EFFECTS OF ARTIFICIAL SWEETENERS ON GUT MICROBIOME, METABOLOME AND INFLAMMATION

by

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(Under the Direction of Travis C. Glenn)

ABSTRACT

Despite wide use in our diet, the effects of artificial sweeteners on human health have been inconsistent, with both beneficial and adverse outcomes being reported. Maintaining the balance of gut microbiota and its metabolic functions is vital for human health. However, this balance can be disrupted by various external factors, including chemicals from the diet. Interestingly, it has been reported that artificial sweeteners, such as saccharin, could alter gut microbiota and induce dysbiosis. Inflammation is one of the most common physical conditions associated with the dysbiosis of gut microbiota, which is involved in several diseases, such as inflammatory bowel disease, diabetes, and obesity. Acesulfame-potassium (Ace-K), sucralose and saccharin are three commonly used artificial sweeteners that are found in many foods and beverages and are generally considered to be safe when consumed within the approved amounts. However, little is known about the functional impact of these artificial sweeteners on gut microbiota. In this dissertation, the effects of Ace-K, sucralose and saccharin on gut microbiota and the changes of fecal metabolic profiles were explored using metagenome sequencing and metabolomics technologies. Also, inflammatory signs were investigated after the consumption of artificial sweeteners. All of these three artificial sweeteners perturbed the gut microbiota and

metabolites in mice and increased the bacterial genes that could induce inflammation. In addition, Ace-K consumption for four weeks increased body weight gain and enriched functional bacterial genes of energy metabolism in CD-1 mice with highly sex-specific effects. Consumption of sucralose and saccharin for six months elevated expression of pro-inflammatory genes in the livers of C57BL/6J male mice. Collectively, the results may provide novel understanding of the interaction between artificial sweeteners and gut microbiota, and their potential role in the development of inflammation.

INDEX WORDS: Artificial sweeteners, Acesulfame potassium, Sucralose, Saccharin, Gut microbiota, Metabolomics, Inflammation, Energy metabolism.

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DEDICATION

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

OVERVIEW ON ARTIFICIAL SWEETENERS, GUT MICROBIOTA AND INFLAMMATION

Artificial sweeteners: common use and potential adverse effects.

Artificial high-intensity sweeteners, are ingredients that can sweeten foods with a much higher intensity of sweetness than table sugar (sucrose) and thus very small amounts of artificial sweeteners can provide the same sweetness as sugar. Usually, artificial sweeteners provide zero or very few calories because of the small amount used in food or drinks. The FDA has approved the use of the following six artificial sweeteners as food additives: saccharin, aspartame, acesulfame potassium (Ace-K), sucralose, neotame and advantame (Table 1.1).

Artificial sweeteners are heavily used sugar substitutes in a variety of food and beverages, which are recommended to the population for the reduction of sugar intake by the American Heart Association and the American Diabetes Association (Gardner et al., 2012). Also, it is stated that artificial sweeteners can be safely consumed within current federal nutrition recommendations by the Academy of Nutrition and Dietetics (Fitch, Keim, Academy of, & Dietetics, 2012). Consumption of artificial sweeteners is common worldwide, and the use increased in the United States according to the National Health and Nutrition Examination Survey (NHANES) data from 1999-2000 to 2007-2008, which showed that the prevalence of beverages containing artificial sweeteners doubled in the US from 6.1% to 12.5% among children and from 18.7% to 24.1% among adults (Sylvetsky, Welsh, Brown, & Vos, 2012). From

1999 to 2004, more than 6000 new products containing artificial sweeteners were introduced into the market in the US, and it was stated that in the year 2008, 65 percent of American households bought products containing sucralose (Yang, 2010).

Sugars provide a large amount of easily absorbed calories, and the replacement of sugars with artificial sweeteners decreases the calories in a diet. Thus, most people choose artificial sweeteners to lose or maintain body weight (Gardner et al., 2012). Despite the broad use of artificial sweeteners as an attempt to reduce calorie intake, their reported effects on human health are not consistent (Bryant, Wasse, Astbury, Nandra, & McLaughlin, 2014; Shankar, Ahuja, & Sriram, 2013). In a study with predominantly female overweight subjects, consuming supplemental drinks and foods with artificial sweeteners was observed to maintain body weight whereas supplemental drinks and foods with sucrose increased body weight (Raben, Tatjana H. Vasilaras, Møller, & Astrup., 2002). Other studies found different results (Ebbeling et al., 2006; Polyak et al., 2010; Tate et al., 2012; Yang, 2010), and Swithers, Martin, Clark, Laboy, & Davidson (2010) went on to show in a study of saccharin in rodents that this decrease in calories may be compensated by an increase in food consumption, leading to more body weight gain. Some artificial sweeteners, such as aspartame, Ace-K and saccharin, were shown to increase appetite and the subsequent energy intake (Yang, 2010) and still others found that the consumption of artificial sweetener could increase body weight in mice even without the compensation from a higher food intake (Polyak et al., 2010). Overall, the effect of artificial sweeteners on body weight are inconsistent.

In addition to obesity, sugar consumption is one of the contributing factors for a series of other health issues, including diabetes and coronary heart disease (Malik, Popkin, Bray, Despres, & Hu, 2010). Artificial sweeteners were also observed to increase the risk of many diseases, such

as diabetes, metabolic syndrome and cardiovascular diseases, in both normal and obese populations (Pepino, Tiemann, Patterson, Wice, & Klein, 2013; Swithers, 2013). In addition, augmented glucagon-like peptide 1 (GLP-1, an incretin hormone that increases glucose-dependent insulin secretion) was found in youths—healthy and with type 1 diabetes, who consumed diet soda before a glucose load (Brown, Walter, & Rother, 2009, 2012). Cong et al (2013) found that male mice exposed to the artificial sweetener Ace-K for 40 weeks had impaired cognitive functions (Cong et al., 2013). Moreover, despite the fact that consumption of artificial sweeteners is increasing in children, the potential long term effects in children are not well understood. Consumption may in fact be problematic since exposure to hyper-sweetened diets at young ages may lead to persistent hyper-sweet preferences throughout adulthood (Swithers, 2015). Thus, a full understanding of the effects of artificial sweeteners on human health is necessary.

Several mechanisms concerning artificial sweeteners and the health problems have been suggested. Artificial sweeteners provide sweetness but not calories, potentially preventing the activation of the food reward pathways completely through neuronal system, which could promote appetite and induce more food intake (Yang, 2010). Also, consumption of artificial sweeteners could change flavor preference and result in more sweetness dependence (Yang, 2010). Another mechanism is that artificial sweeteners can be detected by sweet-taste receptors in both lingual taste buds and gut mucosa, which could affect the metabolic process of the host (Brown, de Banate, & Rother, 2010). In recent years, accumulating evidence suggests that artificial sweeteners could interact with and perturb gut microbiota, which may lead to health issues. For example, some commonly used artificial sweeteners, such as Ace-K, saccharin and sucralose, pass through the intestines un-metabolized (Byard & Golberg, 1973; Roberts,

Renwick, Sims, & Snodin, 2000; von Rymon Lipinski & Gert-Wolfhard, 1985), so they interact with the gut microbiota directly. Consumption of saccharin for 11 weeks was found to change the gut microbiota in mice, which further induced glucose intolerance. In the same study, it was also found that saccharin could modulate the microbiota directly in an in vitro exposure, and the modulated microbiota would induce glucose intolerance in germ free mice (Suez et al., 2014). Abou-Donia, El-Masry, Abdel-Rahman, McLendon & Schiffman (2008) found that products containing sucralose changed the gut microbiota (Abou-Donia, El-Masry, Abdel-Rahman, McLendon, & Schiffman, 2008). Along with the alteration of gut microbiota, signs of inflammation were also found in these artificial sweeteners studies, such as the upregulation of LPS biosynthesis pathway and lymphocytic infiltrates into epithelium (Abou-Donia et al., 2008; Suez et al., 2014). However, more information is needed to identify the impact of artificial sweeteners on gut microbiota and their corresponding function, since the gut microbiota is strongly related to human health.

Gut microbiota and function in human health.

Humans have trillions of microbial cells, including bacteria, fungi, viruses and parasites, inside the gastrointestinal tract, mostly in the distal intestine, and the number of gut microbes is approximately 10 times greater than that of human cells. Only a small fraction of these microbes can be cultured. Recent massive sequencing has allowed for the identification of the gut microbes, and it has been found that over 99% of the metagenome is from over 1000 species of bacteria, encoding 100-fold more genes than the human genome (Qin et al., 2010).

In a healthy condition, the gut microbes confer many benefits to the host, including digestion and fermentation of food, detoxification of xenobiotics, maturation of host immune system, enteric nerve regulation and prevention of colonization by pathogens (Holmes, Li,

Athanasiou, Ashrafian, & Nicholson, 2011). The host, in turn, provides niches and nutrients to the microbes and also secrets antibodies controlling their expansion to form a symbiotic system (Kamada, Seo, Chen, & Nunez, 2013). Increasing evidence indicates that maintaining a healthy gut microbiota is vital for the health of the human body. For instance, germ-free mice showed apparent abnormalities in the intestines and the immune system, and were found to be resistant to diet-induced obesity (Backhed, Manchester, Semenkovich, & Gordon, 2007; Macpherson & Harris, 2004). In humans, Firmicutes and Bacteroidetes are the predominant bacteria representing more than 90% of the total species (Turnbaugh et al., 2009). However, the species in each phyla and their relative proportions are dramatically different between individuals, and microbes with low-abundance also display important functions, such as antibiotics resistance (Arumugam et al., 2011). Gut microbiota varies enormously between individuals according to gene sequencing result, but the functional genes are more similar in the population which means that even individuals sharing few gut microbes might have gut microbiome with similar functions (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012).

As a dynamic system, numerous internal and external factors can modify the gut microbiota and the related functions, such as age, gender and genetic background of the host, diet, use of drugs and antibiotics, states of disease and stress, exercise and exposure to environmental pollutants (Choi et al., 2013; Nicholson et al., 2012). Modifications to the gut microbiota can enhance or disrupt the homeostasis between gut microbiota and the host. If the homeostasis is disrupted, many adverse health results may occur, such as inflammatory bowel disease (IBD), cardiovascular disease, obesity, diabetes, allergies and cancer (Ley, Peterson, & Gordon, 2006). The disturbance of gut microbiota can be revealed from gut microbial, metagenetic and metabolic perspective.

Imbalanced gut microbes and their functional genes were found to be involved in various diseases and toxicological processes. For example, adults with type 2 diabetes have dissimilar gut microbiota from non-diabetic ones (Larsen et al., 2010). Disturbed gut microbiota by bacterial infection have been found to affect the biotransformation of arsenic in mice intestines, which could affect the subsequent toxicological effects (Lu et al., 2013). More Firmicutes and less Bacteroidetes was found in obese mice than in their lean littermates (Turnbaugh et al., 2006), and the obesity-related phenotypes are proven to be transmissible with fecal microbial communities in a study transferring fecal microbes from obese and lean twins to germ-free mice (Ridaura et al., 2013). A remarkable increase in the abundance of one bacteria—Bilophila wadsworthia and metabolite—taurocholic acid induced by high dietary fat diet was found to promote colitis in IL-10 deficient mice (Devkota et al., 2012). Also, the overgrowth of pathogens or opportunistic pathogens resulted in diseases of the host. For example, Escherichia coli in gut could induce the progress of IBD (Arthur et al., 2012). Bacterial genes involved in nitic oxide production were found to increase in obesity and IBD (Greenblum, Peter J. Turnbaugh, & Borenstein., 2012).

Previous studies also revealed that the diversity of the gut microbiota is very important for human health. For example, Turnbaugh et al (2009) found that reduced bacterial diversity was associated with obesity in humans and Abrahamsson et al (2012) found that infants with atopic eczema have lower diversity of gut microbiota compared to healthy subjects.

Besides the gut microbes, large amount of metabolites produced by gut microbes and the host were involved in health issues of the host. Choline, trimethylamine N-oxide (TMAO), and betaine metabolized from dietary lipid phosphatidylcholine by the gut microbes have been shown to be modulated in cases of obesity, diabetes and cardiovascular diseases (Kim, Seo, & Cho,

2011; Wang et al., 2011). In addition, short chain fatty acids (SCFAs) fermented by gut microbes, which can be affected by host, environmental and microbiological factors, can suppress inflammation in the host (Macfarlane & Macfarlane, 2003; Tremaroli & Backhed, 2012).

Gut microbiota related inflammation in diseases.

Interaction between the gut microbiota and the mucosal immune system is continuous before and after the birth of an infant (Jimenez et al., 2008). The gut mucosal system includes the epithelial barrier lined by intestinal epithelial cells (IECs), which are sealed by the apical junctional complex (Turner, 2009), immune tissues and cells, such as immune inductive lymphoid tissue, lymphocytes and plasma cells, and the mucus gel layer, containing secreted mucins and high concentration of antimicrobial molecules such as defensins and secretory IgA (Maloy & Powrie, 2011). Several studies found that the gut microbiota were required for the development of some immune tissues and cells such as gut-associated lymphoid tissues, T helper 17 (TH17) cells, regulatory T (Treg) cells, IgA-producing B cells and plasma cells (Kamada et al., 2013). Extensive deficits in the immune system of germ free mice also support this requirement (Round & Mazmanian, 2009). Gut microbes interact with the host mucosa directly via the recognition of pathogen-associated molecular patterns (PAMPs), such as LPS, flagellin, and bacterial DNA and RNA, by pattern recognition receptors (PRRs) of the mucosa (Maloy & Powrie, 2011), or indirectly through secreted metabolites, such as SCFAs, secondary bile acids, and indole derivatives (Nicholson et al., 2012). In a healthy condition, a homeostasis exists in the system, including a balance within the gut microbes and the physiological interaction between the gut microbiota and the intestinal mucosal system (Cerf-Bensussan & Gaboriau-Routhiau, 2010). Inflammation is one common condition associated with breakdown of this homeostasis.

Several inflammatory diseases are related to gut microbiota, including inflammatory bowel diseases (IBD) and obesity. IBD with two main forms—Crohn's Disease (CD) and Ulcerative Colitis (UC), is a global disease with increasing incidence and prevalence, and it affects more than one million individuals in the United States (Kaplan, 2015; Molodecky et al., 2012). IBD is characterized by acute or chronic inflammation with unclear aetiology, and a disrupted gut microbiota is one of the major triggering factors, in addition to genetic factors and a susceptible immune system (Hill & Artis, 2010; Xavier & Podolsky, 2007). In subjects with IBD, bacterial members in phyla Firmicutes and Bacteroidetes are depleted compared to non-IBD controls, and bacteria such as Faecalibacterium prausnitzii were found to help protecting humans against IBD by induction of IL-10, while some bacteria such as Escherichia coli promote the development of IBD (Frank et al., 2007; Kamada et al., 2013). Accumulating evidence demonstrates that low-grade inflammation induced by gut microbiota is correlated with metabolic diseases, such as obesity and diabetes (Holmes et al., 2011). Low grade inflammation has been found in obese individual in adipose tissue and systematically, and a study with bariatric surgery (an efficient method to reduce body weight in obese individuals) has shown that Faecalibacterium prausnitzii is directly related to the reduction of low-grade inflammation in cases of obesity and diabetes (Furet et al., 2010). Also, experiments with antibiotic treatment in mice indicated that gut microbiota could modulate inflammation in diet-induced obesity and diabetes through production of metabolic endotoxemia and regulation of intestinal permeability (Cani et al., 2008). Moreover, chronic inflammation induced by gut microbes can aggravate the progression of colorectal cancer (Uronis et al., 2009).

Artificial sweeteners, gut microbiota and inflammation.

Artificial sweeteners can disturb the gut microbiota and the corresponding functions, which can serve as inflammation triggers to the host (Figure 1.1). Several signs shown in previous studies provide support for this statement. For example, the bacterial LPS-related gene was sharply increased by saccharin consumption in mice (Suez et al., 2014), and a product containing sucralose was found to alter the gut microbiota and induce inflammatory lymphocytes infiltration (Abou-Donia et al., 2008). The aim of this dissertation is to investigate the effects of artificial sweeteners on the gut microbiome, metabolome and inflammation, and to inspect the relationships among them.

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Tables

Table 1.1 FDA approved artificial sweeteners

	Nutrition	Multiplier of Sweetness Intensity Compared to Table Sugar (Sucrose)	US FDA Acceptable Daily Intake (ADI)
Sucralose	Non-nutritive	600 x	5mg/kg b.w.
Aspartame	Nutritive 4cal/g*	200 x	50mg/kg b.w.
Saccharin	Non-nutritive	200-700 x	15mg/kg b.w.
Acesulfame K	Non-nutritive	200 x	15mg/kg b.w.
Neotame	Non-nutritive	7,000 to 13,000x	0.3mg/kg b.w.
Advantame	Non-nutritive	20,000 x	32.8mg/kg b.w.

*The calories intake is insignificant because of the small amount consumed.

Figures

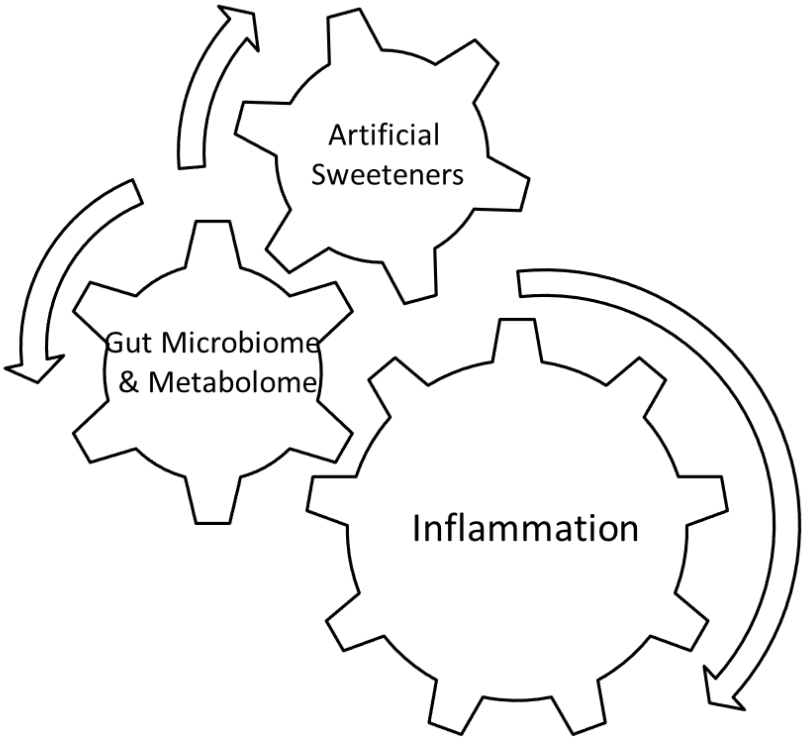

Figure 1.1 Proposed relation between artificial sweeteners, gut microbiome and metabolome, and inflammation.

CHAPTER 2

ARTIFICIAL SWEETENER ACESULFAME POTASSIUM PERTURBS GUT BACTERIA AND ENERGY HOMEOSTASIS IN CD-1 MICE[1]

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Abstract

Artificial sweeteners have been widely used in our diet and the effects of artificial sweeteners on human health have been inconsistent, with both beneficial and adverse outcomes being reported. Obesity and Type 2 diabetes have dramatically increased in the U.S. and other countries over the last two decades. Numerous studies have indicated an important role of gut microbiome in body weight control and glucose metabolism and regulation. Interestingly, it has been reported that artificial sweeteners, such as saccharin, could alter gut microbiota and induce glucose intolerance, raising the question about the contribution of artificial sweeteners on the global epidemic of obesity and diabetes. Acesulfame-potassium (Ace-K), an FDA approved artificial sweetener, is commonly used, but its toxicity tests are considered inadequate. In particular, the functional impact of Ace-K on the gut microbiome is unknown. In this study, we explored the effects of Ace-K on the gut microbiome and the changes of fecal metabolic profiles using 16S rRNA sequencing and GC-MS metabolomics. We found that Ace-K consumption perturbed the gut microbiome in CD-1 mice after a 4-week treatment. Body weight gain, shifts of gut bacterial community compositions, enrichment of functional bacterial genes for energy metabolism, and fecal metabolomic changes were highly-sex specific. Collectively, our results may provide novel understanding of the interaction between artificial sweeteners and the gut microbiome and their potential role in the development of obesity and Type 2 diabetes and associated chronic inflammation.

Introduction

As widely used food additives and sugar substitutes, artificial sweeteners can enhance favor and simultaneously reduce caloric intake. Some epidemiological studies indicate that artificial sweeteners are beneficial for weight loss and also good for those who suffer from

glucose intolerance and type 2 diabetes mellitus (Gardner et al., 2012). However, accumulating evidence in recent years suggests that the consumption of artificial sweeteners could perturb human metabolism, especially glucose regulation (Pepino & Bourne, 2011; Suez et al., 2014). Artificial sweeteners have been found to cause glucose intolerance and induce metabolic syndrome, and they are also associated with higher body weight gain (Brown, De Banate, & Rother, 2010; Dhingra et al., 2007; Fowler et al., 2008; Suez et al., 2014). These findings suggest that artificial sweeteners may increase the risk of obesity and type 2 diabetes. However, the specific mechanism of how artificial sweeteners result in dysregulate of energy balance remains elusive.

Recently, much attention has been paid to the regulating effects of gut microbiota on host health. The gut microbiome is deeply involved in host metabolism and plays a crucial role in food digestion and energy homeostasis in the human body (Flint, Bayer, Rincon, Lamed, & White, 2008; Nicholson et al., 2012; Turnbaugh et al., 2006). Commensal microflora colonization is necessary for immune system development, enteric nerve regulation and pathogen prevention (Heijtz et al., 2011; Hooper, Littman, & Macpherson, 2012; Mazmanian, Liu, Tzianabos, & Kasper, 2005; Taguchi et al., 2002). However, multiple environmental factors, like diet, antibiotics and heavy metals, can disrupt the ecological balance in the gut (Glenn & Roberfroid, 1995; Jakobsson et al., 2010; Yatsunenko et al., 2012). Dysbiosis of the gut microbiome is associated with a series of human diseases, including obesity, diabetes, and inflammatory bowel disease (Guarner & Malagelada, 2003; Turnbaugh et al., 2006). Previous studies found that non-caloric artificial sweeteners, such as saccharin, impaired glucose tolerance by modulating gut bacteria composition, which is also associated with type 2 diabetes (Suez et al., 2014). Likewise, another study found that consumption of Splenda, a nonnutritive sweetener

with 1% sucralose, impaired the growth of gut bacteria in rats (Abou-Donia, El-Masry, Abdel-Rahman, McLendon, & Schiffman, 2008). However, the effects of artificial sweeteners on gut microbiota and its metabolism are still largely unknown. In addition, chronic inflammation commonly occurs in obesity and diabetes, raising the potential role of disrupted gut bacteria in host inflammation.

Acesulfame-K (Ace-K) is one of the major low-calorie artificial sweeteners in our diet. Previous studies has found that Ace-K is genotoxic, and can inhibit glucose fermentation in intestinal bacteria (Bandyopadhyay, Ghoshal, & Mukherjee, 2008; Pfeffer, Ziesenitz, & Siebert, 1985). A recent study found that overall bacterial diversity was different across nonconsumers and consumers of artificial sweeteners, including Ace-K and aspartame after four-day consumption in heathy human adults in the United States (Frankenfeld, Sikaroodi, Lamb, Shoemaker, & Gillevet, 2015), but the doses of artificial sweeteners were only estimated based on a four-day food record. How Ace-K perturbs the gut microbiome and whether it leads to functional changes of the gut is still unknown. In particular, the interaction between host and gut microbiome is complicated, and many host factors could influence the gut microbiome response to external stimuli. Among these factors, sex emerges as important yet largely unexplored one. Previous research demonstrated that females and males have dramatically different physiological conditions and gut microbiome patterns (Clarke et al., 2013; Markle et al., 2013). We have demonstrated sex-dependent gut microbiome response to exposure to xenobiotics, such as arsenic and organophosphates (Chi et al., 2016; Markle et al., 2013). For example, gut bacteria shift and arsenic metabolism pattern are different between male and female mice, which is consistent with sex-specific disease outcomes (X. Cong et al., 2016; Lindberg et al., 2008; Rahman et al., 2006). Therefore, the effects of Ace-K on the gut microbiome of male and female

animals were examined respectively in this study to define sex-specific gut microbiome response, a current gap in microbiome research.

