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# PCB DISRUPTION OF GUT AND HOST HEALTH: IMPLICATIONS OF PREBIOTIC NUTRITIONAL INTERVENTION

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# PCB DISRUPTION OF GUT AND HOST HEALTH: IMPLICATIONS OF PREBIOTIC NUTRITIONAL INTERVENTION

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DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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Lexington, KY

Director: Dr. Bernhard Hennig, Professor of Nutritional Sciences and Toxicology

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2018

ABSTRACT OF DISSERTATION

# PCB DISRUPTION OF GUT AND HOST HEALTH: IMPLICATIONS OF PREBIOTIC NUTRITIONAL INTERVENTION

Exposure to environmental pollutants poses numerous risk factors for human health, including increasing incidence of cardiovascular disease and diabetes. Persistent organic pollutants, such as polychlorinated biphenyls (PCBs) have been strongly linked to the development of these chronic inflammatory diseases and the primary route of exposure is through consumption of contaminated food products. Thus, the gastrointestinal tract is susceptible to the greatest levels of these pollutants and is an important facet to study.

The first two hypotheses of this dissertation tested that exposure to PCBs disrupts gut microbiota directly (*in vitro*) and within a whole body system. PCB exposure disrupted microbial metabolism and production of metabolites (i.e. short chain fatty acids) *in vitro*. These disruptions in microbial populations were consistent in our mouse model of cardiometabolic disease, where we observed reductions in microbial diversity, an increase in the putative pro-inflammatory ratio of Firmicutes to Bacteroidetes, and reductions in beneficial microbial populations in exposed mice. Furthermore, observed greater inflammation was observed both within the intestines and peripherally in PCB exposed mice as well as disruptions in circulating markers associated with glucose homeostasis.

Nutritional interventions high in prebiotic dietary fiber such as inulin may be able to attenuate the toxic effects of pollutant exposure. To test the hypothesis that consumption of the prebiotic inulin can decrease PCB-induced disruption in gut microbial and metabolic homeostasis, $LDLr^{-\backslash-}$ mice were fed a diet containing inulin and exposed to PCB 126. Mice fed an inulin-containing diet and exposed to PCBs exhibited improved glucose tolerance, lower hepatic inflammation and steatosis, and distinct differences

in gut microbial populations. Overall, these data suggests that nutritional intervention, specifically prebiotic consumption, may reduce pollutant-induced disease risk.

KEYWORDS: Gut Microbiota, Cardiometabolic disease, PCBs, Inulin, Prebiotic, Nutrition

Jessie Baldwin Hoffman

November 25, 2018

# PCB DISRUPTION OF GUT AND HOST HEALTH: IMPLICATIONS OF PREBIOTIC NUTRITIONAL INTERVENTION

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# Chapter 1 Literature Review

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Jessie B. Hoffman, Bernhard Hennig [1]

## 1.1 Environmental Pollutants: Overview

Environmental pollution affects nearly every country throughout the world, and exposure to environmental pollutants is involved in the pathogenesis of numerous non-communicable diseases including cardiovascular disease, diabetes, and obesity [2]. Recent evidence from the World Health Organization has revealed that China and India are two of the most affected countries in terms of indoor and outdoor air pollution exposures with approximately 6.5 million associated deaths each year [3]. Pollution from non-airborne exposures (e.g., contaminated foods and waters) are more difficult to determine but should be expected to further increase these high death rates. Despite our knowledge of the adverse health effects of pollution exposure, levels of environmental pollutants have continued to rise over the past few years, especially in developing countries. Great strides have been made over the past decade to substantially reduce the amount of pollution generated and while total elimination is ideal, it is not a realistic or feasible goal in the immediate future [4]. The next question then becomes what can we do now, as individuals, to protect ourselves and future generations from pollutant toxicity and associated diseases? Thus, if we can better understand the mechanisms associated with exposure to environmental pollutants and effects on disease risk we may be able to develop appropriated and effective means to tackle such issues.

### 1.1.1 Effects of Pollutants on the Human Body

Pollutants impact nearly every system of the human body. We are exposed to pollution through a variety of routes including air, the water we drink, and the foods that we consume [2, 5]. These routes of exposure introduce complications throughout the pulmonary system, digestive tract, liver and circulation, and ultimately impact peripheral tissues [2]. The impact of exposure within these systems contributes not only to localized inflammatory responses, but may also influence whole-body metabolic, vascular, and immune health, thus increasing the risk of non-communicable diseases.

*Pulmonary System*

One common route of pollutant exposure is through inhalation, thus exerting detrimental effects on the pulmonary system [5]. Exposure to air pollution has been consistently linked with the development of conditions including asthma, chronic obstructive pulmonary disease, and respiratory infections [6]. There is a well-documented interplay between air pollution exposure and increased airway reactivity in children with asthma, and chronic exposure may also increase the risk of asthma development in children [6, 7]. Furthermore, exposure to air pollutants during gestational and early life has also been associated with disturbances in lung development and prevalence of respiratory conditions in childhood that may persist throughout life [8]. For example, short-term exposure to particulate matter ($PM_{2.5}$), $NO_2$, and ozone, is associated with lower forced vital capacity and lower forced expiratory volume [9]. Additionally, a recent study

reported that in adult patients with acute respiratory distress syndrome, chronic exposure to air pollution (i.e. ozone and particulate matter) is associated with an increase rate of mortality [10]. Importantly, the initiation of inflammatory responses within the lungs from pollutant exposure can impact other organ systems, contributing to further disease pathologies [11]. For example, acute or chronic exposure to airborne particulate matter can accelerate the pathology of atherosclerosis through increases in vascular inflammation, macrophage infiltration, and generation of reactive oxygen species [12, 13].

*Gastrointestinal Tract*

The gastrointestinal tract plays critical roles in human health and has recently garnered more attention in the scientific community, specifically regarding the intestinal barrier and gut microbiome. The principle function of the intestine is to regulate the absorption of water and nutrients into circulation while also serving as a barrier to the infiltration of pathogens and toxic compounds [14]. Additionally, the gut harbors trillions of bacteria that play critical roles in numerous facets of host metabolism and health [15]. Alterations in gut microbial composition is termed “dysbiosis” and has been linked to increased disease risk. These perturbations in gut microbial populations are associated with a decrease in microbial diversity and are observed in conditions of obesity and diabetes [15]. Importantly, there is current evidence that an altered gut microbiome may actually be causal in the development of such chronic inflammatory conditions.[16, 17] Because the primary routes of exposure to many pollutants is through water and foods, the gastrointestinal tract is exposed to high levels of

pollutants and thus be greatly affected. Indeed, it has been observed that exposure to polychlorinated biphenyls (PCBs) disrupts intestinal barrier function through dysregulation of tight junction proteins [18]. Furthermore, oral exposure to the pollutant benzo[a]pyrene resulted in intestinal inflammation and ileal lesions, which was associated with shifts in gut microbial composition [19]. Importantly, Zhang et al [20] have demonstrated that exposure to the persistent organic pollutant tetrochlorodibenzofuran (TCDF), can result in alterations of gut microbial populations at the level of phylum, class, and genus and these changes were associated with disruptions in bile acid and short chain fatty acid (SCFA) metabolism [20]. This study is one of the first to indicate the link between the effects of pollutant exposure in the gut to overall metabolic health.

While it is commonly thought that air pollution only affects lung health, it is important to note that this exposure can also affect the digestive tract and subsequently systemic organ systems. It has been documented that upon inhalation, airborne pollutants are quickly cleared from the lungs and transported to the intestine via mucociliary transport [14]. Therefore, even airborne pollutants can alter overall gut health and microbial populations. For example, it has been demonstrated that ingestion of airborne particulate matter (PM) can cause intestinal inflammation and alter the gut microbiota, contributing to alterations of SCFA production in mice [21]. Additionally, in colonic cells, exposure to PM caused reactive oxygen species production, nuclear factor kappa-b (NF-κB) activation, and disruptions in tight junction proteins, indicating that PM may disrupt intestinal permeability and increase intestinal inflammation [22]. Because

the gut lies at the interface of the outside environment and our internal organ systems, maintenance of a healthy intestinal environment and microbiome may aid in prevention of pollutant-associated diseases.

*Liver*

Many nutrients and toxicants travel from the gut to the liver via the portal vein. Thus, the liver is one of the most critical organs in the body, contributing greatly to metabolism, secretory, excretory, and vascular functions. In the United States, the incidence of liver disease has increased with expanding levels of obesity and is thought to be due to an increase in non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) [23]. While these liver pathologies can be developed through poor dietary and lifestyle choices, there is recent evidence that environmental pollutants can also greatly influence liver disease [24]. The term, toxicant-associated fatty liver disease (TAFLD) is a relatively new labeled type of liver disease and exhibits similar pathologies to other liver diseases [24]. TAFLD and its more severe form, toxicant associated steatohepatitis (TASH), have been observed in workers highly exposed to industrial chemicals such as vinyl chloride [25]. These observations of TASH were associated with increased levels of inflammatory cytokines as well as insulin resistance, indicating the widespread metabolic complications that pollutant exposure can exert via the liver [25]. Furthermore, there is evidence that low-level pollutant exposure may also influence the development of liver pathologies. For example, epidemiological data from the general population revealed associations between PCBs and increased levels of serum alanine aminotransferase (ALT), a widely

used biomarker of liver injury [26]. These findings support a potential association of chronic, low level pollutant exposure and the development of TAFLD and liver disease. Because the liver is such a vital organ for metabolism, environmental exposures affecting the liver may also contribute to obesity and diabetes through disruption in glucose and lipid metabolism. Therefore, further research is warranted to better understand the mechanisms behind pollutant-induced liver injury and avenues of potential protection against these pathologies.

*Vascular Tissues*

Circulating nutrients, toxicants, and their metabolites can modulate vascular responses that can either be pro- or anti-atherogenic. One of the key events in the progression of atherosclerosis is endothelial cell dysfunction. It has been documented that exposure to particulate matter (PM) air pollution can disrupt the vasculature resulting in endothelial cell dysfunction [27]. Furthermore, there is also evidence that arsenic exposure is associated with endothelial cell dysfunction, as evidenced by the presence of circulating soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) in Bangladeshi individuals exposed to arsenic [28]. In support of this, in mouse models of atherosclerosis ($APOE^{-/-}$), exposure to arsenic resulted in increased accumulation of arsenic within the vessel walls as well as exacerbated aortal lesion formation [29]. There is also evidence that persistent organic pollutants (POPs), including PCBs, can increase atherosclerotic risk. Specifically, coplanar PCBs such as PCB 77 and PCB 126 bind to the aryl hydrocarbon receptor (AhR) within vascular endothelial cells, triggering a

cascade of events that leads to production of reactive oxygen species and disruption of cellular redox status [30]. These events ultimately lead to up-regulation of NF-κB resulting in induction of proinflammatory gene products such as cytokines, chemokines, and cell adhesion molecules that contribute to endothelial cell dysfunction and the early stages of atherosclerosis [31]. More recent evidence suggests that dioxin-like pollutant exposure (PCB 126) can result in up-regulation of the enzyme flavin-containing monooxygenase 3 (FMO3) and subsequently increase circulating levels of trimethylamine-N-oxide (TMAO), a biomarker strongly associated with cardiovascular disease [32].

*Peripheral Tissues*

Upon circulation of these environmental pollutants, as discussed above, numerous peripheral tissues become exposed, including highly vascularized adipose tissue [33]. With the worldwide obesity epidemic, research on the toxicological interplay between pollutants and adipose tissue has become increasingly important. It is no longer believed that adipose tissue is solely a storage of excess energy, but in fact plays numerous metabolic and endocrine roles within the body. In regards to pollutant exposure, adipose tissue is capable of storing lipophilic pollutants, specifically persistent organic pollutants (POPs) [33]. While such storage can be protective to reduce availability to other organ systems in situations of acute and high pollutant exposures, there is evidence that a low and chronic release of POPs from adipose tissue can exert some toxic effects to other lipophilic tissues such as the brain and the liver [34]. Furthermore, it has been demonstrated that pollutants can alter adipose tissue

structure and function through metabolic disruption and inflammation, which may increase the risk of chronic metabolic diseases [33, 35]. Some pollutants also have been associated with increases in adiposity and weight gain and are thus termed “obesogens” [36]. These pollutants, including bisphenol A (BPA), benzo[a]pyrene, and certain organophosphate pesticides, are thought to play a role in the development of obesity by disrupting lipid storage and metabolic mechanisms and promotion of adipocyte hyperplasia [36, 37].

As evidenced by the above-discussed findings, pollution has diverse and body-wide effects on human health, thus increasing the risk of disease. Therefore, identifying what makes an individual more at risk and understanding how lifestyle choices influence pollutant-toxicity is of utmost importance.

### 1.1.2 Polychlorinated Biphenyls- An Overview

Polychlorinated Biphenyls, or PCBs are a group of synthetic organic chemicals comprised of carbon, hydrogen, and chlorine atoms. There are over 200 different congeners of PCBs, with their differences being the number and location of the chlorine atoms [2]. Due to their thermally stable and lipophilic nature, PCBs were utilized extensively as lubricants and coolants in electrical equipment and also in industrial products such as pigments and plasticizers. PCBs production was banned in 1979 due to the observed environmental persistence and potential human health effects, however because of their high resistance to degradation, they still are present in the environment [2]. PCBs biomagnify along the food chain and thus pose a risk for human exposure through consumption of contaminated foods. The highest contaminated food

substances include fish such as catfish, salmon, and carp, as well as other animal products including meat and dairy. There is increasing evidence that PCB exposure is associated with adverse health consequences [38]. Some of the most toxic PCB congeners include the dioxin-like PCBs. These PCBs act similarly to the compound tetrachlorodibenzodioxin (TCDD), through strong binding of the aryl hydrocarbon receptor (AhR) which in turn triggers a cascade of events resulting in the upregulation and uncoupling of cytochrome p450 Cyp1a1 resulting in production of reactive oxygen species [39]. This chronic production of ROS causes systemic inflammation and subsequent inflammatory health complications such as cardiovascular disease and diabetes. Evidence of the effects of PCBs on specific organ systems was discussed in a prior section. There is also evidence that PCBs can act through specific molecular mechanisms such as epigenetic regulation and microRNA alterations. For example, it has been demonstrated that exposure to PCB 126, a coplanar dioxin-like PCB, induces inflammation in endothelial cells through epigenetic regulation of the NF-kB p65 subunit [40]. It was also shown in human umbilical vein endothelial cells that the PCB mixture, Aroclor 1260 alters the expression of microRNAs that are associated with vascular diseases, indicating yet another mechanism by which PCBs can induce inflammatory disease [41] Further research is still needed to understand the diverse mechanisms by which PCBs induce health complications and specifically how diet and preexisting disease influence the toxicity of these pollutants.

### 1.1.3 Factors Influencing Pollutant-Induced Disease Risk

One major question to be addressed is why certain people are more susceptible to pollutant-associated diseases? It has been demonstrated that compromised health or sub-optimal lifestyles, may exacerbate the toxicity of environmental pollutants. For example, it has been shown that pollutant toxicity is worsened in animal models with non-alcoholic fatty liver disease (NAFLD) [42]. This illustrates the idea that individuals in poor health or with underlying disease conditions may be more susceptible to environmental insult or other chemical or non-chemical stressors. Additionally, it is now appreciated that events occurring during pregnancy can play a significant role in the future health and disease risk of the offspring [43]. It has been documented that the offspring of mothers exposed to environmental pollutants during pregnancy exhibit effects that may be detrimental and persist into adulthood [44, 45]. For example, particulate matter exposure throughout pregnancy was observed to be associated with an increased fetal C-reactive protein (CRP) level upon delivery, indicating that prenatal pollution exposure may increase fetal inflammatory responses and thus potentially alter long-term health outcomes [46]. Moreover, data from a longitudinal birth cohort study revealed an association between prenatal exposure to dichlorodiphenyltrichloroethane (DDT) and hypertension later in life, further indicating the long-term negative effects of early-life pollutant exposure [47]. Aside from compromised health and prenatal exposures, some individuals may be susceptible to disease development associated with environmental insults due to genetic predisposition to these non-communicable diseases. For

example, certain genetic polymorphisms in oxidative stress genes and inflammatory genes have been demonstrated to influence the respiratory effects and susceptibility to air pollution [48].

