EFFECTS OF TART CHERRY SUPPLEMENTATION

ON MARKERS OF INSULIN RESISTANCE AND GUT

INTEGRITY IN MICE FED WESTERN DIET

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Abstract: **Objectives**: Tart cherries (TC) are rich source of phenolic compounds such as anthocyanins and flavonoids that can promote health by influencing the gut microbiota. This study investigated the effects of TC supplementation on gut health and metabolic parameters in mice fed a western diet (WD).
Methods: Six-week-old male C57BL/6 mice were randomly assigned to dietary treatment groups in a 2x3 factorial design with diet (control [AIN-93M] or WD, 45% fat kcal and 26% sucrose kcal) and TC (0, 5, 10% wt/wt) as factors for 12 wks. At the end of dietary treatment, body composition was assessed, and tissues were collected to evaluate metabolic parameters and markers of gut health. Cecal content was used for bacterial and short chain fatty acid analyses. Data was analyzed using two-way ANOVA with Kruskal-Wallis/Dunn's and Tukey's as *post-hoc* tests for the gut microbiota data and metabolic parameters, respectively.
Results: TC at 10% dose significantly increased the abundance of the beneficial bacterial phylum, Actinobacteria, relative to the unsupplemented groups ($p=0.018$ and 0.010 vs control and WD, respectively). Relative cecal weight ($p=0.007$) and cecal propionic ($p=0.0212$), i-butyric ($p=0.0183$), i-valeric ($p=0.0126$), n-valeric ($p=0.0261$), and n-heptanoic acids were significantly increased with TC supplementation. Histological evaluation revealed reduced ileal villi height ($p=0.0348$), width ($p=0.0042$) and area ($p=0.0132$) with WD and TC did not alter this response. Overall, the expression of genes related to gut health was unaffected by both WD and TC supplementation. Body weight ($p=0.0012$), fat mass ($p=0.007$), fasting blood glucose ($p=0.001$), serum total cholesterol ($p<0.0001$), triglyceride ($p=0.002$), leptin ($p=0.0011$), plasminogen activator inhibitor 1 ($p=0.0344$), and resistin ($p=0.0012$) were increased with WD and TC had no effect on these parameters. Despite modest effects on metabolic parameters, the homeostatic model assessment of insulin resistance, HOMA-IR, a commonly used tool for assessing insulin resistance, was significantly improved by 5% TC ($p=0.0003$).
Conclusion: TC supplementation restored some beneficial bacteria and increased short chain fatty acid production altered by WD. However, these changes in the gut did not translate to improvement in metabolic outcomes except for HOMA-IR. The mechanism by which TC improve HOMA-IR needs to be investigated in future studies.

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CHAPTER I

INTRODUCTION

Diabetes mellitus (DM) is a common public health issue around the world

(Tabish; 2007). DM is defined as a metabolic disorder in which chronic hyperglycemia is

present as a result of impaired insulin action (type 2 diabetes mellitus, T2DM) or

impaired insulin secretion (type 1 diabetes mellitus, T1DM) or both (Goldenberg and

Punthakee, 2013). Among diabetic patients, 90% to 95% people have T2DM and by the

year 2030, 439 million people are estimated to be affected worldwide with T2DM (Wu et

al., 2014). According to the National Health Interview Survey (NHIS) in 2016, 21 million

U.S. adults (8.6%) are suffering from T2DM (Bullard et al., 2018). In 2015, diabetes was

considered as the sixth leading cause of disability, and socioeconomic pressure is

increasing among diabetics due to the management of diabetes (Chatterjee et al., 2017). It

has been estimated that the global costs of diabetes will increase in the U.S. from $1.3

trillion in 2015 to $2.1 trillion in 2030 (Bommer et al., 2018). These statistics indicates a

need for preventative intervention.

Along with inherited factors, a number of lifestyle factors such as cigarette

smoking, consuming alcohol, physical inactivity and sedentary lifestyle are also known to

increase the likelihood of developing T2DM. Additionally, consumption of diet rich in fat

and refined sugars increases obesity (Olokoba et al., 2012) (Cordain et al., 2005), a main risk factor for the development of T2DM. In obese individuals, there is an increase tissue inflammation such as in the adipose tissue, which contributes to insulin resistance and T2DM (Diamant et al., 2011). Among individuals with T2DM, 60% are obese (body-mass index [BMI] ≥ 30 kg/m²) and showing signs of insulin resistance. Therefore, obesity and T2DM are closely linked (Chatterjee et al., 2017).

Recent findings have demonstrated that changes in the gut microbiota are associated to many chronic conditions including obesity, T2DM, colon cancer, inflammatory bowel disease (Young; 2012), and cardiovascular disease (Tang et al., 2017). The gut microbiota is considered a "virtual organ" of the body because the metabolic activity performed by gut bacteria is equal to that of an organ (Clarke et al., 2014). The human gastrointestinal (GI) tract contains about 100 trillion microorganisms and it has been reported that the gut microbiota plays an important role in digestion, absorption, nutrient metabolism and host's immune system maintenance (Valdes et al., 2018). Previous studies reported that plant polyphenols are good substrates for gut microbiota and phenolic acids are produced from colonic fermentation of these polyphenols (Mansoorian et al., 2019). These metabolites have potential health benefits such as antimicrobial activity to pathogenic bacteria (Mansoorian et al., 2019). Additionally, the gut microbiota is responsible for the fermentation of non-digestible food substrates such as dietary polysaccharides and because of this fermentation, multiple groups of metabolites are produced including the short chain fatty acids (SCFAs). Among the SCFAs, acetate, propionate and butyrate are abundantly present ($\geq 95\%$) in the colon (den Besten et al., 2013). Butyrate has been shown to improve gut barrier function

by stimulating the production of intestinal mucus as well as reducing inflammation of the adipose tissue (Bakker and Nieuwdorp, 2017). Increased production of SCFAs is involved in maintaining glucose homeostasis because SCFAs has been shown to increase the secretion of pancreatic insulin by stimulating the release of incretins through its interaction with the receptor GPR43 (Tolhurst et al., 2012). Vilsbøll and Holst (2004) reported that incretin production increases the postprandial response of insulin to glucose.

Diet plays a major role in determining the composition of the gut microbiota and improper nutrition can lead to imbalance in bacterial population leading to dysbiosis. It has been reported that alterations in the gut microbiota due to western diet (45% fat kcal; 22.8 % sucrose kcal) is associated with obesity and increased risk of chronic diseases (Murphy et al., 2015). Cani and colleagues (2008) reported that cecal microbial dysbiosis due to WD was observed together with glucose intolerance, increased body weight, fat accumulation and low-grade inflammation. The alteration in gut microbiota due to WD diet may also induce local (i.e. gut) and systemic inflammation due to increased production of inflammatory cytokines (Guo et al., 2017). Therefore, obesity and its adverse outcomes may be reduced by preserving the balance of the gut microbiota (Cani et al., 2008).

Contrary to the effects of WD, a good quality diet preserves the balance of good bacterial population (Walsh et al., 2014). Food components such as dietary fiber and polyphenols play an important role in the preservation of the gut microbiota (Ercolini and Fogliano, 2018). Dietary polyphenols, like fiber, are reported to exert prebiotic effects and increase the growth of good bacteria while preventing the growth of pathogenic bacteria, resulting to reduced risk of chronic diseases like T2DM (Ercolini and Fogliano,

2018) and obesity (Wang et al., 2014). Both fiber and polyphenol-rich diet can counter the effects of WD diet that is known to disrupt epithelial mucosal barrier and cause low-grade inflammation (Zmora et al., 2018).

Tart cherry (TC) is one of such foods, which are rich in polyphenols and fructooligosaccharides (FOS), that have been reported to be beneficial to health and may modulate the negative effects of WD (Mayta-Apaza et al., 2018). TC (*Prunus cerasus*) is a stone fruit that belongs to the Rosaceae family. Worldwide, the production of TC increased from 2,154,000 to 3,057,000 metric tons due to its higher resistance to environmental factors as well as increased customer demand due to reported therapeutic properties (Mayta-Apaza et al., 2018). The US is one of the top five producers of TC (Mayta-Apaza et al., 2018). TC is rich in polyphenols such anthocyanins and flavonols (Mayta-Apaza et al., 2018). Studies have reported that the polyphenols from TC have antidiabetic, antioxidant, cardioprotective and anti-inflammatory effects (Mayta-Apaza et al., 2018). In addition to these health effects, TC polyphenols have also been reported to inhibit oxidative damage, colon cancer and symptoms of osteoarthritis (Thomas, 2017). Moreover, Seymour and colleagues (2008) reported that supplementation of high fat diet with freeze-dried TC powder (1% wt/wt) reduced hyperlipidemia and modulated blood glucose (Seymour et al., 2009). From these studies, it can be concluded that TC is a functional food that may be beneficial to maintain a healthy gut microbiota (Mayta-Apaza et al., 2018) and improve the health of the host.

Despite reports that polyphenols are important for maintaining a balance in gut microbiota, our current knowledge is lacking on how the consumption of TC in the context of WD diet helps in maintaining the balance of gut microbiota and in improving

markers of glucose homeostasis. Therefore, the **objective** of this study is to evaluate the effects of TC supplementation on the gut (i.e., microbiota, markers of gut integrity and structural changes, SCFAs production) and its impact on glucose homeostasis, body composition, and blood lipids and pancreatic and adipose-derived hormones in mice fed a WD. The **hypothesis** of this study is that TC supplementation, because of its many bioactive components, will dose-dependently maintain the balance of the gut microbiota and retain gut integrity, which will improve blood glucose, and body composition in C57BL/6 mice fed a WD. The **specific aim** of study is to investigate the dose-dependent effects of TC supplementation in the context of control and WD on:

Aim 1: the gut microbial populations and production of SCFAs,

Working hypothesis: TC reported as a potential source of fiber particularly FOS as well as phenolic compounds. Due to these bioactive components, TC will prevent the loss of beneficial bacteria, which is commonly associated with consumption of WD. These microbial changes in the gut will increase the production of SCFAs.

Aim 2: the gut structural changes and expression of genes related to gut barrier integrity,

Working Hypothesis: The increase in the beneficial bacteria due to TC supplementation will help maintain the health of intestinal epithelial cells and gut barrier integrity, in part, by providing nutrients such as SCFAs to the intestinal epithelial cell.

Aim 3: markers of glucose homeostasis, and

Working Hypothesis: The increase production of SCFA due to TC supplementation will stimulate the production of gut hormones that can maintain glucose homeostasis.

Additionally, there will be a decrease in inflammation because TC will maintain gut integrity and prevent entry of inflammatory molecules such as lipopolysaccharides through the gut (i.e. metabolic endotoxemia). This metabolic endotoxemia is known to alter glucose homeostasis and will be prevented by TC.

Aim 4: body composition and serum lipids and pancreatic and adipose-derived hormones.

Working hypothesis: The increase in SCFAs production due to TC supplementation will modulate body composition and serum lipids and hormones. SCFAs acts as signaling molecule and stimulates fatty acid oxidation and inhibits lipogenesis.

CHAPTER II

LITERATURE REVIEW

This literature review includes an overview of the growing trend of obesity and type 2 diabetes mellitus (T2DM), the gut microbiota and influence of nutrition on gut microbial composition, the relationship among gut microbiota, obesity and T2DM, and the nutritional composition and health effects of tart cherry (TC).

Prevalence of Obesity and T2DM

Obesity, defined as having a body mass index (BMI≥30 kg/m²), is a major public health concern worldwide. In the past decades, overweight and obesity has increased in different parts of the world. Two billion individuals were reported to be overweight or obese worldwide and 62% of these individuals were living in developing countries (Ford et al., 2017). Among Asian countries, the magnitude of obesity is reported to be high in Thailand and lowest in India and the Philippines (Ramachandran and Snehalatha, 2010). According to the Working Group for Obesity in China, the prevalence of obesity in 2015 among Chinese boys and girls was recorded as 10.5 % and 7.1 %, respectively (Zhang et al., 2018). In Southern and Eastern part of Europe, women are more affected with obesity (Seidell, 1995). In the past few decades, the global percentage of men and women with BMI greater than 25 kg/m2 increased from 28.8 % to 36.9 % and 29.8% to 38.0 %, respectively (Ford et al., 2017). Nowadays, obesity is also prevalent in poor countries

such as Nepal, India and Bangladesh, where undernutrition is also common (Balarajan Villamor, 2009). In Great Britain, the number of adults with obesity increased from 6% men and 8% women in 1980 to 23% men and 25% women in 2002 (Rennie and Jebb, 2005).

The United States is one of the western countries where obesity is prevalent (Lobstein, 2011) and is continually increasing (Ogden et al., 2006). A study conducted by Hales and colleagues (2017) showed that in the US in 2015-2016, 42.8% of adults aged 40-59 years old, 35.7% of adults aged 20-39 years old and 41.0% of adults aged 60 and older were obese. Among women in 2015-2016, 44.7% of women aged 40-59 years old and 36.5% of women aged 20-39 years old were also suffering from obesity (Hales et al., 2017). The prevalence of obesity was 20.6% among adolescents (aged 12-19 years), 18.4% among school-aged children (aged 6-11 years) and 13.9 % among pre-school children. There was no gender difference in prevalence of obesity and it was significantly increased in both youth and adults from 1992-2000 through 2015-2016 (Hales et al., 2017). Results from these studies support that obesity is a growing global health problem particularly in the US where it is prevalent in all age groups.

Obesity has detrimental effects on individual's health. One effect of obesity is an increase likelihood of developing T2DM. Ogden and colleagues (2006) reported that obesity and T2DM are correlated and obesity is responsible for accelerating the development of T2DM. A meta-analysis of twenty studies demonstrated that more than 85% of individuals with T2DM are overweight or obese (Gao et al., 2018). A report by Wild and colleagues (2004) showed that in 2000, the worldwide prevalence of T2DM was 2.8% and they estimated that it would increase up to 4.4 % in 2030. Similarly,

Forouhi and Wareham (2010) also reported that prevalence of T2DM would increase from 328 million in 2013 to 592 million in 2035. T2DM is more prevalent in men compared to women and in people over the age of 60 years compared to other age groups (Wild et al., 2004).

Similar to the worldwide trend, a significant growing trend over the years in T2DM has been reported in the US. According to the Centers for Disease Control and Prevention, 29.1 million adults (9.3% of total population) in the US were affected by diabetes in 2012 and it is predicted that diabetes will affect one in every three Americans in 2050 (Chaudhury et al., 2017). Rowley and his colleagues (2017) also reported that the number of individuals with diabetes would raise by 54% from 2015 to 2030. According to National Health Interview Survey in 2016, 21.0 million or 90.9% adults among all the diabetic adults were living with T2DM (Bullard et al., 2018). Due to unfavorable outcomes associated with obesity and T2DM, there is an urgent need for intervention strategies to limit the health effects of obesity and T2DM. Researchers and health professionals are continuing to investigate prevention strategies for obesity and T2DM, which will improve the quality of life of many individuals.

The Gut Microbiota

The gastrointestinal (GI) tract is considered the largest interface between the host, environmental factors, and antigens present in the human body (Thursby and Juge, 2017). It also contains plenty of microorganisms and acts as a passage for approximately 60 tons of food throughout the lifespan of an individual. The complex and dynamic population of microorganisms in the human GI tract is known as the "gut microbiota" (Thursby and Juge, 2017). It has been estimated that more than 10^{14} microorganisms are present in the

human GI tract (Bäckhed et al., 2005) and a recent study suggested that the ratio of human cells to bacterial cells is 1:1 (Sender et al., 2016). The gut microbiota species are complex and highly diverse, and only 30% of the species can be enumerated by microscopic observation via culture-based characterization (Moore and Holdeman, 1974).

The growth of human gut microbiota usually starts right after birth and over the 2 to 3 years after birth form a complex adult-like population of organisms that act as a gut barrier against pathogens (Doré and Corthier, 2010). At the early stages of life, the diversity of the gut microbiota is usually low, with Actinobacteria and Proteobacteria being the dominant phyla. The diversity of the gut microbiota increases in the first year of life and grows towards a distinct adult-like microbial profile (Palmer et al., 2007). The composition, functions, and diversity of infant gut microbiota starts to resemble the adult microbial profile at the age of 2.5 years. The composition of the gut microbiota is generally stable in adulthood but it can be altered by life events such as illness, dietary changes, and medication use such as antibiotic treatment (Thursby and Juge, 2017).

Manson et al. (2008) reported that more than 400 bacterial species have been observed in the fecal matter of an individual. Before the development of anaerobic culture-based techniques, only 10-20% of gut microbiota have been identified using culture-based techniques and scientists were not able to isolate anaerobic microorganisms (Jandhyala et al., 2015). In recent years, other microorganisms have been identified with the improvements in anaerobic culture-based techniques and molecular approaches such as metagenomics and single gene approach based on ribosomal RNA. Subsequently, *Bacteriodes, Eubacterium, Ruminococcus, Clostridium* and *Bifidobacterium* were identified as the dominant genera in the gut (Jandhyala et al., 2015).