In this study, we studied the effects of Ace-K on the gut microbiome and the changes of fecal metabolites using 16S rRNA sequencing and GC-MS metabolomics. We found that Ace-K consumption perturbed the gut microbiome in CD-1 mice after a 4-week treatment. Body weight gain, shifts of gut bacterial community compositions, enrichment of functional bacterial genes and fecal metabolomic changes were highly-sex dependent. Specifically, Ace-K increased the body weight gain in male but not female mice. Functional genes involved in energy metabolism were activated and inhibited in male and female mice, respectively. Moreover, differential changes of fecal metabolic profiles were observed between male and female animals. Taken together, these results may provide novel insight into understanding functional interaction between artificial sweeteners and gut microbiome and its role in the development of obesity and chronic inflammation.

Materials and Methods

Animals and exposure. CD-1 mice (~7 week old) were purchased from Charles River and provided standard pelleted rodent diet and tap water ad libitum under the environmental conditions of 22°C, 40–70% humidity, and a 12:12 hr light:dark cycle. All 20 mice (10 male and 10 female) were housed in the University of Georgia animal facility for a week before the experiment was initiated. Then, mice were randomly assigned to control, or Ace-K group (five male and five female mice in each group). Water (control) and artificial sweeteners were administered to mice (~8 weeks old) through gavage for 4 weeks, with 37.5mg/kg body weight being administrated to animals. This dose was equivalent or much lower than previous animal studies using Ace-K (Bandyopadhyay et al., 2008; W. N. Cong et al., 2013). Body weight was

measured before and after the treatment. All experiments were approved by the University of Georgia Institutional Animal Care and Use Committee. The animals were treated humanely and with regard for alleviation of suffering.

16S rRNA gene sequencing. DNA was isolated from frozen fecal pellets collected at different time points using PowerSoil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions, and the resultant DNA was quantified and stored in -80°C for further analysis. A total of 1ng of the purified DNA was used to amplify the V4 region of 16S rRNA of bacteria using universal primers of 515 (5'-GTGCCAGCMGCCGCGGTAA) and 806 (5'-GGACTACHVGGGTWTCTAAT). Individual samples were barcoded and pooled to construct a sequencing library, and then sequenced by Illumina MiSeq at the Georgia Genomics Facility to generate pair-end 250 + 250 (PE250, v2 kit) reads to a depth of at least 25,000 reads per sample. The raw mate-paired fastq files were merged and quality-filtered using Geneious 8.0.5 (Biomatters, Auckland, New Zealand) with error probability limit set as 0.01. The data then were analyzed using quantitative insights into microbial ecology (QIIME, version 1.9.1). UCLUST was used to get the operational taxonomic units (OTUs) with 97% sequence similarity. The data was assigned at five different levels: phylum, class, order, family and genus.

Functional gene enrichment analysis. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Galaxy Version 1.0.0) was first used to analyze the enrichment of functional genes of the microbiome of each group (Langille et al., 2013). PICRUSt can accurately profile the functional genes of bacterial communities based on the marker genes from 16S sequencing data and a database of reference genomes (Bunyavanich et al., 2016; Garcia-Mazcorro et al., 2016; Sheflin et al., 2016). PICRUSt has been widely used in microbiome functional gene enrichment analysis, with ~95% accuracy being reported for

bacterial metagenome (Bunyavanich et al., 2016; Garcia-Mazcorro et al., 2016; Sheflin et al., 2016). Results from PICRUSt were imported to Statistical Analysis of Metagenomic Profiles (STAMP) (version 2.1.3) for further statistical analysis and visualization (Parks, Tyson, Hugenholtz, & Beiko, 2014).

Metabolomics analysis. Metabolites were extracted from fecal samples using methanol and chloroform as described previously (Lu et al., 2014). Briefly, 20mg feces was vortexed with 1ml of methanol/chloroform/water solution (2:2:1) for 1 hour, followed by centrifugation at 3,200 x g for 15 minutes. The resultant upper phase and lower phase was transferred to a HPLC vial and dried for about 4 hours in SpeedVac, and derivatized using N,O Bis(trimethylsilyl)trifluoroacetamide (BSTFA). Agilent 6890/5973 GC-MS system equipped with a DB-5ms column (Agilent, Santa Clara, CA) was used to conduct the metabolomic profiling and capture all detectable metabolites features at mass range of 50 to 600 m/z. The XCMS Online tool was used to pick up and align peaks and calculate the accumulated peak intensity.

Statistical analysis. The difference in the individual gut microbiota between 0-day and 4-week was assessed with Mothur software (Schloss et al., 2009). Heat maps were used to visualize the clustering of functional gene enrichment results. To generate differences of metabolic profiles between the control and artificial sweetener groups, a two-tail Welch's t-test ($p<0.05$) was used. Also, partial least squares discriminant analysis (PLS-DA) was performed to examine the difference of metabolomes in different groups.

Results

Ace-K increased body weight gain of male mice but not female. We first examined the body weight of mice after a four-week treatment. Clearly, male mice had a much higher body

weight gain than control male mice (10.28 g versus 5.44g, $p<0.001$). For female mice, the body weight gain was not statistically significantly different from controls, as shown in Figure 2.1A.

Ace-K altered the gut microbiome components in a sex-specific manner. Given the different body weight gain induced by Ace-K consumption and the crucial role of gut bacteria in host energy homeostasis, we further explored whether the Ace-K caused different effects on gut microbiota in male and female mice. Figure 2.1B and 2.1C show the genera of gut bacteria which have been significantly changed ($p<0.05$) in female and male mice. Notably, genus *Bacteroides* has been highly increased in Ace-K-treated male mice, which is consistent with previous studies (Gardner et al., 2012). Two other genera *Anaerostipes* and *Sutterella* also have significantly changed, as shown in Figure 2.1C. However, for female mice, four-week Ace-K consumption dramatically decreased the relative abundances of multiple genera, including *Lactobacillus*, *Clostridium*, an unassigned genus in *Ruminococcaceae* and an unassigned genus in *Oxalobacteraceae*, whereas *Mucispirillum* was increased, as shown in Figure 2.1B. These results indicated that the effects of Ace-K on gut microbiome composition were sex-dependent in mice.

Energy metabolism related functional genes have opposite alteration in male and female mice after Ace-K consumption. Gut microbiome composition profiles are generally associated with different functional gene pools. Therefore, we further investigated the functional gene alterations induced by Ace-K consumption. In Ace-K treated female mice, the genes in key energy metabolism pathways were widely decreased (Figure 2.2), which was consistent with the decrease of multiple genera in female mice. The relative abundance of numerous genes involved in carbohydrates absorption or transportation were significantly reduced, such as glucose uptake protein, lactose permease, monosaccharide-transporting ATPase, components in multiple sugar

transport system and D-allose transport system, as well as different types of PTS system. Likewise, multiple polysaccharide hydrolysis or degradation genes, like L-xylulokinase, D-xylonolactonase and alpha-amylase were also decreased in female animals administrated to Ace-K. However, the carbohydrates absorption and metabolism pathways were activated in treated male animals, which corresponds to a large increase of *Bacteroides*. Specifically, genes involved in sugar and xylose transportation and glycolysis and TCA cycle were increased, as shown in Figure 2.3A and 2.3B. And, the abundances of multiple genes in carbohydrates metabolism and fermentation pathways were also consistently increased (Figure 2.3C). Taken together, functional gene changes enriched by PICRUSt indicate that Ace-K consumption can induce sex-specific responses in carbohydrate metabolism of the gut microbiome.

Ace-K increased the gene abundances related to lipopolysaccharide synthesis.

Because the gut microbiome and metabolic syndrome are generally associated with systemic chronic inflammation, we investigated whether the perturbation of gut microbiota and its functional genes would contribute to inflammation. As shown in Figure 2.4A and 2.4C, genes involved in LPS synthesis have been widely increased in mice after Ace-K consumption. For example, in Ace-K treated female mice, LPS synthesis related genes were significantly increased, including UDP-glucose:(heptosyl) LPS alpha-1,3-glucosyltransferase, ADP-L-glycero-D-manno-heptose 6-epimerase, -amino-4-deoxy-L-arabinose transferase, UDP-D-GlcNAcA oxidase and UDP-GlcNAc3NAcA epimerase, and LPS exporting genes like lipopolysaccharide export system protein LptA and lipopolysaccharide export system permease protein, as well as a LPS-assembly protein. In addition, multiple genes encoding flagella components were also increased, including flagella basal body P-ring formation protein FlgA, flagellar L-ring protein precursor FlgH, flagellar P-ring protein precursor FlgI and flagellar FliL

protein, as shown in Figure 2.4B. For male mice, two genes participating in LPS biosynthesis, glycosyltransferase and UDP-perosamine 4-acetyltransferase, were upregulated (Figure 2.4C). Moreover, Ace-K consumption increased the abundance of one bacterial toxin synthesis gene, thiol-activated cytolysin, as shown in Figure 2.4D. These data suggest that the perturbation of gut microbiome by Ace-K consumption enriches the LPS synthesis related genes, which may increase the risk of occurrence of chronic inflammation in the host.

Ace-K significantly changes the fecal metabolites. Metabolites serve as signaling molecules for the complex crosstalk between host and gut bacteria. Therefore, we further investigated whether Ace-K consumption disturbed the fecal metabolic profiles. The cloud and PLS-DA plots, as shown in Figure 2.5, reveal that the gut microbial metabolome in Ace-K administrated animals were different from controls regardless of sex. However, the changes of specific metabolites were largely different in male and female mice, with the majority of metabolites being down-regulated or up-regulated in female and male mice, which is consistent with differential changes of gut bacteria community components and functional genes in male and female animals. Table 2.1 and 2.2 listed typical metabolites that were identified for female and male mice, respectively. Notably, multiple bacteria metabolism-related metabolites, such as lactic acid and succinic acid were decreased in female mice, as shown in Figure 2.6A. 2-Oleoylglycerol also largely decreased in females. In males treated with Ace-K, the concentration of pyruvic acid, a central metabolite of energy metabolism, was significantly higher than controls (Figure 2.6B). Interestingly, cholic acid (CA) was increased, whereas deoxycholic acid (DCA) was dramatically declined in feces of male mice (Figure 2.6B). Fecal metabolomics profiling results suggest that Ace-K consumption can significantly change the gut metabolic profiles, which could influence the crosstalk between host and gut microbiome.

Discussion

In this study, we applied DNA sequencing and metabolomics approaches to characterize the sex-specific effects of Ace-K consumption on gut microbiota and its metabolism. Ace-K consumption altered the gut bacteria compositions and metabolism profiles, and the perturbations was highly sex-dependent. Specifically, Ace-K increased the body weight gain in male mice but not in female. Ace-K induced differential gut bacterial composition changes in male and female mice. In addition, functional gene enrichment analysis shows a significant sex-specific effect, with numerous bacterial genes involved in energy metabolism being activated in male mice, but inhibited in females. Moreover, Ace-K may also increase the risk of development of chronic inflammation via disrupting gut bacteria and associated functional pathways. This study could provide novel insight into the effects of artificial sweetener consumption on host health, and highlight the role of gut microbiome and bacterial products in regulating host metabolic homeostasis. In addition, previous studies about effects of artificial sweeteners on gut microbiota and host health rarely considered the contribution of sex, however, our results emphasize the importance of sex in mediating gut microbiome and host response to compounds such as artificial sweeteners.

Although artificial sweeteners have been considered safe, accumulating evidence indicates that artificial sweeteners can induce glucose intolerance and disturb energy homeostasis in the human body. In particular, it has been demonstrated that the gut microbiome play a role in these processes (Gardner et al., 2012; Suez et al., 2014). In this study, the components of the gut microbiome changed in Ace-K treated mice (Figure 1B and 1C). Notably, we found that the abundance of genus Bacteroides was much higher in male mice treated with Ace-K than controls. This result is consistent with previous studies on artificial sweeteners (Suez et al.,

2014). For example, saccharin consumption was found to induce the over-growth of Bacteroides in male mice. Bacteroides is one of the most abundant and well-studied members of the commensal microbiota. Many Bacteroides species have extensive capability to utilize glycan and can produce fermentative end products, short-chain fatty acids (SCFAs), to supply nutrition and other beneficial properties to the host (Comstock, 2009; Turnbaugh et al., 2006). Likewise, Anaerostipes also increased in Ace-K treated male mice. As one of the major members of Firmicutes, genus Anaerostipes contains multiple SCFA-producing species like Anaerostipes butyraticus sp. nov. and Anaerostipes caccae (Eeckhaut et al., 2010; Remely et al., 2014; Sanz, Santacruz, & De Palma, 2008). High abundance of Bacteroides and Anaerostipes in the gut microbiome generally reflects a high capacity for energy harvest and is associated with obesity (Turnbaugh et al., 2006). Corresponding to the increase of these genera, functional gene enrichment analysis indicated that a number of genes involved in carbohydrates absorption, degradation and fermentation were consistently increased in Ace-K treated male mice. Moreover, pyruvate was also significantly increased male mice administrated with Ace-K (Figure 2.6B). Pyruvate is one of the key metabolites related to energy metabolism and can be further fermented to various short-chain fatty acids, such as propionate and butyrate (Cummings & Branch, 1986). Therefore, the significant increase of fecal pyruvate, as well as the Bacteroides and the genes in energy metabolism pathways, suggest that energy metabolism and harvest capacity was increased by Ace-K consumption in male mice. These results are consistent with a significantly higher body-weight gain in Ace-K treated males when compared to controls (10.28 g vs 5.44 g, $p<0.001$).

However, interestingly, the response of gut microbiota in female mice was remarkably different, or even opposite when compared to male mice. Four-week Ace-K consumption caused

decrease of multiple genera in gut bacteria, such as *Lactobacillus* and *Clostridium*, as well as an unassigned genus in *Ruminococcaceae* and in *Oxalobacteraceae*, as shown in Figure 2.1B. According to previous studies, bacteria in these genera play a crucial role in food digestion and polysaccharide fermentation (Ben David et al., 2015; Crittenden et al., 2002; Flint et al., 2008). For example, *Rumintoceaceae* is considered a major bacterial member to hydrolyze complex food fibers and gut fermentation, which plays important role in host energy utilization (Biddle, Stewart, Blanchard, & Leschine, 2013). Likewise, some species in *Clostridum* such as *Clostridium thermocellum* can produce glycoside hydrolase and participate in polysaccharides digestion (Flint et al., 2008). Moreover, functional gene enrichment analysis with PICRUSt show that the carbohydrates absorption, degradation and fermentation pathways were widely decreased in female mice after Ace-K consumption (Figure 2.3). Therefore, the significant decline of these bacterial components and functional genes indicated that Ace-K consumption impaired polysaccharides digestion and fermentation ability of gut microbiome in female mice, which could further influence the host energy harvest. This conclusion has been further supported by our GC-MS results that various fermentation products, such as lactic acid and succinic acid, were decreased in female mice treated with Ace-K (Figure 2.6A). Consequently, in contrast to the large increase of body weight gain in male animals, Ace-K consumption didn't significantly affect the body weight gain of female mice.

Type 2 diabetes and obesity are generally associated with systemic chronic inflammation. It has been well documented that toxic products generated by gut bacterial could enter into body circulation and induce chronic inflammation (Cani et al., 2008; Deitch, 2002; Wright, Ramos, Tobias, Ulevitch, & Mathison, 1990). In fact, our results on gut bacterial compositions and functional genes show that the Ace-K induced gut bacteria perturbation may increase the risk of

chronic inflammation. As shown in Figure 2.1C, *Bacteroides* and *Sutterella* were significantly increased in Ace-K treated male mice. A significant rise of *Bacteroides* genus not only increases the glycan-utilization ability, but also possible promotes the development of chronic inflammation. A previous study found that multiple selected commensal species *Bacteroides* species including *B. thetaiotaomicron* and *B. vulgatus* could induce colitis in mice with certain genetic backgrounds(Bloom et al., 2011). *Bacteroides fragilis* with enterotoxin gene sequences has been found widely present in patients with inflammatory bowel disease (Prindiville et al., 2000). Similarly, a recent in vitro study found that *Sutterella spp.* have pro-inflammatory capacity and adherence ability to intestinal epithelial cells, indicating they may play a role in host immunomodulation (Hiippala, Kainulainen, Kalliomäki, Arkkila, & Satokari, 2016). Likewise, *Mucispirillum* was highly increased after Ace-K consumption in female mice. The relationship between *Mucispirillum* and intestinal inflammation has been widely demonstrated in previous studies. It has been found to be increased in acute inflammation, active colitis and chemically induced colitis (Rooks et al., 2014; Schwab et al., 2014; Selvanantham et al., 2016). Another study also found that the abundance of *Mucispirillum* had a strong positive correlation with inflammation-related genes *Slc25a25* in inguinal adipose tissue (Ravussin et al., 2012). Taken together, these results show that Ace-K consumption may increase the risk of development of inflammation by disrupting the gut bacterial compositions.

Functional gene enrichment analysis also support that Ace-K may increase inflammation. The significant increase of lipopolysaccharide synthesis or modification genes are observed after Ace-K consumption, especially in female mice (Figure 2.4A and 2.4C). LPS, as an endotoxin, is a major component of the outer membrane in Gram-negative bacteria and the high concentration of LPS in blood can trigger inflammation and metabolic disorders. Moreover, thiol-activated

cytolysin also increased in Ace-K treated male mice (Figure 2.4D). Thiol-activated cytolysin is a prominent type of toxin of Gram-positive bacteria and is known as an important virulence factor (Billington, Jost, & Songer, 2000). It can stimulate the expression of inflammatory mediators, like cytokines, and induce inflammatory responses. Bacteria flagella is another factor that can influence the pathogenicity of gut microbiota and trigger host immune responses (Ramos, Rumbo, & Sirard, 2004). Multiple genes coding flagella components have been found to be increased in Ace-K treated female mice, such as FlgA, FlgH, FlgI and flagellar FliL protein (Figure 2.4B). This may indicate that the abundance of species with flagella have been increased after Ace-K consumption, which also potentially increases the risk of inflammation.

Moreover, Ace-K consumption might increase the risk of inflammation by weakening the anti-inflammatory mediators in gut. For example, *Lactobacillus* are beneficial probiotic bacteria to human in many aspects (Bernet, Brassart, Neeser, & Servin, 1994; Liu, Fatheree, Mangalat, & Rhoads, 2010; Madsen, Doyle, Jewell, Tavernini, & Fedorak, 1999; Peran et al., 2006), including anti-inflammatory effects. Previous studies found that *Lactobacillus* species could reduce colonic inflammation in rat with colitis (Peran et al., 2006) and also prevented colitis in *IL-10*–deficient mice (Madsen et al., 1999). Likewise, species in genus *Clostridium* also has similar effects, as shown by Koji Atarashi et al (Atarashi et al., 2011). Notably, we detected a significant decrease of 2-oleoyglycerol (2-OG) in Ace-K treated female mice (Figure 2.6A). Previous studies demonstrated that 2-OG stimulated the release of glucagon-like peptide-2 (GLP-2). GLP-2, secreted from enteroendocrine L-cells, can increases barrier function and decrease gut permeability and the LPS levels in host, which is beneficial to reduce inflammation (Duca & Lam, 2014). Therefore, the decline of *Lactobacillus* and 2-OG in Ace-K treated female mice suggests that the anti-inflammatory capacity may be impaired after consuming Ace-K.

Interestingly, the intensities of several bile acids in fecal samples from Ace-K treated male mice was affected (Figure 2.6B). It is known that bile acids participate in the lipid absorption and cholesterol homeostasis. Multiple previous studies show that normal bile acids metabolism plays a role in inflammatory response regulation (Duboc et al., 2013; Wang, Chen, Yu, Forman, & Huang, 2011). For example, Henri Duboc et al found that inflammatory bowel diseases were associated with bile acid dysmetabolism (Duboc et al., 2013). In addition, they observed that bacteria-catalyzed transformation from CA to DCA was dramatically decreased and the second bile acids ratio was significantly lower in germ free mice than conventional ones (Duboc et al., 2013). In this study, we found that CA and DCA was increased and decreased, respectively, after Ace-K treatment (Figure 2.6B).

Collectively, our data support that Ace-K consumption can lead to adverse effects on the gut microbiome in mice. Perturbation of gut bacteria could further influence the energy homeostasis and the risk of development of inflammation. Notably, distinct sex-specific effects were observed in this study. Ace-K consumption leads to a significant increase of body weight in male mice through disrupting the gut bacterial and activating bacterial energy harvest pathways. However, no significant increased body weight gain was observed for female mice. Down-regulated energy metabolism related pathways and short-chain fatty acids were found in Ace-K treated female mice, which could prevent the occurrence of the obese phenotype in female mice. Although gut microbiome plays a key role in regulating host metabolism, we could not rule out other mechanisms that may also contribute to Ace-K-induced alterations of energy homeostasis and body weight gain in mice. For example, some studies suggest that nonnutritive sweeteners could interfere with physiological responses that regulate energy homeostasis (Mattes & Popkin, 2009; Pepino & Bourne, 2011). Evidence also supports that artificial sweeteners may interact

with sweet taste-like receptors to influence the incretin release and further disturb energy homeostasis (Jang et al., 2007; Mattes & Popkin, 2009). Interestingly, 2-OG, which decreased in Ace-K treated female mice (Figure 2.6A), could activate GPR119 to stimulate the release of glucagon-like peptide-1(GLP-1), an incretin involved in the regulation of energy homeostasis (Hansen et al., 2011). GLP-1 promotes the glucose-induced insulin secretion and also can enhance satiety and reduces food intake by nutrient-sensing mechanisms (Duca & Lam, 2014; Everard et al., 2013). Taken together, gut microbiome responses and complex regulation might be involved in energy metabolism of host after artificial sweetener consumption. Future studies are warranted to further elucidate mechanisms involved.

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Tables

Table 2.1. Identified significantly changed metabolites ($p < 0.05$, compared to controls) in fecal samples of Ace-K consumed female mice.

Metabolites	Fold	p value	m/z	Retention time	Pathways or Functions
Phosphoric acid	2.43	0.034	310.1000	12.24	-
Urea	-1.35	0.035	132.1000	11.85	Urea cycle
Stearic acid	-1.40	0.018	271.2000	25.64	Fatty acids
Palmitic acid	-1.49	0.033	75.1000	23.40	Fatty acids
Isoferulic acid	-1.53	0.034	190.1000	23.92	Isoferulic acid
Linoleic acid	-1.58	0.014	475.0000	25.26	Fatty acids
Succinic acid	-1.74	0.031	133.1000	15.73	TCA cycle
Uracil	-2.28	0.039	190.1000	13.12	Pyrimidine
2-Oleoylglycerol	-2.44	0.0041	218.2000	30.28	Monoacylglycerol
Sitosterol	-2.50	0.024	247.2000	35.55	Sterol
Oleic acid	-2.58	0.041	340.3000	30.31	Fatty acids
Lactic acid	-2.87	0.017	240.3000	29.08	Fermentation
Cholesterol	-2.96	0.031	143.1000	33.99	Sterol
2-Pentanone	-3.12	0.0017	76.1000	8.87	-
N-Acetyl-D-glucosamine	-4.14	0.027	282.1000	23.67	Glucose derivative
Tyrosine	-4.75	0.0049	356.1000	20.04	Amino acids

Table 2.2. Identified significantly changed metabolites ($p < 0.05$, compared to controls) in fecal samples of Ace-K consumed male mice.

