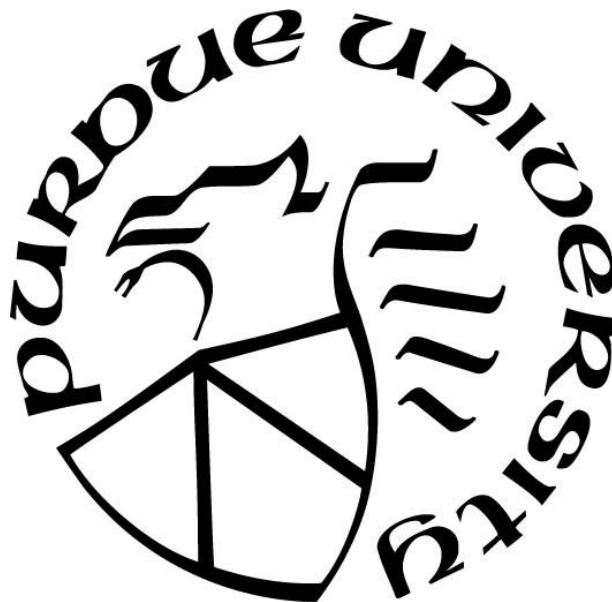# VITAMIN E DELTA-TOCOTRIENOL AND METABOLITE: MODULATION OF GUT MICROBIOTA AND CHEMOPREVENTION OF COLORECTAL CANCER

by

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To my beloved parents and Hung-Li Wang for their unconditional love and support.

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ABSTRACT

Colorectal cancer is one of the leading causes of cancer deaths in the United States and multiple modifiable factors contribute to colorectal carcinogenesis. Gut microbiota are believed to play key roles in colon cancer development. Dietary factors may modulate gut microbiota composition, which may potentially have impact on carcinogenesis. Thus, it is reasonable to develop dietary interventions to effectively prevent colorectal cancer development through alteration of gut microbiota. In this thesis, the first objective is to evaluate the effect of vitamin E forms and metabolites, i.e., δ-tocotrienol (δTE), γ-tocotrienol (γTE) and δTE-13'-COOH (δTE-13'), respectively, on gut microbiota in mice. Healthy male balb/c mice were supplemented with a δTE/γTE mixture or δTE-13' by gavage for two weeks, while control mice received soybean oil. We isolated DNAs from fecal samples and used 16S rRNA gene sequencing to evaluate the impact of these compounds on gut microbiota compositions. Further, we also examined the effect on short chain fatty acids (SCFAs). We observed that supplementation of δTE-13' increased microbial richness using the Faith index. On the other hand, supplementation did not separate the microbial communities from the control group. But, these compounds managed to alter the relative abundances of several taxa that might present chemopreventive activities against colon cancer. Specifically, *Desulfovibrio*, a sulfur-reducing bacterium, was decreased after δTE/γTE supplementation. *Eubacterium coprostanoligenes* group, a group of microbes that can reduce circulating cholesterol, was increased after δTE/γTE supplementation. In addition, several members from the *Lachnospiraceae* family were elevated under δTE/γTE and δTE-13' supplementation, and these microbes are known to produce SCFAs and maintain colonic health. However, the measurement of SCFAs showed that supplementation of δTE/γTE and δTE-13' did not change SCFAs compared with controls. In the second project, I investigated anti-proliferative effects of combining δTE or δTE-13' with sodium butyrate (NaBu) on human colorectal carcinoma HCT116 cells. Our data showed promising additive effects against cell growth. Collectively, these results indicate that δTE/γTE and δTE-13' can modulate gut microbiota under healthy conditions, which provides insights into potential chemopreventive activities of these vitamin E forms. Our cell-based studies also showed additive anticancer effects of combining δTE or δTE-13' with NaBu, which provides rationale to further develop combination of butyrate producers with vitamin E forms for cancer prevention.

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer deaths in the United States (Siegel et al., 2020). It is essentially considered to be a genetic disease that is caused by the accumulations of oncogenic mutations (Fearon, 2011). However, there are multiple intrinsic and extrinsic factors that are highly involved in the development of CRC. For example, lifestyle (e.g. diet choices, physical activities and smoking), environmental exposures (e.g. pathogens) and gut microbiome. In addition, lifestyle and environmental changes would all lead to potential gut microbiome modulation which presents either pathogenic to CRC development or beneficial to the chemoprevention of CRC (Saus et al., 2019). To be more specific, the metabolic interplay between the microbiota and the host metabolism is responsible of manipulating host's health status. For example, extreme environmental and dietary triggers might cause dysbiosis of the gut microbial composition and generate toxic metabolites which further induce intestinal inflammation and CRC development. On the other hand, there are beneficial host-microbe metabolic interactions which can be further applied as effective chemoprevention methods. For example, fermentation of dietary fiber in the gut results in producing various short-chain fatty acids (SCFAs). Among the predominant SCFAs that generated in colon, butyrate processes anti-cancer ability by regulating immune responses and downstream tumor-suppressor gene expressions. Therefore, various dietary interventions are performed to investigate their anti-cancer abilities while also interested in microbiota-related modulation.

The vitamin E family has eight naturally occurring fat-soluble antioxidants, i.e., αT, βT, γT, and δT and αTE, βTE, γTE, and δTE (Jiang, 2017). Among the different isoforms of vitamin E, αT is the predominant vitamin E in plasma and tissues. In contrast, other vitamin E forms are substantially metabolized in the liver through hydroxylation and oxidation to generate 13'-hydroxychromanol (13'-OH) and 13'-carboxychromanol (13'-COOH). αT showed disappointing results in large clinical trials while other vitamin E forms show superior biological functions in disease prevention. In addition, long-chain metabolites of vitamin E forms, 13'-COOHs, serve stronger inhibition effects which prevent colon cancer development and progression (Jiang et al., 2008). We previously observed that δTE and its metabolite δTE-13'-COOH supplementation

alleviated colon cancer burdens and also observed changes in microbiome composition *in vivo.* To be more specific, the microbial communities of δTE and δTE-13'-COOH supplementation groups were separated from the groups that received normal chow diet. In addition to the separation, the supplementation increased the relative abundance of several beneficial microbes. For example, *Roseburia,* which is often found decreased in inflammatory bowel disease patients (Machiels et al., 2014), was observed increased in the δTE-13'-COOH supplementation group. Next, *Eubacterium coprostanoligenes* group, which is a group of anaerobe that acts to reduce cholesterol to coprostanol (Ren et al., 1996), was found increased in δTE group. Last but not least, *Lactococcus,* a common probiotic species that produces lactic acid (Kimoto-Nira et al., 2007) was found interestingly increased in both δTE and δTE-13'-COOH supplementation groups. Collectively, we observed that the vitamin E supplementation alleviated CRC progression and also modulated gut microbiome profile.

However, previous observations were all under disease animal model (AOM/DSS). Thus, we cannot clearly understand whether vitamin E affect directly affect microbiome under healthy condition. Therefore, we investigated and compared the effect of δTE/γTE and δTE-13'-COOH supplementation on healthy Balb/c mice on microbiome diversities, taxa differences and metabolic SCFA productions to serve as future insights on CRC chemoprevention. To answer this question, three specific aims are followed. First, we performed the microbiome analyses and analyzed microbial diversities across treatment groups to examine whether the vitamin E supplementation under healthy condition would affect the microbial community profile. Second, we would like to pinpoint differential microbial taxa across treatment groups. Last, we would like to investigate whether there were differences of the microbial metabolic endpoints across treatments. Therefore, the concentrations of predominant SCFAs, including acetate, propionate and butyrate, were measured and compared.

CHAPTER 1.   LITERATURE REVIEW

1.1 Colorectal Cancer (CRC)

1.1.1 The Prevalence and Significance of Colorectal Cancer

Colorectal cancer (CRC) is one of the leading causes of cancer death in developed countries including the United States. According to the latest population-based statistical analysis of cancer occurrence from The American Cancer Society, the expected new cases of colorectal cancer to be diagnosed in the United States will be 52,340 and 52,270 in men and women respectively and the total estimated deaths are the third leading cause among cancers in both sex (Siegel et al., 2020). This phenomenon suggested that therapeutic methods have not reached to the point to effectively treat this disease. Therefore, more attention is required on the development of prevention and effective treatment on colorectal cancer.

CRC is essentially a genetic disease and accumulation of oncogenic gene mutations leads to autonomous colonic epithelial cells proliferation and further results in colon adenoma and further cancer development (Fearon, 2011). Among the colorectal cancer cases, only 20% have a familial basis, such as hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis (Rustgi, 2007). In most colorectal cancer cases, colorectal cancer development linked to environmental events rather than heritable genetic changes. Environmental factors that trigger tumorigenesis included mutagens, pathogens, and colonic inflammation (Terzić et al., 2010) may all in turn trigger the alteration of gut microbiome which lead to cancer development . In addition, according to the report from Siegel et al. which analyzed the incidence pattern of CRC in the United States from 1974 to 2013 (Siegel et al., 2017), the CRC risk of young adults around 30 years old has escalated back to the level of adults born in 1980 (Figure 1.1). To be more specific, the age-cohort CRC risk of adults that age 20 to 39 years old was increased by 1.0% to 2.4%. Collectively, this information strongly suggested other critical environmental factors that play an etiologic role in colon carcinogenesis.

1.1.2 Inflammation-associated Colon Cancer

The connection between inflammation and tumorigenesis is supported by various studies from genetic, pharmacological, and epidemiological data (Terzić et al., 2010). Colitis-associated cancer (CAC) is a subtype of CRC which is associated with inflammatory bowel disease (IBD), which is hard to treat and has a high mortality. More than 20% of IBD patients develop CAC within 30 years and 50% of these patients will die from CAC development. In addition, IBD patients those who had family history of CRC have 2-fold higher risk of developing CRC, suggesting overlapping mechanisms of CRC and CAC (Askling et al., 2001). Multiple studies showed that CRC tumors and also cancer cell lines show activation of transcription factors that are involved in multiple inflammatory pathways, for example nuclear factor-kB (NF-kB) and signal transducer and activator of transcription 3 (STAT3) (Sakamoto et al., 2009). One study found out that when inhibiting the activation of NF-κB, the level of several chemokines that is related to angiogenesis were decreased, which suggested that NF-κB activation is involved in angiogenesis of colon cancer and NF-κB inhibition may represent a potent treatment against colorectal cancer (Sakamoto et al., 2009).

Inflammatory responses and their products are known to involved in cancer development. For example, pro-inflammatory enzymes cyclooxygenases (COXs) and 5-lipoxygenase (5-LOX), as well as their products eicosanoids, are known to involved in colon tumorigenesis, angiogenesis, and even metastasis (Wang & DuBois, 2010). Study discovered that transition from adenoma to carcinoma requires increased expression of COX-2 (Koehne & Dubois, 2004). Elevated COX-2 expression was also detected in most colorectal cancer tissues and in experimental models of CAC (Sheehan et al., 1999). Pro-inflammatory pathways generate prostanoids, as indicated as PGE2, which has a predominant role in promoting tumor growth and is also found in many human malignancies including colorectal cancer (Rigas et al., 1993).

1.1.3 Gut Microbiota Involved in CRC Development

Various environmental factors including dietary choices and extrinsic exposures to potential antigens and mutagens were examined to alter the composition and functionality of gut microbiota and hence lead to either facilitating or preventing the development of CRC (Saus et al., 2019). To

begin with, the human intestinal tract consists both carcinomas and the microbiota under colorectal cancer condition, which highly suggested that these microbes are involved in carcinogenesis. One interesting study illustrated elevated colorectal cancer development in germ-free mice treated with fecal microbiota transplant from CRC patients under cancer induction compared with another group of germ-free mice inoculate with fecal samples from heathy individuals (Wong et al., 2017). While in another study, germ-free mice showed less carcinogenesis development compared with gene knockout-engineered murine model when triggered with inflammation (Sears & Garrett, 2014). Both evidences strongly suggested microbiota played a critical role in colonic carcinogenesis.

The interaction between the microbiome and the host metabolism plays a role in manipulating host's health status. In addition, numerous environmental factors would alter the microbiota composition and further affect host metabolism and disease development. In some extreme cases, the symbiotic relationship would be disturbed and further promote disease or even cancer development. Imbalance of the microbial composition, known as dysbiosis, is highly associated with the development of multiple diseases and even cancer. From a large population-based medical record database, microbial dysbiosis that was caused by repeated antibiotic medication can enhance the incidence of colorectal carcinomas development (Boursi et al., 2015). Dysbiosis of gut microbiota can also cause intestinal inflammation through activation of pattern recognition receptors or through pathways like endocytosis, adherence, and secretion of toxins or invasion to the gut environment (Sansonetti & Medzhitov, 2009). In addition, pathogenic and genotoxic bacteria may infiltrate inside host tissue under impaired barrier function scenario and further induce inflammation and result in colorectal carcinogenesis initiation (Zitvogel et al., 2016).

In addition to dysbiosis, differences in the microbial composition are also highly correlated with cancer development. Higher microbial richness is observed in the mucosal biopsy samples of adenoma cases comparing with non-adenoma controls (Dejea et al., 2013). Therefore, the changes might be caused by the elevation of cancer-related microbes and reduction of health beneficial ones. Various studies had aligned potential cancer-associated microbes, namely *Streptococcus gallolyticus, Enterococcus faecalis, Enterotoxigenic Bacteroides fragilis, Escherichia coli*, and the under-representation of beneficial bacteria, such as *Faecalibacterium, Parabacteroides,*

Akkermansia, Alistipes, and *Lachnospiraceae* (Sears & Garrett, 2014). In addition, studies have identified enrichment of *Fusobacterium spp.* in CRC mucosa from normal colon tissue (Castellarin et al., 2012), which is consistent with another study that also found increased *Fusobacterium spp.* in fecal samples from CRC patients compared with healthy individuals (Ahn et al., 2013).

