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Investigating the effect of sodium benzoate on immune cells and microbial populations in the small intestine of murine species.

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Investigating the effect of Sodium benzoate on immune cells and microbial populations in the small intestine of murine species.

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of the Undergraduate College of Biology

James Madison University

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Abstract

Dietary ingredients can influence the mucosal surface morphology and mucosal immunity of the gastrointestinal tract. Additional health concerns and behavioral changes have been attributed to the consumption of foods containing preservatives and additives. Sodium benzoate (**SB**) is a commonly used bacteriostatic in food and beverages. This study investigates the effects of SB on the gut bacteria and mucosal health in the gastrointestinal tract of laboratory mice. The extent of lymphocytic infiltration in intestinal villi and granular density of Paneth cells in the ileum were used as evaluators of mucosal immunity. Adult C57BL/6 mice were randomly assigned to two groups. The control group (n=14) and SB treated group (n=15) received standard rodent chow. The SB treated group received 1% SB treated water. Food and water were available to animals *ad libitum* for the experimental period of 30 days. Animals were monitored for body weight and food/water intake. Ileal samples for histological evaluation and caecal contents for microbial analyses were collected at the end of the experimental period. Paneth cell granular density and lymphocytic infiltration into the lamina propria were evaluated by double blind scoring systems on a scale from 1-4. Culture and PCR analysis from pooled samples (n=6 control, n=6 SB) were used to determine the effects of SB treatment on the presence and prevalence of target species of gut bacteria. Statistical significance was declared at $p<0.05$. There were no changes observed in the granular density of PCs. There was statistically significant lymphocyte infiltration in response to SB suggesting possible alteration in mucosal immunity of the gut. Sodium benzoate increased the food intake and changed the gut microbial population compared to the controls. *Bacteroidetes* and *Firmicutes* decreased while *Enterobacter* increased in relative abundance. In conclusion, SB consumption may influence gut microbial population

and mucosal immunity in murine species. Further studies should be conducted to better understand the mechanisms and long-term effects and SB on the body.

Introduction

Sodium benzoate as a food preservative

Preservatives are used in many food products for the prevention of chemical alterations and microbial contamination. Urticaria, angioedema, asthma, and hyperactivity are some of the symptoms found to be linked with the consumption of food additives (Verhagen 1996). Benzoate preservatives act as bacteriostatic and fungistatic agents under acidic conditions. Sodium benzoate (**SB**), the sodium salt of benzoic acid, is widely used in acidic foods, carbonated beverages, and cosmetics (Lennerz et al., 2014). Safe ingestion is currently regulated by the Food and Drug Administration (FDA) which recommends consumption of SB concentrations no greater than 0.1% in food (FDA, 2017). The World Health Organization (WHO) deemed the compound safe when consumed at 5mg/kg body weight per day (Wibbertmann et al., 2005).

It is known that when benzoate reacts with ascorbic acid (which is commonly present in many food items) it produces the carcinogenic compound benzene (Gardner et al., 1993). Additionally, benzene is metabolized in the mitochondria of liver cells, producing hippurate, which is then cleared from the body by the kidneys. Increased concentration of hippurate in the body may lead to many metabolic diseases like obesity and diabetes (Lee et al., 2013). Another study by Lennerz et al. (2014) found that ingestion of SB in humans significantly increased the levels of several metabolites in the blood including hippurate, acetylglycine and anthranilic acid. This suggests that chronic exposure to SB could potentially lead to diabetogenic effects.

Effects of SB may not be all bad as SB has been found to be an effective off-label treatment for hepatic encephalopathy, a serious neurological complication from cirrhosis, by decreasing the ammonia build-up in the bloodstream (Misel et al., 2013). Sodium benzoate

supplemented diets have also been shown to improve feed efficiency, diarrhea, and intestinal microbiota in piglets (Diao et al., 2014).

Paneth cells and mucosal immunity

Paneth cells (**PC**s) are highly specialized cells located in the epithelium of the small intestine where they influence the microbial composition and inflammatory responses of the innate immune system (Elphick et al., 2005). Paneth cells produce mediators that provide protection for intestinal stem cells and therefore contribute to the maintenance of intestinal mucosa. This single-cell layer of epithelial cells of the intestinal mucosa is adapted to boost nutrient absorption and electrolyte transport, but this also makes it particularly susceptible to microbial infiltration and overgrowth. When exposed to bacteria, PCs secrete granules containing antimicrobial peptides (AMPs) such as defensins and lysozymes (Elphick et al., 2005). This mechanism protects intestinal crypts from overgrowth of opportunistic bacteria. Secretions of PC granules is dependent on stimulation of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing molecules (NODs) located on epithelial surfaces (Elphick et al., 2005). Bacterial glycolipids are also able to induce granule production of PCs upon recognition (Tanabe et al., 2005).

The AMPs that are present in the intestinal epithelium vary with bacterial composition of the gut. Assessment of AMP responses to many different gram-positive and gram-negative bacteria shows an increase in the Paneth cell antimicrobial defenses (Tanabe et al., 2005). In contrast, diseases such as Crohn's disease induce chronic inflammation in the gastrointestinal tract by weakening AMP responses of PCs (Armbruster et al., 2017). Histological analysis of complicated celiac disease cases showed reduced number of granules, but not the proliferative

activity of Paneth cells (Sabatino et al., 2008). Therefore, quantifying PC granular density in ileal tissue can be used as an indicator of gut mucosal immunity.