Metabolites	Fold	p value	m/z	Retention time	Pathways or Functions
Pyruvic acid	2.31	0.0065	300.1000	9.22	Fermentation
Cholic acid	1.92	0.044	185.2000	34.96	Bile acids
α-Glyceryl stearate	1.87	0.0064	83.2000	30.85	Glycerolipids
1,3-Dipalmitin	1.85	0.017	240.3000	29.08	Glycerolipids
Glutamic acid	1.73	0.0097	345.2000	17.54	Amino acids
Linoleic acid	1.33	0.048	263.3000	25.28	Fatty acids
Phosphoric acid	-1.32	0.047	119.1000	12.13	-
Cholesterol	-1.47	0.034	142.2000	34.01	Sterol
Lysine	-1.60	0.021	419.3000	21.89	Amino acids
Glycolic acid	-1.61	0.039	178.1000	9.05	-
Campesterol	-1.73	0.044	260.3000	34.82	Sterol
Isoleucine	-1.90	0.035	231.2000	12.42	Amino acids
Ornithine	-2.23	0.036	142.2000	20.49	Amino acids
Deoxycholic acid	-5.33	0.0084	319.2000	35.04	Bile acids

Figures

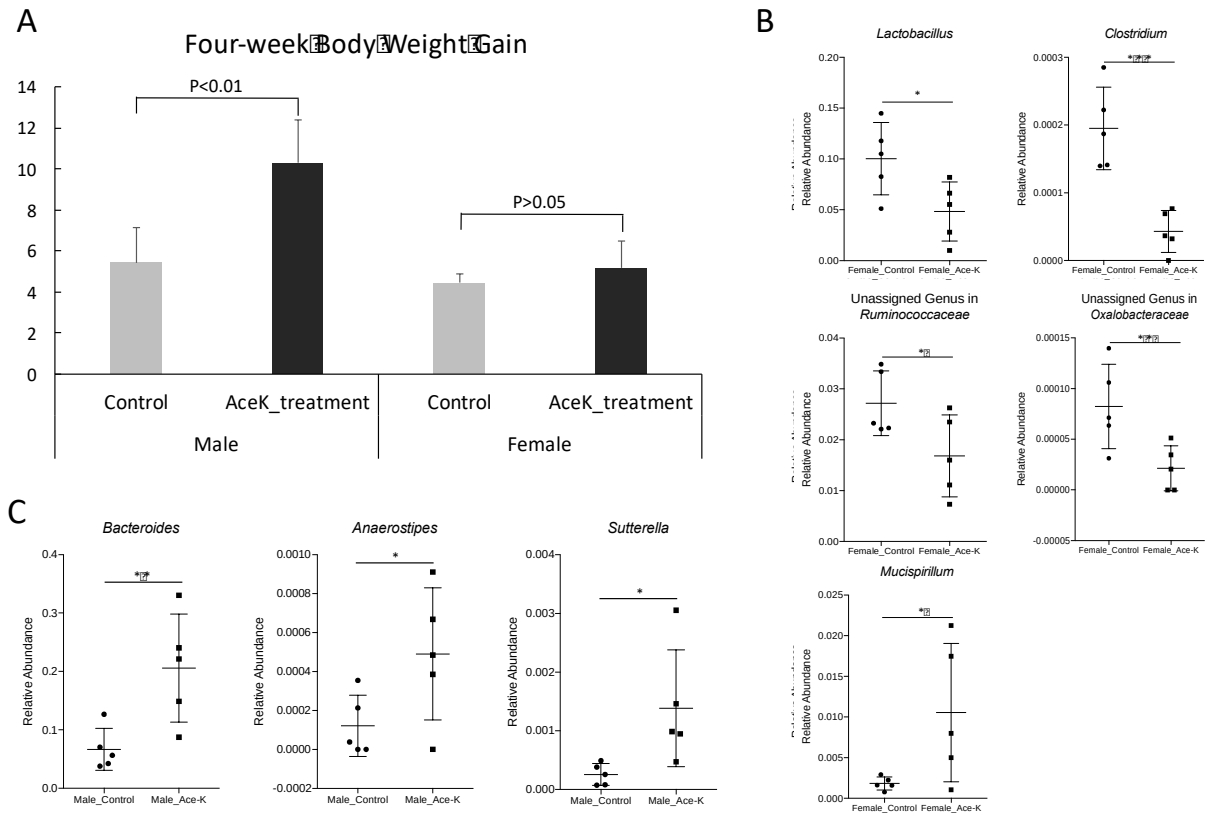

Figure 2.1. The effects of four-week Ace-K consumption on body weight gain and gut microbiome compositions of CD-1 mice. (A). Body weight gain of Ace-K treated male mice was significantly higher than control male mice, while the body weight gain of female mice was not significantly different from controls. (B). Ace-K consumption altered the gut bacteria composition in female mice. *Lactobacillus*, *Clostridium*, an unassigned genus in *Ruminococcaceae* and an unassigned genus in *Oxalobacteraceae*, were significantly decreased, while *Mucispirillum* was increased (C). Ace-K consumption altered the gut bacteria composition in male mice. The abundance of *Bacteroides*, *Anaerostipes* and *Sutterella* was significantly increased after Ace-K consumption. (*p<0.05, **p<0.01, ***p<0.001, N.S. p>0.05)

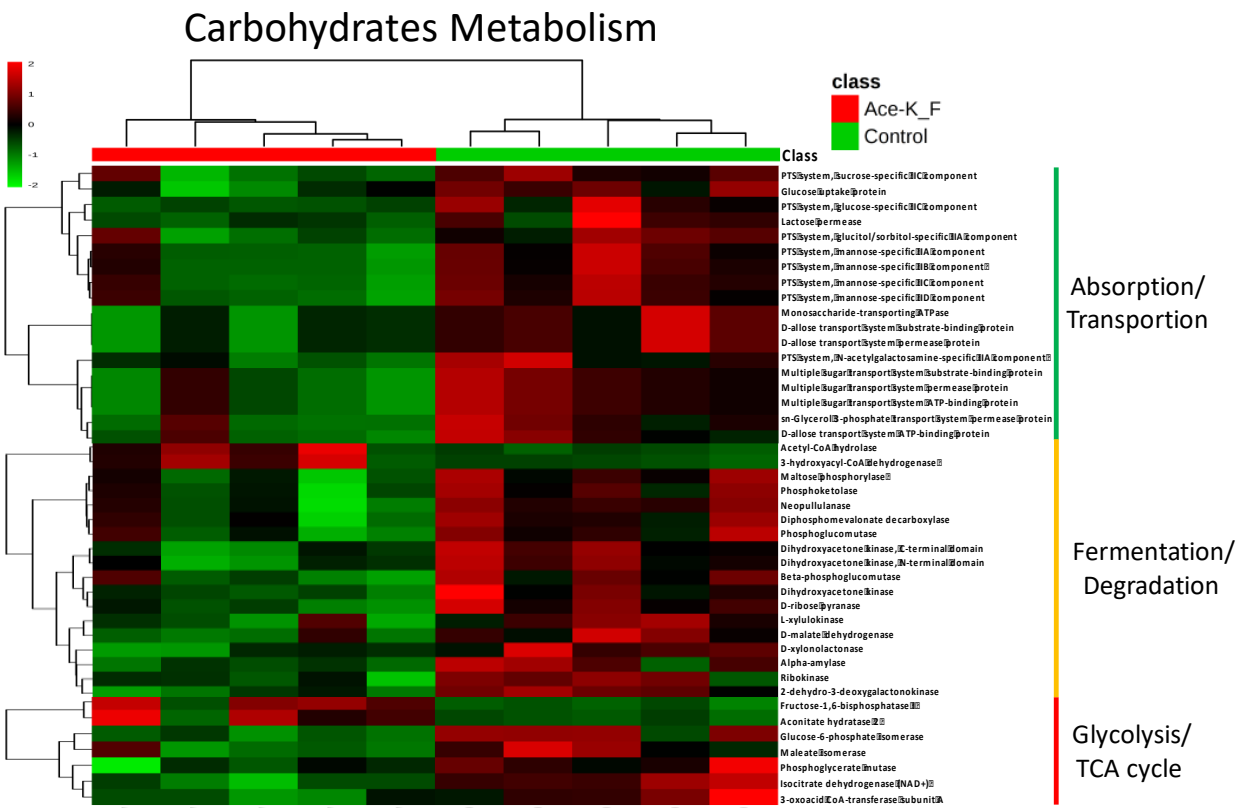

Figure 2.2. Functional gene enrichment analysis show that carbohydrates metabolism related functional genes have been widely and significantly decreased in Ace-K treated female mice (all the genes listed here with $p<0.05$).

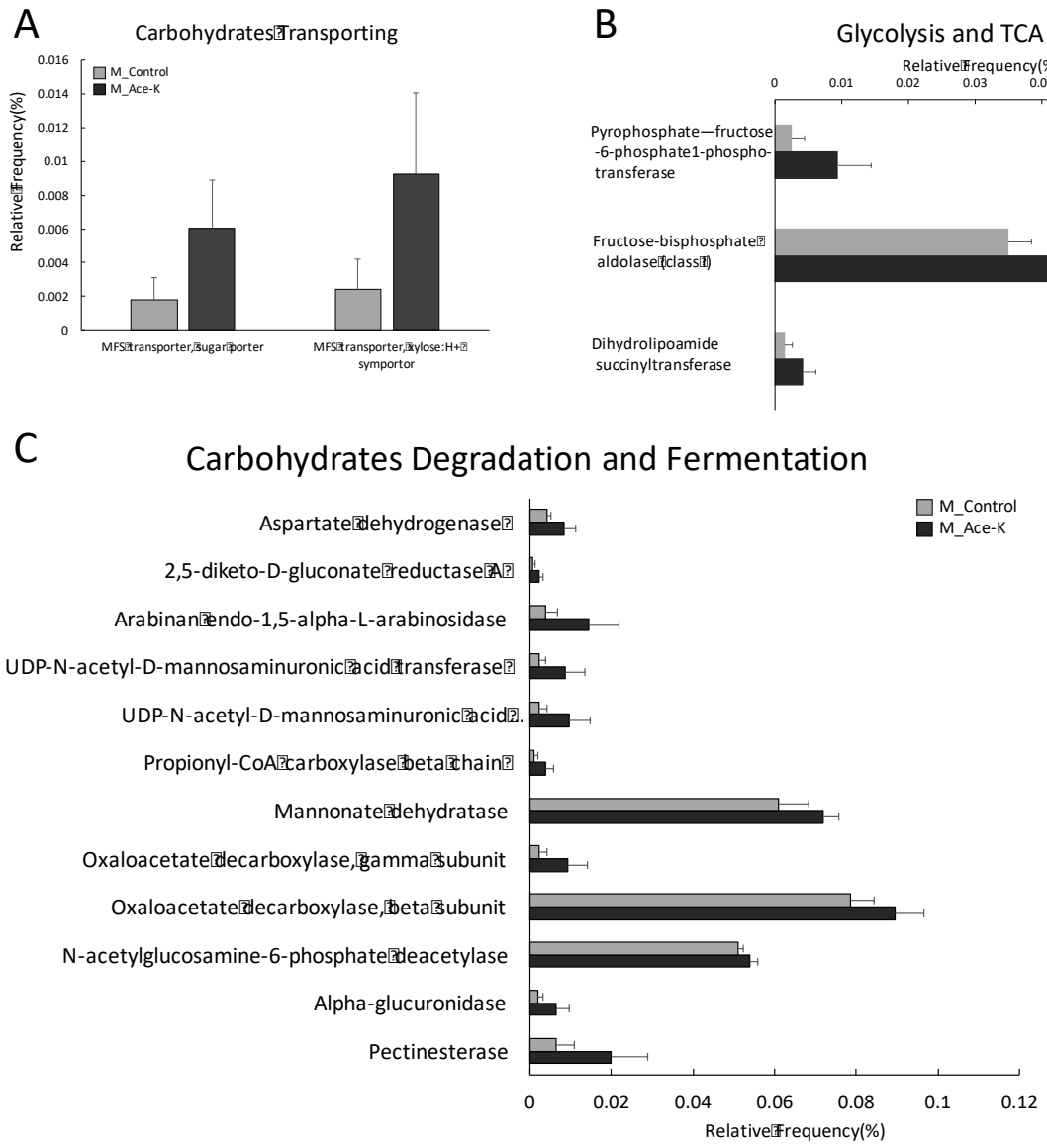

Figure 2.3. Functional gene enrichment analysis show that carbohydrates metabolism related functional genes were significantly increased in Ace-K treated male mice ($p<0.05$). Genes involved in carbohydrates transporting (A), glycolysis and TCA cycle (B), and carbohydrate degradation and fermentation (C) were consistently increased.

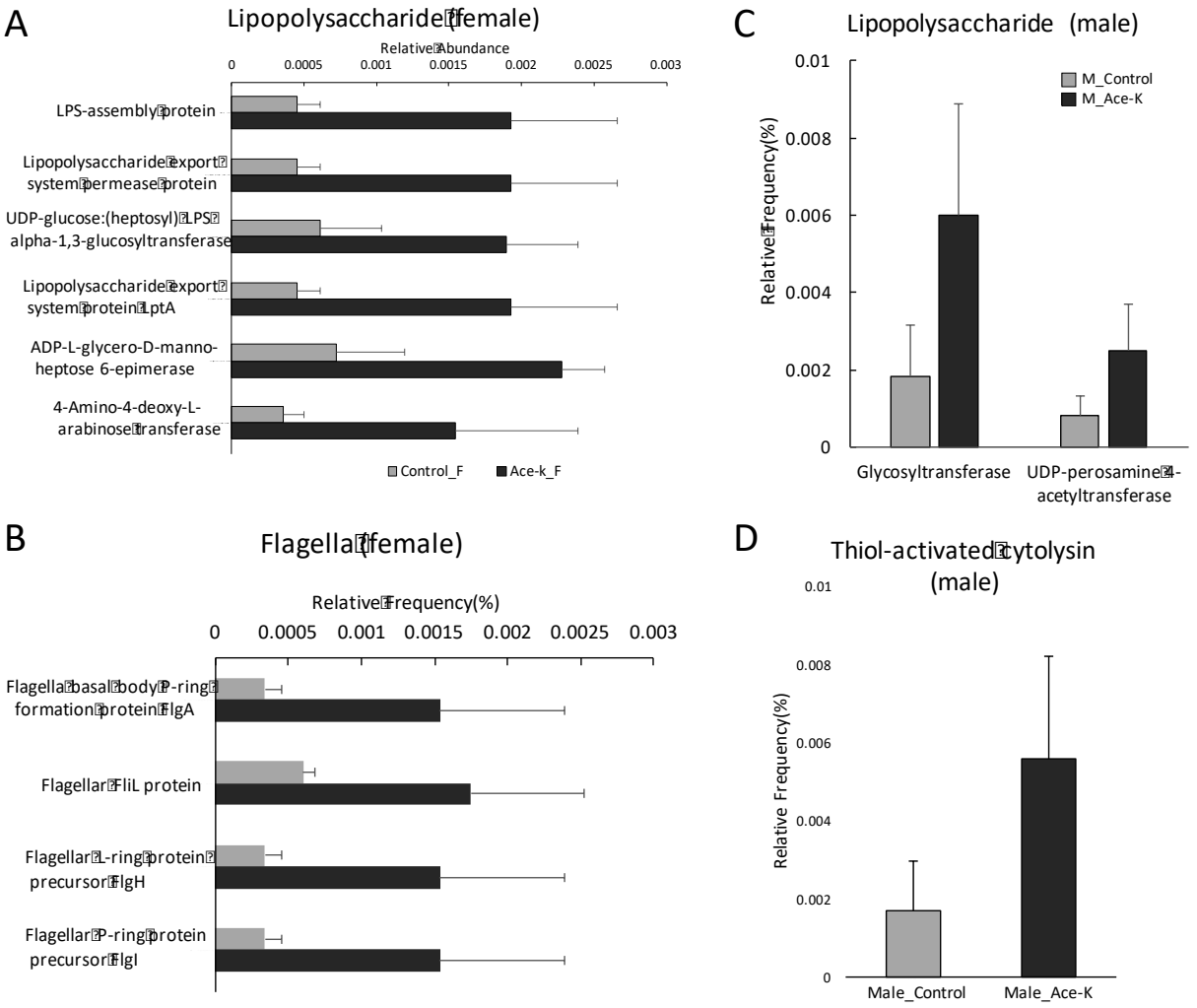

Figure 2.4. Multiple genes encoding pro-inflammatory factors were significantly increased in male and female mice after Ace-K consumption ($p<0.05$). Genes encoding LPS metabolism proteins (A) and flagella components (B) were increased in Ace-K treated female mice. Genes encoding LPS metabolism proteins (C) and thiol-activated cytolysin (D) were increased in Ace-K treated female mice.

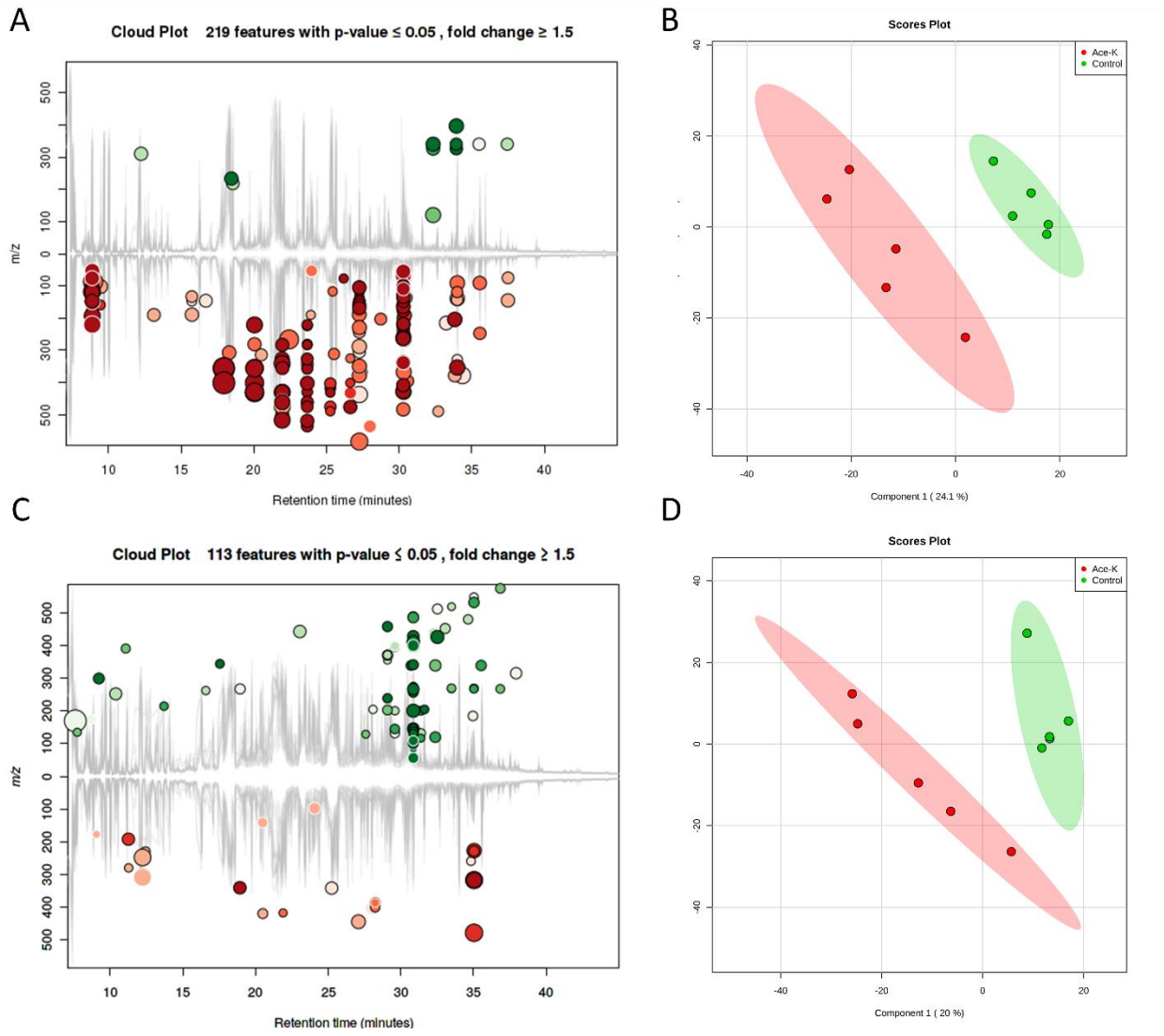

Figure 2.5. Ace-K consumption changed the fecal metabolome of female (A, B) and male (C, D) mice, as illustrated by the **Cloud** and PLS-DA plots.

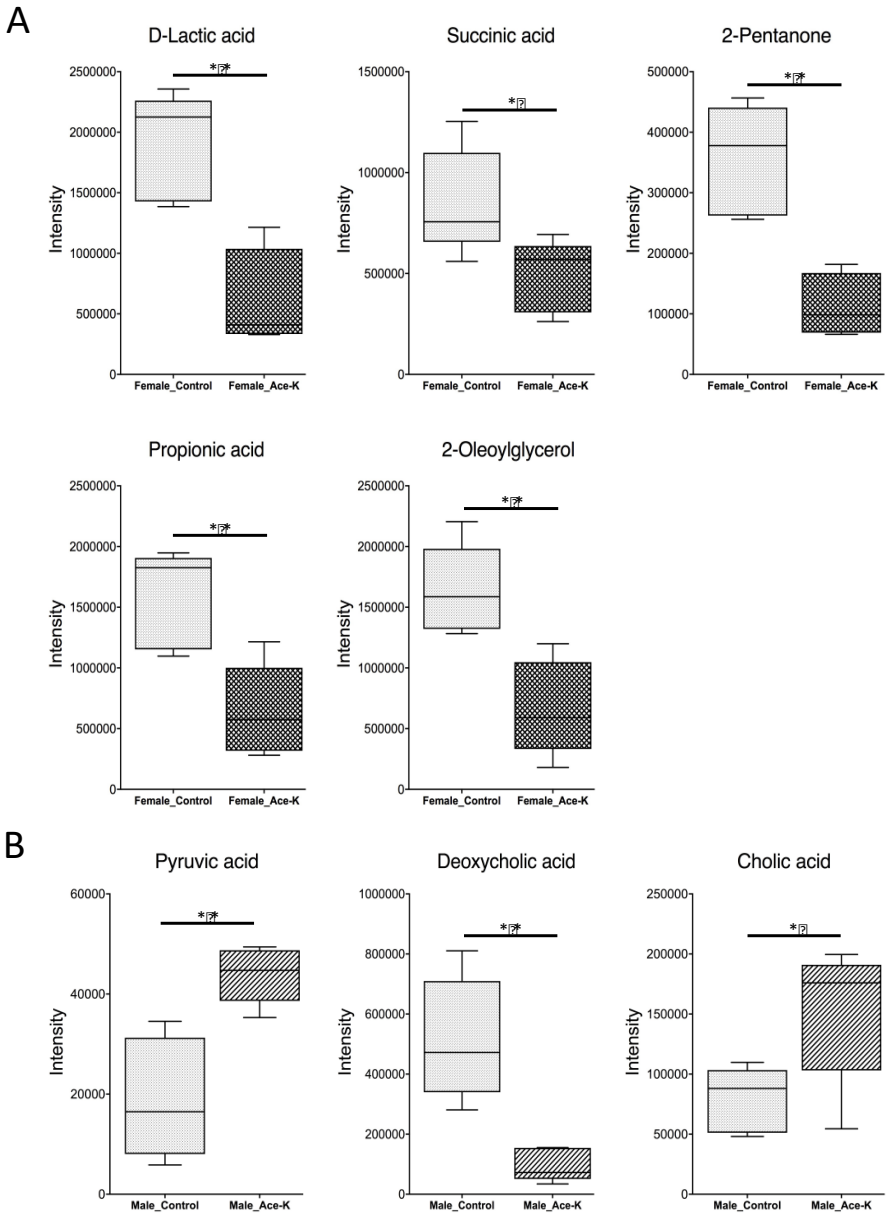

Figure 2.6. Ace-K consumption significantly altered key fecal metabolites in female (A) and male (B) mice (*p<0.05, **p<0.01).

