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ENVIRONMENTAL RISK FACTORS FOR INFLAMMATORY BOWEL DISEASE: TRICLOSAN AND OTHER CONSUMER ANTIMICROBIALS

A Dissertation Presented

by

KATHERINE ZABALA SANIDAD

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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Molecular & Cellular Biology

ENVIRONMENTAL RISK FACTORS FOR INFLAMMATORY BOWEL DISEASE: TRICLOSAN AND OTHER CONSUMER ANTIMICROBIALS

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DEDICATION

To my parents, my greatest role models
To everyone who supported me

ACKNOWLEDGMENTS

ABSTRACT

ENVIRONMENTAL RISK FACTORS FOR INFLAMMATORY BOWEL DISEASE: TRICLOSAN AND OTHER CONSUMER ANTIMICROBIALS

SEPTEMBER 2019

KATHERINE ZABALA SANIDAD, B.S., STONY BROOK UNIVERSITY

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Inflammatory bowel disease (IBD) has become a serious health problem since the incidence and prevalence of IBD has dramatically increased throughout the world. There is evidence that environmental factors are primarily responsible for the increase of IBD, therefore, it is important to identify novel environmental risk factors to reduce the risk of IBD and its associated diseases. Antimicrobials used in consumer products might serve as environmental risk factors for IBD and its associated diseases. Triclosan (TCS), triclocarban (TCC), benzalkonium chloride (BAC), benzethonium chloride (BET), and chloroxylenol (PCMX) are widely used antimicrobial ingredients in consumer products and are ubiquitous contaminants in the environment. In 2016, the FDA removed TCS and TCC from over-the-counter handwashing products while allowing additional time to develop new safety and efficacy data for BAC, BET, and PCMX. Therefore, it is important and timely to better understand the effects of these antimicrobials on human health. Currently, there not much known about how chronic exposure to low-dose consumer antimicrobials affects gut health. Here, using various *in vitro* and animal models, we found that: 1) TCS is metabolically re-activated in the gut by the actions of gut microbiota, leading to the accumulation of microbiota-derived toxic metabolites in

the colon and resulting in gut-specific toxicity; 2) exposure to low-dose TCC exaggerated the severity of colitis and exacerbated the development of colitis-associated colon tumorigenesis, via gut microbiota-dependent mechanisms; and 3) exposure to low doses of BAC, BET, and PCMX, increases dextran sodium sulfate (DSS)-induced colonic inflammation and exposure to BAC increases azoxymethane (AOM)/DSS-induced colon tumorigenesis in mice. Together, these results support that chronic exposure to consumer antimicrobials could be a novel risk factor for colitis and colitis-associated colon cancer through gut microbiota-dependent mechanisms. A better understanding of the impact antimicrobials on human health, specifically gut health, could lead to significant influence on public health and regulatory policies.

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INTRODUCTION

1.1 Environmental risk factors for inflammatory bowel disease

Inflammatory bowel disease (IBD), characterized by chronic inflammation within the small and large intestines, has become a serious health problem. The incidence and prevalence of IBD has dramatically increased in the United States and other countries, and currently there is no cure for IBD [1]. Another concern for IBD patients is that they have an increased risk for developing colon cancer. In fact, more than 20% of IBD patients will develop and more than 50% of those patients will die from colon cancer [2]. There is evidence that environmental factors are primarily responsible for the increase of IBD [3-5]; therefore, it is important to identify novel environmental risk factors to reduce the risk of IBD and its associated diseases.

One environmental factor suggested to increase IBD are antimicrobial agents through modulation of the gut microbiome. It is well established that oral antibiotic drugs, at therapeutic doses, can modulate gut microbiota to regulate colonic inflammation [6,7]. However, it is not known how chronic exposure to low-dose consumer antimicrobials affects gut health. Here, we will focus on the consumer antimicrobials triclosan (TCS), triclocarban (TCC), benzalkonium chloride (BAC), benzethonium chloride (BET), and chloroxyenol (PCMX) (see chemical structures in Figure 1). We will discuss their exposure into the environment as well as their effects on the gut microbiome and gut health in animal models and humans.

1.2 Antimicrobials in the consumer products and the environment

During the past two decades, antimicrobial-containing products have increased in the U.S. and other countries [8]. The two most common antimicrobials highly produced are triclosan (TCS) and triclocarban (TCC). TCC was introduced in 1957 as an antifungal and antibacterial compound specifically used against resistant forms of *Staphylococcus aureus* (MRSA) and *Enterococci* (VRE) [8]. Later in 1964, TCS was introduced as a broad-spectrum bacteriostatic and fungicidal agent. TCS has antimicrobial activity due to its inhibition of the FabI enzyme which inhibits fatty acid synthase in microorganisms [9]. TCC and TCS are incorporated into consumer products like soap, toothpaste, mouthwash, clothes, kitchenware, and toys. They are also used in deodorants, polymers, and fibers [10].

When antimicrobial compounds are typically disposed, they usually are transported through drainage systems to wastewater treatment plants (WWTPs) where they can be removed from water that will end up being discharged into surface waters [8]. When released into the environment, TCS has been shown to be transformed into methyl-TCS by certain bacteria such as *Rhodococcus*, *Acinetobacter*, and *Mycobacterium* under aerobic conditions, and TCS can also go through photodegradation [11-14].

Despite their fates in WWTPs, TCS and TCC have been reported to break through WWTPs to disperse into surface waters at ng/L concentrations. In addition, the two antimicrobials are can also accumulate in carbon- and lipid-rich sewage sludge, which is then deposited in landfills or applied onto land [15]. Because of this, TCS and TCC has been detected in the environment in the United States and various other countries. In 2014, they were known to be part of the top 10 contaminants in U.S. rivers [8]. TCS has been detected in Indiana stream waters [16]. In Germany, TCS and methyl-TCS was

detected in the surface water, fish, and suspended particulate matter in rivers [17,18]. TCS has been found in the surface waters of Washington D.C. and Switzerland [19,20]. Both TCS and TCC were also detected in the soil of fields that receive sewage sludge in Ohio and the United Kingdom [13,21]. They were also detected in earthworms and biosolids in Ottowa, Canada [22]. TCS can also be detected in biosolids in North Carolina and California that could be potentially used for farming [23]. TCC has been detected in Lake Greifensee sediments in Switzerland [24].

Both TCS and TCC appear to last long in the environment. TCS detected in the surface water in Germany was found to have an 11 day half-life [17,18]. Another study showed that the TCS and TCC that were detected in field soil had a half-life of 20-58 days and may last even longer [13,21]. Interestingly, TCC, TCS, and methyl-TCS were detected in earthworms four years after applying TCS- and TCC- containing biosolids into an environment in Ottowa, Canada [22].

Due to their widespread contamination and longevity in the environment, TCS and TCC were detected in both aquatic life and food crops. TCS was found in salmon [25,26] while TCC was absorbed by freshwater mussels [27]. Both TCS and TCC have been reported to accumulate in crops and vegetables like soybean plants that were planted in contaminated soil and irrigated with contaminated water [28-31]. TCS and TCC were also found in the roots and shoots of crops such as broccoli, potato, beats, cabbages, and peppers. The root tissues of the plants accumulated greater than 100 ppm TCC, while onions accumulated greater than 800 ppm in their bulbs. TCS and methyl-TCS was found in the roots of certain vegetables [32].

Benzalkonium chloride (BAC), benzethonium chloride (BET), and chloroxylenol (PCMX) are other antimicrobial compounds that are potential replacements of TCS and TCC in consumer products. BAC, BET, and PCMX are antimicrobial ingredients used in many cleaning products and surface disinfectants that are widely used in public spaces including workspaces, hospitals, and homes [33,34]. They are also used in hand hygiene or skin antiseptics, with concentrations of 250-500 mg/L [34-36]. In addition to being used in hand soaps and dish soaps, PCMX has also been used as a pesticide [34]. Previous studies have shown that BAC and BET were also frequently detected at high levels in grapefruit seed extract (GSE), a common dietary supplement, with 5 out of 6 commercial GSE products containing 1.3-10% of BET[37,38]. Because of the widespread use of these compounds, BAC and BET were also detected in various environments. They were detected in river water, sewage effluent, urban estuarine sediment, and stormwater samples [39-41]. The release of these antimicrobials into the environment raise concerns regarding their exposure to and impacts on human health and the environment [37-45].

1.3 Human exposure to antimicrobials

Various antimicrobials have been detected frequently detected in the human body. Between 2003-2004, TCS was detected in 75% of urine samples of individuals in the United States (TCS concentration = 7.9 nM-13.1 μM) [46]. TCS has also been detected in urine samples (TCS concentration = 0.08-0.71μM) in Queensland, Australia [47] and in Quebec, Canada [48]. In 2013-2014, 36.9% of urine samples in the United States contained >0.1 μg/L of TCC [49]. Both TCS and TCC were detected in the urine samples of people in China [50] as well as in the serum of women [51]. TCC reportedly reached a concentration of

~500 nM in the plasma of humans using TCC-containing personal care products [52]. Both TCS and TCC were also found in the blood of pregnant women and their newborns in an urban population from Brooklyn, NY [53] and in the urine of pregnant women in Canada [54]. BAC has also been detected in the serum of a patient who accidentally ingested the antimicrobial [43]. There have been no recent studies about whether BET or PCMX have been found in humans, but that may change with the increase use of these antimicrobials.

The average human intake levels of TCS from using consumer products was estimated to be 0.047-0.073 mg/kg/day; in mice it would be equivalent to 0.56-0.88 mg/kg. Men, women, and children, are exposed to 0.047, 0.065, and 0.073 mg/kg/day, respectively [55]. Data from previous studies was used to extrapolate TCS tissue exposure levels from consumer products: according to data from two studies, dermal exposure to TCS can lead to 0.4-64 nmol TCS/mg tissue [56-58]. Because antimicrobials are found quite often in human tissues, it is important to understand different routes by the compounds that might lead to human exposure.

Because TCS is used in certain toothpastes, exposure to the antimicrobial can occur orally in humans [26]. Previous human studies had shown that using TCS-containing toothpaste daily for weeks increased the plasma concentrations of TCS (a combination of TCS and TCS-glucuronide) from 0.03–2.7 nM to 90–1,000 nM [59,60]. Other studies also showed that oral exposure to TCS via TCS-containing toothpaste or mouth rinse can increased plasma levels of TCS in human subjects [61,62]. Interestingly, a study showed that mothers using TCS-containing toothpaste for 6-12 weeks had 0.4-38 ng/g TCS (a combination of TCS and its conjugates, TCS-sulfate and TCS-glucuronide) in their plasma and 0.022-0.95 ng/g TCS in their breast milk [63]; this suggests that the young

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infants being breastfed by those mothers can be orally exposed to TCS. In fact, two studies reported that infants were orally exposed to TCS via the breast milk of mothers exposed to TCS-containing personal care products [64,65].

Oral exposure to antimicrobials might also occur via food consumption. As mentioned before, TCS and TCC has been reported to accumulate in crops and vegetables [28]. TCC was also shown to be uptaken by freshwater mussels [27]. TCS, TCS-conjugates (TCS-glucuronide, TCS-sulfate), and methyl-TCS found in catfish, salmon, and other fish [18,25,66]. BAC was found in food additives in minced meat and raw sausage batters [44]. Previous studies have shown that BAC and BET were frequently detected at high levels in grapefruit seed extract (GSE), which is a common dietary supplement, though these compounds are not permitted to use as food additives [37,38]. This suggests that suggests humans can be exposed to antimicrobials via the consumption of food and dietary supplements.

Antimicrobials are also found in many different consumer products for topical application. TCS is in deodorants thus allowing people to be exposed to the antimicrobial via their skin [67]. TCS absorption into the body can occur through dermal exposure as shown in an *in vitro* study using human epidermal membranes [68]. Another study also showed TCS absorption through dermal exposure with TCS on human skin reaching millimolar levels [57,69]. The estimated TCS concentration in tissues after dermal application is 14-67% of applied TCS dose [70].

TCS and TCC were active ingredients in many soaps which would allow another route of dermal exposure; however, it was shown that TCS- and TCC-containing soaps had no additional health benefits compared to soap without those antimicrobials [71], and

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were therefore no longer allowed to be sold. The antimicrobials BAC, BET, and PCMX are still allowed in hand hygiene or skin antiseptics, such as hand and dishwashing soaps, suggesting a higher chance of dermal exposure to these compounds [34-36]. They are also used in many cleaning products and surface disinfectants [33,34].

Humans can ultimately be exposed to antimicrobials through the environment, use of antimicrobial-containing PCPs, as well as ingestion of certain food products. Because this exposure in humans is prominent, it is important to understand their effects on human health.

1.4 Effects of antimicrobials on health

There have been various *in vitro* and animal studies to understand the effects of antimicrobials on human health. In fact, many of these studies have focused on antimicrobials as endocrine disrupting chemicals; as agents that can facilitate developmental and reproductive defects; as toxicants that can increase genotoxicity, inflammation, and cancer; and as facilitators of antibacterial resistance to themselves as well as other antibiotics.

Antimicrobials were shown to act as EDCs and increase developmental and reproductive defects. TCS and TCC have been shown to be endocrine disrupting chemicals (EDCs). In fact many studies have shown that both TCS and TCC can increase androgenic activity in various *in vitro* assays and animal models [72-77]. One study even showed that TCC, TCS, and methyl-TCS can be EDCs in frog and mammalian cell culture systems [78]. Exposure to TCS resulted in sperm toxicity in male rats [79], while

exposure to BAC and BET showed developmental defects in rodents and caused reproductive dysfunction in mice [80,81].

Antimicrobials were also shown to increase genotoxic and inflammatory responses. Mice that were dermally exposed to TCS had increased immune cell infiltration into the skin draining lymph node, suggesting a potential for allergic reactions [82]. Previous studies have shown that BAC increased pro-inflammatory cytokines in immortalized human conjunctival and corneal epithelia cells [83], and also increased proinflammatory cytokines, phagocytosis, and migration in THP-1 macrophages [84]. BAC also showed genotoxic and inflammatory effects in mammalian and plant cells and rainbow trout at environmentally relevant concentrations [85,86]. As mentioned before, BAC, BET, and PCMX are three possible replacement antimicrobials for TCS and TCC. Interestingly, one study had shown that these three antimicrobials have comparable toxicities to TCS in C. elegans, greater toxicity in zebrafish embryos, and induce similar neurotoxicity to TCC in fish embryos [87]. In addition, PCMX has been shown to cause DNA damage to erythrocytes of rainbow trout [88].

TCS and TCC have been shown to increase the progression of certain cancers. TCS increased liver cancer in mice [89]. TCS also increased ovarian cancer growth and the progression of breast cancer via an estrogen receptor-dependent pathway in cellular and mouse xenograft models [90,91]. TCS can also increase cancer by potentiating the epithelial-to-mesenchymal transition (EMT) in human lung cancer cells [92]. TCS has also been shown to promote cancer by stimulating the secretion of vascular endothelial growth factor (VEGF) by human prostate cancer stromal cells [93]. Chronic exposure to TCC was demonstrated to promote carcinogenesis of human breast cells *in vitro* from non-

cancerous to pre-malignant [94], though more studies should be conducted to support its carcinogenic effects.

Some antimicrobials have been associated with increased antibacterial resistance. A study showed that TCS can contribute to antimicrobial resistance in *Escherichia coli* strains [95]. Another study showed that TCS can cause FabI mutations and antimicrobial resistance in MRSA, clinical isolates of *Staphylococcus aureus, E. Coli*, and *Acinetobacter baumannii* [96-100]. TCS can also cause resistance to antibiotics in *Pseudomonas aeruginosa* by selecting for mutant strains overexpression multidrug efflux pumps [101]. TCS exposure can increase its own resistance in benthic bacterial communities [102,103]. Aside from TCS, BAC and PCMX were also found to increase antibacterial resistance. BAC was found to increase cross-resistance in strains and isolates of *Pantoea*, *Enterobacter*, *Staphylococcus*, and *Escherichia* species to some antibiotics and other biocidal agents [104]. Environmental isolates of staphylococci in Massachusetts were also found to be resistant to BAC, as well as erythromycin, penicillin, and ampicillin [105]. In addition, BAC, used in disinfectants in the food industry, was well tolerated by *Listeria monocytogenes* isolates from some retail foods [106]. PCMX was also tolerated and did not appear to have an effect on certain *Pseudomonas* species found in industrial environments [88]. These results suggest that the application and exposure of these antimicrobials in the environment are associated with antibacterial resistance. It would be interesting to see if the antimicrobials would increase antibacterial resistance in the gut microbiomes of animals and humans as well.

There have also been a few human studies of the effects of these antimicrobials, mostly TCS. Human studies have shown and suggested that TCS exposure is associated

with allergies and asthma [107-109], which is supported by a study that showed TCS-induced aggravation airway hyperresponsiveness in aeroallergen-sensitized mice [110]. Interestingly, one study also suggested that BAC can be a novel allergen, showing that between 1998-2010 the rate of allergic patch tests to BAC had increased [111]. TCS was also shown to be associated reproductive defects since a human study provided evidence that increased TCS in the urine was associated with abnormal morphology in sperm [112]. This study supports the previous *in vitro* and animal studies showing TCS causing reproductive defects. Another study suggested that increased serum concentrations of TCS may influence the thyroid function of children, but not the women who were pregnant with them [113], again, similar to s previous *in vitro* and animal studies supporting TCS as an EDC. More human studies are needed to really investigate the effects of antimicrobials in human health.

1.5 Effects of TCS on the gut microbiome and gut health

IBD has become a serious health problem; the incidence and prevalence of IBD has dramatically increased throughout the world [1]. Considering the growth rate of IBD and the lethal consequence of IBD-associated colon cancer, it is of critical importance to identify novel environmental risk factors to help reduce the risks posed by these diseases. Modulation of the gut microbiome has been implicated in a lot of diseases, and gut microbiota has been shown to contribute to colon inflammation [114,115]. Here, we will discuss consumer antimicrobials as environmental factors that can increase IBD, as well as their effects on the gut microbiome and gut health.

1.5.1 Effects of TCS on animal and human gut microbiomes

Many studies showed that TCS exposure is capable of modulating and disturbing the gut microbiome in various animal models. In fathead minnows exposed to TCS for one week, their gut microbial communities experienced a decrease in alpha diversity and a modulation of their composition compared with gut microbial communities in unexposed fish [116]. The gut microbial communities of zebrafish exposed to TCS for one week experienced a decrease in species richness [117]. Gut microbiomes of adolescent rats exposed to TCS were also had a reduction in the relative abundance of bacteria from the phylum Firmicutes and an increase in bacteria from the phylum Bacteroidetes compared with the gut microbiomes of unexposed rats [118]. TCS exposure for 13 weeks decreased species richness and also significantly modulated 9 groups of bacterial families in the gut microbiomes of TCS-exposed mice compared with control mice [119]. In other animal studies, TCS exposure was found to also perturb the gut microbiome in aeroallergen-sensitized mice and BALB/c mice [110,120]. Consistent with these previous studies, our recent study showed that a 3-week treatment of TCS via diet reduced the diversity and altered the composition of the gut microbiome in C57BL/6 mice [121]. Altogether, these results show that TCS can perturb the gut microbiome in various animal models.

In this study, the fish were exposed to low doses of TCS (0.1-1 ppb which is equivalent to 0.35-3.45 nM) to mimic the levels of TCS detected in the aquatic environment [116]. The dose used in fathead minnows is quite low compared to the TCS levels found in humans after toothpaste use (~90-1000 nM) [59,60], yet there was still a potent effect on the gut microbiomes of these animals, suggesting that there may be a similar effect in humans.

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Recent human studies support that routine exposure to TCS, through the use of TCS-containing personal care products (PCPs) or consumption of TCS-containing breast milk, can alter the human gut microbiomes. In one study, recent mothers who reported using PCPs containing TCS provided both their breast milk and their infant stool samples for evaluation of the infants' gut microbiomes [64]. Interestingly, the gut microbiomes of infants who received breast milk containing TCS had significantly lower alpha diversity compared with infants who received breast milk with non-detectable levels of TCS. In addition, the relative abundances of certain bacteria were modulated in the infants who were fed TCS-containing breast milk [64]. Another study, recent mothers were given different TCS-containing PCPs that exposed individuals to TCS orally or dermally, and the adult and infant gut microbiomes were evaluated [65]. There was a decrease in the diversity of mothers' gut microbiomes and an increase in the relative abundance of broadly antibiotic-resistant *Proteobacteria* species in adults [65]. However, we need to acknowledge that there are also inconsistent results. Some infants who consumed TCS-containing breast milk had similar gut microbiomes to infants receiving breastmilk not containing TCS [65]. Also, the gut microbiomes of human subjects exposed to TCS via PCPs for 4 months were not significantly different from unexposed human subjects [122].

Altogether, animal studies, and some human studies, show that environmentally relevant concentrations of TCS has been shown to modulate the gut microbiome. However, these studies do not go further to discuss what the modulation of the gut microbiome in these animal studies could mean in terms of animal and human health. Modulation of the gut microbiome has been implicated in a lot of diseases including metabolic diseases [114]. Gut microbiota has also been shown to contribute to colon

inflammation [115]. Therefore, our study aimed to further understand the effects of TCS on the gut microbiome and how the gut microbiome plays a role in the pathogenesis of colitis and colon cancer.

1.5.2   Effects of TCS on gut health

Our study sought to understand how TCS could affect diseases, specifically colitis and colon cancer, through its modulation of the gut microbiome [121]. Our recent study showed that exposure to 10-80 ppm TCS via diet induced colonic inflammation, exaggerated *dextran sodium sulfate* (DSS)- or Interleukin 10 (IL-10) knockout-induced colitis, and exacerbated the development of azoxymethane (AOM)/DSS-induced colon tumorigenesis in mouse models, suggesting that TCS has potential adverse effects on gut health [121]. Gut microbiota has been shown to contribute to colon inflammation [115], so we evaluated the gut microbiome's role in TCS-enhanced colitis. Compared with unexposed mice, TCS-exposed mice had a decrease in the alpha diversity in their gut microbiomes and a modulation of their gut microbial composition at phylum and genus levels [121]. These results are consistent with the previous animal studies summarized previously [116-119]. Due to this, we wanted to see if this modulation of the gut microbiome by TCS played a role in the proinflammatory effects of TCS.

Regarding the mechanisms by which TCS exposure exaggerates colonic inflammation and colon cancer, our study supports that the gut microbiome plays an essential role. Indeed, TCS exposure induces colonic inflammation in conventionally raised mice, but not in mice lacking a gut microbiome (germ-free mice) or mice lacking Toll-like receptor 4 (*Tlr4*$^{-/-}$ mice), a critical regulator of host-microbiome interactions.

These results support that the gut microbiome is required for the pro-inflammatory effects of TCS [121]. Again, since substantial studies, including our current study, have shown that TCS has potent effects to alter gut microbiome in animals [116-119] and human subjects [64,65], we think that it is of critical importance to reassess the potential adverse effects of TCS on the gut microbiome and gut health, in order to generate science-based regulatory policies.

A critical question is whether the results observed in animal models could reflect the responses of human exposure to TCS. The doses of TCS used in our study (10-80 ppm in diet) are among the lowest doses used in animal experiments of TCS [121,123]. Using LC-MS/MS, we found that after mice were exposed to 10–80 ppm TCS via diet for 3 weeks, the concentrations of TCS and its metabolite in mouse plasma were comparable to the concentrations reported in the plasma of TCS-exposed human volunteers [59,60]. In addition, we treated mice for several weeks, while humans could be exposed to TCS for many years. We have to point out that there are many challenges to use mouse models to study human exposure to consumer chemicals, and there could be fundamental differences in absorption, metabolism, secretion, and biological responses to TCS between mice and humans [123]. Together, these results support that it is essential to evaluate the impact of TCS on gut health further.

1.6 Significance and hypothesis

The incidence and prevalence of IBD are dramatically increasing in the United States and other countries, making IBD a serious health problem [1]. Emerging evidence supports that environmental factors are primarily responsible for the growing incidence

of IBD [3-5]. Considering the growing incidences and potential lethal consequence of IBD-associated colon cancer, it is of practical importance to identify novel environmental risk factors, in order to reduce the risks posed by IBD and associated diseases.

Triclosan (TCS) is an antimicrobial compound incorporated into many consumer products, is frequently detected in the human body, and is an environmental contaminant in the US [8,46]. Our recent study showed that exposure to low-dose TCS exaggerates colonic inflammation and exacerbates the development of colitis-associated colon tumorigenesis in mouse models, suggesting that TCS could have adverse effects on gut health [121]. Exposure to TCS alters the gut microbiome and fails to induce colonic inflammation in mice lacking a gut microbiome (germ-free mice), supporting that the gut microbiome contributes to the pro-inflammatory effect of TCS [121]. However, the functional roles of the gut microbiota, as well as specific gut bacteria involved, in the pro-inflammatory effects of TCS are unknown.

One functional role of the gut microbiota is its ability to metabolize xenobiotics. Because TCS contains a phenolic structural moiety (-Ph-OH), it is highly susceptible to Phase II metabolizing enzymes such as glucuronyl transferase and sulfotransferase. Indeed, *in vivo* studies showed that TCS is rapidly metabolized by Phase II enzymes [124], leading to the formation of TCS glucuronide and TCS sulfate which are water-soluble, biologically inactive, and are rapidly secreted out of the body. However, TCS metabolism in the colon has not been characterized. Determining the metabolites of TCS in the colon, along with gut microbiota's role in TCS metabolism, is important for understanding its pro-inflammatory effects.

The long-term goal of our research is to identify novel environmental risk factors for IBD and associated colon cancer, in order to prevent these diseases. To achieve this, the objective of this research is to validate the effects and mechanisms of triclosan, as well as other consumer antimicrobials, and the gut microbiome on IBD. We hypothesize that the gut microbiota is responsible for colonic metabolism of TCS, and that TCS itself, but not its metabolites, are responsible for its biological activity. In addition, we hypothesize that other consumer antimicrobials such as TCC, BAC, BET, and PCMX are also able to have detrimental effects on gut health.

To test these hypotheses, we propose the following specific aims:

Aim 1. To characterize the major metabolites of TCS in the colon, determine the role of the gut microbiome in TCS metabolism, and identify specific bacteria involved in TCS metabolism.

Aim 2. To compare the biological effects of TCS and its metabolites.

Aim 3. To determine the effects of other antimicrobials (TCC, BAC, BET, and PCMX) on gut health.