Inflammatory infiltration in the intestinal mucosa

The intestinal mucosa acts as a contact barrier between the environment and the body and contains a large community of immune cells functioning to combat pathogenic microorganisms and antigens. As a normal housekeeping process, inflammatory cells infiltrate tissues to clear dead or damaged cells from the site. Microscopic enteritis (ME) for gluten-related conditions such as celiac disease, gluten sensitivity, wheat allergy, autoimmune enteropathy, and dermatitis herpetiformis have been assessed by lymphocyte infiltration at sites of damaged tissue in the gastrointestinal system (Ierardi et al., 2017). When assessing gastrointestinal mucosa, abnormal infiltration of intraepithelial lymphocytes (IELs) is an important indicator of secondary diseases such as irritable bowel syndrome, a few autoimmune conditions, infections, and immunoglobin deficiencies. Therefore, grading the severity of inflammatory infiltrates can be used as a tool to evaluate the degree of damage to the intestinal epithelial tissue. A study by Pongsavee (2015) found that treating lymphocytes with increasing concentrations of SB elevated micronucleus formation and chromosomal breakage, demonstrating the cytotoxic effects of SB in lymphocytes. As mentioned before, SB is a known precursor to the carcinogen benzene; therefore, it is expected that physiologically the body will respond to these harmful materials with increasing inflammatory responses. On this premise, grading the intensity of inflammatory infiltrates in the intestinal epithelia may be an adequate model to detect damage in epithelial cells caused by SB intake.

Gut microbial community

Intestinal microbiota contributes to homeostasis in the body due to diversified functions of intestinal microbes. The small intestine consists of a large community of diverse microorganisms which occupy a variety of niches; commensal, symbiotic, opportunistic, or pathogenic. The gastrointestinal tract is a reservoir for the largest and most diverse of these communities of microbiota; capable of undergoing alterations depending on age, diet, antibiotic-exposure, and environmental factors (Conlon and Bird, 2014). Changes made in the intestinal microbiota can affect a host organism in different ways, so comprehension of these interactions is important. There is an increasing body of evidence showing the relationship between gut microbiota and diet related health and behavioral changes. Comparison of normal corn starch, resistant starches HA7, and octenyl-succinate HA7 diets in mice revealed respective microbiome composition corresponding with a unique gut microbiota (Lyte et al., 2016). Fluctuations in the microbiome of the body can also have implications on body function and play a significant role in the immune system, obesity, cardiovascular disease, and brain activity (Lee and Hase, 2014). Inflammatory bowel disease, a very common intestinal condition, is associated with host mucosal function and diminishing commensal microbes. This relationship results in potential mucosal inflammation and impaired regeneration reinforced by the increased presence of Toll-like receptor 4 (TLR4). Increased TLR4 receptor signaling is associated with impaired epithelial barrier and altered microbial population compared to wild-type (WT) littermates, suggesting that intestinal immune signaling is capable of modulating gut bacterial communities in addition to diet (Dheer et al., 2016). Intestinal bacteria in monogastric animals are concentrated in the distal gut. Caecum contents harbor an accurate representation of existing microbiome and create an accurate depiction of any changes in the microbial population in response to diet. For this

experiment, all strains were selected on the basis of naturally occurring microorganisms in the murine model gastrointestinal system (Canny and Mccormick, 2008).

Rationale and objectives

Incorporation of preserved foods and carbonated beverages in regular diets have increased around the world, yet there is limited information on the short- and long-term cumulative effects of oral exposure to the SB in these foods. Likewise, there is very little information available on the effects of SB on gut health and on the changes in gut bacteria in response to SB intake. The purpose of the proposed study is to investigate whether consumption of SB alters the mucosal immunity and microbial population in the small intestine using a mouse model. The hypothesis for this experiment was that SB intake will alter the normal gut microbial population and induce inflammatory response in the small intestinal mucosa. Measurable changes in histology, and microbial population analysis in the gut following exposure to SB can be used to predict the impact of short and long-term ingestion of this commonly consumed preservative. The specific objectives of this study are:

1. To determine the effect of SB on Paneth cell granular density in the ileum.

2. To determine the effect of SB on leukocyte infiltration in the villi of ileum.

3. To determine the effect of SB on the presence and prevalence of a commonly found intestinal bacterial strains in the cecum.

Material and Methods

Animals and experimental design

All animal experiment protocols followed the regulations specified by the Institutional Animal Care and Use Committee (IACUC) of James Madison University. Adult C57BL/6 mice (The Jackson laboratory) were obtained and maintained under a controlled environment on a 12-hour light and dark cycle with monitored temperature and humidity. All animals had access to food and water *ad libitum*. Animals were randomly assigned to either control (n=14) or treatment groups (n=15). The treatment group received standard rodent chow and drinking water concentrated with 1% SB (Lab Grade Powder, Fisher Science Education, USA). This concentration was determined based on calculations equivalent to 5mg/kg for body weight of the mice. The control group did not consume any SB and received standard rodent chow and normal drinking water. Individual animals were weighed weekly, and the total food and water intake was measured daily. At the end of 30 days, all animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Necropsies were performed for any gross lesions in major organs in addition to the gastrointestinal tract and stored for further analysis.

