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Irreversible effects of trichloroethylene on the gut microbial community and gut associated immune responses in autoimmune-prone mice.

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Abstract

The developing immune system is especially sensitive to immunotoxicants. This study assessed trichloroethylene (TCE)-induced effects on the gut microbiome and cytokine production during the development in mice. Mice were exposed to TCE (0.05 or 500 μg/ml) at the levels that approximate to environmental or occupational exposure, respectively. Mice were subjected to a continuous developmental exposure encompassing gestation, lactation and continuing directly in the drinking water postnatally for 154-days (PND154) or PND259. To observe persistence of the effect TCE was removed from the drinking water in a subset of mice on PND154 and were provided regular drinking-water until study terminus (PND259). Abundance of total tissue-associated bacteria reduced only in mice exposed to TCE until PND259. Ratio of *Firmicutes/ Bacteroidetes* did not alter during this continuos exposure, however, cessation of high-dose TCE at PND154 resulted in the increased abundance *Bacteroidetes* at PND259. Furthermore, high-dose TCE-exposure until PND259 resulted in a lower abundance of the genera *bacteroides* and *lactobaccilus* and increased abundance of genus *bifidobactrium* and bacterial family *Enterobactereace*. TCE exposure until PND154 showed significant changes in the production of IL-33; that might play a dual role in maintaining the balance and homeostasis between commensal microbiota and mucosal health. At PND259, IL-3, GM-CSF and Eotaxin were altered in both, the continuous exposure and cessation groups, whereas only cessation-group had higher level of KC that may facilitate infiltration of neutrophils. The irreversible effects of TCE after a period of exposure cessation suggested a unique programming and potential toxicity of TCE even at the environmentallevel exposure.

Short Abstract:

Exposure to TCE with levels relevant to human exposure initiated a shift in the commensal bacterial population and expression of inflammatory respose related genes in a dose dependent manner in an irreversible manner. Even after TCE exposure cessation for 105 days, the animals

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were not able to return to the population distribution found in the control animals as was evident by the shift in the ratio of *Bacteroidetes* and *Firmicutes*, and increased abundance of genus *bifidobactri,um* and bacterial family *Enterobactereace*. Furthermore, levels of IL-3, GM-CSF and Eotaxin did not reach to control level after the cessation of TCE, thus indicating irreversible effects of this toxicant at the levels equivalent to environmental exposure.

Keywords

bacteria; Bacteroidetes; Firmicutes; gastrointestinal; immune; intestine; microbiome; TCE; toxicity; xenobiotic

Introduction

Trichloroethylene (TCE) is an industrial solvent and widespread water pollutant most strongly linked to immunotoxicity and autoimmune disease in experimental rodent models (Cai et al., 2008; Cooper, Makris, Nietert, & Jinot, 2009; Griffin, Blossom, Jackson, Gilbert, & Pumford, 2000; Griffin, Gilbert, Lamps, & Pumford, 2000). There is also evidence to suggest that TCE promotes immune-mediated inflammatory diseases, hypersensitivity and autoimmunity in humans (Cooper et al., 2009; Dai et al., 2015; EPA, 2011; Huang et al., 2015; Khader, Gaffen, & Kolls, 2009; Parks & De Roos, 2014; Zhao et al., 2016).We and others have shown the effect of continuous developmental exposure to TCE in mice encompassing gestation, lactation, and early life generated $CD4^+$ T cell alterations and/or early signs of tissue inflammation in both normal and autoimmune-susceptible mouse strains (Blossom, Melnyk, Li, Wessinger, & Cooney, 2017; Gilbert, Woodruff, & Blossom, 2014; Peden-Adams et al., 2006). We recently reported that continuous developmental exposure to TCE beginning at gestation generated autoimmune hepatitis (AIH)-like tissue pathology at postnatal day (PND) 259 even when TCE was removed from the drinking water at PND 154 (Gilbert, Bai, Barnette, & Blossom, 2017).

In our model of TCE-induced AIH, immune cells infiltrate the liver leading to a cascade of inflammatory events that are counter-balanced by an increase in IL-6-related signaling molecules that are important in liver regeneration and repair (Gilbert et al. 2014). Similar findings have been reported idiopathic AIH in humans (Nguyen Canh et al., 2017). The bi-directional interaction between the gut and liver has been documented. The blood supply for the liver emanates from the gut via the portal vein that carries/translocates gastrointestinal tract-derived bacterial products [(e.g., lipopolysaccharide (LPS)] and other immune mediators such as chemokines MCP-1, and RANTES (Seki & Schnabl, 2012). While several mechanisms have been postulated to play a role in TCE-induced AIH in this mouse model (Gilbert et al., 2009), one new area yet to be explored is the role of TCE exposure on the microbiome, and correlation of this outcome with the gut-associated immune response.

There is evidence that the developing immune system is especially sensitive to microbial exposure (Kelly, King, & Aminov, 2007; Lee & Mazmanian, 2010). The intestinal mucosa, which is the largest immunologically active area in the body, is covered with a mucus layer. This mucus layer serves as a protective layer for epithelial cells and forms a niche for commensal microbes. The commensal microbes may also affect mucus-secreting goblet cell

dynamics directly by the release of bioactive molecules, or indirectly by activation of immune cells (Deplancke & Gaskins, 2001; Viggiano et al., 2015; Wells et al., 2017).

There is predominance of specific bacterial species in the different regions of the intestine that play specific roles in their anatomic location. The intestinal microbial population helps in the digestion and fermentation of food material. This metabolic process leads to the production of short chain fatty acids (SCFA) and gases such as hydrogen and carbon dioxide. Hydrogen is further metabolized by colonic bacteria for acetogenesis, methanogenesis, and dissimilating sulfate reduction (Christl, Murgatroyd, Gibson, & Cummings, 1992; Conway de Macario & Macario, 2009; Frey et al., 2010; Nakamura, Lin, McSweeney, Mackie, & Gaskins, 2010; Roccarina et al., 2010). Various external factors such as xenobiotic compounds and environmental toxicants affect the microbial population in the intestine. TCE is a known inhibitor of the hydrogen consumption by methanogens and acetogens (Florin, 1997). Thus, in order to better understand the role of TCE on the intestinal microbial population and associated gut immune response, this study evaluated whether continuous developmental exposure to TCE altered predominant microbial population and immunoregulatory cytokines in the gut. This study also examined persistence of effects to determine if removal of TCE would reverse these effects.

Materials and Methods

Mouse treatment

This study was conducted under an approved Animal Use Protocol by the Animal Care and Use Committee at the University of Arkansas for Medical Sciences. Eight-week-old female MRL+/+ mice (Jackson Laboratories; Bar Harbor, ME) were used for the study. This strain was selected based on many years of study where we and others showed that TCE exposure accelerated immunotoxicity and autoimmunity in lupus-prone MRL+/+ mice. These mice develop autoimmune hepatitis as primary pathology and inflammatory lesions in lung, pancreas, and kidney (Cai et al., 2008; Griffin, Gilbert, et al., 2000). In contrast, in the NZB/NZW lupus model, unlike in the MRL strain, idiopathic disease is fast-progressing and more severeconsequently, TCE had little to no disease-promoting effects (Keil, Peden-Adams, Wallace, Ruiz, & Gilkeson, 2009). Thus, among the autoimmune-prone strains, MRL+/+ mice are ideally suited to study environmentally-triggered autoimmunity involving chronic exposures. The female mice were paired with male MRL+/+ mice as described (Gilbert et al., 2017). The mice were randomized and divided into 3 groups (vehicle, 0.05, or 500 μg/ml TCE); 10 dams per group. All animals were administered ultrapure unchlorinated drinking water. Controls were given water containing 1% Alkamuls EL-620, the reagent used to solubilize the TCE. Water was changed 3 times/week to offset degradation of TCE. Maternal exposure continued during gestation and lactation. Thus, pups were exposed from gestational day 0 to postnatal day (PND) 0 *in utero* and PND 0-PND 21 by lactation. Female pups were weaned at PND 21 and exposed to TCE in the drinking water until PND 154 or PND 259. One half of the female offspring (8/group) were euthanized on PND 154. The other half of the pups were maintained on drinking water without TCE until study terminus [(PND259 (8/group); cessation group]. PND 259 was selected because this age was similar to mice shown previously to develop liver pathology following adult exposure to TCE

(Gilbert et al., 2009). The TCE concentration used in the study approximated human occupational (76 mg/kg/day) and potential levels that could be encountered through human environmental exposure (0.01 mg/kg/day), respectively (Gilbert et al., 2017).