How do these microbes play in cancer development? Various biological activities of the intestinal microbiota are proposed to be involved in CRC carcinogenesis (Sears & Garrett, 2014). For example, some bacteria would generate metabolites like secondary bile salt or various kinds of reactive oxygen species and further cause negative impacts on host health (Hill, 1975). In addition, Rubinstein et al. demonstrated *Fusobacterium nucleatum* promotes oncogenic and inflammatory responses in cancer cells and tissues by invasion and adhesion via activation of the FadA adhesin/E-Cadherin/β-catenin signaling cascade (Kostic et al., 2013). Another evidence also showed the relationship between microbes, immune responses and cancer development. One study has found *F. nucleatum* was negatively associated with the level of CD3+ T cells in colorectal carcinoma tissue in CRC patients, which promote colonic neoplasia (Mima et al., 2015; Zitvogel et al., 2016). In addition, virulence factors derived from *F. nucleatum* are known to inhibit T-cell activity and block their cytotoxic activity on tumor cells (Gur et al., 2015).

1.1.4 The Chemoprevention of CRC

As mentioned earlier, the development of effective prevention and treatment on colorectal cancer is needed. Chemoprevention, the usage of specific compounds to prevent, inhibit, or reverse carcinogenesis (Chauhan, 2002). Drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), are available for the treatment of colorectal cancer development. Celecoxib, a potential chemopreventive agent that inhibits COX-2 activity, showed promising effect against colon carcinogenesis in preclinical and clinical trials, which significantly reduced the number of colorectal polyps in human subjects (Sporn & Suh, 2002). Besides targeting at pro-inflammatory enzymes, other agents would inhibit proinflammatory pathways, as indicated NF-κB, which regulates downstream gene expressions that involved in inflammation. For instance, curcumin (diferuloylmethane), which is a yellow substance that extracted from the root of the plant *Curcuma longa* Linn. It has been examined to inhibit the carcinogenesis of murine skin, stomach, intestine and liver. However, most of the drugs have inconsistent outcomes on patients and even combine

with unwanted side effects (Dubois et al., 2004). Another well-known NSAID, aspirin, possesses as a chemopreventive agent and shows a modest protective effect in some clinical trials for colorectal cancer prevention in long-time usage. However, a consistent association was only seen with higher usage of 300 mg or more of aspirin a day, lower doses require further investigation to examine its effect (Flossmann & Rothwell, 2007). On the other hand, studies have shown regular aspirin usage is associated with unfavorable side-effects, such as gastrointestinal bleeding, and risk appears to be more strongly related to higher-dose usage (Huang et al., 2011). Thus, the optimal dosage for cancer prevention and the precise mechanisms underlying aspirin's anticancer effects require deeper investigations. In conclusion, the discovery and deeper exploration of other compounds for chemoprevention are needed.

In addition to inhibit inflammation as a CRC prevention method, modulating gut microbiome has also being highly investigated aspect when dealing with CRC (Saus et al., 2019). To begin with, some commensal and symbiotic microbes present tumor-suppressive activities in ways far beyond than inhibiting pathogens (Nicholson et al., 2012). To be more specific, these microbes present their ability to metabolize dietary components that escaped from host's digestion and generate various bioactive metabolites which further participate in CRC prevention (Tremaroli & Backhed, 2012).

1.2 Vitamin E

1.2.1 Vitamin E Forms and Food Sources

Vitamin E family comprises of eight structurally related lipophilic antioxidants, including α-, β-, γ-, δ-tocopherols (αT, βT, γT and δT) with saturated 16-carbon phytyl-like side chain and corresponding α-, β-, γ-, δ-tocotrienols (αTE, βTE, γTE and δTE) with three double bonds in the side chain (Jiang, 2014). The four different forms of tocopherols and tocotrienols (α, β, γ and δ) differ by the degree of methylation on the 5- or 7-position of the chromanol ring (Figure 1.1, Jiang, 2014). Vitamin E was first discovered by Evans and Bishop in 1922 as an essential nutrient for reproductive function in rats (Evans & Bishop, 1922).

Natural forms of vitamin E are synthesized in photosynthetic organisms. For example, tocopherols have been detected in various plant parts although the content and composition are highly heterogenous. Most vitamin E forms are commonly found in plant seeds (Sundl et al., 2007), almonds, hazelnuts, peanuts, pine nuts and sunflower seeds are good sources of αT. On the other hand, γT is rich in flaxseeds, pecans, pistachios, pumpkin seeds, walnuts and sesame seeds (Thomas & Gebhardt, 2006), whereas barley, palm oils, and oats are excellent sources of tocotrienols especially γTE (Irías-Mata et al., 2017; Walde et al., 2014) and δTE can be extracted from annatto seeds (Zabot et al., 2018). The majority of vitamin E consumed in the U.S diet comes from corn and soybean oil (Jiang et al., 2001), and the predominant form of vitamin E consumed in a typical U.S. diet is γT, which accounts for ~60-70% (Jiang, 2014; Sears & Boiteau, 1989). Although the level of γT is relatively high in the U.S. diet, αT is the predominant form of vitamin E that retains in the body, the differences in tissue retention between αT and other vitamin E forms are likely due to the different binding affinity of liver proteins for αT, which plays an important role in vitamin E transportation and metabolism.
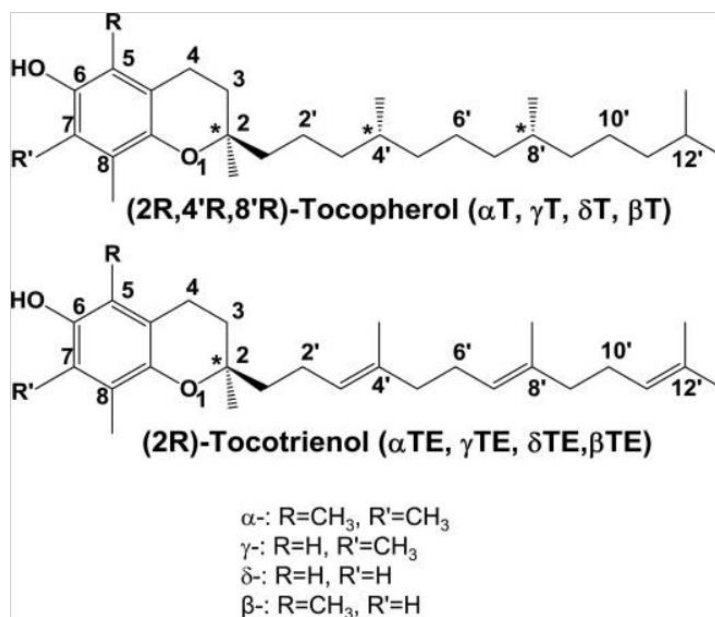
Figure 1.1 Structures of Natural Vitamin E Forms (Jiang, 2014)

1.2.2 Absorption, Metabolism and Excretion of Vitamin E

Dietary vitamin E forms are absorbed along with dietary fat and being packaged in chylomicron particles in the intestine cells and further transported with other lipid molecules to the parenchymal cells of liver via the lymphatic system (Drevon, 1991). Although specific receptors for the uptake

of vitamin E has not been identified, all lipoprotein receptors should have the ability to pick up vitamin E. It is known that the absorption of vitamin E is mediated by cholesterol membrane transporters including the scavenger receptor class B type 1 (SR-BI) (Reboul et al., 2006), ATP-binding cassettes A1 and G1 (ABCA1 and ABCG1) (Reboul et al., 2009), Niemann-Pick C1-like transporter 1 (NPC1L1) (Reboul et al., 2012) and CD36 molecules (Goncalves et al., 2014).

Among the different vitamin E forms, αT is the predominant form found in plasma and tissues due to its high binding affinity to the hepatic α-tocopherol transfer protein (αTTP) that transfers αT outside the liver and prevents it from being metabolized in the liver (Jiang, 2014). On the other hand, other vitamin E forms has relatively lower binding affinity to αTTP comparing with αT, and thus they are being extensively metabolized in the liver by an enzyme cytochrome P450 (CYP450) via α-hydroxylation and oxidation to generate 13'-hydroxychromanol (13'-OH) and 13'-carboxychromanol (13'-COOH), which can be further metabolized via β-oxidation to other shorter-chain carboxychromanols in other subcellular compartments of hepatocytes (Figure 1.2, Jiang, 2014) (Sontag & Parker, 2002).

Fecal excretion is proposed the major way of vitamin E excretion since a large amount of tocopherols, tocotrienols, and their metabolites were found in feces of rats supplemented with γT, δT, γTE, and δTE (Jiang et al., 2015; Zhao et al., 2010), whereas short-chain metabolies, for example CEHCs and conjugated CEHCs, are mainly excreted in the urine (Chiku et al., 1984; Lodge et al., 2001; Swanson et al., 1999). In addition, unmetabolized vitamin E forms were found be eliminated in bile (Yamashita et al., 2000).
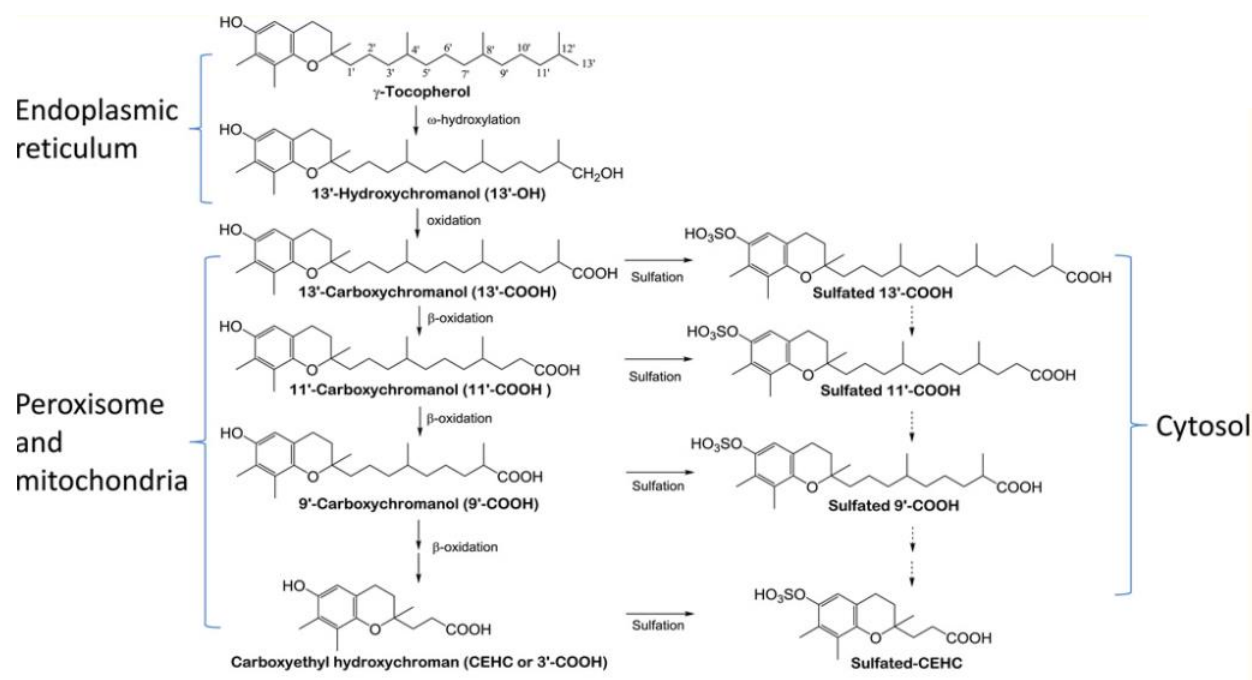
Figure 1.2 Molecular Mechanism of Vitamin E Metabolism and the Formation of Metabolites (Jiang, 2014)

1.2.3 Biological Functions of Vitamin E Forms

αT showed disappointing results in large clinical trials(Jiang et al., 2008). Although other vitamin E forms has lower bioavailability comparing with αT as previously described, these vitamers show superior biological functions in disease prevention. In addition, long-chain metabolites of vitamin E forms, 13'-COOHs, serves stronger inhibition effects on pro-inflammatory enzymes, cyclooxygenases (Jiang et al., 2008; Wallert et al., 2015) and 5-lipoxygenase (Jiang et al., 2011), which prevent the cancer development and progression. Moreover, recent study shows that 13'-COOHs could induce apoptosis and autophagy in human colon cancer cells through the modulation of sphingolipids, which leads to the suppression of colon tumor development in mice (Jang et al., 2016).

1.2.3.1 Antioxidant Activities of Vitamin E Forms and Carboxychromanols

The vitamin E family is a group of vitamers that possess antioxidant potentials which can scavenge lipid radicals by donating hydrogen atom from the phenolic group on the chromanol ring of the vitamer structures (Jiang, 2014). Among the vitamin E forms, for example, γT, that possesses an

unsubstituted 5-position are suggested to trap reactive oxygen species superiorly than αT. For example, γT is more superior in detoxifying NO₂ by forming 5-nitro-γT (Christen et al., 1997). Tocotrienols are suggested to have superior antioxidant activities comparing with tocopherols (i.e. αT) since they are more evenly distributed in the phospholipid bilayer, which is speculated to have a better interaction with free radicals in the cellular environment (Packer et al., 2001; Wong & Radhakrishnan, 2012). In addition to tocopherols and tocotrienols, long-chain metabolites, 13'-OH and 13'-COOH, which are from δT or δTE have also been reported to have promising antioxidant properties against lipid peroxidation in vitro (Terashima et al., 2002). To sum up, various vitamin E forms including their long-chain metabolites have promising antioxidant activities.

1.2.3.2 Anti-inflammatory Activities of Vitamin E Forms and Carboxychromanols

Inflammation, which is a series of processes that involved the activation, recruitment of immune cells and further the actions of innate and adaptive immunity, is important for tissue repair, regeneration, and remodeling for the regulation of tissue homeostases (Greten & Grivennikov, 2019). On the other hand, inflammation can also lead to the development of various disease including asthma, inflammatory bowel disease, rheumatoid arthritis and cancer (Grivennikov et al., 2010; Murdoch & Lloyd, 2010; Szkaradkiewicz et al., 2009).

