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Metabolic fate of environmental chemical triclocarban in colon tissues: roles of gut microbiota involved

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Abstract

Metabolic transformations play critical roles in the bioavailability and toxicities of environmental pollutants and toxicants. However, most previous research has focused on the metabolic reactions in host tissues, the gut microbiota-mediated biotransformation of environmental compounds is understudied. Using triclocarban (TCC) as a model environmental compound, here we study the

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Credit Author Statement

Zongwei Cai, Guodong Zhang: Conceptualization, Supervision; **Guangqiang Wang, Hongna Zhang, Jianan Zhang:** Data curation, Investigation, Writing- Original draft preparation; **Katherine Z. Sanidad, Vladimir Yeliseyev, Julie Parsonnet, Thomas D. Haggerty:** Methodology, Software, Investigation; ***Haixia Yang, Lianzhong Ai, Minhao Xie***: Validation, Writing- Reviewing and Editing.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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metabolic fate of TCC in gut tissues and determine the roles of gut microbiota involved. We find that compared with other tissues, the colon tissue has a unique metabolic profile of TCC, with high abundance of the parent compound TCC and its free-form metabolites. Using a variety of approaches including antibiotic-mediated suppression of gut bacteria *in vivo*, germ-free mice, and *in vitro* culture of fecal bacteria, we found that the unique metabolic profile of TCC in the colon is mediated by the actions of gut microbiota. Overall, our findings support that gut microbiota plays important roles in colonic metabolism of TCC, highlighting the importance to consider the contributions of gut microbiota in toxicology evaluation of environmental compounds.

Keywords

gut microbiota; triclocarban (TCC); biotransformation; metabolism

Introduction

Exposure to environmental pollutants and toxicants is associated with increased risks of many human diseases such as cancer, inflammation, and metabolic diseases (Ananthakrishnan, 2013; Holtcamp, 2012; Irigaray et al., 2007). It is important to better understand the mechanisms underlying the toxicities of environmental compounds, to accurately assess their toxic potentials and develop strategies to reduce their health risks. Metabolic transformations play critical roles in the bioavailability and toxicities of environmental compounds (Dekant, 2009). While most previous research has focused on metabolism in host tissues (Xu et al., 2005), the biotransformations of environmental compounds by the gut microbiota are considerably understudied (Claus et al., 2016). It is important to better understand the roles of gut microbiota involved, since the gastrointestinal tract is a major route of entry for environmental compounds and also allows these compounds to interact with gut microbes. In addition, emerging research has shown that gut microbes can catalyze unique metabolic reactions that are distinct from, or even opposite to, those catalyzed by the host enzyme systems (Koppel et al., 2017). However, few studies have investigated the metabolic fates of environmental compounds in gut tissues, and the roles of gut microbiota involved are also largely unknown.

Triclocarban (3,4,4′-trichlorocarbanilide, TCC) is a high-volume chemical used as an antimicrobial ingredient in many consumer and industrial products (Halden, 2014). The National Health and Nutrition Examination Survey showed that TCC was detected in ~37% of US populations (Ye et al., 2016). Our recent research showed that exposure to TCC increased the severity of colitis and exacerbated the development of colon tumorigenesis in mouse models, illustrating its potential gut toxicity (Xie et al., 2019; Yang et al., 2020). Furthermore, we showed that the presence of gut microbiota is required for the gut toxicity of TCC, since co-administration of a broad-spectrum antibiotic cocktail (ABX), which suppresses gut bacteria, abolished the colitis-enhancing effects of TCC in mice (Yang et al., 2020). However, the functional roles of the gut microbiota involved are unknown.

We hypothesize that gut microbiota contributes to colonic metabolism of TCC, resulting in a unique metabolic profile of TCC in gut tissues and results in its subsequent gut toxicity. To

test this hypothesis, here we used liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis to compare the metabolic fates of TCC in gut *vs.* other tissues, then used a variety of approaches including ABX-mediated suppression of gut microbiota *in vivo*, germ-free mouse models, and culture of gut bacteria *in vitro*, to determine the functional roles of gut microbiota involved.

Materials and Methods

Chemicals

TCC (99% purity) was purchased from Alfa Aesar (Haverhill, MA). TCC metabolites, including 2′-hyhroxy-TCC (2′-OH-TCC), 3′-hyhroxy-TCC (3′-OH-TCC), 2′-hyhroxy-TCC (2′-OH-TCC), 6-hyhroxy-TCC (6-OH-TCC), 3′,4′-dichloro-4′-hydroxy-carbanilide (DHC), and 2′-hydroxy-TCC-Sulfate (2′-OH-TCC-Sulfate), were synthesized in house as described previously (Zhang et al., 2020). LC–MS-grade solvents were purchased from Fisher Scientific and other chemicals were from Sigma Aldrich (St. Louis, MO), if not otherwise indicated.

Animal experiments

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts (UMass) Amherst and the Massachusetts Host-Microbiome Center at the Brigham and Women's Hospital in Boston MA.

Animal protocol 1: metabolic profile of TCC in mouse tissues—Swiss Webster mice (age = 6 weeks) were purchased from Charles River (Wilmington, MA) and housed in a specific pathogen-free (SPF) animal facility at UMass. After acclimation, the mice were housed in a standard cage (2 mice per cage), treated with 80 ppm TCC via a modified AIN-93G diet (administering TCC at a dose of ~8 mg/kg/day, the dose is calculated based on a diet of 3 g daily chow) for 4 weeks, then the mice were sacrificed and tissues including blood, liver, heart, kidney, lung, bile, small intestine and colon (both mucosa and digesta) were collected for LC-MS/MS analysis (n = 11 mice per group). We have determined the administration method and dosage of TCC, based on our previous studies (Xie et al., 2019; Yang et al., 2020).

Animal protocol 2: roles of gut microbiota in gut metabolism of TCC—Swiss Webster mice were given drinking water with or without ABX containing 1.0 g/L ampicillin and 0.5 g/L neomycin for the whole duration of the experiment. This ABX strategy was used in previous studies by us and others (Cani et al., 2008; Vijay-Kumar et al., 2010; Wang et al., 2020; Yang et al., 2020). After 7 days, the mice were treated with vehicle (Veh) polyethylene glycol 400 (EMD Millipore, Billerica, MA) or 80 ppm TCC via diet, as described above and our previous reports (Xie et al., 2019; Yang et al., 2020). After 28 days of diet treatment, the mice were sacrificed to collect tissues (colon digesta, cecum digesta and feces) for LC-MS/MS analysis (n = 6 mice per group for Veh or Veh + ABX group, and n = 11 mice per group for TCC or TCC + ABX group).