Gastrointestinal tissue collection

The animals were fasted overnight prior to euthanasia. At necropsy, three pieces of about 3 cm section (each) of ileum were collected in a cryo-vial from each animal for DNA, RNA or protein extraction. These tissues were flash frozen for further use.

Extraction of DNA from ileal tissue

For DNA extraction, the ileal tissue was minced and transferred to a bead beat tube. Tissue lysate was prepared using a Fast Prep Machine as described earlier (Williams et al., 2015). The tube containing lysate was incubated at 65°C for 20 minutes with proteinase K, followed by another incubation at 37°C for 15 minutes with RNAse-A. An equal volume of phenol–chloroform–isopropanol was added to the tube containing cell lysate and mixed thoroughly. This tube was centrifuged at 12 000x g for 30 minutes. After centrifugation, the aqueous layer was transferred to a new tube, and DNA was precipitated by adding 3M sodium acetate, isopropanol and polyacryl carrier. The cell pellet was washed with 70% ethanol. After washing the tubes were air dried and DNA was suspended in DNase- and RNase-free water.

Extraction of DNA from fecal samples

Bacterial community was analyzed in the fecal samples of all the animals. Fecal DNA was extracted following the protocol described earlier with slight modifications (Khare et al., 2004). In brief, about two to three fecal pellets (about 100 mg) were suspended in PBS (1 ml). The diluted fecal mixture was mixed well to produce a homogeneous suspension. This suspension of fecal sample was transferred to the bead-beating tubes and fixed in the Fast Prep, bead beater (MP Biochemicals, Santa Ana, CA) and operated at speed 6 for 40 s. Ten µl of proteinase K (final concentration, 200 µg) was added to the same bead beat tube and incubated at 65°C for 20 min followed by 95°C for 10 min. Three µl of RNase A (Promega Corporation, Madison, WI.) was added to the cooled suspension and further incubated for 15 min at 37°C. The DNA extraction from this mixture was carried out essentially using the same protocol as described earlier (Khare et al., 2004) The DNA pellet was suspended in the DNAse and RNAse free water and used as template for bacterial community analysis.

Real-time PCR for identification of bacterial groups

To identify the predominant phyla and representative genera and species of bacteria present in intestinal mucosa during the exposure of TCE real time PCR was used. The primers used in this study to target bacterial groups were essentially the same as used earlier (Williams et al., 2015). These targets included detection of entire bacterial population (Universal), two phyla (*Bacteriodetes* and *Firmicutes*), predominant genera (*Bacteroides, Lactobacillus* and *Bifidobacterium*) and *Enterobacteria* family. The ABI 7500 machine was used to conduct real-time PCR. The reaction mix contained a total volume of 22 µl with DNA template, SyBr Green master mix and 900nM (each) forward and reverse primers. The optimal assay

conditions were as follows: initial activation of AmpliTaq Gold at 90 °C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 minute. The Threshold cycle (Ct) was defined as the cycle at which the fluorescence was significantly higher than the average standard deviation of the earlier cycles, and the sequence detection application began to detect the increase in signal associated with an exponential growth of the PCR product.

Normalization and presentation of data

Tissue associated microbial population data were normalized with the *β-actin.* The measurement of total/specific bacterial population is represented as fold change when compared to the control group. The results show the relative fold change in the entire bacterial population (Universal), two phyla (*Bacteriodetes* and *Firmicutes*), predominant genera (*Bacteroides, Lactobacillus* and *Bifidobacterium*) and *Enterobacteria* family. The results of the predominant phyla are represented with a 100% stack bar diagram, in which each assessed phyla shows the contribution to the sum of the two predominant bacterial phyla. The changes in the bacterial genes representing the genus *Bacteroides, Lactobacillus* and *Bifidobacterium*, and *Enterobacteria* family were calculated as the fold difference in treated animals when compared to control animals.

A comparative analysis was also conducted to assess the comparative dominance of bacterial phyla, genus and family in fecal and tissue samples. In this analysis, the data normalization was carried out using the quantile method (Mar et al., 2009; Pabinger, Rodiger, Kriegner, Vierlinger, & Weinhausel, 2014).

Protein Extraction from the ileal tissue:

Mouse ileal samples were thawed and the excess fat was removed. Ileal tissue was weighed and transferred to gentleMACS Tube (Miltenyi Biotec Inc. San Diego, CA). Lysing solution was added to the ileal tissue at a concentration of 10 mg tissue / 100 μL. Tissue extract was made in the gentleMACS Dissociator (Miltenyi Biotec Inc. San Diego, CA). Lysate was centrifuged at 4°C for 10 minutes at 750 rpm. Clear homogenate was transferred to 2 mL Eppendorf tubes. Eppendorf tubes were centrifuges at 4°C for 15 minutes at 12,000 rpm. The clear supernatant was transferred into new 2 mL Eppendorf Tubes and these were stored at −80°C until cytokine profiling. The protein concentration was measured in the spectrophotometer using BioRad Protein Assay reagent.

Multiplex cytokine assay in Bioplex:

Gut associated mucosal chemokine and cytokine levels were evaluated in the tissue lysate using the Bio-Plex Mouse Cytokine multiplex kits (Bio-Rad, Hercules, CA) following the manufacturer's instructions. These kits contain several cytokines and chemokines that are relevant to the mucosal immunity (IL-1α, IL-1ß, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1 (MCAF), MIP-1α, MIP-1ß, RANTES, TNF-α, IL-17F, IL-21, IL-22, IL-23p19, IL-31, IL-33 and MIP-3α). In brief, cytokines were measured in duplicate using a novel multiplexed, bead-based, flow cytometric assay that utilizes anti-cytokine monoclonal antibodies attached to microspheres incorporating distinct proportions of two fluorescent

dyes. The assay enables quantification of several cytokines/chemokines in a small volume of protein lysate. Manufacturer provided standard for each cytokine were used to make the standard curve (concentration range 2–00 to 32,000–00 pg/ml). The minimum detection limit for this assay was <10 pg/ml.

Data was exported to Bioplex Manager. Comparisons of different treatment groups were performed using the Mann-Whitney test. All statistical tests were two-sided, and $p<0.05$ was considered significant.

RESULTS:

Effect of TCE on the ileal mucosa associated bacteria

In mice euthanized at PND 154, there was an increase in the total bacterial population in all the animals relative to controls, regardless of TCE concentration (Figure 1). However, when this exposure was prolonged for an additional 105 days (PND 259), the bacterial population in the intestine in both TCE groups decreased significantly. When the TCE exposure was removed from drinking water at PND 154 the intestinal bacterial population appeared to recover to levels found in the continuously exposed animals by PND 259. (Figure 1).

Next, we examined whether or not there was a TCE-induced shift in the bacterial population that represented Gram positive (Phyla *Firmicutes*) or Gram negative (Phyla *Bacteroidetes*) bacteria. Figure 2 depicts 100% stack column for the abundance of bacteria representing *Firmicutes* and *Bacteroidetes* phylum. Control animals were predominantly colonized with *Firmicutes*. Continous exposure of TCE until PND 154 caused a minor reduction in the abundance of *Firmicutes* in low dose group. However, continuous treatment of TCE till PND 259 caused a decrease in the abuundance of *Bacteroidetes* both in low and high dose-treated animals, where as the abundance of *Firmicutes* increased. To our surprise, exposure cessation on PND 154 caused an incresed abundance of Gram negative bacteria (*Bacteroidetes*) in the high dose group animals when examined at PND 259.

We further investigated if TCE had effects on the bacteria representing particular genus (Figure 3). On PND 154 the abundance of bacteria that belong to genus bacteroides were almost same in animals exposed to low dose TCE as the control. However bacteria representing the *Lactobacillus* and *Bifidobactrium* genus were higher than controls. Both of these genera belong to the phylum *Firmicutes*. In contrast, when animals were continuouly exposoed to low dose TCE until PND 259, bacterial populations representing *Bacteroides, Lactobacillus* and *Bifidobactrium* were increased. In contrast, the high dose exposure, decreased abundance of *Bacteroides* and *Lactobaccilus* and increased abundance of *bifidobactrium* in this group of animals (Figure 3) although there was sustantial sample to sample variability was observed in all experimental groups.