## 1.2 Nutritional Modulation of Disease Risk and Pollutant Toxicity

As discussed above, the risk of pollutant-induced toxicity may be increased by underlying diseases, prenatal exposures, and genetic predispositions. Because certain unhealthy lifestyle choices can predispose an individual to disease, it can be expected that this may allow for greater pollutant induced toxicity. Thus, finding ways to achieve a healthy lifestyle and therefore reduce disease risk are significant and highly important.

Recent evidence has shown that while genetics do play a role in disease risk, it may be smaller than once thought. In a recent study published by Khera et al. [49] it was observed that in participants with a high genetic risk of coronary artery disease, those living a “favorable lifestyle” (i.e. no obesity, no smoking, regular physical activity, and a healthy diet) had approximately a 50% lower relative risk of coronary artery disease compared to those living an “unfavorable lifestyle” [49]. These findings demonstrate just how influential our lifestyle choices are on overall disease risk independent of genetic risk. For individuals looking to achieve “favorable lifestyle” choices, a logical place to start is examination of dietary habits. Because nutrition is such a critical aspect of life with food being a fundamental component for survival, understanding how nutrition affects our health is essential. Research has established that certain nutrition practices can worsen health outcomes while others can provide benefits. This is well

evidenced with the present global obesity epidemic, which has been attributed to a reliance on processed, high fat, and nutrient poor foods that contribute to excess energy intake relative to actual nutrient requirements [50]. While body fat gain is the first visible complication of this chronic positive energy balance, the internal inflammatory processes and metabolic complications elicited pose a greater threat to overall well-being. These less visible metabolic complications can even occur prior to any weight gain. For example, consumption of high fat diets, with greater proportions of saturated, trans, and omega-6 fatty acids (e.g., linoleic acid) can increase inflammatory processes rapidly and thus long-term cardiovascular disease risks [51, 52]. Additionally, research examining the effects of consumption of processed meats has become a source of great interest and controversy in the field of nutrition. As defined by the American Institute for Cancer Research, processed meats are “meats preserved by smoking, curing or salting, or addition of chemical preservatives”. Interestingly, a meta-analysis conducted by Micha et al [53] revealed that a single serving per day of processed meat was associated with a 42% increase in risk of coronary heart disease and a 19% higher risk of diabetes mellitus [53].

On the other hand, it is well appreciated that many chronic diseases, such as cardiovascular disease and type 2 diabetes, can be prevented or attenuated with healthful dietary practices. This includes diets similar to that of the Mediterranean diet, rich in omega-3 fatty acids, fruits, vegetables, and whole grains [54, 55]. These dietary components, which are rich in antioxidant and anti-inflammatory compounds (e.g., phytochemicals and polyphenols), may reduce or

prevent the proinflammatory events associated with chronic metabolic diseases [54, 55]. Additionally, there is also evidence that consumption of polyphenolic compounds such as resveratrol, tea polyphenols, and curcumin can protect against inflammation and chronic disease through alterations in proinflammatory gene expression both directly and through epigenetic modifications.[56] For example, curcumin, a principle component of the spice turmeric, has been demonstrated to suppress or inhibit the expression of tumor necrosis factor-a (TNF-α) as well as cyclooxygenase 2 (COX-2), the target of nonsteroidal anti-inflammatory drugs (NSAIDs) [57, 58]. Additionally, the consumption of table grapes, rich in polyphenolic compounds including resveratrol and anthocyanins, have been shown to attenuate systemic inflammatory responses, hepatic lipogenesis, and adiposity in mice fed a high fat diet [59]. Thus, dietary choices are very powerful, and when chosen appropriately can vastly influence the development of numerous metabolic diseases.

The underlying denominator of non-communicable diseases, such as those observed from pollutant exposure, is oxidative stress and inflammation [60]. An inflammatory process usually precedes other health effects, and thus it is a critical step to understand and identify means to protect against inflammation. Similar to the way in which nutrition can contribute to or protect from non-communicable diseases, nutrition can also modulate the toxicity of environmental pollutants, thereby altering overall disease risk.

### 1.2.1 Poor Nutrition Can Exacerbate Pollutant Toxicity

It has been established that certain unhealthy nutrition practices can actually worsen the toxicity of pollutant exposure. The overconsumption of processed and refined foods contributes to diets high in inflammatory fatty acids, which not only exacerbate diet-induced metabolic complications, but also pollutant-associated inflammatory processes [61, 62]. These high-fat, processed foods are also low in protective bioactive nutrients, thus leading to increased oxidative stress and inflammation. Additional stressors, such as environmental insults, added to this may result in a greater inflammatory response, which could be due to the fact that the mechanisms of pollutant-induced toxicity and dietary-induced inflammatory responses are similar [62]. This detrimental interplay of nutrition and pollutants was illustrated comparing the effects of PCB exposure in mice fed corn oil (high in linoleic acid) versus mice fed olive oil (high in oleic acid). In corn oil fed mice, PCB exposure resulted in further increases in expression of aortic vascular cell adhesion molecule 1 (VCAM-1). Importantly, this observation was not observed in mice fed olive oil, indicating the selective interaction of specific dietary fats with PCB inflammatory processes.[63] Others have also observed this toxic interplay of pollutants and nutrition. For example, it has been shown that high fat diets can exacerbate arsenic-associated liver inflammation and fibrosis.[64] Additionally, diet-induced obesity and non-alcoholic fatty liver disease (NAFLD) are worsened in the presence of PCB 153, further illustrating the detrimental interplay of poor nutrition and pollutant exposure.[65]

### 1.2.2 Healthful Nutrition Can Protect Against Pollutant Toxicity

As discussed above, individuals consuming a healthful diet already have a lower risk of chronic inflammatory disease and thus may be less susceptible to environmental insults and associated disease risks. This is important when considering the observed increase in air pollution in many overpopulated parts of the world and the link to risks of pulmonary and peripheral diseases. In fact, biological and epidemiological evidence suggests that exposure to particulate matter and related components of air pollution can contribute to oxidative stress and inflammation, suggesting that intake of diets enriched in antioxidant and anti-inflammatory nutrients should be advised to down-regulate pulmonary disease risks [66-68]. There is evidence that specific nutrients can actually reduce the toxicity and health complications associated with pollutant exposure. Diets rich in bioactive food components such as omega-3 fatty acids and polyphenols, contain high levels of antioxidant and anti-inflammatory compounds that are capable of blunting the toxic and inflammatory effects of pollutant exposure [69]. Resveratrol, a polyphenol found abundantly in grapes, berries, and other plants, has been shown attenuate hepatic steatosis and oxidative stress in mice exposed to 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD)[70]. Furthermore, resveratrol has also been documented to combat PCB-induced disruptions in adipocyte glucose homeostasis, which may protect against the development of type 2 diabetes associated with pollutant exposure [71]. Another phenolic compound, epigallocatechin gallate (EGCG), found in green tea, is capable of attenuating cardiovascular inflammation and toxicity associated with arsenic

exposure [72]. In support of this, our lab has observed that consumption of green tea can reduce oxidative and inflammatory responses associated with PCB 126 exposure through up-regulation of antioxidant enzymes [73]. Importantly, EGCG exhibits epigenetic regulation of NF-κB target genes, reducing PCB 126 induced endothelial cell inflammation mechanisms [74].

### 1.2.3 Nutritional Practices to Reduce Pollutant Body Burden

In addition to combatting pollutant-induced inflammation and toxicity directly, there is evidence that certain nutritional components can actually aid in reducing overall body burden of pollutants. Interestingly, it has been observed that individuals consuming vegetarian or vegan diets exhibit trends towards lower body burden of organochloride compounds [75]. These diets tend to be high in polyphenols and antioxidant compounds due to their plant-based focus, and thus it is possible that specific bioactive and plant-derived compounds may be promoting greater excretion of pollutants. However, these associations need to be further explored to determine if this is indeed the case. Because nutrition and pollutants often interact with and alter similar mechanistic pathways, it is possible that nutritional targeting of some of these pathways may be able to modulate pollutant metabolism and excretion. One example of the overlap in nutritional and pollutant interactions can be observed between arsenic and folate. For the body to facilitate excretion of arsenic, it must undergo methylation, with S-adenosylmethionine (SAM) functioning as the methyl donor. Interestingly, synthesis of SAM relies on a one-carbon metabolism that is folate-dependent [76]. Therefore, supplementation with folate may enhance the methylation and

subsequent excretion of arsenic. Indeed, Peters et al. recently observed that 12 and 24-week supplementations of 800μg/day of folic acid significantly reduced blood arsenic concentration in an arsenic-exposed Bangladeshi population [76]. This finding is especially important due to the prevalence of chronic arsenic exposure worldwide and supports the importance of fostering a better understanding of the metabolism and excretion pathways of pollutants as potential targets of intervention. In addition to nutritional modulation of molecular mechanisms to enhance pollutant excretion, direct disruption or binding of these pollutants via the enterohepatic circulation may also provide a means of intervention to reduce body burden. For example, during this process, these pollutants become exposed to the intestinal environment, thus providing an avenue for potential dietary intervention. Indeed, it has been observed that consumption of the dietary fat substitute, Olestra, a sucrose polyester which can enhance the excretion rate of PCBs and hexachlorobenzene in mice and also in highly exposed individuals [77-79]. This enhanced excretion is believed to be through interference with the enterohepatic circulation of these pollutants. Because of these findings, there is the potential that other nutritional components may act similarly and be able to bind and increase pollutant excretion and ultimately reduce the body burden of certain pollutants.

The levels of environmental pollution are on the rise worldwide, especially in densely populated areas including China and India. Environmental pollution impacts virtually every aspect of the human body, including the pulmonary and gastrointestinal systems, liver, vasculature, and other peripheral tissues such as

the adipose tissue. The diverse and detrimental impacts that pollutant exposure has on these systems contribute to the increasing occurrence of chronic inflammatory conditions observed worldwide. While efforts to reduce and remediate pollution are underway, means of immediate protection at an individual level is of upmost importance. Nutrition can have a drastic impact on the development of or protection against non-communicable diseases, such as those associated with pollutant exposure. There is increasing evidence of the protective mechanisms of healthful nutrition on pollutant toxicity, specifically regarding components of the Mediterranean diet (i.e. omega-3 fatty acids and anti-inflammatory polyphenols), suggesting a means by which individuals may be able to control the development of pollutant-associated disease risks. Because nutrition is such a critical aspect of daily life, it is important to continue to foster a better understanding of the ways in which the foods we consume can impact the toxicity of environmental pollutants. Positive lifestyle changes such as healthful nutrition (e.g., diets high in phytochemicals or polyphenols) may provide the most sensible means to develop primary prevention strategies of diseases associated with many environmental toxic insults.

### 1.3 The Gut Microbiome and Effects on Host Health

The term gut microbiota refers to the complete population of microorganisms colonizing the intestines, and as current knowledge stands, is comprised of over 1000 species of bacteria [80]. The gut microbiome has been defined using parameters including diversity, richness, and even distribution of microbial communities. To classify these parameters, 16S rRNA gene sequencing or more

global scale metagenomic analyses are often implemented to compare bacterial populations most commonly from stool samples, but also from intestinal biopsies [81].

Over recent years, interest has grown significantly on understanding the ways in which gut microbiota influence overall host health. Importantly, the gut microbiome has been associated with numerous diseases and health conditions including inflammatory bowel diseases, neurological illnesses, and metabolic diseases [80]. Currently, the scientific community defines a “healthy” gut microbiome as one with greater richness and bacterial diversity, as a reduced bacterial diversity is often observed in obesity, diabetes, and other diseases [80].

Within the intestinal environment there are five main phyla of bacteria: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia [80]. It has been observed that in situations of obesity and chronic inflammatory diseases, the ratio of Firmicutes to Bacteroidetes is increased and that this ratio may not only be a result of these conditions but may actually be causal [80]. Studies using fecal microbiota transplantation (FMT) have been instrumental in the scientific communities understanding of how the gut microbiome impacts overall host health. For example, it has been demonstrated that performing FMT from mice that were obese into lean mice lead to a significant increase in weight gain in the mice receiving the obese microbiota [82]. Importantly, the reverse has also been demonstrated in obese mice transplanted with lean mice microbiota. Upon transplantation, the obese mice lost weight and had improvements markers of glucose intolerance. While these experimental

observations have been made, it is important to understand the mechanisms that drive these findings. Therefore, current microbiota research focuses on identifying core microbial species or genera that exert specific functions that may aid in metabolism, contribute to immunity, reduce inflammation, or function in communication with other organs (e.g. gut-brain axis). For example, *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Eubacterium*, *Rosburia*, and *Clostridium* clusters IVa and XIVa have been associated with optimal gastrointestinal functions and overall health in humans [83]. However, it is important to note that these classifications are still very broad and thus making generalizations at this level of taxonomy may not be most appropriate.

### 1.3.1 Microbial Metabolite Production

The primary end products of bacterial saccharolytic fermentation of non-digestible carbohydrates are short chain fatty acids (SCFAs). These SCFA are formed when carbohydrates escape digestion and absorption in the small intestine and thus persist into more distal regions of the digestive tract where they are metabolized by the residing gut microbiota [84]. The primary SCFA formed by bacterial fermentation are acetate, propionate, butyrate, and formate. Lactate is also produced by several bacterial groups including lactic acid bacteria, *Bifidobacteria*, and Proteobacteria, primarily from rapid fermenting non-digestible carbohydrates [85]. In addition to non-digestible carbohydrates, amino acids may also be fermented and contribute to SCFA production, although this is to a much smaller degree [84]. Through advanced metagenomic analyses, characterization of specific bacterial species responsible for SCFA production

has been made possible. While acetate is a common metabolite for most bacterial species, lactate, propionate, and butyrate have been demonstrated to be more specific with regards to both bacterial species and substrate [86]. Within the the human microbiome, it has been demonstrated that a few species seem to be main contributors to butyrate production, including *Eubacterium rectale*, *Eubacterium hallii*, *Faecalibacterium prausnitzii*, and *Ruminococcus bromii* [87]. Furthermore, *Akkermansia muciniphila*, a mucin degrading bacterium has been identified as a large contributor to propionate production [88]. This bacterium has garnered much attention in the scientific community due to it being linked with improved intestinal health and metabolic parameters in type 2 diabetic and obese subjects, but there is still some debate on solely classifying this bacterium as beneficial [88]. Nevertheless, *A. muciniphila* has been demonstrated to attenuate the high fat-induced endotoxemia that occurs as a result of an impaired intestinal barrier [89]. These protective effects on the intestinal barrier have also been demonstrated *in vitro*, where it was shown that this bacterium binds to the extracellular matrix protein laminin and strengthens the enterocyte monocyte layer [90]. While it still remains unknown, there is potential that some of these effects mediated by *A. muciniphila* are resultant of SCFA production. These discussed specific bacterial species noted for SCFA production is by no means exhaustive but demonstrates how metagenomic analysis has allowed the functional characterization of certain bacterial species residing in the human intestinal environment.

SCFAs exert effects locally within the intestine and systemically and serve a variety of purposes [91]. For example, butyrate serves as a preferential energy source for the colonic epithelial cells, facilitating the maintenance of an impermeable intestinal barrier through regulation of tight junction proteins such as ZO-1, occludins, and claudins [91]. A strong intestinal barrier is critical for preventing leakage of bacteria and toxins into systemic circulation, which can contribute to chronic inflammatory responses that can influence the development of disease [91, 92]. Additionally, SCFA concentrations are sensed by specific G-protein coupled receptors (GPRs), GPR41 and GPR43, which are involved in numerous systemic processes including the regulation of glucose and lipid metabolism [91].