To validate the bacteria-depleting effect of the ABX, we analyzed total fecal microbial biomass (Faith et al., 2011). Briefly, mouse fecal samples were collected, and total fecal DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction with the addition of bead-beating step. The quantity of the extracted DNA was measured using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Animal protocol 3: roles of gut microbiota in time-dependent gut metabolism of TCC—Swiss Webster mice were supplied with drinking water with or without the ABX for 7 days, then the mice were treated with a one-time oral gavage of 8 mg/kg TCC which was dissolved in polyethylene glycol 400. At t = 2, 6, 12, and 24 h post the oral gavage, the mice were sacrificed to harvest tissues (colon and cecum digesta) for LC-MS/MS analysis (n = 5 mice per group for each time point).

Animal protocol 4: comparison of TCC metabolism in conventional mice vs. germ-free mice—Conventional or germ-free Swiss Webster mice were treated with a one-time oral gavage of 8 mg/kg TCC which was dissolved in polyethylene glycol 400. At t = 6 h post the oral gavage (a time point determined from our time-course study in animal protocol 3), the mice were sacrificed to harvest tissues for LC-MS/MS analysis (n = 5 mice per group).

Collection of human stool samples

Human stool samples from a previous study were reused (Poole et al., 2016). Briefly, healthy human volunteers were given TCC-containing or non-TCC-containing household and/or personal care products for up to 4 months (4 human subjects in TCC group and 7 human subjects in control group). Stool samples were collected once a month from the human volunteers during the 4-month period. This study was approved by the Institutional Review Board of Stanford University (ClinicalTrials.gov identifier NCT01509976).

Extraction of TCC metabolites

Mouse tissues and human stool samples (about 100 mg) were added into homogenizer tubes, 1 mL methanol was added, homogenized using an OMNI bead ruptor (OMNI International, Kennesaw, GA), then centrifuged at 7000 × *g* for 3 min. The supernatant was transferred to a fresh tube, centrifuged again at 13,000 × *g* for 5 min, and 500 μL of the supernatant was transferred to a fresh tube and vacuum centrifuged to dryness. Bacterial broth (50 μL) 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 proportional to sample weights, the supernatants were collected for LC-MS/MS analysis after centrifugation (15,000 × *g*, 10 min, 4 °C).

Detection of TCC and its metabolites by LC-MS/MS

TCC and its metabolites were quantified using a Dionex Ultimate 3000 ultrahigh performance liquid chromatography system coupled to a TSQ Quantiva Triple Quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA), as we described previously

(Xie et al., 2019). Chromatographic separation was accomplished on an ACQUITY UPLC BEH C18 column (1.7 μm particles, 2.1 mm × 100 mm, Waters). The mobile phases consisted of solvent (A) 2 mM ammonium acetate in water and (B) acetonitrile with a constant flow rate of 0.3 mL/min. The gradient was started at 15% B for 1.0 min, increased to 80% B at 2.0 min and kept for 3.0 min, ramped to 100% B at 5.5 min and held for 1.0 min, then reverted to 15% B at 7.0 min and reconditioned for 2.0 min. The mass spectrometer was operated under the electrospray (ESI) negative ionization and multiple reaction monitoring (MRM) mode. The following MRM transitions were used for quantification: TCC, 313.11/160; 2′-OH-TCC, 328.89/168; 3′-OH-TCC, 328.89/168; 6-OH-TCC, 328.89/202; DHC, 295/134.11; OH-TCC-Sulfate, 409/329; OH-TCC-glucuronide, 505/329; *N*-TCC-glucuronide, 489/336; *N*′-TCC-glucuronide, 489/302. Other MS parameters were as follows: spray voltage, 2600 V; sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary units; ion transfer tube temperature, 350 °C; vaporizer temperature, 300 °C. The limits of detection (LODs) of TCC, 2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, and DHC in fecal samples were 0.3, 0.3, 0.9, 0.5, 1.8 pmol/g, respectively (Xie et al., 2019). The LOD of OH-TCC-Sulfate was 0.1 pmol/g. OH-TCC-glucuronide, N-TCC-glucuronide, and N′-TCC-glucuronide were semi-quantified with the peak area and their LODs were not available.

Effects of fecal bacteria on catalyzing biotransformation of TCC *in vitro*

Samples of mouse feces or human stool were dissolved in sterile phosphate buffered saline (PBS) containing 0.05% L-cysteine, then centrifuged at 900 × *g* for 5 min. The supernatant which contained culturable bacteria was fermented at 37 °C in deMan, Rogosa and Sharpe (MRS) broth. When the OD_{600} value reached 0.5, the bacteria culture was inoculated 1:10 into MRS broth containing either vehicle or the extract of small intestine digesta from TCC-treated mice. After 24 hours, the samples were collected, extracted and subjected to LC-MS/MS analysis.

Statistical Analysis

Data are expressed as mean ± SEM. Shapiro–Wilk test was used to verify the normality of data and Levene's test was used to assess equal variance of data. Statistical comparison of two groups was performed using Student's t-test, or Wilcoxon–Mann–Whitney test (when normality test fails). Statistical analysis for the interactions between TCC treatment and ABX treatment was performed by two-way ANOVA, followed by Tukey Kramer's method. The statistical analyses were performed using SAS statistical software (SAS Institute) and SigmaPlot software (Systat Software, Inc), and $P < 0.05$ was considered statistically significant.

Results

Unique metabolic profile of TCC in mouse gut tissues

To our knowledge, a detailed profiling of TCC metabolism in different animal tissues was not attempted. To this end, we treated mice with TCC via diet, then used LC-MS/MS to quantitatively measure TCC and its metabolites, including its free-form metabolites (2′-OH-

TCC, 3′-OH-TCC, 6-OH-TCC, and DHC) and conjugated metabolites such as OH-TCC-Sulfate, in various mouse tissues (Fig. 1A).