CHAPTER 3

GUT MICROBIOME RESPONSE TO SUCRALOSE AND ITS POTENTIAL ROLE IN INDUCING LIVER INFLAMMATION IN MICE[2]

[2] Bian, X, Bei, G, Chi, L, Tu, P, Ru H and Lu, K. Submitted to *Frontiers in Physiology*, 04092017.

Abstract

Sucralose is a broadly used artificial sweetener. Numerous studies have concluded that sucralose is safe, however, previous studies indicated that sucralose consumption could alter the gut microbiota. The gut microbiome plays a key role in host health, such as digestion and fermentation of food, development of immune cells, and regulation of the enteric nervous system. Inflammation is one of the most common physical conditions associated with the dysbiosis of the gut microbiome, which is associated with a series of human diseases, such as diabetes and obesity. The aim of this study is to investigate the structural and functional effects of sucralose on the gut microbiota and associated inflammation in the host. In this study, C57BL/6 male mice received sucralose in drinking water for six months. Differences in the gut microbiota and metabolites between controls and sucralose-treated mice were determined using 16S rRNA gene sequencing, functional gene enrichment analysis and metabolomics. Expression of inflammatory genes in tissues was determined with RT-PCR. Alteration of bacterial genera showed that sucralose affects the gut microbiota. Enrichment of bacterial genes of pro-inflammatory mediators and fecal metabolites suggest that sucralose consumption may increase the risk of developing tissue inflammation via disrupting the gut microbiota, which is consistent with elevated pro-inflammatory gene expression in the livers of sucralose-treated mice.

Introduction

Artificial sweeteners are commonly used food additives that have a much higher intensity of sweetness than table sugars and are recommended to the population for the reduction of sugar intake for health reasons (Gardner et al., 2012). Consumption of artificial sweeteners is increasing in the United States, and in 2008, the prevalence of consumption of beverages containing artificial sweeteners was 24.1% among adults. Sucralose, with 600 times sweetness than sucrose, is one of

the most important artificial sweeteners in the market for its most sugar-like taste, no bitterness aftertaste, high stability at high temperatures, and long shelf-life (Grice & Goldsmith, 2000; Sylvetsky, Welsh, Brown, & Vos, 2012). A number of previous studies concluded that sucralose is safe for its intended use as artificial sweetener and brings no calorie to the body (Grotz & Munro, 2009; Sylvetsky et al., 2012). Most of ingested sucralose will not be absorbed and metabolized, but goes through the gastrointestinal tract unchanged (Roberts, Renwick, Sims, & Snodin, 2000). However, this does not prove that sucralose is inert to the gut microbiota. One study showed that a product containing sucralose could alter rat gut microbiota and induce inflammatory lymphocytes infiltration (Abou-Donia, El-Masry, Abdel-Rahman, McLendon, & Schiffman, 2008), but the study were considered to be deficient in several aspects (Brusick et al., 2009), including exposed to mixture but not pure sucralose and high doses. Another study focuses on the metabolic effects of sucralose on environmental bacteria and shows that sucralose can inhibit the growth of certain bacterial species (Omran, Ahearn, Bowers, Swenson, & Coughlin, 2013). Therefore, sucralose may inhibit bacteria in intestine and alter the gut microbiota, and this alteration could affect the health of the host.

The mucosal surfaces of the human intestines are host to more than 100 trillion microbes (including bacteria, fungi, viruses and parasites) from more than 1000 species (Ley, Peterson, & Gordon, 2006; Qin et al., 2010). Gut microbes interact with the host mucosa directly via the recognition of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, flagellin, and bacterial DNA and RNA, and this recognition is accomplished by the pattern recognition receptors (PRRs) of the mucosa (Maloy & Powrie, 2011). Alternatively, the interaction can occur indirectly through secreted metabolites (Nicholson et al., 2012). These interactions could achieve a symbiotic homeostasis. Increasing evidence indicates that this homeostasis is vital for

human health (Holmes, Li, Athanasiou, Ashrafian, & Nicholson, 2011; Tremaroli & Backhed, 2012). In a state of good health, the gut microbes make contributions to the host, including digestion and fermentation of food, development of immune cells, enteric nerve regulation and prevention of colonization by pathogens (Holmes et al., 2011). The host, in turn, provides habitat and nutrients and also secrets antibodies to inhibit the aggressive expansion of microbes (Kamada, Seo, Chen, & Nunez, 2013). Being highly diverse, the gut microbiota can be shaped by various factors, including aging, diet, drugs, antibiotics, diseases, stress, exercise and environmental pollutants (Ley et al., 2006; Nicholson et al., 2012), and if homeostasis is disrupted as a result of this shaping, many adverse outcomes may occur, such as cardiovascular disease, obesity, diabetes, allergies and cancer (Ley et al., 2006). For example, an increased ratio of Firmicutes to Bacteroidetes was found in obese mice compared to their lean littermates (Turnbaugh et al., 2006), and obesity-related phenotypes were found to be transmissible in a study in which fecal microbes from obese and lean human twins were transferred to germ-free mice (Ridaura et al., 2013). Likewise, a remarkable increase of taurocholic acid and Bilophila wadsworthia induced by dietary fat promotes colitis in IL10 deficient mice (Devkota et al., 2012). Metabolites from dietary choline by the gut microbes have shown to be modulated in obesity, diabetes and cardiovascular diseases (Kim et al., 2010; Z. Wang et al., 2011).

Inflammation is one of the most common physical conditions associated with the dysbiosis of gut microbiota. For example, acute or chronic inflammation is the central characterization of inflammatory bowel diseases (IBD) (Xavier & Podolsky, 2007), of which a disrupted gut microbiota is one of the major triggers in addition to genetic factors and the host immune system, while the precise etiology remains unclear (Hill & Artis, 2010). Moreover, increasing evidence demonstrates that low-grade chronic inflammation induced by disrupted gut microbiota is

associated with metabolic diseases (Holmes et al., 2011). Obesity and diabetes are associated with low-grade inflammation not only in adipose tissues, but also systemically. A study of bariatric surgery, a method to reduce body weight for obese individuals, has shown that one species of gut microbes, *Faecalibacterium prausnitzii*, is directly related to the reduction of low-grade inflammation in obesity and diabetes (Furet et al., 2010). A dyslipidaemia induced by a high fat diet results in increased levels of lipopolysaccharide (LPS), which is a pro-inflammatory mediator (Holmes et al., 2011). Moreover, chronic inflammation induced by gut microbes can drive the progression of colorectal cancer from adenoma to invasive carcinoma (Uronis et al., 2009). Thus, inflammation can be triggered and modulated by altered gut microbiota, and exposure to compounds that can alter the gut microbiota may induce inflammation in the host.

In this study, we first used 16S rRNA gene sequencing to examine the effects of sucralose on the gut microbiome of C57BL6/J mice over a 6-month administration. Next, we used metabolomics to profile fecal metabolome changes associated with perturbed gut microbiome. Finally, we assessed several biomarkers of inflammation to define the effects of sucralose consumption on host tissues. Our results show that sucralose altered the gut microbiome and associated metabolic profiles, which could contribute to inflammatory responses in liver of mice.

Materials and Methods

Animals and exposure. Male C57BL/6J mice (~8 weeks old) purchased from Jackson Laboratories (Bar Harbor, ME) were used in this study. Twenty male mice were housed in the University of Georgia animal facility for a week before the study, then assigned to the control and treatment groups (ten mice in each group), which received tap water or sucralose (Sigma-Aldrich, MO) in drinking water respectively for six months. Concentration of sucralose were 0.1mg/ml, which was equivalent to FDA approved acceptable daily intake (ADI) in human

(5mg/kg/day). Fresh solutions were made every week and the consumption of water was measured for both groups. Standard pelleted rodent diet and tap water ad libitum were provided to the mice in the environmental conditions of 22°C, 40–70% humidity, and a 12:12 hr light:dark cycle before and during the experimentation. Body weight was measured before and after the treatment. Fecal pellets were collected at baseline, three and six months of post-treatment. All experiments were approved by the University of Georgia Institutional Animal Care and Use Committee. The animals were treated humanely and with regard for alleviation of suffering.

16S rRNA gene sequencing of the gut microbiota. The gut microbiota was investigated through 16S rRNA gene sequencing in fecal samples at different time points. Fecal DNA from individual mouse was isolated using a PowerSoil DNA Isolation Kit (MO BIO Laboratories) according to the manufacture's instruction, and the resultant DNA was quantified and stored in -80 °C for further analysis. V4 region in the 16S rRNA gene was targeted using universal primers of 515 (5'-GTGCCAGCMGCCGCGGTAA) and 806 (5'-GGACTACHVGGGTWTCTAAT). For each sample, 1ng of the purified fecal DNA was used as template for the amplification, then barcoded with specific indexes individually. Then, the amplified products were normalized, pooled and sequenced by an Illumina MiSeq at the Georgia Genomics Facility. Pair-end 250 + 250 (PE250, v2 kit) reads were generated to a depth of at least 25,000 reads per sample. Geneious 8.1.5 (Biomatters, Auckland, New Zealand) was used to process the raw fastq files, and the mate-paired files were trimmed to dispose of bases with error probability higher than 0.01, and merged. Then, the data were analyzed using quantitative insights into microbial ecology (QIIME, version 1.9.1) (Caporaso et al., 2010), and UCLUST were used to get the operational taxonomic units (OTUs) with 97% sequence similarity against Greengenes database

13.8. The matched sequencing were assigned at five different levels: phylum, class, order, family and genus.

Functional gene enrichment analysis. An open-source R package, Tax4Fun, was first used to analyze the enrichment of functional genes of the microbiome of each group (Asshauer, Wemheuer, Daniel, & Meinicke, 2015). The output from QIIME with a SILVA database extension (SILVA 119) was applied. Tax4Fun can predict the functional genes of bacterial communities based on the 16S rRNA sequencing data and provide good approximation to the gene profiles obtained from metagenomic shotgun sequencing methods. Results from Tax4Fun were applied to Statistical Analysis of Metagenomic Profiles (STAMP) (version 2.1.3) (Parks, Tyson, Hugenholtz, & Beiko, 2014) for further statistical analysis.

Fecal metabolomics analysis. Metabolites of fecal samples collected at six-month were extracted using methanol and water as described (Lu et al., 2014). In brief, 20mg feces was disrupted in 1ml of methanol/water solution (1:1) with TissueLyser at 50Hz for 5 minutes, followed by centrifugation at 12,000rpm for 10 minutes. The resultant upper phase was collected and dried using SpeedVac, then dried samples were re-suspended in 20% Acetonitrile for MS analysis. Metabolomic profiling was conducted using a quadrupole-time-of flight (Q-TOF) 6520 mass spectrometer (Agilent Technologies, Santa Clara, CA) with an electrospray ionization source interfaced with Agilent 1200 HPLC system. The Q-TOF was calibrated with standard tuning solution (Agilent Technologies) daily to ensure a mass accuracy that is less than 5ppm. An YMC Hydrosphere C18 column was used to separate the metabolites and all detectable molecular features at mass range of 30 to 2000 m/z was captured in the positive mode.

Metabolomics data processing and metabolite identification. Data obtained from the HPLC-Q-TOF system was processed and analyzed as described (Lu, Knutson, Wishnok, Fox, &

Tannenbaum, 2012). Briefly, the raw .d data were convert to .mzdata format using MassHunter Workstation software (Agilent), and only signals with intensity higher than 1000 counts were included in the following analysis. Peak alignment, intensity calculation and comparison between the control and treatment group were performed using the XCMS Online tools. Significantly changed molecular features were profiled, and searched against the Human Metabolome Database (HMDB) (http://www.hmdb.ca) and METLIN (http://metlin.scripps.edu). The matched molecular features were fragmented using MS/MS in the Q-TOF 6520 mass spectrometer to get the product ions and the spectra were compared with the HMDB and METLIN MS/MS database to identify significantly altered metabolites.

Quantitative real-time polymerase chain reaction (qPCR). Liver samples treated with RNAlater (Thermo Fisher Scientific) were used to isolate RNA with a RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacture's instruction, and the resultant RNA was digested with DNA-free™ DNA Removal Kit (Thermo Fisher Scientific) to remove genomic DNA contamination. Then, cDNA was synthesized from 1ug total RNA using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, CA) and the products were diluted to 1:5 before use in subsequent reactions. Quantitative real-time PCR was performed on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). The sequences of the primers used for quantitative PCR were as follows: TNF-α 5-CCCTCACACTCAGATCATCTTCT and 5-GCTACGACGTGGGCTACAG; IL-6 5-TAGTCCTTCCTACCCCAATTTCC and 5-TTGGTCCTTAGCCACTCCTTC; IL-1β 5-GCAACTGTTCCTGAACTCAACT and 5-ATCTTTTGGGGTCCGTCAACT; iNOS 5-GTTCTCAGCCCAACAATACAAGA and 5-GTGGACGGGTCGATGTCAC; MMP-2 5-CAGGGAATGAGTACTGGGTCTATT and 5-

ACTCCAGTTAAAGGCAGCATCTAC); MMP-9 5-ATCTCTTCTAGAGACTGGGAAGGAG and 5-AGCTGATTGACTAAAGTAGCTGGA); MMP-13, 5-GTGTGGAGTTATGATGATGT and 5-TGCGATTACTCCAGATACTG and β-actin 5-CGTGCGTGACATCAAAGAGAA and 5-TGGATGCCACAGGATTCCAT. All results were normalized to β-actin gene (endogenous control). The fold change over control samples was calculated using ΔΔCT method by CFX manager software (Bio-Rad). The qPCR conditions were 95 °C for 10 min, 40 cycles of 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C followed by 65 °C to 95 °C increment by 0.5°C for 0.05s. Potential genomic DNA contamination was controlled by the use of DNase digestion and No-RT control, and technical contamination was controlled by the induction of No-template control.

Data analysis. The difference in individual gut bacterial component between controls and sucralose-treated mice at different time points was assessed with the Mothur software. A two-tail Welch's t-test ($p<0.05$) was used to compare the difference of metabolites between the control and sucralose-treated mice. Also, principle component analysis (PCA) was used to examine the intrinsic clusters and outliers. Partial least squares discriminant analysis (PLS-DA) and hierarchical clustering heat map were performed to visualize metabolomics difference in different groups.

Results

Sucralose altered the developmental dynamics of gut microbiome. The gut microbiome is a dynamic system and the bacteria are shifting over time. Maintaining normal developmental trajectories of gut microbiome is critical for its functions. Feces collected at baseline, three and six-month post-administration in both groups were employed to investigate the effects of sucralose on altering the gut microbiome. Through 16S rRNA gene sequencing, we found that 14 genera exhibited different changing patterns over time in sucralose-treated mice

compared to controls, as shown in Figure 3.1. These bacterial genera had no significant difference in abundance at baseline, but were significantly different after three and/or six-month of treatment, indicating sucralose disrupts the developmental dynamics of gut bacteria. The genera included *Turicibacteraceae turicibacter*, *Lachnospiraceae ruminococcus*, *Ruminococcaceae ruminococcus*, *Verrucomicrobiaceae akkermansia*, *Staphylococcaceae staphylococcus*, *Streptococcaceae streptococcus*, *Dehalobacteriaceae dehalobacterium*, *Lachnospiraceae anaerostipes*, *Lachnospiraceae roseburia*, and unclassified members in Family *Clostridiaceae*, *Christensenellaceae*, *Peptostreptococcaceae*, *Erysipelotrichaceae* and Order *Bacillales*. In particular, several of these genera, such as *Ruminococcaceae ruminococcus*, *Streptococcaceae streptococcus*, *Dehalobacteriaceae dehalobacterium*, *Lachnospiraceae anaerostipes* and *Lachnospiraceae ruminococcus*, have been linked to inflammation in previous studies (Collins et al., 2014; Fernández et al., 2016; Munyaka, Rabbi, Khafipour, & Ghia, 2016; Willing et al., 2010).

Sucralose increased gene abundances related to bacterial pro-inflammatory mediators. Next, we examined whether the changes of gut microbiome compositions were associated with functional perturbations of the gut bacteria. Indeed, a number of bacterial functional genes were enriched in sucralose-treated mice. For example, the functional gene enrichment analysis of the gut microbiome showed that genes related to bacterial pro-inflammatory mediators were highly elevated in sucralose-treated mice, as shown in Figure 3.2. Specifically, genes related to lipopolysaccharide (LPS) synthesis were significantly increased after the six-month treatment. In addition, multiple genes related to flagella protein synthesis increased in sucralose-treated mice. Likewise, genes involved in fimbriae synthesis increased in

sucralose-treated mice. Numerous genes of bacterial toxins were also elevated in sucralose-treated mice.

Sucralose changed the fecal metabolome. We next conducted metabolomics profiling to examine the functional impact of sucralose on fecal metabolome. The combination in feces of a large quantity of gut bacteria and their metabolic products creates an ideal biological sample to assess functional changes of the gut microbiome. A total of 13,611 molecular features were detected in fecal samples, among which 1764 features were significantly different ($p<0.05$ and Fold change>1.5) between sucralose-treated mice and controls (Figure 3.3A), clearly indicating that sucralose perturbed the fecal metabolome. PLS-DA plot (Figure 3.3B) showed a separation in molecular patterns of the two groups and the hierarchical clustering heat map (Figure 3.3C) was consistent with this result. Molecular features matched with the METLIN and HMDB database were used for metabolite identification via MS/MS. We tentatively identified 66 metabolites that includes quorum sensing compounds, amino acids and derivatives, lipids, fatty acids, bile acids, and nucleic acids, etc (Table S3.1).

Sucralose altered quorum sensing signals. Bacteria control multicellular behaviors, such as growth and development of biofilm, horizontal gene transfer, host-microbe cross-talk and microbe-microbe interaction by the cell-cell signaling process called quorum sensing. Four acyl homoserine lactones, quorum sensing signals, were identified, including N-butanoyl-l-homoserine lactone, N-(3-oxo-hexanoyl)-homoserine lactone, N-tetradecanoyl-L-Homoserine lactone, N-pentadecanoyl-L and Homoserine lactone. The reduced abundance of these quorum sensing signals in sucralose-treated mice (Figure 3.4) indicates that sucralose disrupts quorum sensing signaling.

Sucralose altered amino acids and derivatives. The gut microbiome is highly involved in the synthesis and regulation of amino acids. Amino acids, such as L-tryptophan, L-tyrosine, L-leucine and L-isoleucine, as well as their derivatives (Table S3.1) were affected by sucralose treatment. For tryptophan metabolism, four compounds were identified, including L-tryptophan (Trp), quinolinic acid, kynurenic acid and 2-aminomuconic acid, as shown in Figure 3.5A. Compared to controls, Trp, quinolinic acid, and 2-aminomuconic acid increased by1.71-, 5.45- and 2.09-fold in sucralose-treated mice, while kynurenic acid reduced by 2.45-fold in sucralose-treated mice. For tyrosine metabolism, while L-tyrosine increased (1.62-fold), two metabolites, p-hydroxyphenylacetic acid and cinnamic acid decreased by 4.63 and 1.53-fold, respectively (Figure 3.5B).

Sucralose altered bile acids. The gut microbiome can transform primary hydrophilic bile acids into secondary hydrophilic bile acids in the large intestine through deconjugation, dehydroxylation and dehydrogenation. Bile acids not only facilitate fat and fat-soluble vitamin absorption and maintain cholesterol homeostasis, but also are viewed as signaling molecules that bind to nuclear receptor FXR and the G-protein-coupled receptor TGR5. Several bile acids were significantly different between the controls and sucralose-treated animals (Figure 3.6). 3-Oxo-4,6-choladienoic acid was increased in sucralose-treated mice while others were reduced, including 3 beta,7 alpha-Dihydroxy-5-cholestenoate, 3a,7b,12a-Trihydroxyoxocholanyl-Glycine and lithocholic acid/isoallolithocholic acid/allolithocholic acid/ isolithocholic acid.

Sucralose induced elevated pro-inflammatory gene expression in liver. As described above, sucralose could increase the production of bacterial pro-inflammatory mediators, which may lead to inflammatory responses in host tissues after being translocated into the host circulation. In fact, sucralose-treated mice had elevated gene expression of pro-inflammatory

biomarkers in liver (Figure 3.7), such as Matrix Metalloproteinase 2 (MMP-2) and inducible nitric-oxide synthase (iNOS).

Discussion

The gut microbiota is a dynamic system and maintaining a healthy balance is vital for the host (Nicholson et al., 2012). Previous studies have demonstrated that changes to the gut microbiota affect the host in many ways, such as immune system development, energy metabolism and absorption, and diseases in and beyond the GI tract (Holmes et al., 2011). Xenobiotics in food or environment consumed by the host can affect the gut microbiome and host health (Gao, Bian, Mahbub, & Lu, 2017; Lu et al., 2014; Suez et al., 2014). One of the prevalent arguments to support sucralose safety is that the majority of sucralose is not absorbed or metabolized in the body (Grice & Goldsmith, 2000). However, we demonstrate herein that sucralose can affect the gut microbiome, its metabolic functions and host even though it goes through the GI tract unchanged.

Specifically, we investigated the effect of sucralose consumption on mouse gut microbiota and host using 16S rRNA gene sequencing, functional gene enrichment analysis, metabolomics and real time PCR. As a result, sucralose consumption for six months changed gut microbes, fecal metabolites and pro-inflammatory gene expression in liver. The alterations induced by sucralose consumption have potential effects on the development of inflammation, and could further influence other physiological functions in the body. This study would provide new understanding of the effect of artificial sweeteners on the gut microbiota and host health.

Sucralose has been tested to be safe using different endpoints in previous studies, but very few study reported its effects on the gut microbiome and, particularly, its functions (Grotz & Munro, 2009). In this study, we examined the functional impact of sucralose consumption on

the gut microbiome and the risk of developing inflammation in mice. Altering the gut bacterial compositions may confer an increased risk of developing inflammation in sucralose-treated mice. For example, among the 14 changed genera, several of these genera were found to be associated with host inflammation. *Ruminococcaceae Ruminococcus*, which were more abundant in sucralose-treated mice, were found to be more abundant in colonic Crohn's Disease than in healthy samples in a previous study (Willing et al., 2010); *Streptococcaceae Streptococcus*, *Dehalobacteriaceae Dehalobacterium*, *Lachnospiraceae Anaerostipes* and *Lachnospiraceae Ruminococcus*, which were reduced in sucralose-treated mice, were found to be negatively associated with inflammation in previous studies (Collins et al., 2014; Fernández et al., 2016; Munyaka et al., 2016; Willing et al., 2010). The functional impact of these altered gut bacteria remains to be further elucidated in the future. Nevertheless, altered gut microbiome composition may lead to differential functional bacterial metagenomes and metabolic capacities of the gut microbiome.