Bacteria belonging to *Enterobacteriace* family represent several pathogenic and non pathogenic bacteria. Mice exposed until PND 154 with low dose TCE had lower abundance of these bacteria, whereas the high dose of TCE resulted in a higher abundance of these bacteria. Similarly, mice continuously exposed to high dose TCE until PND 259 resulted in a

higher abundance of these bacteria (Figure 4). Interestingly, this effect was not reversed by exposure cessation.

Orthogonal Partial least squares regression (PLS regression) analysis of bacterial population to predict the effect of TCE on mucosa-associated and fecal microbiome

PLS regression analysis is an effective statistical prediction tool for small sample size with many (possibly correlated) variables. A PLS regression analysis was conducted to analyze how the bacterial population is affected in the mucosa and fecal samples of mice exposed to TCE with different concentration as well as for different time-period; this analysis predicts how diverse the effect of any perturbation is. Furthermore, it provides the alpha diversity and beta diversity in the experimental groups.

The PLS plot reveal that sample-to sample variation is more diverse in the tissue associated bacteria (Figure 5a) as compared to the fecal microbiome (Figure 5b) in the control animals. This variation was more prominent at PND 154 in the tissue-associated bacteria as indicated by the red circles. By PND 259, the diversity of microbial population is very similar in tissue and feces of control animals. Microbial population within the TCE exposed group animal did not differ much from animal to animal in low dose exposed TCE for 154 days (gray circles), moreover, by PND 259 the inter-animal variation was almost minimal in the low dose TCE group. Importantly, the cessation of high dose TCE indicated by yellow circles could not revert the tissue-associated bacterial population to control levels.

Comparative analysis of bacterial population present ileal mucosa and in the mice feces

TCE is a known inhibitor of the hydrogen consumption by methanogens and acetogens present in the colon. Thus, we hypothesized that TCE would have a more drastic effect on the bacterial population present in the feces obtained from the colon as compared to the ileal tissue associated bacteria. To investigate this, a comparative analysis of the bacterial population in the ileum and colon was assessed. The results clearly showed some similarities and some obvious differences (Figure 6). The heat map depicts the normalization of the data using the quantile method, which assumes that usuallythe distribution of bacteria within a community remains almost constant across all samples. Furthermore, this analysis takes into consideration the relative bacterial population shift; if the abundance of one bacterial population increases, the abundance of another bacterial population decreases.

When comparing total bacterial population number (universal) in control animals, there was increased abundance in fecal samples relative to tissues. When animals were exposed to of TCE until PND 154, regardless of dosethere were little to no changes in the overall bacterial population in the fecal samples. However there was a comparative higher abundance of bacteria belonging to the *Enterobacteriace* family in tissue associated bacterial population.

When specific genus (*Bacteroides, Lactobacillus* and *Bifidobacterium*) were compared, continuous exposure of high dose of TCE until PND 259 caused an increased abundance of tissue-associated bacteria representing genus *Lactobaccilus* which belongs to *Firmicutes* phyla. In contrast, in feces, bacteria representing genus *Bacteroides* were abundant. In most of the animals the low dose TCE exposure until PND 259 did not change thetotal bacterial population in the fecal population. However, low dose TCE exposure until PND 259 in

tissue increased abundance of the total bacterial population commensurate with increased abundance of bacteria representing genus *Bacteroides*.

In both tissue and feces, nimals in the cessation group exposed to the high dose showed a shift in the bacterial population. In other words, the bacterial population did not revert back to animals continuously exposed to TCE. Also noteworthy in the cessation group was the higher abundance of bacteria belonging to *Bifidobacterium* genus in tissue, but not feces.

Effect of TCE exposure on cytokine levels in the intestinal tract—Overall, mice continuously exposed to TCE until PND 154 showed very little effect of gut cytokines relative to controls (Table 1a). However, one cytokine, IL-33, was significantly decreased ($p=0.016$) as compared to controls. TCE exposure had a greater impact on animals continuously exposed until PND 259, with treatment resulting in decreased levels of several key cytokines (Table 1b). In both low and high dose TCE exposure groups, the animals showed significantly lower levels of IFN-γ ($p = 0.004$, and 0.007, respectively). Similarly, IL-3 was significantly decreased in high dose animals relative to controls ($p=0.052$). Other important gut-associated immunoregulatory cytokines including IL-12(p40) ($p = 0.009$), IL-17F ($p = 0.021$), and chemokines GM-CSF ($p=0.006$), Eotaxin ($p = 0.034$) and MIP-1α ($p = 0.024$) were decreased with TCE exposure relative to controls. Notably, one of the ileal samples from the high dose experimental group appeared inflamed during tissue collection and was removed from the analysis.

In the cessation group at PND 259, prior TCE exposure had more effect on gut cytokines than controls indicating persistence of TCE effects. Changes in treated groups were primarily decreased compared to controls (Table 1c). In the high dose experimental group, a number of cytokines were significantly affected, the levels of which were decreased compared to controls. These mediators included Eotaxin ($p = 0.016$), GM-CSF ($p = 0.004$), IFN-γ ($p =0.03$), IL-17 ($p = 0.04$), IL-1b ($p = 0.05$), IL-3 ($p < 0.005$), MCP-1 ($p = 0.03$), and MIP-1a ($p = 0.03$). Despite the overall decrease in cytokines, there was one notable exception. In the low dose experimental group, cytokine IL-31 was significantly increased compared to controls ($p=0.039$).

Discussion

TCE is a halocarbon best known for its use as an industrial solvent and metal degreaser. Due to improper disposal over the years, TCE has contaminated many of the water systems in the US. Because TCE exposure will continue to be a problem, a better understanding of health risks associated with TCE exposure are needed.

One of the most sensitive outcomes associated with human TCE exposure in humans is immunotoxicity (EPA, 2011). $CD4^+$ T cells are especially sensitive, and even if overt disease is not diagnosed, altered numbers of activated $CD4^+$ T cells are often found in blood of humans exposed to TCE (Bassig et al., 2016; Hosgood et al., 2012; Lan et al., 2010) As shown by ourselves and others, TCE exposure increased the percentage of IFN-γ- and IL-17-secreting $CD4^+$ T cells in mice that went on to develop $CD4^+$ T cell-mediated AIH (Gilbert et al., 2009; Wang, Wang, Ma, Ansari, & Khan, 2013).

More recently, our lab demonstrated that continuous exposure to levels of TCE described here was sufficient to promote autoimmune disease in female mice at PND 259. Remarkably, the autoimmunity in the form of AIH tissue pathology and anti-liver antibodies was detected 15 weeks after exposure ended, indicating that removal of TCE does not prevent immune-mediated liver pathology (Gilbert et al., 2017).Given the correlation between gut dysbiosis and autoimmunity, our goal in the current study was to expand this research to assess the impact of developmental TCE exposure on the gut microbiome and gut-associated immune responses. The link between developmental TCE exposure and long-lasting effects on the microbiome and gut associated immune responses has not been previously characterized. Potentially relevant to the results reported here, TCE contaminated drinking water has been shown to be associated with colon cancer (Paulu, Aschengrau, & Ozonoff, 1999). The potential association between TCE-induced immunotoxicity and gut dysbiosis is underscored by recent reports that gut microbes and their circulating microbial products as a “microbial reservoir of potent stimulatory entities” for the activation of immune cells (Czaja, 2016, 2017; Seki & Schnabl, 2012). In rats, oral TCE exposure resulted in severe damage to the brush border membranes of the intestine, and altered carbohydrate metabolism (Khan et al., 2009) . As the commensal microbiota resides on the ileal mucosa, it was reasonable hypothesize that oral exposure of TCE may impact the intestinal microbiome and gut associate immune responses.