The human gut microbiota is reported to contain bacteria that belong to the phyla Firmicutes, Bacteriodetes, Actinobacteria, Proteobacteria, Fusobacteria, Cyanobacteria and Verrucomicrobia (Fraher et al., 2012). The phyla Firmicutes and Bacteriodetes constitute about 90% of the gut microbiota followed by Actinobacteria and Verrucomicrobia (Fraher et al., 2012). The phylum Firmicutes is composed of more than 200 different genera with the predominant species *Faecalibacterium prausnitzii, Ruminococcus albus* and *Ruminococcus flavefaciens* (Rinninella et al., 2019). The phylum Actinobacteria is less dominant (Jandhyala et al., 2015) and include the genus *Bifidobacteria* and *Collinsella-Atopobium* group (Doré and Corthier, 2010). Bacterial species ocassionally found in the gut include *Eubacterium cylindroides, Sporomusa, Verrucomicrobium, Clostridium ramosum, Selenomona* and *Clostridium ramosum* (Doré and Corthier, 2010).

The presence and function of the microbiota vary in each segment of the gastrointestinal tract with over 70% of all the microorganisms in the human body are present in the large intestine (Jandhyala et al., 2015). Besides the beneficial bacteria, the colon also contains pathogenic species such as *Escherichia coli, Salmonella, Bacteroides fragilis, Vibrio cholera* and *Campylobacter,* but these are present in very low amount (0.1% or less of the total gut microbiota) (Gevers et al., 2012).

There is a mutual relationship between diet and the gut microbiota. Colonization of the gut begins from birth. The initial development of the gut microbiota is influenced by numerous factors such as, breast-feeding vs. formula feeding, mode of delivery, timing of the introduction of solid foods and termination of milk feeding (Fallani et al., 2011). Pediatric studies showed that during lactation, *Bifidobacterium* is the dominant gut

bacteria of an infant (Tanaka and Nakayama, 2017). Fats, proteins, carbohydrates and immunoglobulins are the major components of human milk. The oligosaccharides such as galactooligosaccharides in human milk have potential role in the growth of beneficial microbes in the gut of infants. With the introduction of solid food in the diet, adult-like complex and more stable gut microbiota is established and the Bacteroidetes and Firmicutes become dominant. Different components of the diet such as non-digestible food components and polyphenols influence the gut bacterial population (Tanaka and Nakayama, 2017). The ability to isolate and assess the bacterial populations in our gut is important in building our knowledge about their role in health and disease.

Methods of Assessing the Gut Microbiota

Gut mucosal and stool samples are most commonly used for the analysis of the gut microbiota. Previous studies showed that the biodiversity of fecal and gut mucosal microbiota is different from each other (Panek et al., 2018). Feces, which is an easily available metabolic waste, is found to have more potential for determining disease risk and therapeutic intervention in both human and animal models (Panek et al., 2018). One advantage with using fecal sample is ease of collection compared to biopsy samples of the gut mucosal surface and cecum content (Panek et al., 2018). One more advantage of fecal sample is that it is easy to obtain series of sample from one animal over time, unlike the case with cecal samples where the samples can be obtained only single time-point by sacrificing that animal (Panek et al., 2018). Early approach to assessing gut microbial population used bacterial culture, but this method provided only limited information about the structure of the gut microbiota. Cost effectiveness is a major advantage of culture-based approaches for identification of bacteria but these methods are time

consuming (Jandhyala et al., 2015). One limitation of using bacterial culture is its inability to identify anaerobic bacterial population that are difficult to culture (Jandhyala et al., 2015). However, recent advances in novel techniques make it possible to identify previously uncultured species.

Recently, new approaches that are culture-independent have been developed to analyze the composition of the gut microbiota in a given sample. These methods involve 16S ribosomal RNA-based sequencing of bacterial gene. The 16S ribosomal RNA region is a small and most conserved region with higher variable sites that are enough to differentiate several bacterial species than the 5S and 23S rRNA genes. The V3, V4, V6 and V8 regions of 16S rRNA are common for the identification of bacteria (Hamady et al., 2008). Although 16S rRNA is an advanced and commonly used technique to analyze the composition of gut microbiota, it cannot explain biological or clinical significance of the association between a disease and a particular microbial pattern. To overcome this limitation of 16S rRNA technology, metagenomics is the latest advancement to study the gut microbiota. This technique is able to explain the next step of detecting which microbes are present and what they do (**Table 1**) (Fraher et al., 2012). These culture-independent approaches deliver further comprehensive information of the gut microbiota such as microbial diversity, quantitative and qualitative knowledge of microbes and alteration in gut microbiota due to diseases.

The denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH), temperature gradient gel electrophoresis (TGGE) and DNA microarray and sequencing techniques are defined as culture-independent approaches

(Fraher et al., 2012). The description with advantages and disadvantages of these techniques are highlighted in **Table 1**.

Effects of Diet on Gut Microbiota

Non-digestible food components are beneficial for the host's health because these stimulates the growth of beneficial bacteria (Carlson et al., 2018). Non-digestible food components are resistant to digestive enzymes but can be metabolize by colonic bacteria. These components pass through colon very slowly and interacts with the microbiota, which stimulates fermentation (Jacobs et al., 2009). Thus, slow transit time, favorable pH and readily available nutrients provide favorable environment for the growth of bacteria in the colon (Slavin, 2013). Non- digestible plant food components provide food for microbiota with complete or partial fermentation in the colon. Non-digestible food components include non-digestible carbohydrates such as oligosaccharides, polysaccharides, resistant starch and non-carbohydrate compounds such as polyphenols and lignin (Saura-Calixto et al., 2009).

Bacterial fermentation of non-digestible food components in the colon produces short-chain fatty acids (SCFAs) such as butyrate, acetate and propionate. SCFAs plays an important role in the physiology of the host (Baxter et al., 2019). Additionally, these SCFAs serves as energy source for the gut microbiota and improve the growth of many beneficial bacteria, which can produce other nutrients such as vitamins (Sivaprakasam et al., 2016)**.** The SCFA butyrate has been described to improve the beneficial bacteria in the gut, while acetate and propionate can be used for energy by the liver and adipose tissue (Roy et al., 2006).

Table 1: Techniques for the assessment of gut microbiota (Fraher et al., 2012)

Technique	Description	Advantages	Disadvantages
Culture-based	Selective media used for bacterial isolation	Cost-effective, semi-quantitative	Labor intensive, limited culturable organisms
qPCR	16S rRNA amplification and quantification. Reaction mixture contains a compound that fluoresces when it binds to dsDNA	Phylogenetic identification, fast, quantitative	PCR bias, unable to identify unknown species
DGGE/ TGGE	Gel separation of 16S rRNA amplicons using denaturant/temperature	Fast, semi-quantitative, bands can be excised for further analysis	No phylogenetic identification, PCR bias
T-RFLP	Fluorescently labelled primers are amplified and then restriction enzymes are used to digest the 16S rRNA hybridization occurs, fluorescence can be enumerated using flow cytometry	Fast, semi-quantitative, cheap	No phylogenetic identification, PCR bias, low resolution
FISH	Fluorescently labelled oligonucleotide probes hybridize complementary target 16S rRNA sequence with DNA probe. When hybridization occurs, fluorescence can be quantified using flow cytometry	Phylogenetic identification, semi-quantitative, no PCR bias	Dependent on probe sequences, cannot identify unknown species
DNA microarrays	Fluorescently labelled oligonucleotide probes hybridize with complementary nucleotide sequences. Fluorescence detected with a laser	Phylogenetic identification, semi quantitative, fast	Cross hybridization, PCR bias, species present in low levels can be difficult to detect
Cloned 16S rRNA gene sequencing	Cloning of full-length 16S rRNA amplicon, Sanger sequencing and capillary electrophoresis	Phylogenetic identification, quantitative	PCR bias, laborious, expensive, cloning bias
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons	Phylogenetic identification, quantitative, fast, identification of unknown bacteria	PCR bias, expensive, laborious
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome (e.g. 454 pyrosequencing or Illumina)	Phylogenetic identification, quantitative	Expensive, intense computational data analysis

Abbreviations: qPCR: quantitative polymerase chain reaction, DGGE: denaturing gradient gel electrophoresis., TGGE: temperature gradient gel electrophoresis, T-RFLP: terminal restriction fragment length polymorphism, FISH: fluorescence in situ hybridization

The increase production of SCFAs by the gut microbiota also results in decrease in the pH of the colon. Thus, high fiber intake could decrease the pH of the colon. Flint and his colleagues (2012) reported that a major shift in the configuration and metabolic output of human gut microbiota has been observed in pH between 5.5 and 6.5. One unit decrease in pH (6.5 – 5.5) has been shown to have a potential effect on gut microbiota with tendency to suppress *Bacteroides spp.* and to increase gram-positive bacteria, which are responsible to produce butyrate (Flint et al., 2012).

Nowadays, people are consuming western diet (WD) that is high in fat and simple sugar and dietary fiber intake that is not sufficient to meet the recommended dietary allowance. A low-fiber diet is known as fiber gap and it can cause alterations in the composition of the gut microbiota and other beneficial metabolites such as SCFAs, which are important in the host's intestinal mucosal immunity (Rinninella et al., 2019). A low fiber diet can cause significant decrease in the proportion of Bifidobacteria and butyrate-producing bacteria of the family Lachnospiraceae. The loss of gut microbial diversity and beneficial metabolites has negative effects on host health. Thus, non-digestible food components, changes in pH and transit time can influence the composition of the gut microbiota (Rinninella et al., 2019).

Polyphenols are naturally present in plants as secondary metabolites. These chemical compounds contain more than one hydroxyl group attached to a benzene ring (Scalbert and Williamson, 2000). Polyphenols are generally classified as phenolic acid, flavonoids, stilbenes and lignans. These classes of polyphenols are different in their carbon skeleton and among these polyphenols, the flavonoids are the most abundant in our diet (Scalbert and Williamson, 2000). Flavonoids can be further classified as

anthocyanin, isoflavones, flavonols, flavanones, flavanols and flavones. Many studies have shown that polyphenols play an important role as antioxidants, which helps in the prevention of many diseases such as cancers, diabetes, cardiovascular diseases, neurodegenerative diseases and osteoporosis (Scalbert et al., 2005).

Just like dietary fiber, plant polyphenols are good substrates for the gut microbiota. A small portion of polyphenols is absorbed in the small intestine and most of the polyphenols (over 90%) reach the colon along with dietary fiber (Mansoorian et al., 2019). Unlike dietary fiber, polyphenols are not used for the production of SCFAs. Instead, phenolic acids are produced from polyphenols by colonic fermentation and these acids are available for colonic absorption. These metabolites have potential health benefits such as antimicrobial activity to pathogenic bacteria (Mansoorian et al., 2019). Phenolic acids can be determined in both urine and plasma after dietary intake of foods rich in polyphenols (Edwards et al., 2017).

Several studies have reported that polyphenols play an important role in the development of the gut microbiota (Ozdal et al., 2016). Some phenolic compounds have been considered as antimicrobial agents due to their bacteriostatic or bactericidal activities. Ozdal and colleagues (2016) reported that quercetin supplementation reduce the growth of bacterial species, which are responsible for diet-induced obesity such as *Bacillus spp., Erysipelotrichaceae* and *Eubacterium cylindroides*. Hostetler and colleagues (2017) reported that the growth of pathogenic bacteria could be inhibited by flavonoids such as diosmetin, which are present in citrus fruits. Thus, polyphenols can influence gut bacterial population and influence the health of the host.

Role of the Gut Microbiota in Nutrition and Health

Various potentially bioactive components are released and transformed by the gut microbiota. For example, SCFAs are produced from microbial fermentation of non-digestible carbohydrates in the colon (Flint et al., 2012). An additional energy is provided to the host from the absorption of these SCFAs and serves as a substrate for the survival of the gut microbiota. Thus, the gut microbiota contributes in 'energy harvest' from the diet, and this condition might be beneficial under conditions of food scarcity (Flint et al., 2012). The host's energy supply from microbial contribution depends on the amount of non-digestible carbohydrates in the diet and the extent of microbial fermentation and absorption of SCFAs (Blaut and Klaus, 2012).

In addition to fermentation of non-digestible carbohydrates, the gut microbiota is also responsible for the biotransformation of polyphenols into their simpler metabolites, thus affecting polyphenol absorption and bioavailability. Braune and Blaut (2016) reported that *Clostridium* and *Eubacterium* have been identified as involved in the metabolism of some phenolic compounds such as isoflavone, flavonol, flavonone and flavan-3-ol. In addition to this, fermentation of polyphenols by colonic bacteria yield a broad spectrum of biotransformation products such as phenylbutyric acid, phenyl-propionic acid, valeric acid, phenylacetic acid, phloroglucinol, urolithin A and urolithin B (Hervert-Hernandez and Goñi, 2011). Thus, the biotransformation and promotion of polyphenol absorption by the gut microbiota may be vital in assessing the extensive health benefits of these plant chemicals.

The dominance of beneficial bacteria in the gut provides health benefits to the host while other bacterial species are linked to diseases (Hervert-Hernandez and Goñi, 2011). For example, *Bacteroides, Clostridium* and *Eubacterium* has been found to be

increased in gastrointestinal disorders (Saito et al., 1992). On the other hand, some species such as *Lactobacillus* and *Bifidobacterium* are considered beneficial bacteria due to their ability to modulate the gut microbiota. These species inhibit pathogens and are commonly used as probiotics (Hervert-Hernandez and Goñi, 2011). Other health properties attributed to the beneficial bacteria in the gut include improvement of lactose digestion, reinforcement of intestinal epithelial cell tight junctions, reduction of serum cholesterol, stimulation of anti-inflammatory cytokine production, and increased mucus secretion (Hervert-Hernandez and Goñi, 2011).

Metabolism and Benefits of Short Chain Fatty Acids (SCFAs)

SCFAs are carboxylic acids with aliphatic tails of 1-6 carbons and its production largely depends on dietary intake of non-digestible carbohydrates such as resistant starch and fiber (Venegas et al., 2019). Acetate, propionate and butyrate are the major SCFAs produced from colonic fermentation of dietary fiber. The production of these SCFAs also depends on microbiota composition and environmental conditions such as availability of substrate and pH (Venegas et al., 2019).

Butyrate is mainly produced by bacteria belonging to the phylum Firmicutes, in particular *Faecalibacterium prausnitzii* and *Clostridium leptum* of the family *Ruminococcaceae* (Louis and Flint, 2009). Propionate is produced by colonic bacteria using different pathways such as succinate pathway, acrylate pathway and propanodiol pathway and several Firmicutes are involved in the production of propionate (Ríos-Covián et al., 2016).

SCFAs have a wide range of beneficial effects both within and outside the gut. Fermentation of carbohydrates producing 400-600 mmol SCFAs per day can provide

approximately 10% of the daily caloric requirements (den-Besten et al., 2013). Microbial production of SCFAs may play a vital role in the regulation of plasma glucose levels by increasing the gut hormones, peptide YY (PYY) and glucagon like peptide-1 (GLP-1) via activation of the free fatty acid receptor (Ffar)-2 and Ffar-3 (Karaki et al., 2008, Tazoe et al., 2009). GLP-1 regulates blood glucose levels by increasing the secretion of insulin and decreasing the secretion of glucagon by the pancreas (Barrera et al., 2011). Previous *in vitro* studies showed that propionate decreases the activity of hepatic 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), thus it reduces the rate of cholesterol synthesis (den-Besten et al., 2013). SCFAs such as acetate, propionate and butyrate are associated with activation of G-protein coupled receptor 43 (GPR43) on immune cells and mediates anti-inflammatory activity (Li et al., 2018).

The Gut Microbiota in Obesity and T2DM

Obesity is one of the world's fastest growing health challenges and it is prevalent in both developed and developing countries. Obesity may increase the risk of many health problems, including T2DM, cardiovascular diseases and certain cancers (Segula, 2014). Gut microbes impact host metabolism by affecting signaling pathways and it has been reported that obesity is associated with alteration of gut microbiota including higher Firmicutes/Bacteroidetes ratio (Kinlen et al., 2017). However, the exact mechanism linking gut microbiota to obesity and T2DM is not very clear mainly due to the diversity and complexity of the gut microbiota. Baothman and collegues (2016) reported that the composition of the gut microbiota is different in obese as compared to lean individual. The bacteria from phyla Firmicutes is increased and the bacteria from phyla

Bacteroidetes is decreased in obese individuals. This change in bacterial ratio has been shown to increased low-grade inflammation (Baothman et al., 2016).