Another group of microbial metabolites that have been recently studied are involve the compound L-tryptophan. L-tryptophan and its metabolites have been demonstrated to be involved in gut immune function and also play a role in several immune diseases [93]. Endogenous tryptophan metabolism is conducted through two main pathways: the kynurenine pathway and the serotonin pathway, which occur primarily in the liver [93]. Interestingly, the gut microbiota can utilize tryptophan, limiting the availability of this compound to host tissues. It has been estimated that the gut microbiota can metabolize 4-6% of dietary tryptophan, resulting in the production of several compounds such as indole, indicant, tryptamine, and skatole, as well as other indole acid derivatives [93]. The understanding of which microbes possess the enzymes to metabolize tryptophan is currently limited, however it is known that members of Firmicutes including

*Clostridium sporogenes* and *Ruminococcus gnavus* are capable of producing tryptamine and skatole can be produced from indole acetic acid by bacteria belonging to the genera *Lactobacillus*, *Clostridium*, and *Bacteroidetes* [93]. Several other bacteria undoubtedly possess the capabilities to produce tryptophan metabolites and as technology and understanding of the gut microbial field progresses, the characterization of these microbes can be anticipated. Studies have demonstrated that these microbial-derived tryptophan metabolites can exert effects on host health, specifically through actions as ligands of the aryl hydrocarbon receptor (AhR) [94-96]. AhR is critical to maintenance of intestinal immunity and it has been demonstrated that excessive degradation of AhR ligands can induce detrimental effects on immune homeostasis [97]. It has been shown that during acute colitis, dietary tryptophan can alleviate colitis symptoms in a DSS-inducible intestinal injury mouse model and that these effects are due to microbial metabolites of tryptophan, not the parent compound [95]. A diet containing high amounts of tryptophan can actually increase AhR mRNA expression and the microbial metabolites that activate AhR result in the increase in colonic expression of IL-22 [98]. Recently IL-22 has gotten a lot of attention due to its discovered involvement in the maintenance of gut barrier function and immune function in the intestine [99]. Furthermore IL-22 has also been shown to play a role in anti-bacterial immunity, increasing clearance of specific pathogenic bacteria, further supporting the therapeutic potential of diets rich in tryptophan [100]. While research on this topic has progressed significantly over the past few

years, there still is much research needed to investigate the potential other roles that microbial-derived tryptophan metabolites play in gut and host health.

Some bacterial metabolites can also be detrimental and potentially contribute to disease risk. The bacterial derived metabolite trimethylamine n-oxide (TMAO) has recently been linked to coronary artery disease in the human population [101]. TMAO is formed from quaternary nitrogen containing compounds such as dietary choline, phosphatidylcholine, and carnitine. When ingested, these dietary compounds are metabolized by the gut microbiota to generate trimethylamine (TMA) and subsequently the liver enzyme flavin-containing monooxygenase 3 (FMO3) oxidizes it to form TMAO [101]. Evidence has shown that there are several bacterial families involved in the production of TMA/TMAO. In human and preclinical models, Deferribacteraceae, Prevotellaceae, Enterobacteriaceae, and Anaeroplasmataceae have been demonstrated to contribute to the formation of TMA [102]. Furthermore, in vitro, several species have been identified as TMA producers including *Anaerococcus hydrogenalis*, *Clostridium sporogenes*, *C. hathewayi, Escherichia fergusonii*, and others [103, 104]. Circulating levels of TMAO have been associated with numerous deleterious disease states, from cardiovascular disease, to chronic kidney disease, to type 2 diabetes [102]. Despite these findings, the debate still remains whether or not TMAO itself is contributing to disease progression or if it is solely a biomarker that is a result of a different diseae mechanism [105]. Additionally, recent research utilizing gut microbial modulation to attempt to reduce circulating TMAO levels have been

ineffective and inconclusive [106]. Due to these inconsistencies and debates, there is a great need for more research in this area.

### 1.3.2 Modulation of the Gut Microbiome

The gut microbiome can be affected by to numerous factors, perhaps the most notable being dietary habits. Diet has been demonstrated to rapidly and consistently exert changes on gut microbiota, with some of these alterations occurring within hours of dietary changes [107]. Studies examining the impacts of dietary components on the gut microbiota have focused mostly on high fat diets and high fiber diets, specifically looking at high fat diets as detrimental and high fiber diets as beneficial for gut health and host outcomes. The evidence behind each of these foci is discussed below.

High fat diets (HFD) have been consistently shown to negatively impact gut microbial populations [108]. Generally, it has been shown that HFD consumption can lead to a decrease in Bacteroidetes and increase in Firmicutes, which has been associated with numerous chronic disease states [108]. One of the mechanisms linking the consumption of a HFD to chronic disease is hypothesized to be disruptions in gut permeability and increases in intestinal inflammation [108]. For example, HFDs have been shown to reduce intestinal tight junction proteins, which can increase bacterial translocation, elevate circulating levels lipopolysaccharide (LPS) and contribute to inflammatory outcomes [109]. Furthermore, HFDs have been shown to increase inflammatory mediators within the small and large intestine of conventionally raised mice, but not germ-free mice, indicating an involvement of the microbiota in HFD-induced

inflammatory response [110]. It is important to note that high fat diets are often low in dietary fiber, which is known to be a positive modulator of the microbiota. Importantly, the detrimental impacts of a HFD on gut microbiota is not conclusive. For example, gut microbiota research on the ketogenic diet, which focuses on very high fat and low carbohydrate consumption, has shown to actually be beneficial for microbial populations [111]. For example, it was shown that ketogenic diet-fed mice had increased abundance of *Akkermansia muciniphila* and *Lactobacillus* and reduced levels of supposed pro-inflammatory taxa such as *Dulsufovibrio* [112]. Due to these inconsistencies in the literature regarding high fat diets and effects on the gut microbiota, more research is needed to elucidate the specific effects of HFD on microbiota and health outcomes in a variety of populations.

Diets high in fiber have been consistently demonstrated to modulate the microbiota. High fiber diets are well understood to be beneficial for host health, specifically in regard to cardiovascular disease and diabetes prevention [113]. Previously, it was believed that these beneficial effects were solely mediated through regulation of cholesterol levels and glucose homeostasis [113]. However, as understanding of the gut microbiota has increased, new research has discovered that high fiber-induced modulation of the gut microbiota may play a role in these previously observed effects [114-116]. One mechanism behind the beneficial effects of diets high in fiber is increases in short chain fatty acid (SCFA) production. Certain fibers are highly fermentable, meaning that they are metabolized by the gut microbiota to produce metabolites, primarily SCFA, that

can then be absorbed by the host. As discussed previously, SCFA have diverse impacts both locally within the gut and systemically as metabolic substrates as well as through ligand binding to specific receptors (e.g. GPR41 and GPR43) [85]. Another mechanism by which high fiber diets may contribute to host health is through immune modulation [117]. For example, it was recently shown that fiber consumption protects against diet-induced obesity and systemic inflammation through restoration of interleukin-22 (IL-22), which functions in restoration of enterocyte function and fortification of the intestinal barrier [116]. These observations of fiber-induced beneficial effects involving SCFA and immune modulation have been observed with highly fermentable fibers (e.g. inulin), while research examining minimally fermented fibers (e.g. cellulose) has not shown strong effects [118]. The fibers that are highly fermentable are termed prebiotics, and is discussed extensively in later sections.

More recently, great interest has developed into specific modulation of the microbiota with the intention to benefit gut and host health. This can be achieved through use of probiotics, prebiotics, and synbiotics. Probiotics are defined as actual live bacteria that when administered in adequate amounts, confer a health benefit on the host [119]. In preclinical and clinical trials, the use of probiotics to improve conditions including inflammatory bowel diseases, neurological conditions, and diabetes have showed some promise [120, 121]. Unfortunately, the benefits of probiotic supplementation do not always seem to translate to healthy individuals, indicating that there may be only specific populations that can benefit from probiotic use [122]. One common downfall of probiotic

supplementations is that supplements in general are not regulated by the Food and Drug Administration (FDA), which calls into question the quality, purity, and actual bacterial content of probiotic supplements [119]. Another issue with consumption of probiotics involves the potential side effects of gastrointestinal upset and bloating, which varies between individuals and ultimately counteracts the goal of these supplements as therapeutic agents [123]. There clearly needs to be more research into the therapeutic potential and uses of probiotic supplements, specifically focusing on the mechanistic roles of specific bacterial species. Prebiotics will be discussed in detail in the following section.

## 1.4 Prebiotics: An Overview

The term prebiotic is defined as “a selectively fermented ingredient that allows specific changes both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” [124]. The most well-known group of prebiotics are dietary fibers, however other dietary components may also fit this definition, seeing that they satisfy the functional criteria. Currently, the literature shows that all prebiotics are fiber, however not all fibers are prebiotic. It is recommended that humans consume at least 28g and 35g of fiber per day for females and males, respectively [125]. However, in the U.S., less than 3% of all Americans are meeting these recommended intakes [125]. Prebiotics that have been documented in the literature as having functional properties include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), some disaccharides, and some nonstarch

polysaccharides [126]. Prebiotics undergo microbial fermentation in the colon yielding SCFAs, of which up to 95% are absorbed in the colon and therefore may modulate host metabolic and inflammatory responses [126]. Consumption of a diet rich in prebiotics is capable of reversing dysbiosis and attenuating associated inflammatory conditions. It has been documented that prebiotic consumption may reduce the risk of cardiovascular disease and diabetes, primarily through the actions of the SCFAs discussed above [126]. Further research is still needed to understand the diverse role of prebiotic consumption on gut microbial composition, SCFA production, and associated host-responses. As with probiotics, one complication with consumption of prebiotics is the potential for gastrointestinal discomfort, especially in individuals that may have a microbial composition unable to fully ferment the prebiotic [127]. As the literature regarding the efficacy and health benefits of both prebiotics and probiotics expands, a movement towards personalized recommendations of dietary and bacterial supplemental protocols can be anticipated, much like the current movement in the medical and nutrition field.

### 1.4.1 Inulin

Understanding the action and benefits of specific prebiotics is necessary to better elucidate the manipulability of the gut microbiota and how this influences host health. The prebiotic inulin is an inulin type fructan (ITF) containing linear chains of fructosyl groups that are linked by $\beta$(2-1) glycosidic bonds and terminated with an $\alpha$-D(1-2)-glucopyranoside ring group on the reducing end [128]. The chain length of inulin varies from having two to more than 100

fructose units, which results in differences in fermentation by gut microbiota. ITFs include inulin, oligofructose, fructooligosacchardies (FOS), and bifidogenic oligo- or polysaccharide chains [129]. The latter compounds are smaller in size and thus, have a smaller degree of polymerization (DP). Inulin itself has been minimally processed so has a DP greater than 10 while the aforementioned molecules have a DP less than 10. Notably, the greater the DP, the greater effects will be in the distal colon with a smaller DP having more effect towards the proximal colon [129]. One of the most common forms of inulin that is available in food products or supplements is derived from chicory root, but inulin is also found in garlic, onions, and other vegetables. Inulin is one of the most studied prebiotics and its consumption has been associated with a host of health benefits ranging from improved gastrointestinal function, improved glucose control, and improvements in energy metabolism [129-131]. Specifically, administration on inulin has been demonstrated to reduce adiposity and parameters associated with metabolic syndrome [132, 133]. The mechanisms behind the beneficial actions of inulin are believed to include alterations in gastrointestinal peptide and short chain fatty acid production, regulation of the immune system, and modulation of lipid metabolism. While humans lack the enzymes to metabolize and garner energy from inulin, the production of SCFA is thought to contribute to approximately 1 kilocalorie per gram of fiber for the host [118]. Furthermore, it was recently shown that inulin supplementation protected against diet-induced obesity through improving gut microbiota populations and restoring enterocyte function through effects on IL-22 [116]. These observed

mechanisms are also dependent and linked to alterations in gut microbiota. Inulin is known to have strong stimulatory prebiotic activity amongst specific bacterial groups including *Bifidiobacterium spp.*, *Anaerostipes* spp., as well as *Lactobacillus gasseri*, and *Faecalibacterium prausnitzii.* [134] The latter two bacteria are of interest and importance due to their known production of butyrate and potential therapeutic contribution to host health. Furthermore, it was recently observed that inulin administration resulted in reduction in *Bilophila*, a member of the *Desulfovibrionacaeae* family [134, 135]. This is important because *Desulfovibrionacaeae* are known sulfate-reducing bacteria that produce hydrogen sulfide (H2S), a genotoxic gas that can hamper intestinal health. As such, reductions in *Bilophila* spp. following inulin administration has been associated with improved gut barrier function [134]. These observations offer another gut microbial dependent mechanistic insight into the health benefits of inulin consumption.

With regards to overall human health and disease outcomes, studies have examined the effects of inulin consumption on outcomes related to diabetes and cardiovascular diseases. For example, in prediabetic patients, inulin supplementation aided in weight reduction and reduced intrahepatocellular and intramyocellular lipid content, which the authors suggest contributed to observed improvements in glucose homeostasis [136]. Inulin also has potential applications in the prevention of cardiovascular disease with several studies finding an improvement in blood lipids and lipoproteins with inulin supplementation. Importantly, in a meta-analysis, it was found that the most

profound effect of consumption of inulin-type fructans was on lowering of low density lipoprotein (LDL) levels, an effect well evidenced to lower the risk of adverse cardiovascular events [137]. Because it is still unknown if these discussed clinical findings are solely gut-microbial dependent, more research is needed to elucidate the protective mechanism of inulin on diabetic and cardiovascular outcomes.

## 1.5 Scope of Dissertation

### 1.5.1 Aims of dissertation

The overall objective of this project is to elucidate the effects of PCB 126 on gut microbial homeostasis and cardiometabolic disease and how nutritive measures may be able to protect against the effects of pollutant exposure. PCB 126 was chosen as the model pollutant in this study due to its known effects on human health as well as a substantial quantifiable prevalence in human samples[138].

Our *central hypothesis* is that exposure to PCB 126 disrupts host health and increases the risk for cardiometabolic disease in a gut-microbial dependent manner and that prebiotic (i.e. inulin) consumption will be able to attenuate these effects and reduce the risk of disease. As such, we will test our central hypothesis by these three specific aims:

**Specific Aim 1.** Test the hypothesis that PCB 126 directly disrupts gut microbial fermentation and viability.

**Specific Aim 2.** Test the hypothesis that PCB 126 negatively alters murine gut microbial populations and/or gut health, exacerbating inflammatory and metabolic complications involved in cardiometabolic disease.

**Specific Aim 3.** Test the hypothesis that consumption of the prebiotic inulin can decrease PCB-induced disruption in gut microbial and metabolic homeostasis and ultimately protect against cardiometabolic disease.

# Chapter 2 Environmental pollutant-mediated disruption of gut microbial metabolism of the prebiotic inulin

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## 2.1 Synopsis

Exposure to environmental pollutants is associated with a greater risk for metabolic diseases including cardiovascular disease. Pollutant exposure can also alter gut microbial populations that may contribute to metabolic effects and progression of inflammatory diseases. Short-chain fatty acids (SCFAs), produced from gut fermentation of dietary carbohydrates, such as inulin, exert numerous effects on host energy metabolism and are linked to a reduced risk of diseases. The hypothesis was that exposure to dioxin-like pollutants modulate gut microbial viability and/or fermentation processes. An inulin-utilizing isolate was collected from murine feces, characterized and used in subsequent experiments. Exposure to polychlorinated biphenyl, PCB 126 impeded bacterial viability of the isolate at concentrations of 20 and 200 μM. PCB 126 exposure also resulted in a significant loss of intracellular potassium following exposure, indicating cell membrane disruption of the isolate. Furthermore, total fecal microbe samples from mice were harvested, resuspended and incubated for 24 hours in anaerobic media containing inulin with or without PCB 126. HPLC analysis of supernatants revealed that PCB 126 exposure reduced succinic acid production, but increased propionate production, both of which can influence host glucose and lipid metabolism. Overall, the presented evidence supports the idea

that pollutant exposure may contribute to alterations in host metabolism through gut microbiota-dependent mechanisms, specifically through bacterial fermentation processes or membrane disruption.