In addition to biological effects, in the environment, several microorganisms are involved in the biotransformation and bioremediation of TCE, such as Dehalococcoides sp., Pseudomonas *fluorescens*, Nitrosomonas *europaea* and Xanthobacter *autotrophicus* (Guan, Liu, Xie, Zhu, & Han, 2013; Y. Zhang et al., 2011). X. *autotrophicus* has been reported to metabolize about 50% of TCE into CO and CO_2. In fact, X. *autotrophicus* share homology with the subunits of acetone carboxylase (encoded by acxABC) of *Helicobacter pylori,* a common gut pathogen and an etiological agent of peptic ulcer disease (Brahmachary et al., 2008). In the current study, abundance of the microbial population was assessed in feces, as well as, bacteria attached to ileal tissue. The abundance of the bacterial population was less variable in fecal samples compared to ileal tissue. This finding could be due to the flushing of ileum with saline at necropsy; as this process might have an effect on the bacterial population that is loosely attached to ileum. In addition to marked differences in microbial abundance at the two sites examined, there were clear differences with TCE exposure. At PND 154 the total bacterial population (TAB) increased with exposure to both doses of TCE, whereas prolonged exposure (PND259) decreased the bacterial population in a dose dependent manner (higher the dose->lower the total bacterial counts). The cessation of TCE exposure allowed continued proliferation of the bacterial population seen at PND 154 relative to the controls in a dose-dependent manner. However, after the cessation there was a shift in the ratio of *Bacteroidetes* and *Firmicutes* in the high dose animals. At the genus level, there was no adverse effect of TCE on the tested genera at 154 days (both low and high dose), and low dosed animals of 259 and cessation group; however, there was decrease in the bacterial population that belong to genus *Bifidobacterium* in high dosed animals after cessation. Moreover, TCE exposure increased abundance in the bacterial population that belongs to family *Enterobacteriaceae* that was most evident at PND 259 Overall, the pattern of abundance of tested bacteria revealed occurrence of dysbiosis due to TCE exposure.

Moreover, even after stopping the TCE exposure for 105 days, the animals were not able to return to the population distribution found in the control animals as was evident by the shift in the ratio of *Bacteroidetes* and *Firmicutes*, as well as the higher abundance of tested genus and family. We cannot rule out the possible changes in other bacterial populations, as this study was aimed to a test the effects of TCE on specific bacterial groups.

Several intestinal cytokines were altered by TCE exposure. Out of several tested cytokines, IL-33 was the only one that was significantly decreased in animals exposed to the higher dose of TCE at PND 154. IL-33 has several functions. It is known to mediate IgA production from B cells, and relevant to this study, plays a role in maintenance of the balance and homeostasis between commensal microbiota and mucosal health by regulating intestinal inflammation and maintaining mucosal homeostasis (Sun et al., 2017). Earlier reports documented that IL-33-deficient mice displayed intestinal dysbiosis characterized by increased levels of colitogenic and mucolytic bacteria (Malik et al., 2016). IL-33 is also known to be a critical regulator of intestinal Th17/Treg balance and adaptive immunity (Hodzic, Schill, Bolock, & Good, 2017).

In terms of other cytokines, the levels of IL-17 were reduced even after TCE was removed from the drinking water; though the levels of IL-33 were not significantly different than the control animals under these conditions. At the mucosal surface, Th17 cells play a very critical role to maintain a balance between the commensal microbial population and pathogenic invaders (Khader et al., 2009).Th17 and IL 17 mediated immune responses play central role in the pathogenesis of intestinal fibrosis (Ray et al., 2014). It has been reported that TCE could have profibrogenic effects leading to hepatic fibrosis in genetically predisposed mice (Kopec, Sullivan, Kassel, Joshi, & Luyendyk, 2014). The reduced levels of IL-17 in the high dosed animals thus reflect a resistance for the development of fibrosis; however, if the shift in the bacterial population is also responsible for this resistance is not known. Based on these findings, it is plausible to hypothesize that the dysbiosis that occurred with continuous developmental TCE exposure until PND 154, initiated a cascade of cytokine production that may have dampened the immune responses by gut-associated lymphoid tissue.

Likewise, our results clearly showed a significant decrease in the CC chemokines [Eotaxin, Monocyte Chemoattractant Protein 1 (MCP-1), Macrophage Inflammatory Proteins (MIP) −1α]. These chemokines are involved in the attraction of eosinophils (Eotaxin), monocytes (MCP-1) and macrophages (MIP-1α) at the gastrointestinal mucosa. In addition, the cytokines produced by these cells (IFN-γ, IL-1ß and IL 3) were also significantly lower, thus it is tempting to speculate that the decrease immune cell number may also lead to the lower cytokines levels.

The mechanism behind the decreased levels of the gut cytokines and chemokines is not known. However, the biological consequence of lower levels of these mediators in the gut may play a protective role by the host. For example, IFN-γ is known to induce translocation of bacteria across gut epithelial cells (Clark, Hoare, Tanianis-Hughes, Carlson, & Warhurst, 2005). The lower level of IFN-γ may protect the translocation of altered bacterial population to intestinal mucosa.

Despite the overall impact of the TCE-mediated decrease of gut cytokines and chemokines, there was one notable exception. The CXC chemokine (KC) was significantly increased in the TCE cessation group only. It has been suggested that neutrophil chemokine KC is produced by gastrointestinal epithelial cells in response to inflammatory mediators (Song et al., 1999). One of such inflammatory mediator could be IL-31 (Yagi et al., 2007), the levels of which were increased upon cessation of TCE. Furthermore, TCE may be functioning directly or indirectly as an inflammatory mediator. The increased number of Gram negative bacterial population (*Bacteroides* Genus) could contribute to lipopolysaccharide (LPS) production that could trigger a proinflammatory reaction. Interestingly, our study also shows a simultaneous increase in the bacteria that belong to *Lactobacillus* genus. The mutual alteration in the bacterial population representing Genus *Bacteroides* and *Lactobacillus* could reflect a balancing effect to control intestinal inflammation (L. Zhang et al., 2006)

There were a few mediators in the gut that remained unchanged when comparing continuous TCE exposure vs. cessation at PND 259. The levels of GM-CSF were significantly lower in continuously exposed mice at PND 259, and removal of the TCE had no effect on this marker. The production of GM-CSF in the intestinal mucosa aids in the recruitment of dendritic cells, thus contributing protection against enteric bacterial pathogens (Coon, Beagley, & Bao, 2009; Hirata, Egea, Dann, Eckmann, & Kagnoff, 2010). The lower level of GM-CSF may contribute to the higher abundance of bacteria representing *Enterobacteriaceae* family. However, the pathogenic bacterial population could be kept at bay by increased levels of KC that may facilitate infiltration of neutrophils. Microbiota homeostasis in the intestinal mucosal tissue is proposed to be maintained by neutrophil gelatinase-associated lipocalin protein (Mori et al., 2016). Thus, it is enticing to postulate that KC could be a very significant player to distinguish TCE mediated gut-associated responses during continuous exposure or exposure cessation.

In a case-control study, close association was reported between the development of primary pneumatosis cystoides intestinalis (PCI) and occupational exposure to TCE (Yamaguchi et al., 1985). The PCI is characterized by the presence of gas cysts in the intestinal wall that are usually produced by bacteria. In the high dosed animals, there was a higher occurrence of gas cysts (data not shown); however, it was not determined if it was in the lumen or lumen wall. In any case, the presence of gas cyst in the high dosed animals may be reflective of either a direct effect of high dose TCE animals on PND 259 or could be due to the bacterial dysbiosis.

In conclusion, perturbation of the gastrointesinal tract with levels of TCE relevant to human exposure initiates a disturbance in the commensal bacterial population, which appeared to modify the protective inflammatory respose in a dose dependent manner. These alterations in the cytokines/chemokines may compromise the intestinal barrier function. Importantly, because many of these observations were observed after a period of exposure cessation, it suggests a potential programming effect and underscores the need for additional studies to understand how these effects persist after TCE is removed.