During inflammation, over-production of reactive oxygen/nitrogen species and inflammatory mediators like cytokines, prostaglandins, and leukotriences would exacerbate inflammation and provide the microenvironment for disease development and further causing damage to host tissues (Belardelli, 1995; Williams & Shacter, 1997; Yokomizo et al., 2001). Promisingly, accumulative studies showed vitamin E forms and their long-chain metabolites not only possess antioxidant properties but can also inhibit the formation of inflammation mediators which prevent further disease development (Figure 1.3, Jiang et al., 2011) (Jiang et al., 2008; Jiang et al., 2011). For example, prostaglandin E₂ (PGE2), which is one of the most abundant prostaglandins that produced in the body through arachidonic acid oxidization and plays a role in inducing disease progressions. The oxidization process that generate PGE2 is catalyzed by cyclooxygenase-1/2 (COX-1/2), which exhibits multiple biological functions (Ricciotti & FitzGerald, 2011). As mentioned earlier, excess formation of PGE2 would worsen inflammation (Funk, 2001). Vitamin

E forms show differential properties in PGE2 inhibition. Jiang et al. were the first to identify that γT potently reduce PGE2 formation in LPS-stimulated RAW264.7 murine macrophages and IL1β-treated A549 human lung epithelial cells (Jiang et al., 2000). Later, they discovered γTE and δT showed superior inhibition activity of PGE2 production than γT in IL-1β-induced A549 cells without affecting COX-2 expression. None of these vitamin E forms showed to inhibit the activity of purified COX-2, suggesting that although these vitamin E forms can inhibit PGE2 formation but are weak COX-2 enzyme inhibitors. On the other hand, long-chain metabolized vitamin E form 13'-COOHs are examined to both inhibit PGE2 formation and COX activities (Jiang et al., 2008). From enzyme kinetic data reveal that 13'-COOH compete with arachidonic acid at the substrate-binding site and plays as competitive inhibitor of both COX-1 and COX-2 (Jiang et al., 2008). These data consistent with computer simulation, which support that 13'-COOH binds to the substrate binding site of COX enzymes by forming hydrogen-bonds with Tyr355 and Arg120 with the carboxyl group of 13'-COOH. Furthermore, the chromanol ring of 13'-COOH appears to provide further interaction with Phe209, Phe381, and His226 via hydrophobic interaction and hydrogen bond formation (Jiang et al., 2008). Another example, interleukin-6 (IL-6), which is secreted from stimulated macrophages, is a pro-inflammatory cytokine and contributes to various disease development. Vitamin E forms possess ability to suppress CCAAT-enhancer binding protein β (C/EBPβ), nuclear factor (NF)- κB and JAK-STAT6/3 (signaling signal transducer and activator of transcription) pathways (Ahn et al., 2007; Wang & Jiang, 2013; Wang et al., 2012; Yang & Jiang, 2019). To be more specific, mechanistic studies illustrate γTE inhibited IL-6 by suppressing LPS-induced activation of NF-κB and up-regulation of C/EBPβ and C/EBPδ (Wang & Jiang, 2013). Moreover, γTE inhibits TNFα-triggered activation of NF-κB by up-regulation of an anti-inflammatory molecule, A20, which induce cellular stress and modulation of sphingolipid metabolism (Wang et al., 2015).
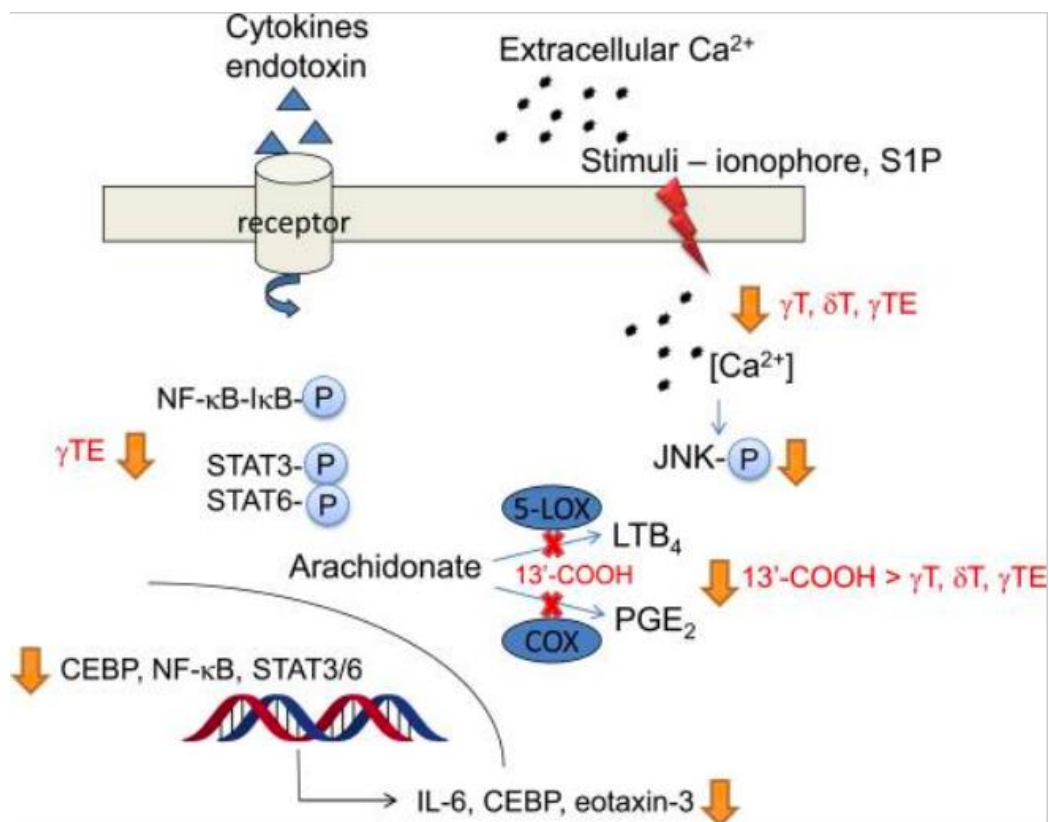
Figure 1.3 Mechanisms of the Anti-inflammatory Properties of vitamin E Forms and Long-chain Carboxychromanols Metabolites (Jiang et al., 2011)

1.2.3.3 Gut Microbial Communities Influenced by Vitamin E

One study illustrated that low supplementation of αT (0.06 mg/20 g of bw per day, total 34 days) had different ratio of *Firmicutes* to *Bacteroidetes* compared to the control group and the high amount of αT group 0.18 mg/20 g of bw per day. However, no further microbial diversity related analyses and taxonomic differences were examined. Therefore, further investigations between Vitamin E family and gut microbial profile are needed (Choi et al., 2019)

1.2.4 Vitamin E Serves as a Chemopreventive Reagent for CRC

Family of different vitamin E forms and their corresponding metabolites can inhibit the formation of eicosanoids like prostaglandins and leukotriences, which are known to exacerbate inflammation and provide the microenvironment for disease development. Accumulative studies indicated non-αT forms of vitamin E and their long-chain metabolites are potent chemopreventive agents against CRC. In one study, 0.3% γT-rich mixed tocopherol (γTmT) enriched diet effectively inhibited

colon carcinogenesis in azoxymethane (AOM)/ dextran sulfate sodium (DSS) treated mice, including lowering the level of PGE2, LTB4, and the formation of both adenocarcinomas and adenomas in the colon tissue (Ju et al., 2009). In a similar research, 0.1% supplementation of γT in diet suppressed colon tumorigenesis promoted by moderate colitis, which was induced by one cycle of DSS. On the other hand, this treatment was not effective against severe colitis, which was promoted with three DSS cycles (Jiang et al., 2013).

Various vitamin E forms are examined to reduce aberrant crypt foci (ACF) formation, two studies reported that 0.2% δT, γT and γTmT diet significantly reduced formation (Guan et al., 2012; Newmark et al., 2006). Among the treatment, δT shows the most significant anticancer activity followed by γT and γTmT in this AOM-induced rat model, while αT is not effective. Also, in another study that applied different colon cancer model which induced carcinogenesis with 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), supplementation of 0.2% δT and γT but also not αT, showed the ability to reduce colon tumor formation and suppressed the activation of NF-κB and STAT3 signaling pathways in the animal colon tumors and adjacent tissues (Chen et al., 2017)

1.3 Butyrate

1.3.1 Butyrate Synthesis and Physiological Functions

According to global epidemiological and scientific studies, data suggested that fiber-rich diet is associated with low risk of developing colon cancer. Dietary variation would further modulate the microbial composition and affect the downstream microbial activities which play important role in the colon and body metabolic needs of host. Fermentation of dietary fiber in the gut results in producing various short-chain fatty acids (SCFAs), predominately acetate (two-carbon), propionate (three-carbon), and butyrate (four-carbon). Among these SCFAs, butyrate, not only has remarkable colonic health-promoting properties but also processes the ability to suppresses inflammation and carcinogenesis through affecting immune responses and downstream gene expressions (O'keefe, 2016).

1.3.1.1 Butyrate Synthesis, Absorption and Transport

Butyrate is formed by bacterial fermentation in the human lumen. In healthy man, the SCFAs production is mainly contribute to energy needs, plays a key role in gut homeostasis maintenance. Indigestible carbohydrates that escaped from human digestion are further being broken down by hydrolytic and non-hydrolytic bacteria which can generate enzymes and convert carbohydrate monomers into a variety of intermediates as butyrate, acetate and propionate. These microbes gain energy by phosphorylation during oxidative substrate breakdown of the carbohydrates (Encarnação et al., 2015; Louis & Flint, 2009).

Butyrate can be synthesized through acetyl-CoA, glutarate, lysine and succinate (Figure 1.6) (Encarnação et al., 2015). Among these pathways, the acetyl-CoA pathway is the major one. The usage of glutarate and lysine, shows that protein can also have an important role in synthesizing butyrate (Vital et al., 2014), and demonstrate that the microbiota can adjust to different nutrition source environment. There are disagreements regarding the transport and absorption of butyrate. Paracellular transport is facilitate by the pH in the colon, it falls in the range that the SCFAs will be dissociated, which supported its transportation through the cells. In addition, the gradient difference between the lumen and blood, which both electrical and chemical features favor absorption. In the other hand, the transcellular transport can be supported by the increased sodium absorption (Daly et al., 2005; Mortensen & Clausen, 1996)

1.3.2 Butyrate Biological Properties and Further Anticancer Effects

Butyrate and other SCFAs can be oxidized by colonic epithelial cells for energy usage. Although colon cells can also utilize glucose and other compounds, approximately 60-70% of the energy requirement comes from these bacterial fermentation products through oxidation followed by the tricarboxylic acid (TCA) cycle (Roediger, 1982). Butyrate also plays a role in regulating cell growth and differentiation. It arrests cell growth in the G1 phase of the cell cycle, together with the induction of cellular differentiation, and modulation of gene expression (Macfarlane & Macfarlane, 2012).

Butyrate provides multiple beneficial properties as to prevent tumorigenesis on top of providing energy and homeostasis of colonocytes. To begin with, butyrate plays an important role in maintaining colonic mucosal health (Roediger, 1982). For example, it reinforces the mucosal barrier by increasing the expression of mucin-encoding genes and the induction of antimicrobial peptides (Schauber et al., 2003; Willemsen et al., 2003). Also, it mediates immune cell responses as indicated Treg cell activation of FOXP3 and IL-10 cytokine expression (Smith et al., 2013). Treg activation also helps host against colitis development, which is proposed via the interaction with free fatty acid receptor 2 (also known as GPR43) and further direct inhibition of histone deacetylase (Smith et al., 2013).

In addition, butyrate plays a paradoxical role besides support mucosal proliferation under certain condition. To be more specific, butyrate can also act as an antineoplastic agent, which potentially suppresses cancer cell growth. Studies have shed light on the mechanisms behind the paradox, further illustrated that the butyrate amount is the key of this phenomenon. Low colonic concentration of butyrate showed no histone deacetylase inhibitory effect. However, excess amount of butyrate would not being properly metabolized and accumulate in cells due to the Warburg effect (Vander Heiden et al., 2009), cancerous cells shift metabolism from oxidative metabolism to glycolysis formation with lactic acid formation Butyrate further acts as a histone deacetylase inhibitor (HDACi), which further increase gene expression related to facilitates histone acetylation and increases cell differentiation and apoptosis while along with other beneficial biological activities and also suppresses the proliferation of cancerous colonocytes (Donohoe et al., 2012; Sengupta et al., 2006).

1.3.2.1 Butyrate Serves as a Chemopreventive Reagent for CRC

From a metagenomic analysis of both CRC patients and healthy individuals, a high fiber diet has been shown to be correlated with elevated levels of short chain fatty acid (SCFAs) and reduced overall risk of CRC development (Yu et al., 2017). Butyrate, which is a microbial fermentative SCFA, which provides multiple beneficial properties as to prevent tumorigenesis and further carcinogenesis (Roediger, 1982).

SCFAs are known to reduce epithelial inflammation and trigger cancer cell apoptosis via p21 activity, providing an important defensive capacity against colorectal carcinogenesis. Among the SCFAs, butyrate is known to inhibit histone deacetylase, which leads to increased histone acetylation and further regulates the transcriptional activity of various tumor suppressors, thereby reducing inflammation and CRC risks (Wang et al., 2019). In addition to inhibit histone deacetylase, there are multiple signaling pathways in CRC cells are investigated to be mediated by butyrate, which also play important roles in CRC prevention (Figure 1.7) (Wang et al., 2019). To begin with, butyrate reduces the expression of neuropilin and growth factor that are involved in angiogenesis. One study showed that butyrate inhibited the transactivation of Sp1, which reduced the mRNA and protein level of the neuropilin-1 (NRP-1) and vascular endothelial growth factor (VEGF) in CRC cell lines as indicated as HCT116, HT-29, and Caco-2 cells (Danny et al., 2010). In addition, butyrate is examined to induce tumor-suppressing gene expressions and further inhibited tumors growth. A study has shown that the mRNA and protein expression levels of p21, waf-1 and bax in HT-29 cells was influenced by butyrate (Liu et al., 1995). Furthermore, butyrate possesses the ability to modulate microRNA (miRNA) expressions, which also play important roles in the progression and metastasis stages of cancer development. For example, butyrate decrease the level of miR-203, which is the tumor suppressor miRNA, and inhibit the development of tumors (Tiwari & Gupta, 2014). miR-92a, which shown to over-express in CRC patients and promote tumor metastasis, has significantly decreased its expression level in human CRC cells as indicated as HCT116 and HT-29 after butyrate treatment (He et al., 2005). One interesting study did a series of gene expression microarray analyses with colonic epithelial cells under butyrate treatment while comparing with other NSAIDs. Butyrate was most similar to curcumin treatment on Caco-2 cell line with G0-G1 arrest and least similar to the G2-M arrest. While in another comparison that was made between butyrate and sulindac, although identified gene clusters showed similar responses, as indicated as G0-G1 arrest, elevation of b-catenin-Tcf signaling, and apoptotic cascade expression, there were extensive differences in the functional effects of the two agents. Two above-mentioned comparisons strongly suggested butyrate presented the similar or different ability to fight against CRC (Mariadason et al., 2000).