Sample Collection and Analysis

Histological analysis

Small intestinal tissues (ileum) were collected consistently from the same anatomical location and flushed with phosphate buffered saline to wash off the lumen contents. Tissues were then fixed in 10% normal buffered formalin. Routine tissue processing was performed for paraffin embedding and sections of 7μm thickness were stained with hematoxylin (Modified Mayer's Hematoxylin, Richard-Allan Scientific, USA) and eosin (Eosin Y, Fisher Scientific,

Belgium). Random coding during embedding of tissue was established to eliminate subconscious bias during histopathological assessment. Photomicrographs of high-quality sections containing complete villi were used for histological evaluations. Paneth cells were graded from a total 40 crypts per animal using a scoring system for granules. Granular concentration was graded on a scale from 1-4 according to concentration differences (Fig 1; Podany et al., 2016). Inflammatory infiltration was graded on a scale from 1-4 based on the infiltrating lymphocytes into the lamina propria and submucosal layers from 40 villi per animal (Fig 2; Erben 2014). All samples were graded by two individuals separately using double blind analysis.

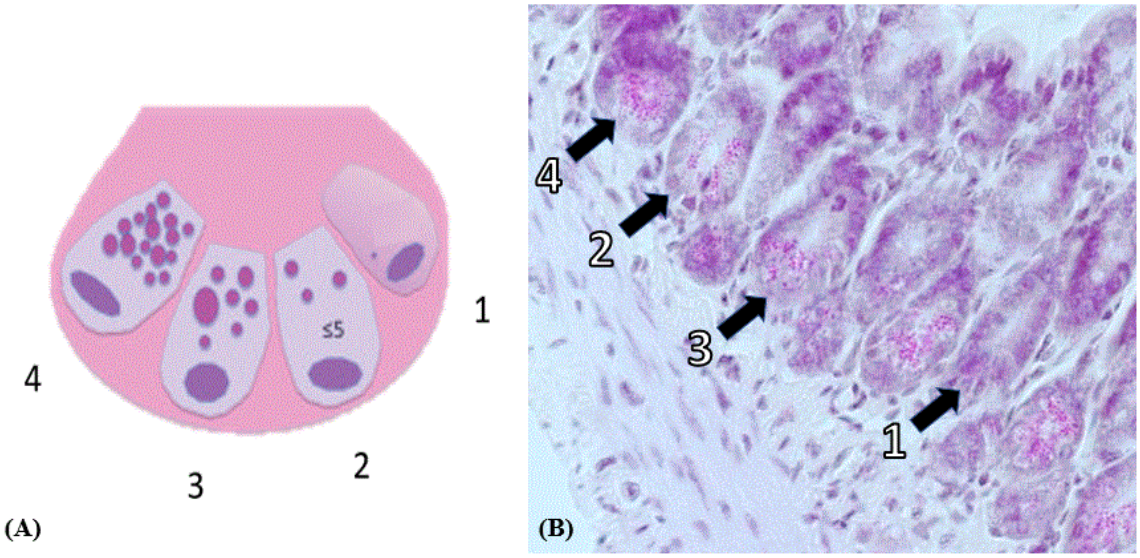

Figure 1. Paneth Cell Grading method. (A) Illustration of PC degranulation scoring criteria (adapted from Podany et al., 2016); (B) A sample of H&E stained ileal tissue from the experimental mice (100X objective). Scores were assigned as follows; Score 1: little to no granules present; Score 2: very few granules present; Score 3: moderate number of granules; Score 4: high density of granules.

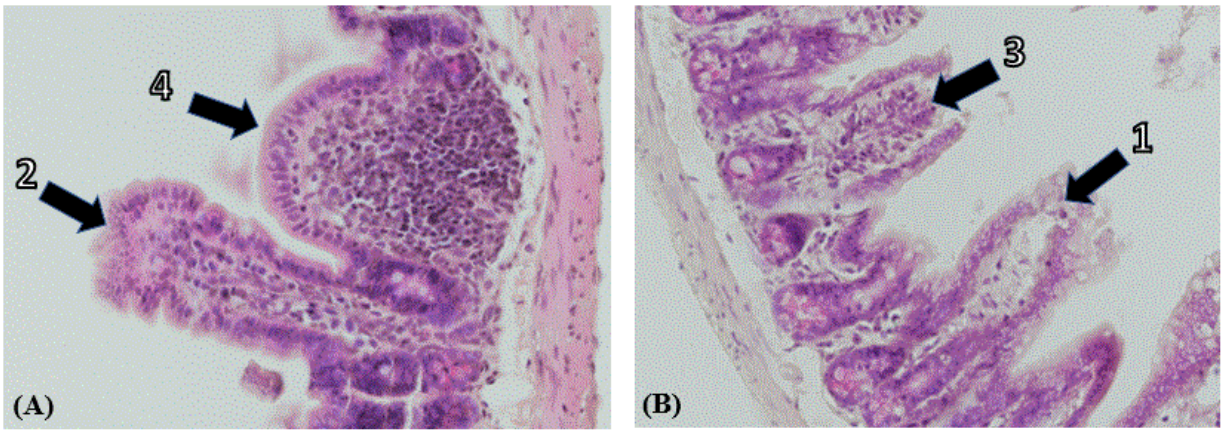

Figure 2. Scoring scale of lymphocyte infiltration (Erben et al., 2014). A and B shows samples of H&E stained ileal tissue from the experimental mice (100X objective). Score 1: Intact villi and minimal inflammatory cell infiltration; Score 2: Mild inflammatory infiltration in the epithelial layer and villi deformation; Score 3: Moderate inflammatory infiltration, villi distorted; Score 4: Villi distortion and obvious tissue necrosis.