## 2.2 Introduction

Exposure to environmental pollutants can illicit numerous deleterious health effects. Polychlorinated biphenyls are a class of persistent organic pollutants that increase development of chronic inflammatory diseases such as diabetes and atherosclerosis, primarily through inflammatory mechanisms [2]. Due to their lipophilic nature, they accumulate in the adipose tissue of living organisms and bioaccumulate along the food chain. Thus, one of the primary routes of human exposure is through the ingestion of contaminated foods including fatty meats, fish, and dairy [2]. This exposure route is unique in that it has the ability to directly impact the gastrointestinal tract and gut microbiota.

The gut microbiota can be impacted by outside influences including diet and environmental exposures [80]. Over the past decade, greater appreciation and understanding has come about regarding the gut microbiota and its influence on overall host health and wellbeing. The gut microbiota exerts impacts on neurological, metabolic, and inflammatory bowel diseases, highlighting the diverse and wide reaching impact of our intestinal bacterial residents [140]. Fermentation is an innate aspect of gut microbial metabolism and the metabolites produced can have diverse impacts on host health. The primary end products of bacterial saccharolytic fermentation of non-digestible carbohydrates are short

chain fatty acids (SCFAs). These SCFA are formed when carbohydrates escape digestion and absorption in the small intestine and thus persist into more distal regions of the digestive tract where they are metabolized by the residing gut microbiota [91]. The primary SCFA formed by bacterial fermentation are acetate, propionate, and butyrate, although there are numerous other fermentation acids produced. SCFA exert effects both locally, within the intestine, and systemically, and they serve a variety of biological purposes [91]. For example, butyrate serves as a preferential energy source for the colonic epithelial cells, facilitating the maintenance of an intestinal barrier through regulation of tight junction proteins such as ZO-1, occludins, and claudins [86, 91]. A strong intestinal barrier is critical for preventing leakage of bacteria and toxins into the systemic circulation, which may contribute to chronic inflammatory responses that can influence the development of disease [91, 92]. Additionally, SCFA concentrations are sensed by specific G-protein coupled receptors (GPRs), GPR41 and GPR43, which are involved in numerous systemic processes including the regulation of glucose and lipid metabolism [86, 91].

The prebiotic inulin is a polymer of fructans containing linear chains of fructosyl groups that are linked by B(2-1) glycosidic bonds and terminated with an alpha D(1-2)-glucopyranoside ring group on the reducing end [118]. The chain length of inulin varies from having two to more than 100 fructose units, which results in differences in fermentation by gut microbiota [118]. In preclinical and clinical models, inulin has been demonstrated to have beneficial effects on several disease states including cardiovascular disease and diabetes [129, 135, 141].

Inulin is fermented by certain members of the gut microbial community to produce short chain fatty acids including acetate, propionate, and butyrate, which is believed to be one of the mechanisms of action in which inulin exerts host health effects [130]. In the human nutrition community, inulin, is commonly referred to as a dietary fiber based on its inability to be metabolized by mammalian enzymes and thus is not absorbed by the human body [142]. Due to the observed health effects of inulin consumption, it is commonly added to numerous food products to increase the dietary fiber content while adding slight non-caloric sweetness.

Our lab has demonstrated previously that PCB 126 exposure decreases gut microbial diversity, reduces specific bacterial populations, and impacts overall host metabolism in a mouse model [143]. However, little is known about the mechanisms by which PCBs impact the gut microbiota and how this may translate to effects for the host. Therefore, the objective of this study was to examine the impacts of PCB 126 exposure on gut microbial viability and fermentation utilizing the prebiotic inulin as a substrate.

## 2.3 Materials and Methods

### 2.3.1 Materials and Chemicals

Inulin from chicory was purchased from Sigma Aldrich (St. Louis, MO, USA). 3,3',4,4',5-pentachlorobiphenyl (PCB 126) was obtained from AccuStandard Inc. (New Haven, CT, USA).

### 2.3.2 Animals and Fecal Collection

Male C56BL6/J mice were fed a standard chow diet *ad libitum*, housed at 22 °C with 50% humidity, and exposed to a 12-h light/ 12-h dark cycle. When fecal samples were needed for *in vitro* experiments, fresh fecal pellets were collected from mice and immediately placed in a sterile microcentrifuge tube. Samples were quickly transferred in an insulated container at 37 °C for immediate experiment setup to ensure bacterial viability. For fermentation experiments, fecal cell suspensions were made from pooled samples of three mice and repeated a total of three times. The University of Kentucky animal care and use committee approved all animal procedures.

### 2.3.3 Media and Anaerobic Technique

Growth medium was prepared, and cultures were transferred using the Hungate anaerobic method. Growth medium contained (per 1 L) 240 mg $KH_2PO_4$, 240 mg $K_2HPO_4$, 480 mg NaCl, 480 mg $(NH_4)_2SO_4$, 100 mg $MgSO_4$ $7H_2O$, 64 mg $CaCl_2$ $2H_2O$, and 600 mg cysteine hydrochloride [144]. Media was adjusted to a pH of 6.7 by adding NaOH, autoclaved to remove $O_2$, and cooled under $CO_2$, at which point 4.0 g $Na_2CO_2$ was added. Media was then anaerobically transferred to Hungate tubes, capped with rubber stoppers and aluminum seals, and autoclaved for sterilization. Inulin was prepared anaerobically and added aseptically at a concentration of 4 g/L unless otherwise indicated. Cultures were routinely transferred with anaerobic technique using a tuberculin syringe and incubated at 37°C.

### 2.3.4 Inulin-Fermenter Enumeration, Isolation, and Characterization

Fecal samples (175 mg) were placed in anaerobic chamber (Coy Labs, Grass Lake, MI; 95% $CO_2$, 5% $H_2$), suspended in 1 mL dilution buffer (PBS), and subjected to 10-fold serial dilution in dilution buffer using sterile anaerobic techniques. Samples (200 µL) from the dilutions were plated on basal medium agar (15 mg/mL) containing 4g/L of inulin for bacterial isolation. Dilution plates were incubated in an anaerobic chamber for 72h at 37 °C. Following incubation, colonies were isolated and grown in liquid growth media (4 g/L inulin) at 37 °C for 24-48h. Cultures were then characterized by Gram staining and light microscopy. Phylogenetic identity was determined via 16S rRNA sequencing. Genomic DNA was extracted (QiaAMP DNA Mini Stool Kit; Qiagen, CA, USA) and amplified by PCR using PuReTaq™ Ready-to-Go™ PCR beads (GE Healthcare, Buckinghamshire, United Kingdom) and universal 16S primers (10 pmol; Integrated DNA Technologies, Coralville, IA, USA; Forward-5'-AGAGTTTGATCCTGGCTCAG-3', Reverse – 3'-ACGGCTACCTTGTTACGACTT-5'). The PCR cycles were: denaturing (94 °C; 5 min), 35 cycles (94 °C, 0.5 min; 55 °C, 1 min; 72 °C, 1 min), and final extension (72 °C; 5 min). PCR products were sequenced by the University of Kentucky, sequencing core facility (Lexington, KY, USA) using an ABI 3730 DNA Analyzer (Applied Biosystems, CT, USA). The closest phylogenetic relatives were identified using a BLAST search of GenBank [145]. The 16S sequences were aligned and a phylogenetic dendrogram was assembled using the Neighbor-Joining method in Geneious (version 10.2.5). The bacterial isolate was further

characterized for substrate utilization using an API 20A kit (BioMeriux, Marcy l'Etoile, France).

### 2.3.5 Growth Experiments

Growth experiments were conducted in growth media (9mL, pH 6.7) in Hungate tubes. Stock solutions of PCB 126 were solubilized in DMSO and the same amounts of DMSO as in PCB-treated cultures were added to control cultures. The levels of DMSO in cultures was less than 0.05%. PCB 126 (0.02 μM) or control (DMSO) was added to growth media prior to inoculation with JB12 cultures. The dose of 0.2 μM was chosen as a level that has been documented a in exposed human populations. Each tube was inoculated with (1% v/v) from stationary phase (16 h) cultures and incubated at 37*C. Optical densities were determined via spectrophotometry (600 nm) to quantify bacterial growth.

The effect of increasing concentrations of PCB 126 on the growth of the inulin-fermenting isolate was conducted via broth dilution (10-fold increments). Isolates were transferred in growth media (9 mL, pH 6.7) containing 4 g/L of inulin that was prepared using sterile anaerobic technique. PCB 126 was added to growth media via a Hamilton syringe in 10-fold dilutions (0.02, 0.2, 2, 20, 200 μM). Each tube was inoculated with a stationary phase from 16h cultures and incubated at 37 °C. Optical densities were recorded 24h after inoculation and the degree to which growth was observed was documented. All growth experiments were conducted in triplicate.

### 2.3.6 Intracellular Potassium Quantification

Intracellular potassium was quantified using a method described in Flythe et. al.2007 [146]. Growing cultures were energized with glucose for 25min, at which point PCB 126 or vehicle control (DMSO) were introduced. Concentrations and preparations of PCB 126 and DMSO were as described above. At each timepoint, cells were separated from supernatant via centrifugation (13000*g*, 1 min) through silicon oil using a 50:50 mixture of Dexter Hysol 550 and 560. Cell pellets were removed with dog nail trimmers, and subsequently digested in 3 N $HNO_3$ (25 °C for 72h). Insoluble cell material was removed via centrifugation (13,000*g*, 2 min) and the potassium concentrations were determined using flame photometry (Cole-Parmer 2655-00 Digital Flame Analyzer; Cole-Parmer Instruments, IL, USA).

### 2.3.7 Fecal Cell Suspensions

Fecal samples (175 mg) were placed in an anaerobic chamber and suspended in 9mL of basal medium[144]. Samples were centrifuged (340g, 5 min) to remove major undigested material. Supernatants were collected and centrifuged (25,000g, 10 min) to pellet bacteria. Bacterial pellets were resuspended in 9mL of growth medium and split into 3 tubes each containing 2 mL of bacterial suspension. PCB 126 (0.02, 0.2 or 2μM final conc.) or vehicle control (DMSO) and inulin (4 g/L or 10 g/L final conc.) were added to the bacterial suspensions. The preparation of PCB 126 and DMSO were the same as described above. The pH and optical density (OD; absorbance at 600 nm)

were quantified to ensure normalization. Samples were incubated for 0h or 48h at 37 °C, centrifuged (21,000*g*, 2 min) to collect supernatant for fermentation end product quantification, and frozen stored at -20 °C until analysis.

### 2.3.8 Fermentation End Product Quantification

Fermentation end product quantification was conducted as discussed previously.[144] Supernatant samples were thawed and clarified in a microcentrifuge (21,000*g*, 2 min) for short-chain fatty acid (SCFA) quantifications. SCFAs were quantified using as Summit HPLC (Dionex; CA, USA). Extracts (100 µL) were injected into an anion-exchange column (Amine HP-87H; Bio-Rad, Hercules, CA) at 50 °C, separated isocratically with 5 mM sulfuric acid (0.4 mL/min flow rate), and subsequently detected via refractive index and UV absorption at 210 nm.

### 2.3.9 Statistical Analyses

Each experiment was performed in triplicate. Data were input into GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) for statistical analyses and graphing. Growth inhibition, intracellular potassium, and fermentation acid data were analyzed by Student's t-test to calculate comparisons of means between treatment groups. All data are presented as means $\pm$ SEM and results were considered statistically significant with an observed p-value $<0.05$.

## 2.4 Results

### 2.4.1 Identification of an inulin-fermenting isolate

An inulin fermenting microorganism (JB12) was isolated mouse feces. The isolate formed smooth, white opaque colonies approximately 1mm in diameter on basal medium agar with 4 g/L inulin. Culture purity was confirmed through repeated isolation streak. Sequence homology (16S rRNA) revealed that the closest characterized relative of JB12 was *Acutalibacter muris* (96% identity), a member of the *Ruminococcaceae* (**Figure 2.1**). JB12 was Gram-positive, and, unlike *A. muris*, it formed central endospores. In addition to inulin, JB12 metabolized glucose, saccharose, maltose, salicin, xylose, cellobiose, mannose, and trehalose (**Table 2.1**)[147-149].

### 2.4.2 Effect of PCB 126 on bacterial growth

To elucidate the effects of PCB 126 on an isolate JB12, experiments examining growth effects were conducted. For growth curve experiments, PCB 126 (0.02 μM) or control (DMSO) was added to growth media (9 mL) and subsequently inoculated with a stationary phase (16h culture; 1% v/v) of JB12 cultures. Cultures were incubated at 37*C and growth was quantified over time via optical densities (600 nm). JB12 grew rapidly in the growth medium with inulin as the substrate and reached an optical density of ~2.6 in 9h. **(Figure 2.2).** PCB 126 exposure partially inhibited growth of JB12, reaching an optical density of ~1.6 in 9h.

To examine the effects of PCB 126 dose on JB12 growth, increasing doses of PCB 126 were added to tubes containing basal media which were subsequently inoculated with a stationary phase (16h) culture of JB12 and incubated at 37°C

for 24h. 24h after inoculation, growth was evaluated via optical density. We observed a significant reduction of bacterial growth at the two highest PCB 126 concentrations (20 μM and 200 μM) compared to control ($P$<0.05) **(Figure 2.3)**. Apparent reduction in bacterial growth at lower PCB concentrations less than *20 μM* were not significant.

### 2.4.3 Disruption of bacterial cell membrane by PCB 126

When cell suspensions of the inulin-fermenting isolate were energized with glucose, they accumulated intracellular potassium. When PCB 126 was added to the cell suspension, energized cells continuously lost intracellular potassium while a control that received only vehicle continued to accumulate intracellular potassium **(Figure 2.4)**. Significantly lower levels of intracellular-potassium observed at 35, 45, 55, and 65 minutes compared to vehicle control.

### 2.4.4 PCB 126-induced modulation of fermentation acid production from inulin substrate

Fecal cell suspensions were grown and maintained using inulin as a substrate (4g/L or 10g/L) and exposed to PCB 126 at varying doses or DMSO control for 48h. Examination of the fermentation acid production from inulin at 4g/L revealed that the highest concentrations of PCB 126 (0.2 μM and 2 μM) significantly reduced production of succinate (**Figure 2.5**). Additionally, PCB 126 exposure significantly increased propionate concentrations in the 2μM exposed cells. The cells that were not exposed to PCB 126 produced 46 mM total fermentation acids, including 11 mM propionate, 12 mM butyrate, and 21 mM

acetate. To further understand the effects of PCB 126 on fermentation acid production, we performed a second experiment in which the inulin substrate concentration was increased to 10g/L. At this increased concentration of inulin, PCB 126 exerted no significant effects on fermentation acid production at any of the exposure levels (**Figure 2.6**).

## 2.5 Discussion

The objective of this study was to examine the impacts of PCB 126 exposure on gut fermentation utilizing the prebiotic inulin as a substrate. Previous results indicated that the dioxin-like PCB altered the population sizes of a variety of phylogenetic groups in the hindgut when mice received the toxicant *in vivo* [143]. The current study follows up by mechanistically examining the effects of PCB 126 on bacteria isolated from murine feces. The effects on uncultivated hindgut bacterial fermentation *ex vivo* are also shown. Bacterial fermentation is a key metabolic process allowing bacteria to extract energy from compounds that the host takes in [86, 91]. Products of fermentation of indigestible carbohydrates, such as inulin, include various fermentation acids or short chain fatty acids. SCFA are common, easily-quantifiable products of bacterial fermentation and thus are a good measure to examine potential pollutant-induced effects [86, 91].