Our research can lead to the further understanding of the effects of these antimicrobials on gut health, which may lead to a significant impact on regulatory policies of these antimicrobials. Furthermore, this information may help us understand more about what environmental factors play a role in IBD.
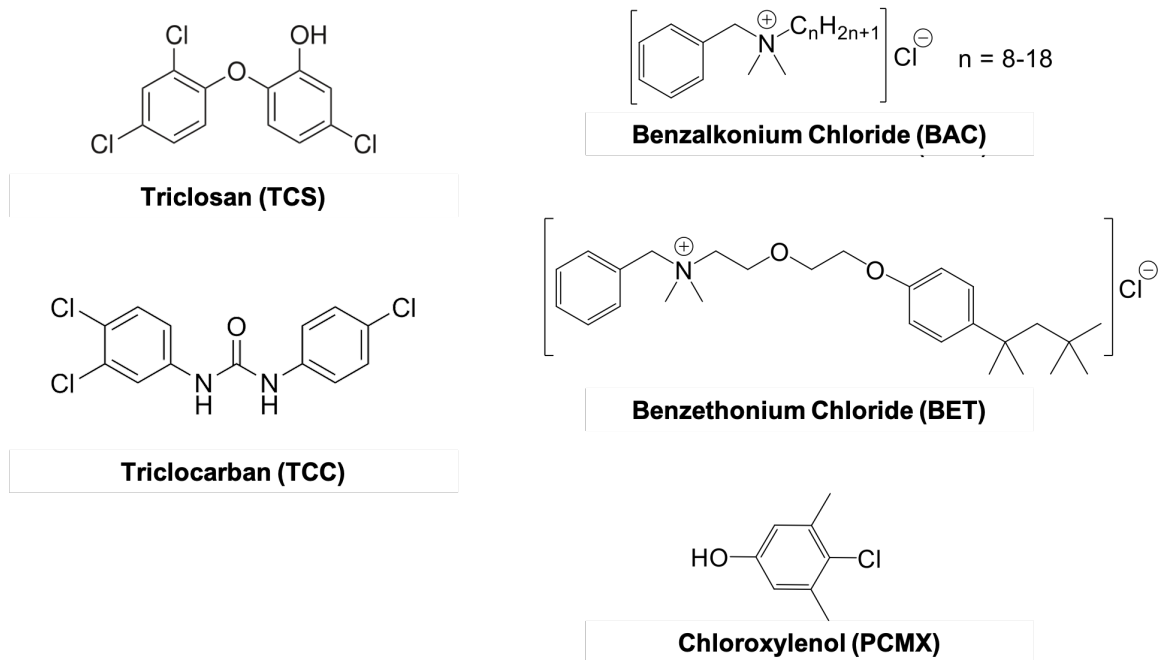
Figure 1.1: Chemical structures of triclosan (TCS), triclocarban (TCC), benzalkonium chloride (BAC), benzethonium chloride (BET), and chloroxylenol (PCMX).

CHAPTER 2

METABOLIC REACTIVATION OF THE ENVIRONMENTAL TOXICANT

TRICLOSAN BY GUT MICROBIOTA

2.1 Introduction

Previous research regarding the metabolism of environmental toxicants and

pollutants has mainly focused on the metabolic processes in the mammalian host tissues.

Indeed, substantial research has shown that once the environmental compounds enter the

body, they are rapidly metabolized by Phase I and Phase II metabolizing enzymes, which

are commonly expressed in various host organs, resulting in the formation of

glucuronide- and sulfate-conjugates. These conjugates are usually water-soluble,

biologically inactive, and are rapidly removed from the body, leading to inactivation and

detoxification of the environmental compounds [125]. Based on these findings, some

previous studies had suggested that due to the rapid metabolism and low stability of many

environmental compounds, exposure to low doses of these compounds is not likely to

cause adverse effects *in vivo* [126-129]. However, most previous research has only focused on

the metabolic reactions in the host tissues [125], while the gut microbiota-mediated

biotransformation is understudied [130,131].

Emerging research supports that gut microbiota play critical roles in xenobiotic

metabolism [130,131]. The microbiota can catalyze highly unique metabolic transformations,

such as hydrolytic (e.g. de-glucuronidation and de-sulfatation), reductive (e.g. C=C or

N=N bond reduction), functional group transfer (e.g. methyl or acetyl transfer), and

radical-mediated reactions [131]. Many of these metabolic reactions are distinct from

reactions of the host enzymes, resulting in colonic accumulation of microbiota-derived metabolites which could have unique activities and toxicities [131]. These studies suggest that compared with other organs, the gut tissue could have a completely different profile of xenobiotic metabolism due to the presence of gut bacteria, resulting in potential gut-specific toxicology. However, to date, the metabolism of environmental toxicants and pollutants in the gut tissue and the roles of gut microbiota involved are mostly unknown.

Triclosan (TCS) is a high-volume chemical used as an antimicrobial ingredient in more than 2,000 consumer products: every year, several million pounds of TCS are used in the United States [8]. Due to its widespread application and persistence, it has caused pollution ubiquitously in the environment and is frequently detected in the human population, raising concerns about its impacts on the environment and human health [8]. Our recent research shows that exposure to TCS, at doses relevant to human exposure, enhances basal colonic inflammation, increases the severity of inflammatory bowel disease (IBD), and exaggerates IBD-associated colon tumorigenesis in mice [121]. The adverse effects of TCS require the presence of gut microbiota since TCS exposure fails to promote colonic inflammation in germ-free mice [121]. However, the functional roles of the gut microbiota involved are unknown. In this study, we used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to profile TCS metabolism in mouse models, *in vitro* culture of gut bacteria, and human subjects, to study the functional roles of gut microbiota in the gut metabolism of TCS.

2.2 Materials and Methods

2.2.1 Chemicals

Triclosan (TCS, 99% purity) was purchased from Alfa Aesar (Haverhill, MA). TCS glucuronide (95% purity) and TCS sulfate (95% purity) were purchased from Toronto Research Chemicals (Toronto, ON, Canada).

2.2.2 DNA extraction

DNA was extracted from mouse fecal samples and human stool using QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA) following instructions from the manufacturer with an additional bead-beating step. The quantity of the extracted DNA was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific), and the quality was verified using gel electrophoresis. The DNA was then subjected to further analysis.

2.2.3 Real-Time PCR analysis of *16S rRNA* gene and strain-specific bacteria primers

DNA extracted from mouse fecal samples were subjected to qPCR analysis using a DNA Engine Opticon system (Bio-Rad Laboratories, Hercules, CA). 20 μL PCR reactions were made using the Maxima SYBR_green Master Mix (Thermo Fisher Scientific), and DNA was normalized to 5 ng/μL per reaction. The 16S rRNA primers and strain-specific bacteria primers are listed in Table 2.1.

2.2.4 Isolation of bacteria from mouse tissues and human stool samples

Mouse tissues (small intestine, cecum, colon, and feces) were harvested from mice, and human stool samples were collected. The tissues and stool were dissolved in sterile PBS with 0.05% L-cysteine, then centrifuged at 900xg for 5 min. The supernatant containing culturable bacteria was then fermented at 37 ^{O}C in MRS broth in an anaerobic cabinet (Whitley A35 anaerobic work station, Don Whitley Scientific) under an atmosphere of 85% N2, 10% CO2, and 5% H2. In addition, the remaining supernatant (~0.6 mL) containing culturable bacteria was then mixed with sterile 50% glycerol (0.3 mL) and stored at -80 ^{O}C as stock for future experiments.

2.2.5 *In vitro* fermentation of bacteria

The bacterial strains (*Lactobacillus* strains: *L. brevis* 14869*, L. Sakei* 11146*, L. reuteri* DSM 20016*, L. rhamnosus* ATCC 7469*, L. gasseri* 33323*, L. reuteri* DSM 20053; *Bifidobacterium* strains: *B. animalis subsp. animalis* 25527*, B. breve* 15700*, B. bifidum* 29521*, B. infantis* 15697*, B. longum* 15707*, B. pseudocatenulatum*) were obtained from the Agriculture Research Service Culture Collection (NRRL). Strains and isolated bacteria from mouse tissues and human stool samples were fermented at 37 ^{O}C in MRS broth in an anaerobic cabinet (Whitley A35 anaerobic work station, Don Whitley Scientific) under an atmosphere of 85% N2, 10% CO2, and 5% H2. When the OD600 reached 0.5, bacteria were then inoculated 1:10 into MRS broth containing either DMSO vehicle, 20 μM TCS, or 20 μM TCS glucuronide. After 48 h, the samples were collected and stored in -80 ^{O}C for further analysis.

2.2.6 Growth curves of pure bacteria

Pure strains of bacteria (listed above) were fermented at 37 $^{\text{o}}$C in MRS broth in an anaerobic cabinet (Whitley A35 anaerobic work station, Don Whitley Scientific) under an atmosphere of 85% N_2, 10% CO_2, and 5% H_2. When the OD600 reached 0.5, bacteria were inoculated 1:100 into MRS broth containing either DMSO vehicle, TCS (0.1-1 µM), or TCS glucuronide (20 µM) in a 96-well plate. The plate was entered into a plate reader, and the OD600 was measured from 0-30 h.

2.2.7 Human urine and stool collection

Human urine and stool samples from a previous study [122] were utilized (schematic of human urine and stool sample collection is shown in Figure 2.9A). Briefly, healthy human volunteers from Stanford University were given personal care products either containing or not containing TCS. Urine and stool samples were then collected from the volunteers before and 1-4 months after using the personal care products. The study was approved by the Institutional Review Board of Stanford University (ClinicalTrials.gov identifier NCT01509976).

2.2.8 Extraction of TCS metabolites

Mouse tissues and human stool were placed in homogenizer tubes with beads and 1 mL methanol, then homogenized using a bead-disruptor (OMNI International, Kennesaw, GA). Samples were centrifuged at 10,000 rpm at room temperature for 3 min. The supernatant was collected and then centrifuged again at 14,000 rpm at room temperature for 5 min. 500 µL of the supernatant was then collected and vacuum-

centrifuged to dryness. For bacterial broth (50 µL) and human urine (100 µL), each sample was combined with 1 mL methanol and placed on ice. After 10 min on ice, samples were centrifuged at 14,000 rpm at room temperature for 5 min. 500 µL of the supernatant was then collected and vacuum- centrifuged to dryness. The extracts were re-dissolved in methanol with amount that was proportional to sample weights or volumes, then centrifugated (14,000 rpm, 15 min, 4°C) before the LC-MS/MS analysis.

2.2.9 Detection of TCS and TCS metabolites by LC-MS/MS

TCS, TCS glucuronide, and TCS sulfate in the samples were quantified using a Thermo Scientific Dionex Ultimate 3000 ultrahigh performance liquid chromatography (UHPLC) system coupled with a TSQ Quantiva Triple Quadrupole Mass Spectrometer. ACQUITY UPLC C18 column (1.7 µm particles, 2.1 mm × 100 mm, Waters) was used for chromatographic separation. Data acquisition was performed by multiple reaction monitoring (MRM) mode.

Hydroxyl-TCS, hydroxyl-TCS glucuronide, and hydroxyl-TCS sulfate were monitored by a Thermo Scientific Dionex Ultimate 3000 UHPLC system coupled with an Orbitrap Fusion Tribrid Mass Spectrometer. Chromatographic separation was achieved on an ACQUITY UPLC HSS T3 column (1.7 µm particles, 2.1 mm × 100 mm, Waters). Targeted MS2 mode was used for metabolite identification. Details of the instrumental methods are provided in Table 2.2-3 The data were analyzed using Xcalibur software (version 4.1, Thermo Fisher Scientific).

2.2.10 Cell culture and treatment with TCS, TCS glucuronide, and TCS sulfate

MC38 mouse colon cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 ^{O}C under an atmosphere of 5% CO2. MC38 cells were seeded at 20% confluency and left to settle overnight. Cells were then treated with either 1 μM TCS, TCS glucuronide, TCS sulfate, or vehicle (DMSO). After 48 h, cells were harvested for RT-qPCR analysis, and cell medium was collected for ELISA analysis.

2.2.11 Reverse-Transcriptase-qPCR of inflammatory biomarkers

Total RNA was isolated from them using Trizol reagent (Ambion) according to the manufacturer's instruction. RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystem) according to the manufacturer's instructions. 20 μL PCR reactions were prepared using the Maxima SYBR_green Master Mix (Thermo Fisher Scientific), and qPCR was carried out using a DNA Engine Opticon system (Bio-Rad Laboratories). Mouse-specific primer sequences (Thermo Fisher Scientific) used to detect inflammatory biomarkers are listed in Table 2.1. *Gapdh* expression was used as an internal control.

2.2.12 ELISA of inflammatory biomarkers

Cell medium from MC38 colon cancer cells treated with either 1 μM TCS, TCS glucuronide, TCS sulfate, or vehicle (DMSO) for 48 h was collected, and 30 μL from each sample was used. Cytokine concentrations in the medium were determined using the CBA Mouse Inflammation Kit (BD Sciences) according to the manufacturer's

instructions. Data were acquired using a BD LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

2.2.13 *In vitro* **fermentation of human stool bacteria for 16S rRNA sequencing**

Human fecal samples were collected from 8 individuals (4 male and 4 female, age = 25-30 years, Asian, healthy, without pre-, pro-, or anti-biotics for at least 3 months). Bacteria from the samples were isolated and cultured at 37 $^{\text{o}}$C in Gifu Anaerobic (GAM) broth (HIMedia Laboratories, West Chester, PA), in an anaerobic cabinet (Whitley A35 anaerobic workstation, Don Whitley Scientific, UK) under an atmosphere of 85% N2, 10% CO2, and 5% H2. Bacteria were inoculated 1:10 (v/v) into GAM broth containing TCS (0.1 and 1 µM) or 0.1% DMSO vehicle and incubated at 37 $^{\text{o}}$C in the anaerobic cabinet for 48 h. After incubation, bacteria were collected for DNA extraction and 16S rRNA sequencing.

2.2.14 **Microbial phylogenetic profiling by 16S rRNA sequencing**

All PCR reactions were performed in a 96-well plate on a T100$^{\text{TM}}$ thermal cycler (Bio-Rad) with 2X KAPA HiFi Hotstart ReadyMix (KAPA Biosystem, Wilmington, MA). First, PCR was performed to amplify DNA of the bacterial community with primers (see Table 2.1) that bound the V3-4 regions of the 16S rRNA gene. The primers also incorporated the Illumina overhang adaptor. After purification with AMPure XP beads (Beckman Coulter, Danvers, MA), limited cycle PCR was performed using the Nextera XT Index Kit (Illumina, San Diego, CA) to attach dual indices and Illumina sequencing adapters. After another purification with AMPure XP

beads, the quantity of the purified PCR products was measured by Qubit dsDNA BR

Assay kit (Life Technology, Carlsbad, CA) and the amplicon quality was estimated by

ScreenTape Assay on Tape Station 2200 (Agilent Technologies, Santa

Clara, CA). Samples were then pooled in equimolar amount and pair-end 2*300bp

sequencing was performed using a MiSeq reagent kit V3 (5% PhiX) (Illumina, San

Diego, CA) on Illumina MiSeq platform. Sequencing was performed at Genomics

Resource Laboratory, University of Massachusetts Amherst.

Sequencing data was processed using the QIIME^{TM} software pipeline v1.9.1

[132]. Generally, the high- quality sequence data (quality value ≥ 30) was demultiplexed.

Sequences were then clustered into operational taxonomic unites (OTUs) using the

open reference OTU picking against Greengenes bacterial 16S rRNA database (13_8

release) with 97% similarity threshold. The α-diversity (the diversity within sample

community species richness) was determined with ten iterations at a maximal sequence

depth where all samples could be included.

2.2.15 Data and Statistical Analyses

Data are expressed as mean ± SEM. The statistical comparison of two groups

was analyzed using Student's t-test. The statistical comparison of three or more groups

was analyzed using one-way ANOVA. The analysis of TCS metabolites in colon

digesta of mice (Figure 2.2) according to antibiotic treatment and TCS treatment was

performed using two-way ANOVA. The statistical analyses were performed using SAS

statistical software and SigmaPlot. $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Profiles of TCS metabolites in mouse tissues

To our knowledge, detailed profiling of TCS metabolism in animal tissues has not been attempted. To this end, we treated mice with TCS via diet for 4 weeks, then used LC-MS/MS to profile TCS metabolites in various tissues. In agreement with previous studies [123], we found that, in most organs, such as the liver, bile, heart, and small intestine (mucosa and digesta), the dominant TCS metabolites were its glucuronide- and sulfate-conjugates (Figure 2.1). However, in the digesta of the cecum and colon tissues, the dominant metabolite was free-form TCS, with very low concentrations of the conjugates. Indeed, the relative percentage of free-form TCS, TCS glucuronide, and TCS sulfate in the colon digesta were 99.1 : 0.69 : 0.23 (Figure 2.1), illustrating a different profile of TCS metabolism.

2.3.2 Role of gut microbiota in the colonic metabolism of TCS in mice

Compared with other organs such as the liver, a major difference of the colon tissue is that the colon harbors a large number of gut bacteria [130]. To determine whether gut microbiota mediate the unique profile of TCS metabolism in the colon digesta, we tested whether antibiotic cocktail-mediated suppression of gut microbiota alters TCS metabolism in the colon digesta (see the scheme of the animal experiment in Figure 2.2A). We used an antibiotic cocktail from previous studies [133,134] and found that treatment with the cocktail caused a dramatic reduction of fecal bacteria, as assessed by

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qPCR analysis of the *16S rRNA* gene (Figure 2.3), validating the depleting effects of the cocktail on gut microbiota.

We found that treatment with the antibiotic cocktail reduced the concentration of free-form TCS (~50% reduction), while enhancing the concentration of TCS glucuronide (~6-fold increase) and TCS sulfate (~5-fold increase) in the colon digesta (Figure 2.2B), see complete LC-MS/MS results in Table 2.4). Two-way ANOVA analysis showed that there was a significant interaction between antibiotic cocktail treatment (antibiotic cocktail versus water) and TCS treatment (TCS versus vehicle) on both free-form TCS (P < 0.001) and TCS-conjugates (P = 0.009). These results support that gut microbiota mediate the conversion of TCS glucuronide and/or TCS sulfate conjugates to free-form TCS, contributing to the colonic metabolism of TCS.

Using MS/MS-based fragmentation analysis, we also identified some novel metabolites, including hydroxyl-TCS, hydroxyl-TCS glucuronide, and hydroxyl-TCS sulfate, in the colon digesta (see mass spectrum of these compounds in Figure 2.4), and found that the profiles of these metabolites were also dependent on gut microbiota. Consistent with our results above, the antibiotic cocktail treatment reduced the concentration of free-form hydroxyl-TCS while increasing the concentration of hydroxyl-TCS glucuronide and hydroxyl-TCS sulfate in the colon digesta (Figure 2.2C-E, see complete LC-MS/MS results in Table 2.5). Two-way ANOVA analysis also showed that there was a significant interaction between antibiotic cocktail treatment and TCS treatment on these novel metabolites (P < 0.001 for all three metabolites). These results further support the roles of gut microbiota in colonic metabolism of TCS.

2.3.3 Effects of gut bacteria on the metabolism of TCS *in vitro*

To further validate the roles of gut microbiota in TCS metabolism, we cultured gut bacteria under anaerobic conditions and studied their effects on TCS metabolism *in vitro*. Our results above support that gut microbiota convert TCS glucuronide to free-form TCS *in vivo* (Figure 2.2), and we tested whether gut microbiota can directly catalyze this reaction *in vitro* (see the scheme of the experiment in Figure 2.5A). With the presence of gut microbiota from mice feces and human stool, TCS glucuronide was converted to free-form TCS, while this reaction was not observed without the presence of the bacteria (Figure 2.5B). These results support that the gut microbiota can catalyze the de-glucuronidation reaction *in vitro*.

To explore the specific gut bacteria involved, we cultured pure bacteria strains and tested their effects on TCS metabolism. Out of 12 strains, we found that 3 strains, including *Lactobacillus brevis*, *L. rhamnosus*, and *Bifidobacterium breve*, were capable of converting TCS glucuronide to free-form TCS *in vitro* (Figure 2.5C). Some strains, such as *L. reuteri* (DSM 20053 and DSM 20016) and *B. longum*, showed no effect to metabolize TCS glucuronide *in vitro* (Figure 2.5C). To validate that the lack of metabolic activity was not due to suppressed bacterial growth, we tested the effects of TCS glucuronide on bacterial growth and found that TCS glucuronide had no effect on bacterial growth (Figure 2.6).

To better understand the interactions between TCS and TCS-metabolizing bacteria (e.g. *L. brevis*, *L. rhamnosus*, and *B. breve*), we tested the effect of free-form TCS on the growth of these bacterial strains. Compared with vehicle (DMSO), treatment with TCS (concentration = 0.1-1 μM) did not affect bacterial growth *in vitro* (Figure 2.7).

Next, we tested whether TCS exposure could change the abundance of these strains in mouse gut microbiota. We used strain-specific primers [135,136] to perform qPCR analyses and found that treatment with TCS (80 ppm in diet) for 4 weeks appeared to increase, but not significantly, the relative abundance of these bacteria in mouse fecal microbiota (Figure 2.8).

Besides the de-glucuronidation reaction, we also tested whether gut bacteria can catalyze the conversion of TCS to hydroxyl-TCS. We incubated TCS with gut bacteria from the small intestine, cecum, colon, and feces from the mice, as well as gut bacteria from human stool. LC-MS/MS analysis showed no formation of hydroxyl-TCS (Table 2.6). This result suggests that at least the culturable gut bacteria cannot convert TCS to hydroxyl-TCS *in vitro*.

2.3.4   Profiles of TCS metabolites in human subjects

We analyzed the profiles of TCS metabolites in the urine and stool of TCS-exposed human subjects. To this end, we utilized human samples from a previous study (ClinicalTrials.gov identifier NCT01509976), in which the human subjects were exposed to TCS by using personal care products for up to 4 months (see scheme of experiment in Figure 2.9A) [122].

LC-MS/MS showed that the dominant metabolites in human urine are the conjugates: after 4 months of exposure, the relative percentages of TCS conjugates (a combination of TCS glucuronide and TCS sulfate) and free-form TCS in the urine was ~99.5 : 0.5 (Figure 2.9B, see complete LC-MS/MS results in Table 2.7-8). However, in the stool, the dominant metabolite is free-form TCS: after 4 months of exposure, the

relative percentages of TCS conjugates and free-form TCS in the feces was ~1.7 : 98.3

(Figure 2.9C, see complete LC-MS/MS results in Table 2.9-10). These results are in

agreement with the mouse study as described above, which showed that colon digesta (or

feces) versus other tissues have distinct profiles of TCS metabolites.

2.3.5 Effects of TCS and its conjugates on inflammation *in vitro*

Our results above support that gut microbiota convert TCS conjugates to free-

form TCS in the colon. To explore the biological significance of the microbiota-mediated

metabolism, we studied the comparative effects of TCS conjugates (TCS glucuronide and

TCS sulfate) versus free-form TCS. We treated MC38 colon cancer cells with 1 µM of

each compound and studied the inflammatory responses. We determined the

concentration using our results above which showed that the concentration of free-form

TCS in the stool of TCS-exposed human subjects was up to ~1100 pmol/g (calculated to

be ~ 1 µM, see Figure 2.9C and Table 2.9). Using qRT-PCR analysis, we found that

treatment with TCS increased the gene expression of pro-inflammatory cytokines (*Il6*,

Ifng, *Il1b*, and *Mcp1*) in MC38 colon cancer cells, while TCS glucuronide and TCS

sulfate had no such effects (Figure 2.10A). Furthermore, ELISA analysis showed that

treatment with TCS, but not the conjugates, increased concentrations of pro-inflammatory

cytokines (IL-6, IFN-γ, and TNF-α) and reduced an anti-inflammatory cytokine IL-10 in

the medium of MC38 cells (Figure 2.10B). Together, these results suggest that the gut

microbiota convert TCS conjugates, which are biologically inactive, to generate free-

form TCS, which has potent and direct pro-inflammatory effects, supporting the

biological significance of the microbiota-mediated metabolism.

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After demonstrating that free-form TCS had direct pro-inflammatory effects, we further studied the direct effect of free-form TCS on gut microbiota *in vitro*. Previous studies showed that exposure to TCS altered gut microbiota in animals and humans [65,116-119,121]; however, it remains unknown whether TCS has a direct effect on gut microbiota. We cultured human fecal microbiota derived from 8 healthy volunteers, treated with TCS (concentration = 10 nM, which represents the low concentration of free-form TCS in the stool of TCS-exposed human subjects, see Figure 2.9C and Table 2.9) or vehicle (DMSO) under anaerobic conditions, then analyzed gut microbiota using 16S rRNA sequencing (see scheme of experiment in Figure 2.11A). At this low concentration, TCS treatment had no significant effect on the diversity of the microbiota (Figure 2.11B). Regarding microbiota composition, TCS treatment altered bacterial abundance at both the phylum and genus levels (Figure 2.11C-D and Figure 2.12). Notably, TCS treatment increased the relative abundance of Proteobacteria, which is shown to be increased in IBD patients and is positively associated with the pathogenesis of IBD (Figure 2.11D) [137]. Together, these results support that free-form TCS has a potent and direct effect of altering gut microbiota, resulting in a potential pro-inflammatory phenotype.

2.4 Discussion

Previous research regarding the metabolism of TCS, as well as many other environmental compounds, has only focused on the metabolic processes in mammalian host tissues (e.g. liver), while the gut microbiota-mediated metabolism is understudied. Notably, previous studies showed that after TCS enters the body, it is rapidly metabolized by phase II detoxification enzymes, resulting in the formation of TCS glucuronide and

TCS sulfate, which are water-soluble, biologically inactive, and are quickly removed from the body [123]. Here, our central finding is that gut microbiota catalyzes unique metabolic conversions of TCS, resulting in colonic re-generation of free-form TCS species which has potent and direct effects on both colon epithelial cells and gut bacteria to induce a pro-inflammatory phenotype (see proposed model in Figure 2.13). This leads to the accumulation of biologically active free-form TCS in the gut, and this could lead to gut-specific toxic effects. These results highlight the critical importance of gut microbiota in the metabolism and bioactivity of TCS. In addition, these results suggest that, besides TCS, many other environmental chemicals could also be metabolically re-activated in the gut by the actions of gut microbiota, resulting in the accumulation of microbiota-derived toxic metabolites in the colon and leading to gut-specific toxicity.

Our results are in agreement with previous studies which showed that β-glucuronidase-expressing gut bacteria could catalyze de-glucuronidation reactions [138]. We identified three strains of bacteria, including *L. brevis*, *L. rhamnosus*, and *B. breve*, that were capable of converting TCS glucuronide to free-form TCS *in vitro*. This is consistent with previous studies, which showed that these strains have expression of β-glucuronidase [139-141]. Interestingly, we found that some β-glucuronidase-expressing gut bacteria, such as *B. bifidum* and *L. gasseri* [142,143], can't convert TCS glucuronide to free-form TCS, suggesting that only specific β-glucuronidase isoforms recognize TCS glucuronide as a substrate. It is feasible that, upon TCS exposure, individuals with different profiles of gut bacteria or activities of gut metabolizing enzymes could have varied colonic metabolism of TCS, resulting in inter-individual variations in biological responses to TCS exposure. Notably, previous studies showed that the fecal β-

glucuronidase activity was significantly increased in colon cancer patients (~12-fold increase of the enzymatic activity compared with healthy controls) [144], suggesting an enhanced metabolic re-activation of TCS, which has colon cancer-enhancing effects [121], in colon cancer patients. Elucidation of the roles of gut bacteria in the metabolism and bioactivity of TCS could help to design better human studies to clarify the health impacts of TCS.