CHAPTER 2. MATERIALS AND METHODS

2.1 Preparation of Vitamin E Forms

For cell study, δ-tocotrienol (δTE) was a gift from BASF (Florhan Park, NJ) while δTE-13'-COOH (δTE-13'), which is a long-chain metabolites of δTE, was synthesized according to a published method (Maloney & Hecht, 2005). Dimethyl sulfoxide (DMSO) and [3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Sodium butyrate (NaBu) was also purchased from Sigma and was dissolved in phosphate-buffered saline (PBS) buffer before usage.

As for animal study, the δTE/γTE (8:1) mixture was gifted from American River Nutrition (Hadley, MA), and contains total tocotrienols at 70%. δTE-13', was synthesized according to a published method (Maloney & Hecht, 2005). Both δTE/γTE (8:1) mixture and the δTE-13'-COOH compound were dissolved in soybean oil before gavaging.

2.2 Cell Cultures and Treatment

HCT-116 human colorectal carcinoma cells were obtained from American Type Culture Collection (Manassas, VA) and routinely cultured in McCoy's 5A modified medium containing 10% fetal bovine serum (FBS) in $75cm_2$ culture flask in 37_oC, 95% air and 5% CO_2 humidified incubator. Cells were plated in McCoy's 5A modified medium containing 10% FBS in 24-well plates with the density of 4 x 10_4 cells per well. Cells were attached after overnight incubation before treatment. Next, plated cells were treated with different concentrations of δTE, δTE-13' and NaBu and their combination in fresh, and relative cell viabilities were measured for three consecutive post-treatment time points as indicated as 24hr, 48hr and 72hr.

2.3 MTT Assay for Cell Viability Evaluation

Relative cell viabilities under different treatments after 24, 48 and 72hr were examined by MTT assay. In living cells, the mitochondrial dehydrogenase reduces yellow MTT into purple insoluble formazan, which can be further dissolved in DMSO and then measured at a wavelength of 570nm by using a microplate reader (Spectra MAX 190, Molecular Devices, Sunnyvale, CA)

2.4 Animal Study

This animal study was approved by Purdue Animal Care and Use Committee (PACUC). Male BALB/c mice were purchased from Envigo (San Diego, CA), and the mice were housed in Purdue Life Science Animal Facility for a week for adaptation before daily gavaging and then randomly grouped by body weight to meet even distribution. Mice received Teklad rodent diet (8604) from Envigo (San Diego, CA) throughout the entire study, there were 24.3% crude protein, 4.7% fat and 4.0% crude fiber in the rodent diet. Diet and water was fed *ad libitum*. In addition, mice were given δTE/γTE (8:1) mixture or δTE-13' by gavaging using soybean oil (0.2 mL) as the vehicle control (n = 8 in each group). Fecal samples were collected at three time points. The first collection was done before the first day gavaging, the second one was done after one-week's gavaging, and the last one was done after two-week's gavaging. The mice were euthanized after two-week gavaging, and plasma, liver, spleen, kidney, feces and cecum content were collected. All samples collected were frozen at -80$_o$C until use.

2.5 Microbiome Analyses

2.5.1 Microbial DNA Extraction, Amplification and Sequencing

Fecal microbial DNA was extracted using Fast DNA Soil Spin kit (MPbio, Irvine, CA), and approximately 40mg of fecal samples from each animal were used. After the genomic DNA was extracted, the quality was checked by running agarose gels and used Nanodrop One while the quantity of the extracted DNA were measured using NanoDrop 3300 fluorospectrometer (Thermo Scientific, Wilmington, DE) with the usage of Hoechst dye, which further assist in aiming 10ng of DNA per sample for PCR amplification before performing sequencing. Usage of specific primers to target and amplify V3-V4 region of 16S rRNA (IL-V3-343F: 5'TACGGRAGGCAGCAG3', IL-V4-803R: 5'CTACCRGGGTATCTAATCC 3').

In addition to amplifying a specific 16S rRNA gene region, Illumina adaptor and dual-index barcodes were applied to allow samples to be pooled together for sequencing. QuantiFluor® dsDNA System (Promega, Madison, WI) was used for amplicon quantification. Last, based on the quantification results of the tagged amplicons, the actual input DNA amount of each sample was 28.2 ng. Since the final quantified concentrations of each sample were different, the input volumes

of samples were calculated. Each sample were pooled together and sent to the Purdue Genomics Core Facility (West Lafayette, IN) for sequencing using a MiSeq instrument, and 2x250 paired-end sequencing was performed.

2.5.2 Microbial DNA Mock Standard

Since one of the major challenges that would occur during the microbiome analyses is the bias and errors introduced in the complex workflows, as to assure the analyses pipeline was appropriate, ZymoBIOMICS Microbial Community DNA Standard #D6305 (Irvine, CA), which contains known microbes (eight bacteria species and two fungi species) (Table 2.1) with precise composition was used to serve as a positive control of PCR and sequencing processes.

Table 2.1 The Microbial Composition of ZymoBIOMICS Microbial Community DNA Standard #D6305

Defined Microbial Community (species)
Bacillus subtilis
Listeria monocytogenes
Staphylococcus aureus
Enterococcus faecalis
Lactobacillus fermentum
Salmonella enterica
Escherichia coli
Pseudomonas aeruginosa
Saccharomyces cerevisiae
Cryptococcus neoformans

2.5.3 QIIME 2 Microbiome Analyses Pipeline

The primer tags, low-quality sequence reads (quality scores that were below 30), were first trimmed from the raw sequences during DADA2 denoise step (Callahan et al., 2016). After importing raw sequences to QIIME 2 pipeline (Jovel et al., 2016). Diversity analyses were available through the q2-diversity plugin, which performed alpha diversity (Pielou's evenness and richness of observed OTUs) and beta diversity metrics (Bray Curtis and Jaccard). Phylogenetic diversity analyses were also made for Faith's Phylogenetic Diversity (alpha diversity- richness) and weighted and unweighted UniFrac (beta diversity) (Hamady et al., 2010; Jovel et al., 2016; Lozupone et al., 2011; Lozupone et al., 2007). Furthermore, SILVA_132 marker gene reference

database and Naive Bayes classifier were used to perform taxonomic analyses (Quast et al., 2012). To be more specific, SILVA_132 99% and 97% reference databases and different parameter settings of Naive Bayes classifier were conducted to optimize the taxonomic analyses of the mock microbial standard. The default setting of the k-mer, which is the computation approach of taxonomic classification, was 7-mer. Besides this default setting, the 11-mer was also applied as to increase the recall of classification, which measures the completeness, or sensitivity of the classifier (Bokulich et al., 2018).

2.6 Analysis of Short Chain Fatty Acids (SCFAs) by GC-FID

Three short-chain fatty acids, acetate (catalog number: A38S), propionate (catalog number: A258) and butyrate (AC108111000) were purchased from Fisher Scientific (Rockingham County, NH) and served as external controls. In addition, 4-methylvaleric acid was used as internal control which was added in samples and external controls. The fecal and cecum feces samples were diluted in deionized water and centrifuged at 13,000 rpm for 10 min, two extractions were done for each sample. Supernatants were collected and injected into a gas chromatograph with a fused silica capillary column (NukonTM, Supelco No: 40369-03A, Bellefonte, PA) and a flameionization detector (GC-FID 7890A, Agilent Technologies, Inc., Santa Clara, CA). A SCFA standard mix, which contained known concentration of acetate, propionate and butyrate, was used as the external standards.

2.7 Statistical Analyses

For cell studies, one-way ANOVA was first performed to examine whether the distribution across groups are equal or not. Student t-test was used to examine whether the combination effect is significant different comparing with the individual ones. In addition, student t-test was also performed to examine whether the combination effects were additive or synergistic. To be specific, the cell viability differences between the vehicle-treated cells and the combination-treated one were calculated, which is the actual combination effect (abbreviate as actual effect in further paragraphs). On the other hand, the difference between the vehicle-treated cells with either δTE/δTE-13' or NaBu were summed up to make the theoretical combination effect (abbreviate as theoretical effect in further paragraphs). Comparing the actual and the theoretical effect, it would

be synergistic effect if the actual effect is significantly higher than the theoretical effect. In addition, if the extent is not high enough to be significant, it can just be concluded as additive effect. All data in graphs and tables are expressed as mean ± SEM. $P < 0.05$ was considered significant.

For animal and further microbiome analyses, Kruskal-Wallis tests of the alpha diversity, including the Faith (Faith, 2002), observed OTUs (Edgar, 2017), Pielou's evenness (Pielou, 1966) and Shannon (Shannon & Weaver, 1949) were done among different groups and consequent pairwise of tests was also conducted. On the other hand, beta diversity was determined using perMANOVA (Anderson, 2005), which is a non-parametric multivariate statistic, and PERMDISP (Anderson, 2006) computes variances based on two types of tests, using either centroids or spatial medians of multivariate dispersions, which was used to ensure significant differences were not due to differences in dispersion.

As to identify features that are differentially abundant across samples, linear discriminant analysis (LDA) effect size (LEfSe) was performed to identify microbial taxa that differentiated between two or more treatment groups. First, it performed non-parametric Kruskal Wallis ranking test (Kruskal & Wallis, 1952) in different treatment groups (classes) to examine all features, testing whether their relative abundances were differentially distributed. Second, features that were differentiated between groups were further analyzed with pairwise Wilcoxon test (Wilcoxon, 1992) between subclasses of different classes. Since my samples did not have different subclasses under treatment classes. Thus, the Wilcoxon test step of LEfSe analysis was not performed. Last, LEfSe applied LDA (Fisher, 1936) to calculate the effect size of each differentially abundant feature (Segata et al., 2011). Since we were more interested in the differences between treatment groups (δTE/γTE and δTE-13') and the control group, after LEfSe picked the differential taxa, Mann-Whitney test (McKnight & Najab, 2010) was further performed between two treatment groups.

CHAPTER 3.   RESULTS

3.1 Illumina 16S rRNA Sequencing Results

The paired-end sequencing was performed on Illumina MiSeq (2x250), the total counts from all samples were 19,128,219. The minimum counts from samples were 32,412 while the maximum counts were 177,578. Next, the minimum unique reads (i.e. features) in each sample were 16,570 while the maximum features were 104,987. The reads of each sample were present in table 3.1. In addition, figure 3.1 illustrated the distribution of the sequence reads and the features from all samples. The sequence depth for further sequencing analyses was based on the minimum features per sample which was 16,570 and its Good's coverage was assured by rarefaction curve (Figure 3.2).

Table 3.1 Sequence Reads and Feature Numbers from Samples

The raw sequence reads and feature counts for each sample after Illumina 16S sequencing, from GMM1-1 to GMM1-24 and also the mock sample.

Sample name	Sequence reads	Features counts
GMM1-2	177578	104987
GMM1-22	133322	70718
GMM1-15	113774	57946
GMM1-11	94826	51223
Mock	92662	47486
GMM1-16	88369	49414
GMM1-13	88233	51048
GMM1-14	87892	50967
GMM1-23	86721	48094
GMM1-18	84414	48800
GMM1-5	84375	39694
GMM1-7	84163	47060
GMM1-20	80259	44397
GMM1-8	71494	38750
GMM1-21	70009	40195
GMM1-9	60750	35143
GMM1-24	60730	34491
GMM1-17	60077	37353
GMM1-10	54342	29439
GMM1-19	50773	31001

GMM1-1	44477	28071
GMM1-3	39135	23422
GMM1-12	38516	21943
GMM1-6	33526	19641
GMM1-4	32412	16580
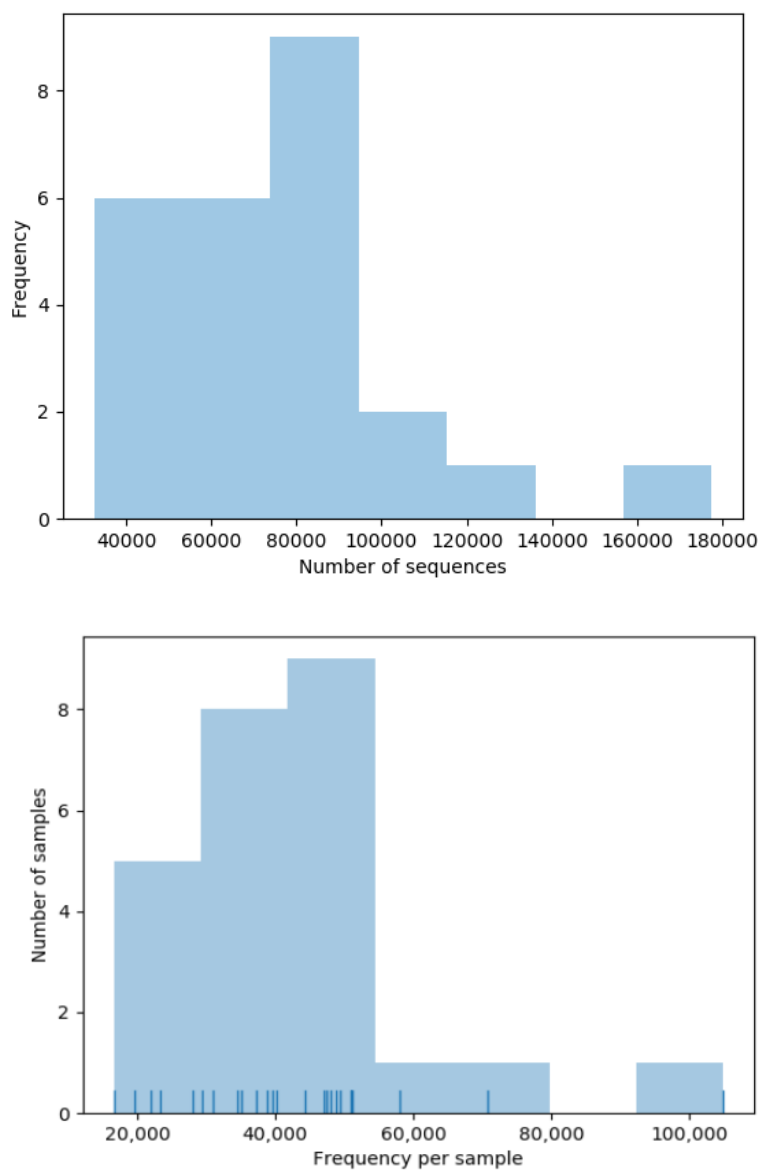
Figure 3.1 Distributions of Sequence Counts, Features from Samples after Sequencing
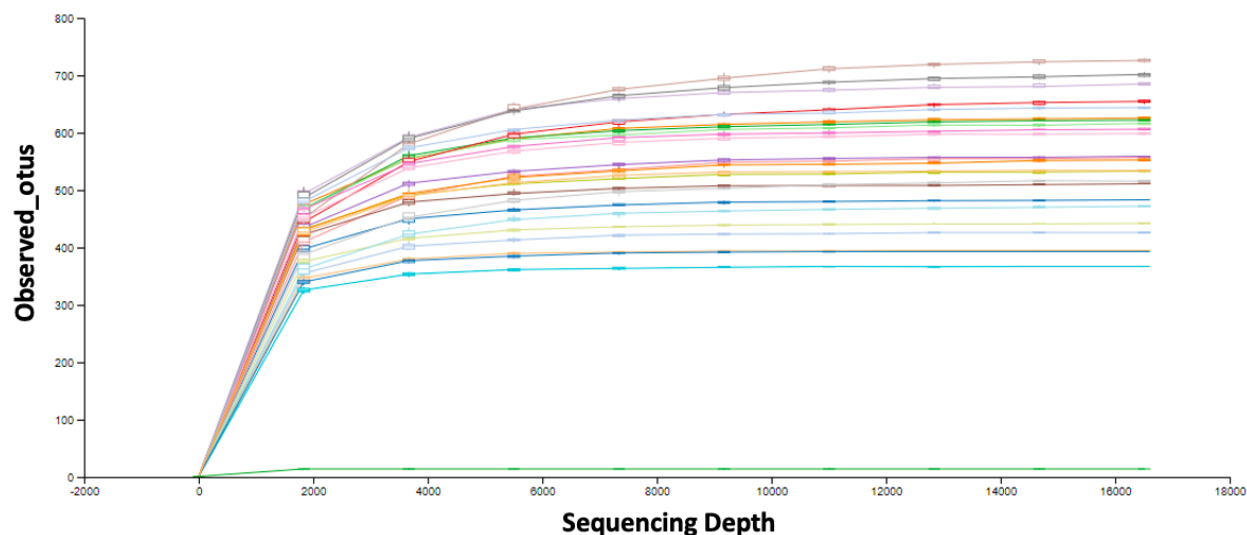
Figure 3.2 The Rarefaction Curve on Sequence Depth 16,570