Previous studies have demonstrated that functional genes of the bacterial community are related to the 16S rRNA marker genes, which provides the opportunity to survey the functional capacities of the gut microbiome through 16S rRNA gene sequencing (Asshauer et al., 2015). From the functional gene enrichment analysis, a number of genes related to bacterial pro-inflammatory mediators were significantly increased in sucralose-treated gut microbiome, which included lipopolysaccharide (LPS) synthesis, flagella protein synthesis, fimbriae synthesis, bacterial toxins and drug resistance genes. LPS, flagella and fimbriae are known pathogen-associated molecular patterns (PAMPs) that can trigger pathological inflammation in the host, and the various toxins produced by bacteria can induce toxicity to the host. LPS, a known endotoxin from the outer membrane of Gram-negative bacteria, can initiate inflammatory events,

such as secretion of pro-inflammatory cytokines like interleukin-6 or tumor necrosis factor TNF-α (de La Serre et al., 2010). Flagella protein levels are low in a healthy gut and high levels of flagella is associated with gut mucosal barrier breakdown and inflammation in previous studies (Cullender et al., 2013). Fimbriae play an important role in the bacterial adhesion to and invasion of epithelial cells, and is a known virulence factor (Nakagawa, 2002). Also, multidrug resistance genes were increased in sucralose-treated gut microbiome, and the increase of multidrug resistance genes and/or multidrug resistant bacteria may lead to a more hostile gut environment (Marshall, Dorothy J. Ochieng, & Levy, 2009). These data indicate that the sucralose consumption for six months increased pro-inflammatory products of the gut microbiome and its ability to potentially induce systemic inflammation.

Likewise, identified metabolites from fecal samples suggested a potential role of these metabolites in regulating inflammation. For example, several amino acids were perturbed in sucralose-treated fecal metabolites in this study. In particular, we found that tryptophan metabolism was disturbed by sucralose, and the disturbance is related to the change of functional genes of the gut microbiome. As shown in Figure S3.1, several genes related to tryptophan metabolism were elevated while the abundance of tryptophan and its metabolites were altered in the fecal samples. The four metabolites identified are involved in kynurenine pathway, which is the most important tryptophan pathway consuming 95% of the tryptophan (Keszthelyi, Troost, & Masclee, 2009). Balance between two metabolites, quinolinic acid and kynurenic acid in this pathway, plays an important role in mediating inflammation and excitability of cells, such as enteric neurons. Quinolinic acid is pro-inflammatory and excitotoxic, whereas kynurenic acid is anti-inflammatory and neuroprotective (Keszthelyi et al., 2009). Herein, we found an elevated level of quinolinic acid and a decreased level of kynurenic acid. This indicates that sucralose

may shift the cells leaning to a pro-inflammatory state. Likewise, tyrosine, and two metabolites from tyrosine, p-hydroxyphenylacetic acid and cinnamic acid, were found to decrease the reactive oxygen species (ROS) production in neutrophils in a previous study (Beloborodova et al., 2012), and the reduced level of both compounds in our study indicated a possibility of increased ROS in the sucralose-treated mice. As the response to ROS, bacterial genes of antioxidative enzymes, such as catalase and catalase-peroxidase, were significantly enriched in sucralose-treated mice (Figure S3.2). ROS can stimulate the pro-inflammatory cytokine release (Chapple, 1997), therefore, the decrease of these two compounds may contribute to the appearance of a pro-inflammatory state. Also, secondary bile acids that have antimicrobial effects were decreased, and this would provide opportunity for pathogens growth (Begley, Gahan, & Hill, 2005).

Pro-inflammatory mediators, such as LPS, and metabolites can translocate into host circulation and tissues, leading to systemic inflammatory responses (de La Serre et al., 2010). In accordance with this expectation, real time PCR results showed that expression of matrix metalloproteinase 2 (MMP-2) and inducible nitric-oxide synthase (iNOS) were elevated in the liver of sucralose-treated mice. MMP-2 is highly related to inflammatory responses, because it can cleave and activate TNF-α and IL-1β, which are both pro-inflammatory cytokines that contributes to the induction of inflammation (Medina & Radomski, 2006; L. Wang et al., 2006). Likewise, iNOS-derived NO regulates pro-inflammatory genes and significantly contributes to inflammatory liver injury. iNOS plays an important role in the progression of inflammatory conditions in multiple liver diseases, such as increasing liver inflammatory response, promoting the induction of liver tumors and contributing to liver fibrosis from chronic viral infection (La Mura et al., 2014; Sass, Koerber, Bang, Guehring, & Tiegs, 2001). Both the expression of MMP-

2 and iNOS were found to be increased in liver of sucralose-treated mice compared to controls, and this indicates that the exposure to sucralose increase the risk of developing inflammation in liver. In particular, most of consumed sucralose passes the GI track unabsorbed and unchanged (Roberts et al., 2000), therefore, inflammatory response in liver was not likely to be stimulated by sucralose directly.

Taken together, as illustrated in Figure 3.8, sucralose consumption alters the gut microbiome and its functions in mice. In particular, enrichment of gut microbial pro-inflammatory genes and fecal metabolites suggests that sucralose alters the gut environment to release more pro-inflammatory mediators and metabolites, which may contribute to the increased expression level of pro-inflammatory markers in liver, such as iNOS and MMP-2. Noteworthily, the majority of the ingested sucralose goes through the GI tract unabsorbed but it does disrupt the gut microbiota, therefore, our results highlight the role of sucralose-gut microbiome interaction in regulating host health, such as chronic inflammation.

Abbreviation list

PAMPs: pathogen-associated molecular patterns

PRRs: pattern recognition receptors

IBD: inflammatory bowel disease

LPS: lipopolysaccharide

iNOS: inducible nitric-oxide synthase

MMP-2: Matrix Metalloproteinase 2

ROS: reactive oxygen species

GI: gastrointestinal

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Tables

Table S3.1. Significantly altered fecal metabolites in sucralose-treated mice compared to controls.

Identified metabolites	Fold Change	p-value	m/z	retention time
Quinolinic acid	5.45	0.0000	168.0287	8.0
2,3-Didehydro-pimeloyl-CoA	4.01	0.0205	908.1711	11.1
3-(3-Ethyloxiranyl)-2-propenal	3.32	0.0246	149.0582	10.9
N-Acetylhistidine	3.06	0.0000	236.0416	2.1
5,8,11,14-all-cis-tetracosanoylethanolamide	2.82	0.0183	426.3371	17.8
17,17-dimethyl-5,8,11,14-all-cis-docosatetraenoylethanolamide	2.82	0.0183	426.3371	17.8
(16,16-dimethyldocosa-cis-5,8,11,14-tetraenoyl) ethanolamine	2.82	0.0183	426.3371	17.8
2-Oxosuccinamate	2.66	0.0002	169.9847	2.0
Ne,Ne dimethyllysine	2.58	0.0238	213.0984	1.5
L-gamma-glutamyl-L-isoleucine	2.21	0.0066	261.1453	8.5
L-gamma-glutamyl-L-leucine	2.21	0.0066	261.1453	8.5
PG(18:4(6Z,9Z,12Z,15Z)/0:0)	2.15	0.0468	505.2557	4.0
2-aminomuconic acid	2.09	0.0014	158.0456	2.8
L-prolyl-L-proline	2.01	0.0344	213.1250	7.9
(all-Z)-7,10,13-Docosatrienoic acid	1.99	0.0080	357.2790	15.2
L-Isoleucine/L-leucine	1.94	0.0006	132.1015	31.5
Citrulline	1.87	0.0057	214.0568	2.1
N-oleoyl leucine	1.87	0.0118	396.3509	19.1
N-oleoyl isoleucine	1.87	0.0118	396.3509	19.1
N6-Acetyl-L-lysine	1.76	0.0082	189.1232	4.1
LysoPE(0:0/14:0)	1.75	0.0364	426.2635	17.6
L-Tryptophan	1.71	0.0087	205.0972	8.2
L-Tyrosine	1.62	0.0003	182.0818	11.5
3-Oxo-4,6-choladienoic acid	1.52	0.0298	371.2560	12.4
Cinnamic acid	-1.53	0.0324	149.0605	10.2
3 beta,7 alpha-Dihydroxy-5-cholestenoate	-1.54	0.0015	455.3111	11.6
Acetylagmatine	-1.57	0.0413	195.1217	2.2
Adenosine	-1.60	0.0364	268.1037	4.4
Palmitaldehyde	-1.60	0.0055	263.2370	18.1
Palmitelaidic acid	-1.67	0.0213	255.2317	17.5
Pyridoxal	-1.72	0.0048	168.0648	8.3
C17 Sphinganine	-1.83	0.0404	288.2896	13.7

N-(3-oxo-hexanoyl)-homoserine lactone	-1.85	0.0339	236.0897	4.7
3-Dehydrosphinganine	-1.86	0.0467	300.2902	14.0
N,N-Dimethylsphingosine	-1.90	0.0146	328.3210	15.2
Adenine	-1.97	0.0253	136.0618	4.6
Lithocholic acid /isoallolithocholic acid /allolithocholic acid/ isolithocholic acid	-2.00	0.0270	399.2907	11.3
Guanine	-2.02	0.0023	152.0565	6.8
N-pentadecanoyl-L-Homoserine lactone	-2.03	0.0101	364.2260	18.2
Nonadeca-10(Z)-enoic acid	-2.04	0.0363	297.2780	31.2
N-tetradecanoyl-L-Homoserine lactone	-2.05	0.0354	350.2123	17.7
L-Dopa	-2.05	0.0004	198.0762	3.0
L-2,3-Dihydrodipicolinate	-2.11	0.0225	208.0023	2.2
2-Furoylglycine	-2.11	0.0225	208.0023	2.2
3-Dehydroquinate	-2.17	0.0060	191.0539	12.0
N(6)-Methyllysine	-2.18	0.0330	161.1270	1.5
Kynurenic acid	-2.25	0.0064	190.0495	12.0
N-butanoyl-l-homoserine lactone	-2.35	0.0003	172.0977	9.5
3a,7b,12a-Trihydroxyoxocholanyl-Glycine	-2.55	0.0363	466.3139	12.6
Hydroxychlorobactene glucoside	-2.61	0.0070	735.4571	11.2
Sterol	-2.92	0.0161	271.2057	14.1
N,N-dimethyl arachidonoyl amine	-2.95	0.0381	332.2957	21.3
DAT(16:0/23:0(2Me[S],3OH[S],4Me[S],6Me[S]))	-3.02	0.0092	997.7105	10.3
PC(P-17:0/0:0)	-3.42	0.0407	494.3631	21.3
LysoPC(14:1(9Z))	-3.51	0.0000	466.2910	11.0
PA(12:0/18:0)	-3.96	0.0193	659.4018	12.3
PA(15:0/15:0)	-3.96	0.0193	659.4018	12.3
N-linoleoyl taurine	-4.09	0.0378	426.2039	26.9
PA(13:0/20:5(5Z,8Z,11Z,14Z,17Z))	-4.45	0.0046	675.4003	11.8
PA(20:5(5Z,8Z,11Z,14Z,17Z)/13:0)	-4.45	0.0046	675.4003	11.8
p-Hydroxyphenylacetic acid	-4.63	0.0101	153.0550	11.0
(20R/S)-24-Hydroxygeminivitamin D4	-9.27	0.0012	519.4065	12.5

Figures

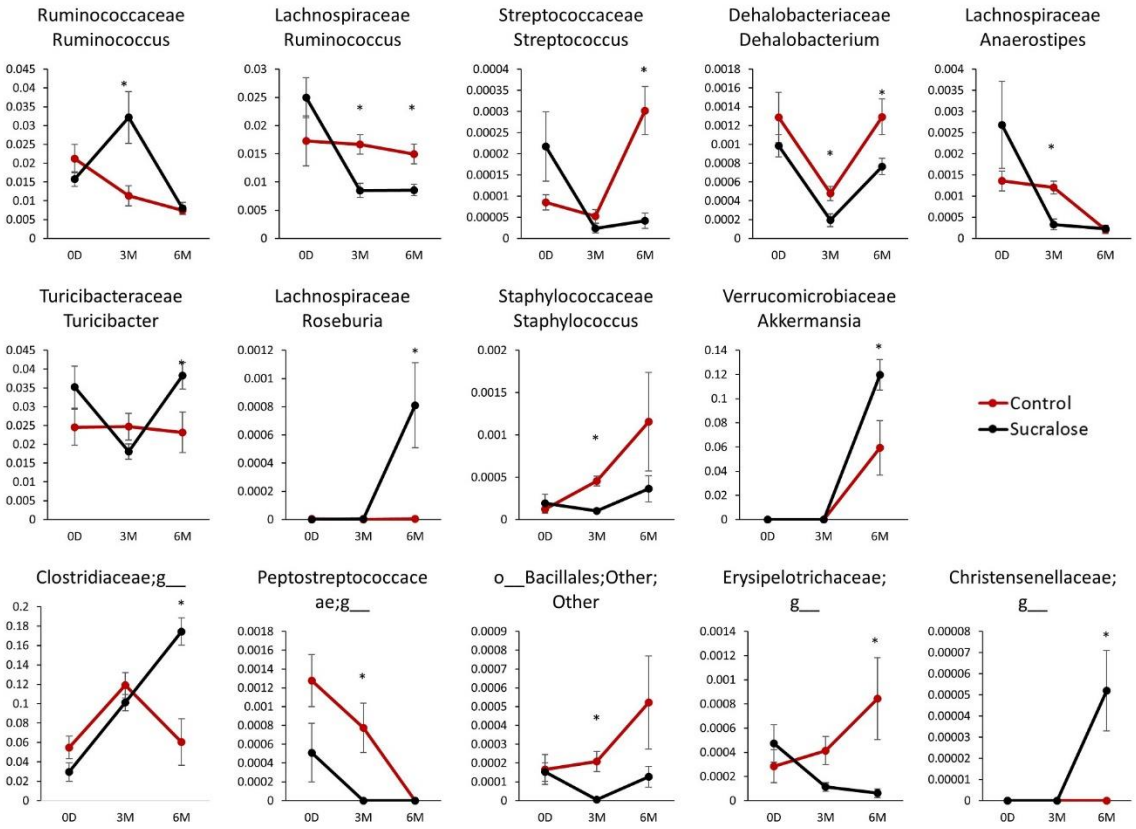

Figure 3.1. Sucralose altered the dynamics of gut microbiome development in C57BL6/J mice. Bacterial genera exhibited different changing patterns over time between the controls and sucralose-treated mice. X-axis represents time after the exposure, and y-axis represents relative percentage of bacteria. (*$p<0.05$).

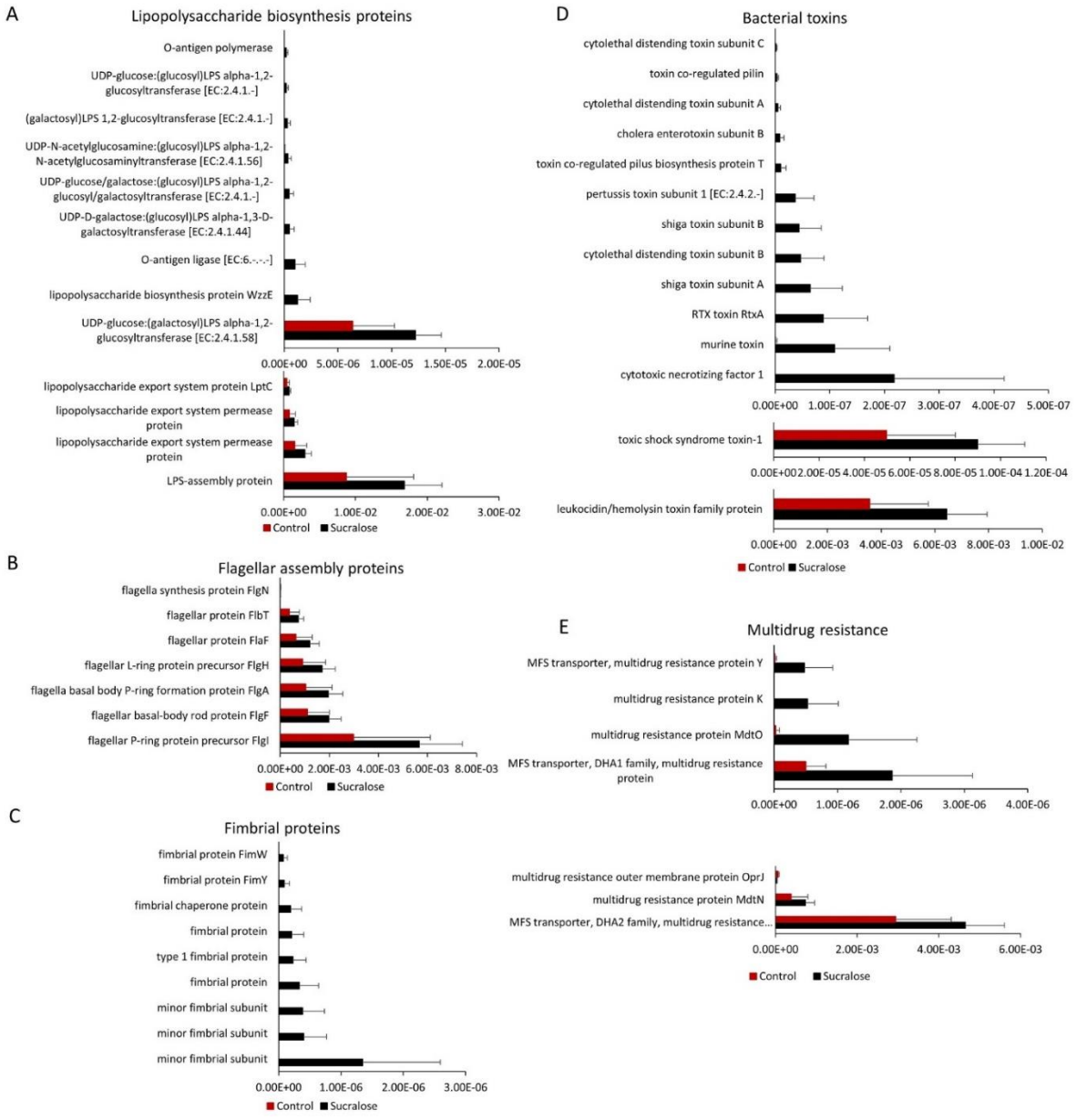

Figure 3.2. Enrichment of bacterial genes (Tax4Fun) of pro-inflammatory mediators after six-month sucralose treatment ($p<0.05$), including LPS (A), flagella (B), fimbriae (C), toxins (D) and multidrug resistant genes (E).

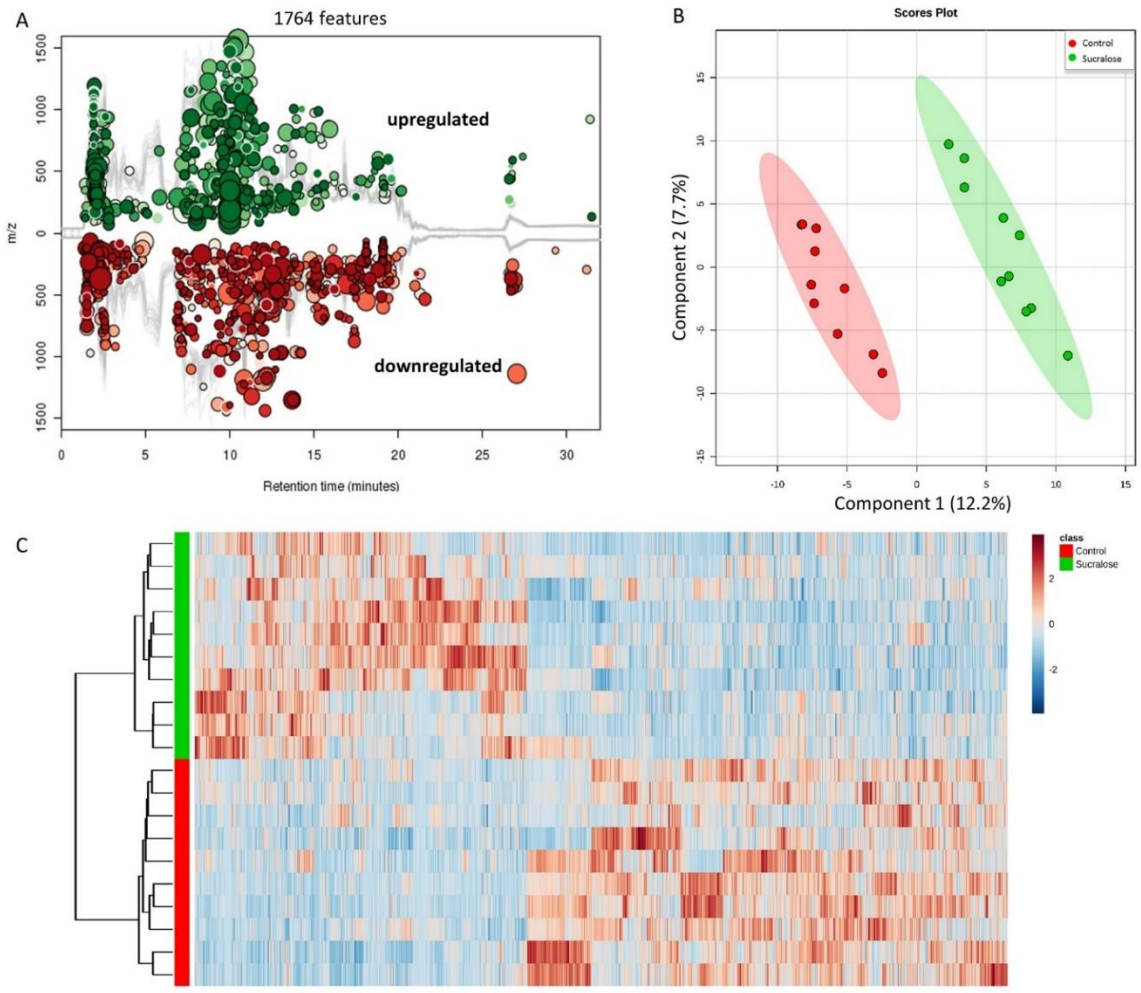

Figure 3.3. Sucralose changed the fecal metabolome, as illustrated by the Cloud plot (A), PLS-DA plot (B) and heat map (C). 1,764 molecular features were significantly different ($p<0.05$ and Fold change>1.5) between sucralose-treated mice and controls.