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Disclaimer:

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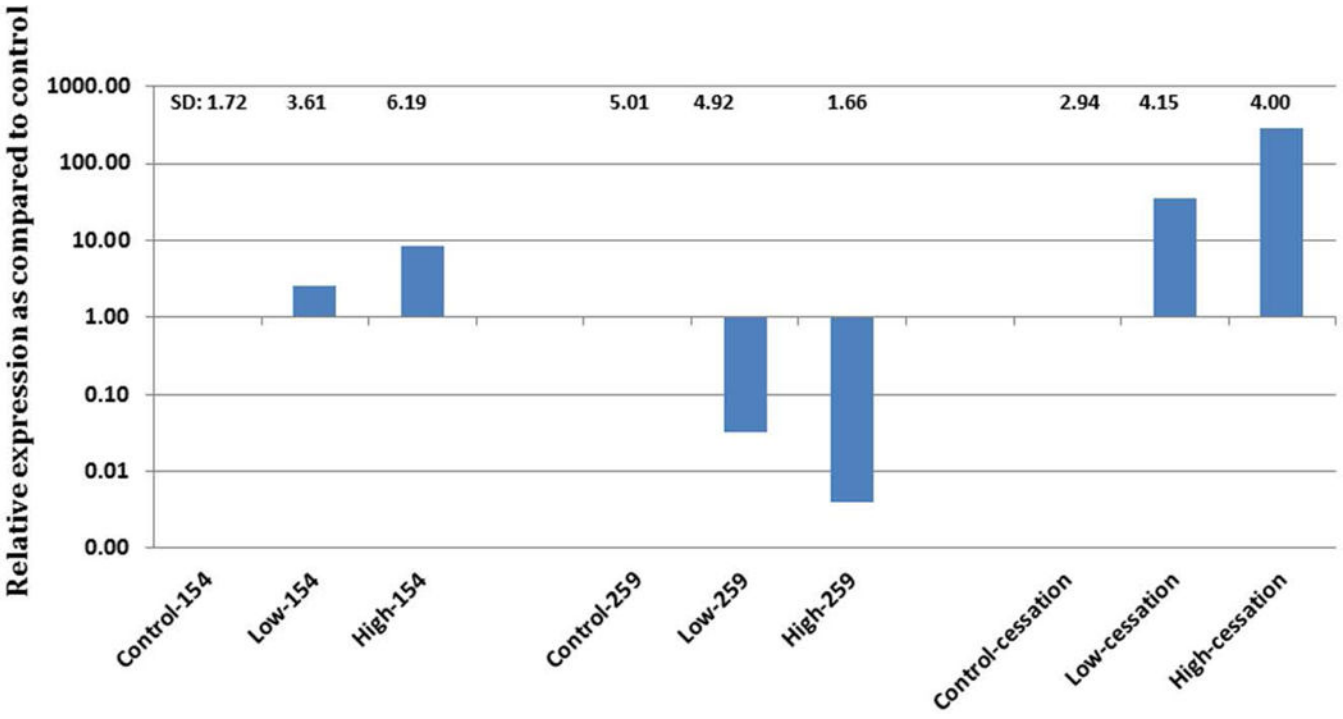

Figure 1. Relative expression of total bacteria associated with ileal mucosa.

Tissue associated total microbial population were assessed using 16s universal primer in Control (untreated), Low dose (0.05 μg/ml) or High dose (500 μg/ml) . Animals were sacrificed at 154 days or 259 days after continuous exposure to TCE in drinking water. A group of animals from each dose were provided regular water after 154 days of TCE exposure and sacrificed on day 259 (Cessation group). The data was normalized with the β-actin. The gene expression of the total bacterial population is represented as fold change when compared to the control group (n-6 in each group).

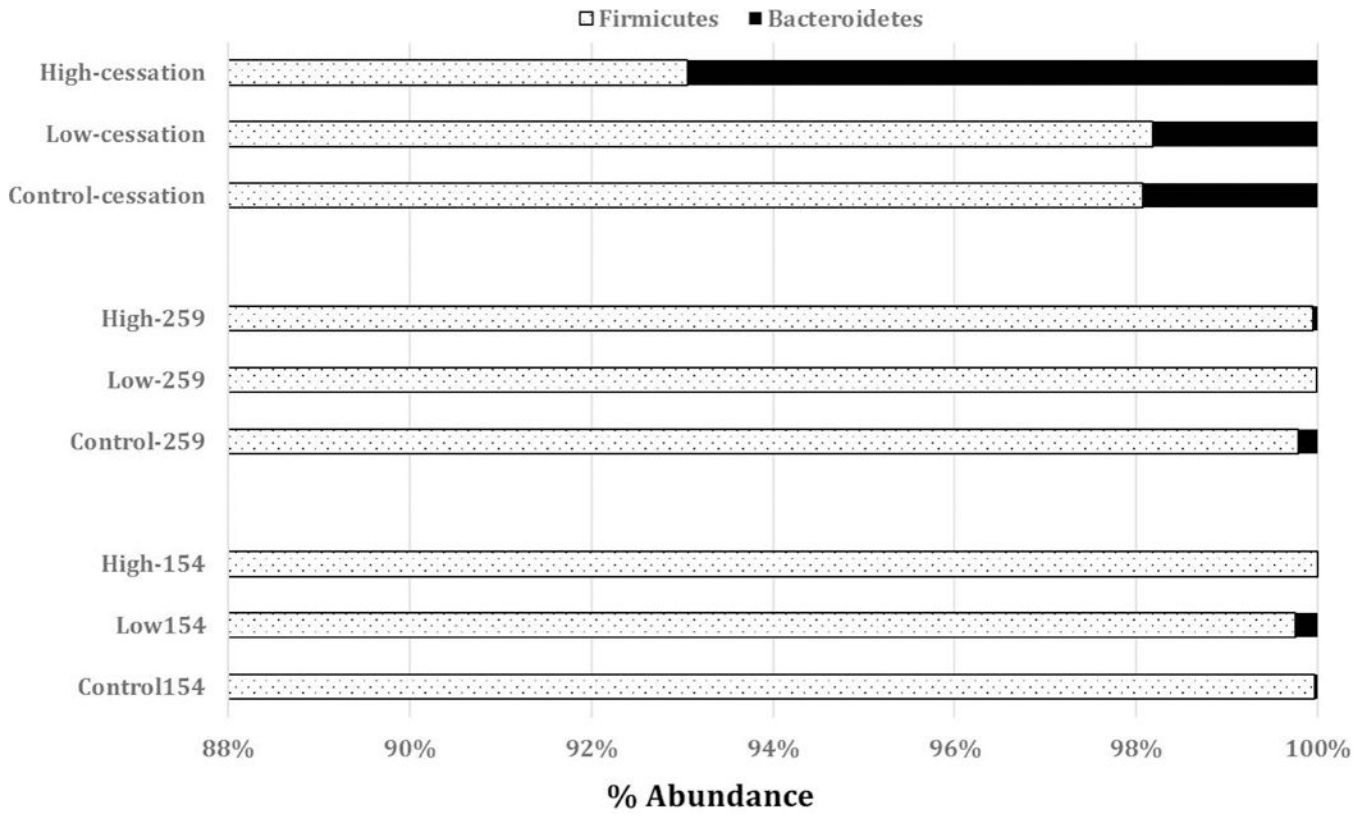

Figure 2. Percent abundance of bacterial population representing major phyla associated with ileal mucosa.

Two major phyla Bacteroidetes and Firmicutes were assessed using specific primer in Control (untreated), Low dose (0.05 μg/ml) or High dose (500 μg/ml). Animals were sacrificed at 154 days or 259 days after continuous exposure to TCE in drinking water. A group of animals from each dose were provided regular water after 154 days of TCE exposure and sacrificed on day 259 (Cessation group). The data was normalized with the β-actin. The y axis reveals 100% stack bar to highlight the ratio of bacterial phyla Firmicutes and Bacteroidetes. Please note that to show a clear difference the stack bar is shown from 88% onwards. The stack bar value are average of six experimental animals.