Diet-induced obesity has been reported to increase the proportion of *Eubacterium dolichum,* belonging to the Firmicutes phylum (Turnbaugh et al., 2008). Obese Zucker rats had reduced Bifidobacterium counts as compared to lean rats (Waldram et al., 2009). Similar alteration in proportions of Firmicutes and Bacteriodetes have been found in the feces of obese humans. After the consumption of hypoenergic diet (low carbohydrates and low fat) for a year, fecal proportion of Bacteroidetes were significantly increased with weight loss (Ley et al., 2006). A large-scale intervention trial demonstrated that consumption of hypoenergic diet and increased physical activity could change the composition of the gut microbiota of obese adolescents (Ley et al., 2006). After intervention, the proportion of *Clostridium histolyticum, Clostridium lituseburense* and *Eubacterium rectale* was significantly decreased, while the proportion of the Bacteroidetes–Prevotella group increased with significant weight reduction (8.1% of their body weight) (Nadal et al., 2009). Alteration in gut microbiota due to excess weight may be seen in early life as Kalliomäki and colleagues (2008) reported that during infancy, children maintaining normal weight had higher number of Bifidobacterium, whereas overweight children had an increased number of Staphylococcus aureus in their feces.

WD-induced obesity is characterized by low-grade inflammation, which has been related to alteration in the composition of the gut microbiota and increased plasma lipopolysaccharide (LPS) (Sanz et al., 2010). The transit of LPS into circulatory system known as metabolic endotoxemia, reflects the passage of bacterial fragments across the intestinal epithelial layer into the systemic circulation due to increased intestinal

permeability. Endotoxemia is associated with the reduction of *Bifidobacterium,* which helps in maintaining mucosal barrier function against pathogens (Sanz et al., 2010). A mouse model chronically infused with a dose of LPS to reach the same plasma LPS level as those measured in the WD-fed mice, was characterized with fasting hyperglycemia, obesity, steatosis, hepatic insulin resistance and hyperinsulinemia (Cani and Delzenne, 2009).

Previous studies reported that T2DM is also associated with gut dysbiosis (Adachi et al., 2019). The concentration of butyrate-producing bacteria such as *Roseburia intestinalis* and *Faecalibacterium prausnitzii* has been found to be lower in T2DM, while Lactobacillus species and some pathogens such as *Clostridium hathewayi, Clostridium ramosum*, *Clostridium symbiosum*, and *E. coli* were higher in T2DM. Yassour and colleagues (2016) reported that lower concentration of *Akkermansia muciniphila* in gut could be a marker of glucose intolerance. In support of this finding, Zhang and colleagues (2013) found that there was decreased abundance of *Akkermansia muciniphila* in individuals with prediabetes. Individuals with T2DM showed an increased amount of *Lactobacillus* and reduced amount of *Prevotella* in feces as compared to healthy individuals (Sircana et al., 2018*)*. The results of these studies reveal that the gut microbiota might play a significant role in the pathogenesis of obesity and T2DM.

Nutrition and Health Value of Tart Cherry

Tart cherry (TC), also known as *Prunus cerasus*, is a stone fruit belonging to the Rosaceae family (Kelley et al., 2018). Although, there are more than a hundred cultivars of cherries, they are grouped into two major types, the sweet and sour tart cherries.

Montmorency is the most commonly grown cultivar of TC in the US (Kelley et al., 2018). The main characteristics related to cherry fruit are sweetness, color, sourness and firmness. Sweetness in cherries is mainly due to glucose and fructose, while sourness is primarily due to the presence of malic acid (Ferretti et al., 2010). Unlike flavonoid-containing green tea, TC has excellent palatability and familiarity to U.S. consumers due to its popularity in foods, such as pie, pastry fillings and juice products (Jayarathne et al., 2018).

The nutritional composition of cherries depends on the varieties of cherries (Commisso et al., 2017). The total fiber content of TC is approximately 1.1 g fiber per 100 g of edible portion while its fructoologosaccaride (FOS) content of TC has been reported to be approximately 0.32 g per 100 g of edible portion (Jovanovic-Malinovska et al., 2014) and (USDA, 2014). Cherries contain both fat-soluble and water-soluble vitamins and some carotenoids such as beta-carotene (Ferretti et al., 2010). TC contains significantly higher amount of vitamin A and beta-carotene as compare to sweet cherries. Cherries also contain minerals such as magnesium (10 mg/100 g), calcium (14 mg/100 g), potassium (200 mg/100 g) and phosphorous (20 mg/100 g) (Ferretti et al., 2010).

During ripening, cherry changes from the initial green color to red with the accumulation of polyphenolic compounds (Serrano et al., 2005). Polyphenols are mainly concentrated in the skin and contribute to sensory and organoleptic qualities such as taste (Serrano et al., 2005). Polyphenols as previously discussed are considered as bioactive compounds that play an important role in maintaining the balance of gut microbiota, thus preventing immune dysregulation. Both sweet and sour cherries contain phenolic

compounds such as peonidin 3-rutinoside, cyanidin 3-glucoside, pelargonidin 3-rutinoside, cyanidin, 3-sophoroside, pelargonidin 3-glucoside and cyanidin 3-rutinoside (Ferretti et al., 2010). Sour cherries contain two to three times higher phenolic content compare to sweet cherries (Jayarathne et al., 2018). A study which investigated the total anthocyanins in sweet and sour cherries reported that total anthocyanins of sweet cherries were between 30 and 79 mg cyanidin-3-glucoside equivalents (CGE)/100 g, whereas total anthocyanins of sour cherries were between 45 and 109 mg CGE/100g (Serrano et al., 2005).

The antioxidant capacity of sour cherry extract has been investigated using an ORAC (oxygen radical absorbance capacity) assay and it has been reported that the antioxidant capacity ranges from 1,145 to 1,916 μmol Trolox equivalents/100 g of sour cherry. These values are comparable to some berry fruits such as strawberry and are higher than apple and kiwi fruit (Ferretti et al., 2010). *In vivo,* an animal model given sour cherry juice showed an increased activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase and a decrease in lipid peroxidation (Šarić et al., 2009). In addition to this, a human study reported that consumption of TC juice (240 ml twice daily for 14 days) decreases the level of F(2)-isoprostane, a marker of oxidative damage (Traustadottir et al., 2009).

Obesity and T2DM is associated with low grade inflammation. In an animal model, TC-enriched diet reduced tissue inflammation and plasma level of pro-inflammatory molecules such as interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α) (Seymour et al., 2009). These alterations were associated with reduced levels of serum glucose, cholesterol and triglycerides (Seymour et al., 2009). Another study also reported

that consumption of TC juice by overweight and obese subjects for 4 weeks decrease very low density lipoprotein (VLDL) and triglyceride/high density lipoprotein (TG/HDL) ratio (Martin et al., 2011). In diabetic women, hemoglobin A1C and fasting blood glucose significantly decreased with consumption of TC juice at 40 ml/day for 6 weeks (Ataie-Jafari et al., 2008). Another study reported that TC extract prevents alloxan-induced diabetes in rat and mice (Lachin, 2014). Due to these reported health benefits of TC, this study will further investigate the effects of TC on body composition, markers of glucose homeostasis, insulin resistance, and gut integrity in WD- fed mice. We will explore whether TC is able to prevent the adverse health effects of WD. If our findings are positive, TC will be an affordable alternative in managing WD-induced obesity and T2DM.

CHAPTER III

MATERIALS AND METHODS

Animals and Treatment Groups

Seventy-two 6-week old male C57BL/6 mice were purchased from Charles River

Laboratory (Portage, MI). Mice were housed (3 mice per cage) at Oklahoma State

University's environmentally controlled Laboratory Animal Research Facility. Following

a 1-week acclimatization period, mice were randomly assigned to the following treatment

groups (**Table 2**) in a 2 x 3 factorial design with diet (AIN93-M control diet or Western

Diet, WD) and tart cherry (TC) (0 %, 5% and 10% TC, wt/wt) for 12 weeks

(n=12/group). For the TC diet, Montmorency TC powder was provided by Cherry

Marketing Institute and added at 5% and 10% wt/wt. The macronutrient as well as fiber,

calcium and phosphorus contribution of TC was accounted for such that all TC diets have

the same amounts of these nutrients to either control or WD (**Table 3**). Mice had access

to food and deionized water *ad libitum.* Food intake was monitored daily and body

weights were recorded on weekly basis.

Necropsy and Tissue Processing

At the end of the 12 weeks' treatment, mice were fasted for 3 hours but mice had

access to water. After fasting, body composition (i.e., lean mass, fat mass and body fat)

was assessed using a whole body Piximus scan (GE Medical System Lunar, Madison,

Table 2: Treatment groups

Group	Dietary Treatment (n=12 mice/group)
1	Control diet (C) (AIN-93M)
2	C +5% (wt/wt) Tart cherry (TC)
3	C +10% (wt/wt) TC
4	Western diet (WD) (45% fat kcal and 35% sucrose kcal)
5	WD + 5% (wt/wt) TC
6	WD + 10% (wt/wt) TC

WI) and blood was collected from the carotid artery. Blood was allowed to clot and serum samples were obtained by centrifugation of whole blood samples at 4°C for 10 minutes at 1500 x g. An aliquot of each serum sample was transferred into microcentrifuge tubes and stored at -80°C for further analyses.

The intestines, liver, white adipose tissue, pancreas, spleen, cecum, heart and thymus were collected, weighed and snapped-frozen. Colon length was measured with a ruler. The ileum and colon were flushed with ice-cold saline (0.9% NaCl), and a small portion of ileum and colon was cut and stored in 10 % neutral buffered formalin (NBF) for histological analysis. From the remaining ileum and colon, the lamina propria was removed and collected in microcentrifuge tubes and stored at -80°C for gene expression analyses. In addition, the cecum was harvested and its content was flushed with ice-cold saline into pre-weighed 15 ml centrifuge tubes. Cecal tissue was weighed and snap-frozen. Flushed cecal content was centrifuged at 4°C for 5 minutes at 1200 rpm, supernatant was discarded, weighed and kept frozen at -80°C for analysis of cecal microbiota.

Table 3. Diet composition

Ingredients	Control (C)	C+ 5%TC	C+ 10%TC	Western diet (WD)	WD + 5%TC	WD + 10% TC
			g/kg diet			
Tart cherry[1]	-	50	100	-	50	100
Corn Starch	466	433.3	400.6	34	34	34
Sucrose	100	100	100	308.5	308.5	308.5
Dextrinized Cornstarch	155	155	155	123.5	90.8	58.1
Casein	140	138.09	136.18	173	171.09	169.18
Soybean Oil	40	39.728	39.45	49.4	49.128	48.855
Lard	0	0	0	188.9	188.9	188.9
Cellulose	50	46.9	49.46	61.7	61.165	60.63
Mineral Mix[2]	35	35	35	16.54	16.54	16.54
Vitamin Mix(AIN 93VX)	10	10	10	12.3	12.3	12.3
Calcium carbonate	12.5	12.45	12.399	15.42	15.37	15.32
Tertbutylhydro quinone (TBHQ)	0.008	0.008	0.008	0.008	0.008	0.008
L-cystine	1.8	1.8	1.8	2.2	2.2	2.2
Sucrose	1.1	1.19	1.28	1.37	1.45	1.54
Choline bitartrate	2.5	2.5	2.5	3.1	3.1	3.1
Sodium phosphate, monobasic	5.6	5.528	5.461	6.905	6.837	6.77
Potassium phosphate, monobasic	2.4	2.378	2.349	2.97	2.941	2.912

[1]Tart cherry composition (TC, %): moisture, 14.2; carbohydrates, 65.4; protein, 3.82; fat, 0.545; fiber, 1.07; ash, 16.0; calcium, 0.0403; phosphorus, 0.0754; (NP Analytical Laboratories, St. Louis, MO).[2]Complete mineral mix (TD94049, Harlan-Teklad Laboratories) was used for the control diet and a calcium and phosphorus deficient mineral mix (TD 98057, Harlan-Teklad Laboratories) was used for the TC and WD diets.

Gut Microbiota Analysis

For the determination of possible changes in the gut microbiota affected by TC supplementation, frozen cecal samples were shipped on dry ice to University of California at Davis' Mouse Metabolic Phenotyping Center (MMPC) and Host Microbe Systems Biology Core. Total DNA was extracted using Mo-Bio (now Qiagen) PowerFecal kit. Sample libraries were prepared and analyzed by barcoded amplicon sequencing. In brief, the purified DNA was amplified on the V4 region of the 16S rRNA genes via PCR using the following primers: F319 (5'ACTCCTACGGGAGG CAGCAGT-3') and R806 (5'GGACTACNVGGGTWTCTAAT-3'). High-throughput sequencing was performed with Illumina MiSeq paired end 250-bp run. The data derived from sequencing was processed using QIIME2 for 16S based microbiota analyses. Demultiplexed paired end sequences that already had barcodes and adapters removed were analyzed using Qiime 2 version 2018.4. For quality filtering and feature (operational taxonomic unit; OTU) prediction, we used DADA2 (Callahan et al., 2016). Upon reviewing the sequence quality data, we trimmed 0 nucleotides (nts) from the 5' end of the forward and 0 nts from the reverse reads. Forward reads were truncated to 270 nts and reverse reads to 220 nts. Representative sequences were aligned using MAFFT (Katoh and Standley., 2013).

A phylogenetic tree of the aligned sequences was made using FastTree 2 (Price et al., 2010). OTUs/features were taxonomically classified using a pre-trained Naive Bayes taxonomy classifier. The classifier was trained using the Silva 128 97% OTUs (Quast et al., 2013) for the 319F-806R region. Tables of taxonomic counts and percentage (relative frequency) were generated. Diversity analyses were run on the resulting OTU/feature

.biom tables to provide both phylogenetic and non-phylogenetic metrics of alpha and beta diversity (Lozupone et al., 2011).

Short Chain Fatty Acids (SCFAs)

Cecal and fecal SCFAs (i.e., acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic and heptanoic acids) content were determined according to a previously published method (Ojo et al., 2016). Cecal samples and fecal samples were suspended in ice-cold Millipore water. An internal standard (10 mM of 2-ethylbutyric acid in 12% formic acid) was spiked into the sample suspension resulting in a final concentration of 1 mM internal standard. The pH of the resulting cecal and fecal homogenates was adjusted to 2 -3 using 5M hydrochloric acid. Samples were incubated at room temperature for 10 min followed by centrifugation. The resulting supernatants were filtered using 0.45 mm polytetrafluoroethylene syringe filters. Gas chromatographic analyses was done at Robert M. Kerr Food and Agricultural Products Center (Oklahoma State University, Stillwater, OK). Gas chromatographic (GC) analysis was performed using an Agilent 6890N GC system with a flame ionizable detector and an automatic liquid sampler (Agilent Technologies) as previously described (Ojo et al., 2016). A 5-point calibration was done by use of standard solutions of acetic, propionic, butyric, valeric, isovaleric, isobutyric, caproic and heptanoic acids (Sigma-Aldrich).

Gene Expression by Real-time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Relative expression of genes related to gut barrier integrity (zonulin-1, ZO-1; claudin; occludin), SCFAs receptor (G-protein coupled receptor, GPR43), mucus layer formation (mucin2, MUCN2), antimicrobial peptide (regenerating islet-derived protein 3-

gamma, Reg3- γ; regenerating islet-derived protein 3-beta, Reg3-β) and pro-

inflammatory cytokines (tumor necrosis factor-α, TNF-a; interleukin-6, IL-6) in each

treatment group was determined from the colon and ileum lamina propria using qRT-

PCR. Total RNA was extracted from the colon and ileum lamina propria using Trizol

reagent (Sigma-Aldrich). The concentration of the extracted RNA was verified using a

nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and agarose gel

electrophoresis was used to verify the quality of the 18S and 28S rRNA. The qRT-PCR

analysis was performed using SYBR green chemistry on an ABI 7900HT sequence-

detection system instrument and 2.4 SDS software (Applied Biosystems, CA). The

relative mRNA abundance was calculated by use of the $2^{-\Delta\Delta Ct}$ method, and the invariant

control gene was cyclophilin. Primer sequence used are shown in **Table 4**.