In agreement with previous studies (Schebb et al., 2012; Schebb et al., 2011; Schebb et al., 2014), we found that, in most host tissues, such as blood, liver, heart, kidney, lung, bile, and small intestine (both mucosa and digesta), the dominant TCC metabolites were its conjugated metabolites (Fig. 1B and Fig. S1). For example, the relative molar concentration of free-form TCC compounds (a sum of TCC, 2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, and DHC) vs. conjugated TCC metabolites (using OH-TCC-Sulfate as a representative metabolite) in the blood was 24% vs. 76% (Fig. 1B). However, in the gut tissues, including cecum and colon (both mucosa and digesta) and feces, the dominant TCC metabolites were its free-form metabolites. Indeed, the relative molar concentration of free-form TCC compounds vs. conjugated TCC metabolites in the colon digesta was 85% vs. 15% (Fig. 1B). Overall, these results demonstrate that compared with other tissues, the gut tissues have a unique metabolic profile of TCC, with high abundance of the parent compound TCC and its free-form metabolites.

Unique metabolic profile of TCC in human stool samples

To better understand the gut metabolism of TCC, we analyzed the metabolic profiles of TCC in human stool samples, serving as a proxy of TCC metabolic profile in the gut tissues. To do so, we utilized human samples from a previous study (ClinicalTrials.gov identifier NCT01509976), in which the human subjects used personal care products, with or without TCC, for 4 months (see scheme of experiment in Fig. 2A) (Poole et al., 2016). We would like to point out that humans are mainly exposed to TCC via dermal absorption of TCC from consumer products such as soaps (Halden, 2014), and this route of administration is different from the animal experiment above.

First, we compared total TCC levels (a sum of all detected TCC compounds, including TCC, 2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, DHC and OH-TCC-Sulfate) in the stool samples of the human subjects in control group (human subjects used products without TCC) vs. TCC group (subjects used products with TCC). LC-MS/MS showed that after even 1-month usage of consumer products, compared with control group, the human subjects in TCC group had higher concentrations of total TCC in their stool samples (Fig. 2B). The concentrations of total TCC in the stool samples of TCC-exposed human subjects can reach up to ~500 pmol/g tissue (Fig. 2B). One human subject in the control group showed the presence of TCC compounds at t = 2 month, and this could be because of the ubiquitous presence of TCC in the environment. This result suggests that after human exposure to TCC through using TCC-containing products, TCC and/or its metabolites can be detected in the gut tissues.

Next, we analyzed the metabolic profile of TCC in the stool samples of TCC-exposed human subjects. LC-MS/MS showed that the dominant TCC compounds in human stool samples were TCC and its free-form metabolites, with low abundance of conjugated metabolites (Fig. 2C–D, see complete LC-MS/MS analysis result in Table S1). The relative concentration of free-form TCC compounds (a sum of TCC, 2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, and DHC) vs. conjugated TCC metabolites (using OH-TCC-Sulfate as a representative metabolite) was 97.1 ± 1.3% vs. 2.9 ± 1.3% (mean ± SEM). These results are

consistent with the animal study above (Fig. 1B), further illustrating a unique metabolic profile of TCC in the gut.

Effects of antibiotic-mediated suppression of gut microbiota on gut metabolism of TCC

Our results above showed that after TCC exposure, the dominant compounds in most host tissues (*e.g.*, liver, blood, and small intestine) are its conjugated metabolites, while the dominant compounds in the colon tissues are TCC and its free-form metabolites (Fig. 1B). This leads to our hypothesis that gut microbiota catalyzes deconjugation reactions: the gut microbes convert the conjugated TCC metabolites, which are derived from host metabolism, to generate TCC and its free-form metabolites. To test this hypothesis, we tested whether ABX-mediated suppression of gut microbiota alters the concentrations of free-form vs. conjugated TCC compounds in gut tissues of mice (see scheme of experiment in Fig. 3A). We used an ABX composition from previous studies (Cani et al., 2008; Vijay-Kumar et al., 2010), and our own studies have shown that this ABX strategy can efficiently deplete gut bacteria in mice (Wang et al., 2020; Yang et al., 2020). To further validate this, we analyzed total fecal microbial biomass (Fig. S2A) (Faith et al., 2011) and found that the ABX treatment caused a dramatic reduction of fecal bacteria in the mice (Fig. S2B–C), validating the microbiota-depleting effects of the ABX strategy.

LC-MS/MS showed that ABX-mediated suppression of gut microbiota reduced the concentrations of TCC and its free-form metabolites (2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, and DHC), while increased the concentrations of its conjugated metabolites (N-Gluc-TCC, OH-TCC-Gluc, and OH-TCC-Sulfate) in colon digesta of mice (Fig. 3B–I). Two-way ANOVA analysis showed that there was a significant interaction between ABX (drinking water with ABX vs. normal drinking water) and TCC treatment (TCC vs. vehicle) on gut concentrations of TCC and its metabolites (interaction $P < 0.05$). Among the detected metabolites, only the concentration of N′-Gluc-TCC was not significantly altered by the ABX treatment (Fig. 3J). Besides colon digesta, a similar result was also observed in cecum digesta and feces of the treated mice (Fig. S3–4). Together, these results support our hypothesis that gut microbiota catalyzes deconjugation reactions, converting conjugated TCC metabolites to its free-form metabolites and playing critical roles in colonic metabolism of TCC.

To further validate the roles of gut microbiota in gut metabolism of TCC, we performed a time-course study. We treated mice, which was pre-treated with or without ABX, with a one-time oral gavage of TCC (dose = 8 mg/kg, this dose is similar to the dose used in the diet studies in Fig. 3), then analyzed metabolic profile of TCC in gut tissues at t = 2, 4, 6, 12, and 24 h (see scheme of experiment in Fig. 4A). Consistent with the results in Fig. 3, we found that ABX-mediated suppression of gut microbiota reduced the concentrations of TCC and its free-form metabolites (2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, and DHC), while increased the concentrations of its conjugated metabolites (N-Gluc-TCC and OH-TCC-Gluc) in colon digesta, in a time-dependent manner (Fig. 4B–H). A similar result was also obtained in the cecum digesta (Fig. S5). Together, these results further support that gut microbiota mediates colonic metabolism of TCC.

Gut metabolism of TCC in conventional mice vs. germ-free mice

To validate the roles of gut microbiota in colonic metabolism of TCC, we compared the gut metabolism of TCC in conventional mice vs. germ-free mice. To do so, we treated conventional or germ-free mice with a one-time oral gavage of TCC (dose = 8 mg/kg, the dose is the same as the time-course study in Fig. 4), then analyzed the metabolic profile of TCC in gut tissues at t = 6 h (Fig. 5A). We determined this time point (t = 6 h), based on the time-course study above which showed that: (i) TCC and its metabolites reached maximum concentrations in the gut tissues at t = ~ 6 h, and (ii) ABX-mediated suppression of gut microbiota alters colonic metabolism of TCC at this time point (Fig. 5).