Inulin was chosen as the host-indigestible carbohydrate substrate due to its increased popularity in the food and supplement industry as well as the strong basis of scientific evidence linking inulin consumption to beneficial host health outcomes [130, 150-152]. United States citizens ingest 1-4g of inulin-type fructans per day, which can be anticipated to rise with increasing popularity[153].

Administration of inulin has been demonstrated to reduce adiposity and parameters associated with metabolic syndrome [129, 135, 152]. The mechanisms behind the beneficial actions of inulin are believed to include alterations in gastrointestinal peptide and short chain fatty acid production, regulation of the immune system, and modulation of lipid metabolism [152]. For example, Weitkunat et. al. found that gnotobiotic mice colonized with a human microbiota and fed an inulin-enriched diet had significantly greater amounts of acetate, propionate and butyrate in the cecum as well as elevated levels of acetate and propionate in the plasma of the portal vein compared to mice fed cellulose [154]. Inulin feeding also altered hepatic genes associated with lipogenesis and fatty acid elongation, which was hypothesized to be due to the differences in short chain fatty acid levels. These data highlight the fermentative capability of inulin and the role that fermentation products (SCFA) play on overall host health, making inulin a relevant substrate to study the effects of pollutant exposure.

An inulin-utilizing microorganism was isolated from feces in order to examine the inhibitory effects of PCB 126 at a physiological level. The isolate (JB12) was most closely related to *Acutalibacter muris* which was previously isolated from murine feces [149]. Isolate JB12 grew rapidly in the growth medium when inulin was provided as the substrate, but growth slowed in an apparent stationary phase in the presence of PCB 126 at 0.2 μM. However, the growth appeared to be diauxic because greater optical densities were observed at 24h (Figure 3). At 24h, there was a dose-response inhibitory effect of PCB 126 on the culture.

Diauxic, or biphasic, growth is not surprising because inulin is not truly a single substrate. It is composed of oligofructose of different chain lengths, which are fermented at different rates and to different extents [155]. When polymers of different chain lengths are fermented as though they were different substrates, classical diauxic growth could occur [156]. It was also observed that concentrations greater than 20 μM of the polychlorinated biphenyl reduced the growth of JB12. These results are consistent with the previous *in vivo* data that showed PCB 126 exposure disrupted bacterial populations and decreased bacterial diversity [143]. However, the antimicrobial mechanism of action of PCB 126 on gut bacteria was unknown. Previously, research was conducted on the effects of PCBs and other environmental pollutants on soil microbes [157]. It has been discussed that there is no convincing evidence that PCBs exert genotoxicity in bacteria, such as *Salmonella enterica* [158]. Additionally, there is some evidence that PCBs may be membrane active within specific soil microbial populations [157]; therefore, we examined the effect of PCB 126 on the intracellular potassium of isolate JB12. Potassium is the main intracellular cation in bacterial cells and serves important roles such as acting as a cofactor for certain enzymes [159]. Quantification of intracellular potassium is a method that allows examination of potential membrane disruptions that that result in the leakage or loss of this intracellular cation. We observed that upon exposure to PCB 126, the bacterial cells rapidly lost potassium, which is consistent with a loss of cell membrane integrity. This result indicates that bacterial cell

membrane disruption is a potential antimicrobial mechanism of action by which polychlorinated biphenyls could disrupt gut microbial communities.

To better understand how these findings may impact the gut microbiota as a whole, we examined the effects of PCB 126 on fermentation acid production in a fecal microbial cell suspension. PCB 126 addition (0.2 μM and 2 μM) to the fecal cell suspension completely inhibited succinate production and increased propionate production (2 μM). This is important for the host due because succinate is a substrate in intestinal gluconeogenesis. Impaired intestinal gluconeogenesis was shown to impact systemic glucose tolerance [160]. Furthermore, microbiota-derived succinate was shown to improve glucose and insulin tolerance in wild-type mice [161]. It has been documented that PCB 126 disrupts glucose tolerance, yielding a diabetic phenotype in mouse models, thus this new finding of microbial-derived succinate loss with PCB 126 may play a role in this previously observed phenotype [162]. An increase in propionate was also observed in the fecal cell suspensions exposed to PCB 126, which may have implications in alterations in host hepatic metabolism. In support of this data, previous *in vivo* evidence indicated an increase in propionate producers including Clostridiales [163] and *Akkermansia* [88] in cecum samples of mice exposed to PCB 126 [143]. Propionate can affect hepatic *de novo* lipogenesis as well as lipolysis and in humans an increased level of propionate has been observed in individuals with non-alcoholic fatty liver disease [164]. It is well documented that exposure to dioxin-like pollutants increases hepatic lipid content and exacerbated non-alcoholic fatty liver disease [42, 165]. Therefore, the effects observed on

propionate production have the potential to contribute to these well-known hepatic consequences of pollutant exposure.

The modulatory effects of PCB 126 on fermentation acid production were not observed when the inulin concentration was increased to 10 g/L. This is not surprising because all of the microorganisms in the gut microbiome were not sensitive to PCB 126 at the concentrations tested. At higher inulin concentrations, lactate was a major end product. Lactic acid production is common when easily fermented carbohydrate is in excess. Many Firmicutes (*e.g.* streptococci) and Proteobacteria (*e.g. E. coli*) possess a 1,6- fructose bisphosphate (FBP)-dependent lactate dehydrogenase (LDH) [166]. Rapid membrane transport of sugars leads to an increase in cellular FBP, which triggers LDH and homolactic metabolism [166]. Abundant lactate production regardless of PCB 126 suggests that one or more fructanolytic lactic acid bacterial species were not sensitive to PCB 126. It is important to note that increased lactate production from fructans fermentation is not always beneficial. For example, in horses, excess lactate production and resultant drop in pH in the hindgut can increase intestinal permeability and contribute to the development of laminitis, resulting in required euthanasia [144]. Our present data do not warrant a suggestion for excess consumption of inulin, but rather serves to highlight that the bacterial populations that are able to grow at higher concentrations of inulin are not sensitive to PCB 126. These data indicate that PCBs might be selectively toxic to specific gut microbial populations.

Although this present study is novel and adds to the growing body of literature examining the impacts of pollutant exposure on gut microbial populations, there are a few limitations that should be noted and addressed in subsequent studies. First, the mechanistic aspects of this study focus on a single isolate and thus the translatability of our findings to other microbial species is limited but should be examined in future experiments. Additionally, It has been reported that serum concentrations of PCBs can be found at approximately 3μM in exposed individuals [167]. Due to the primary route of exposure (i.e. food consumption), it could be expected then that the intestinal environment sees similar or higher levels. Thus, the 0.02μM, 0.2μM and 2μM concentrations used for most experiments in this manuscript are physiologically relevant, especially in heavily exposed populations. However, the highest concentrations in utilized in the inhibition experiment (Figure 3) are supraphysiological. Use of these higher levels, while not found in human populations, is important to help characterize and better understand the microbial impact of PCB 126. Furthermore, the experiments conducted in the present study are representative of acute exposures while humans often see more chronic exposures due to the primary route of exposure of PCBs (i.e. food) and their participation in enterohepatic circulation. Thus, gastrointestinal microbial populations are continually exposed to these pollutants which could elicit a different and perhaps more detrimental response over time. Finally, it appears that some microbiota are effected by exposure to PCB 126 while others are not. This is the first study to show impacts of a polychlorinated biphenyl compound on fermentation by gut microbiota. It

remains to be seen if this interaction of the native gut microorganisms with an environmental toxicant is beneficial to the host.

PCB 126 decreased the fermentative ability and viability of an inulin-metabolizing murine fecal bacterium. Potassium efflux indicated that the mechanism of action was membrane perturbation. The fermentation of washed fecal microorganisms were also impacted by PCB 126 ex vivo when the inulin concentration was low. However, the toxicant was not inhibitory to fermentation when the inulin concentration was greater. These results indicate that not all species (e.g. the lactic acid bacteria that flourish under carbohydrate excess conditions) were equally sensitive, a conclusion which is consistent with previous results by our research group using culture-independent methods [13].

**Table 2.1.** Substrate utilization profile of isolate JB12 and relatives

| Substrate | Isolate JB12 | *Acutalibacter muris* (Strain Kb18) | *Anaeromassilibacillus senegalensis* (strain mt9$^{t}$) | *Clostridium leptum* (ATCC 29065) |
|---|---|---|---|---|
| Indole | - | - | - | n.d. |
| Urea | - | - | - | n.d. |
| Glucose | + | - | - | - (w) |
| Mannitol | - | - | - | n.d. |
| Lactose | + | - | - | - |
| Saccharose | + | - | - | - |
| Maltose | + | - | + | + |
| Salicin | + | - | n.d. | - |
| Xylose | + | - | n.d. | - (w) |
| Arabinose | - | - | n.d. | - |
| Gelatin | - | - | n.d. | - |
| Esculin | - | + | n.d. | - (w) |
| Glycerol | - | - | n.d. | n.d. |
| Cellobiose | + | - | n.d. | - |
| Mannose | + | - | - | - |
| Melezitose | - | - | n.d. | n.d. |
| Raffinose | - | - | n.d. | - |
| Sorbitol | - | - | n.d. | - |
| Rhamnose | - | - | n.d. | - |
| Trehalose | + | - | n.d. | - (w) |
| | | | | |
| Catalase | - | - | - | - |
| Spores | + | n.d. | + | + |
| Gram Reaction | + | n.d. | - | + |
| Coccus | - | - | - | - |

+ positive reaction; - negative reaction; (w) weakly; n.d. not determined [134,135,136]

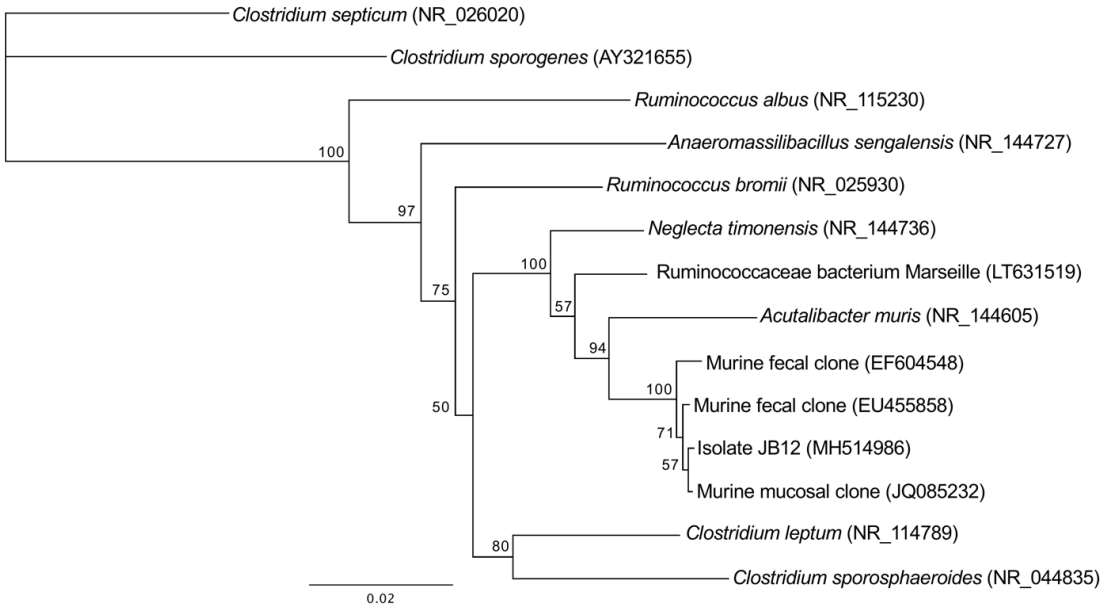


**Figure 2.1**. **A phylogenetic dendrogram of isolate JB12 and close relatives.** Relationships were based on 16S rRNA sequences. The tree was constructed using the neighbor-joining method in Geneious (version 10.2.5) and bootstrap values are expressed as a percentage of 1000 replications. The scale represents a 2% difference in nucleotide sequence.

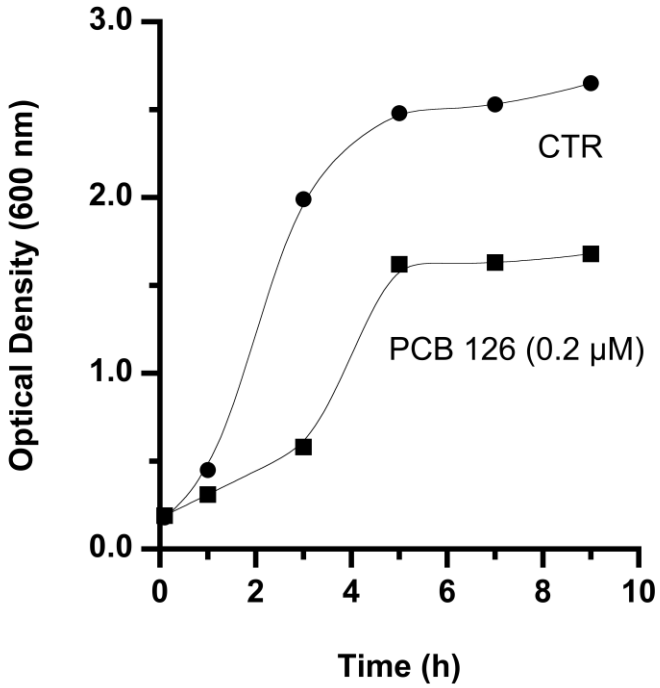


**Figure 2.2. PCB 126 reduces growth of an inulin-fermenting isolate.** A growing culture of JB-12 was inoculated into media containing 4 g/L of inulin with either PCB 126 (0.2 μM) or vehicle control (CTR; DMSO) and incubated at 37°C. Optical density measures were conducted at time intervals to quantify growth. Experiments were conducted in triplicate and representative data are shown.

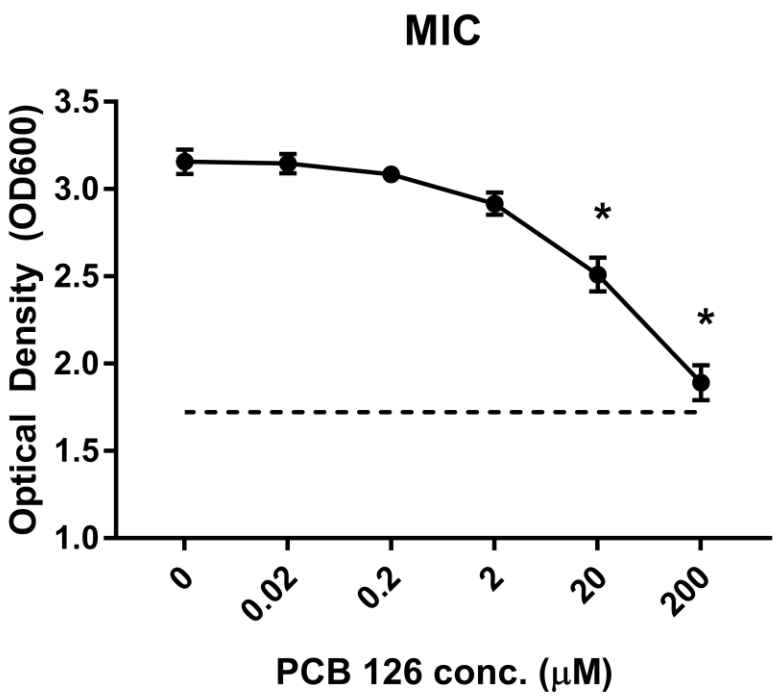


**Figure 2.3. PCB 126 is an inhibitor to an inulin-utilizing bacteria.** Growing cultures of isolate JB12 were exposed to increasing concentrations of PCB 126 (0.02, 0.2, 2, 20, 200 μM) and optical density was quantified after 24 hours of incubation (n=3). PCB 126 impeded bacterial growth at concentrations of 20 and 200 μM ($p<0.05$). All data are presented as means $\pm$ SEM. *Statistically different compared to 0 μM PCB 126 control ($p<0.05$; Student's t-test).