Our results support that due to the unique metabolic reactions catalyzed by gut microbiota (e.g. de-glucuronidation reaction), microbiota-derived toxic metabolites (e.g. the free-form parent compound) could be accumulated in the gut tissues, resulting in gut-specific toxicity. This suggests that during an evaluation of the toxicology of environmental toxicants and pollutants, it is of critical importance to study the metabolism and toxicity in the gut tissues. Indeed, our recent study showed that TCS exposure, at low doses, enhances basal colonic inflammation, increases the severity of IBD, and exaggerates colon tumorigenesis in mice [121]. Previous studies have shown that TCS exposure could also cause adverse effects in other tissues such as liver [145]; however, it is noteworthy that the reported TCS doses to cause liver toxicity are higher than the doses needed to exacerbate gut toxicity [121,145]. This could be, at least in part, caused by the different fate of TCS in the liver and gut tissues: in the liver, the dominant metabolites are glucuronide- and sulfate-conjugates, which are usually biologically inactive; in the gut, the dominant metabolites are free-form TCS species, which have direct and potent effects on both colon epithelial cells and gut bacteria to induce a pro-inflammatory phenotype. Indeed, in our study, after a 4-week exposure to TCS in mice, the average concentration of free-form TCS (the putative biologically active compound)

was ~7.3 pmol/mg tissue in the liver versus ~282.8 pmol/g tissue in the colon digesta,

suggesting an accumulation of toxic compounds in the colon which could result in colon-

specific toxicology. Besides TCS, many other environmental toxicants and pollutants

could also be metabolically re-activated by gut microbiota and result in gut-specific

toxicity, highlighting the critical importance of evaluating the impacts of environmental

exposure on gut health.

In summary, our results, using LC-MS/MS profiling in mouse models and human

subjects, as well as *in vitro* culture of gut bacteria, support that gut bacteria mediates

unique metabolic reactions of environmental toxicant TCS in the gut, resulting in

metabolic re-activation of TCS and potentially gut-specific toxicity. Based on our study,

it is of critical importance to better understand the toxicology of environmental toxicants

and pollutants in the gut tissues.

Table 2.1. Sequences of primers for qPCR analysis.

Gene	Forward primer sequence	Reverse primer sequence
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Il6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Ifng	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
Il1b	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Mcp1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
16S rRNA	CCTACGGGTGGCTGCAG	GACTACTAGGGTATCTAATCC
B. breve	ATGGCAAAACCGGGCTGAA	GCGGATGAGAGGTGGG
L. brevis	CTTGCACTGATTTTAACA	GGGCGGTGTGTACAAGGC
Primers for sequencing		
16S rRNA	TCGTCGGCAGCGTCAGATGTGTAT AAGAGACAGCCTACGGGNGGCWGC AG	GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACHVGGGTAT CTAATCC

Table 2.2. Instrumental method for the quantification of TCS, TCS glucuronide, and TCS sulfate.

Instrument	Thermo Scientific Dionex Ultimate 3000 UHPLC system coupled with a TSQ Quantiva Triple Quadrupole Mass Spectrometer
Analytical column and temperature	ACQUITY UPLC C18 column (1.7 µm particles, 2.1 mm × 100 mm, Waters), 30 °C
Mobile phases	A. Acetonitrile B. Ammonium acetate (2 mM) in water

Gradient profile	Time (min)	Percentage A (%)	Flow rate (mL/min)
	0.0	15	0.30
	1.0	15	0.30
	2.5	80	0.30
	5.0	80	0.30
	5.5	100	0.30
	6.5	100	0.30
	7.0	15	0.30
	9.0	15	0.30

Injection volume	10 µL
MS scan mode	Multiple reaction monitoring (MRM)

Monitored MRM transitions	Analytes	MRM transition (m/z)	Collision Energy (eV)
	TCS	$286.89 \rightarrow 35.22$	16
		$288.89 \rightarrow 35.22$	16
	$^{13}C_{12}$-TCS	$299.00 \rightarrow 35.22$	16
		$301.00 \rightarrow 35.22$	16
	TCS glucuronide	$463.00 \rightarrow 175.00$	15
		$463.00 \rightarrow 287.00$	15
	TCS sulfate	$366.89 \rightarrow 286.89$	15
		$368.89 \rightarrow 288.89$	15

MS/MS parameters	Electrospray ionization (ESI): negative ionization mode; Capillary voltage (kV) = 2.5; Sheath gas (arbitrary units) = 40; Auxiliary gas (arbitrary units) = 10; Ion transfer tube temperature (°C) = 350; Vaporizer temperature (°C) = 300.

Table 2.3. Instrumental method for the identification of hydroxyl-TCS, hydroxyl-TCS glucuronide, and hydroxyl-TCS sulfate.

Instrument	Thermo Scientific Dionex Ultimate 3000 UHPLC system coupled with an Orbitrap Fusion Tribrid Mass Spectrometer (UHPLC–Orbitrap Fusion MS)
Analytical column and temperature	ACQUITY UPLC HSS T3 column (1.7 µm particles, 2.1 mm × 100 mm, Waters), 30 °C
Mobile phases	A. Methanol B. Ammonium acetate (5 mM) in water

Gradient profile	Time (min)	Percentage A (%)	Flow rate (mL/min)
	0.0	10	0.30
	1.0	10	0.30
	6.0	80	0.30
	9.0	80	0.30
	10.0	100	0.30
	12.0	100	0.30
	12.5	10	0.30
	15.0	10	0.30

Injection volume	10 µL
MS scan mode	Targeted MS^2

Ion transitions for metabolite identification	Analytes	Parent ion (*m/z*)	Product ion (*m/z*)
	hydroxyl-TCS	302.9388	266.9627, 160.9561, 156.9703
	hydroxyl-TCS glucuronide	478.9709	302.9388, 266.9627, 175.0254, 160.9561, 156.9703, 113.0239
	hydroxyl-TCS sulfate	382.8956	302.9388, 266.9627, 160.9561, 156.0703, 79.9574

MS/MS parameters	*Global MS parameters*
	Spray voltage (kV) = 3.0;
	Sheath gas (arbitrary units) = 50;
	Auxiliary gas (arbitrary units) = 15;
	Ion transfer tube temperature (°C) = 285;
	Vaporizer temperature (°C) = 300.
	Targeted MS^2 parameters
	Isolation widow (m/z) = 0.8;
	Activation type: high-energy collision dissociation (HCD);
	HCD collision energy (%) = 25;
	Orbitrap resolution = 30000;
	Scan range (m/z) = 50–600;
	Automated gain control (AGC) target = 5×10^4;
	Maximum injection time (ms) = 100;
	Polarity: Negative.

Table 2.4 Concentrations of free-form TCS, TCS glucuronide, and TCS sulfate in mouse tissues after treatment.
See the scheme of animal experiment in Figure 2.2A. Statistical significance of the interaction effect between antibiotics treatment (antibiotics versus water) and TCS treatment (TCS versus vehicle) on TCS metabolites was determined using two-way ANOVA.

		Treatment								
		Vehicle		Antibiotics		TCS		TCS + Antibiotics		Interaction
	TCS metabolites (pmol/mg of tissue)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	P-value
colon digesta	TCS	0.59	0.06	0.35	0.08	288.27	19.69	147.15	6.91	**< 0.001**
	TCS glucuronide	0.00	0.00	0.01	0.00	0.79	0.30	10.87	4.76	0.155
	TCS sulfate	0.00	0.00	0.01	0.00	1.74	0.24	12.94	0.72	**< 0.001**
colon mucosa	TCS	0.19	0.03	0.12	0.01	7.02	0.67	10.80	0.78	**0.015**
	TCS glucuronide	0.00	0.00	0.01	0.00	0.19	0.15	6.29	0.73	**< 0.001**
	TCS sulfate	0.06	0.02	0.24	0.08	7.85	0.60	20.73	1.03	**< 0.001**
small intestine digesta	TCS	0.26	0.06	0.19	0.03	33.39	3.79	30.77	1.20	0.660
	TCS glucuronide	0.04	0.01	0.03	0.00	50.07	7.78	91.48	5.89	**0.007**
	TCS sulfate	0.05	0.03	0.01	0.00	6.97	0.61	9.07	0.20	**0.030**
small intestine mucosa	TCS	0.37	0.06	0.36	0.09	34.64	4.62	38.53	3.27	0.636
	TCS glucuronide	0.02	0.00	0.02	0.00	36.38	5.73	58.75	5.55	0.063
	TCS sulfate	0.11	0.02	0.02	0.00	8.35	0.72	13.68	0.42	**< 0.001**
liver	TCS	0.04	0.00	0.04	0.00	7.25	0.68	12.01	0.72	**0.003**
	TCS glucuronide	0.01	0.00	0.01	0.00	1.41	0.21	4.89	0.46	**< 0.001**
	TCS sulfate	0.04	0.01	0.09	0.01	28.01	2.46	43.65	3.06	**0.011**
	TCS	3.09	0.48	1.88	0.40	26.74	3.43	33.09	3.49	0.294

bile	TCS glucuronide	0.05	0.01	0.05	0.01	142.27	22.13	276.15	35.60	**0.036**
	TCS sulfate	0.13	0.03	0.05	0.01	69.40	5.26	105.20	6.41	**0.006**
heart	TCS	0.07	0.01	0.09	0.01	0.90	0.10	0.66	0.06	0.137
	TCS glucuronide	0.00	0.00	0.01	0.01	1.23	0.15	2.14	0.37	0.122
	TCS sulfate	0.01	0.00	0.27	0.18	8.79	0.52	11.83	0.37	**0.006**

Table 2.5. Peak areas of Hydroxyl-TCS, Hydroxyl-TCS glucuronide, and Hydroxyl-TCS sulfate in mouse tissues after treatment.

See the scheme of animal experiment in Figure 2.2A). Statistical significance of the interaction effect between antibiotics treatment (antibiotics versus water) and treatment (TCS versus vehicle) on Hydroxyl-TCS metabolites was determined using two-way ANOVA.

| | Hydroxyl-TCS metabolites (Peak area/mg of tissue) | Treatment | | | | | | | | Interaction *P*-value |
| | | Vehicle | | Antibiotics | | TCS | | TCS + Antibiotics | | |
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
colon digesta	Hydroxyl-TCS	24967	11521	1086	809	31197734	2000424	3979604	611500	**< 0.001**
	Hydroxyl-TCS glucuronide	4009	615	7623	2358	5240	931	1693280	203969	**< 0.001**
	Hydroxyl-TCS sulfate	21042	9132	24517	7872	10358930	1906931	36357272	3841162	**< 0.001**
colon mucosa	Hydroxyl-TCS	1246	520	783	503	969808	120281	722270	86344	0.256
	Hydroxyl-TCS glucuronide	2064	1096	11146	2014	11665	3403	1117236	202715	**< 0.001**
	Hydroxyl-TCS sulfate	168897	48036	76284	10974	4478284	659995	22891374	2897998	**< 0.001**
small intestine digesta	Hydroxyl-TCS	3193	1736	5087	2435	887549	157038	133173	10319	**0.003**
	Hydroxyl-TCS glucuronide	43482	7221	25840	12503	1857592	336104	820610	60299	**0.048**
	Hydroxyl-TCS sulfate	27285	9900	45878	7277	17836642	2684111	25807127	929018	0.063
small intestine mucosa	Hydroxyl-TCS	3602	2070	2294	586	534201	140727	151013	29716	0.077
	Hydroxyl-TCS glucuronide	7706	1666	8429	2928	669202	110354	158949	30356	**0.005**
	Hydroxyl-TCS sulfate	30006	10757	20404	4350	11456388	1919544	19445667	1993610	0.055
liver	Hydroxyl-TCS	1699	1292	4712	1749	113643	31205	87821	10827	0.550
	Hydroxyl-TCS glucuronide	8150	2117	9799	3547	167420	48614	28723	3557	0.056
	Hydroxyl-TCS sulfate	5311	1241	5555	484	2460621	446963	4353406	457273	0.050
	Hydroxyl-TCS	34884	15102	31577	22358	347869	49864	89741	22215	**0.004**

bile	Hydroxyl-TCS glucuronide	119330	40916	145415	44422	6850477	1635666	1431632	211132	**0.030**
	Hydroxyl-TCS sulfate	111634	40925	53800	20338	40694806	1599612	56083680	2120354	**< 0.001**
	Hydroxyl-TCS	0	0	0	0	0	0	0	0	1.000
heart	Hydroxyl-TCS glucuronide	0	0	0	0	0	0	0	0	1.000
	Hydroxyl-TCS sulfate	26081	5826	6891	3191	500701	58447	550402	36282	0.494

Table 2.6. LC-MS/MS analysis of TCS metabolism by gut bacteria.

Assay	Reaction product analyzed by LC-MS/MS (expressed as peak area from MS)		
	Hydroxyl-TCS	Hydroxyl-TCS glucuronide	Hydroxyl- TCS sulfate
DMSO, w/o bacteria	<Limit of detection (LOD)	<LOD	<LOD
DMSO, w/ mouse fecal bacteria	<LOD	<LOD	<LOD
DMSO, w/ human stool bacteria	<LOD	<LOD	<LOD
DMSO, w/ bacteria from mouse small intestine	<LOD	<LOD	<LOD
DMSO, w/ bacteria from mouse cecum	<LOD	<LOD	<LOD
DMSO, w/ bacteria from mouse colon	<LOD	<LOD	<LOD
TCS (20 µM dissolved in DMSO), w/o bacteria	<LOD	<LOD	<LOD
TCS, w/ mouse fecal bacteria	<LOD	<LOD	<LOD
TCS, w/ human stool bacteria	<LOD	<LOD	<LOD
TCS, w/ bacteria from mouse small intestine	<LOD	<LOD	<LOD
TCS, w/ bacteria from mouse cecum	<LOD	<LOD	<LOD
TCS, w/ bacteria from mouse colon	<LOD	<LOD	<LOD

Note: in all assays as shown above, we did not detect the formation of hydroxyl-TCS species (while we successfully detected hydroxyl-TCS species in animal tissues, see Figure 2.2), suggesting that at least the culturable bacteria can't convert TCS to hydroxyl-TCS *in vitro*.

Table 2.7. Concentrations of free-form TCS, TCS glucuronide, and TCS sulfate in human urine after treatment.
See the scheme of the human experiment in Figure 2.9A.

Human subject #	Treatment	Treatment time (months)	TCS metabolites (pmol/µL urine)		
			TCS	TCS glucuronide	TCS sulfate
5	no TCS	0	< LOD *	< LOD	< LOD
9	no TCS	0	< LOD	5.52	0.0016
16	no TCS	0	< LOD	0.04	< LOD
14	no TCS	1	< LOD	0.41	< LOD
7	no TCS	3	< LOD	0.02	< LOD
5	no TCS	4	< LOD	< LOD	< LOD
6	no TCS	4	< LOD	< LOD	< LOD
7	no TCS	4	< LOD	< LOD	< LOD
9	no TCS	4	< LOD	< LOD	< LOD
16	no TCS	4	0.0642	0.71	< LOD
3	TCS	0	< LOD	0.18	0.0007
4	TCS	0	< LOD	0.03	< LOD
8	TCS	0	< LOD	< LOD	< LOD
12	TCS	0	< LOD	0.13	< LOD
12	TCS	1	< LOD	6.26	0.0047
12	TCS	2	0.0855	9.53	0.0052
13	TCS	3	< LOD	7.71	0.0016
3	TCS	4	0.1123	13.73	0.0069
4	TCS	4	0.0552	7.75	0.0039
13	TCS	4	0.0306	10.37	0.0026

* LOD: limit of detection

Table 2.8. LC-MS/MS semi-quantification of hydroxyl-TCS, hydroxyl-TCS glucuronide, and hydroxyl-TCS sulfate in human urine after treatment.
See the scheme of the human experiment in Figure 2.9A

Human subject #	Treatment	Treatment time (months)	TCS metabolites (Peak area/ µL urine)		
			Hydroxyl-TCS	Hydroxyl-TCS glucuronide	Hydroxyl-TCS sulfate
5	no TCS	0	< LOD	< LOD	< LOD
9	no TCS	0	< LOD	22627	68139
16	no TCS	0	< LOD	< LOD	< LOD
14	no TCS	1	< LOD	< LOD	< LOD
7	no TCS	3	< LOD	< LOD	< LOD
5	no TCS	4	< LOD	< LOD	< LOD
6	no TCS	4	< LOD	< LOD	< LOD
7	no TCS	4	< LOD	< LOD	< LOD
9	no TCS	4	< LOD	< LOD	< LOD
16	no TCS	4	< LOD	< LOD	< LOD
3	TCS	0	< LOD	< LOD	< LOD
4	TCS	0	< LOD	< LOD	< LOD
8	TCS	0	< LOD	< LOD	< LOD
12	TCS	0	< LOD	< LOD	< LOD
12	TCS	1	< LOD	5683	49464
12	TCS	2	< LOD	31321	148435
13	TCS	3	< LOD	61177	161848
3	TCS	4	< LOD	106569	66047
4	TCS	4	< LOD	8887	28240
13	TCS	4	< LOD	53570	165006

Table 2.9. Concentrations of free-form TCS, TCS glucuronide, and TCS sulfate in human stool after treatment.
See the scheme of the human experiment in Figure 2.9A.

Human subject #	Treatment	Treatment time (months)	TCS metabolites (pmol/ g stool)		
			TCS	TCS glucuronide	TCS sulfate
2	no TCS	0	14.93	< LOD	< LOD
5	no TCS	0	13.15	5.21	< LOD
6	no TCS	0	10.10	< LOD	< LOD
7	no TCS	0	23.67	< LOD	< LOD
9	no TCS	0	540.07	< LOD	< LOD
11	no TCS	0	19.95	< LOD	< LOD
14	no TCS	0	10.11	< LOD	< LOD
16	no TCS	0	57.90	< LOD	< LOD
2	no TCS	1	22.49	< LOD	< LOD
5	no TCS	1	13.96	< LOD	< LOD
6	no TCS	1	18.83	< LOD	< LOD
7	no TCS	1	9.65	4.99	< LOD
9	no TCS	1	25.43	21.75	< LOD
11	no TCS	1	< LOD	< LOD	< LOD
14	no TCS	1	14.00	< LOD	< LOD
16	no TCS	1	317.47	< LOD	< LOD
5	no TCS	2	22.69	< LOD	< LOD
6	no TCS	2	29.34	< LOD	< LOD
7	no TCS	2	19.95	< LOD	< LOD
9	no TCS	2	14.40	< LOD	< LOD
11	no TCS	2	< LOD	< LOD	< LOD

14	no TCS	2	56.73	< LOD	< LOD
16	no TCS	2	96.24	< LOD	< LOD
2	no TCS	3	11.79	17.62	< LOD
5	no TCS	3	7.44	< LOD	< LOD
6	no TCS	3	< LOD	< LOD	< LOD
7	no TCS	3	5.90	< LOD	< LOD
9	no TCS	3	7.60	< LOD	< LOD
11	no TCS	3	7.75	< LOD	< LOD
14	no TCS	3	11.01	16.46	< LOD
16	no TCS	3	105.82	< LOD	< LOD
2	no TCS	4	< LOD	< LOD	< LOD
5	no TCS	4	20.54	< LOD	< LOD
6	no TCS	4	< LOD	< LOD	< LOD
7	no TCS	4	8.75	< LOD	< LOD
9	no TCS	4	7.48	14.59	< LOD

Table 2.10. LC-MS/MS semi-quantification of hydroxyl-TCS, hydroxyl-TCS glucuronide, and hydroxyl-TCS sulfate in human stool after treatment.

See the scheme of the human experiment in Figure 2.9A.

Subject #	Treatment	Treatment time (months)	TCS metabolites (pmol/ g stool) Hydroxyl-TCS	Hydroxyl-TCS glucuronide	Hydroxyl-TCS sulfate
2	no TCS	0	< LOD	< LOD	< LOD
5	no TCS	0	< LOD	< LOD	< LOD
6	no TCS	0	< LOD	< LOD	< LOD
7	no TCS	0	< LOD	< LOD	< LOD
9	no TCS	0	10754	< LOD	< LOD
11	no TCS	0	< LOD	< LOD	< LOD
14	no TCS	0	< LOD	< LOD	< LOD
16	no TCS	0	< LOD	< LOD	< LOD
2	no TCS	1	< LOD	< LOD	< LOD
5	no TCS	1	< LOD	< LOD	< LOD
6	no TCS	1	< LOD	< LOD	< LOD
7	no TCS	1	< LOD	< LOD	< LOD
9	no TCS	1	< LOD	< LOD	< LOD
11	no TCS	1	< LOD	< LOD	< LOD
14	no TCS	1	< LOD	< LOD	< LOD
16	no TCS	1	< LOD	< LOD	< LOD
5	no TCS	2	< LOD	< LOD	< LOD
6	no TCS	2	< LOD	< LOD	< LOD
7	no TCS	2	< LOD	< LOD	< LOD

9	no TCS	2	< LOD	< LOD	< LOD
11	no TCS	2	< LOD	< LOD	< LOD
14	no TCS	2	< LOD	< LOD	< LOD
16	no TCS	2	< LOD	< LOD	< LOD
2	no TCS	3	2410	< LOD	< LOD
5	no TCS	3	< LOD	< LOD	< LOD
6	no TCS	3	< LOD	< LOD	< LOD
7	no TCS	3	< LOD	< LOD	< LOD
9	no TCS	3	554	< LOD	< LOD
11	no TCS	3	< LOD	< LOD	< LOD
14	no TCS	3	< LOD	< LOD	< LOD
16	no TCS	3	< LOD	< LOD	< LOD
2	no TCS	4	< LOD	< LOD	< LOD
5	no TCS	4	< LOD	< LOD	< LOD
6	no TCS	4	< LOD	< LOD	< LOD
7	no TCS	4	< LOD	< LOD	< LOD
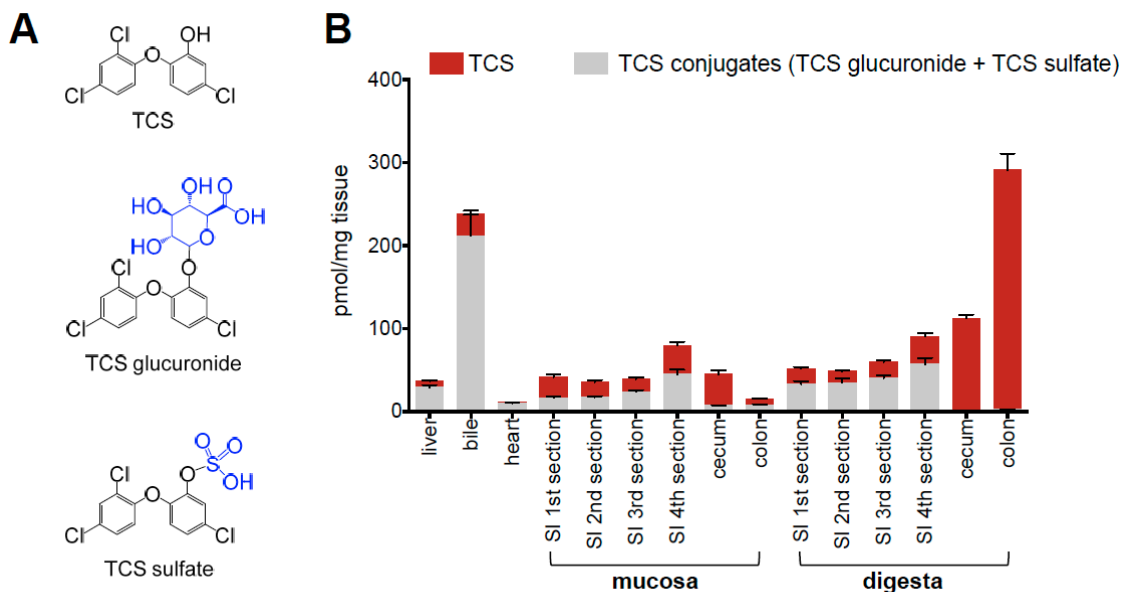
Figure 2.1. Upon TCS exposure, the dominant metabolites in most organs are TCS conjugates (TCS glucuronide and TCS sulfate), while the dominant compound in the digesta of cecum and colon is free- from TCS.