We found that compared with conventional mice, the concentrations of TCC and its free-form metabolites (2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, and DHC) were reduced, while the concentrations of its conjugated metabolites (OH-TCC-Gluc, N-Gluc-TCC and N′-Gluc-TCC) were increased, in the colon digesta of germ-free mice (Fig. 5B). These results are consistent with our results above using the ABX strategy (Fig. 3–4), further supporting our hypothesis that gut microbiota plays critical roles in colonic metabolism of TCC.

Effects of gut bacteria on catalyzing TCC metabolism *in vitro*

Our results above, using antibiotic and germ-free strategies, support our hypothesis that gut microbiota catalyzes deconjugation reactions, converting conjugated TCC metabolites to its free-form metabolites. To further test this hypothesis, we cultured fecal bacteria *in vitro* and tested whether it can directly catalyze the deconjugation reactions. To do so and to mimic the sequential metabolism of TCC in the gastrointestinal tract (e.g., after TCC metabolism in small intestine, the metabolized TCC in the small intestine digesta enters the colon tissue, allowing subsequent colonic metabolism), we cultured fecal bacteria from mice or human *in vitro*, then used the extract of the small intestine digesta from TCC-treated mice as the substrate for the *in vitro* reaction (see scheme of experiment in Fig. 6A).

First, we analyzed the TCC metabolic profile of the extract of the small intestine digesta from TCC-treated mice. Consistent with the result in Fig. 1B, the extract of small intestine digesta contains high abundance of conjugated TCC metabolites. Next, we analyzed the extent to which incubation with fecal bacteria alters the metabolic profile of TCC in the extract. We found that compared with the control reaction (without addition of fecal bacteria), incubation of fecal bacteria from mice or humans increased free-form TCC metabolites (2′-OH-TCC and 6-OH-TCC), while reduced conjugated metabolites (N-Gluc-TCC, OH-TCC-Gluc, OH-TCC-Sulfate, and N′-Gluc-TCC) (Fig. 6B–C). Overall, these results support that gut bacteria can directly catalyze the conversion of conjugated metabolites of TCC to free-form TCC species.

Discussion

Previous research regarding the metabolism of environmental compounds has mainly focused on the metabolic processes in the mammalian host tissues. Indeed, substantial research has shown that once the environmental compounds, such as TCC, enter the body, they are rapidly metabolized by Phase I and/or Phase II enzymes, which are commonly

expressed in many host organs, resulting in formation of glucuronide- and sulfate-conjugates (Schebb et al., 2012; Schebb et al., 2011; Schebb et al., 2014). These conjugates are usually water-soluble, biologically less active or inactive, and are then secreted from the body, leading to inactivation and detoxification of the environmental compounds (Xu et al., 2005). 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* (Ginsberg and Rice, 2009; Lee et al., 2019; Rodricks et al., 2010; Völkel et al., 2002). However, most previous research has focused on the metabolism of environmental compounds in host tissues (Xu et al., 2005), the gut microbiota-mediated biotransformation is considerably understudied (Claus et al., 2016).

Here our central finding is that gut microbiota plays critical roles in the colonic metabolism of TCC, leading to a unique metabolic profile of TCC in the gut. After TCC exposure, the dominant metabolites in gut tissues were the parent compound TCC and its free-form metabolites; in contrast, the dominant metabolites in most host tissues were its sulfate- and glucuronide-conjugated metabolites. These results demonstrate a unique profile of TCC metabolism in the gut tissues. In addition, using a variety of approaches including antibiotic-mediated suppression of gut microbiota *in vivo*, germ-free mouse models, and culture of gut bacteria *in vitro*, we showed that the unique metabolic profile of TCC in the gut tissue was, at least in part, mediated by the actions of gut microbiota. Suppression of gut microbiota, using antibiotic or germ-free approaches, reduced concentrations of TCC and its free-form metabolites, while increased concentrations of its conjugated metabolites, in colon tissues. These results support that gut microbiota can convert the sulfate- and glucuronide-conjugated metabolites of TCC, which are derived from host metabolism, to re-generate the parent compound TCC and its free-form metabolites in the gut tissues (see proposed scheme of TCC metabolism by the host enzymes and gut microbiota in Fig. 7). This notion is further supported by the metabolic reactions using *in vitro* cultured gut bacteria. Overall, these results support that gut microbiota plays critical roles in the metabolism of TCC and potentially other environmental compounds in gut tissues, highlighting the importance of incorporating the contributions of gut microbiota in toxicology evaluation of environmental compounds.

The microbiota-mediated metabolism leads to high abundance of TCC and its free-form metabolites in gut tissues: we found that after usage of TCC-containing products, the concentrations of TCC and its free-form metabolites in the stool samples of human subjects can reach up to ~500 pmol/g tissue (~500 nM). These free-form compounds are usually biologically active: previous studies showed that free-form TCC, at this dose range, has potent and direct effects on tumorigenesis and endocrine function *in vitro* (Huang et al., 2014; Sood et al., 2013). To our best knowledge, few studies have examined the biological actions of the conjugated metabolites of TCC, though previous studies have implicated that many of the glucuronide- or sulfate-conjugates are biologically inactive (Xu et al., 2005). Therefore, the microbiota-mediated metabolism, which leads to accumulation of free-form TCC compounds in the gut, could contribute to the gut toxicity of TCC. In support of this notion, our previous study showed that TCC exposure increased dextran sodium sulfate (DSS)-induced colitis in mice, with reduced colon length, increased colonic infiltration of immune cells, and exacerbated colon tissue damage; however, such effects were abolished

by co-administration of a broad-spectrum ABX which suppresses gut microbiota, suggesting that the presence of gut microbiota is essentially required for the gut toxicity of TCC (Yang et al., 2020). These findings suggest that in a comprehensive evaluation of the toxic potential of environmental compounds, it is important to consider the contributions of gut microbiota involved.