Culture of microbial populations

Bacterial samples for culture were obtained by rinsing cecal contents with 5 ml sterile Phosphate Buffered Saline (PBS) and combined contents were stored on ice. The cecal contents were then serially diluted in PBS (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}). The appropriate final dilutions were plated in duplicate onto Trypticase Soy agar (TSA), De Man, Rogosa and Sharpe (MRS) agar, Eosin Methylene Blue (EMB) agar, and m-Enterococcus (ME) agar for analysis of total bacterial population, *Lactobacillus*, *E. coli*, and *Enterococcus* respectively. Cultures were used in addition to PCR to support bacterial abundance trends. Plates were incubated at 37°C for 24 hours and colonies were counted.

Fecal Microbiota Analysis by Polymerase Chain Reaction

Fresh fecal material was collected from the cecum of each animal after euthanasia, and pooled contents from each cage of animals were stored at -20 °C. Bacterial nucleic acid extraction was performed on approximately 0.3g of mouse cecal contents (n = 6 control, 6 SB)

using a stool DNA kit (OMEGA E.Z.N.A, Norcross, GA). Quality and concentration of total DNA in each sample was determined by nanodrop (Table 1) and the DNA samples were stored at -20^{o}C until PCR analysis. DNA to be used as positive controls were extracted from overnight grown cultures of *Escherichia coli, Bacillus cereus, Enterobacter cloacae, Klebsiella pneumoniae* and *Enterococcus faecalis*, and quality and concentration of DNA was performed (Table 1). The same amount of total DNA (approximately 17 ng/µl) from each sample was used for PCR amplification. Primer pair sequences were based on known published target sequences (Table 2).

Each PCR reaction (25µl) contained 12.5 µl Hot Start Taq 2X master mix (New England Biolabs, Ipswich, MA), 5 µl forward primer (0.5 µM), 5 µl reverse primer (0.5 µM) and 2.5 µl template DNA. The PCR analysis was performed on a Bio-Rad C1000 Touch thermocycler using the following cycling conditions: 95°C for 5 minutes; 34 cycles of 95°C for 30 seconds, 45°C for 30 seconds, 72°C for 30 seconds, 72°C for 5 minutes, and final hold at 4 °C. Agarose gel (2%) electrophoresis and GelRed stain was used to visualize the PCR amplification products (Fig 3). Band densities were measured by digital analysis of the gel images using ImageJ software. Band intensities were measured by individual band pixel density divided by the respective band area. Samples were normalized per gel by dividing each sample density to the marker band to determine the relative abundance of each bacterial species.

Table 1. Quantitation of DNA for all murine fecal samples and bacterial positive controls. C11-C14 and W15-W25 are control and SB treated samples respectively.

Sample	Concentration (ng/ml)	A260/A280	A260/A230
K. pneumoniae	17	2.036	1.405
E. coli	18.5	1.609	1.386
B. cereus	10.4	1.971	1.051
E. faecalis	77	1.692	0.832
E. cloacae	17.5	2.047	1.178
C11	546	2.037	2.318
C12	512	2.149	2.303
C13	441	2.096	2.086
C14	441	2.083	2.164
C26	347	1.994	1.994
C27	174	1.611	1.611
W15	622	2.130	2.160
W16	477.5	2.094	2.023
W17	420.5	2.102	2.046
W18	272	2.084	2.109
W24	89.5	1.925	1.738
W25	210	1.780	2.188

Table 2. Primer pair sequences for PCR of selected species of bacteria from gram-positive and gram-negative groups. All primer sequences used have been published previously.

Bacterial Target	Primer Name	Sequence (5'-3')	Amplicon length (bp)	Citations
Universal Primer (Total DNA)	Eub338F Eub518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	200	Fierer et al. 2005
Bifidobacterium	SQ-F SQ-R	CGCGTCCGGTGTGAAAG CTTCCCGATATCTACACATTCCA	126	Mao et al. 2016
Bacillus	YB-F YB-R	GCAACGAGCGCAACCCTTGA TCATCCCCACCTTCCTCCGGT	92	Diao et al. 2014
Escherichia coli	DC-F DC-R	CATGCCGCGTGATGAAGAA CGGGTAACGTCAATGAGCAA	96	Diao et al. 2014
Lactobacillus	RS-F RS-R	GAGGCAGCAGTAGGGAATCTTC CAACAGTTACTCTGACACCCGTTCTTC	126	Diao et al. 2014
Klebsiella	KlebsiellaF KlebsiellaR	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130	Liu et al. 2008
Actinobacteria	ActinobacteriaF ActinobacteriaR	CGCGTCCGGTGTGAAAG CTTCCCGATATCTACACATTCCA	277	Yang et al. 2015
*Bacteroidetes**	BacteoidetesF BacterioidetesR	GTTTAATTCGATGATACGCGAG TTAASCCGACACCTCACGG	122	Yang et al. 2015
Enterobacter	EnterobacterF EnterobacterR	CAGGTCGTCACGGTAACAAG GTGGTTCAGTTTCAGCATGTAC	512	Fazzeli et al. 2012
Enterococci	EnterococciF EnterococciR	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	Ke et al. 1999
*Firmicutes**	FirmicutesF FirmicutesR	GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	126	Yang et al. 2015