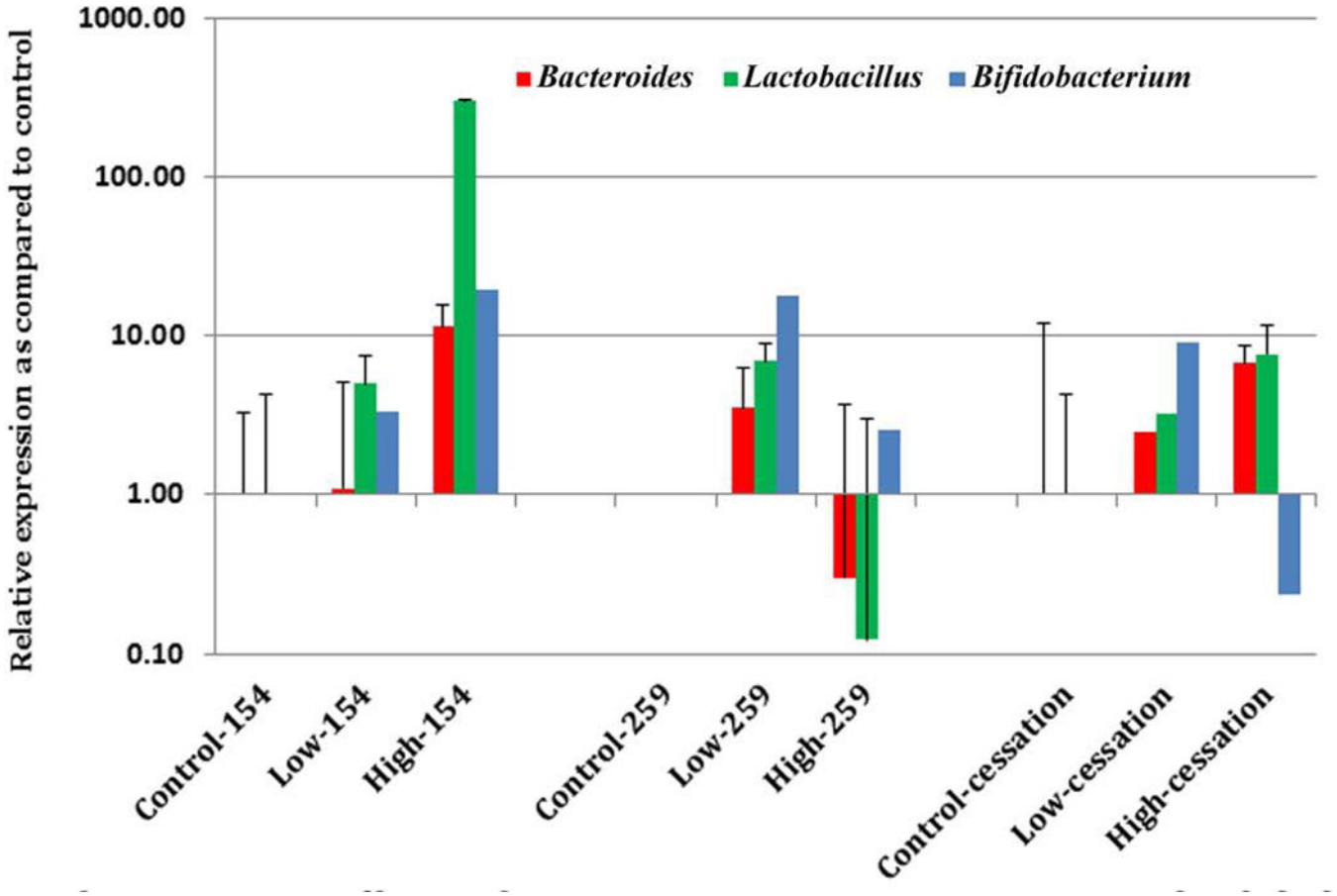

Figure 3. Relative expression of bacterial species representing major Genus associated with ileal mucosa.

Tissue associated microbial population representing three major genera were assessed using specific primers. Animals [Control (untreated), Low dose (0.05 μg/ml) or High dose (500 μg/ml) were sacrificed at 154 days or 259 days after continuous exposure to TCE in drinking water. A group of animals from each dose were provided regular water after 154 days of TCE exposure and sacrificed on day 259 (Cessation group). The data was normalized with the β-actin. The gene expression is represented as fold change when compared to the control group. Error bars represent the mean +SD of 6 animals in each group.

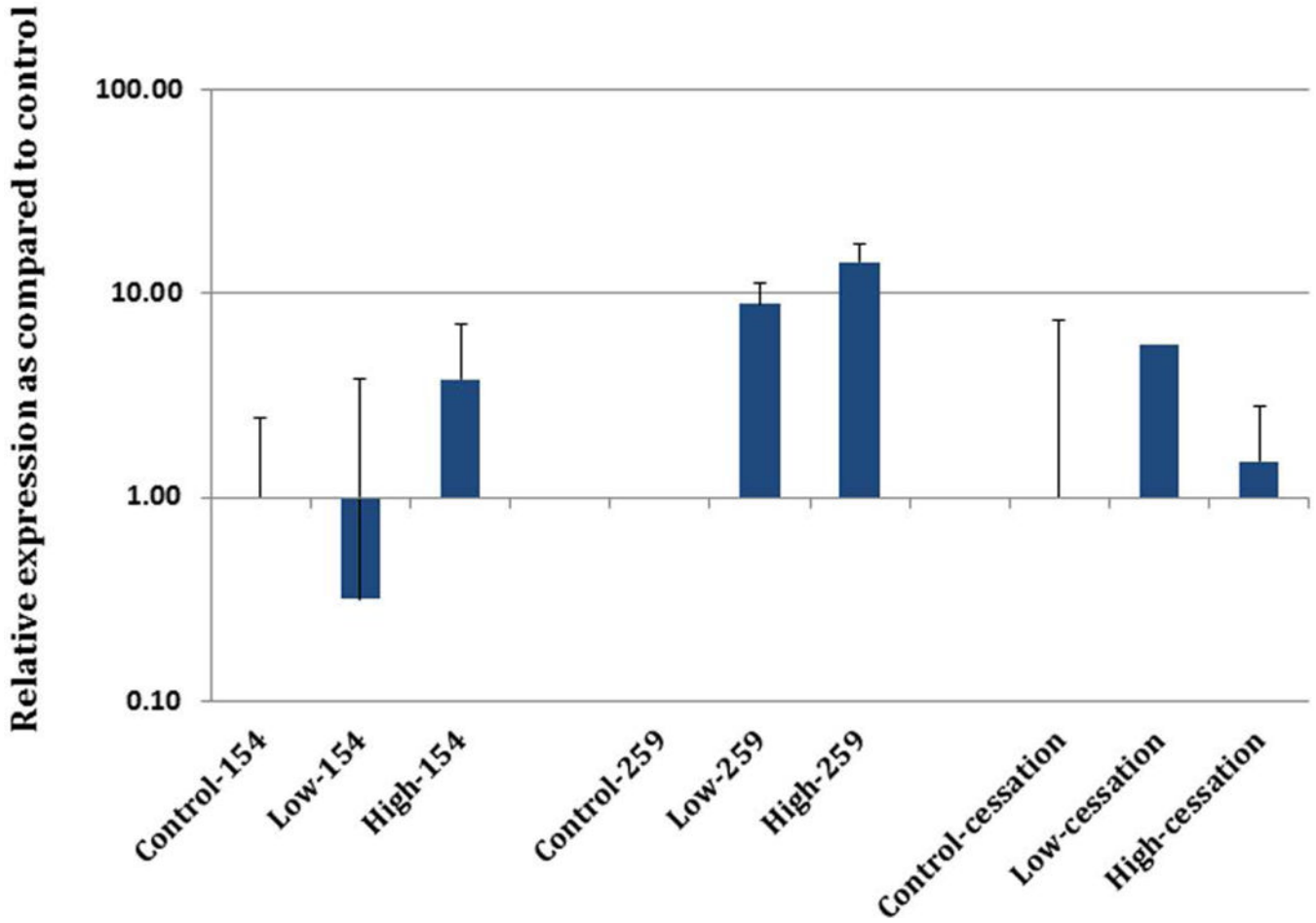

Figure 4. Relative expression of bacterial species representing Enterobacteriaceae family associated with ileal mucosa.