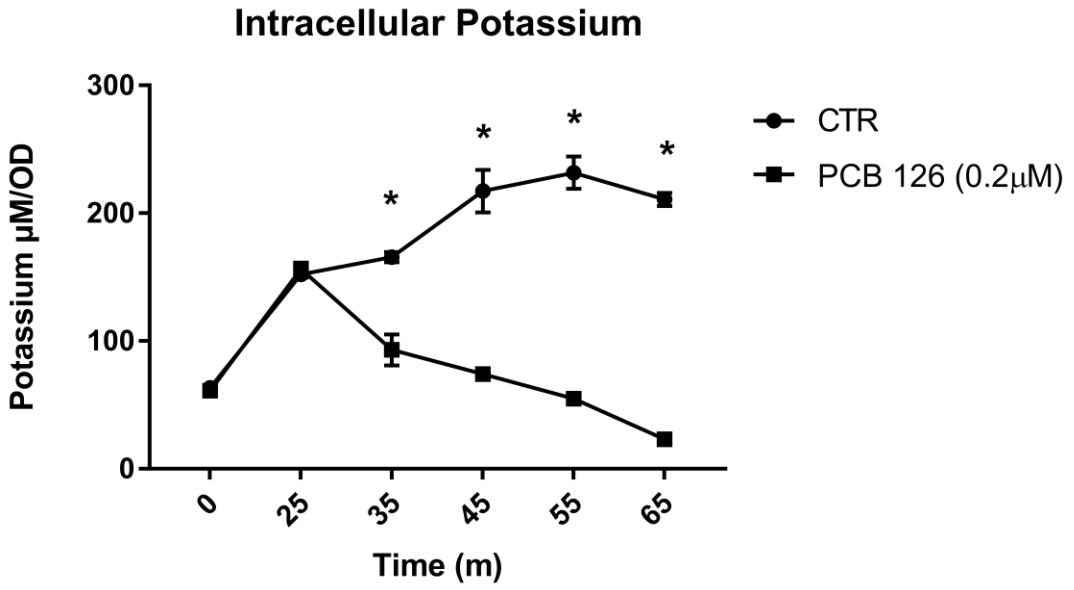


**Figure 2.4**. **PCB 126 exposure decreases levels of intracellular potassium in energized inulin-fermenting strain JB12.** Growing cultures were energized with glucose at time 0 and PCB126 and vehicle control (DMSO) were introduced at 25m (n=3). Samples at each timepoint were subject to centrifugation to remove insoluble cell material and potassium concentration was determined using flame photometry. PCB 126 significantly reduced intracellular potassium levels at 35, 45, 55, and 65 minutes compared to vehicle control. All data are presented as means $\pm$ SEM. * Significantly different compared to vehicle control (DMSO) at specific timepoint ($p<0.05$; Student's t-test)

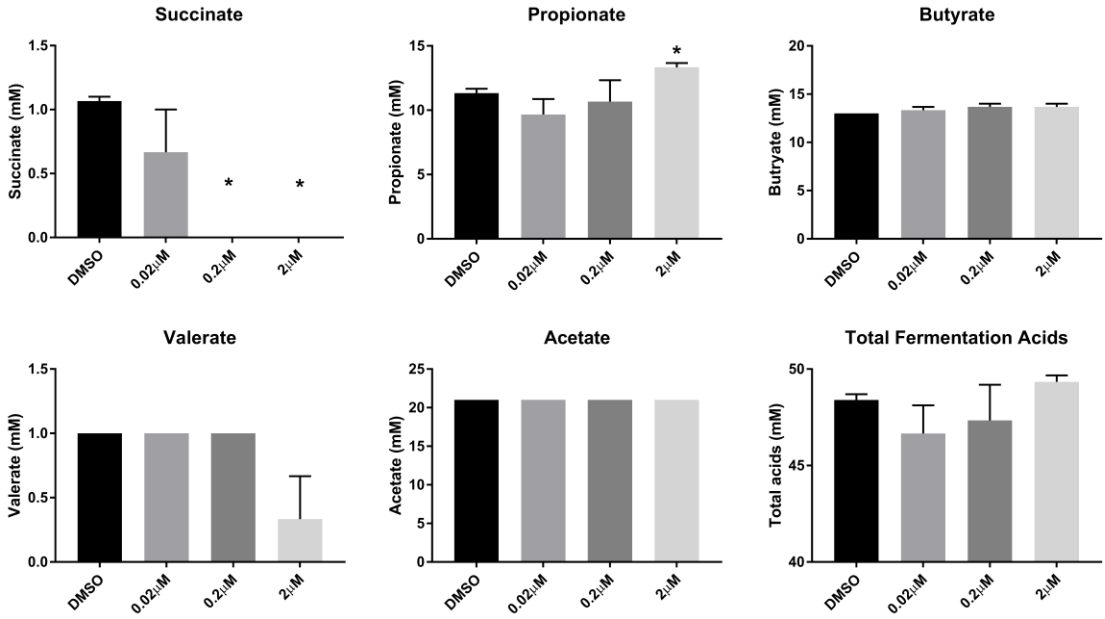


**Figure 2.5. PCB 126 treatment differentially modulates fermentative SCFA from moderate concentrations of the dietary fiber inulin.** Bacterial cell suspensions were maintained on inulin substrate (4 g/L) and subsequently exposed to PCB 126 (0.02 μM, 0.2 μM, 2 μM) or vehicle control (DMSO) for 48h (n=3). HPLC was utilized to quantify fermentation acids in the media after incubation. PCB 126 significantly reduced production of succinate at 0.2 μM and 2μM exposure concentrations and significantly increased propionate production at the 2 μM concentration. All data are presented as means ± SEM. * Significantly different compared to vehicle control (DMSO) ($p<0.05$; Student's t-test).

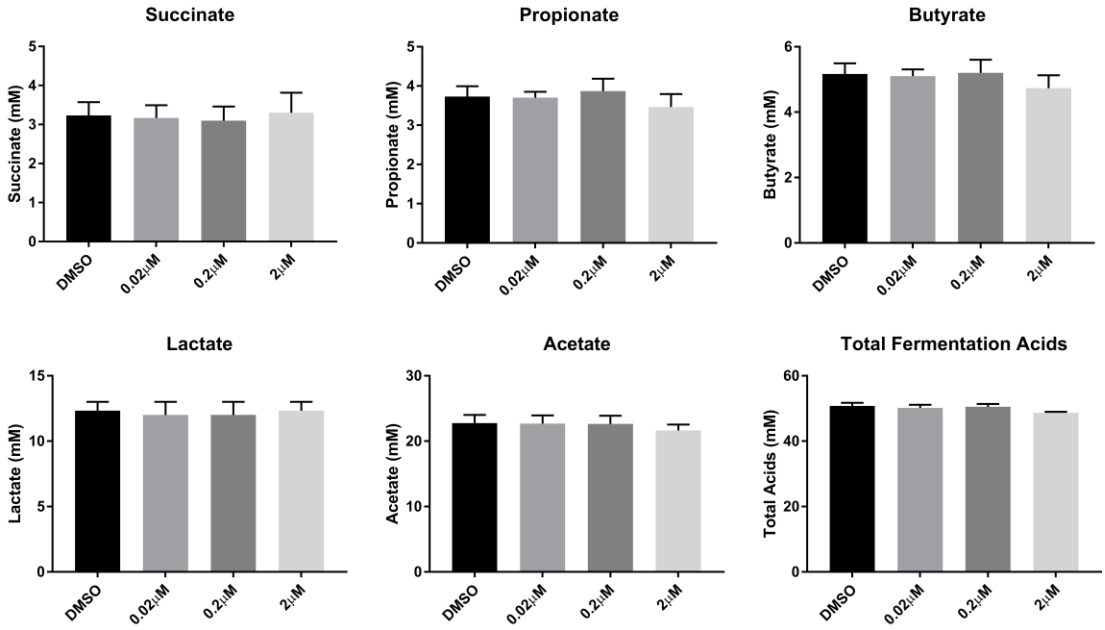


**Figure 2.6**. **Increased inulin provision abolishes effects of PCB 126 treatment on fermentative SCFA production by isolated fecal bacterial.** Bacterial cell suspensions were maintained on inulin substrate (10g/L) and subsequently exposed to PCB 126 (0.02 μM, 0.2 μM, 2 μM) or vehicle control (DMSO) for 48h (n=3). HPLC was utilized to quantify fermentation acids in the media after incubation. PCB 126 had no significant effect on fermentation acid production from a higher inulin substrate concentration (10 g/L). There was a trend towards lower total fermentation acid production ($p=0.055$) in bacterial cultures exposed to higher levels of PCB 126. All data are presented as means ± SEM. * Significantly different compared to vehicle control (DMSO) ($p<0.05$; Student's t-test)

# Chapter 3 Dioxin-like PCB 126 increases inflammation and disrupts gut microbiota and metabolic homeostasis

*Environmental Pollution:* PMID: 30373033

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* both contributed equally to manuscript

## 3.1 Synopsis

The gut microbiome is sensitive to diet and environmental exposures and is involved in the regulation of host metabolism. Additionally, gut inflammation is an independent risk factor for the development of metabolic diseases, specifically atherosclerosis and diabetes. Exposures to dioxin-like pollutants occur primarily via ingestion of contaminated foods and are linked to increased risk of developing cardiometabolic diseases. We aimed to elucidate the detrimental impacts of dioxin-like pollutant exposure on gut microbiota and host gut health and metabolism in a mouse model of cardiometabolic disease. We utilized 16S rRNA sequencing, metabolomics, and regression modeling to examine the impact of PCB 126 on the microbiome and host metabolism and gut health. 16S rRNA sequencing showed that gut microbiota populations shifted at the phylum and genus levels in ways that mimic observations seen in chronic inflammatory diseases. PCB 126 reduced cecum alpha diversity (0.60 fold change; p=0.001) and significantly increased the Firmicutes to Bacteroidetes ratio (1.63 fold change; p=0.044). Toxicant exposed mice exhibited quantifiable concentrations of PCB 126 in the colon, upregulation of *Cyp1a1* gene expression, and increased markers of intestinal inflammation. Also, a significant correlation between

circulating Glucagon-like peptide-1 (GLP-1) and *Bifidobacterium* was evident and dependent on toxicant exposure. PCB 126 exposure disrupted the gut microbiota and host metabolism and increased intestinal and systemic inflammation. These data imply that the deleterious effects of dioxin-like pollutants may be initiated in the gut, and the modulation of gut microbiota may be a sensitive marker of pollutant exposures.

## 3.2 Introduction

Pollutant exposures are associated with numerous chronic inflammatory cardiometabolic diseases including cardiovascular diseases (CVD), obesity, and diabetes [168-170]. Dioxin-like pollutants are a class of highly toxic lipophilic compounds known to accumulate in the adipose tissue of living animals and thus biomagnify up the food chain [168]. Using preclinical animal models including *Ldlr* and *ApoE* deficient mice as a tool to study cardiometabolic disorders, multiple groups have shown that dioxin-like pollutants can impact multiple pathologies through mechanisms that include induction of chronic inflammation in various organ systems [38, 171, 172]. The most common route of human exposure to these pollutants comes from consumption of contaminated food sources and because of this, the intestinal environment is a target organ that deserves further study.

The gut is home to trillions of bacteria which are sensitive to many factors including diet and environmental exposures [173]. Additionally, the gut microbiome plays an essential role in the maintenance of energy metabolism,

immune function, neurological function, and overall host health and well-being [173]. Commensal bacteria exist in a mutualistic relationship with the host, relying on host energy and nutrient intake, and in turn, the gut microbiota protect against pathogen invasion, influence immune function, maintain gut barrier integrity, and produce metabolites that exert various effects on host physiology [173]. Therefore, alterations in gut microbial composition can have deleterious effects on host health. Such alterations are termed “dysbiosis” and have been associated with increased risk of cardiometabolic diseases [174, 175]. Dysbiosis is commonly associated with a decrease in gut microbial diversity, an aspect that has been consistently observed in mice fed atherogenic diets, and human pathological conditions such as atherosclerosis and diabetes [108]. Also, chronic gut and systemic inflammation accompanies the observed disruption of gut flora homeostasis in these pathologies.

Chronic inflammation is an underlying aspect in many non-communicable diseases and has been strongly linked to contributing to the development of cardiovascular diseases (e.g., atherosclerosis) [176]. Atherosclerosis is influenced by inflammation through a variety of mechanisms including cytokine production and release, production of reactive oxygen species, and certain immune responses [176]. Additionally, it is well documented that low-grade chronic inflammation precedes and is predictive of the development of cardiometabolic diseases in adults [177]. Emerging evidence now implicates a critical role of gut microbiota in systemic and intestinal inflammation, which may impact on other peripheral organ systems (e.g., vascular tissue) [174]. The

initiation of these inflammatory processes that exert effects on the cardiovascular and metabolic systems, as well as the gut microbiota can come from behavioral factors such as diet as well as less-modifiable aspects including genetics and environmental pollutant exposure.

Although there is a large body of evidence linking dietary behaviors to modulation of gut microbiota, only a few studies have examined the interplay between persistent organic pollutants (POPs) and the gut microbiota. Zhang et al, found that exposure to 2,3,7,8 tetrochlorodibenzofuran in mice resulted in alterations in gut microbiota, bile acids, and short chain fatty acid (SCFA) metabolism [178]. Additionally, exposure to benzo[a]pyrene induces intestinal inflammation, ileal lesions, and shifts gut microbiota populations [19]. When examining the effects of polychlorinated biphenyls (PCBs), Choi et al. observed disruptions of intestinal barrier function through dysregulation of tight junction proteins [18]. In a later study, Choi et. al found that exposure to a mixture of PCBs (PCB 153, PCB 138, PCB 180) acutely altered the gut microbiota and that exercise was able to blunt these effects [179]. Although pollutant exposures have been shown to modulate gut microbiota in wild-type mice, it is not well established if dioxin-like pollutants can modulate gut microbiota in mice genetically predisposed to cardiometabolic disease. In humans, diabetes and related pathologies regularly accompany or exacerbate atherosclerosis, thus, utilizing *Ldlr* deficient mice is a useful tool to study multiple diseases related to metabolic dysfunction [180]. Therefore, the specific objective of this study was to elucidate effects of the model dioxin-like pollutant PCB 126 on gut health, gut

microbiota, and metabolism in a well-established mouse model of cardiometabolic disease.