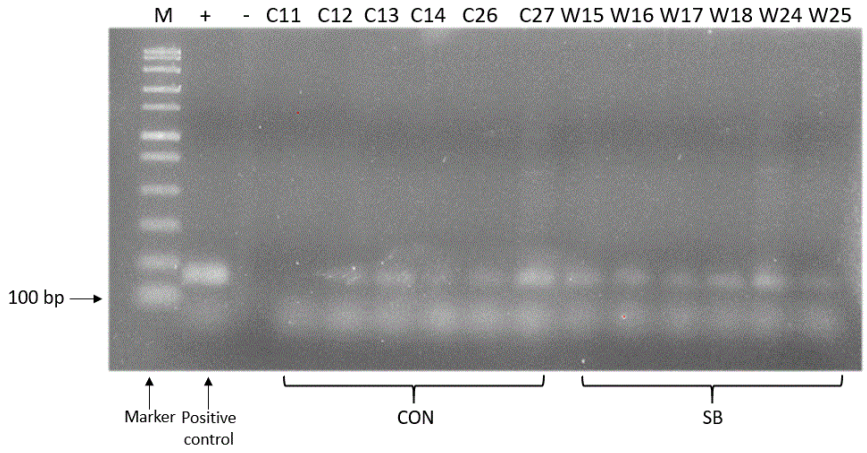

Figure 3. Representative gel electrophoresis image used for density calculations. All the samples and the positive control PCR products were diluted to 17 ng/µl DNA. Sample bands were compared to the 100bp band in the marker to determine normalized band intensity.

Statistical analysis

Histological measurements and microbial population data were analyzed using the nonparametric test with Kruskal Wallis pairwise comparison and the Student *t* test respectively. The experimental unit for histological analyses was the individual animals whereas for culture and PCR analyses pooled samples per cage was treated as experimental unit. Significant difference was declared at a *p* value of less than 0.05. Data are presented as means ± SD.

Results

Food intake, water intake, and body weight gain

Animals that received SB ate more food daily compared to the control mice (4.8 g/d vs. 3.7 g/d for SB and control, respectively; *p<0.01*; Fig 4A.). There was no difference in water intake and body weight gain between the two treatment groups (*p>0.05*, Fig 4B and 4C).

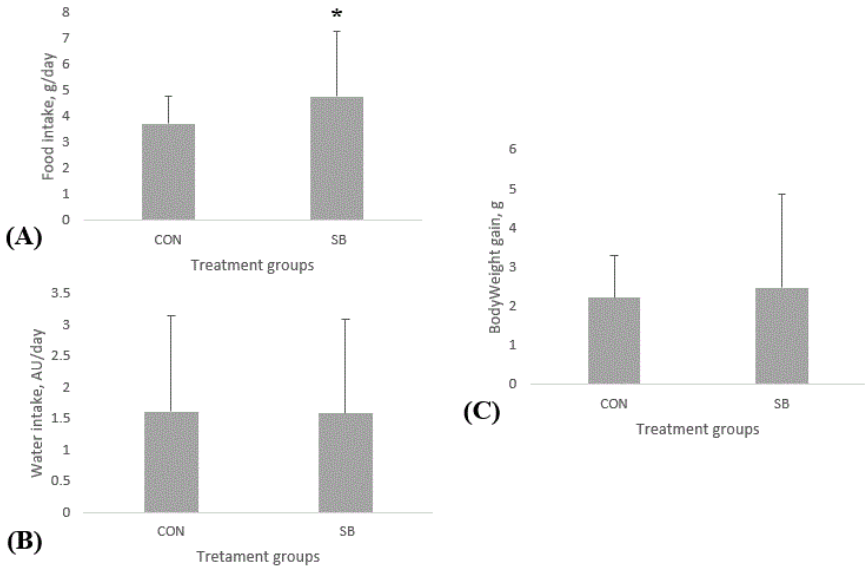

Figure 4. A) The average food intake (g/day), B) average water intake (AU/day), and C) body weight gain (g) for sodium benzoate treated (SB) and control (CON) groups. Data combined from two independent experiments and presented as mean ± SD (n=14 control, n=15 SB). Asterisks indicate statistical significance at *p<0.05*.

Paneth cell granular density in the ileal crypts

The density of the eosinophilic granules in the Paneth cells present in the crypts of ileum were not different between treatments. The mean scores for CON and SB groups were 3.0 and 3.1, respectively (*p>0.05*, Fig 5).

