer, file was opened and pasted into an

excel document. Everything was deleted except the first 3 columns. First three columns were highlighted and custom sorted so that column A goes smallest to largest. In cell D2 type: =ISODD(A2). Command was dragged down the whole column. Columns A-D were highlighted in additional custom sort by column D.

Columns A-D were copied and pasted into qPCR template. Once sorted by template, whole sheet was copied and saved into original excel file. Cycle threshold (CT) data was arranged in triplicate for 50 samples.

Table 1
384 well plate loading layout for qPCR. A1 corresponds to well A1 of 96-well dilution plate used for standardizing samples. Rows M, N, and O were used to load standard curve.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	A1	B1	A2	B2	A3	B3	A4	B4	A5	B5	A6	B6	A7	B7	A8	B8	A9	B9	A10	B10	A11	B11	A12	B12
B	A1	B1	A2	B2	A3	B3	A4	B4	A5	B5	A6	B6	A7	B7	A8	B8	A9	B9	A10	B10	A11	B11	A12	B12
C	A1	B1	A2	B2	A3	B3	A4	B4	A5	B5	A6	B6	A7	B7	A8	B8	A9	B9	A10	B10	A11	B11	A12	B12
D	C1	D1	C2	D2	C3	D3	C4	D4	C5	D5	C6	D6	C7	D7	C8	D8	C9	D9	C10	D10	C11	D11	C12	D12
E	C1	D1	C2	D2	C3	D3	C4	D4	C5	D5	C6	D6	C7	D7	C8	D8	C9	D9	C10	D10	C11	D11	C12	D12
F	C1	D1	C2	D2	C3	D3	C4	D4	C5	D5	C6	D6	C7	D7	C8	D8	C9	D9	C10	D10	C11	D11	C12	D12
G	E1	F1	E2	F2	E3	F3	E4	F4	E5	F5	E6	F6	E7	F7	E8	F8	E9	F9	E10	F10	E11	F11	E12	F12
H	E1	F1	E2	F2	E3	F3	E4	F4	E5	F5	E6	F6	E7	F7	E8	F8	E9	F9	E10	F10	E11	F11	E12	F12
I	E1	F1	E2	F2	E3	F3	E4	F4	E5	F5	E6	F6	E7	F7	E8	F8	E9	F9	E10	F10	E11	F11	E12	F12
J	G1	H1	G2	H2	G3	H3	G4	H4	G5	H5	G6	H6	G7	H7	G8	H8	G9	H9	G10	H10	G11	H11	G12	H12
K	G1	H1	G2	H2	G3	H3	G4	H4	G5	H5	G6	H6	G7	H7	G8	H8	G9	H9	G10	H10	G11	H11	G12	H12
L	G1	H1	G2	H2	G3	H3	G4	H4	G5	H5	G6	H6	G7	H7	G8	H8	G9	H9	G10	H10	G11	H11	G12	H12
M	5ng		-1		-2		-3		-4		-5		-6		-7		-8		-9		-10		NTC	
N	5ng		-1		-2		-3		-4		-5		-6		-7		-8		-9		-10		NTC	
O	5ng		-1		-2		-3		-4		-5		-6		-7		-8		-9		-10		NTC	
P	MNO- target standards + No template controls																							

2.10 Calculate gene copies per ng of sample

The number of gene copies were determined in the standardized volume of 5ng/µL.

Number of copies (N_1) = (amount * 6.022×10^{23}) / (length * 1×10^9 * 650). The amplification efficiency of standards was calculated as follows:

      a. A scatter plot of the standards was created by the log value of the gene copies in sample.

      b. The tread line, equation, and R-value of plot were shown.

      c. If the R-value was close to 0.98 there was successful amplification of standards.

      d. The slope of the standard curve was used to calculate your amplification efficiency (E):

$$A = 10^{-1/slope}$$

Number of amplicons (N_T) was calculated at the fluorescent threshold for each of these standards.

$$N_T = N_1 * (A)^{CT}$$

Using the average fluorescent threshold of these standards, the gene copies (N_0) were determined in each original sample.

$$N_0 = N_T / A^{CT}$$

FINDINGS

Figure 2

This graph shows the standard curve made from known dilutions of cloned plasmids containing *B. wadsworthia* 16S gene.
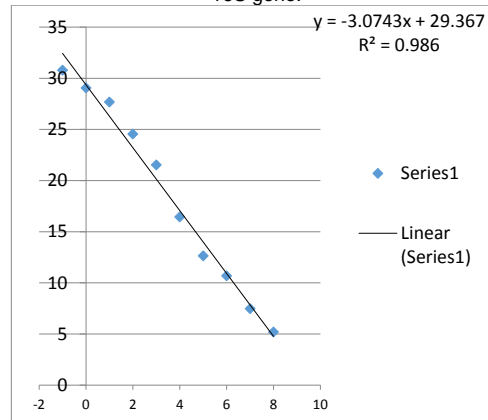
$y = -3.0743x + 29.367$
$R^2 = 0.986$

Table 2

(*B. wadsworthia*) This table compares the significant differences between each of the different variances. The highlighted p value should be less than 0.05 in order to demonstrate significant differences.