In summary, our results support that after TCC exposure, there is a unique metabolic profile of TCC in the gut tissues, and this is caused by the metabolic actions of the gut microbiota. Besides TCC, other environmental compounds could also be metabolized in the gut by the actions of gut bacteria, leading to colonic accumulation of microbiota-derived metabolites and resulting in potential adverse effects on the gut tissues. Further studies are needed to better understand the roles of gut microbiota in the metabolism and toxicity of environmental compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. The colon tissue has a unique metabolic profile of TCC
2. The unique metabolic profile of TCC in colon is mediated by gut microbiota

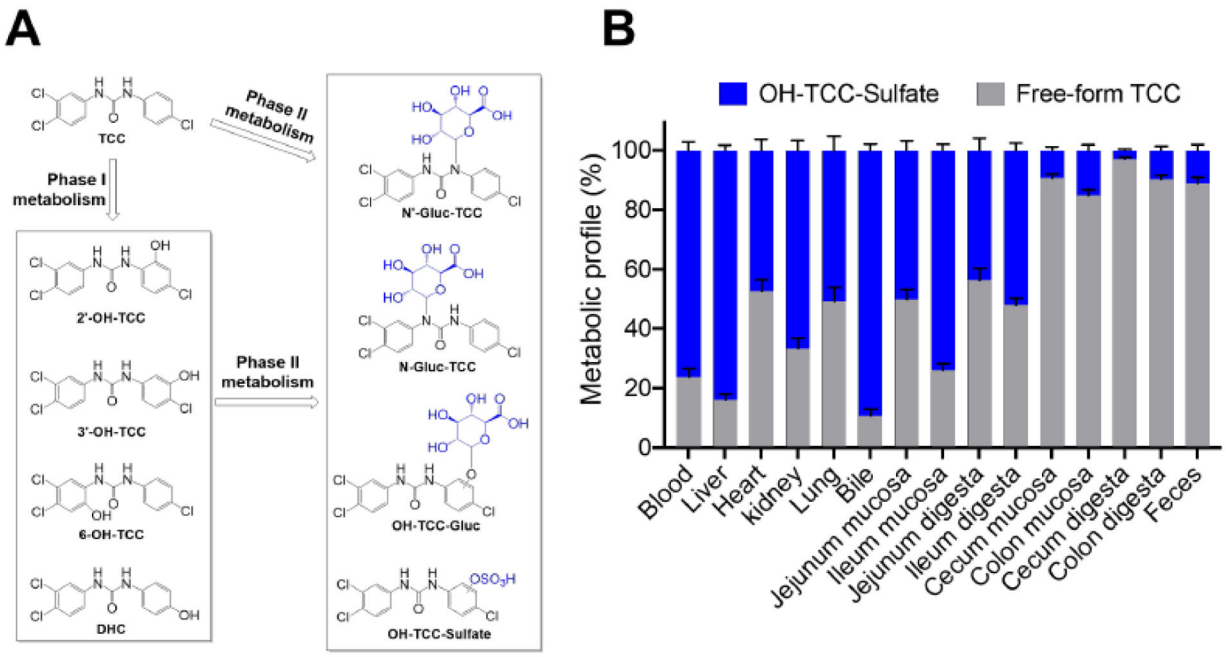

Fig. 1. Metabolic profiles of TCC in mouse tissues after TCC exposure.

(A) Biochemistry of TCC metabolism in host tissues and structures of TCC metabolites. (B) Relative concentration of OH-TCC-Sulfate and free-form TCC (a sum of TCC, 2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, and DHC) in different mouse tissues. The data are mean ± SEM, n = 11 mice per group.

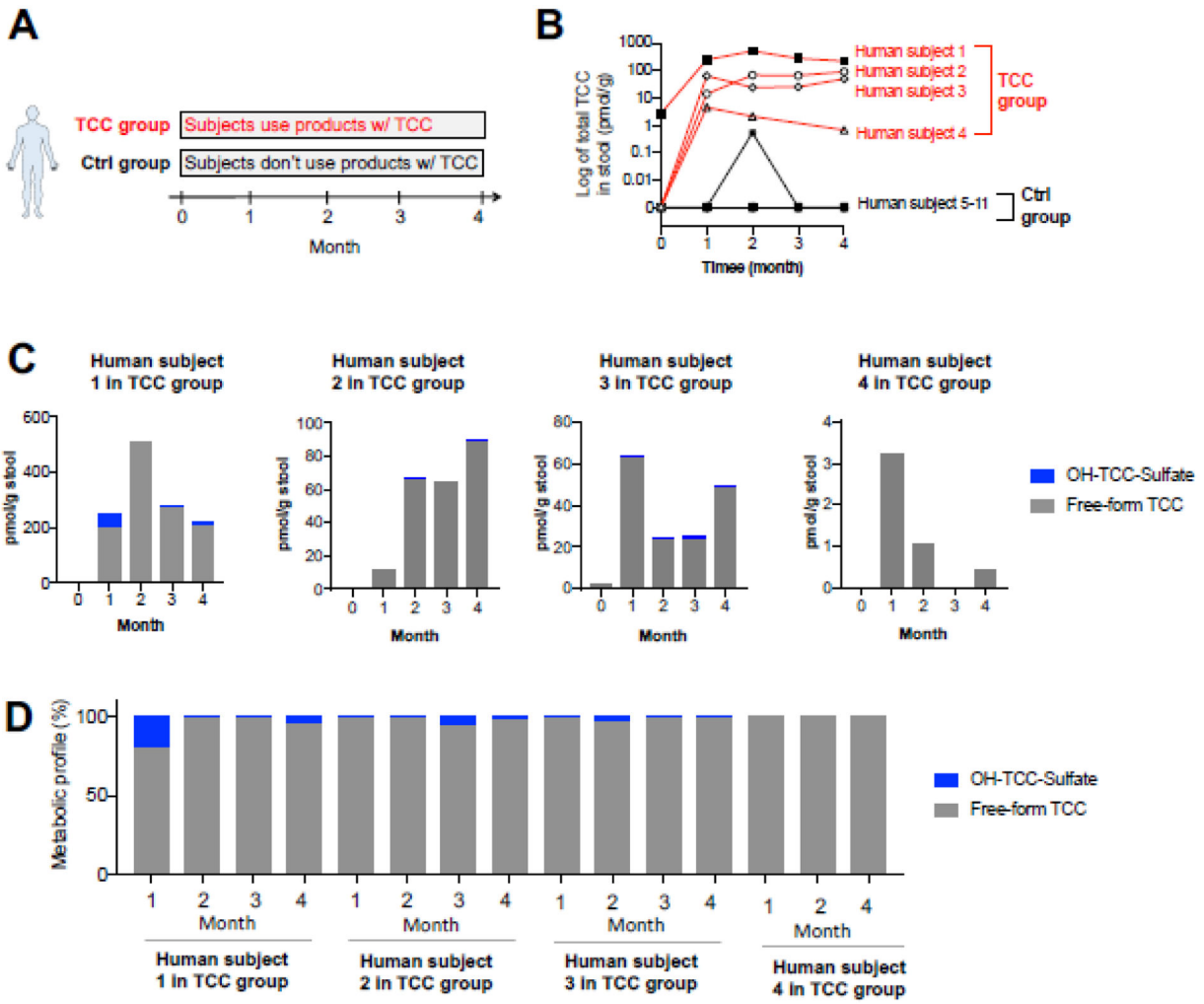

Fig. 2. The metabolic profiles of TCC in human stool samples.