16,570 sequences were randomly picked from each Sample and the corresponding observed OTUs under specific sequencing depth were depicted.

3.2 Optimizing Taxonomic Classification of Microbial DNA Mock Standards

Since the sequences were paired-sequenced, forward and reverse reads need to be merged into one sequence for further diversity and taxonomic analyses. Before merging, the sequences were trimmed based on their quality scores. Initially, no additional bases were trimmed besides the four degenerated bases in both forward and reverse reads. When the sequences were classified through Naive Bayes classifier under SILVA_132 99% reference database with default 7-mer settings. The taxonomic classification failed to identify *Salmonella enterica* and *Lactobacillus fermentum*, both had zero relative abundance. In addition, other species had different relative abundance values compared with the theoretical composition values that were provided by ZymoBIOMICS (Table 3.2). As to optimize the taxonomic classification pipeline, more trimming on low-quality bases were conducted. Interestingly, one-base trimming in the reverse reads successfully identify *Lactobacillus fermentum* (relative abundance 6.1%) but still failed to classify *Salmonella enterica*. Furthermore, when two bases were trimmed, similar output with one-base trimming with no *Salmonella* classification was generated. On the other hand, the SILVA_132 97% reference database was used besides 99% one. Surprisingly, this setting successfully classified *Salmonella* under two-bases trimming.

Besides the default 7-mer parameter setting, which first extract the sequences into various 7 bp features. Next, the substrings of the genomic datasets were computed with the assigned taxa and generate the k-mer probability table (Han & Cho, 2019). In other words, the table presents the probability of the extracted k-mer sequences of taxon. Finally, the sequence would be assigned to the taxa with the highest accumulative probability value. From the above mentioning, the longer the k-mers are, the more likely they can be specifically classified to certain taxa (Bolyen et al., 2018). In other words, longer k-mer increases the recall of the classification Thus, the setting of 11-mer was also applied as to increase the recall of the classification (Bokulich et al., 2018). When classifying the sequences with the combination of SILVA_132 99% reference database and 11-mer parameter settings, it also successfully identified *Salmonella*. Furthermore*,* the relative abundances of the defined microbes are identical with the classification results with SILVA_132 97% reference database and 7-mer setting (Table 3.2).

Table 3.2 Relative Abundance of the Standard Microbial Species under Different Taxonomic Analytical Setting

Defined microbial species	Theoretical relative abundance (%)	No additional trimming (F/R) with 99% SILVA reference	1 base trimming (F/R) with 99% SILVA reference	2 base trimming (R) + 1 base trimming (F) with 99% SILVA reference	2 base trimming (R) + 1 base trimming (F) with 97% SILVA reference	2 base trimming (R) + 1 base trimming (F) with 99% SILVA reference(11mer)
Bacillus subtilis	17.4	32.5	30.6	30.1	30.1	30.1
Listeria monocytogenes	14.1	10.7	10.0	9.8	9.8	9.8
Staphylococcus aureus	15.5	11.4	10.8	10.6	10.6	10.6
Enterococcus faecalis	9.9	7.0	6.6	6.5	6.5	6.5
Lactobacillus fermentum	18.4	0.0	6.1	6.0	6.0	6.0
Salmonella enterica	10.4	0.0	0.0	0.0	14.0	14.0
Escherichia coli	10.1	33.6	31.5	32.6	18.5	18.5
Pseudomonas aeruginosa	4.2	4.8	4.5	4.5	4.5	4.5
Total (%)	100	100	100	100	100	100

3.2 Vitamin E Supplementation Modulate the Relative Abundance of Several Microbial Taxa

Alpha diversity measures richness and evenness within one sample. To be more specific, richness illustrates the number of different biological groups, while evenness focuses on how evenly the microbial taxa are distributed within one sample. From the results of the Faith's phylogenetic diversity, which illustrated richness and also considered the phylogentic relationship of the microbial taxa, showed that δTE-13' supplementation group increased the richness of the taxa in mice comparing with the control group (p=0.05) (Figure 3.3A). However, in another richness index, observed operational taxonomic units (OTUs), did not show differences across supplementation groups (Figure 3.3B). In Pielou's Evenness index and Shannon diversity, which combines the concept of evenness and richness, also did not show differences among different groups (Figure 3.3C-D).

As for beta diversity, it applies various distance matrixes to calculate the distance differences between samples and further examine the differences among samples (Tuomisto, 2010). In addition, Principal coordinate analysis (PCoA) was used to visualize the distance between samples, which presented whether the microbial compositions were separated from the other groups. In PCoA plots, the percentage value on each axis represents the data variation that could be explained by that axis. From the PCoA plots of Bray Curtis, Jaccard, unweighted Unifrac and weighted Unifrac metrics, which the last two considered the phylogenetic relationship of the microbial taxa, the differences from the four analyses were not statistically significant, which indicated there were no separation of the microbial composition across different groups (Figure 3.4 A-D).

Next, taxonomic analyses were performed under SILVA_132 marker gene reference database and Naive Bayes classifier. It's worth mentioning that the reference dataset and parameter settings for the taxonomic classification were decided after the optimization of microbial DNA standards. Thus, SILVA 99% reference database with 11-mer setting were performed during the taxonomic classification. To begin with, total 8 phyla, 12 classes, 17 orders, 35 families, 111 genera and 230 species were identified from total samples included the mock one. Firmicutes and Bacteroidetes were the two major phyla in the samples and the total identified phyla were illustrated in figure

3.5. Furthermore, LEfSe was used to determine taxa that were significantly different among treatment groups. LEfse presented total 17 taxa that were significantly elevated relative abundance from another treatment group, which LDA scores were above or equal 2. While six taxa were identified increased in the control group, seven taxa in the δTE/γTE group, and four taxa in the δTE-13' group (Figure 3.6, Table 3.3). Furthermore, Mann-Whitney tests were performed between pairwise comparisons to examine significances. Those microbes that were increased in the control group in other words meant they were significantly decreased in other supplementation groups. To begin with, *Muribaculaceae mouse gut metagenome* decreased in δTE/γTE supplementation group (P=0.038), and also *Lachnospiraceae NK4A136 group Clostridiales* bacterium *CIEAF020* (P=0.028) and had the trend to decrease under δTE-13' group (P=0.083), similar trend found in *Desulfovibrio* uncultured bacterium and *Desulfovibrio* (genus level). In addition, *RuminococcaceaeUCG-005* was also decreased in the δTE/γTE supplementation (P=0.05). δTE/γTE supplementation group also significantly increased the relative abundance of *LachnospiraceaeUCG-006* (genus level) (P=0.01), *LachnospiraceaeUCG-006* uncultured bacterium (P=0.015), *Eubacterium coprostanoligenes* group (P=0.007) compared with the control group. Relative abundance of *Lachnospiraceae* (family level) (P=0.001), Lachnospiraceae*UCG-006* (genus level) (P=0.038), *LachnospiraceaeUCG-006 Clostridium sp ASF502* (P=0.028) and *Ruminococcaceae UBA1819* (P=0.007) were increased compared with δTE-13' treatment group. While in δTE-13' treatment group, *Lachnospiraceae NK4A136* (genus level) (P=0.005) and *Eisenbergiella* (genus level) (P=0.028) significantly greater relative abundance compared with the control group. While LachnospiraceaeNK4A136group Lachnospiraceaebacterium10_1 (species level) (P=0.05), *Eisenbergiella* (genus level) (P=0.001) and *Enterorhabdus* Ambiguous taxa (P=0.01) were all increased compared with δTE/γTE supplementation group.
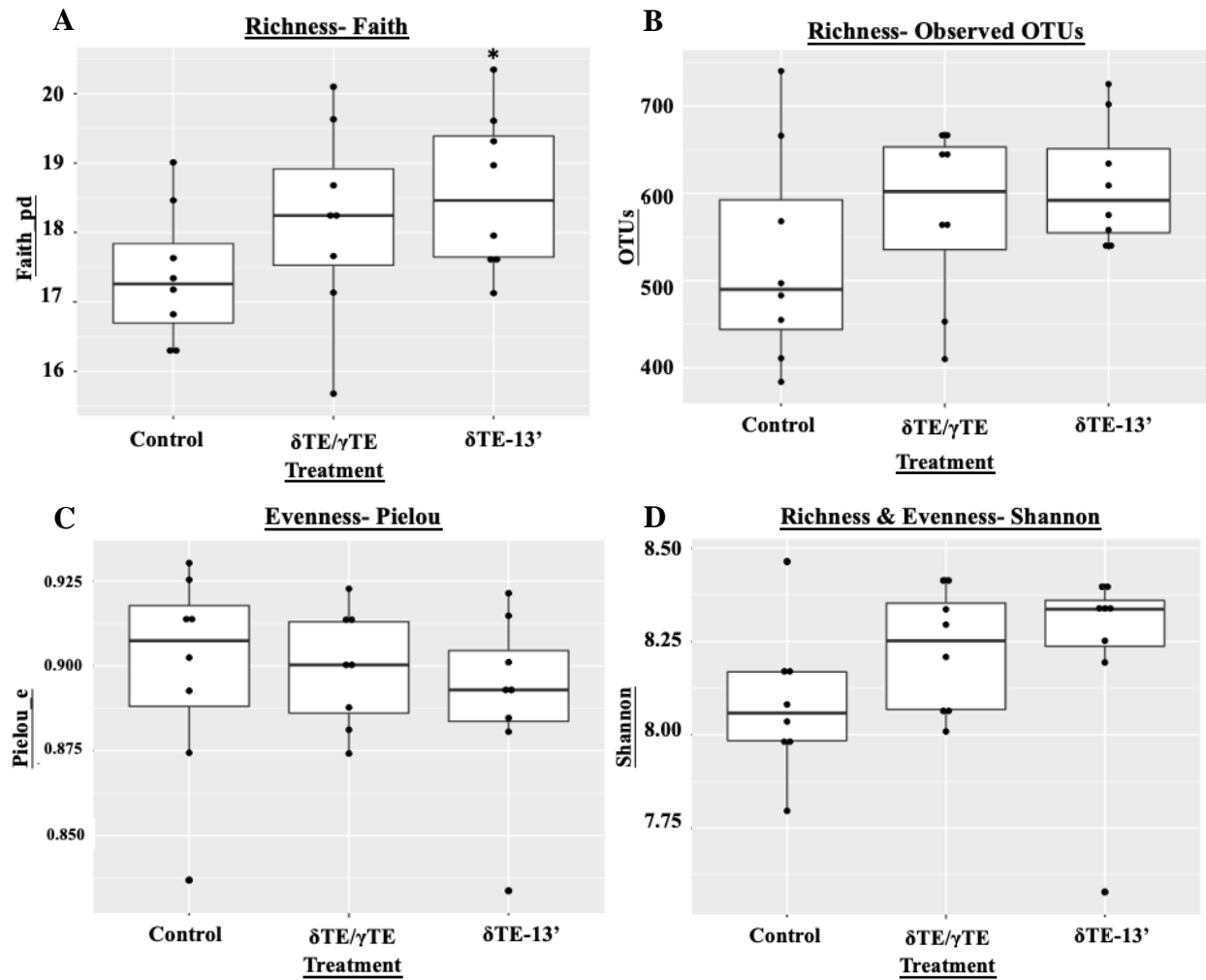
Figure 3.3 The Microbial Alpha Diversity Effects of δTE/γTE and δTE-13' after Two-week Supplementation in BALB/c Mice