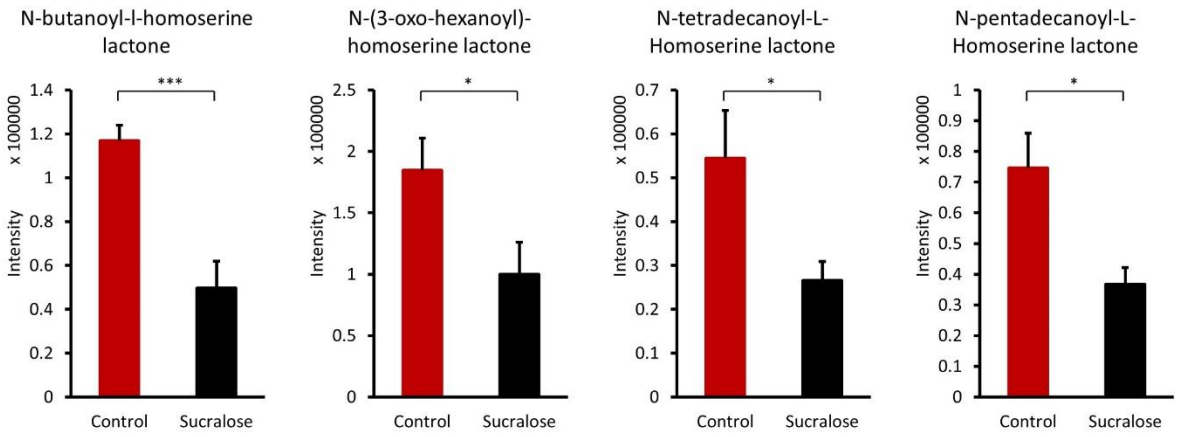

Figure 3.4. Quorum sensing signals were altered by sucralose consumption. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

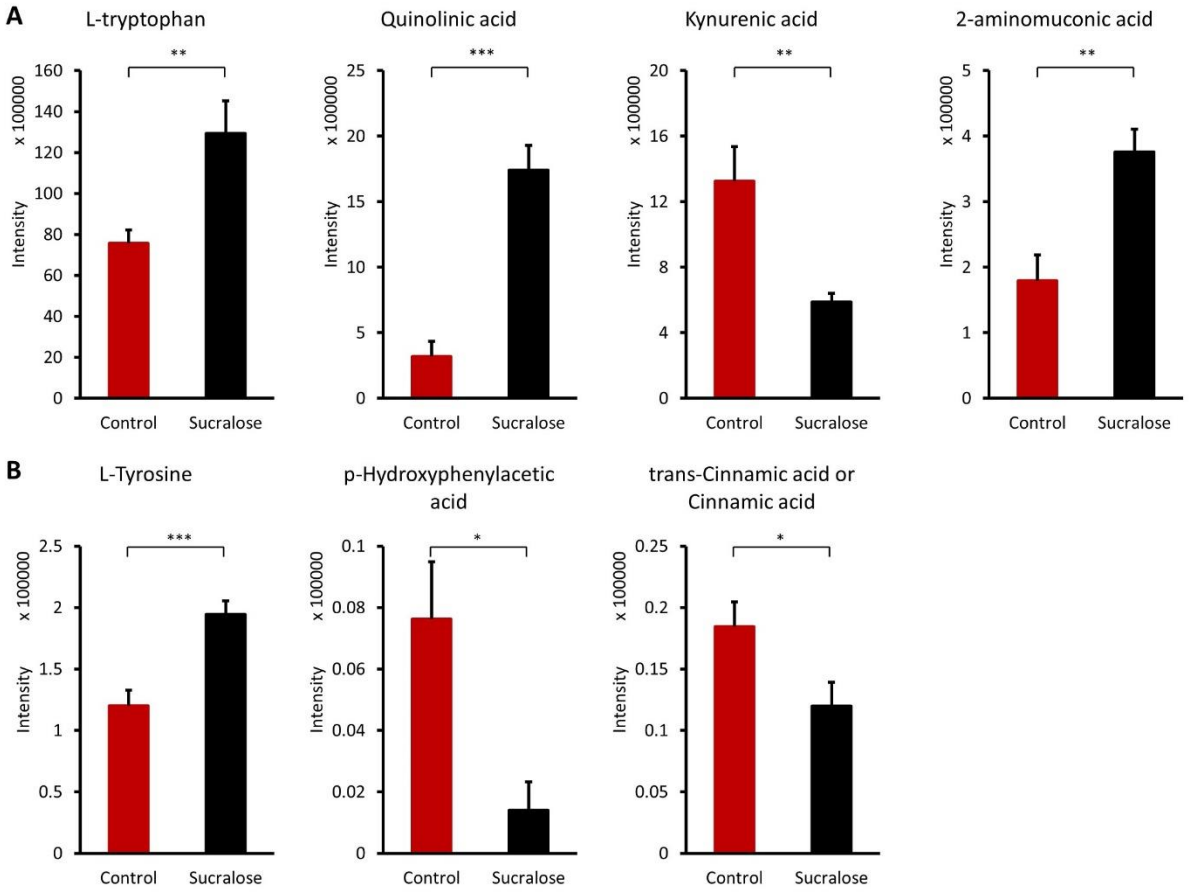

Figure 3.5. Altered amino acids and derivatives by sucralose. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) (A) metabolites of tryptophan metabolism; (B) metabolites of tyrosine metabolism.

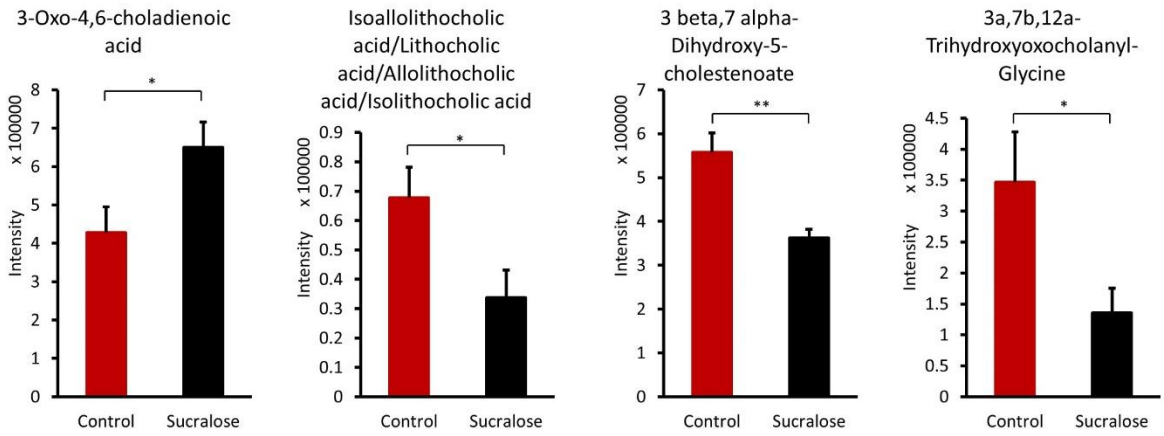

Figure 3.6. Sucralose altered bile acids in the fecal samples of mice treated with sucralose for 6 months. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

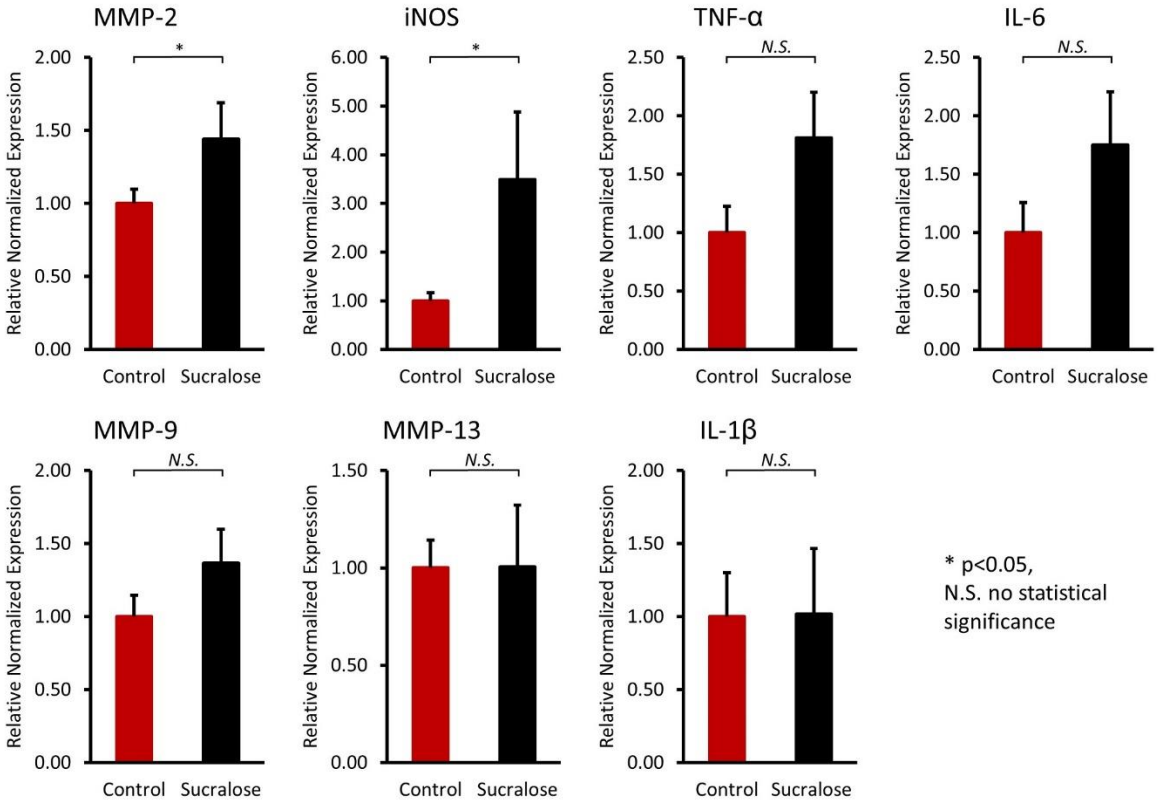

Figure 3.7. Sucralose consumption increased gene expression of inflammatory markers in liver, as examined by qRT-PCR. Matrix Metalloproteinase 2 (MMP-2) and inducible nitric-oxide synthase (iNOS) were elevated in sucralose-treated mice on mRNA level. (*p<0.05, N.S. no statistical significance)

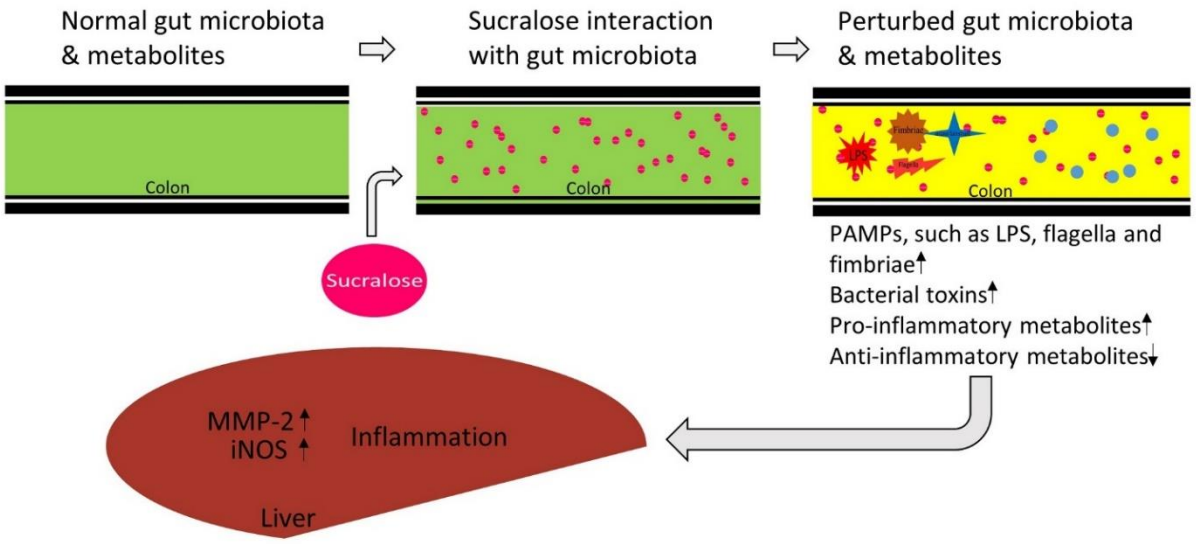

Figure 3.8. Proposed functional link between sucralose-induced gut microbiota alterations and host inflammation. Sucralose perturbs the gut microbiome and its metabolic functions, with enrichment of bacterial pro-inflammatory mediators and disrupted metabolites involved in inflammation regulation. Together, these consequences may contribute to the induction of liver inflammation in the host.

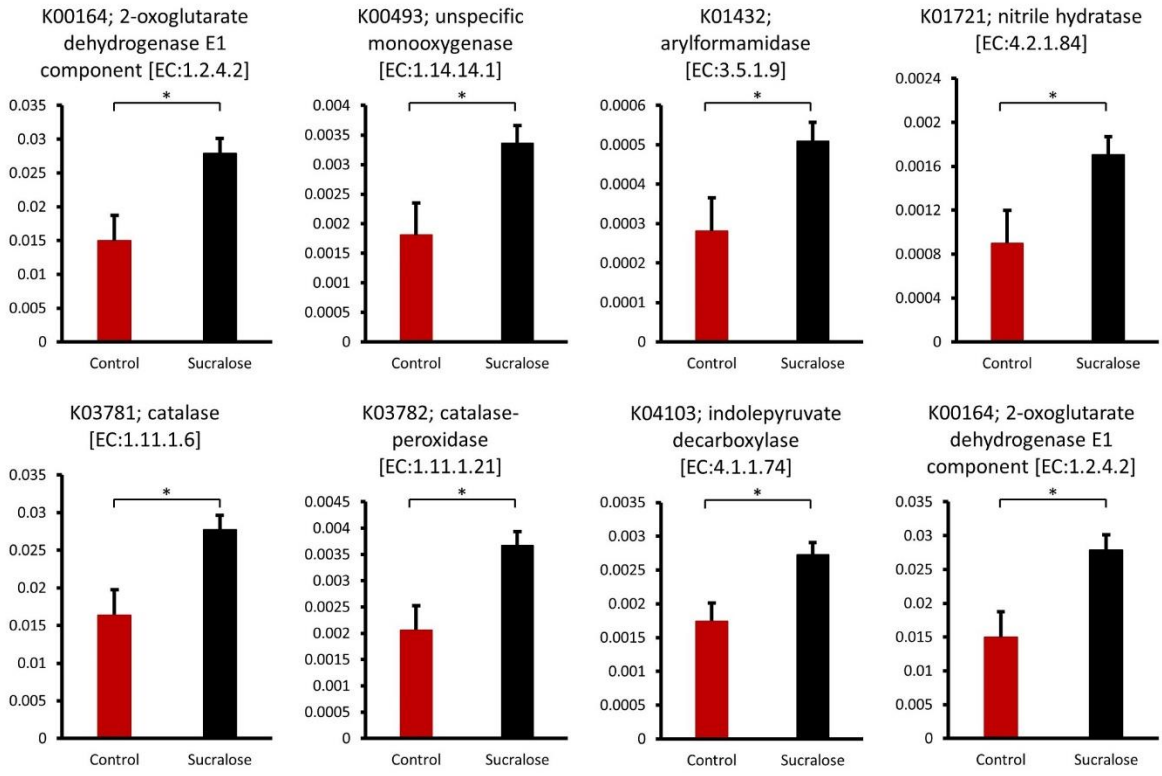

Figure S3.1. Enrichment of bacterial genes related to tryptophan metabolism in the gut microbiome of sucralose-treated mice (*$p<0.05$).

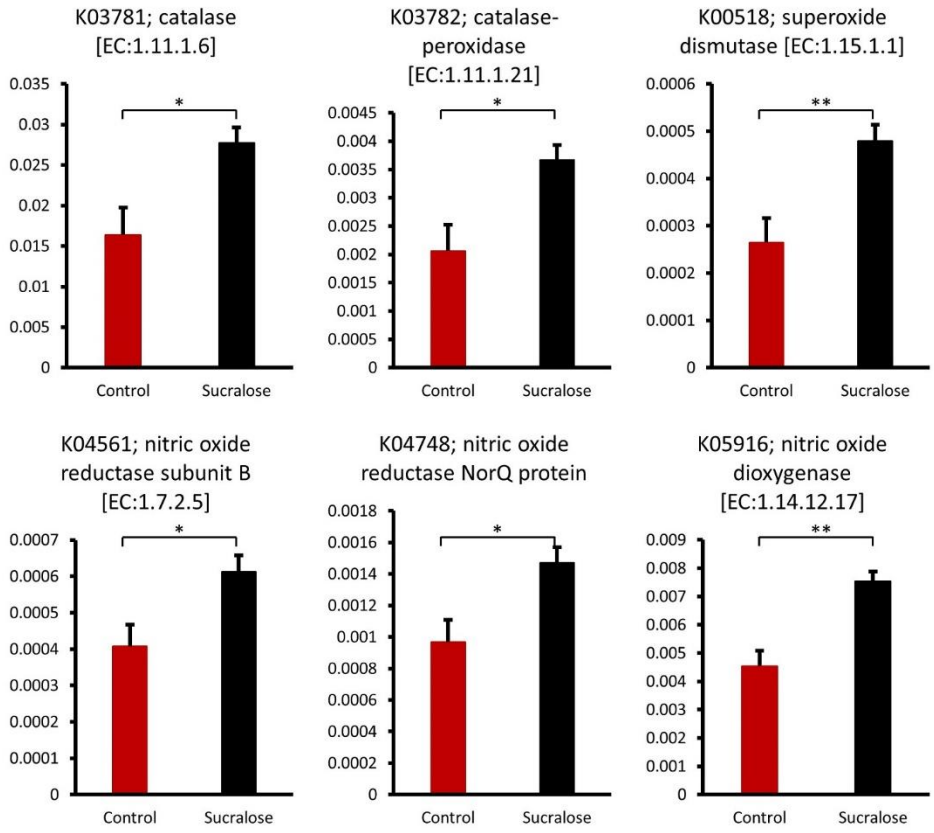

Figure S3.2. Increase of bacterial genes of antioxidative enzymes in the gut microbiome of sucralose-treated mice (*p<0.05, **p<0.01)

CHAPTER 4

SACCHARIN INDUCED LIVER INFLAMMATION IN MICE BY ALTERING THE GUT MICROBIOTA AND ITS METABOLIC FUNCITONS[3]

[3] Bian, X, Tu, P, Chi, L, Bei, G, Ru H and Lu, K. Submitted to *Food and Chemical Toxicology*, 04102017.

Abstract

Maintaining the balance of gut microbiota and its metabolic functions is vital for human health, however, this balance can be disrupted by various external factors including food additives. A range of food and beverages are sweetened by saccharin, the oldest artificial sweetener, which is generally considered to be safe despite controversial debates. Recent studies indicated that saccharin consumption perturbed the gut microbiota hence contributing to dysbiosis. Inflammation is one of the most common physical conditions associated with the dysbiosis of the gut microbiota, which is involved in numerous diseases, such as inflammatory bowel disease, diabetes and obesity. The aim of this study is to investigate the relationship between inflammation and perturbed gut microbiome induced by saccharin. C57BL/6J male mice were treated with saccharin in drinking water for six months. Quantitative-PCRs were used to detect the inflammatory markers in mouse liver, while 16S rRNA gene sequencing and liquid chromatography-mass spectrometry based metabolomics were used to reveal the change of gut microbiota and its metabolomic profiles. Elevated expression of pro-inflammatory iNOS and TNF-α in liver indicated that saccharin induced inflammation in mice. The altered gut bacterial genera, enriched orthologs of pathogen-associated molecular patterns, such as lipopolysaccharide and bacterial toxins, in concert with increased pro-inflammatory metabolites suggested that the saccharin-induced liver inflammation could be associated with the perturbation of the gut microbiota and its metabolic functions.

Introduction

Association between the gut microbiota and host health has raised growing public attention in recent years (Turnbaugh et al., 2007), as the gut microbiota plays an essential role in host-gut interactions, such as energy metabolism, immune response and epithelial homeostasis

(Holmes, Li, Athanasiou, Ashrafian, & Nicholson, 2011). Hence, perturbations on the gut microbiota induced by environmental factors would affect its functional role and lead to gut microbiota-related human diseases, including immune dysfunction, obesity, diabetes and cardiovascular disease, inflammatory bowel diseases and colorectal cancer (Kau, Ahern, Griffin, Goodman, & Gordon, 2011; Kinross, Roon, Holmes, Darzi, & Nicholson, 2008; Tremaroli & Backhed, 2012). It is well documented that the perturbation of relative abundance of gut microbes as well as gut microbial metabolites can contribute to adverse health outcomes. For example, the ratio of Firmicutes and Bacteroidetes was observed to be associated with obesity in mice (Turnbaugh et al., 2006). Likewise, trimethylamine, generated by gut bacteria from dietary choline, can be further metabolized into trimethylamine N-oxide in liver, and the later serves as a predictive maker for cardiovascular disease (Jonsson & Backhed, 2015). Therefore, xenobiotics can potentially lead to diseases by shifting gut microbial composition or altering the microbial metabolism. Thus, it is noteworthy and imperative to investigate the relationship between exposure to xenobiotics and imbalanced gut microbiota.

Artificial sweetners are ubiquitously used food additives, and the US Food and Drug Administration (FDA) has approved the usage of saccharin, sucralose, aspartame, neotame, acesulfame potassium and advantame to date (Spencer et al., 2016). The effects of artificial sweetners on human health are controversial, and particularly, their role in shaping the gut microbiota is barely understood. As the oldest artificial sweetener, saccharin was discovered and used since 1879 (Shankar, Ahuja, & Sriram, 2013), and the FDA approved accepted daily intake (ADI) in human is 15mg/kg body weight/day. Saccharin is generally considered to be safe, partially because of the fact that it is barely metabolized by human body (Spencer et al., 2016). Saccharin goes through the gastrointestinal (GI) tract un-metabolized, and is absorbed slowly

from the intestine but eliminated rapidly in the urine (Renwick, 1985). It has been reported that dietary saccharin could change gut bacterial metabolism in rats by affecting the activity of several bacterial enzymes (Mallett, Rowland, & Bearne, 1985). And saccharin consumption was discovered to be associated with altered amino acids metabolism by gut bacteria (Lawrie, Renwick, & Sims, 1985). Most recently, a study found that saccharin was able to alter the mouse gut microbiota and this alteration led to increased glucose intolerence (Suez et al., 2014). The impact of saccharin on gut microbiota and metabolic profiles leads to a new but underappreciated mechanism of the toxicity of saccharin, which represents a significant knowledge gap in artificial sweetener toxicity research.

Inflammation is one of the most common physical conditions and associated with a number of human diseases, such as inflammatory bowel diseases, obesity, diabetes, atherosclerosis and cancer (Coussens & Werb, 2002; Furet et al., 2010; Holmes et al., 2011; Libby, Ridker, & Maseri., 2002; Uronis et al., 2009; Xavier & Podolsky, 2007). In previous studies, saccharin was recognized to enrich the biosynthesis pathway of lipopolysaccharide (LPS) of the mice gut microbiota (Suez et al., 2014), which is a common trigger of inflammation. In the meantime, it is realized that gut microbiota and metabolites is pivotal for host immune response (Kau et al., 2011). For example, the gut microbiota is involved in the regulation of host inflammatory responses through dietary fermentation product short-chain fatty acids (Maslowski et al., 2009). It is of significance to probe the inflammation-inducing effects of saccharin in view of gut microbial and metabolic changes. In the present study, we used a mouse model to investigate its functional impact on the gut microbiota, particularly the microbiota-related alterations associated with inflammation.

We hypothesized that intake of saccharin is able to induce perturbations of the gut microbiota, regarding bacterial abundance and metabolic profiles, which can potentially elicit inflammation in the host via host-gut microbiota interactions. To test this hypothesis, we exposed C57BL/6J male mice to saccharin at the human ADI level for six months. An integrated approach combining high-throughput 16S rRNA gene sequencing and liquid chromatography-mass spectrometry (LC-MS) metabolomics were applied to investigate the alterations of the gut microbiota induced by saccharin. Results of quantitative real-time polymerase chain reaction (qPCR) revealed increased expression of the inflammation mediators, iNOS and TNF-α, in mouse liver after six-month saccharin exposure. More importantly, both 16S rRNA sequencing and metabolomics profiling revealed a pronounced impact of saccharin on the mouse gut microbiota and metabolites, with many changes linked to pro-inflammatory effects.

Materials and Methods

Animals and exposure. We used C57BL/6J mice (male, ~23g, approximately 8 weeks old) purchased from the Jackson Laboratories (Bar Harbor, ME) for this study. Twenty mice were housed upon arrival in the cages under the environmental conditions (temperature 22°C, 40–70% humidity, and a 12:12 hr light:dark cycle) with standard pelleted rodent diet and water ad libitum for a week before the study. Then, the mice were randomly assigned to the control and treatment group, consisting ten mice in each group. The treatment group received saccharin (Sigma-Aldrich, MO) for six months. Saccharin was dissolved in drinking water to 0.3mg/Ml, which was equivalent to the FDA approved ADI in human. Saccharin solution was made every week. Control mice received drinking water only. Following the treatment, body weight and water consumption were monitored for both groups. The fecal pellets from individual mouse were collected at baseline, three- and six-month and all samples were frozen at −80 °C for

further analysis. This study was carried out in the University of Georgia animal facility. All experiments were approved by the University of Georgia Institutional Animal Care and Use Committee. The mice were treated humanely and with regard for alleviation of suffering.