(A) Scheme of experiment: human subjects used personal care products without TCC (control group) or with TCC (TCC group) for up to 4 months (4 human subjects in TCC group, 7 human subjects in control group). At 0, 1, 2, 3, and 4 months, the stool samples were collected for analysis. (B) Concentrations of total TCC (a sum of all detected TCC species, including 2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC, DHC and OH-TCC-Sulfate) in human stool samples. (C) Concentrations of OH-TCC-Sulfate and free-form TCC compounds in human subjects 1–4 in the TCC group. (D) Relative concentration of OH-TCC-Sulfate vs. free-form TCC compounds in human subjects 1–4 in the TCC group.

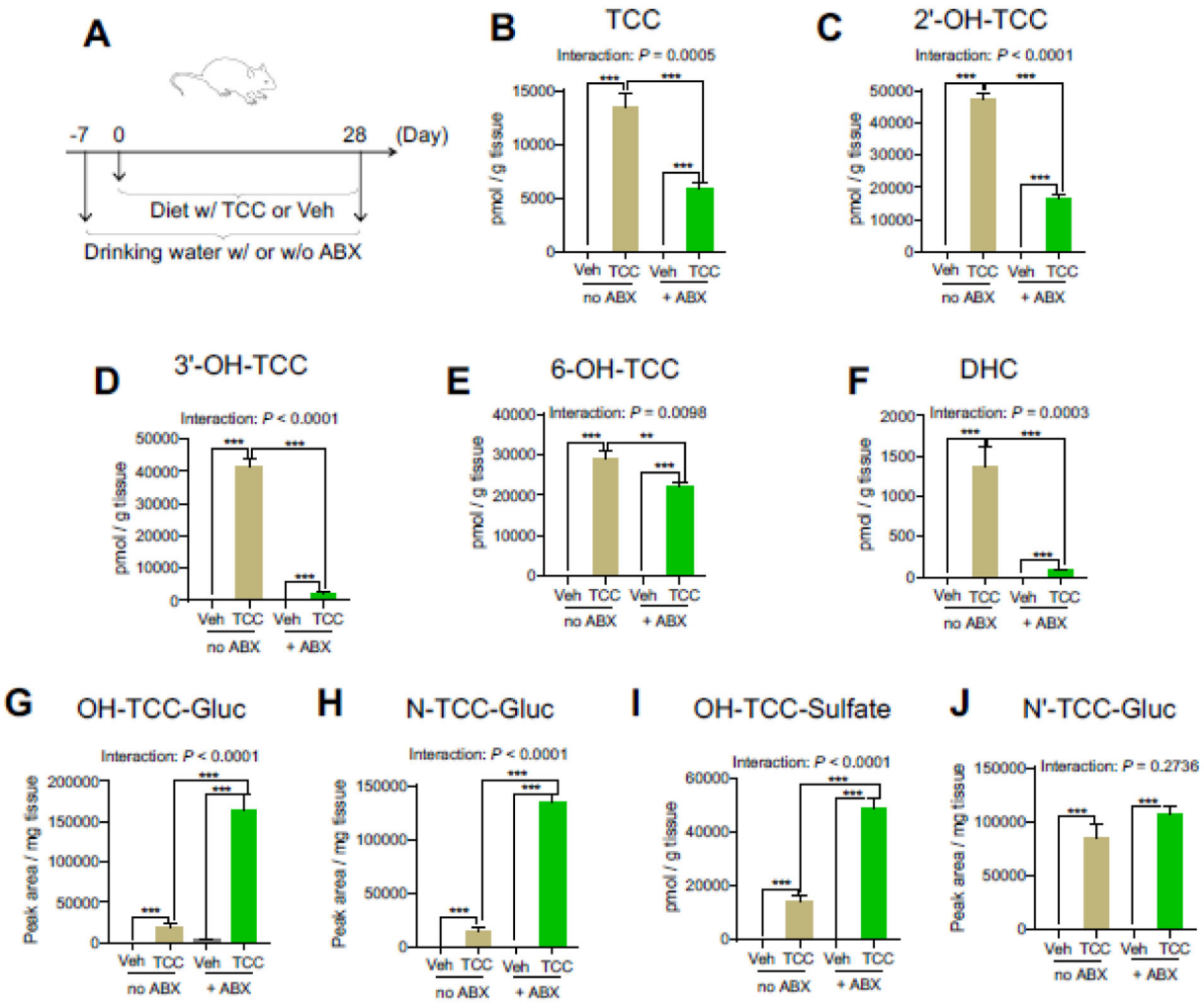

Fig. 3. Effects of ABX treatment on TCC metabolism in colon digesta.

(A) Scheme of animal experiment. (B–J) Concentrations of (B) TCC, (C) 2′-OH-TCC, (D) 3′-OH-TCC, (E) 6-OH-TCC, (F) DHC, (G) OH-TCC-Gluc, (H) N-TCC-Gluc, (I) OH-TCC-Sulfate, and (J) N′-TCC-Gluc in colon digesta. The data are mean ± SEM, n = 6 mice per group for vehicle (Veh) or Veh + antibiotic cocktail (ABX) group, and n = 11 mice per group for TCC or TCC + ABX group. Statistical significance of the interaction effect between ABX (ABX vs. water) and TCC (TCC vs. vehicle) on concentrations of TCC and its metabolites in colon digesta was determined by two-way ANOVA, ** $P < 0.01$, *** $P < 0.001$.