A-B: Mice with δTE-13'supplementation significantly increased the Faith index (richness) than control (A) but did not change another richness index as indicated by Observed OTUs (B). Significant differences at P=0.05 were determined using Mann-Whitney Test pairwise comparison. C-D: Supplementation groups did not affect evenness (C) and Shannon index, which considers both richness and evenness (D).
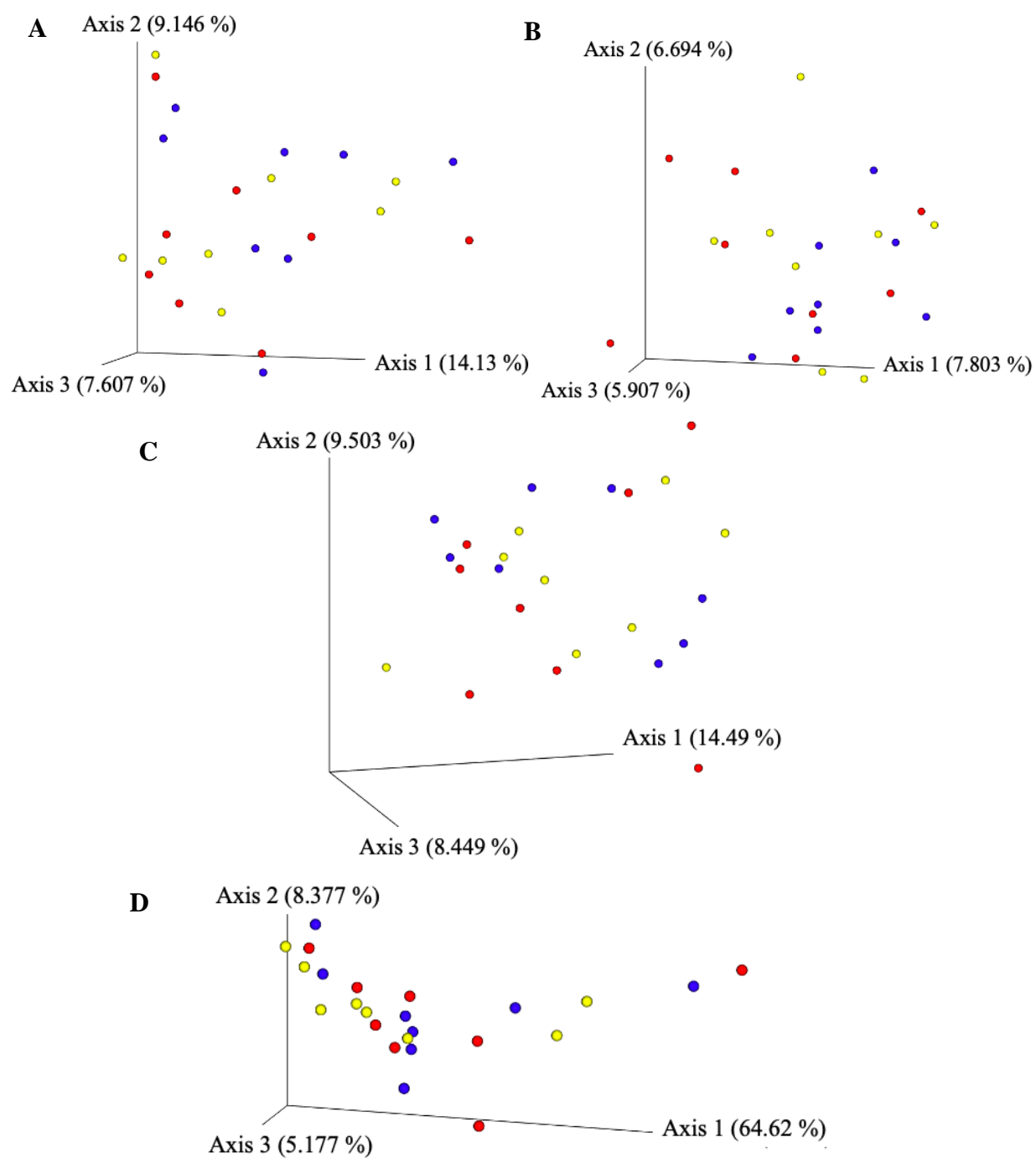
Figure 3.4 The Microbial Beta Diversity Effects of δTE/γTE and δTE-13' after Two-week Supplementation in BALB/c Mice

A-D: Supplementation groups (δTE/γTE and δTE-13') did not separate microbial communities presented in PCoA plots of Bray Curtis (A), Jaccard (B), Unweighted Unifrac (C) and Weighted

Unifrac (D). Each sample presents as a dot (Non-supplementation control group was in red, δTE/γTE group in yellow and δTE-13' group was showed in blue dots.
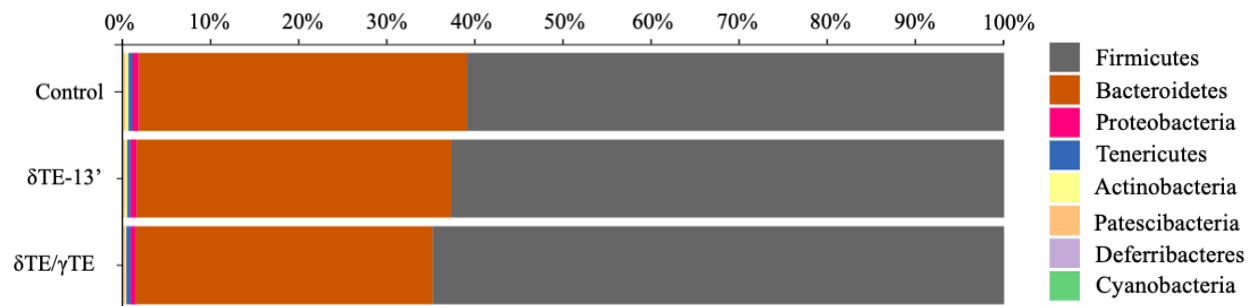
Figure 3.5 Phylum-level Taxonomic Bar Plot of Relative Abundance

Taxa plot based on the 16S amplicon sequencing, processed using QIIME2 with SILVA_132 99% reference database with 11-mer setting. Corresponding phylum of color was depicted in right.
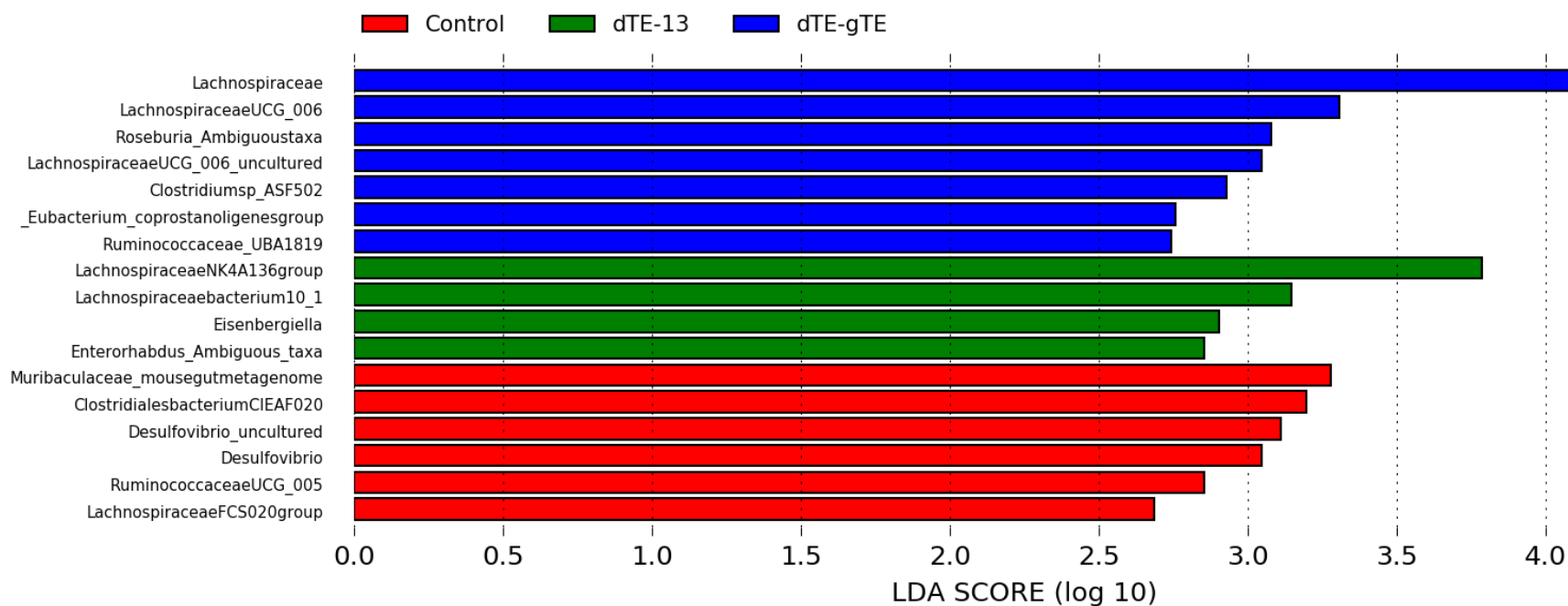
Figure 3.6 Linear Discriminant Analysis Effect Sizes (LEfSe) of the Taxa Differentiating across Groups after Two-week Supplementation.

Taxa with LDA scores >2 in groups after two-week supplementation were showed in bars. δTE/γTE (Blue), δTE-13' (Green) and soybean oil vehicle control (Red).

Table 3.3 Relative Abundance (%) of Bacterial Taxa that Significantly Differed between Treatments.

Differences among the treatment groups were tested using Kruskal-Wallis followed by another between pairwise comparisons. Uncultured taxa are those has yet to be cultured. * represents $P < 0.05$ using Mann-Whitney test by comparing the abundance with control group. # represents the comparison with the control has the trend to be significant.

Family	Genus	Species	Ctrl	δTE/γTE	δTE-13'
Muribaculaceae	mouse gut metagenome	mouse gut metagenome	0.44 ± 0.11	0.11 ± 0.02 *	0.36 ± 0.1
Lachnospiraceae	NK4A136 group	Clostridiales bacterium CIEAF 020	0.49 ± 0.21	0.16 ± 0.11 *	0.17 ± 0.1 #
Desulfovibrionaceae	Desulfovibrio	uncultured bacterium	0.52 ± 0.06	0.29 ± 0.03 *	0.37 ± 0.03 #
Desulfovibrionaceae	Desulfovibrio	N/A	0.52 ± 0.06	0.31 ± 0.03 *	0.37 ± 0.03 #
Ruminococcaceae	UCG-005	N/A	0.11 ± 0.02	0.07 ± 0.01 *	0.1 ± 0.01
Lachnospiraceae	FCS020 group	uncultured prokaryote	0.09 ± 0.03	0.08 ± 0.01	0.02 ± 0.01 #
Lachnospiraceae	N/A	N/A	6.83±1.1	8.25±0.7	5.4±0.47
Lachnospiraceae	UCG-006	N/A	0.45±0.05	0.83±0.11 *	0.43±0.07
Lachnospiraceae	UCG-006	uncultured bacterium	0.35±0.05	0.54±0.07 *	0.34±0.08
Lachnospiraceae	UCG-006	Clostridium sp. ASF502	0.1±0.02	0.17±0.04	0.05±0.02
Ruminococcaceae	Eubacterium	coprostanoligenes group	0.09±0.03	0.19±0.04 *	0.13±0.02
Ruminococcaceae	UBA1819	uncultured bacterium	0.08±0.03	0.12±0.02	0.05±0.01
Lachnospiraceae	NK4A136 group	N/A	1.27±0.25	1.79±0.32	2.5±0.3 *
Lachnospiraceae	NK4A136 group	Lachnospiraceae bacterium 10-1	0.01±0.01	0.0±0.0	0.21±0.11 #
Lachnospiraceae	Eisenbergiella	N/A	0.07±0.02	0.03±0.02	0.15±0.02 *
Eggerthellaceae	Enterorhabdus	Ambiguous taxa	0.06±0.02	0.04±0.02	0.12±0.02 *
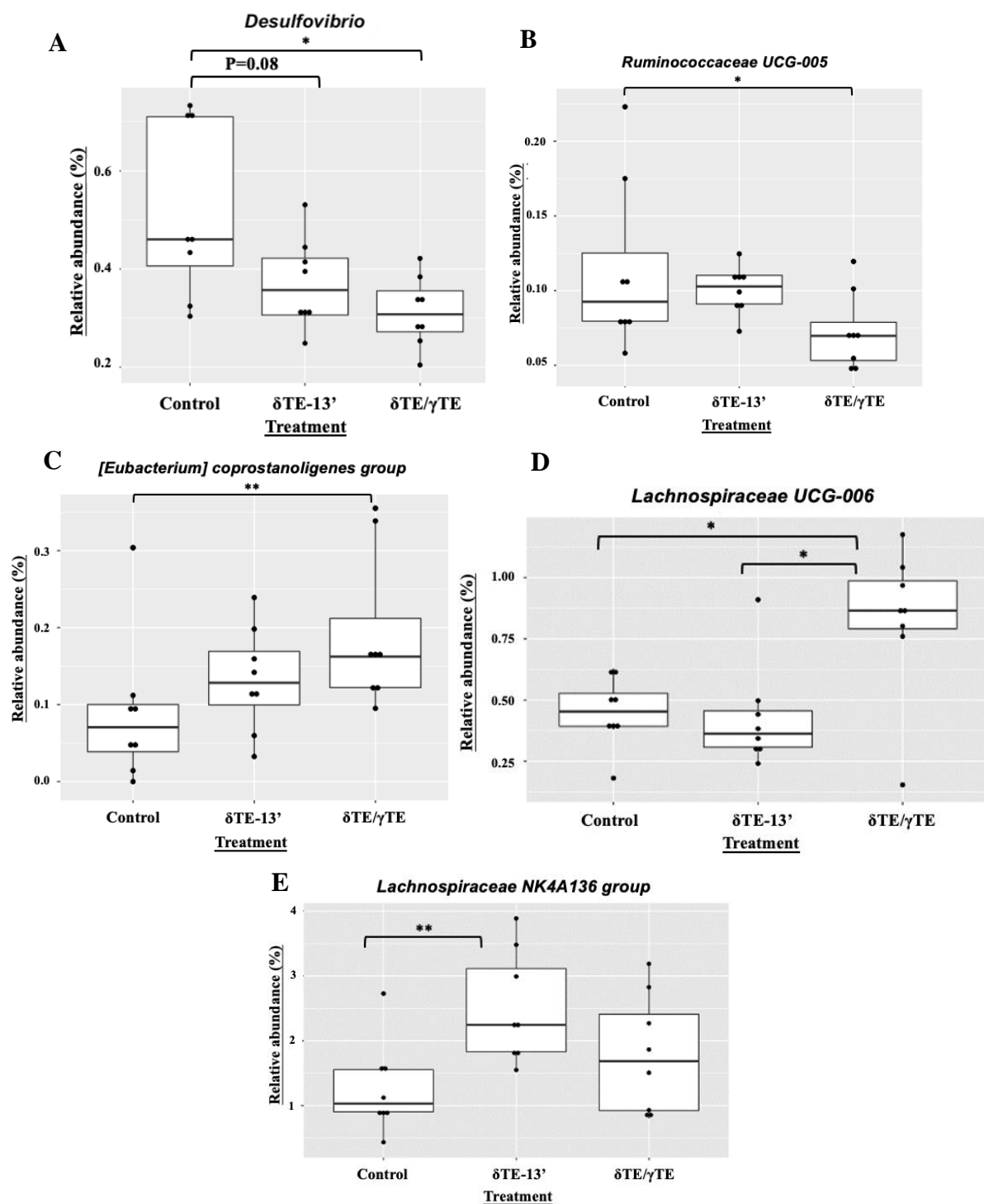
Figure 3.7 Relative abundance (%) of bacterial taxa that potentially involved in CRC development (A) *Desulfovibrio* (genus) (B) *Ruminococcaceae* UCG-005 (C) *Eubacterium coprostanoligenes* group (D) *Lachnospiraceae* UCG-006 (E) *Lachnospiraceae* NK4A136group
* represents $P < 0.05$, ** represents $P < 0.01$ using Mann-Whitney test by performing pairwise comparisons