## 3.3 Materials and Methods

### 3.3.1 Animals, diet, and study design

The diet and dosing schedule utilized for this study was shown previously to be an effective model of PCB 126-accelerated atherosclerosis [181]. The results described herein were collected from a subset of mice from our previous study [181]. Briefly, seven week-old male *Ldlr* -/- mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were randomly divided into 2 groups (n = 10 per group) with one group receiving 1 μmol/kg of PCB 126 (AccuStandard, CT, USA) and the other group receiving safflower oil vehicle (Dyets, Bethlehem, PA, USA) via oral gavage at weeks 2 and 4. This dose produces plasma PCB 126 levels that mimic human exposures of dioxin-like pollutants (Petriello et al. 2017). All mice were fed a low-fat high cholesterol Clinton/Cybulsky diet *ad libitum* (Research Diets, New Brunswick, NJ, USA; Product # D01061401C). Mice were housed at 22ºC with 50% humidity, exposed to a 12-h light/ 12-h dark cycle, and given water *ad libitum*. Fecal samples were collected at 72h, 4 weeks, and at sacrifice (12 weeks) after first PCB 126 exposure. Prior to euthanasia, mice were fasted overnight, anesthetized, and blood was collected using retro-orbital bleed. At the conclusion of the study, intestinal samples, cecum, and cecum contents were also collected and immediately snap frozen in liquid nitrogen and stored at -80ºC

until analysis. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

### 3.3.2 DNA extraction and 16S rRNA amplicon library preparation and sequencing

DNA extraction and 16S sequencing was conducted by the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory and analyzed by the program Quantitative Insights Into Microbial Ecology (QIIME). DNA was extracted using PowerSoil 96-well DNA Isolation Kit (MoBio, Carlsbad, CA, USA), according to the manufacturer's protocol with the addition of a 65ºC heating step after the addition of solution C1. PCR amplicon libraries targeting the 16S rRNA encoding gene present in metagenomic DNA were produced using a barcoded primer set adapted for the Illumina HiSeq2000 and MiSeq, and DNA sequence data was generated using Illumina paired-end sequencing [81]. The V4 region of the 16S rRNA gene (515F-806R) was PCR amplified with region-specific primers that include sequencer adapter sequences used in the Illumina flowcell. The conditions for PCR were as follows: 94°C for 3 minutes to denature the DNA, with 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; with a final extension of 10 min at 72°C to ensure complete amplification. Amplicons were quantified using PicoGreen (Invitrogen, Carlsbad, CA) and a plate reader (Infinite 200 PRO, Tecan, Männedorf, Switzerland) and then pooled into a single tube so that each amplicon is represented in equimolar amounts. This pool was then cleaned up using AMPure XP Beads (Beckman

Coulter, Brea, CA), and quantified using a fluorometer (Qubit, Invitrogen, Carlsbad, CA). After quantification, the molarity of the pool was determined and diluted to 2 nM, denatured, and diluted to a final concentration of 6.75 pM with a 10% PhiX spike for sequencing on the Illumina MiSeq. Amplicons were sequenced on a 151bp x 12bp x 151bp MiSeq run using customized sequencing primers and procedures. Operational taxonomic units (OTUs) were selected using open reference OTU picking against the Greengenes database and picked at 97% sequence identity. OTUs were then filtered based on the default parameters provided by QIIME and sequences were rarified to a sampling depth of 10000 reads per sample. Before statistical analyses, OTUs present in less than 50% of samples were filtered from the OTU table.

### 3.3.3 RNA extraction and qPCR

For mRNA extraction, intestinal samples from jejunum and colon were homogenized in TRIzol (Invitrogen, Carlsbad, CA) and isolated according to manufacturer's protocols. mRNA quality and concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA was generated using the AMV reverse transcription system (Promega, Madison, MI) following manufacturer's protocols. Expression of inflammatory, metabolic, and gut health markers were determined via quantitative qPCR utilizing Taqman fast reagents (Thermo Scientific, Waltham, MA) in an CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA). β-actin was used as the housekeeping gene for jejunum and 18S was used as the

housekeeping gene for colon. Differences in gene expression were calculated using ΔΔCt relative quantification compared to control animals

### 3.3.4 Analyses of circulating cytokines and proteins related to metabolic function

To measure plasma metabolic hormones and cytokines the Milliplex Map Mouse Metabolic Hormone Magnetic Bead Panel- Metabolism Multiplex Assay and the Milliplex Map Mouse Cytokine/Chemokine Magnetic Bead Panel 25-plex (Millipore Corp, Billerica, MA, USA) were used, respectively, following manufacturer's protocols. Panels were measured on the Luminex Xmap MAGPIX system (Luminex Corp, Austin, TX, USA) following manufactures instructions. For statistical analyses, values below the standard curve were represented as zero.

### 3.3.5 Metabolomics analysis

At the conclusion of the study, livers were snap frozen and shipped to Metabolon (Durham, NC) for targeted metabolomics analysis as detailed previously [182]. Briefly, Samples were prepared using the automated MicroLab STAR (Hamilton Company, Reno, NV) and analyzed by Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS). All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer. A series of proprietary standards of known concentration was added to each sample extract. 4 separate analytical methodologies were utilized

which were optimized depending on the characteristics of compounds of interest (e.g., hydrophilic compounds vs. hydrophobic compounds). The MS analysis alternated between MS and data-dependent $MS^n$ scans using dynamic exclusion. For biochemical identification, three criteria were utilized: retention time, accurate mass match to the library, and the MS/MS compared against authentic standards. Peaks were quantified using area-under-the-curve. Short chain fatty acids (SCFAs) were quantitated by the West Coast Metabolomics Center (Davis, CA, USA). Briefly, SCFAs were extracted from feces samples using Tert-butyl methyl ether, 2-ethylbutryic acid, and 4-methylvaleric acid and derivatized with N-tert-butyldimethylsilyl- N-methyltrifluoroacetamide (MTBSTFA). SCFAs were quantitated using GCMS (Agilent GC7890B/5977MS) and absolute concentrations were determined using calibration and internal standards.

### 3.3.6 Quantitation of atherosclerotic lesions and glucose sensitivity

To quantify atherosclerosis, aortic roots were frozen in OCT, sectioned at 10 μm per section, and stained with Oil Red O as described before [181, 183]. Briefly, serial sections were collected as close as possible to the emergence of the three valves, and sections were placed on microscope slides (Probe-on Plus; Fisher Scientific, Pittsburgh, PA) until the aortic valves disappeared. Frozen sections were lipid stained with Oil Red O, and images were taken using a Nikon Eclipse 55iUpright microscope attached to a 12 MP color camera. For the results shown in Suppl. Fig. 3, on average a mean of 6 serial sections per mouse were used for quantification. To quantify glucose sensitivity a glucose tolerance test was

performed at 5 and 12 weeks post initial PCB exposure. Mice were fasted for 6h (7am-1pm), and fasting blood glucose levels were measured with a hand-held glucometer (Accu-check Avivia, Roche,Basel, Switzerland) using 1-2μL of blood collected through the tail vein. Glucose was given via IP injection (2mg/g body weight, sterile saline) and blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes post injection.

### 3.3.7 PCB 126 Quantitation

PCB 126 from colons, livers, and plasma was extracted using a modified dispersive solid phase extraction method as before [181]. Briefly, tissue/plasma, internal standard (13C12-PCB 126; Cambridge Isotopes, Tewksbury, MA), deionized water, and acetonitrile containing 1% acetic acid were added to each tube and homogenized. The upper layer was transferred to an Agilent Bond Elut QuEChERS fatty sample dispersive 2 ml SPE column. PCB 126 was analyzed using an Agilent GC-triple quadrupole MS (GC-MS/MS) 7000C system equipped with a multimode inlet and a HP-5MS UI column (30m, 0.25mm, 0.25μm) in multiple reaction monitoring (MRM) mode. Ion transitions monitored were 325.9/255.9 for PCB 126 and 337.9/267.9 for 13C12-PCB 126 internal standard. Relative quantitation was done by comparing peak area of the sample to peak area of an internal standard sample of known concentration.

### 3.3.8 Lipopolysaccharide Binding Protein Quantitation

Circulating levels of LBP were determined by ELISA per manufacturer's instructions (Cell Sciences, Newburyport, MA, USA). Briefly, plasma samples were diluted 1:800 in dilution buffer and absorbance (450nm) was read using a Cytation5 imaging plate reader (Biotek, Vermont, USA).

### 3.3.9 Statistical analyses

qPCR and MAGPIX results were analyzed using a Student's t-test to compute comparisons of means between treatment groups. Analyses were conducted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Metabolomics results were analyzed using a Welch's two-sample t-test (n=6 per group). These analyses were performed using ArrayStudio (Qiagen, Hilden, Germany) on log transformed data.

For statistical analyses of changes in fecal bacterial genera over time, linear mixed-effects model was utilized with unstructured correlation matrix accounting for dependence among observations over time and within an animal. Tukey's pair-wise multiple comparison procedure followed to assess the significance of change in bacterial genera at each time point. Gut hormones and metabolic markers were correlated with bacterial species using one of the three hypothesized linear models: the full model that accounted for a possible interaction between PCB 126 exposure and a biomarker on the gut hormones level; the reduced model that had an additive effect of PCB 126 exposure and a metabolic biomarker only; and the simple model that did not account for the PCB

126 exposure. The most appropriate model was determined based on the largest value of the adjusted-$R^2$, and its results were Bonferroni-adjusted to account for multiple comparisons. Results were considered statistically significant with an observed p-value <0.05.

## 3.4 Results

### 3.4.1 PCB exposure induces shifts in bacterial populations over time

It is hypothesized that the gut microbiota of *Ldlr* -/- mice will shift as cardiometabolic disease progresses, but it is unknown if pollutant exposures will exacerbate or modify these changes. Using 16S rRNA sequencing and the QIIME analysis platform, we quantified the bacterial populations in fecal samples collected 72 h, 4 weeks, and 12 weeks after first PCB or vehicle gavage (depicted as *phyla* level changes in **Figure 3.1** and *genera* level changes in **Figure 3.2**). At the phyla level, PCB 126 decreased relative Bacteroidetes (0.75 fold change, p=0.011) and increased relative Verrucomicrobia (1.41 fold change, p=0.008) and species in the feces at 4 weeks post gavage, but not at 12 weeks. Using these relative abundances, we then analyzed the Firmicutes to Bacteroidetes ratio over the four timepoints **(Figure 3.1B)**. We found that in general this ratio increased throughout the study for all mice (which was expected as mice were fed the atherogenic diet), but interestingly, PCB treated mice had a significantly higher ratio at the conclusion of the study in both feces and cecum contents (feces: 3.29 fold change, p=0.0332; cecum: 1.63 fold change, p=0.044).

Next, using mixed-effects modeling, we increased the resolution of our QIIME analyses to determine which genera differed between PCB and vehicle-treated mice over time. In general, we determined four major trends with which generas changed over time. The first pattern was one in which PCB-treated mice displayed decreased bacterial genera abundance, while the abundance of vehicle-treated mice was stable across the course of the study. For instance, by the conclusion of the study, *Lactobacillus* genera in PCB-treated mice decreased by 84% relative to the baseline values (Tukey-adjusted $p_{TA} = 0.0021$) and was 80% lower than that of the vehicle-treated mice ($p_{TA} = 0.0339$), among which the abundance of *Lactobacillus* genera did not appreciably change from baseline measurements **(Figure 3.2A).** The second pattern was one in which neither vehicle nor PCB-treated mice displayed a statistically significant deviation from each other or from the baseline genera values. For example, the abundance of Oscillospira genera was not affected by PCB exposure, nor did it change or over time **(Figure 3.2B).** The third pattern was one in which PCB-treated mice displayed increased abundance over time, while the abundance among vehicle-treat mice did not change. For example, by the conclusion of the study, the abundance of *Clostridiales* genera was 420% higher among PCB-exposed mice than among vehicle-treated mice ($p_{TA} = 0.0248$; **Figure 3.2C).** Finally, the last pattern was one in which both vehicle and PCB-treated mice displayed increased abundance over time, but this difference was equivalent between the two groups. For example, relative to the baseline levels, the abundance of *Allobaculum* genera increased over tenfold by the conclusion of the study in both groups

(week 14) (both $p_{TA} < 0.0001$; **Figure 3.2D**). A summary of all temporal results can be found in Supplemental Table 1.

3.4.2 PCB exposure alters cecal microbial diversity and bacterial genera

The cecum contains the greatest abundance of microbiota and is a primary site of fermentation and bacterial metabolism. Using 16S rRNA sequencing and QIIME analysis software, we also examined the bacterial populations in cecum samples collected at the end of the study (week 14). Similarly to the fecal results, PCB 126-treated mice exhibited significantly reduced relative Bacteroidetes (0.38 fold change, p=0.002) and Firmicutes (0.54 fold change, p=0.004) and greater relative Verrucomicrobia (1.71 fold change, p=0.021) abundances compared to vehicle control **(Figure 3.1A)**. Alpha diversity is a common measurement of microbial diversity within a sample and takes into account the richness (count of different microbes) and evenness (distribution of different microbes) of a sample. PCB 126 decreased the alpha diversity (Shannon diversity index) in cecal samples compared to vehicle control (0.60 fold change, p=0.001) **(Figure 3.3A).** We also examined beta-diversity in these samples and determined that PCB-exposed mice exhibited a significant difference in microbial community structures compared to vehicle treated mice (R=0.2924, p=0.010) **(Figure 3.4).** We next examined genera level changes in the cecum contents and determined this source of bacteria to be more sensitive to pollutant-induced changes compared with the fecal samples from the same timepoint (i.e., conclusion of study). The abundance of S24.7 and Clostridiales as well as the genera *Bifidobacterium*, *Lactobacillus, Ruminococcus*, and

*Oscillospira* were all significantly decreased in PCB exposed mice by similar magnitudes compared to vehicle control (0.39-0.90 fold change). Furthermore, PCB 126 increased the abundance of *Akkermansia* (1.71 fold change, p=0.021) in the cecal samples compared to vehicle control (**Figure 3.3B).**

### 3.4.3 PCB exposure increases systemic inflammation and alters cardiometabolic disease parameters

Systemic inflammation may be a marker of altered gut health and has been shown to be increased by PCB 126 [40, 181, 184]. Using MAGPIX multiplex technology, we quantified the levels of circulating cytokines in plasma at the conclusion of the study and found that PCB 126 significantly increased interferon gamma-induced protein (IP-10; **Figure 3.5A).** Additionally, macrophage inflammatory protein-1β (Mip-1β), regulated on activation, normal T cell expressed and secreted (Rantes), and interleukin 12 (IL-12 p70) both increased in PCB 126 exposed mice, however this increase did not quite attain the statistical significance threshold ($0.05 < p < 0.1$). We also examined circulating levels of LBP at the conclusion of the study and determined a slight trend toward increased levels in PCB 126 treated mice (p=0.142; **Figure 3.6**) Since gut health is also directly related to endogenous metabolism, we also quantified levels of circulating mediators of glucose/insulin signaling and determined that PCB 126 significantly increased levels of insulin (2.74 fold change, p=0.019), c-peptide (1.63 fold change, p=0.015), and glucose-dependent insulinotropic peptide (GIP) (1.41 fold change, p=0.046) as well as significantly decreased levels of glucagon-

like peptide 1 (GLP-1**; Figure 3.5B)** (0.31 fold change, p=0,048). At 5 weeks post initial PCB gavage and again at the conclusion of the study, we completed a glucose tolerance test and observed no significant differences in glucose sensitivity during either of the challenges. However, fasting blood glucose of PCB-exposed mice trended higher at both timepoints (p= 0.07, p= 0.16) compared to vehicle treated controls **(Figure 3.7).** Finally, we quantified Oil Red O-staining within aortic roots to examine the extent of atherosclerosis in these mice. We previously showed acceleration of atherosclerosis due to PCB 126 exposure using this same model in studies of shorter duration (e.g., 8 or 10 weeks post first PCB gavage) [181]. We determined in this current study that PCB-exposed mice displayed similar trends as seen before, but the differences in lipid accumulation between the two groups was not as evident as in the shorter studies (Mean control; $2.05x10^5 \pm 2.53x10^4$ $\mu m^2$, Mean PCB; $2.60\ x10^5 \pm 3.42x10^4$ $\mu m^2$, p=0.2; **Figure 3.8).**

### 3.4.4 The intestine is a target of PCB 126 toxicity

There is currently limited evidence that PCB 126 can elicit an inflammatory response in intestinal tissue [18, 185]. Thus, we utilized qPCR to examine gene expression of markers related to inflammation, gut health, and metabolism in the jejunum (small intestine) and the colon (large intestine). In general, PCB 126 elicited similar responses in both the jejunum and colon (**Figure 3.9A** and **3.9B**). PCB 126 significantly increased expression of *Cyp1a1*, a marker of aryl hydrocarbon receptor (AhR) activation, in the jejunum and the colon (11.23 fold

change, $p<0.0001$; 23.00 fold change, $p<0.0001$). Additionally, PCB 126 increased expression of the inflammatory markers hepcidin (*Hamp*) and tumor necrosis factor alpha (*Tnfα*) in the colon and interleukin 6 (*Il-6*) and interleukin 18 (*Il-18*) in the jejunum. Expression of the tight junction proteins occludin (*Ocel*) and claudin (*Cldn3*) were significantly increased only in the colon. Furthermore, mucin 2 (*Muc2*) only trended towards increased expression by PCB 126 in the colon ($0.05 < p < 0.1$). Toll like receptor 4 (*Tlr4*), a transmembrane receptor that functions in pathogen recognition and activation of innate immunity, was significantly decreased in the jejunum and colon of PCB 126 exposed mice. Interestingly, peroxisome proliferator-activated receptor delta (*Pparδ*), an important regulator of intestinal cell differentiation and potential inhibitor of inflammatory bowel disease, was significantly decreased in the colon of PCB treated mice (0.46 fold change, $p=0.008$). Gene expression of glucagon (*Gcg*), the gene responsible for the production of glucagon like peptide 1 (GLP-1), was significantly increased in both the jejunum and colon of PCB treated mice. Finally, to better elucidate the presence of PCB in gut tissue, and possible causative mediator of gut inflammation, we followed up these gene expression observations by quantitating PCB 126 in colon tissue via GC-MS and determined that the average concentration was $0.767 \pm 0.608$ (Mean $\pm$ S.E.M.) pmoles/mg tissue. In comparison, hepatic levels averaged $6.388 \pm 0.688$ (Mean $\pm$ S.E.M.) pmoles/mg and plasma levels were undetectable in most mice analyzed.