Animals [Control (untreated), Low dose (0.05 μg/ml) or High dose (500 μg/ml)]were sacrificed at 154 days or 259 days after continuous exposure to TCE in drinking water. A group of animals from each dose were provided regular water after 154 days of TCE exposure and sacrificed on day 259 (Cessation group). The data was normalized with the β-actin. The gene expression is represented as fold change when compared to the control group. Each bar represent mean +SD of 6 animals in each group**.**

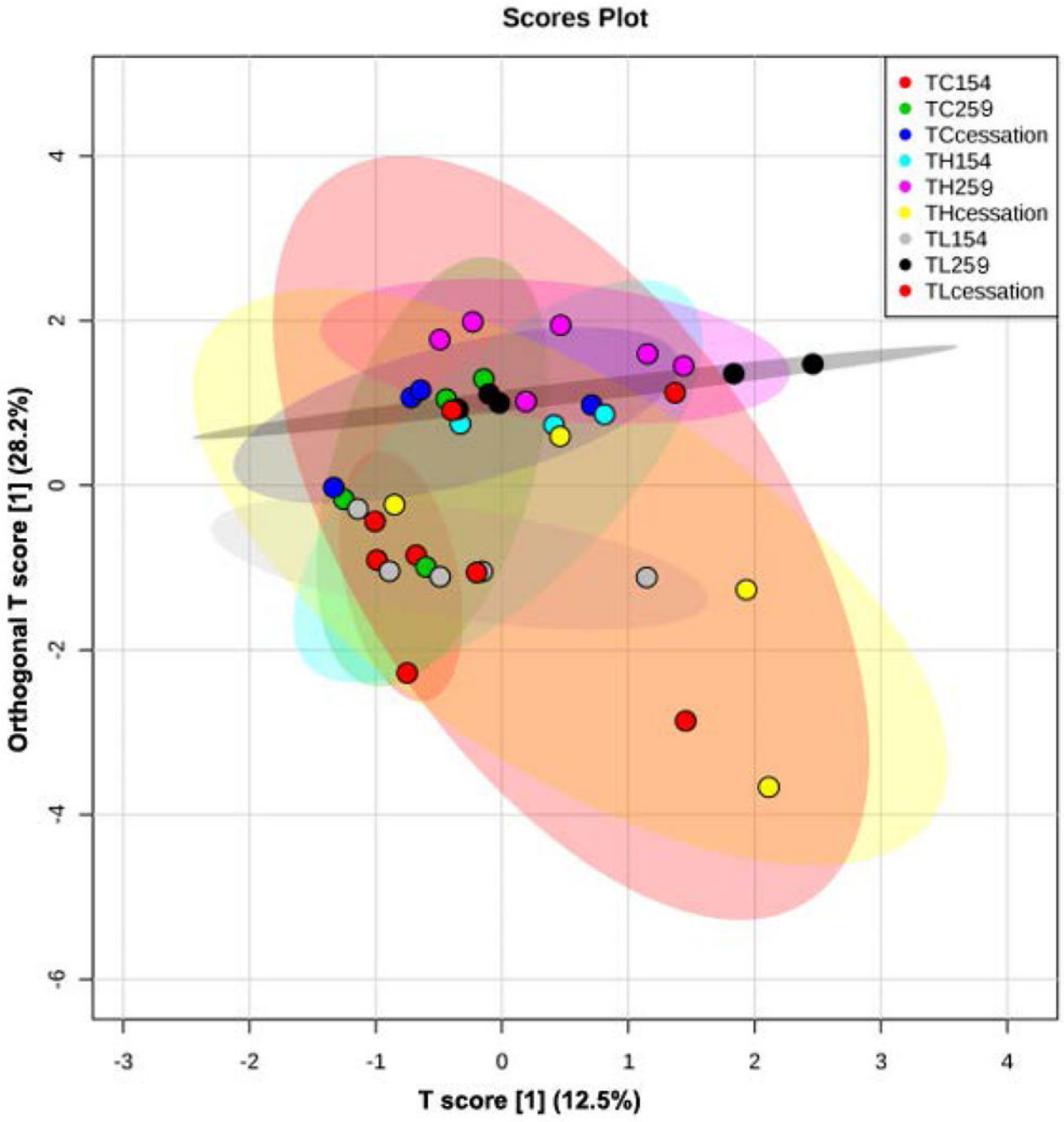

Figure 5a. Orthogonal Partial least squares regression analysis of bacterial population to predict the effect of TCE on mucosa-associated microbiome.

Each colored circle represent one experimental group. TC154=Tissue associated bacteria in control animals on day 154; TC259= Tissue associated bacteria in control animals on day 259; TCcessation= Tissue associated bacteria in control animals after cessation; TH154=Tissue associated bacteria in high dose animals group on day 154; TH259= Tissue associated bacteria in high dose animals on day 259; THcessation= Tissue associated bacteria in high dose animals after cessation; TL154=Tissue associated bacteria in low dose

animals group on day 154; TL259=Tissue associated bacteria in low dose animals on day 259; TLcessation=Tissue associated bacteria in low dose animals after cessation

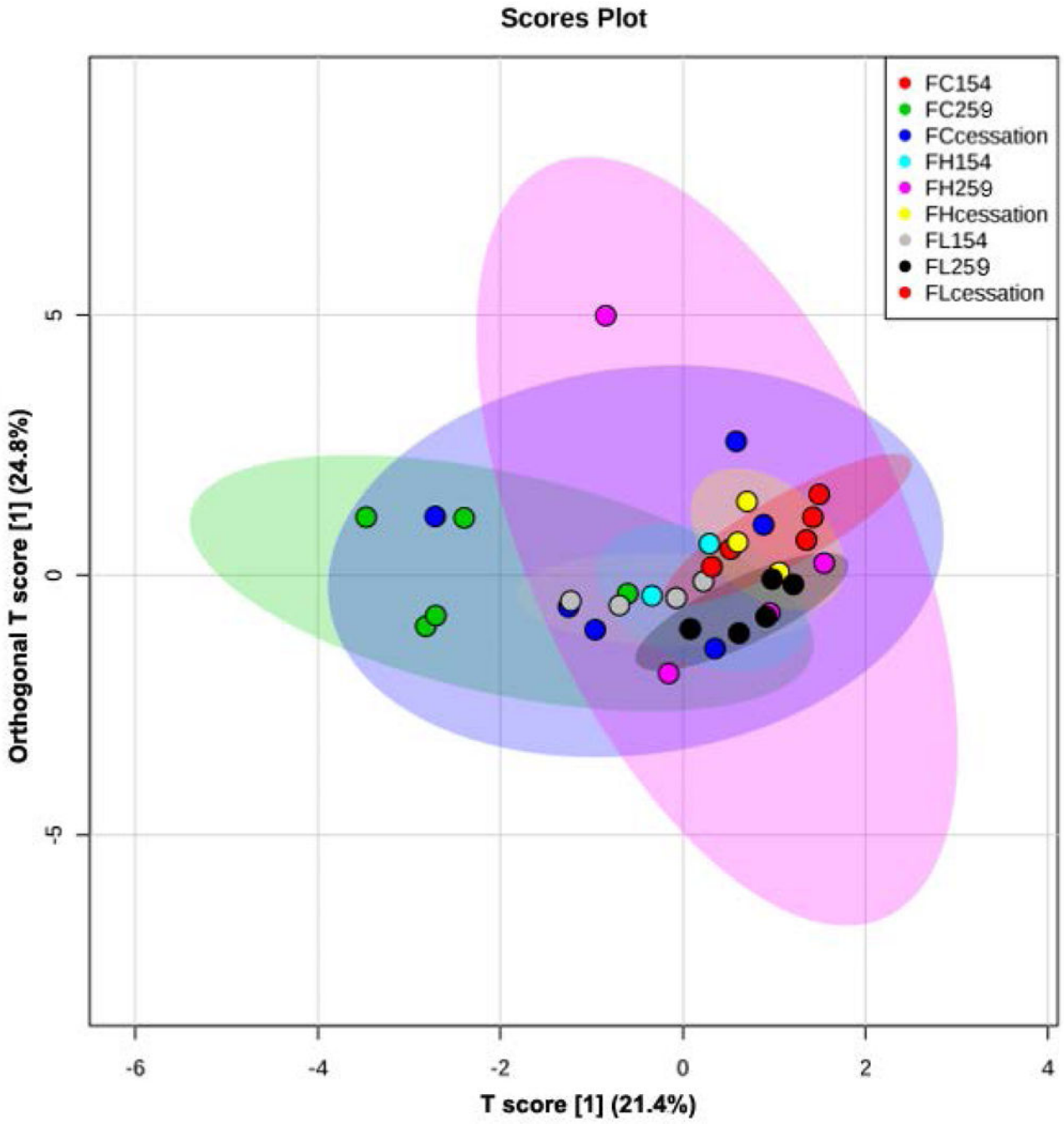

Figure 5b. Orthogonal Partial least squares regression analysis of bacterial population to predict the effect of TCE on fecal microbiome.