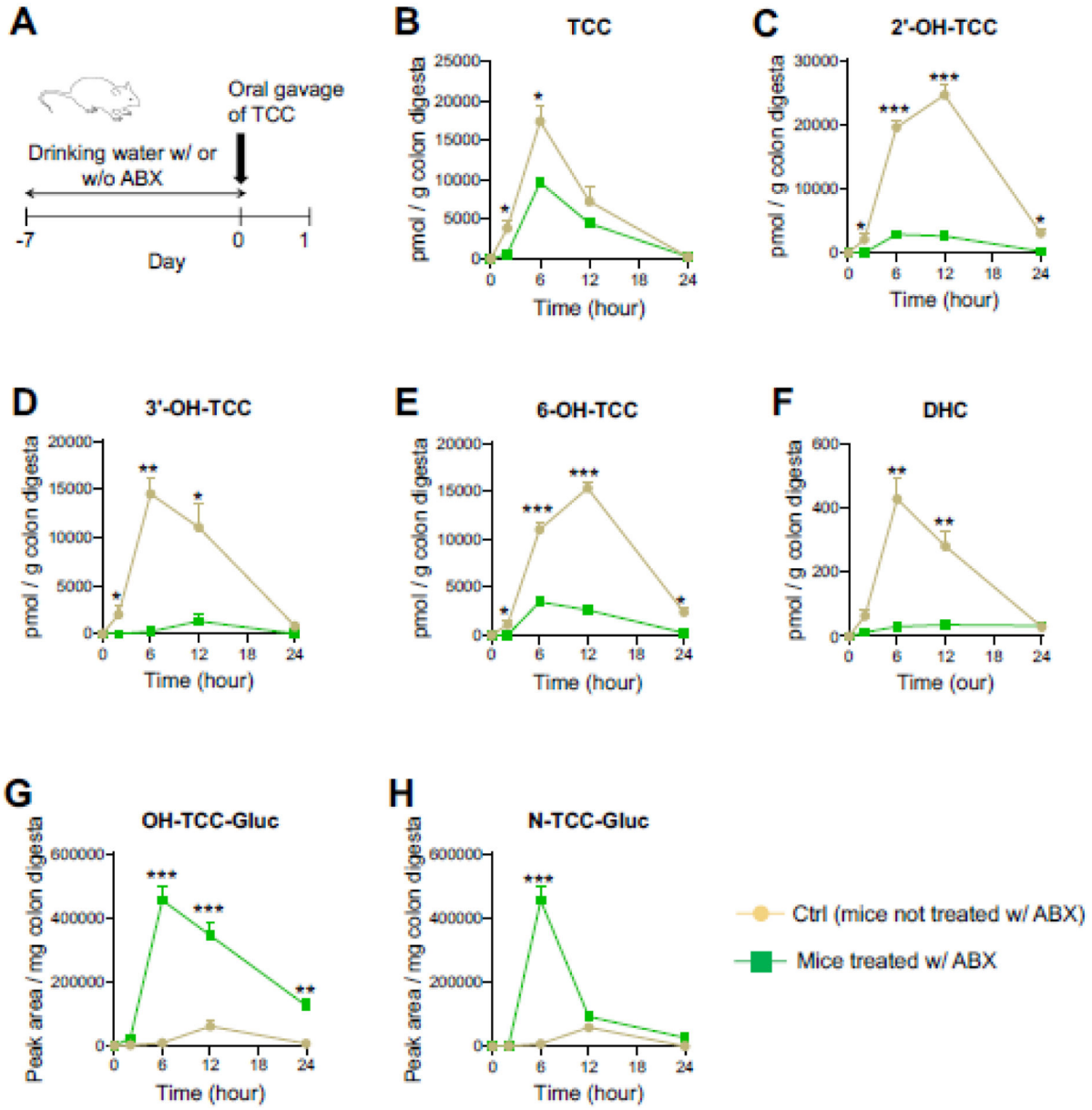

Fig. 4. Effects of ABX treatment on time-course metabolism of TCC in colon digesta.

(A) Scheme of animal experiment. (B–H) Kinetics of concentrations of (B) TCC, (C) 2′-OH-TCC, (D) 3′-OH-TCC, (E) 6-OH-TCC, (F) DHC, (G) OH-TCC-Gluc, and (H) N-TCC-Gluc in colon digesta. The data are mean ± SEM, n = 5 mice per group for each time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

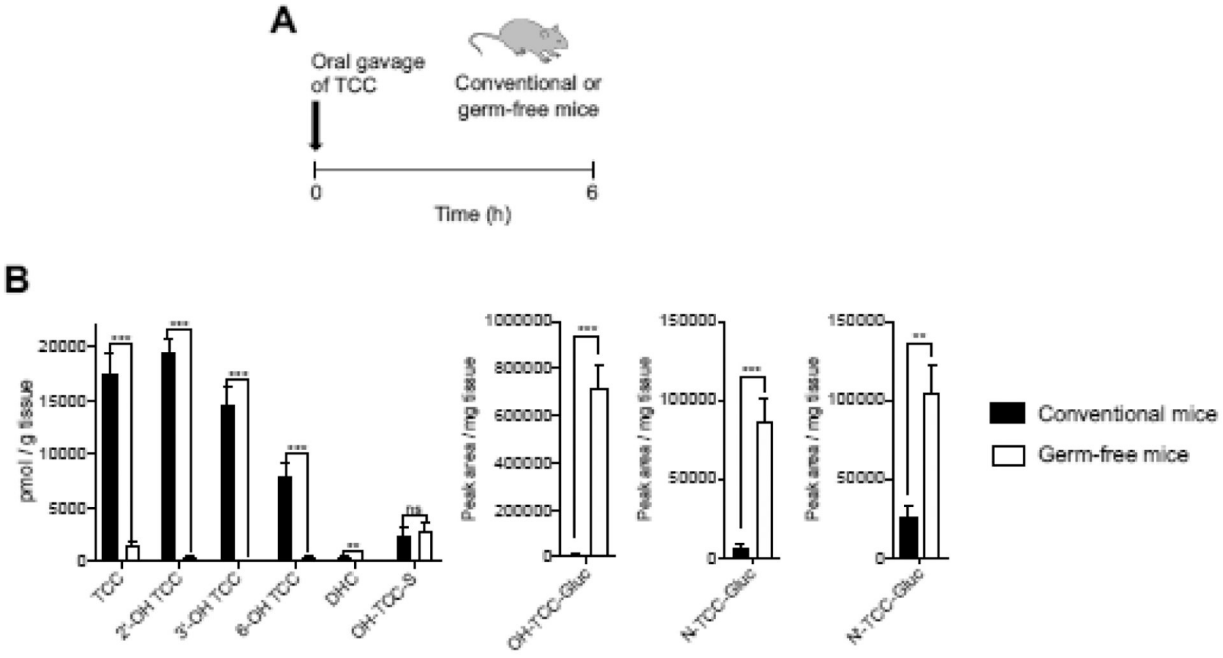

Fig. 5. The metabolic profile of TCC in conventional or germ-free mice.