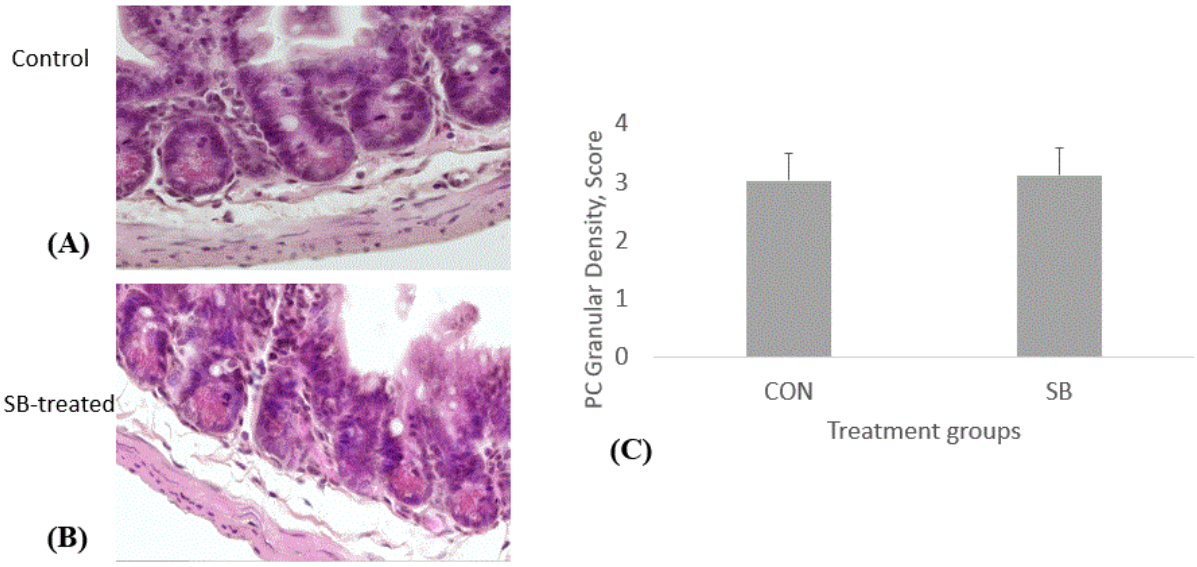

Figure 5. Paneth cell granular density for (A) control (CON) and (B) sodium benzoate treated (SB) mice measured by a 1-4 scoring system. C) Quantitative data from two independent experiments combined and presented as mean ± SD (*p>0.05*; n=14 control, n=15 SB).

Lymphocyte infiltration into the villi

We found a slight increase in lymphocyte infiltration into the lamina propria of villi in the ileum collected from the SB treated mice compared to the control group. The mean infiltration scores for CON and SB groups were 2.3 and 2.5 respectively (*p=0.018*; Fig 6).

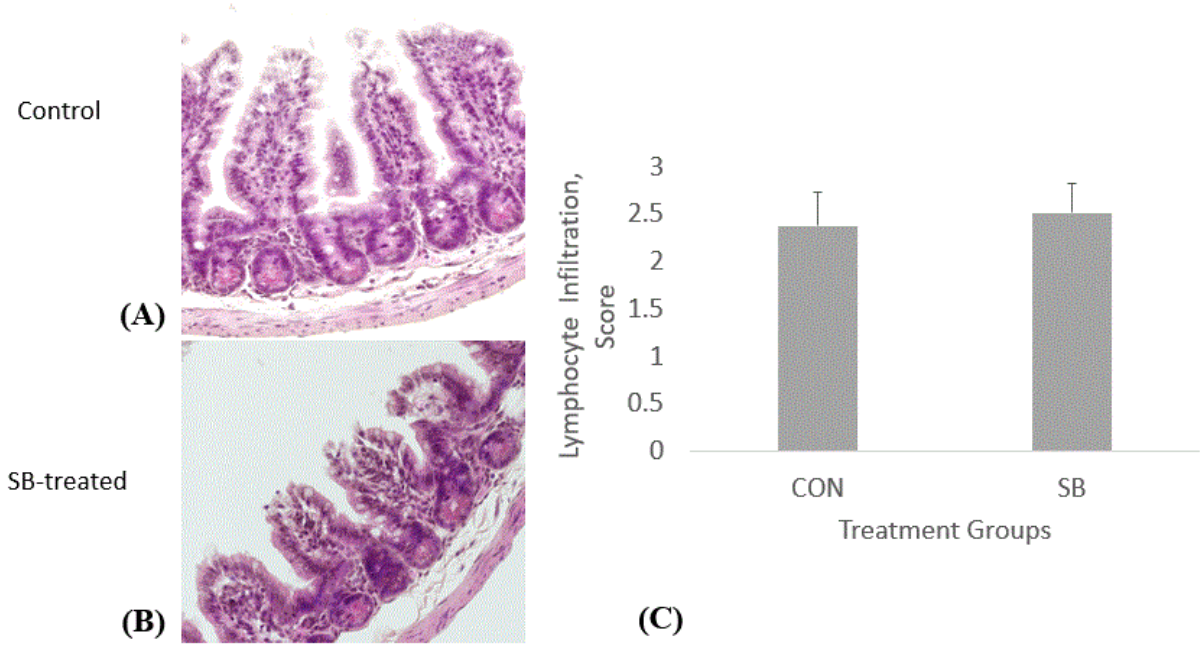

Figure 6. Lymphocyte infiltration into the lamina propria for (A) control (CON) and (B) sodium benzoate treated (SB) mice measured by a 1-4 scoring system. C) Quantitative data from two independent experiments combined and presented as mean ± SD (*p=0.018*; n=14 control, n=15 SB).