(*A*) Chemical structures of TCS, TCS glucuronide, and TCS sulfate. (*B*) LC-MS/MS quantification of the tissue concentrations of free-form TCS and TCS conjugates. SI: small intestine. The data are mean ± SEM, n = 10 mice per group.
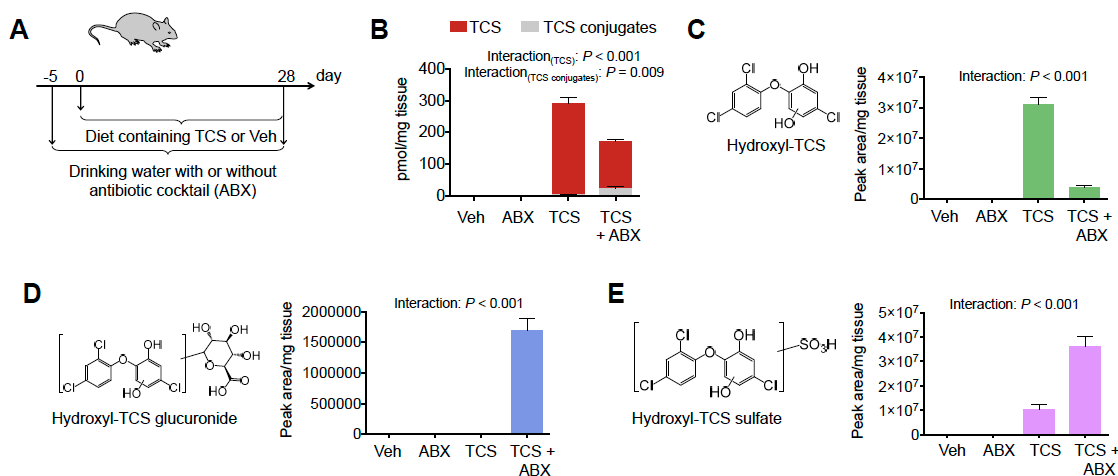
Figure 2.2. Antibiotic cocktail-mediated suppression of gut microbiota reduces concentrations of free-from TCS species, while enhancing concentrations of TCS conjugates in colon digesta.
(A) Scheme of animal experiment. (B-E) LC-MS/MS quantification of (B) free-form TCS, TCS conjugates (a combination of TCS glucuronide and TCS sulfate), (*C*) hydroxyl-TCS, (*D*) hydroxyl-TCS glucuronide, and (*E*) hydroxyl-TCS sulfate in colon digesta. The data are mean ± SEM, n = 5 mice per group for Veh or ABX group, and n = 10 mice per group for TCS or TCS + ABX group. Statistical significance of the interaction effect between ABX (ABX versus water) and treatment (TCS versus vehicle) on TCS species was determined by two-way ANOVA.
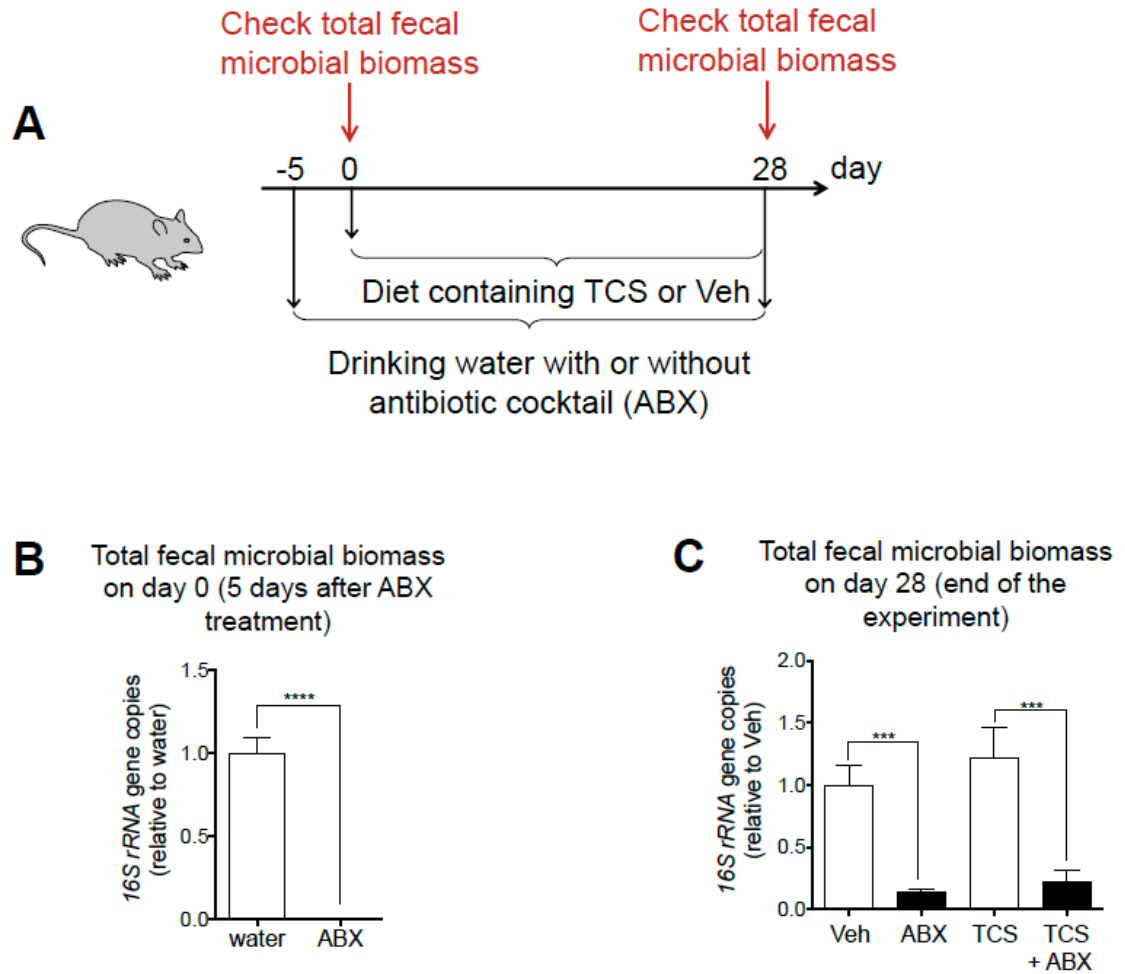
Figure 2.3 Treatment with antibiotic cocktail (ABX) ablated bacteria in colons of mice.
(*A*) Scheme of animal experiment (modified from Figure 2.2A): at day 0 (5 days after ABX treatment) and day 28 (end of the experiment), the total fecal microbial biomass was analyzed by qPCR of 16S rRNA gene. (*B*) 16S rRNA gene copies in mouse feces on day 0 (n = 20 per group). (*C*) 16S rRNA gene copies in mouse feces on day 28 (n = 10 per group). The data are mean ± SEM, ***$P < 0.001$, **** $P<0.0001$.
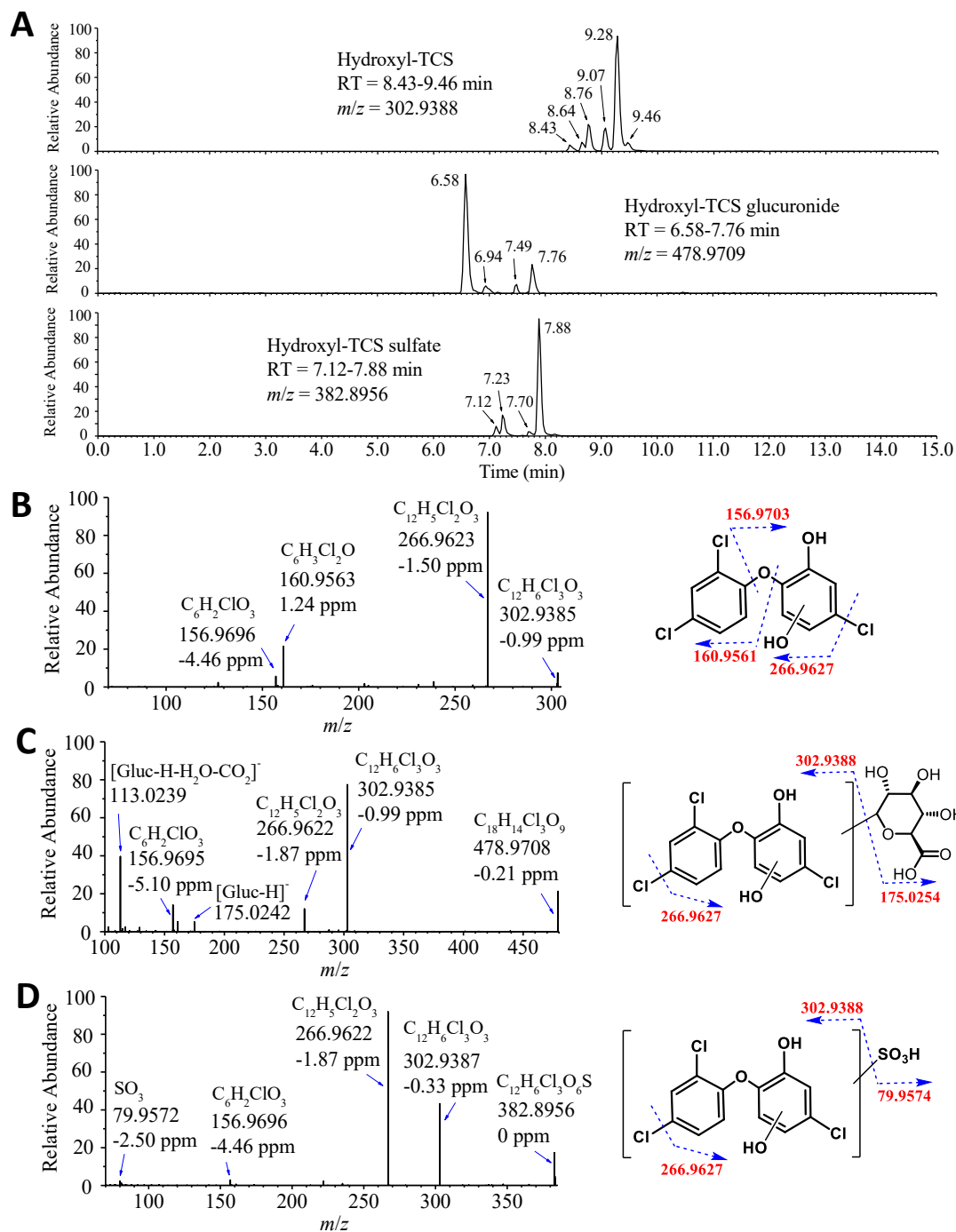
Figure 2.4. Identification of hydroxylated metabolites in colon digesta by UHPLC-Orbitrap Fusion MS.
(*A*) Chromatogram of hydroxyl-TCS, hydroxyl-TCS glucuronide, and hydroxyl-TCS sulfate. (*B-D*) Product ion spectra and proposed structures of *m/z* 302.9388 (hydroxyl-TCS, *B*), *m/z* 478.9709 (hydroxyl-TCS glucuronide, *C*), and *m/z* 382.8956 (hydroxyl-TCS sulfate, *D*).

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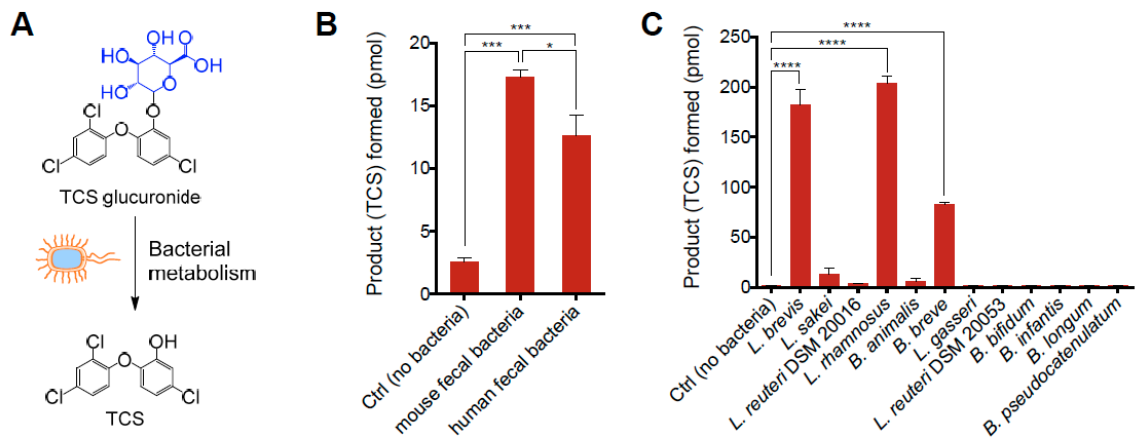
Figure 2.5. Gut bacteria mediates TCS metabolism *in vitro*.

(*A*) Scheme of experiment: TCS glucuronide was incubated with gut bacteria, and the formation of free-form TCS was quantified by LC-MS/MS. (*B*) Effects of fecal microbiota from mice or humans on converting TCS glucuronide to TCS. (*C*) Effects of pure bacterial strains on converting TCS glucuronide to TCS. The data are mean ± SEM, n = 3 per group, *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$. Statistical significance was determined using one-way ANOVA.
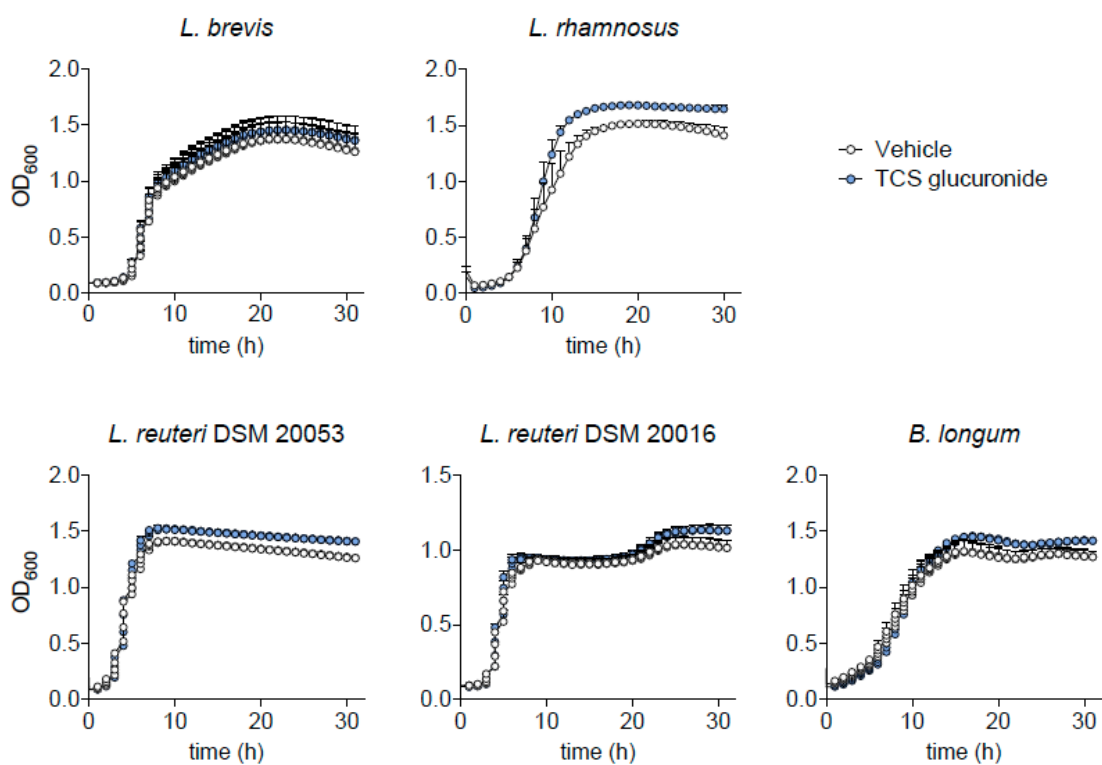
Figure 2.6. TCS glucuronide treatment has no effect on bacterial growth.

Growth curves of *L. brevis, L. rhamnosus, L. reuteri* DSM 20053, *L. reuteri* DSM 20016, and *B. longum* after 30 h treatment with vehicle or 20 µM TCS glucuronide. The data are mean ± SEM, n = 3 from three independent experiments.
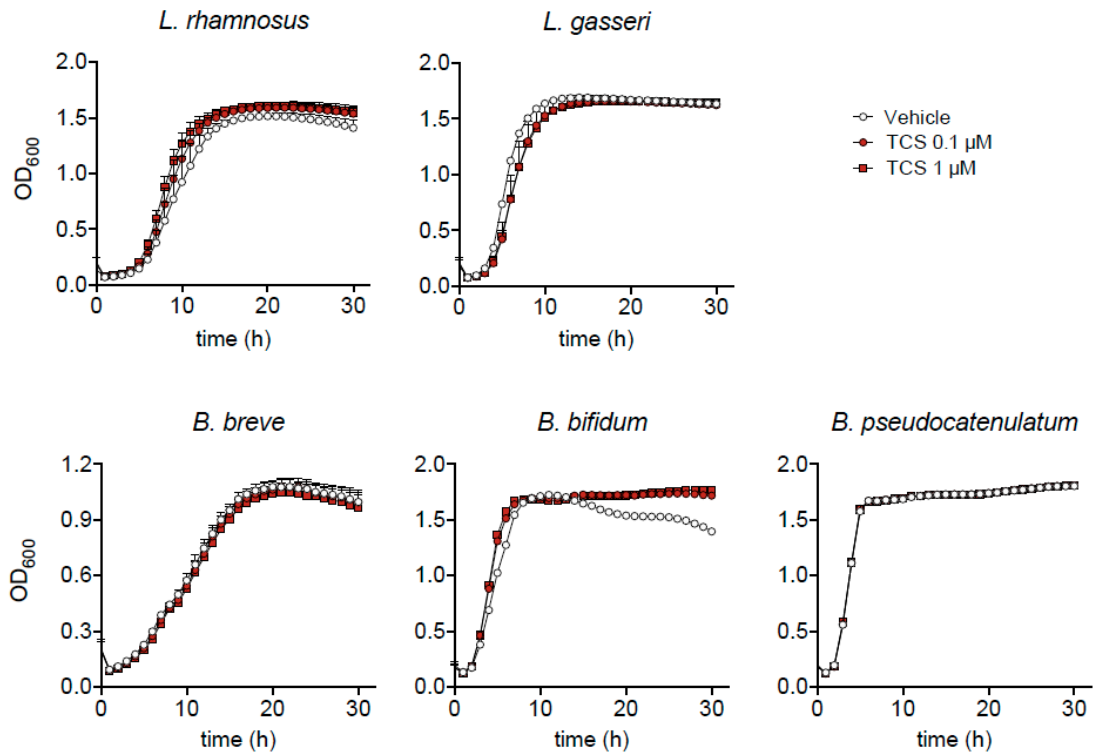
Figure 2.7. TCS treatment has no effect on bacteria growth.

Growth curves of *L. rhamnosus*, *L. gasseri*, B. breve, B. bifidum and *B. pseudocatenulatum* after 30 h treatment with vehicle, 0.1 µM, or 0.1 µM TCS. The data are mean ± SEM, n = 3 from three independent experiments.
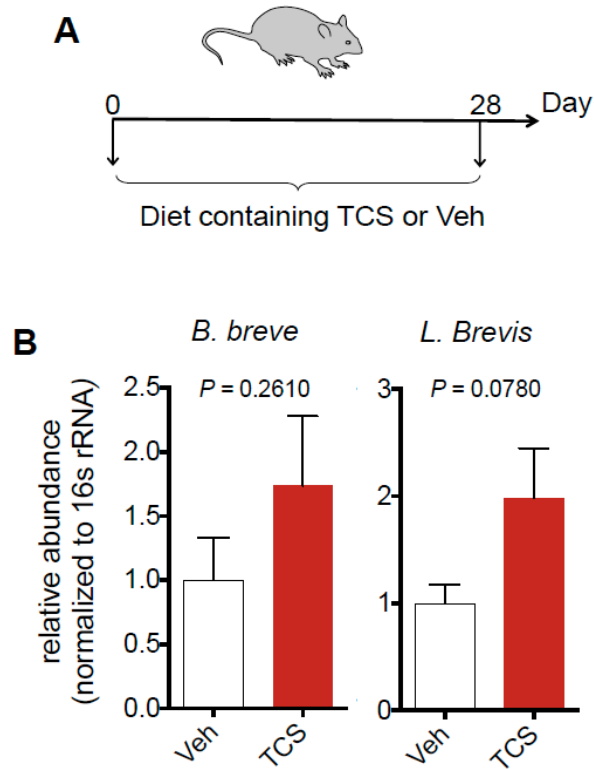
Figure 2.8. Relative abundance of certain bacterial strains after exposure to TCS in mice.

(*A*) Scheme of animal experiment (adapted from Figure 2.2A): the mice were treated with TCS (80 ppm in diet) or vehicle via diet for 4 weeks, then the relative abundance of certain bacterial strains in the mouse feces were analyzed by qPCR using strain-specific primers. (*B*) The relative abundance of *B. breve* and *L. brevis*. The data are mean ± SEM, n = 10 per group, statistical significance was determined using Student's t-test.
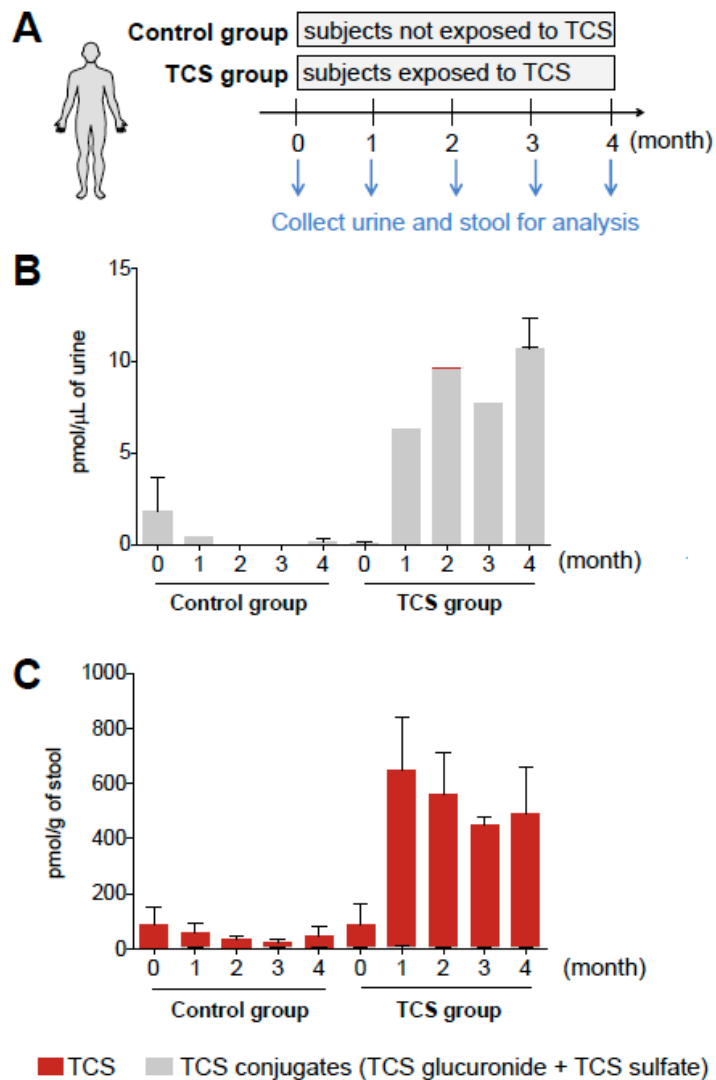
Figure 2.9. Upon TCS exposure, the dominant metabolites in human urine are TCS conjugates, while the dominant compound in human stool is free-form TCS.

(*A*) Scheme of experiment: human subjects were exposed to personal care products without TCS (control group), or exposed to personal care products containing TCS (TCS group) for 4 months. At 0, 1, 2, 3, and 4 months, the urine and stool samples were collected for analysis. (*B*) LC-MS/MS profiling of TCS metabolism in human urine. (*C*) LC-MS/MS profiling of TCS metabolism in human stool. The data are mean ± SEM.
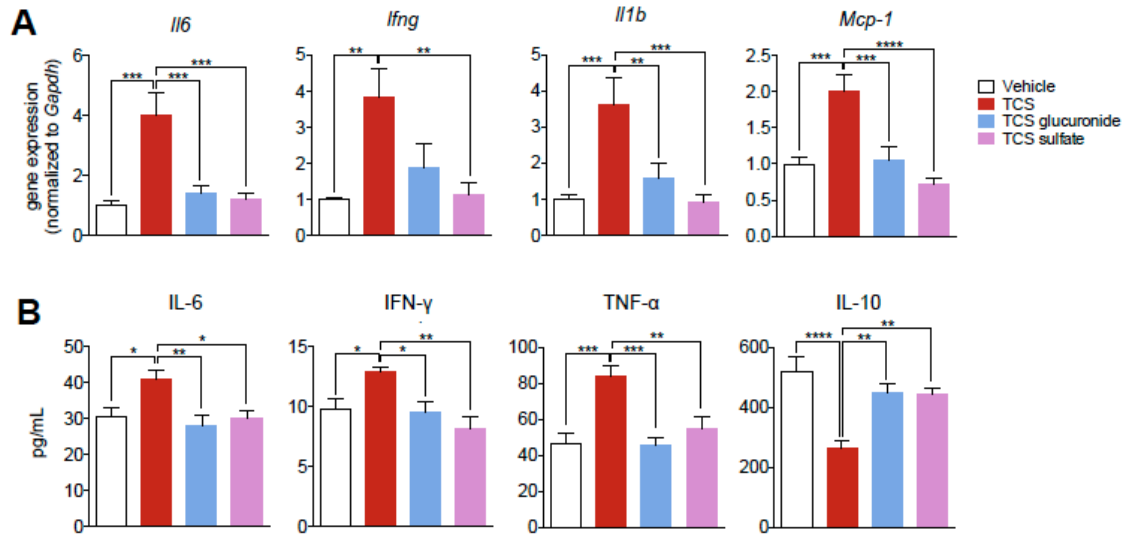
Figure 2.10. TCS, but not TCS glucuronide or TCS sulfate, increases inflammatory responses in MC38 colon cancer cells.

(*A*) Gene expression of inflammatory cytokines and (*B*) ELISA analysis of cytokines in cell culture medium after treatment with vehicle, TCS, TCS glucuronide, or TCS sulfate for 48 h. The data are mean ± SEM, n = 3 per group from three independent experiments, * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Statistical significance was determined using one-way ANOVA.
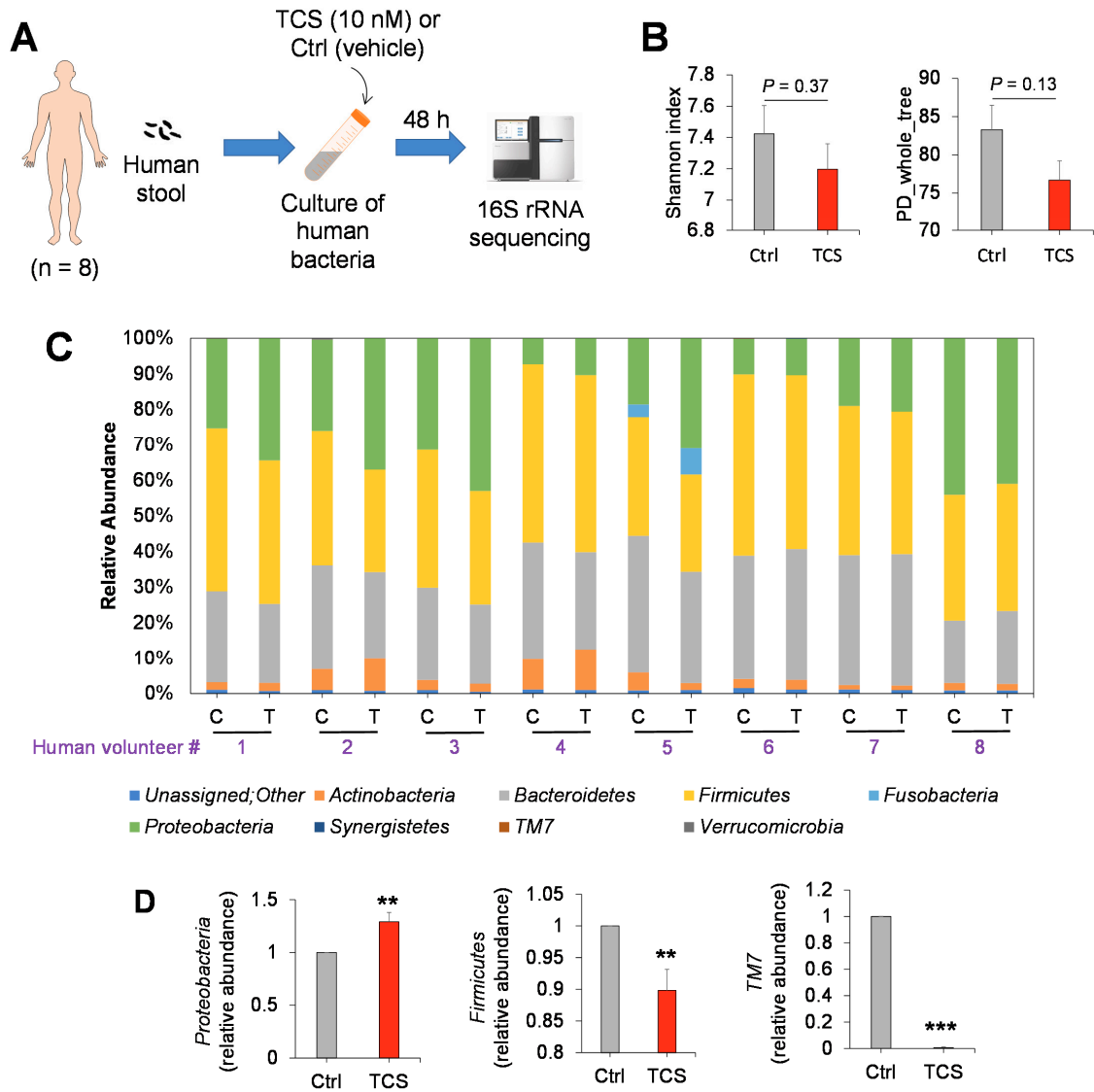
Figure 2.11. Effects of free-form TCS on human gut microbiota *in vitro*.