Each colored circle represent one experimental group. FC154=*Fecal bacteria in control animals on day 154*; FC259= *Fecal bacteria in control animals on day 259*; FCcessation= *Fecal bacteria in control animals after cessation*; FH154= *Fecal bacteria in high dose animals group on day 154*; FH259= *Fecal bacteria in high dose animals on day 259*; FHcessation= *Fecal bacteria in high dose animals after cessation*; FL154= *Fecal bacteria in low dose animals group on day 154*; FL259= *Fecal bacteria in low dose animals on day 259*; FLcessation= *Fecal bacteria in low dose animals after cessation*

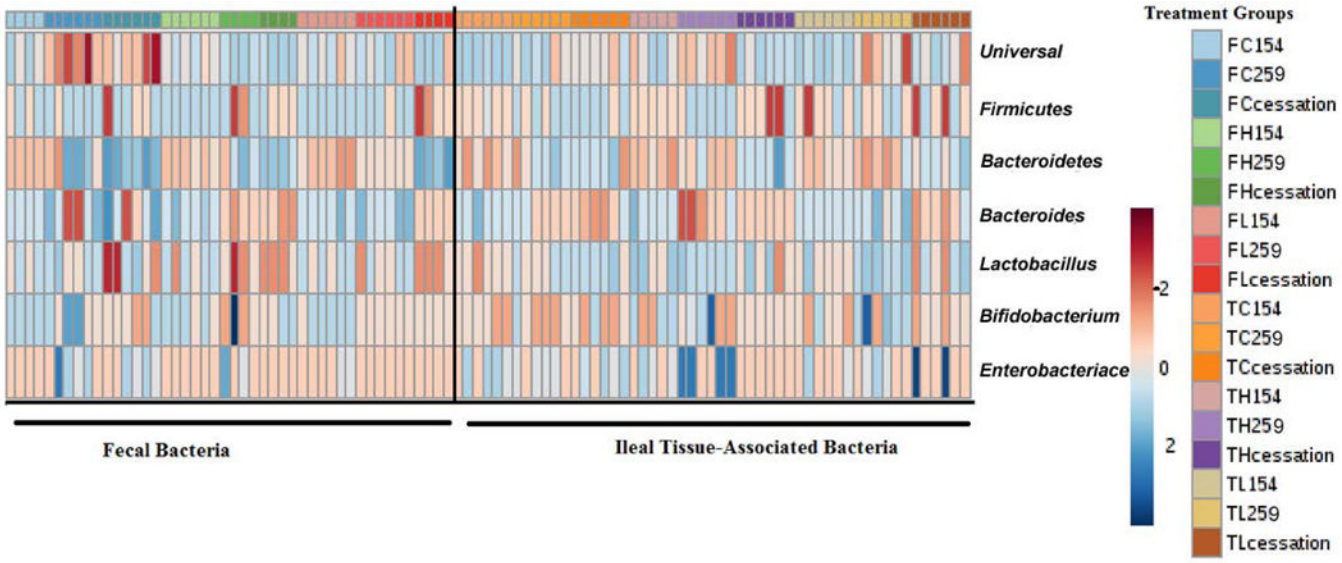

Figure 6. Comparative analysis of bacterial population present ileal mucosa and mice feces.

Heat map showing the abundance of bacterial groups in each sample. Colored short rectangles with in each tick-mark represent one experimental group. Colored long rectangles shows individual samples within one experimental group. Normalization of the data is done using the quantile method, which assumes that usually, the distribution of bacterial gene transcript levels within a community remains also constant across all samples. FC154=*Fecal bacteria in control animals on day 154*; FC259= *Fecal bacteria in control animals on day 259*; FCcessation= *Fecal bacteria in control animals after cessation*; FH154= *Fecal bacteria in high dose animals group on day 154*; FH259= *Fecal bacteria in high dose animals on day 259*; FHcessation= *Fecal bacteria in high dose animals after cessation*; FL154= *Fecal bacteria in low dose animals group on day 154*; FL259= *Fecal bacteria in low dose animals on day 259*; FLcessation= *Fecal bacteria in low dose animals after cessation* TC154=*Tissue associated bacteria in control animals on day 154*; TC259= *Tissue associated bacteria in control animals on day 259*; TCcessation= *Tissue associated bacteria in control animals after cessation*; TH154=*Tissue associated bacteria in high dose animals group on day 154*; TH259= *Tissue associated bacteria in high dose animals on day 259*; THcessation= *Tissue associated bacteria in high dose animals after cessation*; TL154=*Tissue associated bacteria in low dose animals group on day 154*; TL259=*Tissue associated bacteria in low dose animals on day 259*; TLcessation=*Tissue associated bacteria in low dose animals after cessation*

Table 1a.

Effect of TCE exposure on cytokine levels in the intestinal tract after day 154

	Cytokine	Dose	p-value	Fold Change	Mean (pg/mL)	Std. Err.
Day 154	IL-33	Control	---	---	228.49	9.31
	IL-33	High	0.016	−0.606	150.16	22.68
	IL-33	Low	0.697	0.059	237.98	21.4

Table 1b.

Effect of TCE exposure on cytokine levels in the intestinal tract after Day 259

	Cytokine	Dose	p-value	Fold Change	Mean (pg/mL)	Std. Err.
Day 259	IFN-γ	Control	---	---	15.39	0.682
	IFN- γ	High	0.00689	−0.35	12.07	0.662
	IFN- γ	Low	0.00481	−0.325	12.28	0.486
	IL-3	Control	---	---	1.57	0.105
	IL-3	High	0.052	−0.309	1.27	0.084
	IL-3	Low	0.359	−0.112	1.46	0.06
	IL-12(p40)	Control	---	---	32.68	4.59
	IL-12(p40)	High	0.00884	−1.23	13.94	0.889
	IL-12(p40)	Low	0.121	−0.501	23.1	3.21
	IL-17F	Control	---	---	27.26	2.13
	IL-17F	High	0.021	−0.424	20.32	0.598
	IL-17F	Low	0.737	−0.043	26.46	0.854
	GM-CSF	Control	---	---	83.2	0.84
	GM-CSF	High	0.00595	−0.121	76.48	1.45
	GM-CSF	Low	0.187	−0.031	81.46	0.898
	Eotaxin	Control	---	---	1104.19	57.48
	Eotaxin	High	0.034	−0.235	938.41	14.85
	Eotaxin	Low	0.219	−0.12	1016.35	31.87
	IL-31	Control	---	---	419.16	43.45
	IL-31	High	0.052	−0.309	1.27	0.084
	IL-31	Low	0.356	−0.184	368.95	27.56

Table 1c.

Effect of TCE exposure on cytokine levels in the intestinal tract after cessation

	Cytokine	Dose	p-value	Fold Change	Mean (pg/mL)	Std. Err.
Cessation	IL-31	Control	---	---	388.88	79.38
	IL-31	High	0.682	0.162	434.94	74.7
	IL-31	Low	0.039	0.731	645.47	72.77
	KC	Control	---	---	7.27	1.36
	KC	High	0.249	−0.668	4.57	1.72
	KC	Low	0.084	0.507	10.33	0.705
	IFN-γ	Control	---	---	17.2	1.74
	IFN-γ	High	0.03	−0.515	12.03	0.521
	IFN-γ	Low	0.427	−0.139	15.62	0.677
	IL-17	Control	---	---	2.76	0.464
	IL-17	High	0.039	−1.05	1.33	0.37
	IL-17	Low	0.465	−0.224	2.36	0.218
	GM-CSF	Control	---	---	58.56	5.09
	GM-CSF	High	0.00382	-0.789	33.88	4.04
	GM-CSF	Low	0.841	-0.029	57.38	2.55
	Eotaxin	Control	---	---	497.11	52.24
	Eotaxin	High	0.016	−0.656	315.55	19.07
	Eotaxin	Low	0.221	−0.237	421.75	18.7
	IL-1β	Control	---	---	142.75	20.74
	IL-1 β	High	0.058	−0.639	91.69	7.63
	IL-1 β	Low	0.465	−0.189	125.25	8.95
	IL-3	Control	---	---	2.7	0.312
	IL-3	High	0.00512	−1.09	1.27	0.074
	IL-3	Low	0.321	−0.204	2.34	0.108
	MCP-1	Control	---	---	29.06	1.88
	MCP-1	High	0.026	−0.894	15.64	4.09
	MCP-1	Low	0.772	0.044	29.95	2.32
	MIP-1α	Control	---	---	24.34	3.23
	MIP-1 α	High	0.029	−0.735	14.63	1.1
	MIP-1 α	Low	0.654	0.111	26.29	2.74