3.3 Vitamin E Supplementation did not Affect SCFAs' Concentrations Across Groups

As to examine whether the alterations of abundance of several microbes would impact the metabolic activities of fecal and cecal microbiota, we further measured the production of the terminal SCFAs that are most abundantly found in fecal samples (acetate, propionate and butyrate) after the two-week supplementation. From table 3.4, we could tell that total fecal/cecal SCFAs and following acetate, propionate and butyrate concentrations did not show significant differences across treatment.

Table 3.4 SCFA Concentrations of Different Supplementation Group.

Concentration is presented in nmol/g. Data are expressed as mean ± SEM.

SCFAs (nmol/g)	Control	δTE/γTE	δTE-13'
Total fecal SCFA	58.07 ± 3.17	59.14 ± 2.69	53.56 ± 2.96
Fecal acetate	50.3 ± 2.57	51.22 ± 2.2	46.4 ± 2.63
Fecal propionate	3.7 ± 0.32	3.4 ± 0.17	3.19 ± 2.63
Fecal butyrate	4.08 ± 0.62	4.52 ± 0.86	3.97 ± 0.59
Total cecal SCFA	50.55 ± 3.45	46.78 ± 3.4	46.58 ± 4.34
Cecal acetate	36.32 ± 2.2	33.72 ± 2.11	33.66 ± 2.74
Cecal propionate	4.03 ± 0.17	3.92 ± 0.15	3.92 ± 0.2
Cecal butyrate	10.2 ± 1.26	9.14 ± 1.34	9.0 ± 1.55

3.4 The Combination of NaBu and δTE/δTE-13' Inhibit Proliferation of HCT-116 Human Colon Cancer Cells

The individual inhibition effects of NaBu, δTE and δTE-13' on HCT-116 human colon cancer cells were first examined. To begin with, NaBu was examined under 1 and 2 mM, δTE was examined under 5, 6 and 7 μM while δTE-13' was examined under 12.5 and 15 μM. NaBu, δTE and δTE-13' inhibited HCT-116 growth in a dose-dependent and time-dependent manner comparing with vehicle (DMSO) control group (Figure 3.8). Since the inhibition effect of 2mM NaBu was too strong only 1mM NaBu was used for further combination assays.

In order to investigate whether the combination of NaBu and δTE/ δTE-13' has better anti-proliferative activity against HCT-116 human colon cancer cells, the cells were treated with either vehicles or different combinations of NaBu and δTE/ δTE-13'. After 24, 48 and 72 hr treatment, the cell viability was measured by MTT assay as previously described. First, both δTE and δTE-13' could time-dependently significantly inhibit the proliferation of HCT-116 comparing with control. Also, butyrate could inhibit cell proliferation in a time-dependent manner under 1mM concentration comparing with control. As shown in Figure 3.7, control of HCT-116 kept growing from 24 h to 48 hr and doubled from 48 hr to 72 hr. Significant differences (P<0.05) were found in 12.5μM, 15μM of δTE-13' and 5μM of δTE under 24 hr, 48hr and 72 hr treatments when comparing with control on the same post-treatment time. In addition, 1mM of NaBu also showed significant differences under 24 hr, 48hr and 72 hr when comparing with control. Interestingly, the combination effects of either δTE or δTE-13'with NaBu all showed significant inhibition effects comparing with not only control but also individual effects of δTE, δTE-13' and NaBu (Figure 3.9). Overall, the inhibition effect of 1mM Nabu was similar with either δTE or δTE-13'. All these results of MTT assay were in agreement with morphological changes that were observed under the microscope. Smaller and more shrink cells as well as more detached and floating cells were observed under δTE, δTE-13' and NaBu treatments.

To further examine whether the combination effects of δTE/ δTE-13' and NaBu were additive or synergistic effects on antiproliferation, the theoretical and the actual effects that described previously were compared under each post-treatment time. As shown in Table 3.5, the actual effects of δTE-13' (12.5μM) and NaBu (1mM) of post-treatment 24 and 48hr were higher than the

theoretical ones. However, only the actual value of 24 hr was significantly higher than the theoretical one but not for the 48 hr ones. Thus, there was synergistic inhibition effect of δTE-13' and NaBu on HCT-116 proliferation on 24 hr and additive inhibition effect on 48 hr. As for the combination effects of δTE-13' (15μM) and NaBu (1mM), the actual effects under 24 and 48 hr are higher but not significantly comparing with the theoretical ones, which indicated there were additive inhibition effects on HCT-116 carcinoma cells growth. Collectively, there were no additive effects of δTE-13' (12.5 and 15μM) and NaBu (1mM) on 72 hr. In addition to δTE-13', δTE under 5μM also showed interesting combination effects. To be more specific, under 48 and 72 hr, the actual inhibition effects were elevated comparing with the theoretical effects. Although not to the extent of being significant, there was additive inhibition effects of δTE(5μM) and NaBu (1mM) on HCT-116 cell proliferation under 48 and 72 hr treatment times.
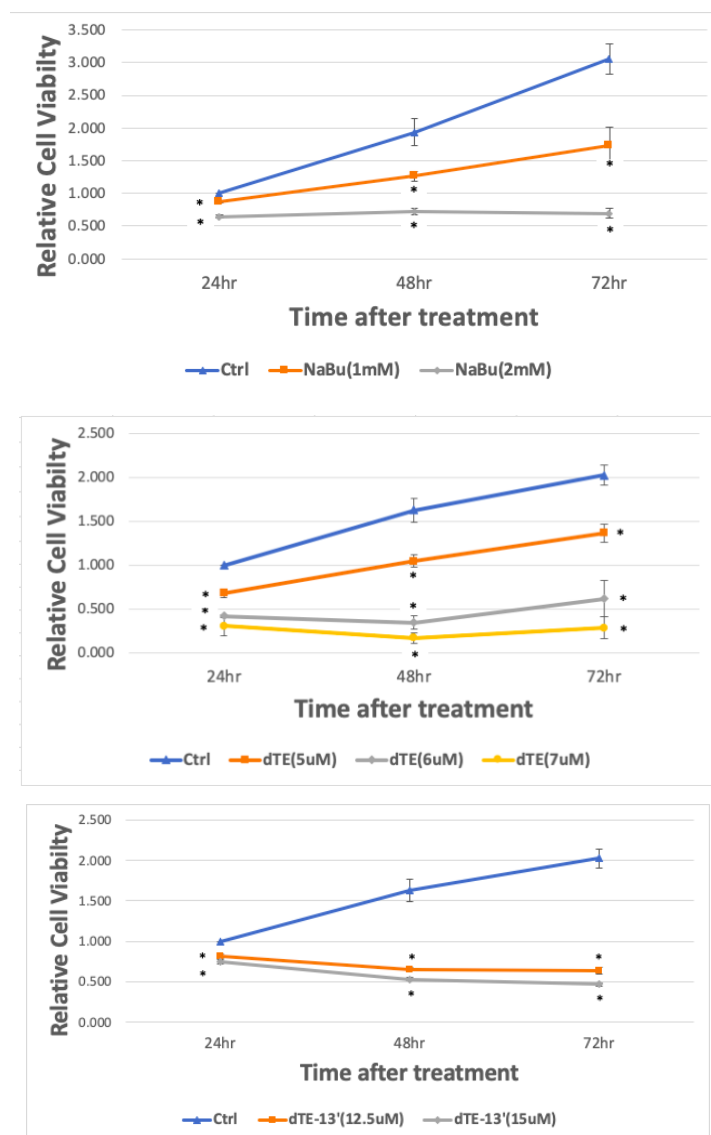
Figure 3.8 The Effects of Sodium Butyrate (NaBu), δTE-13' and δTE on Relative Cell Viability of Human Colon HCT-116 Cells

The relative cell viability was measured by MTT assay after cells were treated with NaBu, δTE or δTE-13' at indicated concentrations and times compared to DMSO controls. Data are presented as mean ± SEM (n=4 for NaBu (1 and 2mM), n=5 for δTE (5 and 7μM), n=4 for δTE(6μM), n=9 for δTE-13'(12.5 and 15μM)). * represents $P < 0.05$ using student's t-test by comparing the relative cell viability of treatment with vehicle (DMSO) treatment.
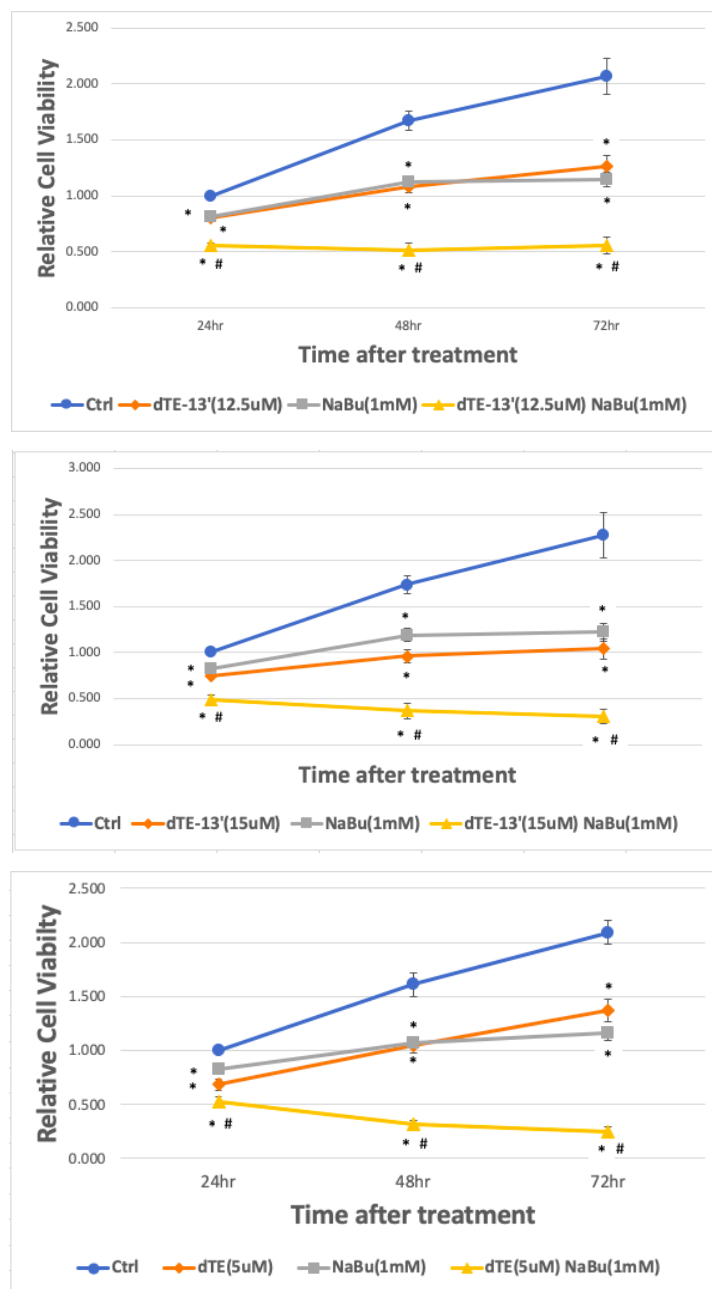
Figure 3.9 The Effect of Sodium Butyrate (NaBu), δTE-13'/ NaBu, δTE and their Combination on Relative Cell Viability of Human Colon HCT-116 Cells

The relative cell viability was measured by MTT assay after cells were treated with individually with NaBu, δTE or δTE-13' and combination of NaBu/δTE or NaBu/δTE-13' at indicated concentrations and times compared to DMSO controls. Data are presented as mean ± SEM (n=9 for δTE-13', n=5 for δTE). * represents P < 0.05 using student's t-test by comparing the relative cell viability of treatment with vehicle (DMSO) treatment.
represents P < 0.05 using student's t-test by comparing the relative cell viability induced by the combination treatment of NaBu and δTE-13' or δTE vs. the individual cell viability after induced by NaBu alone or δTE-13'/δTE alone.

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Table 3.5 Theoretical and Actual Combination Effects of δTE/ δTE-13' and NaBu.

Theoretical effects are the sum of individual inhibition effects comparing with control, while the actual effects are the inhibition effect of the combination treatment comparing with control. Data are expressed as mean ± SEM from different experiments (n=9 for δTE-13', n=5 for δTE). * represents significant differences at P=0.05 using Student t-test pairwise comparison.