Welch F test in the case of unequal variances:	F=2.051, df=20.44, p=0.1248

Tukey's pairwise comparisons: Q below diagonal, p(same) above diagonal

	A	B	C	D	E
A		0.9942	0.9862	0.1476	0.8727
B	0.5728		0.8905	0.06173	0.655
C	0.7179	1.291		0.3595	0.9913
E	1.353	1.926	0.6352	1.983	

Figure 3

This graph shows the average abundance of *B. wadsworthia* in each of the different high-fat and low-fat diets that were fed to the mice.
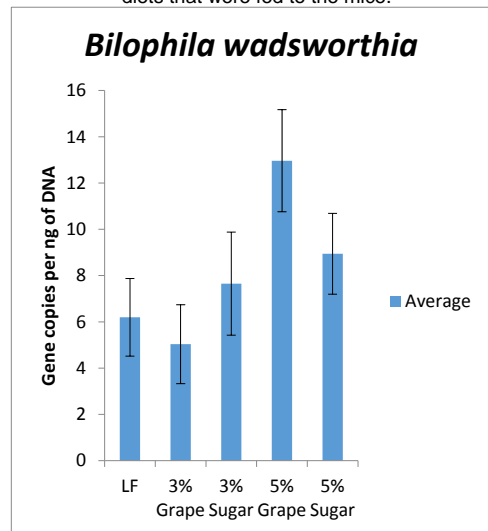
Figure 4

This graph shows the standard curve made from known dilutions of cloned plasmids containing *dsrA* gene.
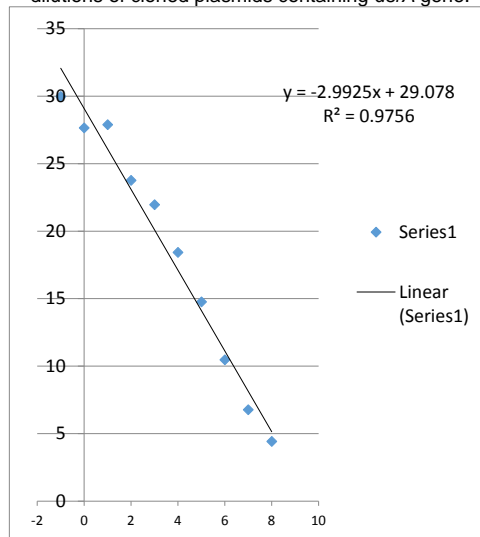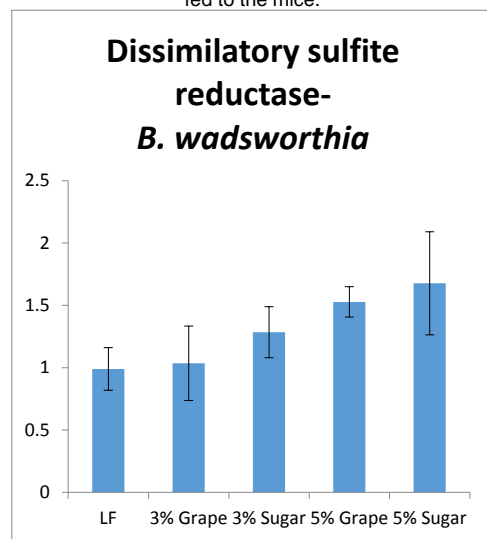
$y = -2.9925x + 29.078$
$R^2 = 0.9756$

Figure 5
This graph shows the average abundance of dsrA (Dissimilatory sulfite reductase- *B. wadsworthia*) in each of the different high-fat and low-fat diets that were fed to the mice.

Dissimilatory sulfite reductase-
B. wadsworthia

Table 3
(*drsA*) This table compares the significant differences between each of the different variances. The highlighted p value should be less than 0.05 in order to demonstrate significant differences. This data is also not statistically significant.

Welch F test in the case of unequal variances:		F=1.683, df=19.77, p=0.1938		
	A	B	C	D	E
A		1	0.9407	0.6408	0.4051
B	0.1674		0.9674	0.713	0.475
C	1.074	0.9067		0.9702	0.8496
D	1.958	1.791	0.884		0.9953
E	2.502	2.334	1.428	0.5436	

One reason for such unusual results could be the microbial population reacted to the grape powder preceding the systemic response. Since samples weren't taken periodically we will never know for sure if that is in fact the case or not. Although, it is possible the hypothesis was proven wrong when it pertains to the change in the B. wadsworthia and dsrA. These data show that other targets may have been more appropriate for this study. Further research should include other targets, including butyryl coA transferase and *Akkermansia muciniphila*. Pyrosequencing is now being conducted in collaboration with researchers at U Chicago to possibly identify microbial targets that do correlate with the protective effects of grapes on development of metabolic imbalance in response to a high fat diet.

CONCLUSIONS

The 3% of grape powder fed to the mice on the high-fat diet did prevent the mice from gaining more body fat. It also prevented increases in WAT (White adipose tissue) mass, liver triglycerides and impaired ZO1 localization. These results do not correlate with the abundance of *Bilophila wadsworthia*. The *B. wadsworthia* abundance was higher in the low-fat diet than the 3% grape powder diet. It was even higher in the 5% grape powder diet. In general these observations were not statistically significant.

ACKNOWLEDGEMENTS

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