Gut microbial population

Bacterial culture results showed no significant differences among the relative abundance of the selected few bacterial populations (data not shown). Polymerase chain reaction results indicated that relative abundance of *Enterobacter* increased while *Bacteroidetes* and *Firmicutes* decreased significantly in SB treated mice ($p<0.01$; Fig 8, Fig 9). There was no difference in the relative abundance of *Eubacteria*, *Bifidobacterium*, *Bacillus*, *Escherichia coli*, *Lactobacillus*, *Actinobacteria*, *Enterococci*, and *Klebsiella* ($p>0.05$; Fig 7, Fig 8, Fig 9).

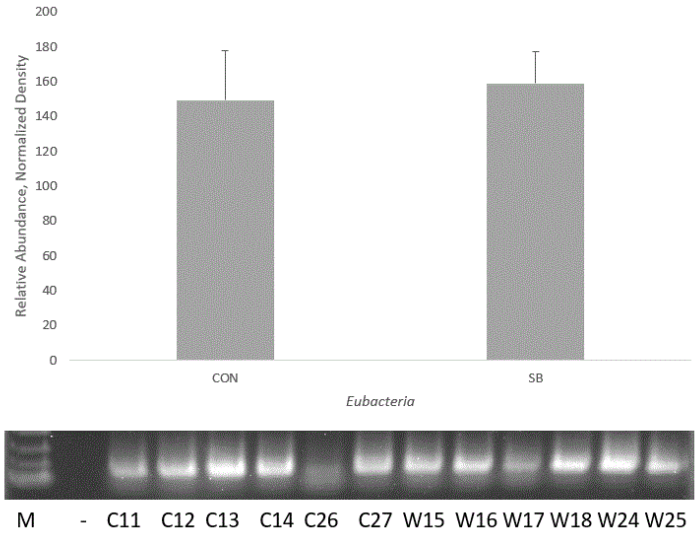

Figure 7. Bar graph showing the average relative abundance of *Eubacteria* in SB treatment in comparison to control group (*p>0.05*). Data combined from two independent experiments and presented as mean ± SD (n=6).

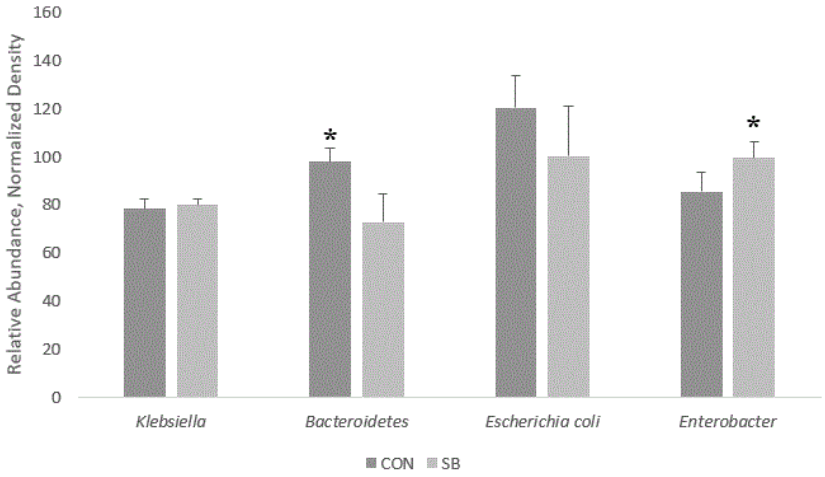

Figure 8. Bar graph showing the average relative abundance of gram-negative bacteria in SB treatment in comparison to control group. Error bars depict one standard deviation. Data combined from two independent experiments and presented as mean ± SD (n=6). Asterisks indicate statistical significance at *p<0.05*.

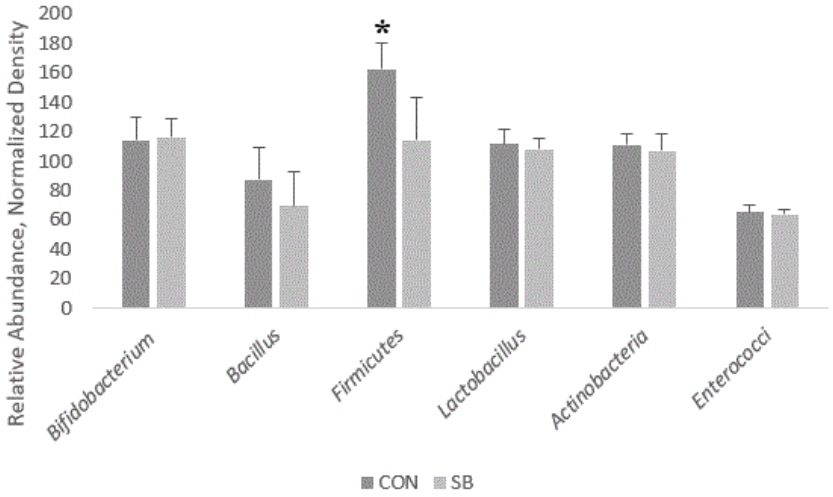

Figure 9. Bar graph showing the average relative abundance of gram-positive bacteria in SB treatment in comparison to control group. Data combined from two independent experiments and presented as mean ± SD (n=6). Asterisks indicate statistical significance at $p<0.05$.