Treatment	Post-treatment time	Theoretical effect	Actual effect
δTE-13' (12.5µM) + NaBu (1mM)	24hr	38.14 ± 3.47	44.76 ± 2.86 *
	48hr	67.22 ± 2.82	68.67 ± 3.54
	72hr	79.4 ± 3.82	70.35 ± 3.56
δTE-13' (15µM) + NaBu (1mM)	24hr	43.12 ± 4.13	51.55 ± 4.88
	48hr	76.38 ± 3.19	79.13 ± 3.96
	72hr	98.90 ± 2.94	86.99 ± 2.54
δTE (5µM) + NaBu (1mM)	24hr	48.72 ± 4.67	47.88 ± 5.46
	48hr	66.59 ± 7.26	79.73 ± 3.75
	72hr	78.07 ± 7.60	87.87 ± 2.89

CHAPTER 4. CONCLUSION, DISCUSSION AND FUTURE DIRECTION

4.1 Different Microbiome Analytical Methods Generate Diverse Taxonomic Classifications of Microbial Standards

In the present study, different microbial mock standards' taxonomic classifications were demonstrated under various reference datasets and parameter settings. To begin with, bases trimming in the beginning of the forward and reverse sequence reads would affect the analyses since the integration of low-quality bases of forward and reverse reads would further affect the accuracy of taxonomic classifications. Merely one base difference in both the forward and reverse reads showed the presence and absence of *Lactobacillus fermentum*, which is a common microbe, suggested of both the importance of proper sequence trimming and the incorporation of microbial standards while performing microbiome analyses.

SILVA_132 reference database contains numerous sequence data that are further being clustered at different identity using UCLUST (Edgar, 2010). For example, SILVA 99% reference is comprised of representative sequences that are first being clustered at 99% similarity. In other words, if two sequences are not 99% similar, they would not be cluster together and present as different sequence in the reference. From our analyses, different reference selections generated underclassification of *Salmonella enterica.* In 99% SILVA reference database default parameter analyses, no classified sequences could be identified as *Salmonella,* with approximately 18.5% of reads fell under *Escherichia-Shigella* genus while 14% were only classified to *Enterobacteriaceae* family, which we initially assumed that total 32.6% of reads belonged to *Escherichia coli*. However, the reads that were first only classified to *Enterobacteriaceae* family were further revealed to belonged to *Salmonella* genus applying SILVA 97% reference database set. One possible explanation to this phenomenon is that the whole collection of *Salmonella* in the SILVA database contains a huge number of sequences with different variances, which fail to generate accurate representative sequence that could represent Salmonella genus under 99% similar clustering. Thus, these unknown sequences in the microbial standard could only be identified to family level but fail to go down deeper. Through combining these findings from microbial

standards suggested that there was no standard pipeline to classify sequence data. Selection of appropriate reference dataset is crucial to classify unknown sequences appropriately.

Along the taxonomic classification, the Naive Bayes classifier was used to perform taxonomic analyses (Quast et al., 2012), when generating the classifier for proper taxonomic analyses, different parameter settings could be installed along the training process. Simply put, the genome sequences in the dataset were first extracted into substring of length based on the k bp of selection. For example, the 7-mer default setting, would first extract the sequences into various 7 bp features and generated the probability tables with the assigned taxa (Han & Cho, 2019). Collectively, the longer the k-mers are, the more likely they can be specific classified to certain taxa (Bolyen et al., 2018), this statement is consistent with our findings, we found out that SILVA 99% reference dataset with 11-mer setting successfully classified all the defined microbes in the microbial standards including the *Salmonella* genus, which the default 7-mer setting failed to do so. However, if the k-mer setting becomes too long, it will fail to generate extracted sequences that can be observed and further assigning to defined taxa (Bolyen et al., 2018). To sum up, different k-mer parameter settings provide various scopes to observe the variation across organisms. Therefore, proper parameter setting would enable the extracted sequences to represent the database appropriately as to further perform taxonomic classification.

In addition to whether the results successfully identified specific taxa, the values of relative composition were also worth discussing. From our findings although the results of the relative abundance from SILVA 99% reference dataset with 11-mer computation setting and 97% reference dataset all successfully classified all the defined microbes in the microbial standards (Table 3.1), the values of the relative abundance of these microbes were still different from the theoretical compositions that ZYMOBIOMICS provided. As to explain this phenomenon, it was examined that different primer sets would generate different results due to primer bias. To be more specific, different regions of genes have different levels of species-level specificity. The GC content of templates and primer DNA, have been reported to influence gene amplification by PCR (Suzuki & Giovannoni, 1996). In addition, different sequencing technologies would also affect the output of the microbiome analyses (Tremblay et al., 2015). To be more specific, ZymoBIOMICS performed shotgun metagenomic sequencing when generating the composition values while we

performed Illumina Miseq sequencing in our study. Thus, different experimental condition should generate various results. In other words, if people are performing same methods when performing microbiome analyses, the microbial compositions results might serve as references for future studies as to further examine whether the experiment and/or the analytical pipeline are appropriate. Thus, these all strongly suggested to incorporate mock microbial standards along unknown samples when performing microbiome analyses.

4.2 Alteration of Microbial Taxa may Play Potential Important Role in Colorectal Cancer Chemoprevention

The alteration of certain microbes might link with the manipulation of host metabolic activities, and further impact health status (Holmes et al., 2011). Surprisingly, several microbial alterations in the present study possibly play a role in disease development. In addition, these microorganisms might impact butyrate production, which potentially possesses the ability of colorectal cancer chemoprevention (Vander Heiden et al., 2009). To begin with, *Desulfovibrio* decreased in δTE/γTE supplementation group and had the trend to decrease in δTE-13' group when comparing with soybean vehicle-supplemented control group (Figure 4.1A). This genus of microbe is a group of specific sulfur-reducing bacteria (SRB), which highly correlated with colorectal cancer development under elevating hydrogen sulfide production. *Desulfovibrio* is also frequently seen in Western populations due to high-protein especially red meat consumption (Christl et al., 1992). Mechanistically, hydrogen sulfide not only deteriorate colon epithelial cells with evidence of genotoxicity using the single-cell gel electrophoresis (Attene-Ramos et al., 2006), this chemical also showed to inhibit the butyrate oxidation in high concentration (Rowan et al., 2009). In addition, another potentially detrimental microbe, *Ruminococcaceae* UCG-005, also decreased in the δTE/γTE supplementation (Figure 4.1B). This group of microbes was examined to represent as a biomarker in gastrointestinal disease patients. This result was generated from a meta-analysis of public datasets obtained from individuals affected by CD, UC, CDI and CRC (Mancabelli et al., 2017). However, mechanistic model that explains the mechanistic connection between *Ruminococcaceae* UCG-005 and colonial diseases has not been elucidated. Thus, further investigations are needed to answer this question.

Another interesting microbe that increased under δTE/γTE supplementation is *Eubacterium coprostanoligenes* group (Figure 4.1C), which possesses the ability to reduce cholesterol to coprostanol and further reduce the cholesterol amount that could be absorbed into circulation (Ren et al., 1996). One study demonstrated that high-fat diet significantly enhanced tumor growth which accompanied with elevating serum LDL cholesterol levels. Thus, lowering the serum cholesterol level would potentially show protective effect against colorectal cancer development. In addition, cholesterol would activate the production of reactive oxygen species and MAPK signaling pathway that are highly involved in colon carcinogenesis, which present consistent concept of lowering cholesterol level would show protective effect against colon cancer (Wang et al., 2017).

Lachnospiraceae family is a group of bacteria that belongs to Firmicutes phylum which possesses the ability of producing butyrate and other SCFAs, which could inhibit intestinal inflammation and maintain colonic health (Faintuch & Faintuch, 2019; Meehan & Beiko, 2014). In addition, average amount of *Lachnospiraceae* members was significantly lower in fecal samples of active Crohn's disease (CD) patients (Geirnaert et al., 2017), which strongly suggested that this specific group of microbes play a role in manipulating colonial disease development, which may in turn show beneficial effects with specific butyrate production. From our present microbial analyses results, members of *Lachnospiraceae* UCG-006 were elevated under δTE/γTE supplementation (Figure 4.1D), while *Lachnospiraceae* NK4A136 increased in δTE-13' group (Figure 4.1E), which indicated the potential in affecting SCFAs' amount. It is known that fiber is the major fermentative source of colonic bacteria and generate various of SCFAs (Topping & Clifton, 2001). Several studies demonstrated that fiber supplementation would elevate the number of specific microbes of the *Lachnospiraceae* family that were interestingly consistent with our findings under vitamin E supplementation including δTE/γTE and δTE-13' Thus, it strongly suggested that the microbial modulation under vitamin E treatments would possible have similar effect. To begin with, *Lachnospiraceae* UCG-006 were found elevated in both preclinical and clinical studies that performed fiber supplementation. To begin with, one study demonstrated that *Phellinus linteus* polysaccharide extract (PLPE) treatment enhanced *Lachnospiraceae* UCG-006 and NK4A136 microbes while also inhibited NF-κB signaling pathway activation in rat model (Liu et al., 2020). Another study performed inulin supplementation in ob/ob mice. Inulin, which is a linear fructose polymer and also a common human prebiotic supplement, this study showed that inulin

supplementation not only alleviated metabolic disorders but also elevated the amount of *Lachnospiraceae* UCG-006 microbes (Song et al., 2019). Lastly, in one clinical randomized intervention study, they substituted refined grains with whole grains in healthy adults for six weeks. Surprisingly, it not only presented elevated amount of *Lachnospiraceae* UCG-006 and NK4A136, but also presented positive correlations between the level of *Lachnospiraceae* and SCFAs concentrations (Vanegas et al., 2017). To sum up, the advancements of specific *Lachnospiraceae* taxa after vitamin E supplementation might have similar effect of fiber treatment and further generate beneficial SCFAs that provide chemopreventive properties.

As to answer the question whether the microbial taxa modulation would lead to SCFAs' manipulation, both the fecal and cecum feces were applied for major SCFA measurements. In the present study, both fecal and cecal SCFAs as well as the following acetate, propionate and butyrate concentrations did not show significant differences across treatment. From the previous assumption, changes in specific microbes' abundances might impact the downstream SCFAs' production. However, our results did not reflect significant changes. When it comes to the microbial functionality, it is critical to consider the whole microbiota as a functional group than merely looking into specific monophyletic since the interplay between diet, microbiota and host physiology is complex. To begin with, more than 80% of the identified fecal microbiota can be classified into three dominant phyla in healthy adults as indicated as Bacteroidetes, Firmicutes and Actinobacteria, which also consistent with our data (data not shown) (den Besten et al., 2013; Lay et al., 2005). Generally speaking, the Firmicutes to Bacteroidetes ratio is regarded to represent the gut microbiota composition. From our results which is shown in table 4.1, the relative amount of Firmicutes and Bacteroidetes and the corresponidng F/B ratio did not reflect the modulation under supplementation treatments. From the above-mentioned concept, the SCFA functionality did not altered might due to the supplementations fail to swift the microbial community enough toward the direction of statistically significant elevated SCFAs as well as the targeted butyrate concentration. In addition, the Bacteroidetes phylum mainly produces acetate and propionate, whereas the Firmicutes phylum produces butyrate as its primary metabolic end product (Macfarlane & Macfarlane, 2003). Thus, as to clarify and advance SCFA production, appropriate end point should be monitoring the whole microbial community rather than only focusing on specific microbes.

When examining the SCFA concentration *in vivo,* it was also important to consider the initial diet effect. To be more specific, the fiber content in the chow diet had a decisive effect of how much SCFA including butyrate would be produced. We supplemented Balb/c mice with Teklad rodent diet (8604), which contains 4% of crude fiber, which would be considered as high fiber diet in many studies. For example, one study supplemented mice with either low (1% cellulose) and high fiber diet (5% inulin) and showed differences of SCFAs, 10umol of butyrate per gram of feces were observed in low fiber group while 30umol/g butyrate in high fiber group (Matt et al., 2018). Thus, it was possible that the initial diet already affected the terminal SCFA amounts. On top of the rodent chow diet, if different supplement intervention studies used different basal chow diet, it would be difficult to conclude the actual supplementation effect since different microbiota and metabolic effects might occur. From our previous animal study, we observed *Lactococcus*, a common probiotic species that produces lactic acid (Kimoto-Nira et al., 2007) was found interestingly increased in both δTE and δTE-13'-COOH supplementation groups. However, in our present study, we did not observe *Lactococcus* in my samples. Collectively, basal diet differences might play as an important factor to affect gut microbial community as well as downstream metabolic interactions.

Table 4.1 Relative Abundance of Firmicutes(F) and Bacteroidetes(B) and Corresponding F/B Ratios among Different Supplementation Group.

Relative abundance is presented in percentage. Data are expressed as mean ± SEM.

	Control	δTE/γTE	δTE-13'
Firmicutes (F)	61.72 ± 3.95	65.38 ± 3.49	62.88 ± 3.54
Bacteroidetes (B)	36.3 ± 3.89	32.97 ± 3.46	35.52 ± 3.54
F/B ratio	1.88 ± 0.26	2.2 ± 0.3	1.95 ± 0.27

4.3 Promising Chemopreventive Mechanisms Underlying the Combination of Vitamin E and Butyrate

In present study, we demonstrated interesting combination effects when combining sodium butyrate (NaBu) with δTE/ δTE-13' in inhibiting HCT-116 colon cancer cell proliferation. Both δTE and δTE-13' that combined with NaBu showed superior inhibition effect comparing with vitamin E forms and NaBu alone. In addition, their results presented additive effects under 48 and 72 hr for the combination of δTE (5μM) and NaBu (1mM) while δTE-13'(12.5μM) and NaBu (1mM) showed synergistic effect in 24hr, additive effect in 48 hr. In addition, δTE-13'(15μM) and NaBu (1mM) showed additive effects in 24 and 48 hr. All the above-mentioned observations were done by comparing the theoretical and the actual combination. Since the combination of NaBu and δTE/δTE-13' have better anticancer efficacies than NaBu or δTE/δTE-13' alone, the interesting phenomenon provide prospective insights of chemopreventive mechanisms that may play a role in tumor-suppressing agent developments.

In our previous studies, both δTE and δTE-13' presented their biological abilities to alleviate colorectal cancer burdens *in vivo*. Also, in one study that administrated *Butyrivibrio fibrisolvens*, a butyrate-producing ruminal bacterium, not only increased the butyrate amount in mice and also decreased aberrant crypt foci formation in mice. Thus, it is promising to combine butyrate and vitamin E forms as indicated as δTE and δTE-13' in future *in vivo* studies.

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