Table 4: Primer Sequence List for qRT- PCR

Symbol	Name	Sequence
Cyclo	Cyclophilin	QF 5'- GGTCTTTGGGAAGGTGAAAGAA -3' QR 5'- GCCATTCCTGGACCCAAAA -3'
IL-6	Interleukin 6	QF 5'- GAGGATACCACTCCCAACAGACC -3' QR 5'- AAGTGCATCATCGTTGTTCATACA -3'
Cldn	Claudin	QF 5'- CTGACCAAGAGCGAACACAA -3' QR 5'- CATGACTGGAGGCAACTGGA -3'
Muc2	Mucin-2	QF 5'- ACCCGAAGAAAGATGGATCG-3' QR 5'- CATAGTCAGATGGGGGGTGGA -3'
Ocln	Occludin	QF 5'- AGCCTGGTTGTTTAGGAGCA -3' QR 5'- CAGAATACGGCTCCTTCCTG -3'
ZO-1	Zonula occluden 1	QF 5'- TGGGAATGGAGTAACAAT -3' QR 5'- GGCAACTTCACCTCACAT-3'
Reg3-β	Regenerating islet derived protein 3 beta	QF 5'- CCATCTTCACGTAGCAGC -3' QR 5'- CAAGATGTCCTGAGGGC -3'
Reg3-γ	Regenerating islet derived protein 3 gamma	QF 5`- CTTCCCGGTGCAGTACAAGT-3` QR 5 - GCTCTTGGGTGAAGTTCTCG-3`
GPR43	G-protein-coupled receptor-43	QF 5'- CTGAGGTCAATCTGCCCAAGTAC -3' QR 5'- CTTCACAGAGCAATGACTCCAAAG -3'
TNF-α	Tumor necrosis factor alpha	QF 5'- CTGAGGTCAATCTGCCCAAGTAC -3' QR 5'- CTTCACAGAGCAATGACTCCAAAG-3'

Histology of Colon and Ileum

Ileum and colon tissues fixed in 10% neutral buffered formalin were dehydrated (Shandon Citadel 2000 Waltham, MA) with 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol, and toluene using an automated tissue processor (Shandon Citadel 2000 Waltham, MA). Next, tissues were embedded in paraffin blocks and 5 μm paraffin section was cut using a microtome (Leica Biosystems Wetzlar, Germany) followed by H&E staining for structural analysis. Image j software (Schneider et al., 2012) was used to analyze villi height, villi width, villi area, and crypt depth.

Glucose Tolerance Test (GTT) and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

Fasting blood glucose was determined from tail blood following food deprivation for six hours. GTT was performed prior to necropsy. Mice were fasted for 6 hours prior to GTT. Each mouse was injected intraperitoneally with a 20% glucose solution at a dose of 2 g/kg body weight. Blood glucose was measured from tail blood samples at 0, 5, 15, 30, 60, and 120 minutes after glucose injection. To examine glucose tolerance, total area under the curve (tAUC) for glucose was calculated (Lucas et al., 2011).

Insulin resistance was calculated by the HOMA-IR equation described previously (Matthews et al., 1985).

HOMA-IR = (fasting insulin (μU/ml) * fasting glucose (mmol/l)/22·5

Serum Lipids

The concentrations of total cholesterol, triglycerides, and non-esterified fatty acids (NEFA) was determined from serum samples using an automated chemistry analyzer (BioLis 24i, Carolina Chemistry, Winston-Salem, NC) following the

manufacturer's instructions. For total cholesterol determination, cholesterol esters are hydrolyzed to free cholesterol and fatty acids by cholesterol esterase. The free cholesterol generated is subsequently oxidized by cholesterol oxidase to cholesterol-4-en-3-one and hydrogen peroxide, which upon the action of peroxidase, forms a quinone dye, whose absorbance can be read at 505 nm giving a proportional value to the total cholesterol present in the sample. The principle of triglyceride determination involves lipase hydrolysis of triglycerides to glycerol and free fatty acids. This is followed by 3 coupled enzymatic steps which uses glycerol kinase, glycerophosphate oxidase and peroxidase, resulting in the formation of a colored complex which forms a red quinoneimine dye whose absorbance can be read at 520 nm with the value directly proportional to the triglyceride concentration in the sample.

NEFA measurement follows the principle of formation of acyl-CoA when NEFA is exposed to acyl-CoA synthetase in the presence of ATP and CoA. Acyl-CoA is oxidized by acyl-CoA oxidase to produce hydrogen peroxide, which allows for the condensation of 3-methyl-N-ethyl-N-(bhydroxyethyl)- aniline with 4-aminoantipyrine in the presence of an added peroxidase to form a purple-colored product whose absorbance can be measured at 550 nm, correlating to the amount of NEFA present in the sample.

Pancreatic-, Gut- and Adipose-derived Hormones

Concentrations of serum hormones (ghrelin, gastric inhibitory peptide (GIP), glucagon, insulin, leptin, plasminogen activator inhibitor 1 (PAI-1) and resistin) were determined using a mouse 8-plex assay kit according to the manufacturer's instructions. Fluorescently dyed nanobeads with unique individual spectral address were used to allow multiple detection of different molecules in a single well of a 96-well plate (Houser,

2012). Analytes were quantified using a Bio-Plex MAGPIX Multiplex Reader (Bio-Rad Laboratories Inc., Hercules, CA). Concentrations of analytes were determined using the Bio-Plex Manager (6.1) software.

Statistical Analyses

For the microbiome data, beta diversity analyses were carried out using the Bray-Curtis matrix. Principal coordinate analyses (PCoA) of the first two coordinates were plotted and comparison between groups was performed in R software (v.3.6.1) with permutational multivariate analysis of variance (PERMANOVA). Using the generated tables of relative phylum and genus abundance, differences between the dietary treatment groups were determined using the Kruskal-Wallis test (SAS version 9.4, NC, USA). Statistically significant results were further subjected to a post-hoc analysis using Dunn's test (Dunn.test package in R software). P-values were false discovery rate (FDR)-corrected with the Benjamini-Hochberg procedure (p.adjust function in R software).

For all other data, a Shapiro Wilks test was performed to assess whether data for continuous variables was normally distributed. After this, normally distributed data was analyzed using 2-way ANOVA, with WD and TC as factors and Tukey was used as a post-hoc test. Analyses were conducted using SAS 9.4 software (SAS Institute, NC, USA). Data presented are means $\pm$ SEM and a P- value <0.05 was considered statistically significant. The P-value for interaction (WD x TC) and main effect P-values (WD, TC) were given.

CHAPTER IV

RESULTS

This study evaluated the effects of tart cherry (TC) supplementation in the context of normal (control) and western diet (WD) on changes in the gut (i.e., microbiota, short chain fatty acids (SCFAs) production and markers of gut integrity) and its impact on body composition, blood glucose, lipid profile, and hormones in C57BL/6 mice.

Body Weight, Food Intake, Body Composition and Tissue Weights

Body weights were similar prior to the initiation of dietary treatments **(Table 5 and Figure 1)**. After only 2 weeks of dietary treatment, the body weight of mice fed the WD was significantly higher ($p<0.05$) as compared to control groups until the end of the 12-week study **(Figure 1)**. TC was not able to prevent the increase in body weight due to consumption of WD. The average food intake per day for the entire duration of the study, showed that there was significant reduction of food intake with WD ($p<0.0001$, **Table 5)** which is most likely due to the higher caloric density of the WD. There was also a significant interaction effect ($p<0.0001$, **Table 5)** on average daily food intake with the addition of TC to the control diet increasing food intake while decreasing if added to the WD.

Relative weight of the spleen, heart, pancreas and thymus were unaffected by WD and TC supplementation **(Table 5)**. Similarly, the length of the colon was unaffected by

WD and TC supplementation. However, relative liver weight was significantly lower in the WD-fed groups (*p=0.001*) while relative cecum weight was significantly increased (*p=0.007*) with TC supplementation in both control and WD-fed groups (**Table 5**). As expected, the relative weights of the abdominal (*p=0.0003*) and peri-renal fat (*p=0.017*) fat were significantly higher in the WD-fed mice compared to those fed the control diet (**Table 5**). Addition of TC to the WD was not able to prevent fat accumulation.

Whole body composition was also assessed by a densitometer (PixiMus) at the end of dietary treatment. WD and TC has no effect on lean mass; however, WD significantly increased total fat mass (*p=0.007*) and percentage body fat (*p=0.045*), but no main effect of TC on these parameters (**Table 5**). There tends to be an interaction effect of WD and TC on lean mass (*p=0.087*), fat mass (*p=0.091*) and % body fat (*p=0.073*). TC tend to increase lean mass in the control group but decrease in WD while decreasing fat mass in the control group and decreasing in the WD-fed groups.

Gut Microbiota Composition

The effect of 12 weeks TC supplementation in preventing gut dysbiosis due to WD feeding was assessed on the cecal content. β-diversity using the Bray-Curtis distance metrics revealed a notable clustering among the TC supplemented groups and the non-supplemented groups (**Figure 2A**). Addition of 5% TC to the control diet did not cause significant alteration in bacterial phylum (*data not presented*) but increased the lactate-producing phyla, Actinobacteria at 10% dose (*p=0.018*) (**Table 6B**). WD significantly decreased the bacterial phyla Proteobacteria (*p=0.017*) and Bacteroidetes (*p=0.033*) while increasing the ratio of Firmicutes to Bacteroidetes (*p=0.038*) in comparison to the control group (**Table 6A**). Bacteriodetes, an acetate and propionate-producing phylum,

was 42% lower ($p=0.033$) in WD group as compared to control (**Table 6A)** and TC

tended ($p=0.055$) to further decreased (74% reduction) these bacterial phyla when added

to the WD (**Table 6D**). Similarly, WD significantly increased Firmicutes/Bacteriodetes

ratio ($p=0.038$) compared to the control (**Table 6A**). This change is associated with low-

grade inflammation and reduction in barrier function, but was further increased

($p=0.048$) by the addition of 10% TC to the WD (**Table 6D**). TC added at 10% level

significantly increased the lactate-producing phyla, Actinobacteria, in both the control

($p=0.018$) and WD ($p=0.010$) (**Table 6B and 6D**). The pro-inflammatory bacterial phyla,

Deferribacteres, tended to be increased by WD ($p=0.094$) (**Table 6B**) which was

significantly reduced by approximately 86% ($p=0.005$) with 10% TC supplementation

(**Table 6D**). Saccharibacteria, responsible for mucosal inflammatory response in T2DM,

tended to increase ($p=0.059$) in WD as compared to control (**Table 6A**) which was

reduced by the addition of 5% TC ($p=0.039$) (**Table 6C**).

   Similar to the phylum level, there is no significant alterations in bacterial genus

with the addition of 5% TC to the control diet (*data not presented*) but the 10% TC dose

significantly reduced Bacteroides ($p=0.028$) (**Table 7B**). Bacteroides was not affected by

WD (**Table 7A**) or 5% TC (**Table 7C**) but the addition of 10% TC to the WD

significantly reduced this genus ($p=0.019$) (**Table 7D**). The Intestinimonas tended

($p=0.073$) to be increased by WD (**Table 7A**) and was significantly reduced by both

doses of TC in WD ($p=0.037$ and $p=0.0003$ for 5% and 10% TC, respectively) (**Table

7C and 7D**). Similarly, WD tended ($p=0.094$) to elevate Mucispirillum (phylum

Deferribacteres) as compared to control, but was only significantly reduced with 10% TC

in both control and WD ($p= 0.093$ and $p= 0.005$) (**Table 7B and 7D**). Lastly, the

WD+10% TC but not the WD+5% TC group had a higher abundance (*p=0.037*) of the Ruminococcaceae UCG-014 genus compared to the WD group (**Table 7D**).

Cecal Content and Fecal Short Chain Fatty Acids

After 90 days of treatment, weight of the cecal content was significantly increased with TC supplementation (*p=<.0001*) in both control and WD (**Table 8**). The SCFAs concentrations of the cecal content and feces were assessed by gas chromatography. TC supplementation was able to increase SCFAs production both in the cecal content and in the feces. In general, WD has no effect on SCFAs of the cecal content but tended to increase n-butyric (*p=0.0778*), i- valeric (*p=0.0893*), and n-valeric (*p=0.0735*) acids. Addition of TC significantly increased propionic (*p=0.0212*), i-butyric (*p=0.0183*), i-valeric (*p=0.0126*) n-valeric (*p=0.0261*), n-heptanoic (*p=0.0485*) acids and tended to increased n-butyric (*p=0.0608*) and n-caproic (*p=0.0942*) acids in the cecal content of both control- and WD-fed groups (**Table 8**).

Fecal SCFAs analysis showed that WD significantly reduced fecal propionic acid (*p=0.0149*) by approximately 25% but this SCFA was not affected by TC (**Table 8**). Fecal concentrations of n-butyric (*p=0.0001*) and n-valeric (*p=0.0011*) acids were reduced by WD and was increased by TC (*p=0.0177* and *p=0.0561* for n-butyric and n-valeric acids, respectively). A significant interaction was observed in fecal n-butyric acid content, with TC supplementation increasing n-butyric acid more in the control group (*p=0.0286*) (**Table 8**).

Relative Expression of Genes Related to Gut Health

The effects of WD and TC on the expression of genes related to gut health were examined by qRT-PCR in both the ileum and colon. Ileal expression of intracellular

scaffolding protein, zonulin-1 (ZO-1) and the short chain fatty acid receptor (G-protein coupled receptor, GPR43) were unaffected by treatments **(Figure 3A)**. Similarly, genes encoding for mucus layer formation (MUCN2) and antimicrobial peptide (regenerating islet-derived protein 3 beta, Reg3-β) were unchanged in the ileum relative to the control group **(Figure 3B)**. To determine if WD contributed to a local inflammatory response in the gut and whether TC affected this response, gene expression of inflammatory mediators was evaluated in the ileum lamina propria. Ileal gene expression of cytokines considered pro-inflammatory (tumor necrosis factor-α, TNF-α and interleukin-6, IL-6) were unaffected by both WD and TC treatments **(Figure 3C)**.

No statistical differences were detected with WD and TC, on the relative expression of genes related to the tight junction proteins (ZO-1, occludin, and claudin), SCFAs receptor (GPR43), antimicrobial peptides (Reg3-γ) and mucus layer production (MUCN2) in the colon **(Figure 4A-C)**.

Gut Structural Analysis

Histological analysis of villi and crypt structures were performed on the ileum and colon. Representative histological images for each of this region of the intestine are shown in **Figure 5**. WD significantly reduced ileal villi height (*p=0.0348*), width (*p=0.0042*) and area (*p=0.0132*), but no change in crypt depth was observed **(Table 9)**. TC supplementation has no effect on villi parameters and crypt depth of the ileum. Similar with the ileum, there was no significant effect of TC on colonic crypt depth but WD tended (*p=0.0804*) to increase this colonic parameter **(Table 9)**.

Glucose and Lipid Parameters

To determine the effects of TC supplementation on glucose homeostasis, a glucose tolerance test was conducted. Additionally, HOMA-IR was calculated from the fasting glucose and insulin concentrations. In a similar pattern to the results of body fat mass and % body fat by densitometry, fasting blood glucose level ($p=0.001$) was significantly increased with WD diet and TC doses were unable to modulate fasting blood glucose **(Table 10)**. To further assess the effects of treatment on glucose homeostasis, a glucose tolerance test **(Figure 6A)** and the glucose total area under curve (tAUC) was calculated **(Figure 6B).** Data from glucose tolerance test showed that WD-fed mice had approximately 24 % higher ($p=0.0007$) blood glucose compare to control prior to glucose injection (baseline, 0 minutes) and 38% higher ($p=0.0053$) blood glucose after 30 minutes of injection as compare to control **(Figure 6A)**. There was a main effect of WD increasing ($p=0.012$) the glucose tAUC after as glucose tolerance test with TC not exerting any effects **(Figure 6B)**. Additionally, insulin resistance was estimated by utilizing the homoeostatic model assessment of insulin resistance (HOMA-IR). Similar to the effects in glucose tAUC, WD significantly increased ($p=0.005$) HOMA-IR and TC significantly improved ($p=0.0003$) this marker of insulin resistance **(Table 10)**. There also tended ($p=0.061$) to be an interaction effects of the WD and TC treatment **(Table 10)**. The 5% TC supplementation tends to be more effective in lowering HOMA-IR both in the control and WD.

Furthermore, the effects of dietary treatment on lipid parameters were assessed by measuring serum cholesterol, triglycerides and non-esterified fatty acids (NEFA) **(Table 10)**. Serum triglycerides ($p=0.002$) and cholesterol ($p<0.0001$) were significantly increased with WD diet and TC supplementation was not able to reduce serum

cholesterol and triglycerides due to WD diet feeding. TC tends ($p=0.062$) to increase

serum triglycerides. Surprisingly, TC supplementation significantly increased serum

NEFA ($p=0.025$) as compare to control and WD diet group **(Table 10)**.

Pancreatic-, Gut- and Adipose-derived Hormones

In order to determine the effect of TC supplementation on pancreatic-, gut- and

adipose-derived hormone, Bioplex Multiplex assay was used to assess the level of these

markers in serum. In this study, dietary treatments had no effect on insulin, ghrelin,

gastric inhibitory peptide (GIP) and glucagon **(Table 11)**. However, serum concentrations

of leptin ($p=0.0011$), plasminogen activator inhibitor 1 (PAI1) ($p=0.0344$) and resistin

($p=0.0012$) were significantly increased with WD diet as compared to control group

(Table 11). TC had not effects on these parameters except for tending to increase

($p=0.0827$) PAI1.