(*A*) Scheme of experiment. (*B*) Effects of TCS on diversity of human stool microbiota. (*C*) Individual relative abundance at phylum level (n = 8 per group). C: control group (treated with vehicle DMSO), T: treatment group (treated with 10 nM TCS). Volunteers 1-4 are male, and volunteers 5-8 are female. (*D*) Effect of TCS on relative abundance of specific bacteria phylum (n = 8 per group). The Y-axis is expressed as relative abundance of treatment group to control group for each individual volunteer. The results are mean ± SEM, ** $P < 0.01$, *** $P < 0.001$.
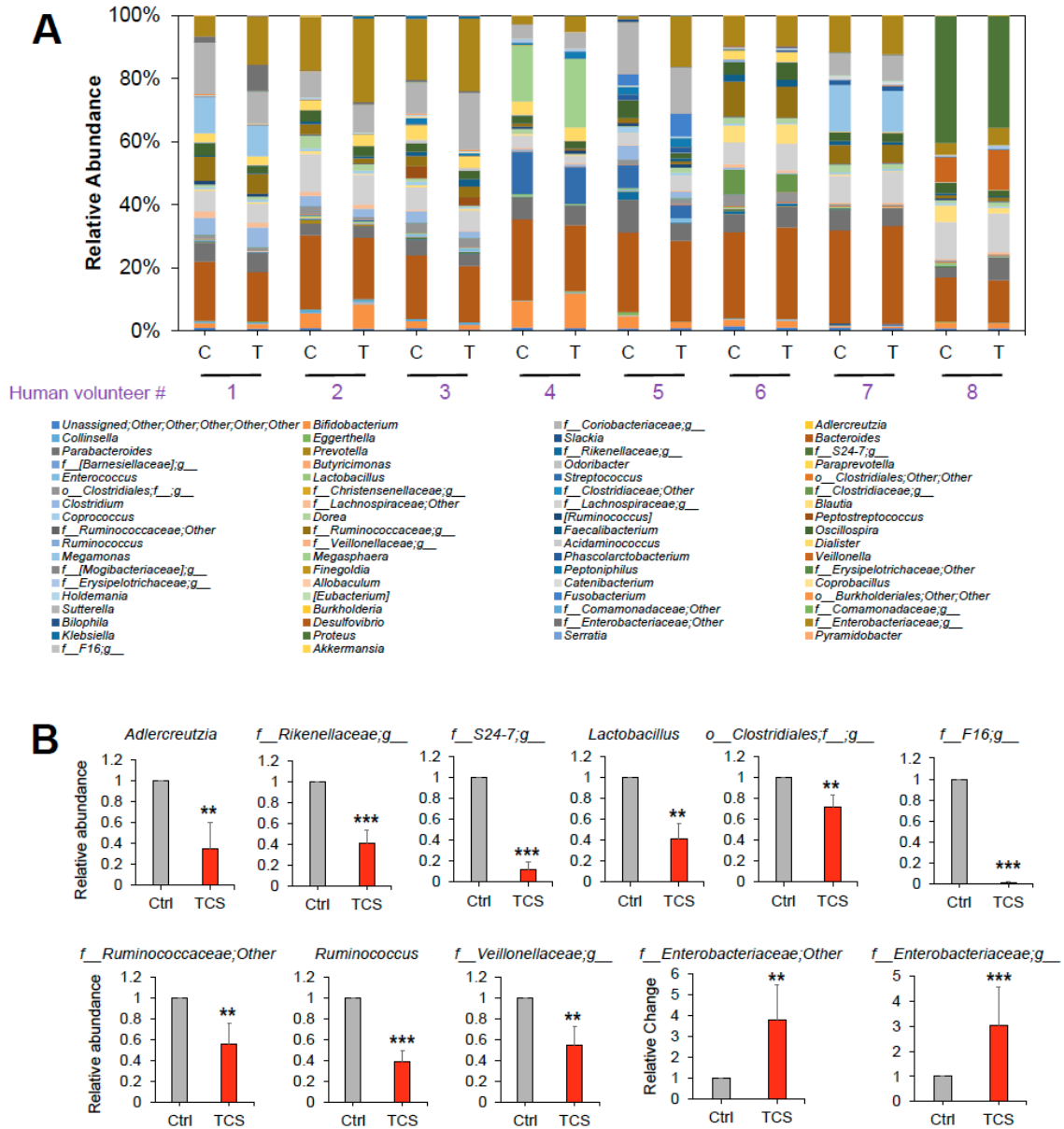
Figure 2.12. Exposure to TCS modulated composition of gut microbiota at genus levels in human fecal bacteria *in vitro*.
(*A*) Individual relative abundance at genus level (n = 8 per group). C: control group (treated with vehicle DMSO), T: treatment group (treated with 10 nM TCS). Volunteer 1-4 are male, and volunteer 5-8 are female. (*B*) Effect of TCS on relative abundance of specific bacteria genera (n = 8 per group). The Y-axis is expressed as relative abundance of treatment group to control group for each individual volunteer. The results are mean ± SEM, ** $P < 0.01$, *** $P < 0.001$.
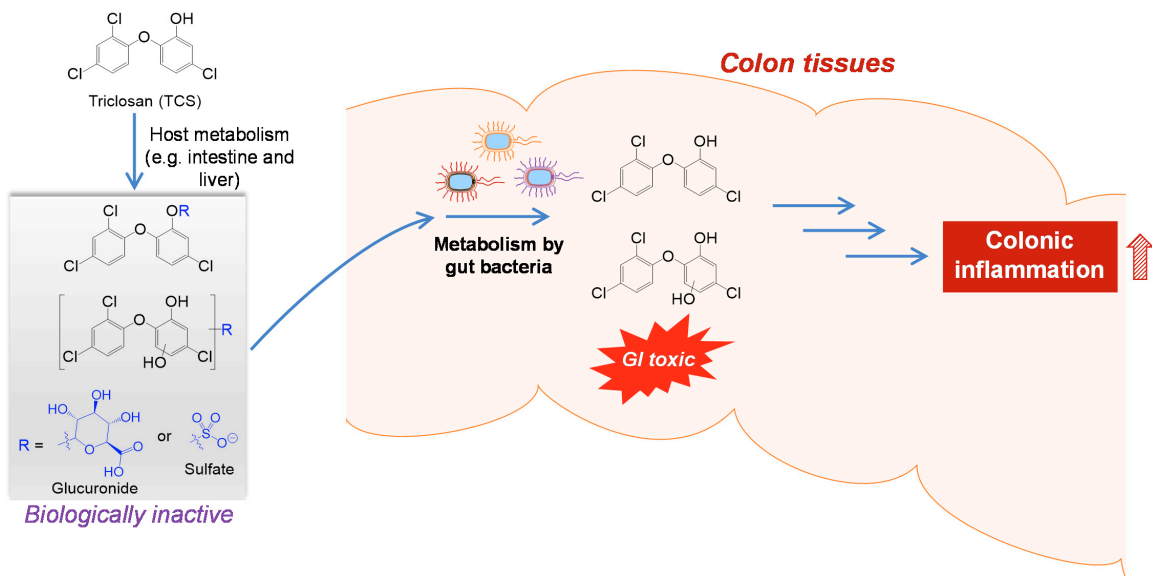
Figure 2.13. Proposed model: gut bacteria mediates colonic metabolism of TCS, leading to metabolic re- activation of TCS and resulting in enhanced colonic inflammation.

CHAPTER 3

TRICLOCARBAN EXPOSURE EXAGGERATES COLONIC INFLAMMATION AND COLON TUMORIGENESIS BY ALTERING GUT MICROBIOTA

3.1 Introduction

Triclocarban (3,4,4'-trichlorocarbanilide, TCC) has been used as an antimicrobial ingredient for more than 60 years, and is incorporated into many consumer products such as bar soaps, deodorants and detergents [8]. Each year, U.S. consumers are exposed to approximate 500,000 pounds of TCC from personal care products [8]. The 2013–2014 National Health and Nutrition Examination Survey showed that 36.9% of urine samples in the United States contained TCC [146]. The majority of used TCC is ultimately released into the environment leading to widespread pollution. As a result, TCC was listed as a top-10 contaminant in U.S. rivers [8]. More alarmingly, recent studies showed that environmental TCC could be efficiently taken up by food crops, leading to the bioaccumulation of TCC and potential human exposure through food consumption. Notably, Mathews et al. showed that some common food crops, such as broccoli, potato, beat, cabbage, and pepper, can accumulate >100 ppm TCC in the root tissues, and onions can accumulate >800 ppm TCC in the bulbs [147]. The results from this study are supported by many other investigations [148-155]. Together, the ubiquitous presence of TCC has raised concern about its impact on the environment and human health.

The regulatory policy of TCC is an intensively debated topic now. In 2016, the FDA removed TCC from over-the-counter handwashing products [156]. This decision was mainly based on recent studies which showed that compared with plain soaps, the

antimicrobial soaps containing TCC did not provide additional health benefits [157]; therefore, high-volume low-value use of TCC in handwashing products was not further allowed by the FDA [156]. This ruling only affects over-the-counter handwashing products, but TCC remains approved by the FDA and the EPA for use in many other consumer products. A better understanding of the impact of TCC on human health could be important to prepare possible further regulatory policies of this compound.

Previous studies for TCC toxicology have focused on endocrine function [158-164], however the effects of TCC on other human disorders are largely unknown. Our recent studies showed that exposure to other consumer antimicrobials, such as triclosan, benzalkonium chloride, and benzethonium chloride, exaggerates the severity of colitis and exacerbates the development of colon tumorigenesis in mouse models, through gut microbiota-dependent mechanisms [121,165]. To date, the effects of TCC on gut health are unknown. Here we studied the actions and mechanisms of TCC on colitis and colitis-associated colon tumorigenesis in animal models.

3.2 Materials and Methods

3.2.1 Animal experiments

All animal experiments were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Amherst. The mice were maintained in a standard specific-pathogen free (SPF) animal facility. To facilitate the microbiota study, after mouse arrival, the mice were rotated between different cages during the adaptation period, then the mice were

randomized to different treatment groups (2-3 mice per cage). These procedures are expected to mitigate potential cage effects on gut microbiota [166,167].

3.2.2 Dextran sodium sulfate (DSS)-induced colitis model

C57BL/6 male mice (six-week-old, Charles River, Wilmington, MA) were randomly divided into control and treatment groups (n = 8 for each group). The mice in the control group were treated with a modified AIN-93G diet (Table 3.1) containing 0.5% v/w polyethylene glycol 400 (PEG 400, EMD Millipore, Billerica, MA), and the mice in the treatment group were treated with the diet containing TCC (99%, Sigma-Aldrich, St. Louis, MO) dissolved in PEG 400 during the whole experiment. After 3 weeks, the mice were treated with 2% DSS (36–50 kDa, MP Biomedicals, Solon, OH) in drinking water to induce acute colitis. After 9 days, the mice were sacrificed, and the blood and colon tissues were collected for analysis.

3.2.3 Interleukin-10 (Il-10)$^{-/-}$ colitis model

Il-10$^{-/-}$ male mice (five-week-old, stock No. 002251, JAX, Bar Harbor, ME) were randomly divided into control and treatment groups (n = 8 for each group). The mice were fed with the modified AIN-93G diet containing PEG 400 (0.5% v/w) or 80 ppm TCC dissolved in PEG 400 during the whole experiment. Standard sterilized water was supplied in bottles *ad libitum*. After 12 weeks, the mice were treated with 200 ppm piroxicam (Sigma-Aldrich, St. Louis, MO) via diet to accelerate development of colitis [168]. After 1 week, the mice were sacrificed, and the blood and colon tissues were collected for analysis.

3.2.4 Azoxymethane (AOM)/DSS-induced colorectal tumorigenesis model

C57BL/6 male mice (six-week-old) were acclimated for 1 week and randomized into control and treatment groups (n = 16 for each group). The mice were fed with the modified AIN-93G diet containing PEG 400 (0.5% v/w) or 80 ppm TCC dissolved in PEG 400 during the whole experiment. After 3 weeks, the mice were treated with 10 mg/kg AOM (Sigma-Aldrich, St. Louis, MO) via intraperitoneal injection. After 1 week, they were given 2% DSS in drinking water for 1 week. At day 50 post the AOM injection, the mice were sacrificed, and the blood and colon tissues of the mice were collected for analysis.

3.2.5 Antibiotic cocktail-mediated suppression of gut microbiota

C57BL/6 male mice (five-week-old) were treated with drinking water with or without a broad-spectrum antibiotic cocktail (1.0 g/L ampicillin and 0.5 g/L neomycin) during the whole experiment [133,134]. After 4 days, the mice were treated with the modified AIN-93G diet containing 80 ppm TCC or vehicle (PEG 400) until the end of the experiment. After 3 weeks of diet treatment, the mice were stimulated with 2% DSS for 8 days in drinking water to induce colitis. At the end of the experiment, the mice were sacrificed, and the blood and colon tissues were collected for analysis.

3.2.6 Flow cytometry analysis

The distal colon tissues from the mice were dissected, washed with cold PBS, and digested with Hank's-balanced salt solution (HBSS, Lonza, Basel, Switzerland) supplemented with 1 mM dithiothreitol (DTT) and 5 mM ethylenediaminetetraacetic acid

(EDTA) for 2 h at 4 °C. The single cell suspensions were filtered through 70 μm cell filters (BD Biosciences, San Jose, CA). The cells were stained with APC-conjugated anti-mouse CD11c antibody, FITC-conjugated anti-mouse CD45 antibody, PE/Cy7-conjugated anti-mouse Ly-6G/Ly-6C (Gr-1) antibody, PerCP/Cy5.5-conjugated anti-mouse F4/80 antibody, and isotype control antibody according to the manufacturer's instructions (BioLegend, San Diego, CA). The stained cells were analyzed using BD LSRFortessa™ cell analyzer (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR). In our analysis, leukocytes were identified as CD45+ cells and macrophages were identified as CD45+ F4/80+ cells.

3.2.7 ELISA analysis of cytokines in plasma

The blood samples were harvested via cardiac puncture, and the plasma fractions were prepared by centrifugation of the blood at 1,500 g for 10 min at 4 ˚C. The concentrations of cytokines in plasma were determined using a CBA Mouse Inflammation Kit (BD Biosciences) according to the manufacturer's instruction.

3.2.8 RT-qPCR analysis

The colon tissues were frozen by liquid nitrogen and ground. Total RNA was isolated from the colon tissues using TRIzol reagent (Ambion, Austin, TX) according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. RT-qPCR was carried out with a DNA Engine Opticon system (Bio-Rad Laboratories, Hercules, CA) with Maxima SYBR-green

Master Mix (Thermo Fisher Scientific). The sequences of mouse-specific primers (Thermo Fisher Scientific) were listed in Table 3.2. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal control.

3.2.9 Hematoxylin and eosin (H&E) staining

The dissected colon tissues were fixed in 4% formalin (Thermo Fisher Scientific) for 24 h, embedded into paraffin (Thermo Fisher Scientific), and sliced into 5-μm sections. For H&E staining, the slides were stained with hematoxylin and eosin (Sigma-Aldrich), and examined with a light microscope. The histological scores were evaluated by blind observers according to the crypt architecture, degree of inflammatory cell infiltration, muscle thickening, and goblet cell depletion of the tissue. The histological damage score is the sum of each individual score.

3.2.10 Immunohistochemistry (IHC)

The sections were prepared and heated in 0.01 M citrate buffer (pH 6.0) for 20 minutes in a PT Module antigen retrieval device (Thermo Fisher Scientific). The antibodies against mouse PCNA and β-catenin (Cell Signaling Technology) were incubated overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated secondary antibodies were then applied to the sections, followed by chromogen 4-diaminobenzidine (Dako, Carpinteria, CA) staining. Sections were then counterstained with hematoxylin for 2 minutes. Positive expression was observed under a light microscope.

3.2.11 16S rRNA sequencing of fecal microbiota

C57BL/6 male mice (six-week-old) were maintained on a modified AIN-93G diet

containing PEG 400 (0.5% v/w) or 80 ppm TCC dissolved in PEG 400 for 3 weeks. The

feces were collected for microbiota analysis. The total fecal DNA was extracted using

QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's instruction with the

addition of the bead-beating step. The quality of the extracted DNA was measured using

a NanoDrop Spectrophotometer (Thermo Scientific) and verified using gel

electrophoresis. PCRs were performed in a 96-well format on a Veriti thermal cycler

(Life Technology) with 2 × KAPA HiFi Hotstart ReadyMix (KAPA Biosystem) using

primers specific for the V3-V4 region of the 16s rRNA gene (see Table 3.2). After

purification with AMPure XP beads (Beckman Coulter), a limited cycle PCR was

performed using the Nextera XT Index Kit (Illumina) to attach dual indices and Illumina

sequencing adapters, followed by an additional purification with AMPure XP bead. The

quantity of the purified PCR products was measured using a Qubit dsDNA BR Assay kit

(Life technology) and the amplicon quality was estimated by ScreenTape Assay on Tape

Station 2200 (Agilent). After quantification and qualification, samples were pooled in

equimolar amount and pair-end 2*300bp sequencing was performed on an Illumina

MiSeq platform using a Miseq reagent kit V3 (8% PhiX) (Illumina). The sequencing data

was processed by QIIME software pipeline v1.9.1. In general, the high-quality sequence

data (quality value $\geq$ 30) was demultiplexed. Sequences were then clustered into

operational taxonomic unites (OTUs) using Open reference OTU picking against

Greengenes bacterial 16S rRNA database (13_8 release) with a 97% similarity threshold.

The α-diversity (the diversity within sample community species richness) was determined

with ten iterations at a maximal sequence depth where all samples could be included. The β-diversity (dissimilarity among different treatment groups) was calculated using weighted and un-weighted UniFrac distances.

3.2.12 Culture of *Bifidobacterium infantis* 272

B. infantis 272 (ATCC, Manassas, VA) was subcultured at 37 °C in MRS broth (Thermo Fisher Scientific) containing 0.5 g/L L-cysteine in an anaerobic cabinet (Whitley A35 anaerobic workstation, Don Whitley Scientific, Shipley, England) under an atmosphere of 85% N_2, 10% CO_2, and 5% H_2. The *B. infantis* 272 were inoculated 1:100 into MRS broth containing TCC or DMSO vehicle and then incubated at 37 °C in anaerobic conditions for 48 h. Bacterial growth was monitored by measuring the turbidity at 600 nm.

3.2.13 Data analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical comparison of two groups was performed using Student's *t*-test or Wilcoxon-Mann-Whitney test, and comparison of three or more groups was analyzed by one-way ANOVA followed by Tukey's post hoc test. Analysis of inflammation in antibiotics-treated mouse experiments was performed by two-way ANOVA, followed by Tukey-Kramer's method, and H&E histology data was analyzed by two-way ANOVA Poisson Generalized Linear Model, followed by the Tukey-Kramer's multiple comparison method. *P* < 0.05 were considered statistically significant.

3.3 Results

3.3.1 TCC increased DSS-induced colitis in mice

To determine the effect of TCC on colonic inflammation, we studied its effect on colitis using a well-established DSS-induced colitis model in C57BL/6 mice [169]. Treatment with TCC via diet (80 ppm in diet, administering TCC at a dose of ~8 mg/kg/day, based on a diet of 3 g daily chow) exaggerated DSS-induced colitis in mice (Figure 3.1). Compared with vehicle control, treatment with TCC exacerbated body weight loss (Figure 3.1A), exacerbated colon length reduction (Figure 3.1B), increased plasma concentrations of the pro-inflammatory cytokine IL-6 (Figure 3.1C), up-regulated the gene expression of *Il-6* in the colon (Figure 3.1D), increased infiltration of leukocytes (CD45+) into the colon (Figure 3.1E), and exaggerated crypt damage in the colon (Figure 3.1F). We also tested the effect of a low-dose TCC (10 ppm in diet) and found that TCC also exaggerated DSS-induced colitis (Figure 3.2). Together, these results demonstrate the pro-colitis effect of TCC *in vivo*.

3.3.2 TCC increased colitis in *Il-10$^{-/-}$* mice

To further validate the pro-colitis effect of TCC, we tested its action on spontaneous colitis using a genetically engineered *Il-10$^{-/-}$* mouse model [168]. Treatment with TCC via diet (80 ppm in diet, administering TCC at a dose of ~8 mg/kg/day, based on a diet of 3 g daily chow) exaggerated colitis in *Il-10$^{-/-}$* mice (Figure 3.3). Compared with the vehicle control, treatment with TCC reduced the colon length (Figure 3.3A, $P < 0.05$), increased the gene expression of *Il-6* in the colon (Figure 3.3B, $P < 0.05$), enhanced infiltration of leukocytes (CD45+) into the colon (Figure 3.3C, $P < 0.01$), and

exaggerated crypt damage in the colon (Figure 3.3D, $P < 0.05$). These results further validate that exposure to TCC exaggerated colitis *in vivo*.

3.3.3 TCC increased AOM/DSS-induced colon tumorigenesis in mice

We tested the effect of TCC on colitis-associated colon tumorigenesis using a well-established AOM/DSS-induced colon cancer model in C57BL/6 mice [170]. Treatment with TCC via diet (80 ppm in diet) increased AOM/DSS-induced colon tumorigenesis in mice (Figure 3.4). Compared with the vehicle control, treatment with TCC reduced overall survival of the mice (Figure 3.4A). Regarding colon tumorigenesis, TCC increased the tumor number, tumor size, and total tumor burden in mice (Figure 3.4B, $P < 0.01$), illustrating its pro-tumorigenic effect. Consistent with enhanced colon tumorigenesis, immunohistochemical staining showed that TCC increased protein levels of proliferating cell nuclear antigen (PCNA, a marker of tumor proliferation, $P < 0.01$) and β-catenin (a marker of the pro-tumorigenic *Wnt* pathway, $P < 0.001$) in colon tumors (Figure 3.4C). In addition, qRT-PCR showed that TCC treatment increased expressions of *c-Myc* and *Axin2* (markers of *Wnt* pathway) in colon tumors (Figure 3.4D, $P < 0.05$), further supporting that TCC enhanced activation of the pro-tumorigenic *Wnt* pathway *in vivo*. Inflammation plays a central role in colon tumorigenesis [171]. Compared with the vehicle control, TCC increased gene expressions of *Il-6* and *Tnf-α* in colon tumors (Figure 3.4D, $P < 0.05$), and enhanced infiltration of leukocytes (CD45+) and macrophages (CD45+ F4/80+) into colon tumors (Figure 3.4E, $P < 0.01$), illustrating its enhancing effect on tumor inflammation. Together, these results demonstrate that exposure to TCC exaggerated colitis-associated colon tumorigenesis *in vivo*.

3.3.4 TCC reduced the diversity and changed the composition of gut microbiota in mice

Gut microbiota plays a central role in regulating colonic inflammation and colon tumorigenesis [172]. We studied the effect of TCC on gut microbiota in C57BL/6 mice. Treatment with TCC via diet (80 ppm in diet) for 3 weeks decreased the α-diversity of the gut microbiota, as assessed by PD-whole tree analysis (Figure 3.5A, $P < 0.001$), and modulated the β-diversity of the microbiota, as assessed by principle coordinate analysis (Figure 3.5B, $P < 0.01$). Regarding the composition of the gut microbiota, exposure to TCC altered the relative bacterial abundance at both phylum and genus levels (Figure 3.5C-D, Table 3.3-3.4). Notably, TCC increased the abundance of *Proteobacteria* (Figure 3.5C, Table 3.3, $P < 0.05$), which has been shown to be increased in inflammatory bowel disease (IBD) patients and is associated with the pathogenesis of IBD [137]. TCC also reduced the abundance of *Bifidobacterium* (Figure 3.5D, Table 3.4, $P < 0.05$), which has been shown to have anti-inflammatory effects [173]. Together, these results showed that exposure to TCC could cause adverse effects on gut microbiota.

3.3.5 TCC inhibited growth of *Bifidobacterium* bacteria *in vitro*

Given our findings that TCC reduced the relative abundance of *Bifidobacterium* in gut microbiota *in vivo*, we studied whether it could directly inhibit the growth of *Bifidobacterium in vitro*. Compared with the vehicle control (DMSO), TCC at a concentration of 100 nM inhibited ~30% of the growth of *B. infantis* 272 (Figure 3.6, $P < 0.05$). This result supports that TCC could have direct effects on gut bacteria.

3.3.6 TCC increased DSS-induced colitis via gut microbiota-dependent mechanisms

To validate the roles of gut microbiota in the biological actions of TCC, we tested whether antibiotic cocktail-mediated suppression of gut microbiota modulates the pro-colitis effect of TCC (see scheme of animal experiment in Figure 3.7A). We used an antibiotic cocktail from previous studies [133,134]. We found that a 5-day treatment with this cocktail caused a >99% reduction of fecal bacteria, as assessed by qPCR analysis of the *16S rRNA* gene (Figure 3.8), validating that this antibiotic cocktail suppressed gut microbiota. Before the DSS stimulation, treatment with TCC and/or the antibiotic cocktail had little impact on mouse body weight (Figure 3.9).

Regarding DSS-induced colitis, two-way ANOVA analysis showed that there was a significant interaction ($P < 0.05$) between TCC treatment (TCC versus vehicle) and antibiotic treatment (antibiotic cocktail versus no antibiotic cocktail) on colonic inflammation (Figure 3.7B-D). Notably, without antibiotic treatment, TCC exposure enhanced crypt damage in the colon while, with antibiotic treatment, the pro-colitis effect of TCC was abolished (Figure 3.7D). These results support that gut microbiota play a critical role in the pro-colitis effect of TCC *in vivo*.