RNA extraction, cDNA preparation, and Quantitative real-time polymerase chain reaction (qPCR). Total RNAs from liver treated with RNAlater (Thermo Fisher Scientific) were extracted using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacture's instruction. The resulting RNAs were treated with DNase (DNA-free™ DNA Removal Kit, Thermo Fisher Scientific) to remove genomic DNA contamination. RNA quality was determined using an Agilent TapeStation (Agilent Technologies). Then, 1µg of total RNA was used for cDNA synthesis using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, CA). The sequences of the primers used for quantitative PCR were listed in Table S4.1 (Alkhouri et al., 2010; Grivennikov et al., 2012; Song et al., 2015; Wang et al., 2006; Xu et al., 2005; Zhang et al., 2015). All results were normalized to the housekeeping gene β-actin as an endogenous control. Each reaction was prepared as instructed in the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) manual. Reactions were run on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System. The qPCR was performed using the following protocol: 95 °C for 10 min, 40 cycles of 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C followed by 65 °C to 95 °C increment by 0.5°C for 0.05s. DNase digestion and no-RT control were used to control the potential genomic DNA contamination, and no-template control was used to control technical contamination. Results were analyzed using ΔΔCT method by CFX manager software (Bio-Rad).

16S rRNA gene sequencing of the gut microbiota and data processing. We used 16S rRNA gene sequencing to investigate the gut microbiota in fecal samples at different time points.

Fecal DNA from individual mouse was extracted using a PowerSoil DNA Isolation Kit (MO BIO Laboratories) according to the manufacture's instruction. The resulting DNA was quantified and stored in -80 °C prior to analysis. For each sample, 1ng of the purified fecal DNA was used as template for the PCR amplification and the barcoded bacterial universal primers of 515 (5'-GTGCCAGCMGCCGCGGTAA) and 806 (5'-GGACTACHVGGGTWTCTAAT) were applied to target variable region 4 of the 16S rRNA gene. Then, the barcoded amplicons from all samples were normalized, pooled and sequenced by Illumina Miseq at the Georgia Genomics Facility. A depth of at least 25,000 reads per sample was prepared by generating pair-end 250 × 250 (PE250, v2 kit) reads. The raw mate-paired files of 16S rRNA gene sequences were first trimmed to dispose bases with high error probability (> 0.01) and merged using Geneious 8.1.5 (Biomatters, Auckland, New Zealand). The pre-processed 16S rRNA gene sequences data were then analyzed using QIIME (Quantitative Insights into Microbial Ecology, version 1.9.1) software package (Caporaso et al., 2010). The operational taxonomic units (OTUs) with 97% sequence similarity against Greengenes database 13.8 were obtained using UCLUST. All OTUs were classified at five different levels: phylum, class, order, family and genus. Mothur software was used to assess the difference in individual gut microbes between controls and treated mice over time.

Functional capability analysis. Tax4Fun, an open-source R package, uses 16S rRNA sequencing dataset to survey the functional profiles of microbial communities (Asshauer, Wemheuer, Daniel, & Meinicke, 2015). It provides functional repertoire with a high correlation coefficient to the corresponding metagenome sequence data based on 16S rRNA datasets (Asshauer et al., 2015). It was used to analyze the enrichment of functional genes of the microbiome in each group to investigate functional responses of bacterial communities to saccharin. QIIME with a SILVA database extension (SILVA 119) was used to pre-process raw

data for Tax4Fun as described previously. Further statistical analysis was investigated using Statistical Analysis of Metagenomic Profiles (STAMP) (version 2.1.3) for results obtained from Tax4Fun (Parks, Tyson, Hugenholtz, & Beiko, 2014).

Fecal metabolite analysis. Extraction of metabolic compounds in fecal samples, collected after exposure to saccharin for six months, was conducted using methanol and water as previously described (Lu et al., 2014). The resultant extracts were suspended in 20% Acetonitrile for MS analysis. A HPLC-Q-TOF (Quadrupole-time-of flight (Q-TOF) 6520 mass spectrometer (Agilent Technologies, Santa Clara, CA) with an electrospray ionization source interfaced with Agilent 1200 HPLC) system was used to conduct metabolomic profiling. The daily calibration of Q-TOF with standard tuning solution (Agilent Technologies) was carried out to ensure a mass accuracy of < 5ppm. Metabolites were separated on an YMC Hydrosphere C18 column and a mass range of 30 to 2000 m/z was employed to capture molecular features in a positive mode. The difference of metabolic profiles between controls and saccharin-treated mice was generated using a two-tail Welch's t-test ($p<0.05$). Partial least squares discriminant analysis (PLS-DA) and hierarchical clustering heat map was performed to show the difference of the metabolomes in different groups. Principle component analysis (PCA) was used to examine the intrinsic clusters and outliers.

Metabolomics data processing and metabolite identification. Data obtained from the HPLC-Q-TOF system was processed and analyzed as described (Lu, Knutson, Wishnok, Fox, & Tannenbaum, 2012). Briefly, the MassHunter Workstation software (Agilent) was first used to convert the raw .d data to .mz data format, and signals with intensity higher than 1000 counts were included for the subsequent analysis. The XCMS Online tools were used to perform peak alignment, intensity calculation and comparison between controls and treatment groups.

Significantly altered metabolic features were profiled and searched against the Human Metabolome Database (HMDB) (http://www.hmdb.ca) and METLIN (http://metlin.scripps.edu). Matched metabolic features with these two database were examined by product ion scan using a MS/MS mode in the Q-TOF 6520 mass spectrometer for each molecular feature, and the spectra were searched against the HMDB and METLIN MS/MS database for tentative identifications.

Results

Effect of saccharin on the inflammation mediators, iNOS and TNF-α expression in mice. To explore the effect of saccharin consumption on chronic inflammation in mice, we examined the pro-inflammatory gene expression on mRNA level. Notably, we found two genes, inducible nitric-oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α), were significantly ($p<0.05$) elevated in saccharin-treated mice liver (Figure 4.1), which suggested a strong link between saccharin consumption and host inflammatory response.

Impact of saccharin on the dynamics of gut bacterial development. Saccharin induced significant changes to the mouse gut microbiota, manifested by the alterations of gut bacteria. As shown in Figure 4.2, the relative abundance of bacteria showed no significant difference between the treatment and control group at baseline, however displayed distinction either at three-month or six-month, or both. Eleven genera were significantly changed after three- and six-month treatment, indicating the effect of saccharin on disrupting the dynamics of gut microbiome development. Specifically, *Sporosarcina*, *Jeotgalicoccus*, *Akkermansia*, *Oscillospira* and *Corynebacterium* were significantly increased after three-month consumption; *Corynebacterium*, *Roseburia* and *Turicibacter* were increased after six-month consumption. *Anaerostipes* and *Ruminococcus* were significantly decreased after three-month consumption; *Ruminococcus*, *Adlercreutzia* and *Dorea* were decreased after six-month consumption. This result indicates that

saccharin consumption could perturb the gut microbiota, which is consistent with a previous report (Suez et al., 2014). Of interest, several bacterial genera are demonstrated to be involved in inflammation. For instance, *Corynebacterium*, *Turicibacter*, *Anaerostipes*, *Dorea*, *Roseburia* and *Ruminococcus* (Bajaj et al., 2012; Chamulitrat et al., 1995; Collins et al., 2014; Fernández et al., 2016; Ng et al., 2013) were related to inflammation and *Adlercreutzia* (Maruo, Sakamoto, Ito, Toda, & Benno, 2008) was related to equol production, suggesting that saccharin-induced gut microbiota changes may be partially responsible for the pro-inflammatory effects of saccharin.

Enrichment of bacterial functional genes of pro-inflammatory mediators. Altered gut microbiota generally functions differently, which can be unveiled by bacterial functional gene profiling. Therefore, we performed the functional enrichment analysis of the mouse gut microbiota based on 16S rRNA sequencing data. The functional comparison of the gut microbiota between the saccharin and control group revealed pronounced difference in term of bacterial inflammation-related pathways. We found that orthologs involved in pro-inflammatory mediators were highly elevated in saccharin-treated mice, as shown in Figure 4.3. At six-month consumption, 6 Lipopolysaccharide (LPS) biosynthesis orthologs, one Flagellar assembly ortholog, eleven fimbrial orthologs, twenty-three bacterial toxin orthologs, and six multidrug resistance orthologs were significantly increased in saccharin-treated mice. Some of the elevated genes were only observed in saccharin-treated mice. These data suggested that perturbation of the gut microbiota by saccharin increased the abundance of bacterial genes that could increase the risk of inflammation in the host, as these pro-inflammatory mediators can translocate into the host circulation to elicit inflammatory response.

Saccharin altered the gut microbial metabolic profiles. Large amounts of metabolites inside the intestine play crucial roles in the communication between the gut microbiota and the

host, which helps keep a healthy gut environment. Given the alteration of fecal microbiota by saccharin and the fact that the gut metabolites are co-produced by gut microbes and host, we further explored the effects of saccharin on gut microbial metabolic profiles. In concert with the perturbation on bacterial abundance, comparative metabolomics analysis indicated that saccharin altered fecal metabolic profiles. Figure 4.4 shows that saccharin altered the gut metabolome with 1743 significantly changed molecular features (Figure 4.4A); and the PLS-DA plot reveals a clear separation between controls and saccharin-treated mice (Figure 4.4B). These results indicated that six-months of saccharin consumption altered the fecal metabolome.

Significantly changed functional metabolites by saccharin. To further investigate the effects of saccharin on chronic inflammation in the host, we identified significantly altered molecular features and obtained a list of changed metabolites (Table S4.2). Notably, some inflammation-related metabolites were identified to be changed. Interestingly, we found that equol, a bacterial metabolite of daidzein, was decreased by saccharin, as illustrated in Figure 4.5A. Meanwhile, daidzein and its major bacterial metabolites, dihydrodaidzein and O-desmethylangolensin, were significantly increased in saccharin-treated mice. Increased daidzein and decreased equol suggest a reduced growth or decreased enzymatic activity of metabolizing bacteria. Likewise, linoleoyl ethanolamide, palmitoleoyl ethanolamide, N,N-Dimethylsphingosine and quinolinic acid (Figure 4.5B), were significantly changed in saccharin-treated mice. The effects of these compounds in mediating inflammation have been demonstrated in previous studies (Ishida et al., 2013; Nishiuma et al., 2008).

Discussion

In this study, we applied qPCR to examine the inflammatory markers in mouse liver and expression of two pro-inflammatory genes were found elevated. Also, we used 16S rRNA gene

sequencing and metabolomics to explore the effects of saccharin consumption on the gut microbiota and its metabolic functions. We have demonstrated that saccharin consumption altered the fecal microbiota and metabolome, indicating a functional impact of saccharin on the gut microbiota and host. Notably, saccharin consumption increased the abundance of some bacterial genes of pro-inflammatory mediators and decreased the level of anti-inflammatory metabolites, which could increase the risk of developing inflammation in the host. This study provides novel insights into saccharin-induced functional changes in the gut environment and a potential link between gut microbiome perturbations and chronic inflammation in the host.

Previous studies have indicated that saccharin may induce inflammation in animals (Suez et al., 2014), and in our study, we found elevated gene expression of iNOS and TNF-α in the liver of saccharin-treated mice. Inducible NOS (iNOS), present in hepatocytes, endothelium and other immune cells, can synthesize high concentration of nitric oxide (NO), and lead to tissue damage in inflammatory processes. Expression of iNOS is highly regulated at the transcriptional level, through cellular receptors, such as CD14, via the activation of NFκB pathway and the expression can be triggered by LPS through CD14 (Aktan, 2004). iNOS plays an important role in the inflammation in multiple liver diseases, such as liver tumors and liver fibrosis from chronic viral infection (La Mura et al., 2014; Sass, Koerber, Bang, Guehring, & Tiegs, 2001). TNF-α, a key cytokine in inflammation, is mainly produced by activated macrophages, induced by pathogen-associated molecular patterns (PAMPs) such as LPS through Toll-like receptors (TLRs) (Wu & Zhou, 2010). Expression of TNF-α can active NFκB pathways and induce damage to cells. Previous studies showed that TNF- α could mediate liver damage, which will lead to inflammation in liver, and anti-TNF antibody treatment could improve steatosis in ob/ob

mice (Garcia-Ruiz et al., 2006). The elevated expression of iNOS and TNF-α herein suggested that saccharin consumption leads to liver inflammation in mice.

Inflammation triggers are common in the GI tract, for instance, living microbes that behave as pathogens or opportunistic pathogens, PAMPs such as LPS, flagellin, bacterial DNA and RNA, and microbial metabolites such as toxins and secondary bile acids. Perturbation of the gut microbiota and metabolites can potentially increase the level of inflammation triggers and lead to inflammation in the host. In this study, we observed changes in the abundances of various bacteria at the genus level between the saccharin and control group. Among changed gut bacteria, several genera were related to inflammation in the host. *Corynebacterium* in the family of *Corynebacteriaceae,* which were found increased in our study, contains some opportunistic pathogenic species. For example, *Corynebacterium parvum* could induce chronic inflammation through the over-production of NO in mouse liver, and lead to hepatic necrosis and death if followed by LPS injection (Chamulitrat et al., 1995). *Anaerostipes*, *Dorea* and *Ruminococcus* in family *Lachnospiraceae* were found decreased in saccharin-treated mice in this study, and the decrease of these three genera were associated inflammation in previous studies: *Anaerostipes*, decreased in a biliary inflammation mouse model, *Dorea* decreased in subjects of irritable bowel syndrome, and loss of *Ruminococcus* was associated with colitis induced by dextran sodium sulfate (Fernández et al., 2016; Ng et al., 2013). Taken together, these bacterial changes may partially contribute to elevated expression of inflammatory genes in mouse liver.

Consistently, the functional enrichment analysis of the gut microbes also revealed a large difference in bacterial functional repertoire between the saccharin and control group. Notably, we found that orthologs related to bacterial pro-inflammatory mediators were significantly increased after saccharin consumption (Fig. 4.3). LPS, flagella and fimbriae, known PAMPs that

can trigger pathological inflammation in the host, were increased in saccharin-treated mice. LPS is an endotoxin from the outer membrane of Gram-negative bacteria, and it can initiate secretion of pro-inflammatory cytokines like interleukin-6 or tumor necrosis factor (TNF)-α via toll-like receptor 4 (TLR4) (de La Serre et al., 2010). Flagella and fimbriae are important bacterial components that are involved in the host inflammatory response modulation (Madianos, Y. A. Bobetsis, & Kinane., 2005). High levels of flagella is associated with gut mucosal barrier breakdown and inflammation (Cullender et al., 2013), while fimbriae play an important role in adhesion and invasion into epithelial cells (Nakagawa, 2002). Likewise, increased bacterial genes involved in multiple bacterial toxins that are also strongly associated with inflammation were found in saccharin-treated mice (Madianos et al., 2005). The enrichment of inflammation-associated orthologs in mouse gut bacterial may result in elevated inflammatory disorders. In addition, after six-month saccharin treatment, the gut microbiota exhibited a higher frequency and abundance of bacterial orthologs of multi-drug resistance. For instance, the frequency of several major facilitator superfamily transporters was higher in mice with saccharin treatment. It is well-documented that multi-drug resistance efflux pumps in bacteria could play an important role in resistance to antibiotics as well as the extrusion of host-produced natural substances and xenobiotics (Marshall, Dorothy J. Ochieng, & Levy, 2009; Piddock, 2006). These results strongly suggested a potential effect of saccharin to induce elevated inflammatory responses through altering the gut microbiota.

Saccharin significantly changed bacterial functional metabolites, which may affect the host. For example, bacterial metabolism of daidzein was impaired by saccharin. Daidzein, an isoflavone found in soy, works as a weak estrogen in the body, and can be metabolized into dihydrodaidzein, O-desmethylangolensin (O-DMA) and equol by gut bacteria (Decroos,

Vanhemmens, Cattoir, Boon, & Verstraete, 2005). Interestingly, the metabolism pattern varies depending on the composition of the gut microbiota, and only about one third of the human population possess gut microbiota that can produce equol and about 80%-90% of the population are capable to produce O-DMA (Atkinson, Cara L. Frankenfeld, & Lampe, 2005). Equol and O-DMA are more biologically active than daidzein as estrogen. Recently, equol was found to have the ability to inhibit LPS-induced oxidative stress in chicken macrophages, inhibit superoxide production in cell culture, protect neurons from neuro-inflammatory injury and suppress inflammatory response in mice and the antioxidant activity of equol is greater than its parent compound daidzein (Gou, Jiang, Zheng, Tian, & Lin, 2015; Hwang, Wang, Morazzoni, Hodis, & Sevanian, 2003; Lin, Yamashita, Murata, Kumazoe, & Tachibana, 2016; Subedi et al., 2017). Therefore, the capacity of equol production from daidzein by gut bacteria can increase its antioxidative and anti-inflammatory ability in the host. In our study, saccharin consumption significantly decreased the production of equol and increased the level of its parent daidzein and other metabolites, O-DMA and dihydrodaidzein (Figure 4.5), which not only demonstrates the impact of saccharin on the metabolic functions of the gut microbiome, but also decreases the ability of the gut microbiome to protect the host from inflammatory challenges. Consistent with the decreased level of equol, we found decreased abundance of *Adlercreutzia* in the gut microbiota (Figure 4.2), which contains equol-producing bacteria in this genus (Maruo et al., 2008). All these results indicate that saccharin consumption can decrease the production of equol, and hence decrease the potential protection from inflammation. In addition, several other anti-inflammatory compounds, palmitoleoyl ethanolamide (PEA), linoleoyl ethanolamide (LEA) and N,N-Dimethylsphingosine, were decreased in saccharin-treated mice. As a fatty acid ethanolamide, PEA was found to inhibit inflammation in human adipocytes and peripheral

tissues through the regulation of pro-inflammatory proteins, nitric oxide, and neutrophils (Ezzili et al., 2010). Likewise, LEA was reported to reduce LPS-induced inflammation in macrophages (Ishida et al., 2013). N,N-Dimethylsphingosine was shown to attenuate airway inflammation in a mouse model (Nishiuma et al., 2008). The reduction of these compounds can impact chemical signaling between the gut microbiome and host, which may contribute to the development of tissue inflammation in the host. Moreover, we found increased quinolinic acid, a metabolite of tryptophan, which served as a pro-inflammatory compound in a previous study (Keszthelyi, Troost, & Masclee, 2009). Chemical signaling of gut microbiome-host interaction likely involves a vast number of functional metabolites, as exemplified by only few compounds briefly described here. Nevertheless, the change of these metabolites indicates that saccharin consumption may increase the risk of host inflammation through altering metabolites produced or regulated by the gut microbiome.

Conclusion

Our results revealed that 6-month saccharin administration in drinking water induced elevated inflammation in mouse liver, which could be functionally associated with saccharin-induced gut microbiome perturbations, exemplified by the alteration of inflammation-related bacterial pathways and metabolites (Figure 4.6). Elevated expression of iNOS and TNF-α in liver supported that saccharin consumption could increase inflammation in mice, and this change may be the consequence of perturbations of the gut microbiota and metabolites arising from saccharin consumption. Our results highlight the role of disrupted gut microbiome in eliciting systemic adverse response in the host. Consequently, this study provides novel insights regarding the toxicity assessment of food additives, such as artificial sweeteners, in view of their effects on gut microbial homeostasis.

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Tables

Table S4.1. Primers used in qPCR.

Gene	Forward primer	Reverse primer	Reference
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	Alkhouri et al., 2010
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC	Alkhouri et al., 2010
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT	Alkhouri et al., 2010
iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC	Alkhouri et al., 2010
β-actin	CGTGCGTGACATCAAAGAGAA	TGGATGCCACAGGATTCCAT	Song et al., 2015;

Table S4.2. Significantly altered fecal metabolites in saccharin mice compared to control.

Identified metabolites	Fold change	p-values	m/z	retention time (minute)
Saccharin	233.08	0.000	184.0062	11.90
Quinolinic acid	6.47	0.000	168.0287	8.05
Phenylalanine	5.33	0.015	166.0857	7.42
Glutaminyl-Phenylalanine/Gamma-glutamyl-Phenylalanine	5.16	0.045	294.1451	8.42
Dihydrodaidzein	4.76	0.025	257.0808	12.49
Daidzein	4.08	0.047	255.0654	12.40
O-Desmethylangolensin	3.83	0.005	259.0972	13.89
Isoleucyl-Tyrosine	3.02	0.019	295.1661	8.28
Kynurenic acid	2.53	0.012	190.0492	11.01
2-aminomuconic acid	2.48	0.002	158.0455	2.81
L-gamma-glutamyl-L-isoleucine	2.20	0.003	261.1467	4.53
L-isoleucyl-L-proline or 1-L-Leucyl-L-Proline	1.80	0.003	229.1544	7.54
m-Aminobenzoic acid or p-Aminobenzoic acid	1.53	0.005	138.0551	8.00
L-Tryptophan	1.50	0.034	205.0972	8.29
Equol	-1.52	0.030	243.1036	13.79
Aminoadipic acid	-1.54	0.015	162.0748	2.36
7Z,10Z-Hexadecadienoic acid	-1.59	0.005	253.2147	13.89
Oleic acid	-1.60	0.030	283.2639	19.09
Palmitoleoyl Ethanolamide	-1.61	0.008	298.2737	19.19
Palmitoyl-L-carnitine	-1.66	0.043	400.3420	15.78
N,N-Dimethylsphingosine	-1.67	0.030	328.3210	15.11
Linoleoyl Ethanolamide	-1.67	0.009	324.2907	19.69
Citrulline	-1.73	0.009	176.1020	2.23
7α-Hydroxy-3-oxo-4-cholestenoate	-2.09	0.007	453.3015	17.40
3-Hydroxyphenylglycine	-2.32	0.000	168.0649	8.36
N(6)-Methyllysine	-2.34	0.014	161.1287	2.19
5'-Deoxyadenosine	-2.50	0.021	252.1095	4.84
Adenine	-2.96	0.014	136.0618	4.84
PI(18:1(9Z)/0:0)	-39.53	0.047	599.3224	14.00

Figures

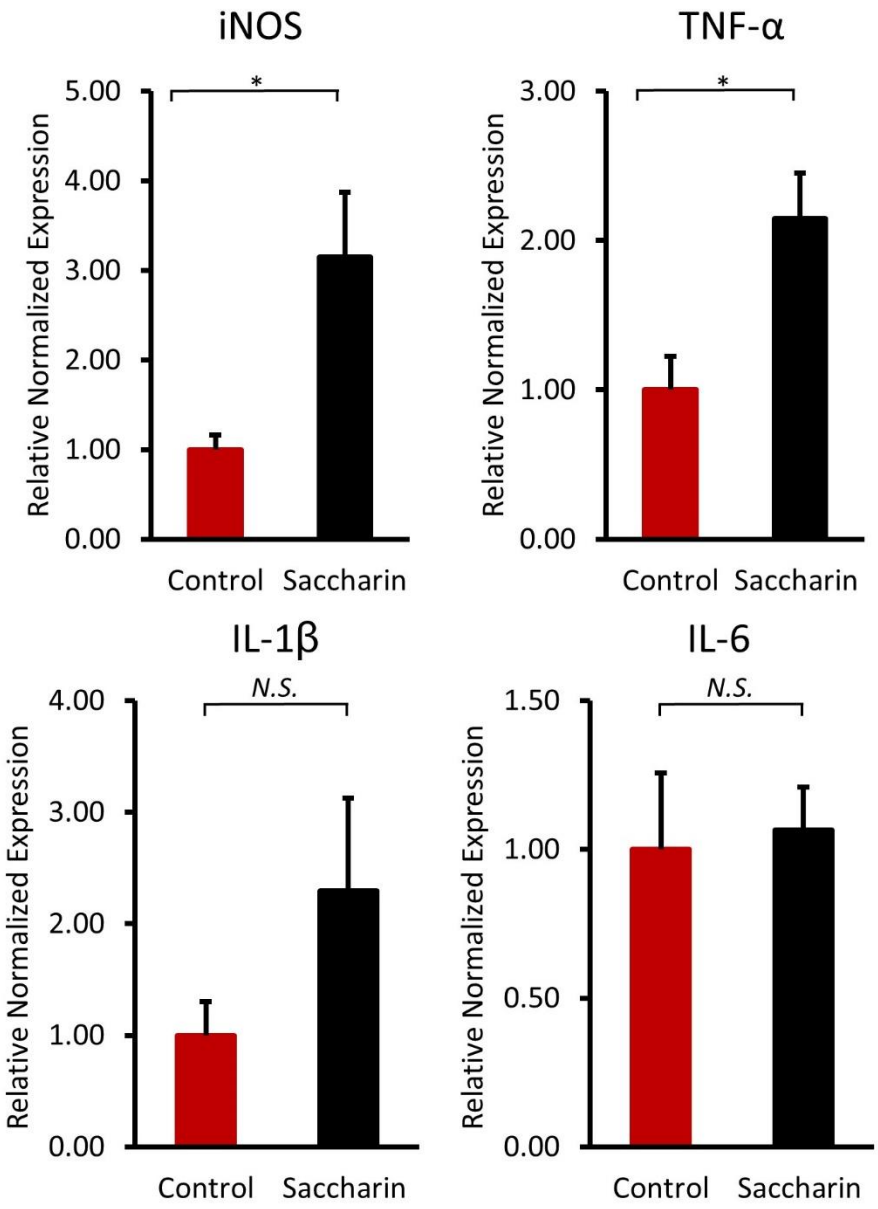

Figure 4.1. Comparisons of pro-inflammatory gene expression on mRNA level in liver of mice. Inducible nitric-oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α) significantly elevated in liver of saccharin-treated mice. (*p<0.05)