(A) Scheme of animal experiment. (B) Concentrations of TCC and its metabolites in colon digesta. The data are mean ± SEM, n = 5 mice per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

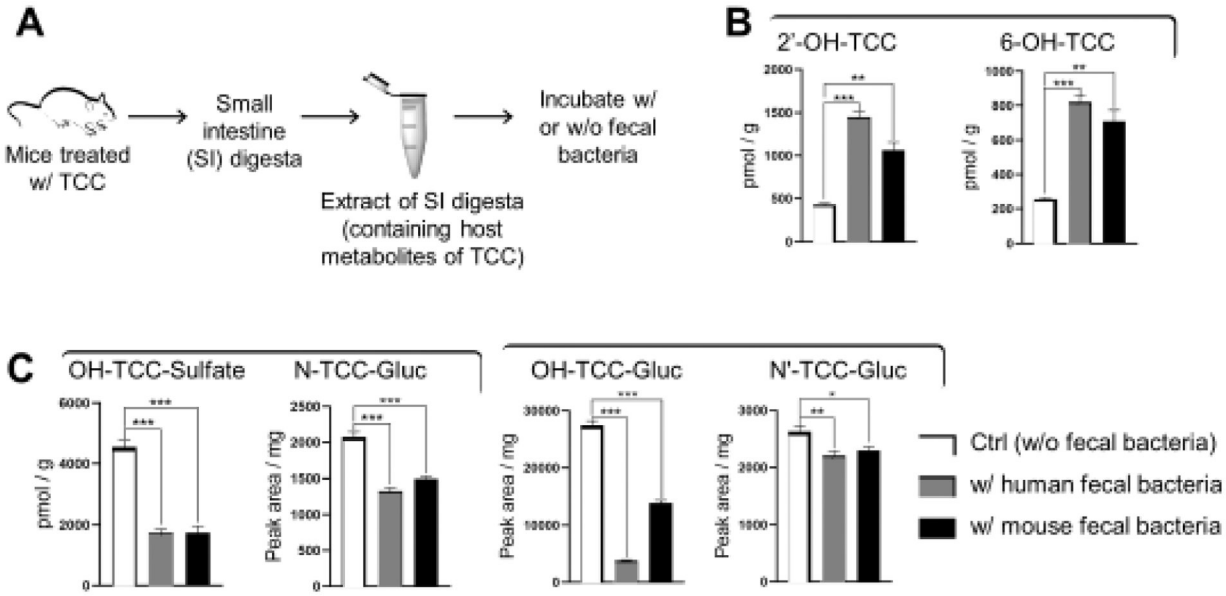

Fig. 6. Effects of gut bacteria on catalyzing TCC metabolism *in vitro*.

(A) Scheme of experiment: we incubated gut bacteria with the extract of small intestine digesta from TCC-treated mice, then analyzed the reaction by LC-MS/MS analysis. (B–C) Concentrations of TCC metabolites. The data are mean ± SEM, n = 5–7 per group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

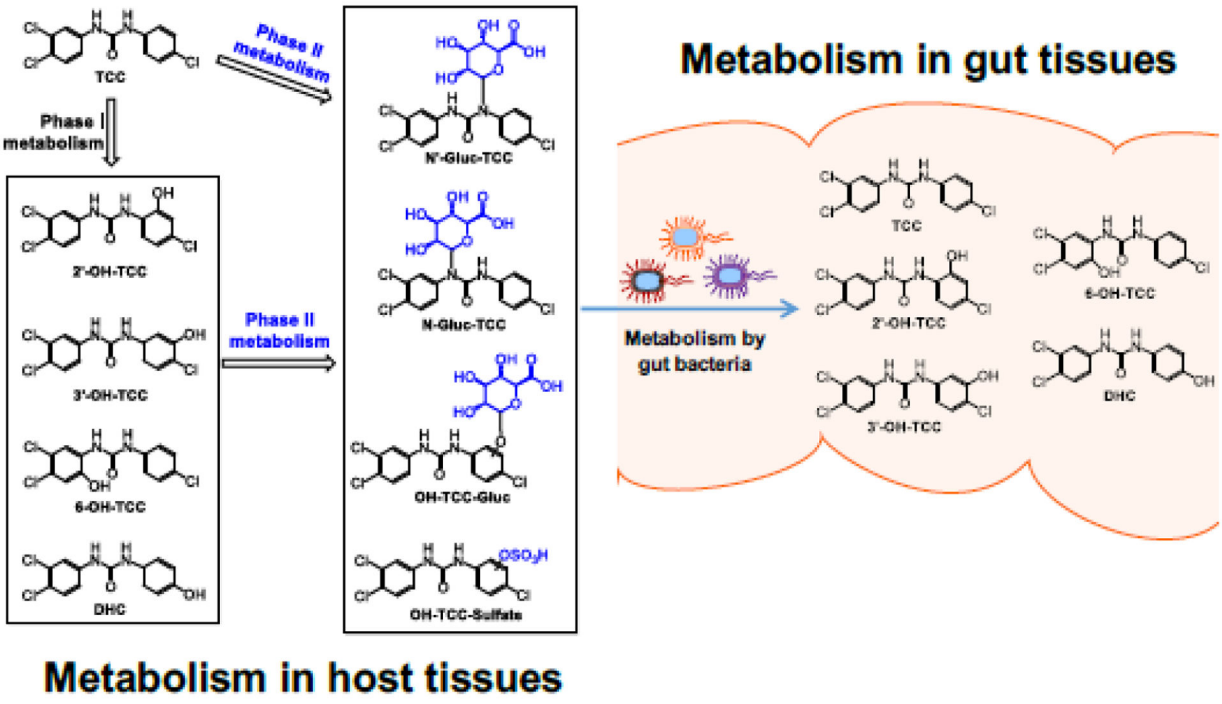

Fig. 7. Proposed model for TCC metabolism in the host tissues and gut microbiota:
gut bacteria mediates colonic metabolism of TCC, leading to colonic accumulation of microbiota-derived TCC metabolites.