Figure 1. Weekly body weights
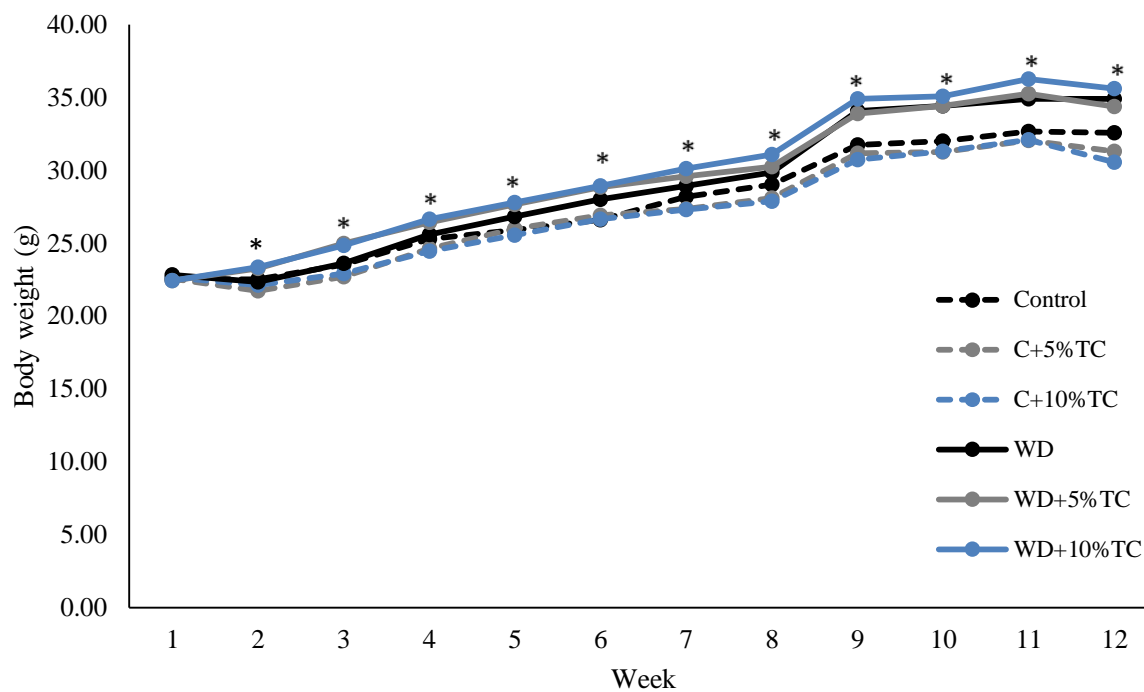
Body weights over the course of the 12-week study. Animals (n=12/group) were assigned to six different treatment groups in a 2x3 factorial design. Factors were diet (control or WD diet) and tart cherry (TC, 0 %, 5%, and 10 % wt/wt). Groups with the same color line had the same level of tart cherry. Baseline weights are indicated by week 0 while final indicates weight at necropsy. Asterisk denote main effect ($p < 0.05$) by WD diet.

Table 5. Food intake, body weight, tissue weight and body composition

|	Control (C)	C + 5% TC	C + 10% TC	WD	WD + 5% TC	WD + 10% TC	P-value WD	P-value TC	P-value TC*WD
Food Intake (g/day/mice)	2.97±0.05^c	3.36±0.06^{ab}	3.47±0.06^a	3.27±0.06^b	2.70±0.04^d	2.83±0.08^{cd}	**<0.0001**	0.1273	**<0.0001**
Body weights									
Baseline (g)	22.52±0.34	22.35±0.37	22.63±0.43	22.84±0.28	22.69±0.38	22.47±0.27	0.5627	0.8912	0.7139
Final (g)	32.55±0.85	31.29±0.91	30.55±0.85	34.40±1.15	34.35±1.19	35.60±1.98	**0.0012**	0.8947	0.4513
Tissue weights (% of total body weight)									
Liver	4.86±0.11	4.60±0.11	4.65±0.09	4.20±0.08	4.24±0.23	4.35±0.24	**0.001**	0.759	0.469
Thymus	0.13±0.01	0.15±0.02	0.13±0.02	0.15±0.02	0.13±0.02	0.16±0.02	0.540	0.867	0.300
Spleen	0.31±0.03	0.35±0.02	0.37±0.02	0.29±0.02	0.34±0.04	0.41±0.10	0.884	0.212	0.807
Pancreas	0.45±0.03	0.50±0.03	0.47±0.01	0.48±0.02	0.48±0.03	0.46±0.02	0.818	0.465	0.639
Heart	0.39±0.02	0.43±0.02	0.48±0.02	0.41±0.02	0.43±0.02	0.41±0.03	0.434	0.186	0.144
Cecum	0.15±0.02	0.15±0.02	0.24±0.02	0.14±0.02	0.16±0.02	0.16±0.02	0.104	**0.007**	0.199
Colon Length	7.12±0.20	7.50±0.27	7.70±0.02	7.09±0.24	7.08±0.31	7.36±0.29	0.21	0.229	0.705
Abdominal fat	3.62±0.30	3.59±0.34	2.68±0.35	4.93±0.30	4.56±0.75	4.74±0.58	**0.0003**	0.4530	0.4752
Perirenal fat	1.78±0.18	1.82±0.20	1.33±0.21	2.19±0.21	2.08±0.40	2.26±0.34	**0.017**	0.754	0.417
Body composition									
Lean mass(g)	22.70±0.59	22.79±0.49	23.91±0.57	24.01±0.60	24.11±0.57	23.14±0.42	0.166	0.947	**0.087**
Fat mass (g)	10.79±0.91	9.40±0.84	7.39±0.68	11.40±1.03	11.46±1.67	13.58±2.13	**0.007**	0.853	**0.091**
% body fat	31.82±2.19	28.78±1.96	23.22±1.79	31.42±1.97	31.20±3.48	34.83±3.91	**0.045**	0.6235	**0.073**

Values are mean ± S.E (n=12/group). Within a row, values with unlike superscript letters are significantly different (p ≤ 0.05) from each other. TC, tart cherry; WD, Western diet.

Figure 2. Gut microbiota composition (n=6 animals per group)

A
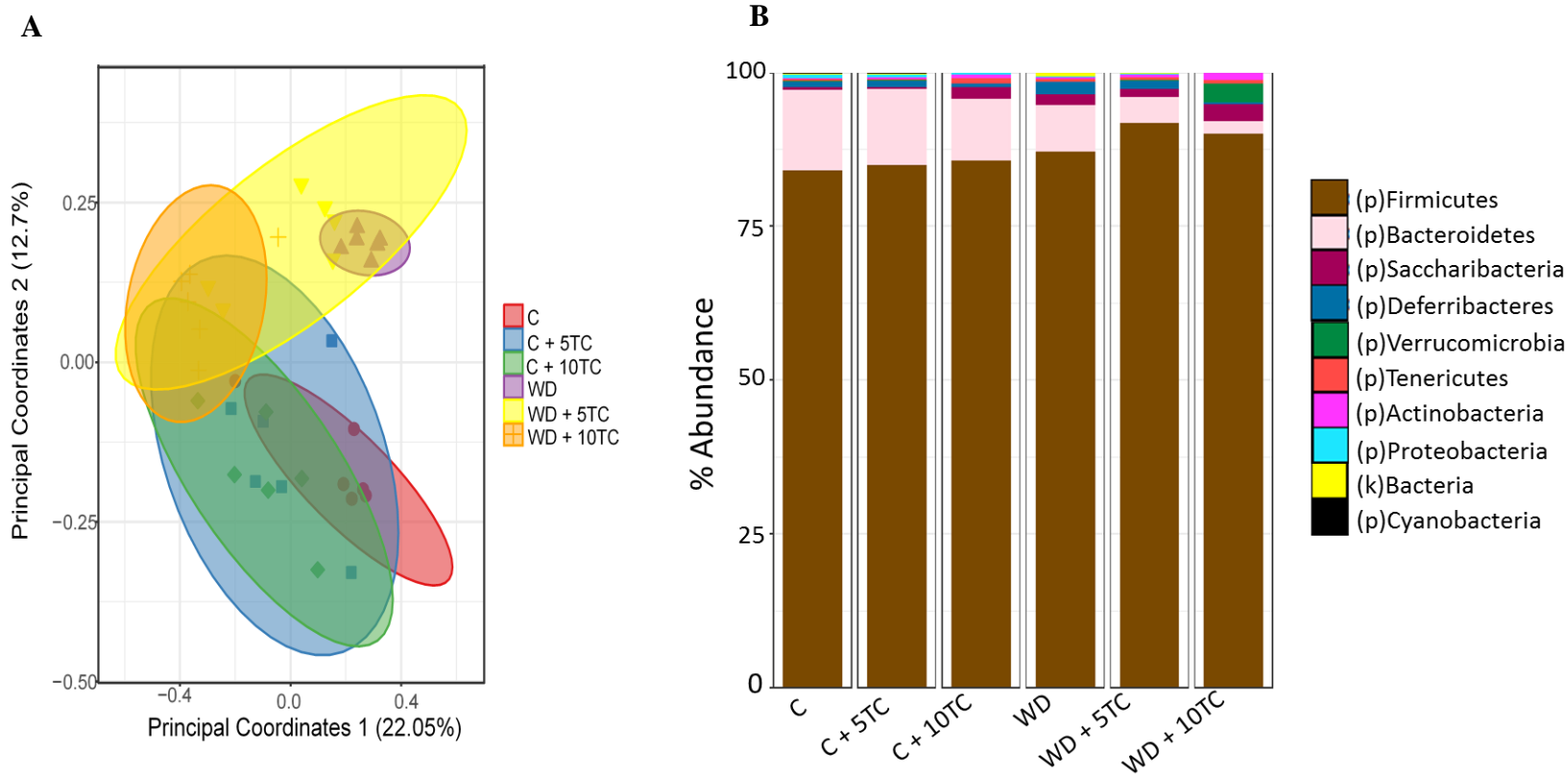
Principal coordinate analysis of gut bacteria based on Bray-Curtis distance of operational taxonomic unit (OTU)

B

Relative abundance of the gut bacterial phyla.

C

Mean percent relative abundance of the most abundant genera

Legend:
- (f)Lachnospiraceae
- (f)Lachnospiraceae;(g)Shuttleworthia
- (f)Ruminococcaceae;(g)Intestinimonas
- (f)Ruminococcaceae
- (f)Ruminococcaceae;(g)Ruminococcaceae UCG−014
- (f)Lachnospiraceae;(g)Lachnospiraceae NK4A136 group
- (f)Bacteroidales S24−7 group
- (f)Peptococcaceae
- (f)Lactobacillaceae;(g)Lactobacillus
- (f)Bacteroidaceae;(g)Bacteroides
- (f)Lachnospiraceae;(g)Eubacterium fissicatena group
- (f)Verrucomicrobiaceae;(g)Akkermansia
- (f)Unknown Family;(g)Candidatus Saccharimonas
- (f)Lachnospiraceae;(g)Ruminococcus gnavus group
- (f)Rikenellaceae;(g)Alistipes
- (f)Lachnospiraceae;(g)Lachnospiraceae UCG−006
- (f)Lachnospiraceae;(g)Coprococcus 1
- (f)Ruminococcaceae;(g)Oscillibacter
- (f)Ruminococcaceae;(g)Ruminococcus 1
- (f)Deferribacteraceae;(g)Mucispirillum
- (o)Bacteroidales
- (f)Ruminococcaceae;(g)Anaerotruncus
- (f)Ruminococcaceae;(g)Ruminococcaceae UCG−009
- (f)Lachnospiraceae;(g)uncultured
- (f)Ruminococcaceae;(g)Ruminococcaceae UCG−003
- (f)Bacteroidales S24−7 group;(g)uncultured bacterium
- (f)Ruminococcaceae;(g)Ruminiclostridium
- (f)Ruminococcaceae;(g)Eubacterium coprostanoligenes group
- (f)Lachnospiraceae;(g)Tyzzerella
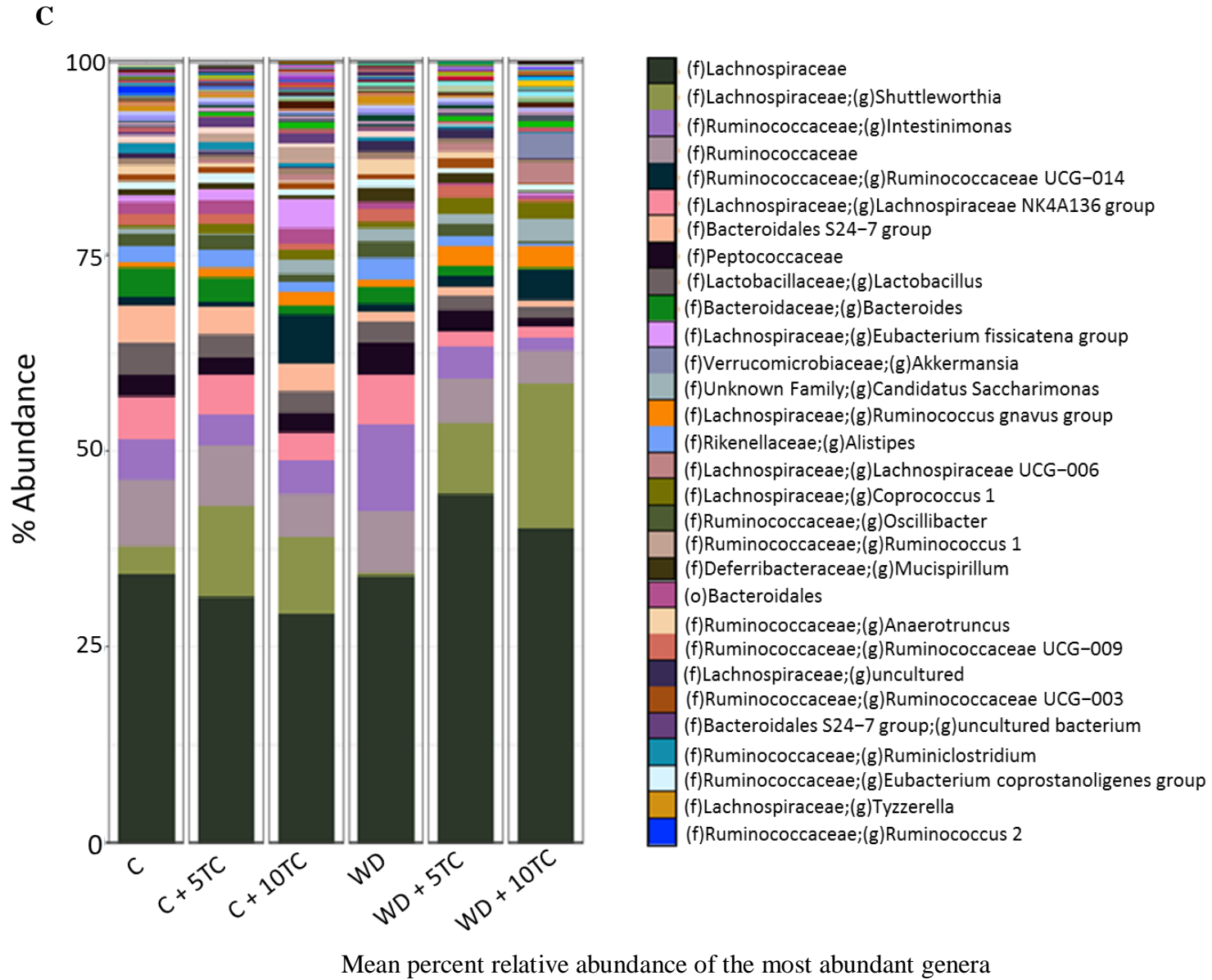- (f)Ruminococcaceae;(g)Ruminococcus 2

45

Table 6. Mean percent relative abundance of cecal bacterial phyla in C57BL/6 mice fed a C or WD supplemented with 5% or 10% TC

A

	Control(C)		Western diet (WD)		C vs WD	
	mean	SEM	mean	SEM	Changes (%)	$P_{adjusted}$
Actinobacteria	0.17	0.06	0.28	0.09	65	0.256
Deferribacteres	0.80	0.14	1.96	0.39	145	**0.094**
Proteobacteria	0.48	0.12	0.14	0.03	-71	**0.017**
Saccharibacteria	0.47	0.11	1.72	0.44	265	**0.059**
Firmicutes	84.08	1.38	87.14	1.01	4	0.152
Bacteroidetes	13.11	1.14	7.65	0.97	-42	**0.033**
Firmicutes/Bacteriodetes ratio	6.75	0.79	12.83	2.41	90	**0.038**

Values are mean percent ± S.E (n=6/group). $P_{adjusted}$ values are based on Kruskal-Wallis rank sum test followed by post-hoc analysis using Dunn's test.