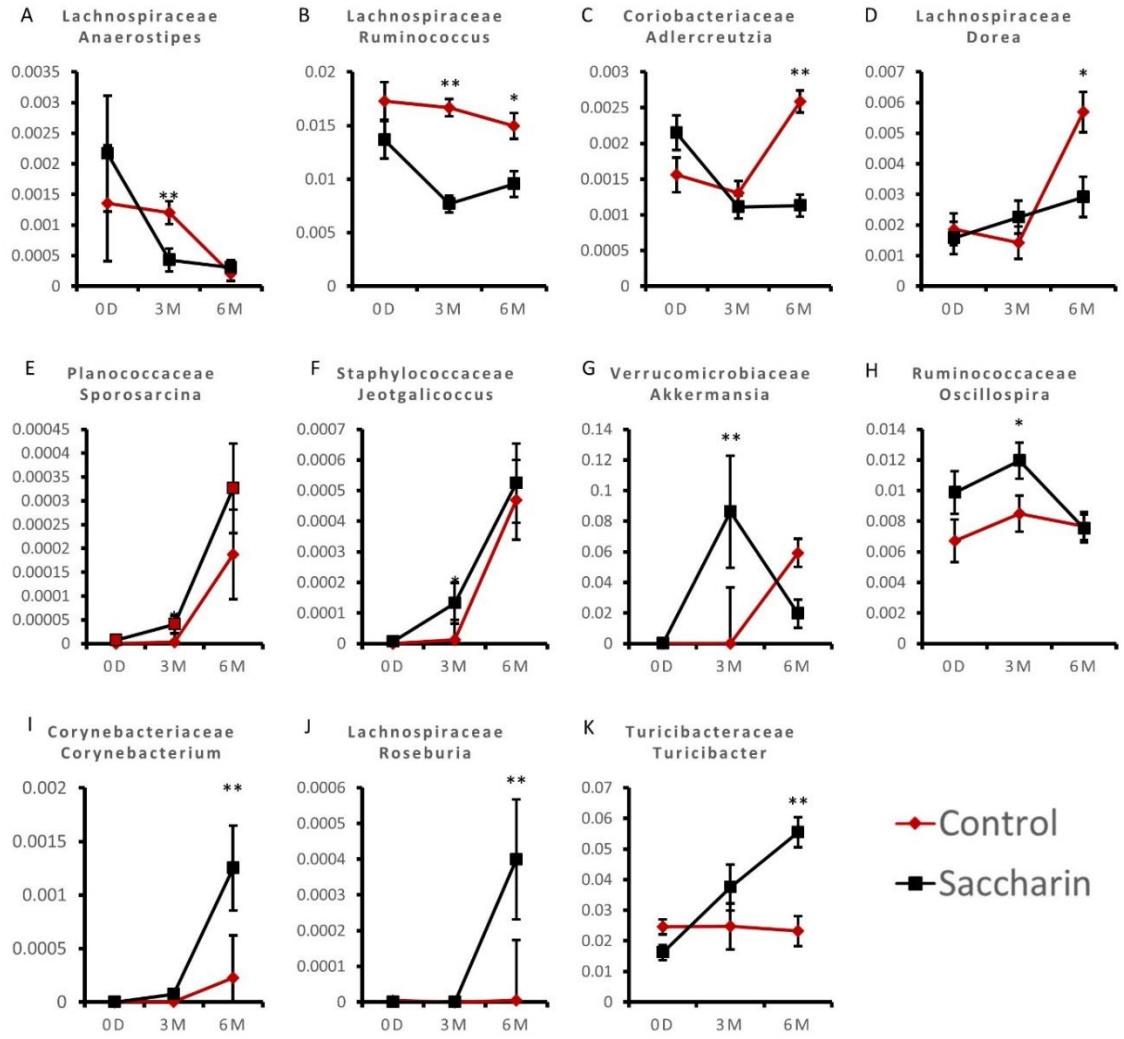

Figure 4.2. Saccharin consumption altered the dynamics of gut microbiome development in male C57BL/6J mice. *Anaerostipes* (A) and *Ruminococcus* (B) were significantly decreased after three-month consumption; *Ruminococcus* (B), *Adlercreutzia* (C) and *Dorea* (D) were decreased after six-month consumption. *Sporosarcina* (E), *Jeotgalicoccus* (F), *Akkermansia* (G), *Oscillospira* (H) and *Corynebacterium* (I) were significantly increased after three-month consumption; *Corynebacterium* (I), *Roseburia* (J) and *Turicibacter* (K) were increased after six-month consumption. X-axis represents time after the exposure, and y-axis represents relative percentage of bacteria (*$p<0.05$, **$p<0.01$)

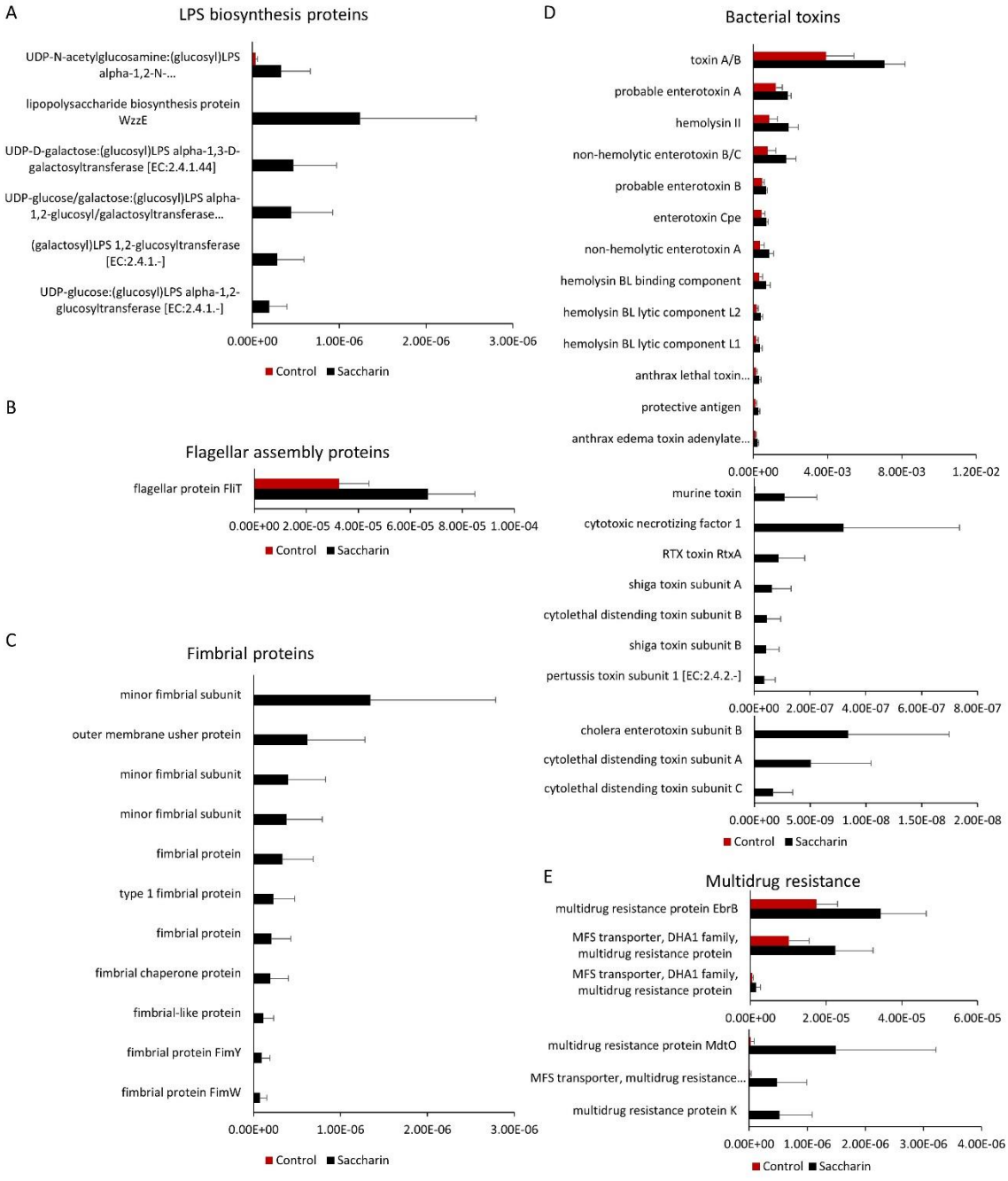

Figure 4.3. Significantly altered orthologs of the gut microbiota based on functional enrichment analysis. Genes of lipopolysaccharide (LPS) biosynthesis genes (A), flagellar assembly (B), fimbrial (C), bacterial toxins (D), and multidrug resistance (E) were significantly increased in saccharin-treated mice ($p<0.05$).

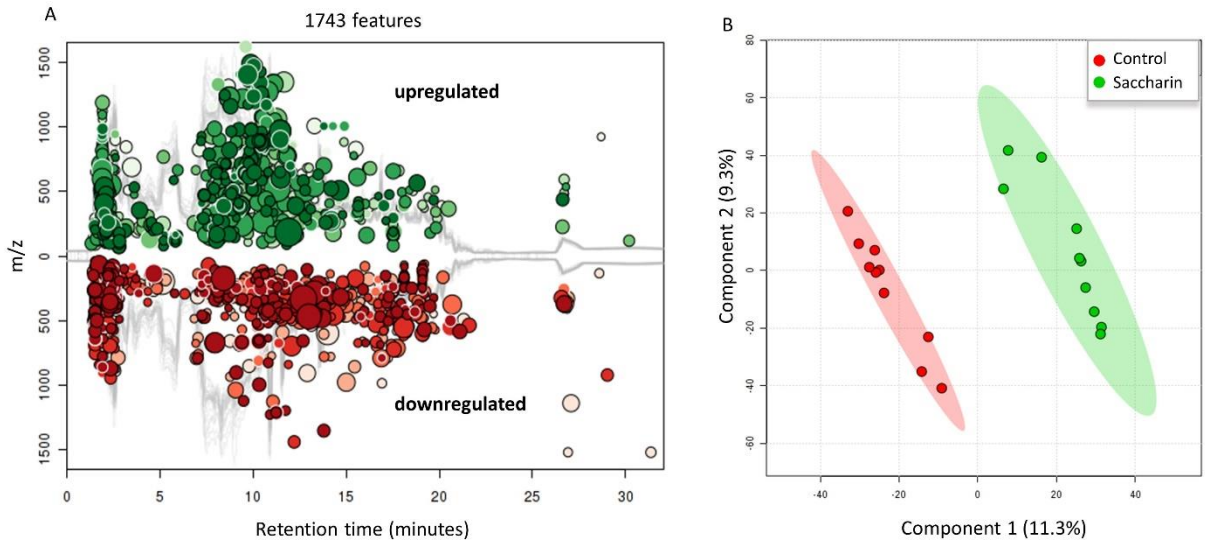

Figure 4.4. (A). Saccharin consumption changed the fecal metabolome in male C57BL/6J mice. 1743 metabolic features were significantly ($p<0.05$ and fold change>1.5) changed compared to controls. (B). Fecal metabolic profiles of controls were separated from those of saccharin-treated mice by PLS-DA.

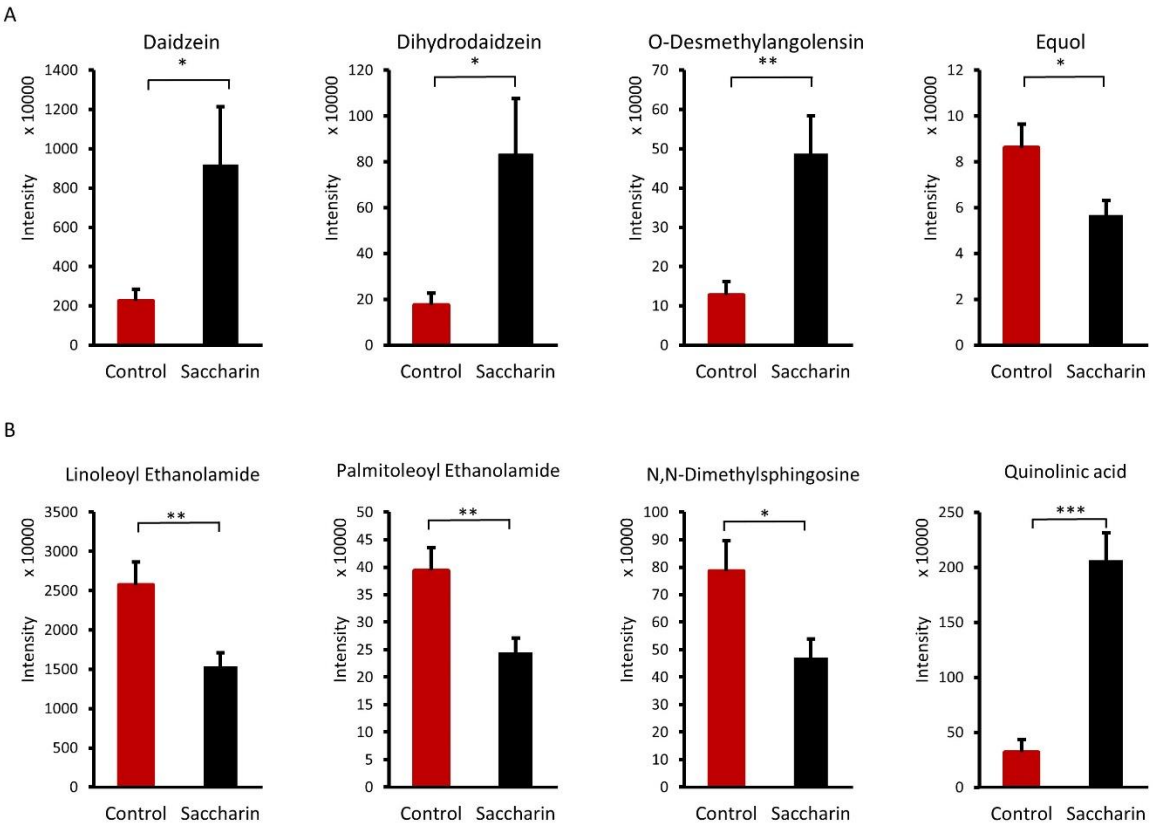

Figure 4.5. Saccharin consumption significantly altered key fecal metabolites in male C57BL/6J mice, as exemplified by equol, a gut microbiome-catalyzed metabolite of daidzein (A) and metabolites involved in mediating inflammation (B). (*p<0.05, **p<0.01, ***p<0.001)

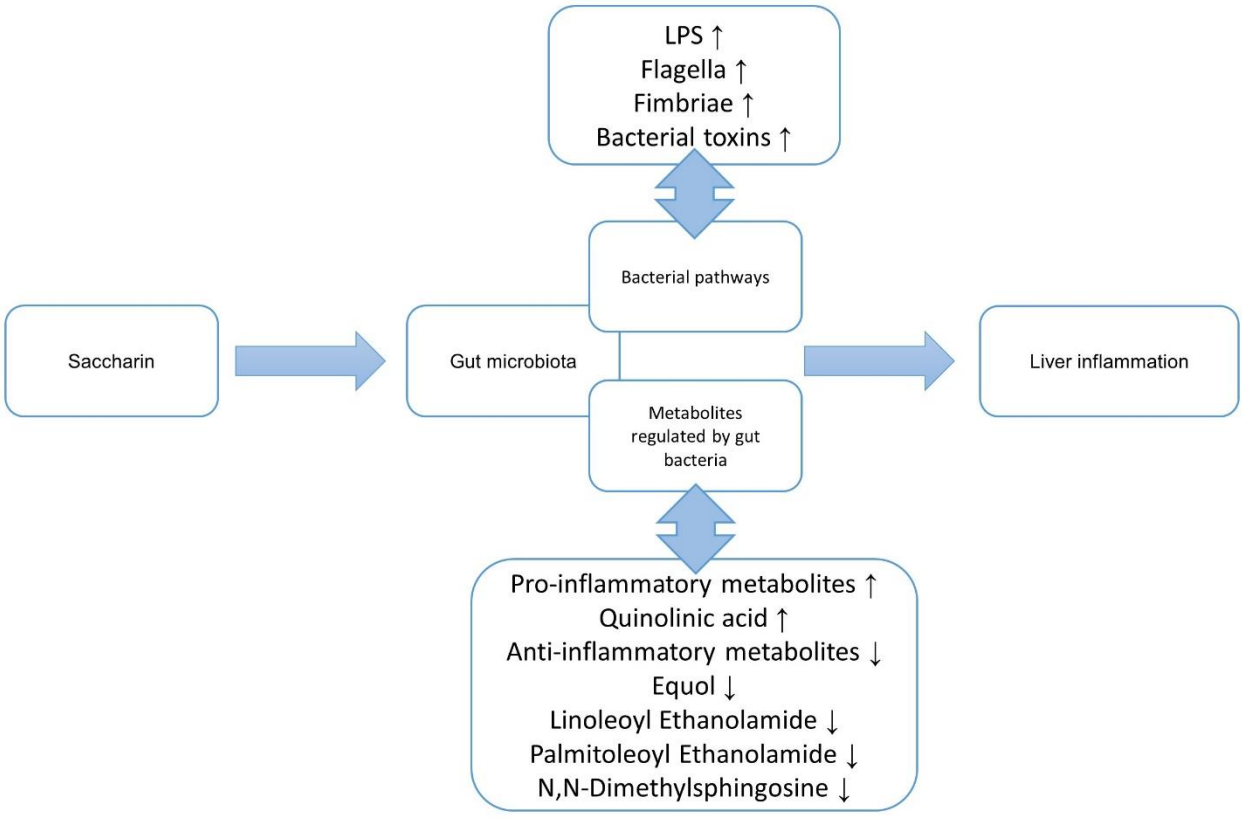

Figure 4.6. The functional link between saccharin-induced gut microbiome perturbations and host inflammation.

CHAPTER 5

CONCLUSION

The safety of artificial sweeteners are of high concern because they are broadly used in everyday life, and previous toxicity tests were mainly focused on the adverse effects to the organs of the body, such as genetic toxicity, immunotoxicity and neurotoxicity. In recent years, the gut microbiota has been gradually recognized as a "super organ" of the host for its profound and extensive influences to the health of the body. In this dissertation, three commonly used artificial sweeteners, acesulfame potassium (Ace-K) (chapter 2), sucralose (chapter 3) and saccharin (chapter 4) were tested to understand their effects on gut microbiota and inflammation, and the results showed that artificial sweeteners perturbed the gut microbiota and metabolites with pro-inflammatory trends and some of the perturbation was sex-specific. So, this dissertation provides new sight into the potential toxic effects of artificial sweeteners to humans through the change of gut microbiota and metabolites.

In chapter 2, Ace-K was dosed to CD-1 mice via oral gavage for four weeks, and the effects on gut microbiota and metabolites were explored using 16S rRNA sequencing and GC-MS metabolomics. Gut microbiota was perturbed with increased bacteria that are positively associated with inflammation and decreased bacteria that weakening inflammation; predicted bacterial gene results also showed augmented genes that contribute to inflammation, and results with metabolites supported the trend with inflammation. In addition, energy metabolism was found to be disturbed with increased body weight and abundances of genes involved in carbohydrate metabolism in male mice and decreased abundances of genes in female mice. All

the findings were highly-sex specific. Overall, this study provides novel understanding of the perturbation of the artificial sweetener Ace-K on gut microbiota and its potential role in inflammation as well as the role of gender-specific toxicity to gut microbiota. Chapter 3 describes the effects of six-month treatment with sucralose in drinking water to C57BL/6 male mice gut microbiota using 16S rRNA sequencing and HPLC-MS metabolomics. Also, qRT-PCR was used to investigate the inflammation status in liver. Elevated expression of pro-inflammatory genes were found in the liver, while perturbed gut bacterial genera, increased bacterial orthologs of PAMPs production, and increased intensity of inflammation-related gut metabolites were observed in sucralose-treated mice. Collectively, sucralose consumption increased inflammation in mouse livers and perturbed the gut microbiota and metabolites at the same time which indicated the relationship between perturbed gut microbiota and inflammation. Chapter 4 shows that six-months of consumption of saccharin in C57BL/6 male mice altered gut microbiota and metabolites, and increased the pro-inflammatory genes in the liver. Increased bacterial orthologs of PAMPs production, such as LPS, were detected in saccharin-treated mice, and more interestingly, decreased production of equol was found in saccharin-treated mice which could decrease the anti-oxidant and anti-inflammatory ability. Techniques used in this chapter were the same as in chapter 3. Together, all these results indicated the link between saccharin-induced alteration in gut microbiota and inflammation in the liver. Overall, perturbation of gut microbiota and metabolites by artificial sweeteners were observed with an obvious trend to inflammation induction after offering the artificial sweeteners to mice for a period of time through oral gavage or drinking water. And these findings could provide novel understanding of the toxicity of artificial sweeteners to humans.

Artificial sweeteners were introduced into the market for people who could not consume sugar but want sweet-taste in their diet, and nowadays, more people choose artificial sweeteners to reduce the energy intake from their diet to keep a "healthier" life style. However, there might be adverse effects from consuming artificial sweeteners, especially through the seldom tested toxicity to gut microbiota. Studies in this dissertation provide some of the first evidence that consumption of artificial sweeteners could perturb the gut microbiota and metabolites which might disturb the normal function of the host. In addition, the consumption of artificial sweeteners was shown to increase the possibility of inflammation at the amount of FDA approved acceptable daily intake (ADI). Chronic inflammation is associated with various human diseases, such as diabetes, obesity and cancer. This finding indicates that instead of a healthier life style, there is an increased possibility to damage health. Furthermore, Ace-K increased the energy metabolism in gut microbiota and body weight in male mice diminishing the act of calorie-reducing from diet. This dissertation also indicates that for researchers concerned about toxicity or beneficial effects of a particular diet, the modulation of gut microbiota in each gender should be taken into account.

In this dissertation, perturbed gut microbiota, increased body weight and inflammation were observed after the consumption of artificial sweeteners, and increased energy metabolism genes, pro-inflammatory bacterial genes and metabolites indicated the relationship between perturbed gut microbiota and the two major outcome –increased body weight and inflammation. However, this does not prove the causation between them, and all these findings might just happen chronologically. Also, other factors might be involved other than the gut microbiota, such as the sweetness sensing receptors in the gut. Further studies should be done to determine

the relationship of artificial sweeteners, altered gut microbiota, increased body weight and inflammation.