Discussion

The role of diet and lifestyle on gut homeostasis and intestinal integrity have gained much attention in recent years (Conlon and Bird, 2014). As a result of lifestyle changes, the amount of consumption of processed and preserved food is increasing across all age groups worldwide. Consumed at low levels, SB is considered as safe at up to 0.1 percent by weight although regulations do not take into consideration the quantity of exposure to products such as soft drinks containing both ingredients ascorbic acid and sodium benzoate. In our study, it is supported that the treated mice were consuming the expected calculated dose of SB because there was no change in water consumption across treatment groups. This study provides a preliminary set of observations on the functional significance of ileal intestinal health in response to the FDA approved limit of SB in the diet.

Paneth cell granular density was not affected by SB consumption

Overall PC production of AMPs were unchanging in our murine model systems. Paneth cells function in innate immunity by releasing different microbicidal peptides against bacteria and bacterial antigens appropriately (Ayabe et al., 2000). In our study, the abundance of two gram-negative and one gram-positive bacterial populations were altered in response to SB. Although there was no induced response of PC granulation in the ileum, these bacterial changes may be changing AMP production more specifically. For example, lysozymes are antibacterial proteins more active against gram-positive bacteria (Elphick et al., 2005). Further studies should be conducted to assess which AMPs are being produced to regulate intestinal microbiome in response to SB.

The effect of SB on leukocyte infiltration in the villi of ileum

Sodium benzoate consumption for 30 days stimulates lymphocyte infiltration into the lamina propria, mimicking trends of conditions such as Helicobacter pylori infection, syphilis, celiac sprue, Menetrier disease, Chron's disease and more (Carmack et al., 2009). Impairment of the immune cells in the small intestine may have adverse health effects as the epithelial wall acts as a microbiological barrier between luminal contents and the rest of the body. If unregulated, this increased inflammatory response poses the threat of compromising the integrity of the intestinal mucosal health. Our data fail to the support the null hypothesis even though the numerical difference between the two treatment groups was very small. This may be due to the large number of villi examined per animal. It is difficult to imply any biological significance for such a marginal difference. A repeated experiment is necessary to confirm our findings.

Sodium benzoate increased the feed intake and altered gut bacterial population

The mechanisms responsible for SB induced increase in feed intake are currently unknown. However, the increase in food intake and lack of change in body weight gain may suggest possible malabsorption or increased metabolism. The intestines contain a large variety of microorganisms of different species important in development, health, and predisposition to disease (Canny and Mccormick, 2008). PCR based techniques are commonly used to provide quantitative information on intestinal microbiota (Huijsdens et al., 2002). The change in bacterial population may be responsible for this lack in body weight gain. Decreased levels of *Bacteroidetes* as observed in our study, can be linked to the increase in food consumption and has been linked to increased BMI (Koliada et al., 2017). Gram-positive and gram-negative bacteria such as *Escherichia coli* and *Bacillus coagulans* are restricted by integrating ε-polylysine into diet that differed further based on subject gender (You et al., 2017). Our study

included both males and females and therefore our results could be confounded because of the gender induced changes in microbial population.

In our study *Bacteroidetes* and *Firmicutes* decreased while *Enterobacter* increased in relative abundance. However, *Enterobacteriaceae* is not a consistent fractional species within intestinal microbiota (Schierack et al., 2007). Both *Firmicutes* and *Bacteroidetes* play a role in healthy gut microbiota and have implications to intestinal diseases (Kaufmann et al., 2007). Decreased *Bacteroidetes* may be responsible for increased food consumption in our animals supported by studies that found higher BMI in animals with this same trend (Koliada et al., 2017). Rats fed high fat diets (HFDs) showed significantly greater presence of gram-positive bacteria, including *Firmicutes*, and animals were reported to be more susceptible to metabolic and gastrointestinal diseases (Crawford et al., 2019). Although we currently do not understand the complexity of these bacterial interactions, the significant changes observed in these two phyla may be of importance.

Summary and Implications

Preservatives are necessary for preventing physical change or spoilage by microbial growth in food products. Our study showed alterations in the relative abundance of a few selected bacterial species in response to SB treatment. Our data also showed increased food intake that correlated with the change in the gut microbes. Future studies are necessary to confirm the changes in mucosal immunity as well as to understand the mechanism of how SB changes the bacterial population. Any changes to behavior or in intestinal metabolite absorption may reveal the role of commensal microorganisms play in the body in response to diet. While these additional studies will be necessary to understand the long-term effects of SB, we predict SB alter gut mucosal immunity mediated though the modified the gut microbial metabolism.

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