B

	Control(C)		C+10 %TC		C vs C+10% TC	
	mean	SEM	mean	SEM	Changes (%)	$P_{adjusted}$
Actinobacteria	0.17	0.06	0.56	0.11	229	**0.018**
Deferribacteres	0.80	0.14	0.54	0.36	-33	**0.093**
Proteobacteria	0.48	0.12	0.19	0.04	-60	0.135
Saccharibacteria	0.47	0.11	1.33	0.57	182	0.221
Firmicutes	84.08	1.38	85.76	1.13	2	0.325
Bacteroidetes	13.11	1.14	10.03	0.85	-23	0.187
Firmicutes/Bacteriodetes ratio	6.75	0.79	8.96	0.95	33	0.187

Values are mean percent ± S.E (n=6/group). $P_{adjusted}$ values are based on Kruskal-Wallis rank sum test followed by post-hoc analysis using Dunn's test. No significant changes were observed at phylum level between C and C + 5% TC. TC, tart cherry; WD, western diet

C

	Western diet (WD)		WD + 5% TC		WD vs WD+5%TC	
	mean	SEM	mean	SEM	Changes (%)	$P_{adjusted}$
Actinobacteria	0.28	0.09	0.45	0.12	61	0.163
Deferribacteres	1.96	0.39	1.24	0.40	-37	0.178
Proteobacteria	0.14	0.03	0.11	0.04	-21	0.265
Saccharibacteria	1.72	0.44	1.45	0.29	-16	**0.039**
Firmicutes	87.14	1.01	91.82	1.25	5	0.107
Bacteroidetes	7.65	0.97	4.30	1.12	-44	0.194
Firmicutes/Bacteriodetes ratio	12.83	2.41	28.77	5.93	61	0.194

Values are mean percent ± S.E (n=6/group). $P_{adjusted}$ values are based on Kruskal-Wallis rank sum test followed by post-hoc analysis using Dunn's test. TC, tart cherry

D

	Western diet (WD)		WD +10% TC		WD vs WD+10%TC	
	mean	SEM	mean	SEM	Changes (%)	$P_{adjusted}$
Actinobacteria	0.28	0.09	1.04	0.21	271	**0.010**
Deferribacteres	1.96	0.39	0.27	0.08	-86	**0.005**
Proteobacteria	0.14	0.03	0.05	0.02	-64	0.101
Saccharibacteria	1.72	0.44	2.45	0.72	42	0.404
Firmicutes	87.14	1.01	90.11	1.83	3	0.199
Bacteroidetes	7.65	0.97	1.98	0.48	-74	**0.055**
Firmicutes/Bacteriodetes ratio	12.83	2.41	59.83	13.10	366	**0.048**

Values are mean percent ± S.E (n=6/group). $P_{adjusted}$ values are based on Kruskal-Wallis rank sum test followed by post-hoc analysis using Dunn's test. TC, tart cherry

Table 7. Mean percent relative abundance of cecal bacterial genera in C57BL/6 mice fed a C or WD supplemented with 5% or 10% TC

A

	Control (C)		Western diet (WD)		C vs WD	
	mean	SEM	mean	SEM	Changes (%)	$P_{adjusted}$
Lactobacillus	4.09	1.09	2.64	1.64	-35	0.171
Parasutterella	0.38	0.11	0.08	0.03	-79	**0.028**
Bacteroides	3.81	0.56	2.23	0.53	-41	0.116
Mucispirillum	0.80	0.15	1.96	0.39	145	**0.094**
Intestinimonas	5.21	1.05	11.02	1.58	112	**0.073**
Ruminococcaceae UCG-014	1.05	0.33	1.00	0.19	-5	0.456

Values are mean percent ± S.E (n=6/group). $P_{adjusted}$ values are based on Kruskal-Wallis rank sum test followed by post-hoc analysis using Dunn's test.

B

	Control (C)		C +10% TC		C vs C+10%TC	
	mean	SEM	mean	SEM	Changes (%)	$P_{adjusted}$
Lactobacillus	4.09	1.09	2.66	0.46	-35	0.438
Parasutterella	0.38	0.11	0.15	0.03	-61	0.179
Bacteroides	3.81	0.56	1.41	0.27	-63	**0.028**
Mucispirillum	0.80	0.15	0.54	0.36	-33	**0.093**
Intestinimonas	5.21	1.05	4.44	1.20	-15	0.389
Ruminococcaceae UCG-014	1.05	0.33	6.27	3.59	499	0.224

Values are mean percent ± S.E (n=6/group). $P_{adjusted}$ values are based on Kruskal-Wallis rank sum test followed by post-hoc analysis using Dunn's test. No significant changes were observed at genera level between C and C + 5% TC.TC, tart cherry; WD, western diet

C

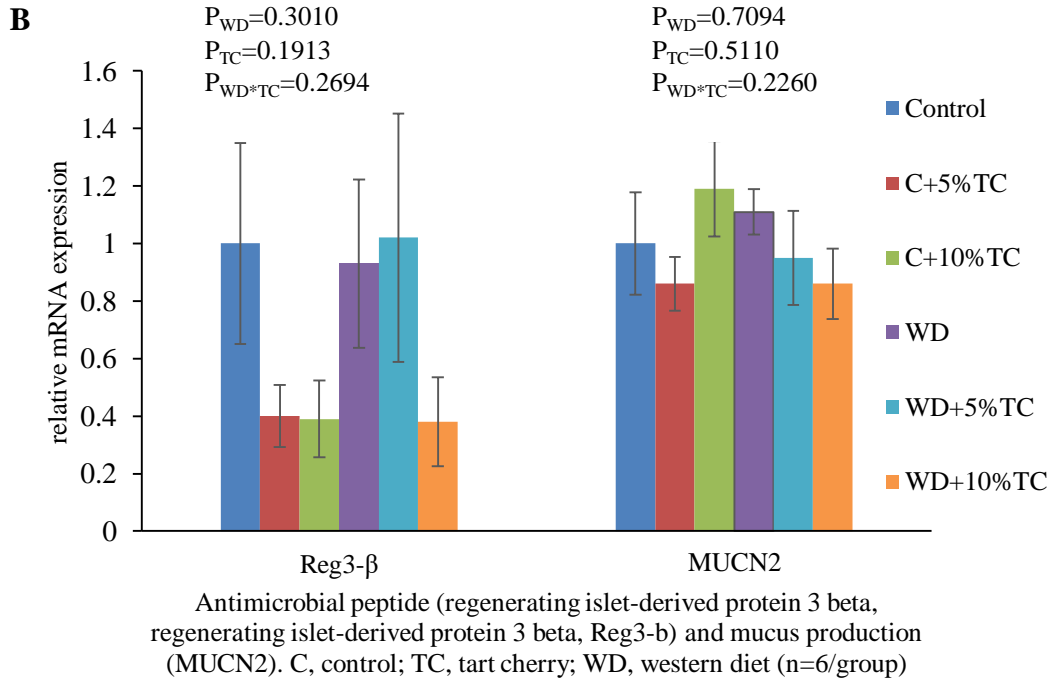|	Western diet (WD)		WD+5 % TC		WD vs WD+5%TC	
	mean	SEM	mean	SEM	Changes (%)	$P_{adjusted}$
Lactobacillus	2.64	1.64	1.80	0.40	-32	0.400
Parasutterella	0.08	0.03	0.05	0.02	-38	0.246
Bacteroides	2.23	0.53	1.19	0.45	-47	0.135
Mucispirillum	1.96	0.39	1.25	0.40	-36	0.178
Intestinimonas	11.02	1.58	4.17	0.96	-62	**0.037**
Ruminococcaceae UCG-014	1.00	0.19	1.57	0.42	57	0.298

Values are mean percent ± S.E (n=6/group). $P_{adjusted}$ values are based on Kruskal-Wallis rank sum test followed by post-hoc analysis using Dunn's test. TC, tart cherry

D

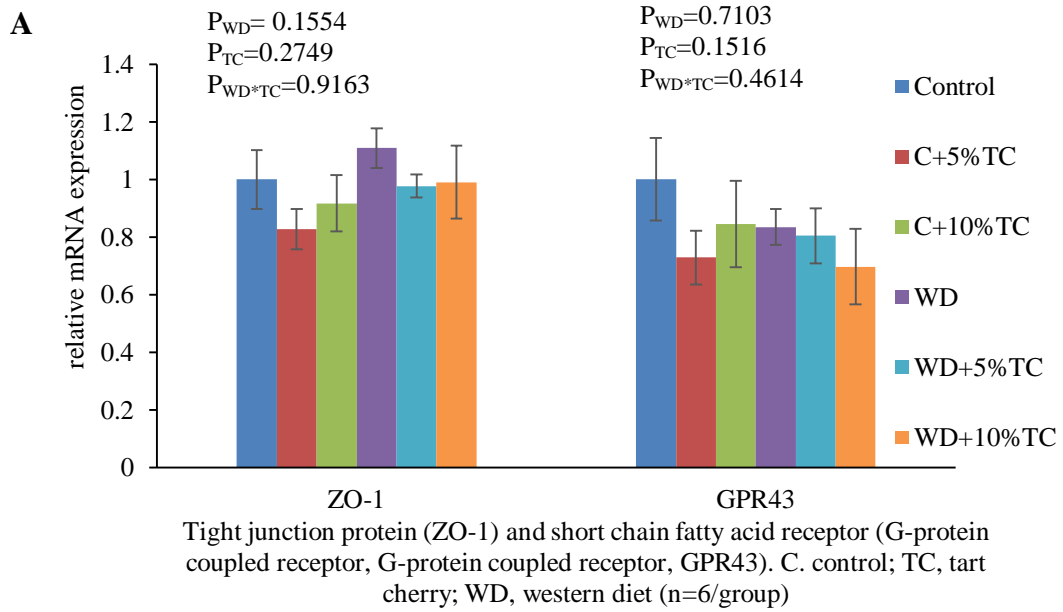|	Western diet (WD)		WD +10% TC		WD vs WD+10%TC	
	mean	SEM	mean	SEM	Changes (%)	$P_{adjusted}$
Lactobacillus	2.64	1.64	1.44	0.38	-45	0.489
Parasutterella	0.08	0.03	0.03	0.02	-67	0.149
Bacteroides	2.23	0.53	0.32	0.13	-86	**0.019**
Mucispirillum	1.96	0.39	0.27	0.08	-86	**0.005**
Intestinimonas	11.02	1.58	1.73	0.79	-84	**0.0003**
Ruminococcaceae UCG-014	1.00	0.19	3.86	0.59	287	**0.037**

Values are mean percent ± S.E (n=6/group). $P_{adjusted}$ values are based on Kruskal-Wallis rank sum test followed by post-hoc analysis using Dunn's test. TC, tart cherry

Table 8. Cecal content weight and short chain fatty acids (SCFAs)

	Control	C + 5% TC	C + 10% TC	WD	WD + 5% TC	WD + 10% TC	P-value WD	P-value TC	P-value TC*WD
Cecal content (mg)	215.0±17.0	228.0±17.0	290.0±17.0	208.0±14.0	231.0±15.0	277.0±12.0	0.672	**<.0001**	0.889
Cecal content SCFAs (nmol/g)									
acetic	2185.0±799.2	2742.4±271.7	2998.4±452.6	1367.3±216.6	3258.5±585.9	2891.9±967.9	0.7885	0.1313	0.5955
propionic	144.35±28.19	333.39±57.16	460.64±106.80	77.64±12.30	567.31±168.72	417.74±167.89	0.6709	**0.0212**	0.3820
i-butyric	13.66±4.78	26.91±6.26	39.33±7.53	6.46±0.69	78.27±25.24	48.48±21.16	0.1193	**0.0183**	0.1141
n-butyric	59.22±6.13	150.39±24.33	284.52±71.67	38.81±9.88	671.97±203.53	447.41±286.98	**0.0778**	**0.0608**	0.2117
i-valeric	21.37±0.63	55.77±9.86	69.67±12.42	22.51±3.93	134.58±43.36	83.39±32.06	**0.0893**	**0.0126**	0.1808
n-valeric	21.55±1.51	56.25±8.37	93.70±23.07	15.42±4.50	204.98±75.04	124.78±58.95	**0.0735**	**0.0261**	0.1334
n-caproic	5.74±1.20	5.13±0.70	7.44±1.28	9.39±2.07	3.91±1.25	5.55±0.81	0.8659	**0.0942**	0.1077
n-heptanoic	5.22±2.83	2.19±1.39	8.73±2.02	6.30±3.17	4.24±0.43	8.36±1.59	0.6043	**0.0485**	0.8290
Fecal SCFA (umol/g)									
acetic	6756.00 ±1470.83	9715.54 ±1886.92	9779.63 ±2101.67	4843.36 ±1255.69	7738.59 ±2762.81	6725.40 ±996.77	0.1414	0.2465	0.9440
propionic	545.78±130.38	620.15±126.19	1081.65±302.50	344.83±106.03	492.11±148.19	339.15±28.23	**0.0149**	0.2861	0.1473
i- butyric	65.88±16.71	82.55±17.92	153.19±53.77	63.00±0.02	88.75±25.54	55.88±4.59	0.1788	0.3639	0.1414
n-butyric	274.76±95.68[c]	374.83±36.71[b]	643.84±92.35[a]	111.93±37.19[c]	237.26±68.60[bc]	159.75±23.52[c]	**0.0001**	**0.0177**	**0.0286**
i-valeric	143.40±35.34	148.41±30.26	301.40±98.13	145.29±37.93	197.84±46.96	132.08±11.23	0.3567	0.3791	0.1058
n-valeric	132.34±34.88	164.33±21.05	251.03±36.71	74.74±22.79	121.28±26.16	96.09±11.88	**0.0011**	**0.0561**	0.1058
n-caproic	10.17±2.78	10.65±1.06	15.82±1.84	6.61±0.88	10.53±1.87	11.63±3.84	0.1759	0.0900	0.6387
n-heptanoic	9.90±6.91	21.63±11.52	19.63±10.99	13.16±3.67	12.74±5.11	24.29±9.82	0.9651	0.5292	0.7030

Values are mean ± S.E (n=6/group). Within a row, values with unlike superscript letters are significantly different ($p \leq 0.05$) from each other. TC, tart cherry; WD, Western diet.