3.4 Discussion

To date, the effects of TCC on human health are not well understood. Previous studies showed that TCC could be a potential endocrine-disrupting compound [158-164]; besides endocrine function, the effects of TCC on other human disorders are largely unknown. Here our central finding is that exposure to TCC exaggerated colonic inflammation and colitis-associated colon tumorigenesis in mice. We found that exposure

to relatively low-dose TCC via diet (10-80 ppm in diet, administering TCC at a dose of ~1-8 mg/kg/day, based on a diet of 3 g daily chow) increased disease developments in multiple animal models, including DSS-induced acute colitis in C57BL/6 WT mice, spontaneous colitis in genetically engineered *Il-10*$^{-/-}$ mice, and AOM/DSS-induced colon tumorigenesis in C57BL/6 WT mice, illustrating its pro-colitis and pro-neoplastic actions. A previous study showed that the no-observed-adverse-effect-level (NOAEL) of TCC was 75 mg/kg/day [174], which leads to a calculated acceptable daily intake (ADI) of TCC to be 0.75 mg/kg/day. This ADI value is comparable to the dose used in our study, as we showed that TCC at a dose of ~1 mg/kg/day exacerbated DSS-induced colitis in mice, supporting the notion that the observed adverse effects of TCC in animal experiments could mimic responses in human exposure to TCC. In addition, previous studies showed that many common food crops could accumulate 100-800 ppm TCC [147], therefore, the administration method (oral administration) and dose regime (10-80 ppm in diet) used in our studies could reflect potential human exposure to TCC. We have to point out that there are many challenges to using animal models to study human exposure to TCC: there could be significant differences when exposed to TCC via oral intake (e.g. consumption of TCC-contaminated water or food) or dermal application (e.g. usage of TCC-containing washing products), and there could be significant inter-individual variations in exposure level, absorption, and metabolism of TCC. Together, our results suggest that TCC could be a novel environmental risk factor for IBD and colitis-associated colon cancer. Due to the ubiquitous presence of TCC in our environment and possibly in our food system, it is of critical importance to better understand the actions of TCC on IBD and colitis-

associated colon cancer, in order to prepare for further regulation policies of this compound.

Our studies support that gut microbiota contributes to the pro-colitis effects of TCC. First, we found that exposure to TCC reduced the diversity of the gut microbiota in mice. This finding is in agreement with a previous study which showed that exposure to TCC caused dysbiosis in rats [175]. Previous studies have constantly shown that compared with healthy individuals, IBD patients have reduced diversity of gut microbiota, suggesting that a reduction of microbial diversity could be correlated with adverse outcomes of gut health [176]. Second, we found that exposure to TCC increased abundance of potentially harmful bacteria, and reduced abundance of beneficial bacteria in mouse gut microbiota. Notably, TCC increased the abundance of *Proteobacteria* phylum, which has been shown to be expanded in the gut microbiota of IBD patients, and associated with the pathogenesis of IBD [137]. In addition, TCC treatment caused a ~75% reduction of the abundance of *Bifidobacterium*, which has been shown to have anti-inflammatory effects [173]. We further found that treatment with TCC at a concentration of 100 nM inhibited the growth of *B. infantis in vitro*, suggesting that TCC could have a direct effect on *Bifidobacterium*. Though there is no study of colonic concentrations of TCC in humans, previous studies showed that after a routine usage of TCC-containing personal care products, the blood concentrations of TCC in humans can reach up to ~500 nM [177], supporting that the dose used in our *in vitro* experiment (100 nM) is biologically relevant. The TCC-induced changes of *Bifidobacterium* and *Proteobacteria* are consistent with the pro-colitis effect of TCC, but more studies are needed to validate the contributions of these gut bacteria in the biological actions of TCC. Finally, we showed that TCC failed to

promote DSS-induced colitis in antibiotic cocktail-treated mice, supporting that gut microbiota is required for the pro-colitis effect of TCC. We have to mention that there are limitations to using the antibiotic cocktail strategy to study the roles of gut microbiota involved [178]. More studies are needed to elucidate the functional roles of gut microbiota, as well as the mucosal immunity, in the biological actions of TCC.

In summary, here our studies showed that exposure to TCC, a widely used antimicrobial ingredient and a ubiquitous contaminant in the environment, exaggerated colonic inflammation and colitis-associated colon tumorigenesis in mice, through the modulation of gut microbiota. These results showed that TCC could be a novel risk factor for IBD and colon cancer. Further studies are needed to better characterize the impact of TCC exposure on gastrointestinal diseases in humans in order to prepare for possible further regulation of this compound.

Table 3.1. Composition of the modified AIN-93G diet used in the animal experiment.

Ingredients	g/kg
Casein	200
L-cystine	3
Sucrose	100
Dyetrose	132
Cornstarch	397.486
Cellulose	50
Mineral mix #210025	35
Vitamin mix #310025	10
Choline Bitartrate	2.5
Corn oil (purified) *	70
Vitamin A Palmitate	0.016

* All ingredients, except corn oil, were purchased from Dyets Inc (Bethlehem, PA). Because many commercial corn oil was already oxidized with varied degrees of lipid peroxidation, we used purified corn oil for our animal experiment. Briefly, commercial corn oil (Mazola®, ACH Food company, Inc., Cordova, TN) was purchased from a local market in Amherst, MA, and purified by a silicic acid-activated charcoal chromatography to remove any pre-existing oxidized compounds, then the purified oil was fortified with 400 ppm tocopherols, flushed with N_2, and stored at -80°C until use.

Table 3.2. Sequences of primers in qRT-PCR and 16S rRNA sequencing.

Gene	Forward	Reverse
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Tnf-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Il-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Ifnγ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
Tlr4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA
Il-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Il-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
C-myc	TGAAGTTCACGTTGAGGGG	AGAGCTCCTCGAGCTGTTTG
Axin 2	TGCATCTCTCTCTGGAGCTG	ACTGACCGACGATTCCATGT
16s rRNA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

Table 3.3. Effects of TCC on composition of the microbiota at phylum levels.
(* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Results expressed as relative abundance (%).

Phylum Name	Ctrl Average	SEM	TCC Average		SEM
Unassigned	1.290	0.111	0.547	***	0.092
Actinobacteria	3.505	0.930	1.091		0.509
Bacteroidetes	52.984	1.841	32.209	**	5.054
Cyanobacteria	0.734	0.163	0.137	***	0.079
Deferribacteres	1.686	0.478	0.004	***	0.002
Firmicutes	38.873	1.670	62.104	***	4.437
Proteobacteria	0.385	0.096	2.048	***	0.567
Tenericutes	0.031	0.004	0.054		0.027
Verrucomicrobia	0.511	0.193	1.806		1.231

Table 3.4. Effects of TCC on composition of the microbiota at genus levels.
The results are expressed as relative abundance (%). (* $P < 0.05$, ** $P < 0.01$, *** $P <$ 0.001)

Genus Name	Ctrl		TCC		
	Average	**SEM**	**Average**		**SEM**
Unassigned	1.290	0.111	0.547	***	0.092
Prauseria	0.004	0.001	0.012	**	0.004
Bifidobacterium	3.352	0.922	0.889	*	0.490
Adlercreutzia	0.145	0.014	0.159		0.034
Eggerthella	0.000	0.000	0.019	*	0.012
o_Bacteroidales;Other;Other	0.022	0.007	0.002	***	0.001
Bacteroides	19.876	2.123	17.980		2.163
Parabacteroides	13.855	2.009	10.540		2.958
f_Rikenellaceae;g	0.336	0.053	0.224		0.065
f_S24-7;g	13.529	3.403	0.004	***	0.002
Butyricimonas	5.362	0.358	3.451		0.935
o_YS2;f_;g	0.734	0.163	0.137	***	0.079
Mucispirillum	1.686	0.478	0.004	***	0.002
f_Bacillaceae;g	0.015	0.002	0.058	*	0.021
Staphylococcus	0.004	0.002	0.117		0.108
Enterococcus	0.020	0.007	0.036		0.023
Lactobacillus	5.887	1.179	2.831	*	1.644
Streptococcus	0.008	0.001	0.097		0.086
Turicibacter	0.139	0.053	0.008	*	0.007
o_Clostridiales;Other;Other	0.037	0.008	0.070	*	0.012
o_Clostridiales;f_;g	11.102	1.453	22.917	**	3.528
f_Christensenellaceae;g	0.110	0.014	0.084		0.020
f_Clostridiaceae;Other	0.014	0.010	0.002	*	0.002
f_Clostridiaceae;g	0.081	0.015	0.390	**	0.112
Clostridium	0.009	0.002	0.013		0.003
Dehalobacterium	0.232	0.027	0.061	***	0.017
f_Lachnospiraceae;Other	0.339	0.064	0.330		0.025
f_Lachnospiraceae;g	3.937	0.554	7.393	**	1.097
Anaerostipes	0.002	0.000	0.011	*	0.003
Blautia	0.001	0.000	0.003		0.001
Coprococcus	0.251	0.024	0.771	***	0.128
Dorea	0.273	0.117	0.541	*	0.134
Pseudobutyrivibrio	0.037	0.009	0.007	**	0.004
Roseburia	0.013	0.007	0.016		0.006
[Ruminococcus]	0.528	0.047	1.369	***	0.211
f_Peptococcaceae;g	0.058	0.012	0.046		0.017
rc4-4	1.163	0.269	1.762		0.962
*f_Peptostreptococcaceae;g_*					
_	0.010	0.006	0.027		0.027
f_Ruminococcaceae;Other	0.806	0.091	0.875		0.169
f_Ruminococcaceae;g	3.514	0.390	7.578	**	1.361

Anaerotruncus	0.013	0.004	0.019		0.007
Oscillospira	5.521	0.531	6.877		0.851
Ruminococcus	3.863	0.422	6.806		2.216
f_[Mogibacteriaceae];g	0.031	0.005	0.017	*	0.004
o_SHA-98;f_;g	0.002	0.001	0.000		0.000
f_Erysipelotrichaceae;Other	0.019	0.006	0.011		0.003
f_Erysipelotrichaceae;g	0.795	0.140	0.873		0.179
Clostridium	0.026	0.006	0.027		0.006
Coprobacillus	0.007	0.003	0.044		0.025
o_RF32;f_;g	0.125	0.040	0.199		0.092
Burkholderia	0.019	0.002	0.082	***	0.044
f_Oxalobacteraceae;Other	0.006	0.001	0.016	**	0.004
f_Enterobacteriaceae;Other	0.000	0.000	0.004		0.002
f_Enterobacteriaceae;g	0.164	0.038	1.395	***	0.458
Enterobacter	0.048	0.031	0.123		0.050
Klebsiella	0.000	0.000	0.187	**	0.127
Stenotrophomonas	0.006	0.001	0.008		0.002
o_RF39;f_;g	0.031	0.004	0.054		0.027
Akkermansia	0.511	0.193	1.806		1.231
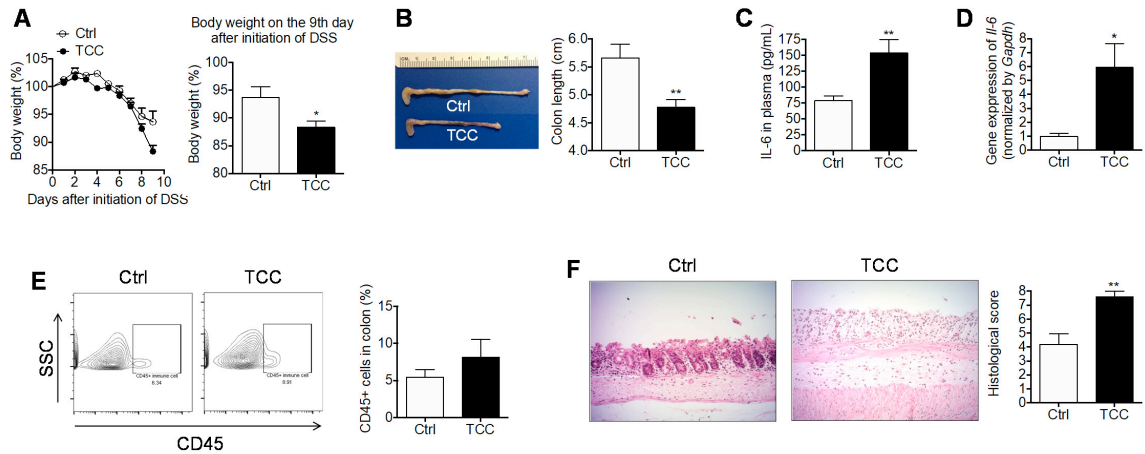
Figure 3.1. TCC increased DSS-induced colonic inflammation in C57BL/6 mice.
(A) Body weight. *Left:* time-course of body weight; *Right:* quantification of mouse body weight on the final day. (B) Colon length. (C) Concentration of IL-6 in plasma. (D) Gene expression of *Il-6* in colon. (E) FACS quantification of immune cell infiltration into the colon. (F) H&E staining of the colon. The data are mean ± SEM, * $P < 0.05$, ** $P < 0.01$, n = 8 mice per group.
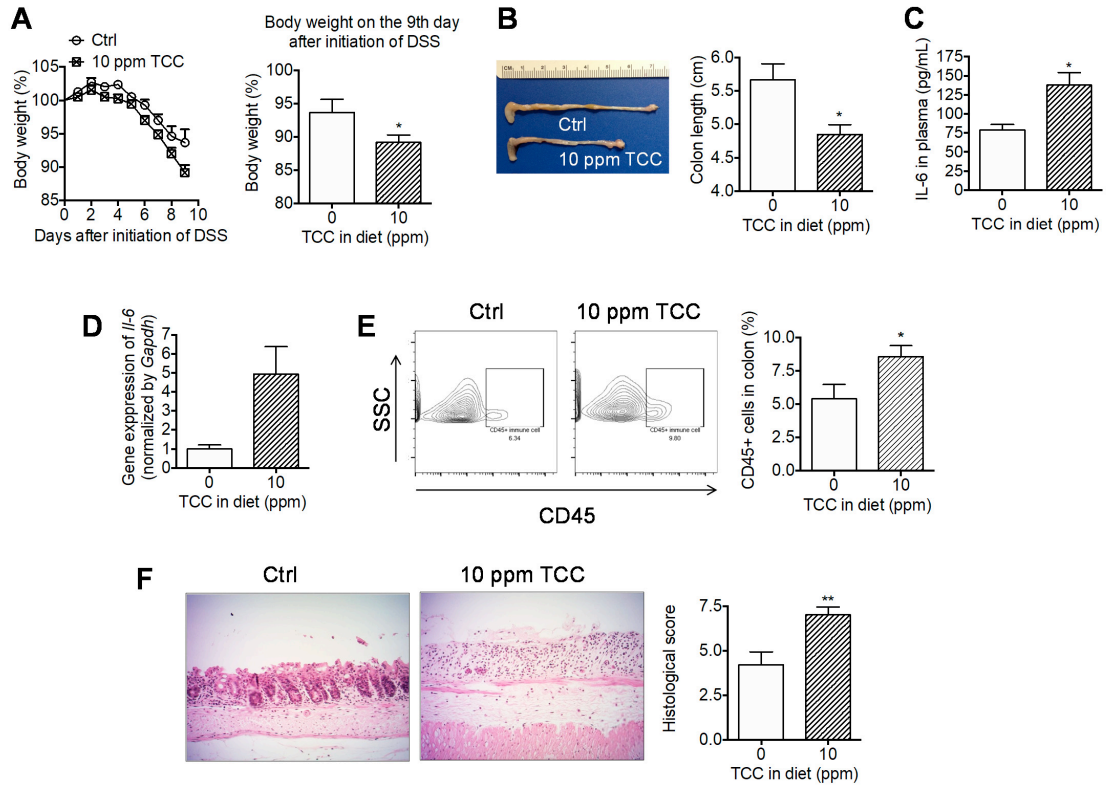
Figure 3.2. Effect of a lower-dose TCC (10 ppm in diet) on DSS-induced colitis in mice.

(A) Body weight. *Left:* time-course of body weight; *Right:* quantification of mouse body weight on the final day. (B) Colon length. (C) Concentration of IL-6 in plasma. (D) Gene expression of *Il-6* in colon. (E) FACS quantification of immune cell infiltration into colon. (F) H&E staining of the colon. The data are mean ± SEM, * $P < 0.05$, ** $P < 0.01$, n = 8 mice per group.
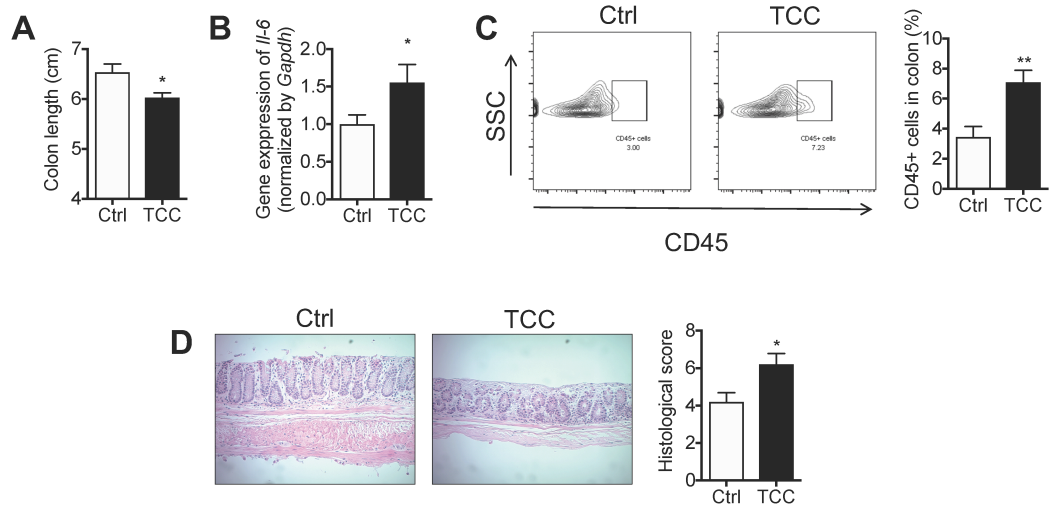
Figure 3.3. TCC increased the colonic inflammation in *Il-10*$^{-/-}$ mice.
(A) Colon length. (B) Gene expression of *Il-6* in colon. (C) FACS quantification of immune cell infiltration into the colon. (D) H&E staining of the colon. The data are mean ± SEM, * $P < 0.05$, ** $P < 0.01$, n = 8 mice per group.
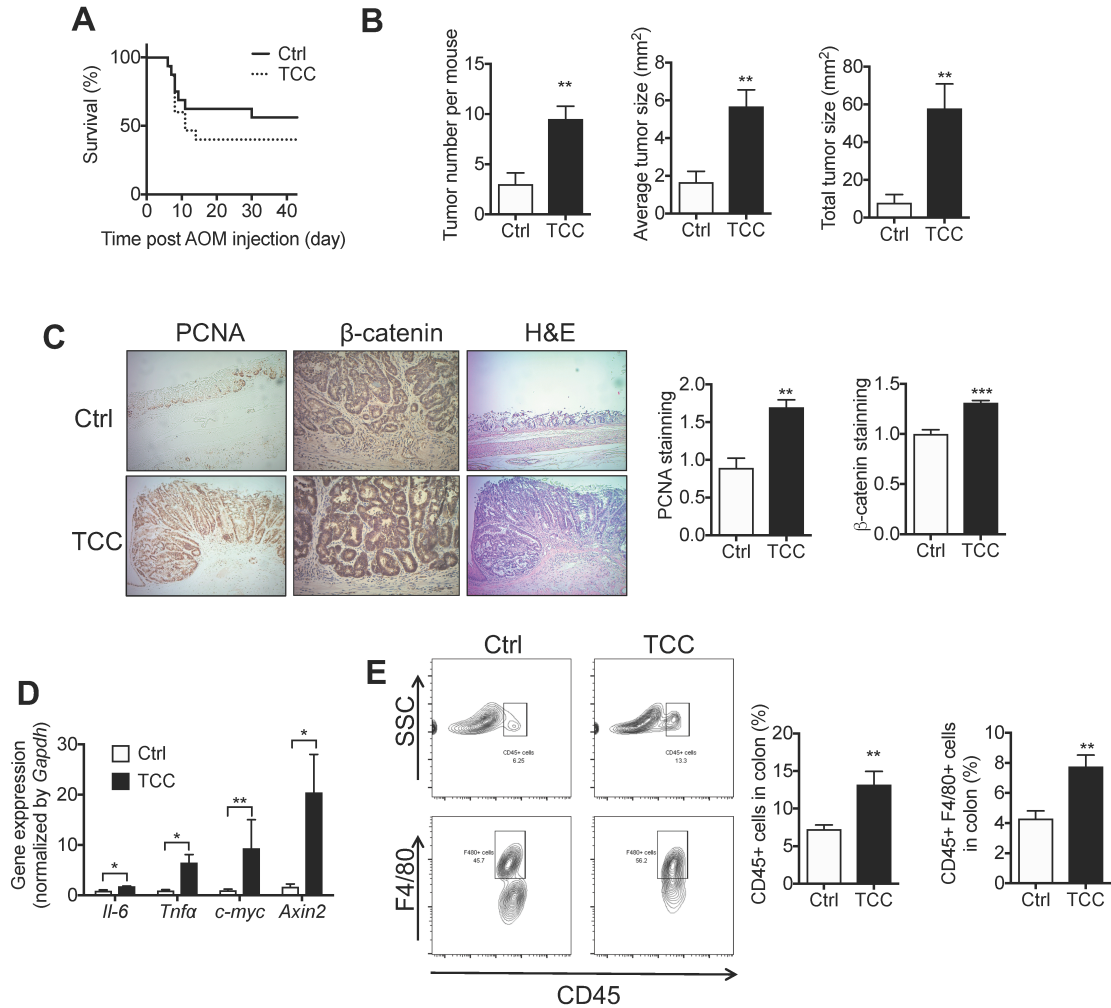
Figure 3.4. TCC increased AOM/DSS-induced colon cancer in C57BL/6 mice.
(A) Survival curve. (B) Quantification of colon tumors in mice. (C) IHC staining of
PCNA and β-catenin in colon tumors. (D) Gene expressions in colon tumors. (E) FACS
quantification of immune cell infiltration into colon tumors. The data are mean ± SEM, *
$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n = 16 mice per group.
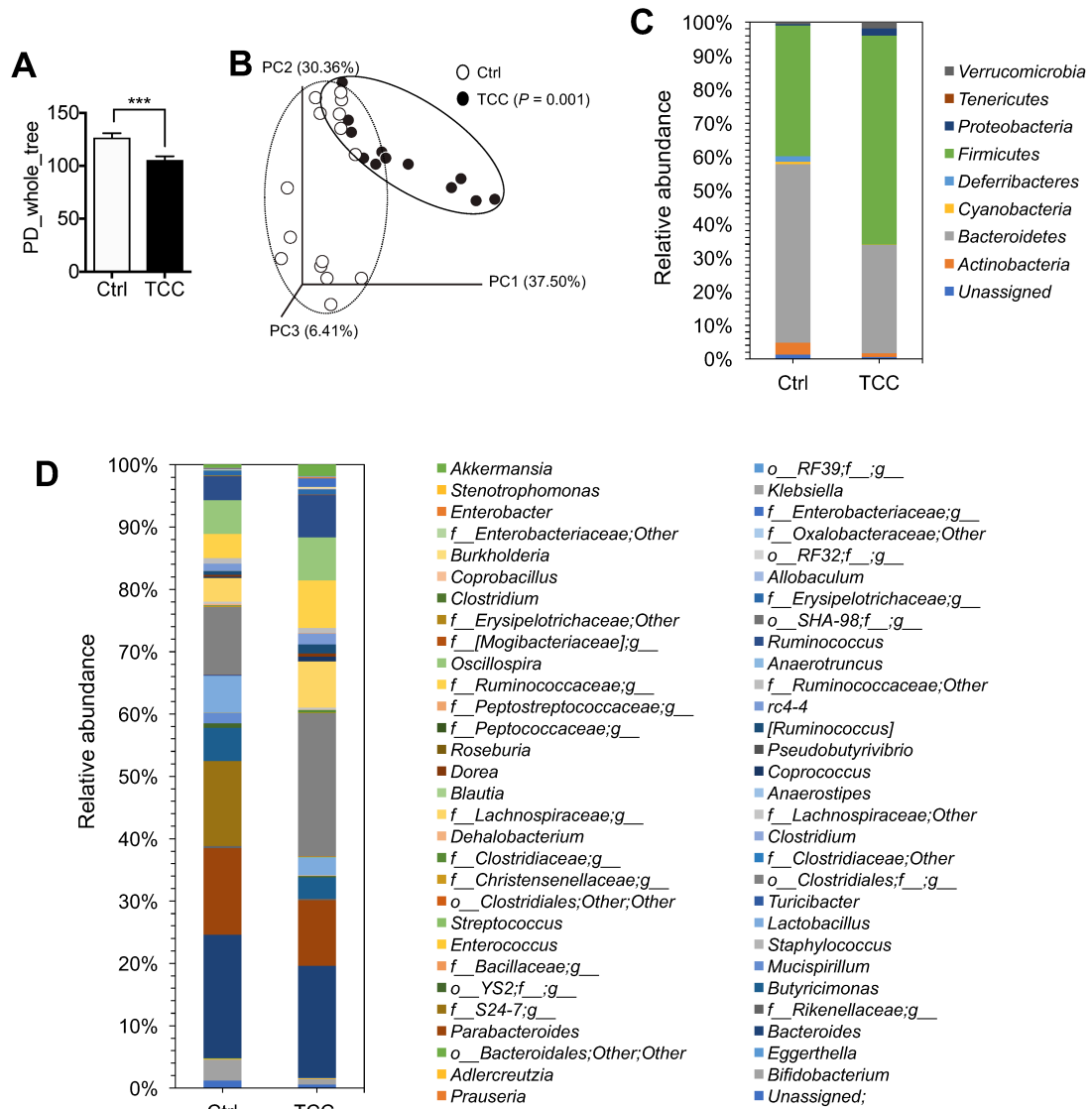
Figure 3.5. TCC reduced the diversity and altered the composition of gut microbiota in C57BL/6 mice.