Figure 3. Relative expression of genes related to barrier integrity, short chain fatty acids (SCFAs) receptor, antimicrobial peptide, mucus layer formation and inflammation in ileum
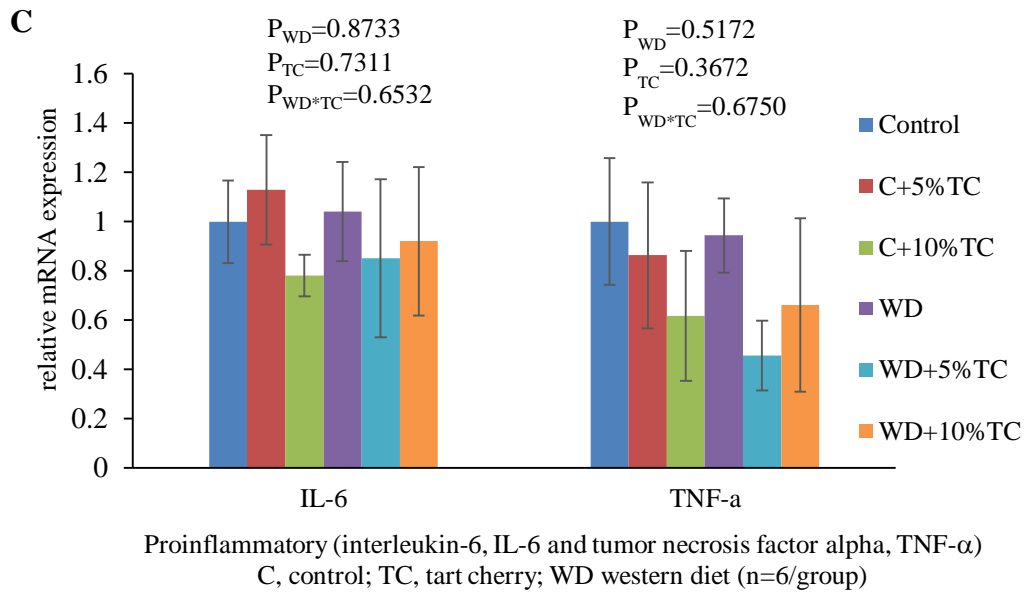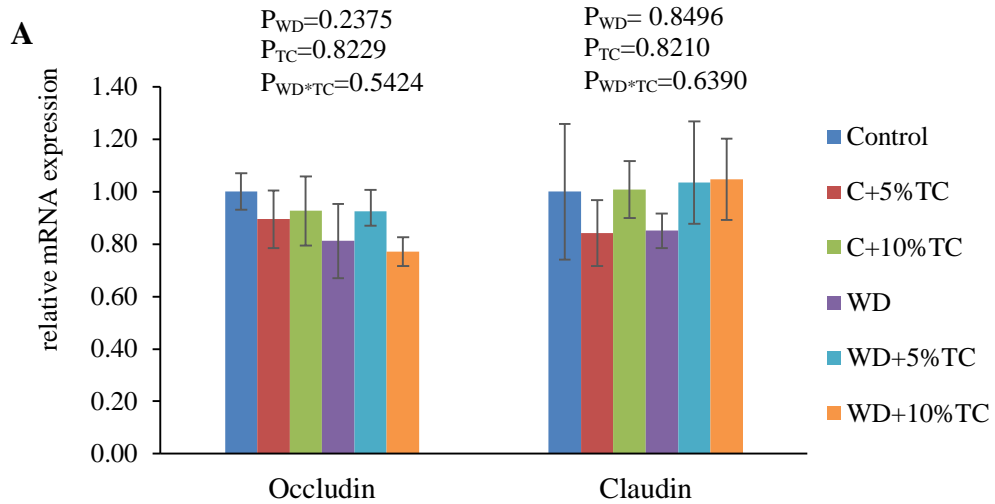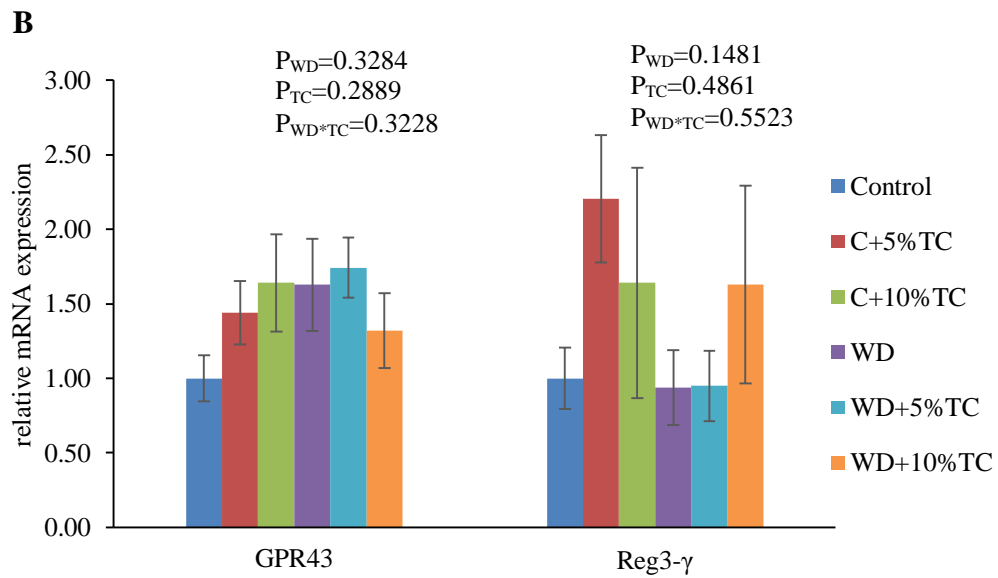

Tight junction protein (ZO-1) and short chain fatty acid receptor (G-protein coupled receptor, G-protein coupled receptor, GPR43). C. control; TC, tart cherry; WD, western diet (n=6/group)

Antimicrobial peptide (regenerating islet-derived protein 3 beta, regenerating islet-derived protein 3 beta, Reg3-b) and mucus production (MUCN2). C, control; TC, tart cherry; WD, western diet (n=6/group)

C

Proinflammatory (interleukin-6, IL-6 and tumor necrosis factor alpha, TNF-α)
C, control; TC, tart cherry; WD western diet (n=6/group)

Figure 4. Relative expression of genes related to barrier integrity, short chain fatty acids (SCFAs) receptor, antimicrobial peptide and mucus layer formation in colon
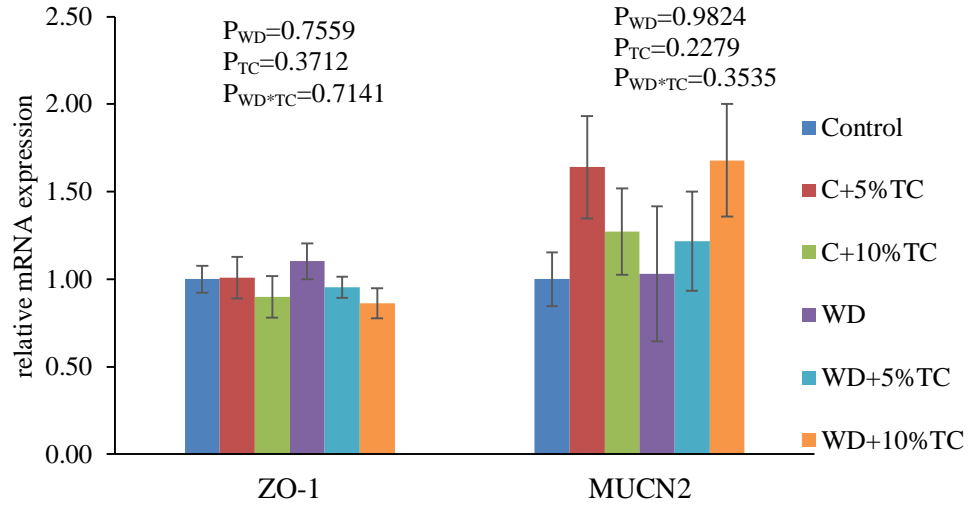
A

$P_{WD}=0.2375$
$P_{TC}=0.8229$
$P_{WD*TC}=0.5424$

$P_{WD}=0.8496$
$P_{TC}=0.8210$
$P_{WD*TC}=0.6390$

Indicators of gut barrier integrity (Occludin and claudin). C, control; TC, tart cherry; WD, western diet (n=6/group)

B

$P_{WD}=0.3284$
$P_{TC}=0.2889$
$P_{WD*TC}=0.3228$

$P_{WD}=0.1481$
$P_{TC}=0.4861$
$P_{WD*TC}=0.5523$

Short chain fatty acid receptor (GPR43) and anti-microbial peptides (Reg3-γ). C, control; TC, tart cherry; WD, western diet (n=6/group)

C

Tight junction protein (zonulin, ZO-1) and mucus production (MUCN2). C, control; TC, tart cherry; WD, western diet (n=6/group)

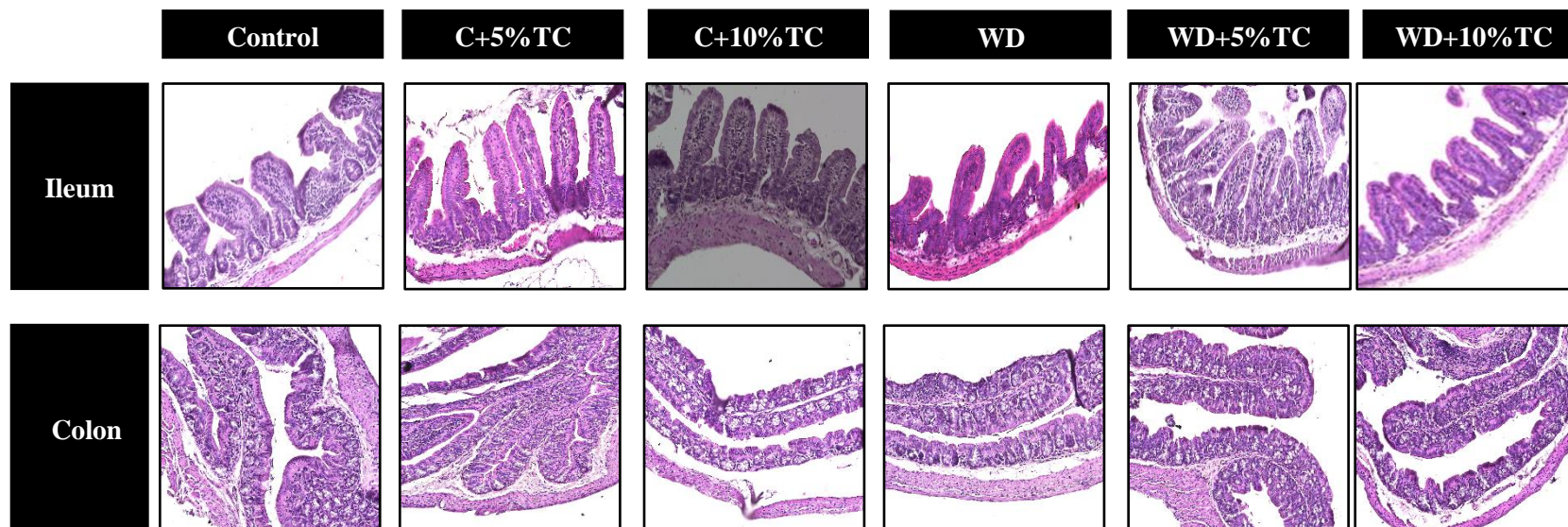**Figure 5. Representative images of histological sections of the ileum and colon (n=6/group)**

Table 9. Villi and crypt structural parameters of the ileum and colon

|	Control	C + 5% TC	C + 10% TC	WD	WD + 5% TC	WD + 10% TC	P-value WD	P-value TC	P-value TC*WD
Ileum									
Villi height (μm)	129.73±10.03	117.83±7.58	100.42±3.96	95.012±5.81	108.14±13.91	99.16±5.04	**0.0348**	0.2265	0.1365
Villi width (μm)	60.81±3.67	56.22±4.96	59.46±4.76	46.06±3.02	52.68±2.89	50.12±1.07	**0.0042**	0.9276	0.3194
Villi area (mm^2)	7.04±1.15	6.31±0.97	5.15±0.50	3.76±0.35	5.13±1.11	4.16±0.60	**0.0132**	0.4409	0.3329
Crypt depth (μm)	47.81±1.29	46.39±1.31	50.75±2.00	46.64±3.98	47.11±6.39	41.63±2.40	0.2616	0.9551	0.3234
Colon									
Crypt depth (μm)	62.92±3.06	51.75±3.40	49.97±2.36	60.03±6.47	67.41±4.93	55.79±3.59	**0.0804**	0.1161	0.1039

Values are mean ± S.E. (n=6/group) TC, tart cherry; WD, Western diet;

Table 10. Fasting blood glucose, and serum lipids

	Control	C + 5% TC	C + 10% TC	WD	WD + 5% TC	WD + 10% TC	P-value WD	P-value TC	P-value TC*WD
Fasting blood Glucose (mg/dL)	113.55±2.57	108.27±5.56	102.83±7.89	131.67±5.66	121.50±4.73	139.55±13.95	**0.001**	0.576	0.278
Insulin (pg/ml)	781.10 ±178.31	352.79 ±63.21	466.34 ±109.83	603.74 ±89.70	537.38 ±198.68	721.23 ±168.67	0.4657	0.2346	0.2887
HOMA-IR	4.05±0.41	2.16±0.35	2.50±0.59	4.77±0.57	2.41±0.40	5.14±0.64	**0.005**	**0.0003**	**0.061**
Serum Lipids									
Cholesterol (mg/dL)	116.50±7.59	108.91±9.53	107.18±5.68	169.58±10.29	161.00±9.25	180.73±10.55	**<.0001**	0.560	0.414
Triglyceride (mg/dL)	51.0±2.96	57.10±3.77	57.22±3.02	64.73±9.30	64.10±5.57	90.67±11.95	**0.002**	**0.062**	0.166
NEFA (mEq/L)	1.12±0.05	1.36±0.07	1.27±0.07	1.21±0.09	1.29±0.06	1.43±0.07	0.273	**0.025**	0.287

Values are mean ± S.E. (n=10-12/group) Within a row, values with unlike superscript letters are significantly different ($p \leq 0.05$) from each other. TC, tart cherry; WD, Western diet; HOMA-IR= Homeostatic model assessment of insulin resistance, NEFA= Non-esterified fatty acids

Figure 6. Glucose tolerance test and glucose total area under the curve (tAUC)
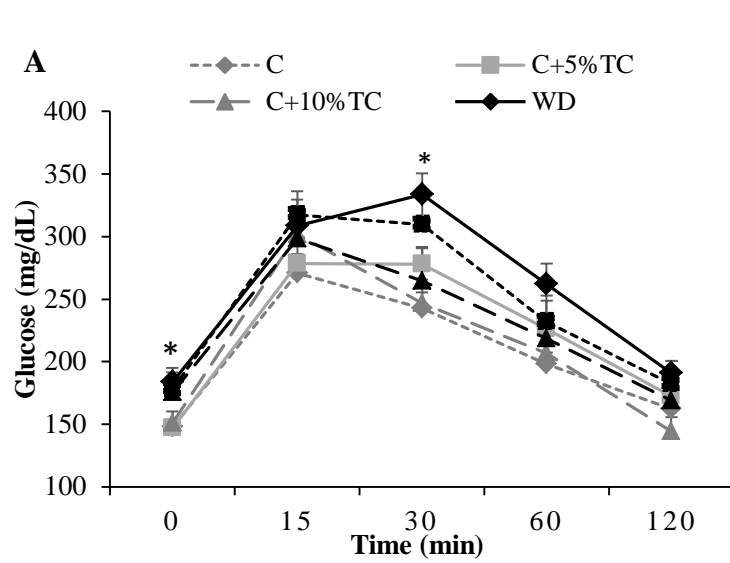
Glucose Tolerance Test (GTT). (n=12/group) Asterisk (*) denote main effect ($p<0.05$) by WD. C, control; TC, tart cherry; WD, western diet
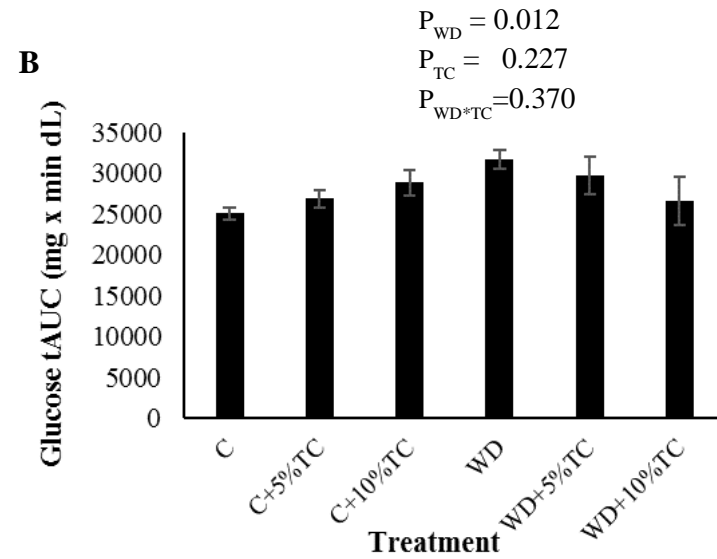
Glucose Total Area Under the Curve (tAUC) (n=12/group) C, control; TC, tart cherry; WD, western diet

Table 11. Pancreatic-, gut- and adipose-derived hormones

	Control	C + 5% TC	C + 10% TC	WD	WD + 5% TC	WD + 10% TC	P-value WD	P-value TC	P-value TC*WD
Ghrelin (pg/ml)	1323.86 ±84.39	1298.20 ±92.49	1115.98 ±98.78	1214.51 ±122.32	1220.51 ±252.18	1053.28 ±59.41	0.4505	0.3104	0.9843
GIP (pg/ml)	132.19 ±7.88	113.61 ±7.55	123.68 ±22.24	127.13 ±11.74	132.02 ±13.37	155.58 ±15.01	0.1904	0.4822	0.4111
Glucagon (pg/ml)	248.21 ±82.47	209.98 ±47.21	227.15 ±45.59	242.42 ±48.91	248.35 ±39.52	405.93 ±127.72	0.2449	0.4538	0.4338
Insulin (pg/ml)	781.10 ±178.31	352.79 ±63.21	466.34 ±109.83	603.74 ±89.70	537.38 ±198.68	721.23 ±168.67	0.4657	0.2346	0.2887
Leptin (pg/ml)	2984.94 ±638.66	1528.41 ±434.89	1333.23 ±276.25	3790.69 ±567.92	3509.91 ±917.87	4962.14 ±1231.29	**0.0011**	0.4928	0.1780
PAI1 (pg/ml)	437.92 ±42.40	472.61 ±32.17	537.33 ±58.69	516.15 ±28.11	552.98 ±30.00	624.83 ±68.56	**0.0344**	**0.0827**	0.9944
Resistin (pg/ml)	25446.8 ±1927.93	19319.43 ±2058.60	18306.82 ±937.92	32084.54 ±3579.36	28840.45 ±4875.31	29705.10 ±4224.79	**0.0012**	0.2553	0.7627

Values are mean ± S.E. (n=8/group) Within a row, values with unlike superscript letters are significantly different (p ≤ 0.05) from each other. TC, tart cherry; WD, Western diet; GIP, gastric inhibitory peptide; PAI1; plasminogen activator inhibitor 1.