(A) α-diversity of the gut microbiota. (B) β-diversity of the gut microbiota, calculated by Principle Coordinate Analysis (PCoA) based on weighted UniFrac distance. (C) Relative abundance of gut bacteria at phylum levels. (D) Relative abundance of gut bacteria at genus levels. The data are mean ± SEM, *** $P < 0.01$, n = 16 mice per group.
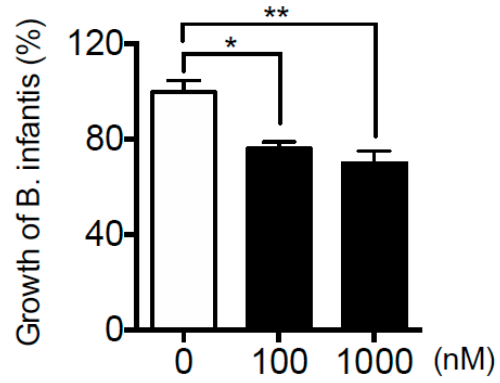
Figure 3.6. Effect of TCC on *B. infantis 272* growth.
B. Infantis 272 was treated with TCC or vehicle (DMSO) under anaerobic conditions for 48 h, then bacterial growth was analyzed by measuring the turbidity at 600 nm. The results are expressed as mean ± SEM, from three independent experiments conducted in triplicates, * $P < 0.05$, ** $P < 0.01$.
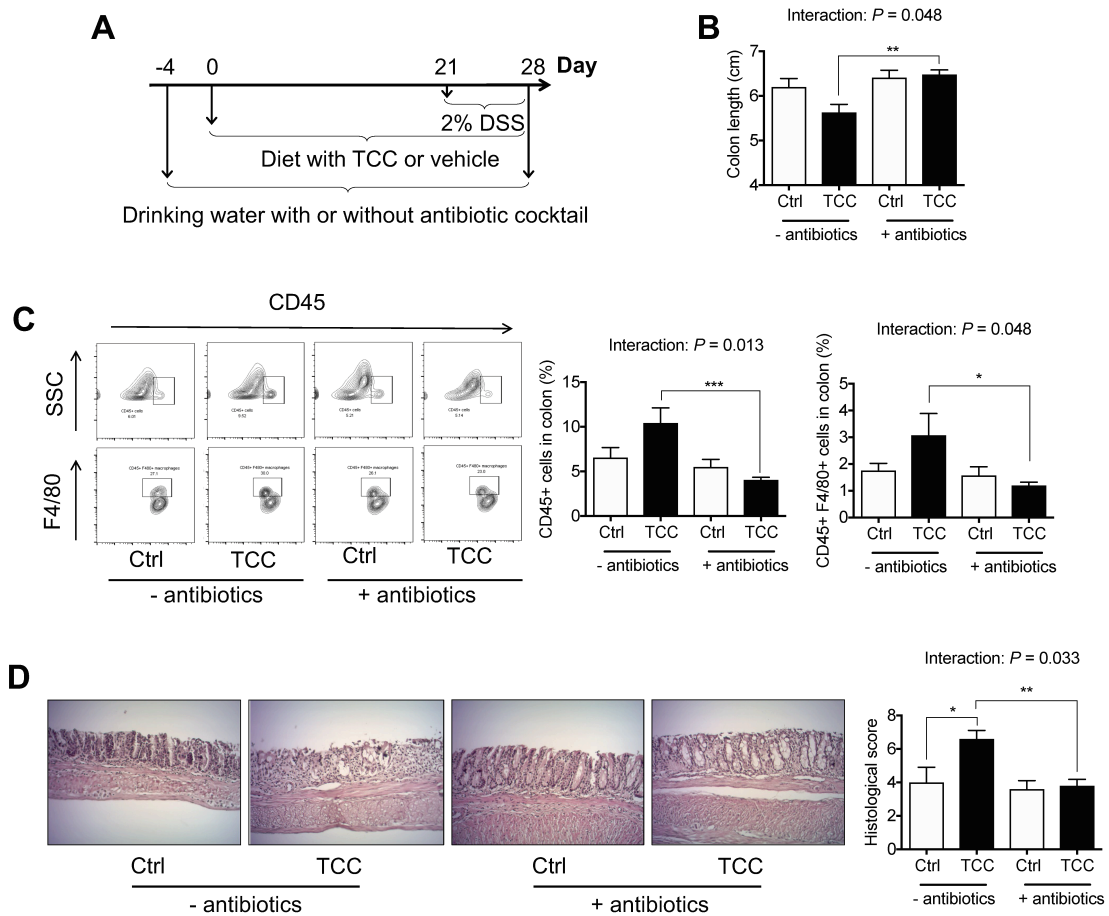
Figure 3.7. TCC increased DSS-induced colitis via gut microbiota-dependent mechanisms.
(**A**) Scheme of animal experiment. (B) Colon length. (C) FACS quantification of immune cell infiltration into colon. (D) H&E staining of colon. The data are mean ± SEM. The statistical significance (P-value) of the interaction effect between TCC treatment (TCC versus vehicle control in the diet) and antibiotic treatment (antibiotic cocktail versus no antibiotic cocktail in the drinking water) on colitis was determined by two-way ANOVA analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n = 8-10 mice per group.
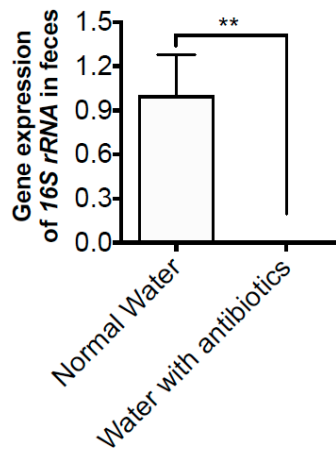
Figure 3.8. Effect of the antibiotic cocktail on fecal bacterial load.

The mice were given drinking water with or without the antibiotic cocktail (1.0 g/L ampicillin and 0.5 g/L neomycin) for 5 days. Feces were collected and *16S rRNA* gene expression in feces were analyzed by qRT-PCR (n = 7 per group). The results are expressed as mean ± SEM, ** $P < 0.01$.
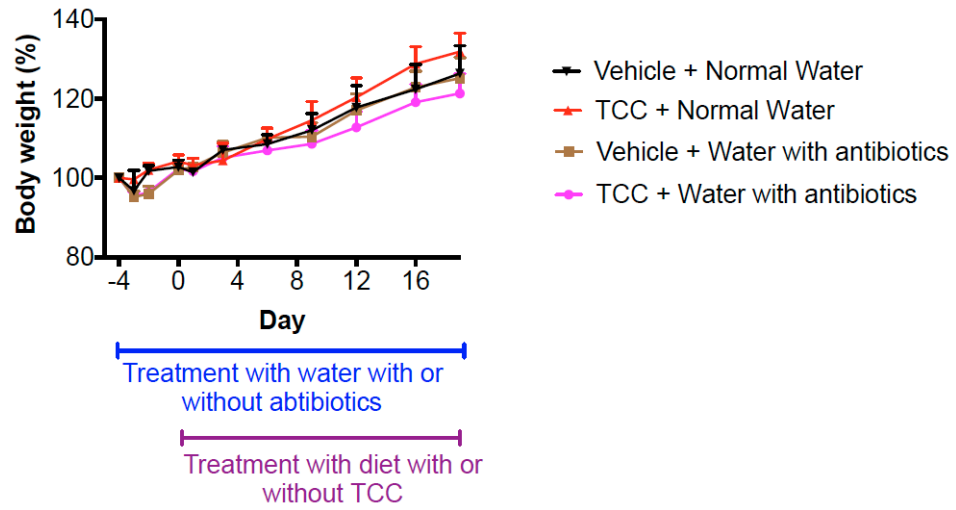
Figure 3.9. Effect of TCC and/or antibiotic cocktail on mouse body weight

Before the DSS stimulation. The results are expressed as mean ± SEM.

CHAPTER 4

EFFECTS OF CONSUMER ANTIMICROBIALS BENZALKONIUM CHLORIDE, BENZETHONIUM CHLORIDE, AMD CHLOROXYLENOL ON COLONIC INFLAMMATION AND COLITIS-ASSOCIATED COLON TUMORIGENESIS IN MICE

4.1 Introduction

Benzalkonium chloride (BAC), benzethonium chloride (BET), and chloroxylenol (PCMX) are chemicals used in high-volume as antimicrobial and antibacterial ingredients in many consumer, industrial, and medical products, including eye and nasal drops, soaps, mouthwash, and cosmetics. Previous studies have shown that these compounds are generally well-tolerated in animal models, though there are reports that these compounds could cause adverse effects, such as inflammatory responses and genotoxic effects [83-85,179], mitochondrial dysfunction [180], reproductive dysfunction [81], and developmental defects [181]. Recent studies show that these compounds, partly due to their high-volume use, are frequently detected in river water, sewage effluent, food products, and human samples of plasma and serum [37-45]. This has raised concern about their impact on the environment and human health. In September 2016, the U.S. Food and Drug Administration (FDA) removed 19 antimicrobial compounds from consumer antiseptic wash products, but deferred rulemaking for BAC, BET, and PCMX to allow additional time to develop new safety and efficacy data for these three antimicrobials [156]. Therefore, it is of critical importance to better understand the effects of these compounds on human health, producing knowledge that could have a significant impact on regulatory policies.

Inflammatory bowel disease (IBD) is characterized by colonic inflammation, and given that it affects ~1.6 million people in the U.S. and ~2.5 million people in Europe [182], IBD is a serious health problem in developed countries. IBD patients have elevated risks of developing colon cancer: it was estimated that over 20% of IBD patients could develop colon cancer within 30 years of diagnosis, and over 50% of these patients could die from colon cancer [2]. Previous studies performed in cultured cells showed that exposure to these compounds caused inflammatory responses and genotoxic effects *in vitro* [83-85]. However, to date, the effects of BAC, BET, and PCMX on inflammation and associated tumorigenesis *in vivo* are unknown. Here, we studied the impact of these three antimicrobial compounds on colonic inflammation and colitis-associated colon tumorigenesis using mouse models. Our results demonstrated that exposure to low doses of these antimicrobial compounds, in particular BAC, increased dextran sodium sulfate (DSS)-induced colonic inflammation and azoxymethane (AOM)/DSS-induced colon tumorigenesis in mice, suggesting that these antimicrobial compounds could have potentially adverse effects on gut health.

4.2 Materials and Methods

4.2.1 Chemicals

BAC (purity ≥95%, catalog # AC215411000), BET (purity 97%, catalog # AC215411000), and PCMX (purity >98.5%, catalog # AC109061000) were purchased from Thermo Fisher Scientific (Waltham, MA). Polyethylene glycol 400 (PEG 400, catalog # PX1286B-2) was purchased from EMD Millipore (Billerica, MA).

4.2.2 Animal experiments

C57BL/6 male mice (age = 6 weeks) were purchased from Charles River (Wilmington, MA) and were maintained in a standard Specific Pathogen Free (SPF) animal facility at the University of Massachusetts Amherst. All animal procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts. At Charles River, bleach- and chlorine dioxide-based disinfectants are used in the animal facility; and at UMass, hydrogen peroxide-based disinfectants are used to clean surfaces in the animal facility (biological safety cabinets, etc.) and BAC-containing products are used to clean the floor of the animal facility. Therefore, there is little or no exposure of the mice to environmental BAC or other antimicrobials.

4.2.3 DSS-induced colitis model

C57BL/6 mice were administered a modified AIN-93G diet (see diet composition in Table 4.1) containing 80 ppm BAC, BET, or PCMX (administered at a dose of ~8 mg/kg body weight/day, based on a diet of 3 g daily chow), or vehicle control (0.5% Polyethylene glycol 400) throughout the whole experiment. The diets were mixed thoroughly so that the compounds were distributed evenly and changed every 3-4 days. After 3 weeks on the diet, the mice were treated with 2% DSS (molecular weight in the range of 36–50 kDa, MP Biomedicals, Solon, OH) in drinking water for 7 days to induce colonic inflammation. At the end of DSS treatment, the mice were sacrificed for analysis of colonic inflammation.

4.2.4 Dose-response study of BAC on DSS-induced colitis

C57BL/6 mice were administered a modified AIN-93G diet containing 5-40 ppm BAC (administered at a dose of ~0.5-4 mg/kg body weight/day, based on a diet of 3 g daily chow), or vehicle control throughout the whole experiment. After 3 weeks on the diet, the mice were treated with 2% DSS in water for 7 days to induce colonic inflammation. At the end of DSS treatment, the mice were sacrificed for analysis of colonic inflammation.

4.2.5 AOM/DSS-induced colitis-associated colon cancer model

The mice were administered a diet containing 40 ppm BAC (administered at a dose of ~4 mg/kg body weight/day, based on a diet of 3 g daily chow) or vehicle throughout the whole experiment. The diets were mixed thoroughly so that the compounds were distributed evenly and changed every 3-4 days. After 3 weeks on the diet, the mice were given intraperitoneal (i.p.) injection of 10 mg/kg AOM (Sigma-Aldrich, St. Louis, MO). After 1 week, they were treated with 2% DSS in water for 1 week. On day 50 post the AOM injection, the mice were sacrificed, and blood and colon tissues of the mice were collected for analysis.

4.2.6 Flow cytometry analysis of immune cell infiltration into colon tissues

Distal colon tissues of the treated mice were dissected, washed with cold PBS, and digested with Hank's-balanced salt solution (HBSS, Lonza, Basel, Switzerland) supplemented with 1 mM dithiothreitol (DTT) and 5 mM ethylenediaminetetraacetic acid (EDTA) for 2 h at 4 °C. The released cells were filtered through 70 μm cell sorters (BD

Biosciences, San Jose, CA) to obtain single cell suspensions, which were stained with FITC-conjugated anti-mouse CD45 antibody, PE/Cy7-conjugated anti-mouse Ly-6G/Ly-6C (Gr-1) antibody, PerCP/Cy5.5-conjugated anti-mouse F4/80 antibody, and isotype control antibody (BioLegend, San Diego, CA). The stained cells were analyzed using a BD LSRFortessa™ cell analyzer (BD Biosciences, San Jose, CA), and data were analyzed using the FlowJo software (FlowJo LLC, Ashland, OR). The leukocytes were identified as CD45+ cells, neutrophils were identified as CD45+, Gr1+ cells, and macrophages were identified as CD45+, F4/80+ cells. Percentages of cells were calculated based on total single cells released from distal colon tissues.

4.2.7   ELISA analysis of cytokines

Colon tissues were cut open longitudinally, washed with PBS buffer containing penicillin (100 U/mL) and streptomycin (100 μg/mL) (Lonza, Basel, Switzerland). Distal colon tissues (25 mg) were placed in RPMI 1640 medium (Thermo Fisher Scientific) with 10% fetal bovine serum (FBS, EMD Millipore, Temecula, CA) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL), and incubated at 37 °C in an atmosphere containing 5% CO_2 for 24 h. The supernatants were collected, and cytokines in the supernatant were measured by the CBA Mouse Inflammation Kit (BD Biosciences) according to the manufacturer's instruction.

4.2.8   Hematoxylin and Eosin (H&E) staining

Dissected colon tissues were cut open longitudinally, washed in ice-cold PBS and fixed in 4% formalin (Thermo Fisher Scientific) at 4 °C. Tissues were gradually incubated with 15% and 30% sucrose (Thermo Fisher Scientific) at 4 °C, then embedded in optimal cutting temperature (OCT) compound (Fisher Healthcare, Houston, TX) and frozen for cryosectioning. OCT-fixed tissue specimens were sliced into 5 μm sections, mounted on glass slides, and air-dried for one hour. Sections were stained with H&E staining (Sigma-Aldrich) and examined with light microscopy. The histological scores were evaluated by a blinded observer and given scores according to the following measures: crypt architecture (scored 0-3 with 0 as normal and 3 as most crypt distortion and loss); inflammatory cell infiltration (0-3 with 0 as normal and 3 as most dense inflammatory infiltrate); muscle thickening (0-3 with 0 as normal and 3 being most muscle thickness); goblet cell depletion (0-1 with 0 as goblet cells present and 1 goblet cells depleted) and crypt abscess (0-1 with 0 as absent and 1 present). The histological score is the sum of each score.

4.2.9   Immunohistochemistry

Formalin-fixed tissue was embedded in paraffin by using TissueWave™ 2 Microwave Processor (Thermo Fisher Scientific), sliced to 5 μm sections on the same slide, dewaxed in serial xylene solutions (Thermo Fisher Scientific), and rehydrated through graded ethanol solutions (Pharmco-Aaper, Shelbyville, KY). Antigen retrieval was performed by heating the sections in 0.01 M citrate buffer (pH 6.0) to 95°C for 10 minutes. Samples were incubated with anti-PCNA antibody (Dako, Carpinteria, CA),

anti-cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, MA), anti-β-catenin antibody (BD Biosciences) overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated secondary antibodies were then applied to the sections, followed by the chromogen 4-diaminobenzidine staining according to the instruction of HRP/DAB (ABC) Detection IHC kit (Abcam, Cambridge, MA). Sections were then counterstained with hematoxylin for 1 min. Positive expression of PCNA, cleaved caspase-3 and β-catenin were observed under light microscope and scored using Fiji software [183]. Briefly, images of the IHC stained tissues were uploaded to Fiji, and the color was deconvoluted. The intensity numbers of the "Colour_2" image (brown staining) were converted to Optical Density (OD) numbers and then scored relative to control.

4.2.10 Quantitative reverse-transcriptase DNA polymerase chain reaction (qRT-PCR) analysis

Distal colon tissues (20 mg) were ground using liquid nitrogen. Total RNA was isolated from the colon tissues using TRIzol reagent (Ambion, Austin, TX) according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. qRT-PCR was carried out with a DNA Engine Opticon system (Bio-Rad Laboratories, Hercules, CA) with PowerUp^{TM} SYBR^{TM} Green Master Mix (Thermo Fisher Scientific). The sequences of mouse-specific primers (Thermo Fisher Scientific) are listed in Table 4.2. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression was used as an internal control.

4.2.11 Toll-like receptor 4 (TLR4) reporter cell assay

HEK-Blue™ mTLR4 cells (InvivoGen, San Diego, CA) were cultured in DMEM medium supplemented with 10% FBS and HEK-Blue™ Selection solution (InvivoGen, a mixture of antibiotics to maintain the expression of transgenes in HEK-Blue™ mTLR4 cells) and incubated in a 37 °C incubator under an atmosphere of 5% CO2, according to the manufacturer's instructions. Briefly, to determine activation of TLR4, 20 μL of mouse plasma sample, and 180 μL of 2 x 10^4 HEK-Blue™ mTLR4 cells suspended in HEK-Blue™ Detection medium, was added into each well of 96-well plate. After incubation at 37 °C for 16 h, the production of secreted embryonic alkaline phosphatase (SEAP) was assessed by reading the absorbance at 620 nm with a plate reader (Molecular Devices, Sunnyvale, CA).

4.2.12 Quantification of LPS in plasma

Plasma LPS levels were quantified using the LPS ELISA kit (MBS261904, MyBiosource, San Diego, CA) following the manufacturer's instructions.

4.2.13 Real-time PCR (qPCR) analysis of *16S rRNA* gene in liver

DNA was extracted from mouse liver samples (~25 mg) using QIAamp DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instruction with the addition of the bead-beating step. The quality of the extracted DNA was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). DNA was subjected to qPCR analysis using a DNA Engine Opticon system (Bio-Rad Laboratories, Hercules, CA) with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific).

DNA was normalized to 5 ng/μL per reaction. The sequences the *16S rRNA* primers (Thermo Fisher Scientific) are listed in Table 4.2.

4.2.14 Data analysis

Data are expressed as the mean ± standard error of the mean (SEM). Group comparisons were carried out using one-way analysis of variance (ANOVA) or Student *t*-test. *P* values less than 0.05 were considered statistically significant.

4.3 Results

4.3.1 Exposure to BAC and BET exaggerated DSS-induced colitis in mice

To study the effects of these antimicrobials on colonic inflammation, we used a well-established DSS-induced colitis model in mice. We treated mice with BAC, BET, PCMX (80 ppm in the diet), or vehicle via diet for 21 days. During this period, treatment with the antimicrobial alone did not affect the mouse body weight (Figure 4.1). We then stimulated the mice with 2% DSS in drinking water for 7 days to induce colonic inflammation (see scheme of animal experiment in Figure 4.2).

Exposure to BAC and BET, but not PCMX, increased DSS-induced colonic inflammation in mice. Compared with vehicle-treated DSS mice, the BAC-treated DSS mice showed increased weight loss on days 6-7 after initiation of DSS stimulation ($P < 0.05$), while BET and PCMX had no such effect (Figure 4.2B). Exposure to all three antimicrobial compounds caused a reduction in colon length ($P < 0.001$, Figure 4.2C), which is a biomarker of colitis. Histology analysis showed that exposure to BAC and BET, but not PCMX, increased DSS-induced colonic inflammation, with increased crypt

damage and inflammatory cell infiltration into colon tissues ($P < 0.05$, Figure 4.2D). We next analyzed the colon at the cellular and molecular level. Immune cells and cytokines play critical roles in the development of colitis [2]. Flow cytometry analysis showed that exposure to BAC and BET, but not PCMX, increased infiltration of immune cells–including leukocytes (CD45+), macrophages (CD45+, F4/80+), and neutrophils (CD45+, Gr1+)–into colon tissues ($P < 0.05$, Figure 4.2E). ELISA analysis showed that exposure to BAC and BET, but not PCMX, increased concentrations of interleukin 6 (IL-6), a pro-inflammatory cytokine, in colonic explant ($P < 0.05$, Figure 4.2F). Consistent with the ELISA result, qRT-PCR analysis showed that exposure to BAC increased gene expression of *Il-6* in colon tissues ($P < 0.01$); BET also increased *Il-6* expression, but the effect was not statistically significant, and PCMX had no effect (Figure 4.2G). These results suggest that BAC and BET, but not PCMX, were able to increase DSS-induced colonic inflammation.

4.3.2 Dose-dependent effects of BAC on DSS-induced colitis in mice

Among the three compounds, BAC showed the most potent effect on DSS-induced colonic inflammation, and we further studied its dose-dependent effect on colonic inflammation (see scheme of animal experiment in Figure 4.3A). The results showed that exposure to 20-40 ppm of BAC in the diet (~2-4 mg/kg body weight/day) caused a reduction in colon length ($P < 0.05$, Figure 4.3B). Flow cytometry analysis showed that exposure to BAC increased immune cell infiltration into the colon in a dose-dependent manner (Figure 4.3C). These results suggest that even at lower doses, BAC was able to increase DSS-induced colonic inflammation.

4.3.3 Exposure to BAC exaggerated AOM/DSS-induced colon tumorigenesis in mice

After demonstrating that exposure to BAC showed the most potent effect on DSS-induced colonic inflammation, we studied its effect on colitis-associated colon tumorigenesis using an AOM/DSS-induced colon tumorigenesis model in mice (see scheme of animal experiment in Figure 4.4A). Compared with vehicle-treated mice, the mice treated with BAC had increased mortality: the overall mortality for the control group was 21% (3 out of 14 mice died in the control group), and mortality for the BAC group was 60% (9 out of 15 mice died in BAC group) (Figure 4.4B). Exposure to BAC increased the spleen weight, suggesting increased systemic inflammation: the spleen weight of vehicle-treated mice was 135 ± 14 mg (mean $\pm$ SEM) versus 191 ± 20 mg for BAC-treated mice ($P < 0.05$, Figure 4.4C). Exposure to BAC also increased the average tumor size (1.8 ± 0.3 cm^2 for the control group versus 3.5 ± 0.5 cm^2 for the BAC group, $P = 0.008$), yet had no effect on the numbers of tumors per mouse (8.6 ± 1.3 for the control group versus 10 ± 1.8 for the BAC group, $P = 0.539$), although the total tumor burden in mice did increase (14.8 ± 3.7 cm^2 for the control group versus 35 ± 8.8 cm^2 for BAC group, $P = 0.033$) (Figure 4.4D). The qRT-PCR analysis showed that treatment with BAC increased the gene expressions of several pro-tumorigenic genes, such as *c-Jun*, *cyclin D*, and *β-catenin*, in colon tumors ($P < 0.05$, Figure 4.4E). Consistent with the qRT-PCR result, immunohistochemistry showed that β-catenin protein levels were increased in colon tumors of BAC-treated mice ($P < 0.01$). In addition, we found that the proliferating cell nuclear antigen (PCNA) protein levels were increased ($P < 0.01$), while

cleaved caspase-3 protein levels were decreased ($P<0.001$), in BAC-treated colon tumors, which suggests that exposure to BAC increased tumor proliferation and decreased tumor apoptosis (Figure 4.4F). Together, these results demonstrate that exposure to BAC increased AOM/DSS-induced colitis-associated colon tumorigenesis in mice.

4.3.4 Exposure to BAC increased activation of Toll-like receptor 4 (TLR4) in systemic circulation

Innate immunity receptors, in particular TLR4, play critical roles in regulating colonic inflammation and associated colon tumorigenesis [184,185]. We studied the effects of BAC exposure on TLR4 signaling, by testing the potential of the plasma from BAC-treated DSS mice on the activation of TLR4. Using a TLR4 reporter cell line (HEK-Blue™ mTLR4), we found that, compared with the plasma from vehicle-treated DSS mice, the plasma from BAC-treated DSS mice caused enhanced activation of TLR4 ($P = 0.013$, Figure 4.5A). Consistent with the TLR4 reporter assay, we found that the BAC-treated DSS mice had higher plasma levels of LPS, a known ligand of TLR4 ($P = 0.040$, Figure 4.5B) [186]. In addition, we found that the liver tissues from BAC-treated DSS mice had higher levels of bacterial DNA, as assessed by qPCR analysis of *16S rRNA* gene ($P = 0.064$, Figure 4.5C). These results demonstrate that exposure to BAC enhanced translocation of bacterial products from the gut into the circulation and other organs. Previous studies showed that an enhanced level of bacterial products in the systemic circulation could be due to compromised mucosal permeability [187]. Therefore, we tested the effect of BAC exposure on the expression of colonic proteins involved in regulating intestinal barrier function. We found that compared with vehicle-treated DSS mice, the

BAC-treated DSS mice had a dramatic reduction of colonic expression of *Occludin* ($P =$ 0.006, Figure 4.5D), which encodes a protein that plays an important role in maintaining mucosal barrier function [188]. Together, these results showed that exposure to BAC enhanced activation of TLR4 in the systemic circulation, in part through impairing intestinal barrier function and enhancing bacterial products in the circulation.

4.4 Discussion

In the last century, there has been a dramatic increase in the incidence and prevalence of IBD in the United States and other countries [1]. This rapid development suggests that environmental factors could contribute to the growing incidence of IBD around the globe [3-5]. In support of this hypothesis, recent studies showed that exposure to certain dietary and environmental compounds increased the development of IBD in animal models [4,5]. Due to the increased incidences of IBD and the potential lethal consequence of IBD-associated colon cancer, it is of practical importance to discover new environmental risk factors for these diseases.

BAC, BET, and PCMX are antimicrobial compounds used in many consumer products. Previous studies showed that these compounds could cause some adverse effects, including inflammatory responses and genotoxic effects [83-85,179], mitochondrial dysfunction [180], reproductive dysfunction [81], and developmental defects [181]. In this study, we studied their effects on IBD and IBD-associated colon tumorigenesis in mouse models. Here, our central finding is that short-term exposure to low-dose antimicrobial compounds, in particular BAC and BET, increased colitis and/or colitis-associated colon tumorigenesis in mice, which suggests potential adverse effects from these compounds.

Among the tested three compounds, PCMX showed the least severe adverse effect; however, in the DSS colitis experiment, exposure to PCMX also increased DSS-induced colon length reduction. This suggests some adverse effects on gut health may result from PCMX. Our results are in agreement with previous studies that illustrated pro-inflammatory effects of BAC *in vitro*. These studies showed that treatment with BAC increased production of pro-inflammatory cytokines in immortalized human conjunctival and corneal epithelial cells, and increased phagocytosis, macrophage migration, and production of pro-inflammatory cytokines in THP-1-derived macrophages [83,84]. Therefore, the enhancing effects of BAC on colitis and colitis-associated colon cancer could be, at least in part, due to its pro-inflammatory effect. To our knowledge, this study is the first animal study to demonstrate that these antimicrobial compounds have adverse effects on gut health. A better understanding of their actions on colonic inflammation and colon cancer could help to establish regulatory policies for these compounds.