CHAPTER V

DISCUSSION

This study was conducted to determine the dose-dependent effects of TC supplementation in preventing gut dysbiosis, the loss of beneficial bacteria and increase in harmful bacteria, due to consumption of WD. Moreover, we also evaluated if TC supplementation will maintain gut integrity and consequently improve body composition and glucose and lipid parameters that was altered by WD. The findings of this study showed that addition of TC to the WD prevented the loss of some beneficial bacteria at both 5% and 10% level of supplementation. However, TC supplementation was not able to modulate the increased body weight and fat accumulation caused by WD. Similarly, body composition, gut morphological parameters and fasting glucose were unaffected by TC supplementation. Although body composition seems to not be affected by TC in the context of WD, it seems to improve lean and fat mass when taken in the context of normal diet. Despite TC not affecting markers of gut barrier integrity and fasting blood glucose, HOMA-IR, a marker of insulin resistance was improved by TC particularly the 5% dose. Additionally, TC-fed mice in the context of both control and WD had higher cecal and fecal SCFAs compared to mice that did not received TC.

It has been demonstrated in several studies in human and animal models that gut microbial dysbiosis contributes to the development of obesity and obesity-related diseases such as T2DM (Muscogiuri et al., 2019). Davis (2016) reported that a high-fat

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and high-sugar WD increases the relative abundance of Firmicutes as compared to Bacteroidetes in animal models. Obese individuals have been reported to have higher concentrations of bacteria that belongs to the phylum Firmicutes and lower concentrations of bacteria that belong to the phylum Bacteroidetes as compared to healthy individuals (Muscogiuri et al., 2019). Larsen and colleagues (2010) also showed that the proportion of bacteria in the phylum Firmicutes was increased in the gut of T2DM adults as compared to non-diabetic adults. *Faecalibacterium prausnitzii*, bacteria belonging to the phylum Firmicutes and one of the most abundant species in the human gut with anti-inflammatory properties, is found to be decreased in people with T2DM (Furet et al., 2010). Bacteria that belong to Lactobacilli and Bifidobacteria are considered good for gut health and they are the usual target for dietary intervention studies to improve health. Elderly individuals that consumed prebiotics such as fructooligosaccharides (FOS) and galactooligosaccharides (GOS) have higher proportions of Bifidobacteria and Lactobacilli (Toward et al., 2012). In addition to this, Colantonio et al (2019) reported that high-fat diet induced loss of Lactobacillus and Bifidobacteria, which was normalized upon prebiotic feeding resulting to a reversal of symptoms and disease progression of T2DM. These studies demonstrate that dietary intervention that modulate gut bacteria improves health outcome.

In our study, we observed an increase in the phylum Actinobacteria with TC supplementation specifically with the 10% dose. The phylum, Actinobacteria is a recently characterized phylum capable of exerting anti-oxidant properties (Dholakiya et al., 2017). It is reported that Actinobacteria increases the production of lactate from carbohydrate fermentation and this lactate can be converted into butyrate by other colonic bacteria

(Rivière et al., 2016). In support of our findings, Actinobacteria was also reported to increase after consumption of 8 oz. TC juice daily for 5 days by healthy adults (Mayta-Apazaet al., 2018). One component of TC that may be responsible for this increase in Actinobacteria is its fiber content such as FOS. Mao and colleagues (Mao et al., 2018) showed that mice fed diets with FOS had increased Actinobacteria. The FOS content of TC has been reported to be approximately 0.32 g per 100g of edible portion (Jovanovic-Malinovska et al., 2014) and the total fiber content of TC is approximately 1.1 g fiber per 100g of edible portion (USDA, 2014). The concentration of FOS in our 10% TC might be sufficient to induced the increase in Actinobactera that we observed in our study.

In addition to Actinobacteria, we also observed an 8-fold decrease in the pro-inflammatory phylum, Deferribacteres with 10% TC supplementation. Deferribacteres is considered pro-inflammatory because it is positively correlated with pro-inflammatory cytokines production such as IL-6 and TNF-α (Li et al., 2019). Li and colleagues (2019) demonstrated that the prebiotic inulin treated diabetic mice significantly reduced Deferribacteres as compared to untreated diabetic mice. The decreased abundance in Deferribacteres we have observed might also be partly attributed to the fiber content of TC. In addition to the production of pro-inflammatory cytokines, one genus that belonged to the Deferribacteres phylum is Mucispirillum that can inhabit the mucus layer of the colon and the capacity to degrade mucus layer (Berry et al., 2012). Thus, the genus Mucispirillum could potentially lead to a significant alteration in the intestinal permeability and can cause gut inflammation (Berry et al., 2012). In this study, the relative abundance of Mucispirillum was significantly reduced in WD+10% TC group as compared to WD, which might indicate gut-protective capacity of TC.

Another bacterial genus that was modulated by the 10% dose of TC is Ruminococcaceae UCG-014. The genus Ruminococcaceae UCG-014, which is related to the family Ruminococcaceae, has been reported to increase the production of acetate with colonic fermentation of resistant starch (Xie et al., 2018) and enhanced proportion of Ruminococcaceae species has been reported in FOS treated C57BL/6J mice after 6 weeks of intervention (Zhu et al., 2017). Thus, the increase in abundance of Ruminococcaceae UCG-014 with the addition of 10% TC to the WD, might be due to the FOS content of TC. Despite the positive changes in certain bacterial phyla and genus with TC supplementation, we observed that TC further increased the ratio of Firmicutes to Bacteroidetes due to WD. Our findings on the increased ratio of Firmicutes and Bacteriodetes due to consumption of WD were in agreement with previous studies (Turnbaugh et al., 2008) and this was reported to be due to the overgrowth of Firmicutes (Marcobal et al., 2006). Increased ratio of Firmicutes and Bacteriodetes is associated with intestinal and systematic inflammation. Firmicutes are reported to extract more calories from the diet leading to obesity (Turnbaugh et al., 2008). At this time, we could not offer any explanation on why TC would further increase the ratio of Firmicutes to Bacteroidetes.

Because changes in gut microbiota results in alteration in SCFAs production, we assessed the concentrations of cecal and fecal SCFAs. We found that TC supplementation enhanced the production of SCFAs in both cecal and fecal samples. The SCFAs, propionic, i-butyric, i-valeric and n-valeric acids were increased with TC supplementation in both the control and WD groups. Increased cecal SCFAs was also reported with 10% TC supplemented control diet as compared to unsupplemented,

control diet-fed obese diabetic (db/db) mice (Garcia-Mazcorro et al., 2018). These SCFAs are reported to have many physiological roles and considered important for gastrointestinal health due to their preferential use by intestinal epithelial cells as energy source, act as signaling molecules and for their anti-inflammatory properties (Rivera-Piza and Lee, 2020). The production of SCFAs is reported to be suppressed by WD (Brinkworth et al., 2009), while prebiotics prevented changes in the gut microbiome and promote the production of SCFAs. In this study, we speculate that the fiber in TC acts as prebiotics, which increased colonic fermentation and hence increased SCFAs production. Rivera-Piza and Lee (2020) reported that propionate activates the G protein-coupled receptors, GPR43 and GPR41. Activation of GPR43 which is found in the ileum and colon, improves glucose tolerance by promoting the secretion of incretins such as glucagon-like peptide-1 (GLP-1) from L-cells and gastric inhibitory peptide (GIP) from K cells (Kimura et al., 2013). However, in our study, ileal and colonic expression of GPR43 and GIP as well as glucose tolerance which was assess by glucose tolerance test was not affected by TC. Therefore, TC may improve glucose homeostasis not by affecting the gut through stimulation of incretin but may be by other mechanisms and should be investigated in future studies.

In addition to the fiber content, TC is also known to be rich in phenolic compounds (Mayta-Apaza et al., 2018). The phenolic compounds present in TC include flavonols, anthocyanins and phenolic acids (Mayta-Apaza et al., 2018). Anthocyanins are one of the major classes of polyphenols present in TC and it has been reported that 100 g of fresh TC contain 12.5-25.0 mg of anthocyanins (Wang et al., 1999). These anthocyanins are reported to improve glucose homeostasis by increased insulin secretion

from β-cell of pancreas (Belwal et al., 2017). After WD consumption, increased plasma free fatty acids (FFA) result in lipotoxicity and increased oxidative stress, which can contribute to β-cell dysfunction and decrease the production of insulin (Oh et al., 2018). Bolleddula and colleagues reported that addition of anthocyanins extracted from TC with high fat diet (1g/kg of high fat diet) prevented the loss of β-cell architecture and increased insulin secretion. The anthocyanins isolated from TC also exhibited *in vitro* antioxidant and anti-inflammatory activities (Wang et al., 1999). Anthocyanins were also reported to improve carbohydrate metabolism by upregulating the translocation of insulin-regulated glucose transporters and increasing the activation of peroxisome proliferator activated receptor-γ (PPARγ) (Różańska et al., 2018). In addition to upregulation of glucose transporters and β-glucokinase, activation of PPARγ plays a vital role in regulating the expression of mitochondrial antioxidants including superoxide dismutase 2 (Sod2) in β-cells and restoring the function of β-cells (Grieco et al., 2019). Thus, activation of PPARγ positively correlates with increased insulin sensitivity. A previous study conducted by Seymour et al (2009) reported that 1% TC supplementation with high fat diet increased PPARγ expression and this could potentially help to decrease insulin resistance. These protective effects of TC were attributed to its anthocyanin content. Although, we did not measure the expression of PPARγ, we assessed insulin resistance by using HOMA-IR, which is a commonly used model for assessment of insulin resistance in laboratory animals (Tomie-Furuya et al., 2005). We observed an improvement in HOMA-IR by TC particularly with 5% dose. Tsuda et al., (2003) reported that TC anthocyanin extract supplementation with high fat diet reduced hyperglycemia and hyperinsulinemia, which are the major characteristics of insulin resistance. Perez and colleagues (2007) also

reported that *Aloe vera* gel, as a good source of polyphenols, improves insulin resistance. The decreased insulin resistance observed in our study might be partly due to the anthocyanins content of TC. However, other components of TC might act synergistically with anthocyanins to improve insulin resistance and this needs to be explored in future studies.

We also investigated the effects of TC on maintaining gut barrier integrity. Intestinal epithelia functions as a biological barrier and maintain homeostasis. Impaired functioning of the gut barrier has been implicated in a variety of disease states including insulin resistance (Bischoff et al., 2014). Tight junction proteins and mucus layer are both important in maintaining gut barrier integrity (Bischoff et al., 2014). Tight junction associated proteins such as ZO1, occludin and claudin acts as gut epithelial paracellular barrier, which prevents the entry of pathogenic bacteria into mucosa and stimulation of inflammatory response (Feng et al., 2018). SCFAs have been reported to protect intestinal barrier functions (Cishing et al., 2015). SCFAs especially butyrate and acetate, are important for regulation of mucus layer by upregulating the transcription of various MUCN genes (Hedemann et al., 2009). Even though, we observed an increase in SCFAs with TC supplementation, TC has no effects on gene expression of tight junction proteins, mucus production as well as structural parameters of the ileum and colon.

Structural changes of the ileum and colon were also investigated to determine the effects of dietary treatments. Villi contain cells specialized for nutrient absorption and crypts contain stem cells such as Paneth cells. These Paneth cells support continual regeneration of epithelium and secretion of antimicrobial peptides (Bevins and Salzman, 2011). Systemic and local inflammation can influence the gut absorptive area, epithelial

cell lining and barrier function by increased production of pro-inflammatory cytokines. With respect to WD consumption, gut structure such as villi height, width and area was negatively altered in ileum as compared to control diet. This is similar to previous study where consumption of 40% fat diet based on saturated fat for 8 weeks significantly reduced villi height in both ileum and jejunum (Goda and Takase, 1994). The evidence provided by a recent study (Yang et al., 2019) also support these results as high fat diet fed mice had reduced villus height in ileum as compared to mice fed with control diet. In our study, TC was unable to modify these effects of WD.

Although TC supplementation restored the loss of beneficial gut bacteria such as Actinobacteria due to WD similar to what has been reported on prebiotics (Everard et al., 2014), TC was not able to prevent weight gain. The highest body weight was recorded in 10% TC supplemented WD group. In support of our findings, a previous study conducted by Chrisfield (2017) showed that addition of 1% TC powder to high fat diet was unable to modify weight gain and increased adiposity in mice fed a high fat diet for 18 weeks. Jayarathne and his colleagues (2018) also reported that 4% TC powder supplemented diet had no effect on body weight of Zucker fatty rats after 8 weeks of dietary treatment. In our study, the increase in body weight of the WD+10% TC group may have been due to some genera from the family Ruminococcaceae, which have been reported to possess strong energy-harvesting capabilities from starch in the colon (Li et al., 2017).

This study also investigated the effects of TC supplementation on serum lipids. Serum NEFA was significantly increased with TC supplementation in both control and WD group. This effect of TC on NEFA might be partly attributed to the increase in SCFAs. A previous study on 3T3-L1 adipocytes reported that propionate and butyrate

increased the rate of lipolysis approximately 2-3 fold (Rumberger et al., 2014). In support of this concept, consumption of propionate-containing bread also increased adipose tissue lipolysis (Triosh et al., 2019). These studies support our finding of increased serum NEFA with TC and that the increased SCFAs particularly propionate may partly be responsible for this effect.

Unlike our findings with NEFA, TC supplementation did not have any significant effect on serum cholesterol. In support of this finding, Martin et al., (2010) reported that daily consumption of TC juice did not have any significant effect on total cholesterol in obese adults as compared to placebo. This observation might be partly explained with changes in SCFAs, particularly acetate and propionate. Gut microbial-produced propionate and acetate are quickly absorbed and metabolized in our body (Louis et al., 2014). Wolever et al (1991) provided rectal infusion of acetate to healthy subjects, and increased levels of acetate and cholesterol were observed. This study supports the evidence that increased acetate metabolism in the liver may increase serum cholesterol as acetate is a precursor of cholesterol synthesis (Bloch, 1965). On the other hand, propionate is considered an inhibitor of incorporation of acetate into cholesterol in liver (Demigné et al., 1995). Our observation that TC did not affect serum cholesterol may be a result of TC's ability to produce sufficient propionate that is capable of decreasing acetate incorporation into cholesterol in the liver.

In our study, serum triglyceride tended to increase with TC supplementation. This again, may partly be attributed to TC's ability to increase SCFAs production and further, these SCFAs might increase the expression of fatty acid synthase. Fatty acid synthase plays an important role in *de novo* lipogenesis and thus increase accumulation of

triglycerides (Yurina et al., 2016). Yu et al (2008) evaluated the effects of the SCFAs acetic, propionic and butyric acids on 3T3-L1 cells and their finding showed that SCFAs promote lipid accumulation by modulating the expression of enzymes related to lipogenesis such as fatty acid synthase. Propionic acid and butyric acid treated cells significantly increased the triglyceride content as compared to control group (Yu et al., 2008). Future studies should measure the effects of TC on enzymes related to lipogenesis such as fatty acid synthase

The current study provides evidence that TC supplementation altered microbial population and increased production of cecal and fecal SCFAs. However, despite these changes within the gut, TC has no effects on markers of gut health and has modest effects on markers of glucose homeostasis. Other physiological effects of TC supplementation such as its effects on serum concentrations of SCFAs, pancreatic beta cells functions as well tissues important in glucose homeostasis including the adipose, liver, and skeletal muscle needs to be explored in future studies. Furthermore, the implication of increased propionate production due to TC supplementation may be further investigated in a model of liver cancer, since previous mice study (Bindels et al., 2012) has shown that propionate counteract malignant cell proliferation in the liver tissue. To our knowledge, this is the first study to show the ability of TC (at 10 % wt/wt dose) to increase the abundance of beneficial gut bacteria due to WD feeding. Leaning on the knowledge that TC is a potential source of various polyphenols (Wang et al., 1999, Mayta-Apaza et al., 2018), it will be interesting to study the possible impact of TC anthocyanins as antioxidant and potential physiological benefits that may be derived from it. Future studies in humans are also needed.

In conclusion, this study showed that TC supplementation modulated gut bacteria by increasing the beneficial bacterial phyla, Actinobacteria and Deferribacteres; b) enhanced SCFAs production; and c) improved the marker of insulin resistance, HOMA-IR particularly the 5% TC dose. However, TC supplementation was not able to modify the adverse effect of WD on gut structure parameters (i.e., villi height, width and area) and improve body weight gain, accumulation of fat, and increased fasting glucose level induced by WD. The increase in bacterial population and SCFA due to TC supplementation in WD may have other potential health benefits that needs to be investigated in future studies.

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