Among the three tested antimicrobial compounds, PCMX showed the least adverse effect on DSS-induced colitis, with little impact on colonic inflammation. There could be several reasons for the different effects of PCMX compared to BAC and BET. PCMX, but not BAC and BET, contains a phenolic group, which is highly prone to rapid metabolism by phase II detoxification enzymes such as glucuronosyltransferase and sulfotransferase, leading to formation of water-soluble conjugated metabolites (glucuronides and sulfates) and excretion via the urine. Indeed, previous studies in dogs showed that PCMX was rapidly metabolized to glucuronide- and sulfate- conjugates and excreted in urine [189]. Therefore, the lack of effect of PCMX on colonic inflammation in our studies could be in part due to its poor metabolic stability. In addition, compared with

BAC and BET, PCMX has a much smaller size, which could impact its absorption and duration in the gastrointestinal tract. To date, the metabolism and pharmacokinetics of these antimicrobial compounds *in vivo* are not well investigated. Further studies could help to address the structure-activity relationships of the antimicrobials, which could help to design better antimicrobial ingredients.

A critical question in this study is whether the effects observed in the animal models represent responses that would arise from human exposure. An examination of the comparability of our administered dosage to investigated Acceptable Daily Intake (ADI) levels can shed light on this question. In our experiment, we found that exposure to 40 ppm of BAC in diet (administering BAC at a dose of ~4 mg/kg/day, based on a diet of 3 g daily chow) increased colitis-associated colon tumorigenesis in mice. The dose used in our animal experiment is the lowest among reported animal studies of BAC [42,190]. To put the dose in perspective, previous studies showed that the No-Observed-Adverse-Effect Level (NOAEL) of BAC was 192 mg/kg body weight/day from a 90-day study in CD-1 mice [191], which leads to a calculated ADI of BAC of ~1.9 mg/kg body weight/day [192]. Therefore, the dose of BAC used in the animal experiments is comparable to previously investigated ADI values. This supports the biological relevance of our findings. We recognize the difficulties in translating the animal data to human studies. There are several challenges to using animal models to study human exposure to these consumer antimicrobial compounds. First, accurate assessment of the degree, type, and variation of exposure levels of these compounds in humans is largely lacking. Second, dermal exposure (e.g. by using soaps) and oral exposure (e.g. by using mouthwash or toothpaste) to these antimicrobial compounds could lead to different biological responses.

Indeed, a recent study showed that usage of antibacterial toothpaste, but not antibacterial soaps, modulated gut microbiota in humans [193]. Third, after exposure to these compounds, there could be a high degree of inter-individual variation in the metabolism and secretion of these compounds. Based on our studies, it is feasible to conclude that long-term high-dose exposure to these antimicrobial compounds could lead to adverse effects on gut health. Previous studies have shown that BAC and BET were frequently detected at high levels in grapefruit seed extract (GSE), a popular dietary supplement, despite the fact that these compounds are not permitted to use as food additives [37,38]. Notably, von Woedtke et al. showed that five out of six commercial GSE products contained 1.3-10% of BET. Frequent consumption of these contaminated dietary products could lead to high-dose exposure to BET and might cause adverse effects on gut health.

TLRs are important innate immunity receptors and play critical roles in regulating colonic inflammation and associated colon tumorigenesis [2,194]. Our results showed that during DSS-induced colitis, exposure to BAC impaired intestinal barrier function and thus enhanced translocation of bacterial products (e.g. LPS and bacteria) from the gut into the systemic circulation and other organs, resulting in enhanced activation of TLR4 in the systemic circulation. Previous studies showed that enhanced activation of TLR4 is associated with increased progression of colitis and colitis-associated colon cancer [185,195,196]. Indeed, compared with WT mice, genetically-engineered mice lacking TLR4 had attenuated, while genetically-engineered mice with overexpression of TLR4 had enhanced, the progression of DSS-induced colitis and/or AOM/DSS-induced colon cancer [184,195,196], though there are inconsistent results [197]. Together, these results support

that TLR4 signaling might play a role in the observed pro-colitis and pro-neoplastic effects of BAC.

In summary, our study showed that exposure to low-dose antimicrobial compounds, particularly BAC, increased colonic inflammation and colon cancer in mice. These studies suggest that further studies are needed to better characterize the effects of these compounds on gut health in order to develop sound regulatory policies for these compounds.

Table 4.1. Composition of the modified AIN-93G diet used in the animal experiments.

Ingredients	g/kg
Casein	200
L-cystine	3
Sucrose	100
Dyetrose	132
Cornstarch	397.486
Cellulose	50
Mineral mix #210025	35
Vitamin mix #310025	10
Choline bitartrate	2.5
Corn oil (purified) *	70
Vitamin A palmitate	0.016

* All ingredients, except corn oil, were purchased from Dyets Inc (Bethlehem, PA). Because commercial corn oil was already oxidized with varied degrees of lipid peroxidation, we used purified corn oil for our animal experiments. Briefly, commercial corn oil was purchased from a local market in Amherst, MA, and purified by a silicic acid-activated charcoal chromatography to remove any pre-existing oxidized compounds [198], then the purified oil was fortified with 400 ppm tocopherols, flushed with N_2, and stored at -80°C until use.

Table 4.2. Sequences of primers in qRT-PCR and qPCR.

Gene	Forward	Reverse
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Il-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Tlr4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA
Axin2	TGCATCTCTCTCTGGAGCTG	ACTGACCGACGATTCCATGT
C-Jun	CCTTCTACGACGATGCCCTC	GGTTCAAGGTCATGCTCTGTTT
Ki67	ATCATTGACCGCTCCTTTAGGT	GCTCGCCTTGATGGTTCCT
C-myc	TGAAGTTCACGTTGAGGGG	AGAGCTCCTCGAGCTGTTTG
CyclinD	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC
Wnt2	CTCGGTGGAATCTGGCTCTG	CACATTGTCACACATCACCCT
β-catenin	CAGCTTGAGTAGCCATTGTCC	GAGCCGTCAGTGCAGGAG
Occludin	ACGGACCCTGACCACTATGA	TCAGCAGCAGCCATGTACTC
16S rRNA	TCGTCGGCAGCGTCAGATGTGTAT AAGAGACAGCCTACGGGNGGCWGC AG	GTCTCGTGGGCTCGGAGATGTGTATA AGAGACAGGACTACHVGGGTATCTAA TCC
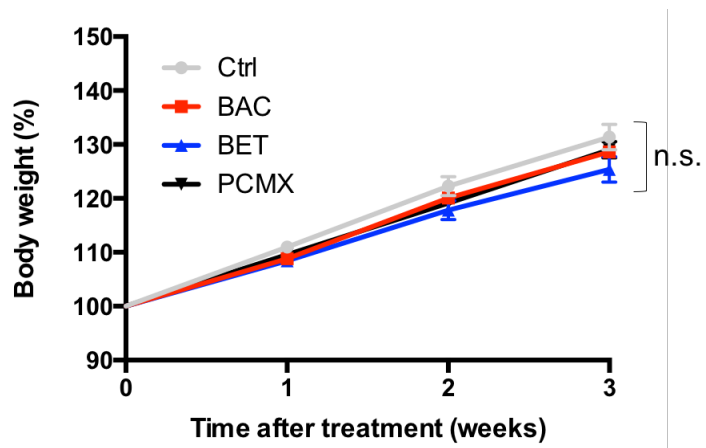
Figure 4.1. Effect of BAC, BET, and PCMX on mice body weight.
The results are expressed as mean ± SEM.
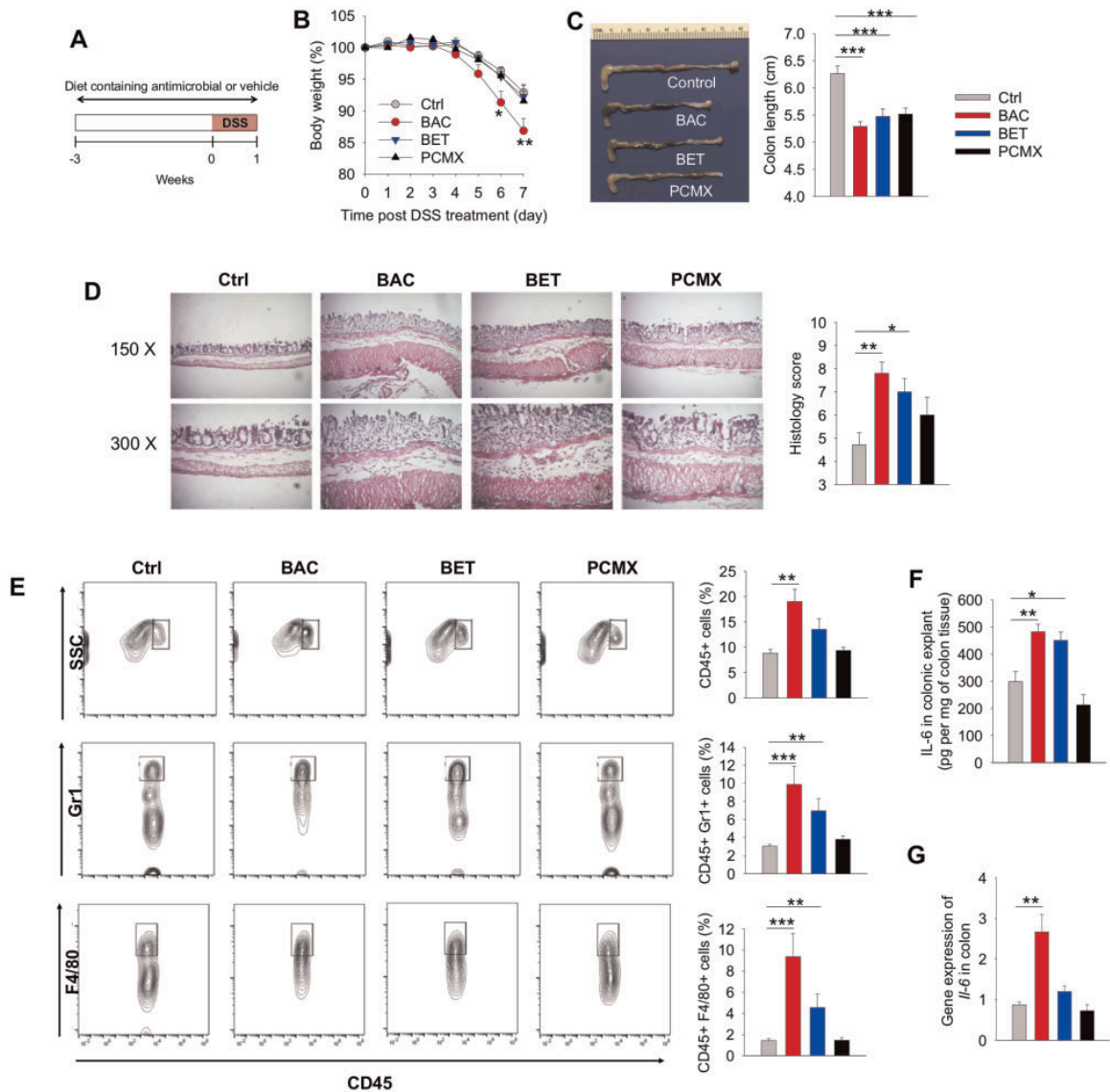
Figure 4.2. Effects of BAC, BET, and PCMX on DSS-induced colitis in C57BL/6 mice.
(A) Scheme of animal experiment. (B) Body weight. (C) Colon length. (D) Representative images of H&E staining of colon sections and histological score. (E) Quantification of immune cell infiltration into colon tissues. (F) ELISA analysis of IL-6 in colonic explant. (G) Gene expression of *Il-6* in colon tissues. The results are expressed as mean ± SEM, n = 12 mice per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
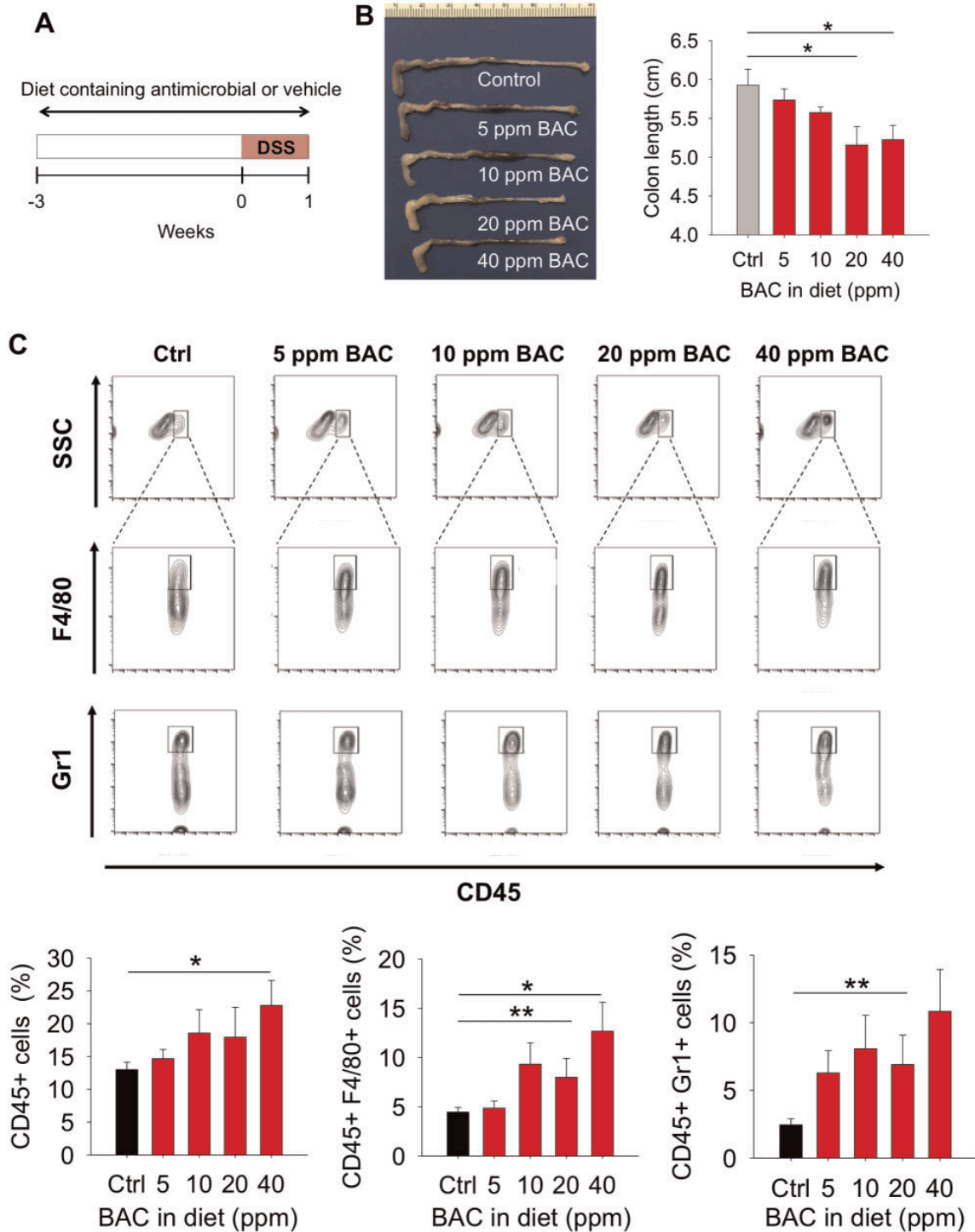
Figure 4.3. Dose-response effect of BAC on DSS-induced colonic inflammation in mice.
(A) Scheme of animal experiment. (B) Colon length. (C) Flow cytometry analysis of immune cells in colon tissues. The results are expressed as mean ± SEM, n = 8 mice per group, * $P < 0.05$, ** $P < 0.01$.

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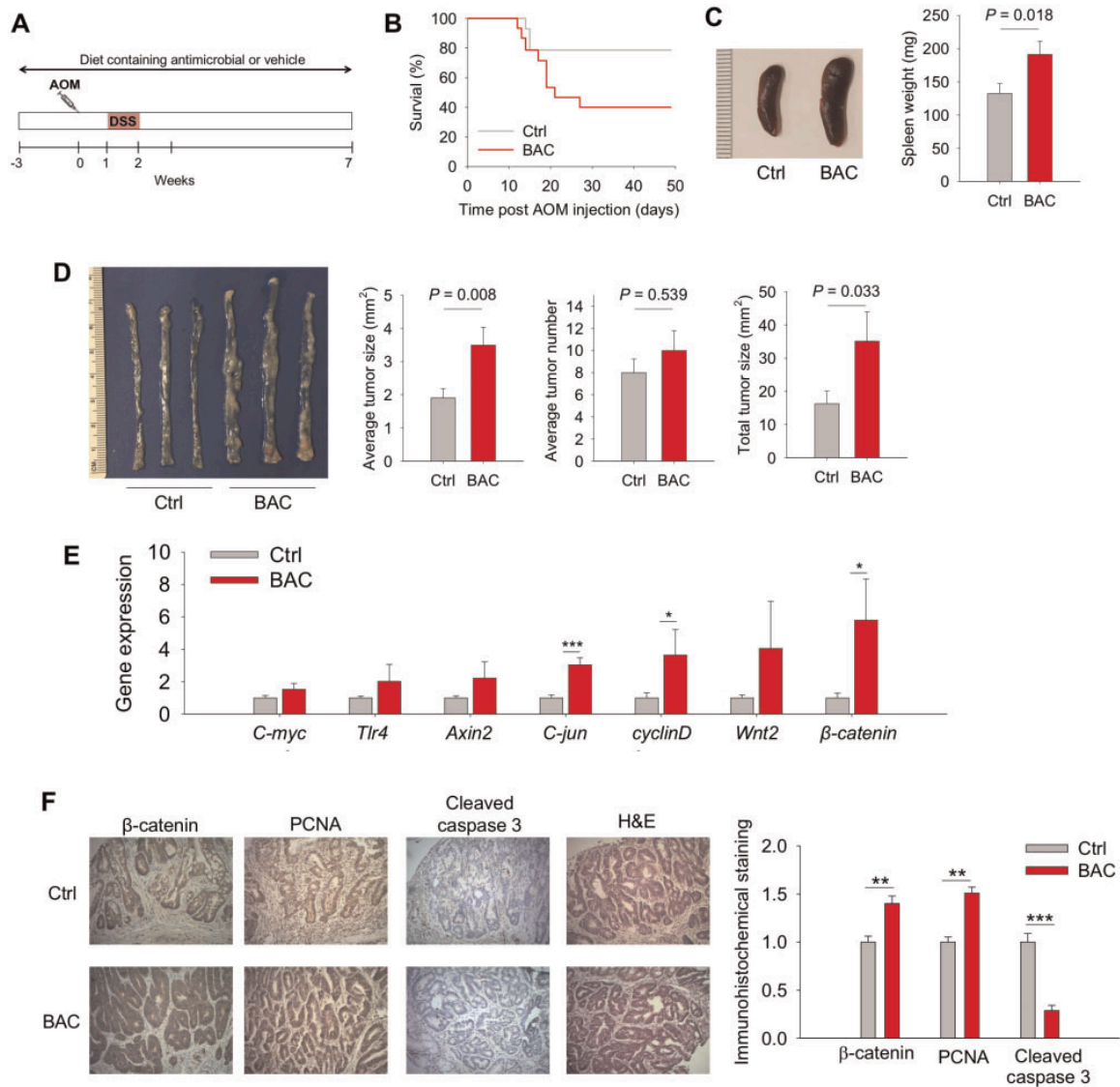
Figure 4.4. Effect of BAC on AOM/DSS-induced colitis-associated colon cancer in C57BL/6 mice.
(A) Scheme of animal experiment. (B) Survival rate. (C) Spleen weight. (D) Representative images of colon tumors and quantification of colon tumorigenesis. (E) qRT-PCR analysis of gene expressions in colon tumors. (F) Representative images of immunohistochemical staining (magnification ×300) of β-catenin, PCNA, cleaved caspase 3, and H&E, and quantification of immunohistochemical images. The results are expressed as mean ± SEM, n = 14-15 mice per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
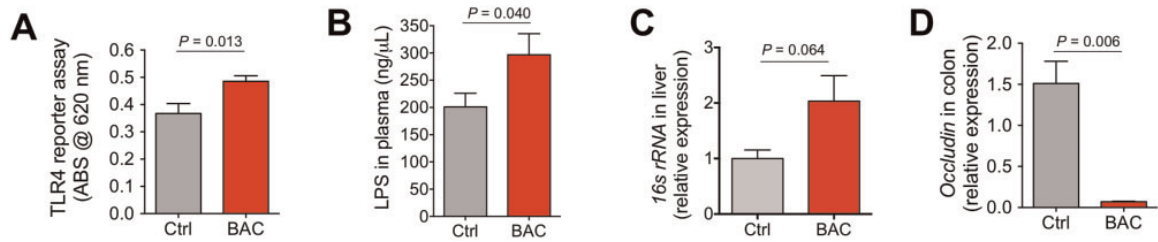
Figure 4.5. BAC exposure increased TLR4 activation in the systemic circulation.
(A) TLR4 reporter assay of the plasma from BAC- or vehicle-treated DSS mice. (B) Concentration of LPS in plasma. (C) Expression of *16S rRNA* gene in liver. (D) qRT-PCR analysis of gene expression of *Occludin* in colon. The results are expressed as mean ± SEM, n = 10-12 mice per group.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

IBD has become a serious health problem since its incidence and prevalence has dramatically increased throughout the world. Antimicrobials used in consumer products might serve as environmental risk factors for IBD and its associated diseases. TCS, TCC, BAC, BET, and PCMX are widely used antimicrobial ingredients in consumer products and are ubiquitous contaminants in the environment. In 2016, the FDA removed TCS and TCC from over-the-counter handwashing products while allowing additional time to develop new safety and efficacy data for BAC, BET, and PCMX. Therefore, it is important and timely to better understand the effects of these antimicrobials on human health. Currently, there is not much known about how chronic exposure to low-dose consumer antimicrobials affects gut health. Here, we validated the effects and mechanisms of triclosan, as well as other consumer antimicrobials, and the gut microbiome on IBD using three studies.

First, we showed that TCS was metabolically re-activated in the gut by the actions of gut microbiota, leading to the accumulation of microbiota-derived toxic metabolites in the colon and resulting in gut-specific toxicity. Upon TCS exposure, the dominant metabolites of TCS in host tissues were its glucuronide- and sulfate- conjugates, which are biologically inactive; however, in the colon digesta, the dominant metabolites were free-form TCS species, which have potent and direct effects on both colon epithelial cells and gut bacteria to induce a pro-inflammatory phenotype. This unique profile of TCS metabolism in the colon digesta was disrupted by treatment with an antibiotic cocktail.

Furthermore, gut microbiota from mice and humans, as well as specific bacteria strains (*Lactobacillus brevis*, *L. rhamnosus*, and *Bifidobacterium breve*), catalyzed TCS biotransformation *in vitro*, supporting that gut microbiota mediates TCS metabolism.

Second, we showed that exposure to low-dose TCC exaggerated the severity of colitis and exacerbated the development of colitis-associated colon tumorigenesis, via gut microbiota-dependent mechanisms. Exposure to TCC increased DSS- and IL-10 knockout- induced colitis, and exaggerated AOM/DSS-induced colon tumorigenesis in mice. Regarding its mechanisms, TCC exposure reduced the diversity, altered the composition of gut microbiota, and failed to promote DSS-induced colitis in mice lacking the microbiota, supporting that the presence of the microbiota is critical for the pro-colitis effects of TCC.

Finally, we reported that exposure to low doses of BAC, BET, and PCMX increased DSS-induced colonic inflammation, and BAC increased AOM/DSS-induced colon tumorigenesis in mice. In addition, we find that exposure to BAC increases activation of TLR4 signaling in the systemic circulation, by disrupting intestinal barrier function and thus enhancing circulating levels of bacterial products.

Our studies support that the gut microbiome is required for the pro-inflammatory effects of these antimicrobials. However, the functional roles of the microbiome, as well as the specific gut bacteria, involved in the biological actions of these antimicrobials are unknown. Elucidation of the specific bacteria involved will help to design human studies to validate the impact of antimicrobial exposure on human health and clarify potential inter-individual variations in metabolism and responses to their exposure.

The regulatory policy of TCS, as well as other antimicrobials, is an intensively debated topic now. Our study, as well as the research from other laboratories, support that TCS and other widely-used antimicrobials could have adverse effects on the gut microbiome and gut health. More studies are needed to better characterize the impact of these compounds on gut health in humans.

There are still more experiments needed to evaluate TCS on gut health. In our study, we treated mice with TCS, and other antimicrobials, for several weeks. Because humans could potentially be exposed to TCS for many years, it is worth pursuing an animal study exposing mice for several months, or longer to further mimic human exposure to TCS. This would be especially interesting due to the fact that we see dramatically enhanced colon damage (>2-fold in the histological score) in mice exposed to TCS for only a relatively short time of 3 weeks [121]. In addition to exposing mice for a longer period, another potential experiment is to expose mice to TCS for a few weeks and then stop exposure for another several weeks to see if this removal of TCS would decrease TCS-enhanced colitis. This is based a previous study which showed that the modulation of the gut microbiomes of TCS-exposed and unexposed fathead minnows disappeared after 2 weeks of depuration [116]; this suggests that the modulation caused by TCS may disappear after removal of TCS, which may in turn lead to a decrease in TCS-enhanced colitis.

It would be ideal to further investigate the identification of which specific bacteria contribute to TCS-enhanced colitis, and whether this is due to TCS promoting antibacterial resistance in specific microbes. There has been concern of TCS promoting antibacterial resistance in specific microbes [199], and some of the studies previously

discussed support this: in zebrafish, the abundance of microbes that are resistant to TCS was increased in the TCS-exposed groups [117]; in infants, there was a positive correlation between urinary TCS levels and Proteobacteria species which is a group associated with an increase in antibiotic resistance genes in the gut microbiota [65]; and in mice, TCS also appeared to enrich genes in bacteria that are involved in TCS resistance, multidrug resistance efflux pumps, and bacterial stress when exposed to mice [8].

Together, our results suggest that these widely used antimicrobial compounds could exaggerate disease development of inflammatory bowel disease and associated colon cancer. Further studies are urgently needed to better characterize the impacts of these compounds on gut diseases in order to develop sound regulatory policies for these compounds.

APPENDIX

PUBLICATIONS

1. **Sanidad KZ**, Zhang H, Zhao E, Yang J, Zhang J, Yang H, Xiao H, Cai Z, and Zhang G. (2019) Metabolic reactivation of the environmental toxicant triclosan by gut microbiota. Submitted, Under review in *PNAS*.

2. Yang H*, **Sanidad KZ***, Wang W, Gu M, Cao X, Yang J, Ozay EI, Minter LM, Xiao H, and Zhang G. (2019) Triclocarban exposure exaggerates colonic inflammation and colon tumorigenesis in mice via altering gut microbiota. Submitted to *Environmental Science & Technology*, *denotes co-first authors).

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