### 3.4.5 PCB 126 alters hepatic metabolism in ways that mirror metabolic diseases

With the close proximity of the gut and liver, hepatic metabolism and gut health are tightly intertwined, exerting continual influence on each other. It is known that changes in hepatic metabolism occur as cardiometabolic disease progresses, but it is unknown if dioxin-like pollutant exposure can modify global metabolomic pathways in mice genetically predisposed to cardiometabolic disease. Thus, using metabolomics-based approaches we next examined the impact of PCB 126 on multiple metabolites related to glycolysis, lipogenesis, and gut microbiota **(Table 3.2)**. PCB 126 exposure significantly decreased glycolytic intermediates including glucose 6-phosphate, 2-phosphoglycerate, and 3-phosphoglycerate and increased glycerol, glycerol 3-phosphate, and 3-hydroxybutyrate. Furthermore, we observed PCB induced alterations in metabolites that can be influenced by interactions between gut microbiota and host systems including reductions in N-acetylphenylalanine, hippurate, and 5-hydroxyindoleacetate, and elevations in dimethylglycine, N-oleoyltaurine, and O-methyltyrosine. There were no differences in other gut microbe-influenced metabolites in the liver including indolelactate, indole-3-carboxylic acid, and 3-indoxyl sulfate. Finally, we completed fecal short chain fatty acid analysis at the conclusion of the study and determined that PCB 126 significantly decreased formic acid concentrations by nearly half ($p=0.002$). Acetic, butyric, isovaleric, or propionic acids were not changed between groups (**Table 3.3**).

3.4.6 PCB 126 induced bacterial alterations associated with metabolic markers

Finally, little is still known about how alterations in gut microbiota are related to overall host health, therefore, using regression analysis we examined the relationship between quantitative markers of inflammation and metabolism with cecal bacterial genera counts. In general, of the correlations examined, there were very few statistically significant associations between cecal genera counts and circulating mediators of metabolism and inflammation. For example, no significant association was observed between any of the cecal generas and *Il-6*, c-peptide, GIP, or insulin. However, we did observe a statistically significant association between absolute counts of *Bifidobacterium* and GLP-1 (**Figure 3.9a)**. Specifically, there was a positive association between *Bifidobacterium* counts and GLP-1 among PCB-treated mice (Bonferroni-adjusted p-value for the number of generas considered $p_{BA} = 0.044$), while among vehicle-treated mice GLP-1 levels were decreasing with an increase in *Bifidobacterium* counts ($p_{BA} = 0.031$). Furthermore, we also observed an inverse association between *Akkermansia* and fasting blood glucose in both PCB 126 exposed mice and vehicle treated mice **(Figure 3.9b)**. Moreover, at the conclusion of the study, overall fasting glucose levels were 23 points higher ($p_{BA} = 0.0342$) among PCB-treated mice than among unexposed mice **(Figure 3.9b)**.

## 3.5 Discussion

Currently, a major exposure source of dioxin-like pollutants such as PCB 126 is through consumption of contaminated foods [2]. Thus, the gastrointestinal

system is susceptible to a relatively high concentration of pollutants but is a comparably under studied target organ. Importantly, the gut can have drastic impacts systemically because of its critical role in maintenance of overall health [92]. We previously showed that PCB 126 could accelerate atherosclerosis and increase systemic inflammation in mice genetically predisposed to cardiometabolic disease, but any impacts of PCB 126 on gut microbiota in this model were yet to be examined [181]. Therefore, in this study we employed the same *Ldlr* -/- mouse model fed a high cholesterol diet to examine the effect of PCB 126 on gut microbiota populations, gut health, and the interplay of these effects with inflammation and metabolic complications. We observed that PCB 126 dramatically altered gut microbial populations, primarily in the cecum, and consistently increased intestinal and systemic inflammation.

The gut microbiota are very sensitive to environmental factors including diet and pollutant exposure. Dysbiosis, or the abnormal distribution of bacterial populations, is associated with numerous health conditions from inflammatory bowel diseases to obesity and cardiometabolic disease [92]. In these conditions, an increase in the ratio of Firmicutes/Bacteroidetes has been consistently observed and has been linked to increased susceptibility to inflammation, infection, oxidative stress, and insulin resistance [186, 187]. In **Figure 3.1B** we showed that this ratio was increased due to PCB 126 exposure by the end of the study in both feces and cecum. Furthermore, individuals with type 2 diabetes, have been demonstrated to have a unique microbial signature comprised of a reduction in the number of *Clostriadiales*, including *Roseburia* spp, and

*Faecalibacterium prausnitzii* [188, 189]. In these patients with diabetes or insulin resistance, it has been observed that these specific alterations in gut microbial composition persist without alterations in carbohydrate metabolism [188, 189]. Research examining the relationship between cardiometabolic disease and gut microbiota has been of great interest in the field lately. Studies have identified a strong link between a gut microbial dependent metabolite, trimethylamine N-oxide (TMAO), and the incidence of cardiometabolic disease [190-192]. In our metabolomics work described herein we did observe an increase in dimethylglycine, a possible TMAO precursor, but hepatic TMAO levels were not significantly different between groups (plasma metabolomics was not completed). Because the microbiota seems to have a strong ability to sense and respond to systemic inflammation, examining known causes of inflammation, such as pollutant exposure, on gut microbiota populations and functionality is greatly needed.

There are only a few studies examining the effects of dioxin-like pollutant exposure on gut microbiota and overall host health. Similarly, to what we observed, Lefever et. al. found that TCDD exposure did not significantly affect blood glucose homeostasis but did find distinct alterations in the gut microbiota including an increase in the Firmicutes:Bacteroidetes ratio, which is commonly seen in inflammatory disease conditions [193]. Additionally, another group showed that exposure to TCDF induced similar changes to bacterial populations as what we observe with PCB exposure [178]. For example, TCDF significantly decreased the abundance of Firmicutes in the cecal contents similarly to what we

observe in the cecal contents of our exposed animals. However, it is important to note that TCDF exposure significantly reduced overall the Firmicutes: Bacteroidetes ratio, opposite to what we observed with PCB exposure. Finally, Choi et. al observed increases in Verrucomicrobia in the feces of PCB-exposed mice, similarly to what we observe in the present study [179]. Although many of these effects are similar to what we see in our current study, some differences may be due to the unique model of cardiometabolic disease that the present study utilizes.

Using 16S rRNA sequencing of fecal samples we examined the change in bacterial populations over time **(Figure 3.2)**. At the conclusion of the study, we determined that PCB treated mice displayed a significant drop in S24.7 (30%) and *Lactobacillus* (80%), as well as a significant increase in *Clostridiales* (80.9%). The repeated collection of feces samples also revealed changes in gut microbiota as cardiometabolic disease progressed in the *Ldlr* -/- mice. For example, irrespective of treatment group, mice showed a significant ~70% decrease in *Lachnospiraceae* (family) counts, a near ablation of *Anaerostipes* counts, and a drastic increase (>1100 times) in *Allobaculum* from baseline to study conclusion (Supplemental Table 1). Surprisingly, few studies in *Ldlr* or *ApoE* -/- mice exist describing genera level changes throughout the progression of cardiometabolic disease. There is some work looking at progressive changes due to ageing and glucose intolerance/diabetes, but more work focused on vascular diseases is needed [194, 195]. In addition to studying gut microbiota changes over time using the collection of feces, we also examined gut microbiota

differences due to PCB 126 at the conclusion of the study in cecum contents. This medium was more sensitive to changes due to PCBs with significant alterations in S24.7, Clostridiales, *Akkermansia*, *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, and *Oscillospira* **(Figure 3.3B).**

Interestingly, all mice, regardless of treatment, exhibited high levels of Verrucomicrobia (phylum level) and subsequently *Akkermansia* (genus level) throughout the entirety of the study compared to what is typically observed in the murine gut microbiota [149]. We hypothesize that the high cholesterol diet all mice received caused an increase in *Akkermansia*. Although *Akkermansia* is usually thought of as a healthful bacterial species, we are not the first to observe increased abundance during high cholesterol feeding. Hamilton et. al reported that an obesogenic diet may increase the level of this bacteria similarly to what we observed [196]. Furthermore Carmody et al. observed a high proportion of Verrucomicrobia and thus *Akkermansia* with high fat, high sucrose feeding [197]. Although the authors of both articles attributed this to the fat content, these diets were also high in cholesterol which may be driving the increase in this bacterium observed in both studies as well as ours. Further research needs to be done to examine this effect.

We did observe that PCB 126 exposed mice exhibited higher cecum levels of the phylum Verrucomicrobia and *Akkermansia,* a genus falling within this phylum*,* at the end of the study, compared to vehicle treated mice. In support of this, a smaller but still significant increase in Verrucomicrobia following PCB treatment was observed by Choi et al [179]. The *Akkermansia* genus contains mucin

degrading bacteria, specifically *A. muciniphila,* and there are reports that an abnormally high abundance of *Akkermansia* may actually be detrimental due to the excessive degradation of the mucus layer lining the intestine in such a way that the mucin producing goblet cells cannot compensate [196, 198]. In support of this, we observed trends toward an increase in mucin (*Muc2*) gene expression in the colon of mice exposed to PCB 126. Although we did collect and histologically stain Ileum tissues (H&E) in the current study, pathology analyses did not reveal any conclusive differences between the two groups (necrosis was observed in 50% of PCB treated mice and 33.3% of vehicle treated mice; data not shown).

Overall, cecum bacterial populations were impacted to a greater degree by PCB 126 exposure compared to feces. The cecum is characterized by a greater abundance of bacterial species and is often more representative of actual gut microbial populations than fecal samples [199]. We observed that PCB 126 exposed mice had reduced levels of the genera *Oscillospira*, *Bifidobacterium*, *Lactobacillus*, and *Ruminococcaceae*. *Oscillospira* has recently been observed to be positively associated with leanness and health while decreases in this genera are observed in inflammatory diseases [200]. In our previous study, utilizing the same mice, we showed that PCB 126 exposed mice had higher body fat and lower lean mass percentages [181]. *Bifidobacterium* and *Lactobacillus*, two of the most commonly recognized genera and prebiotics in the food system, are both associated with improved gut health, glucose tolerance and reduced risk of disease [120]. Specifically, lower levels of *Bifidobacteria* has been observed in

the fecal microbiota of patients with irritable bowel syndrome, a condition characterized by high levels of inflammation, while *Lactobacillus* spp. supplementation has been demonstrated to aid in the prevention of intestinal inflammation [201, 202]. Importantly, both *Bifidobacterium* spp. and *Lactobacillus* spp. have been demonstrated to be increased by prebiotic supplementation, which in turn was associated with improved glucose tolerance and metabolic health [203, 204]. Thus, the reductions in these bacterial genera in PCB treated mice may be associated with the observed alterations in metabolic health.

To link the gut microbiota alterations to systemic inflammation and responses to PCB 126 exposure, we conducted regression analysis examining the relationship between markers of inflammation and metabolism with cecal bacterial genera. Using this approach, we observed an inverse association between *Akkermansia* and fasting blood glucose, regardless of treatment group. This observation has been noted by other groups previously, demonstrating that decreased levels of *Akkermansia* were associated with glucose intolerance and supplementation of *Akkermansia muciniphila* to high fat fed mice improved glucose tolerance [205]. Interestingly we observed a toxicant-dependent association between *Bifidobacterium* and GLP-1, where increased levels of *Bifidobacterium* were associated with increased levels of GLP-1, only in the PCB 126 treated group. This is important due to GLP-1's involvement in managing insulin levels. As such, it has been demonstrated that *Bifidobacterium*

*adolescentis* supplementation improves insulin sensitivity in an animal model of metabolic syndrome [206].

This current study is significant because we were able to examine PCB 126-induced changes in gut health over time. Importantly, we observed that even 10 weeks following a final dose of PCB 126, gene expression of *Cyp1a1* remained highly elevated in the jejunum and colon. *Cyp1a1* is a marker of AhR activation, observed with exposure to PCB 126 and other dioxin-like pollutants. Our observations of quantifiable levels of PCB 126 in the colons of exposed mice at the end of the study highlight that PCBs have a long-lasting impact on the gut. Other investigators have discussed the circulation of these pollutants through enterohepatic circulation, which involves the cycling of drugs and other compounds from circulation into the intestine where reuptake and transport to the liver occurs [207]. Because we observed such an elevation in *Cyp1a1* gene expression, we hypothesize that these compounds are continually being cycled through enterohepatic circulation, thus exerting a continual impact on the gut microbiota and contributing to chronic increases in gut inflammation.

Intestinal inflammation poses a risk for systemic inflammatory and metabolic conditions including diabetes and cardiovascular disease. For example, patients with inflammatory bowel disease have increased endothelial dysfunction and atherosclerotic lipid profiles [208]. This association with inflammatory diseases and risk of cardiometabolic disease is well founded, for example in patients with rheumatoid arthritis and psoriasis [209]. It is important to note that these associations are independent of traditional cardiometabolic disease risk factors.

Importantly, we observed increases in intestinal markers of inflammation including *Tnfα*, *Il-6*, *Il-18*. In our previous study using the same model system, we showed that PCB 126 increased circulating levels of multiple cytokines at 8 weeks post PCB gavage, but Tnf*α* and Il-6 were not changed. More work needs to be completed to better understand how tissue specific inflammation (as we see in the current study in the intestines) is related to an overall systemic inflammatory response. It has been demonstrated that intestinal upregulation of *Il-18* results in production of chemoattractants and cytokines that can reach circulation and initiate inflammatory responses systemically [210]. Interestingly, it has been shown that the gut microbiota and antigen-presenting cells in the intestine interact and stimulate the release of cytokines including TNF-α and IL-6 and that this results in the exacerbation of inflammatory bowel disease as well as whole body inflammation [211]. Although we did see similar trends in our previous work showing PCB 126 accelerated atherosclerosis at earlier time points [181], this was no longer significantly different in mice sacrificed at 12 weeks post first PCB gavage. However, if we combined data points from our previously published work and this current study to increase statistical power, the acceleration of atherosclerosis due to PCB 126 becomes highly significant ($p=0.004$; data not shown). It is not unexpected that as cardiometabolic disease progresses in all of the *Ldlr* deficient mice, the impact of PCB 126 on atherosclerosis is blunted at later time points. To investigate the impact of dioxin-like pollutants in future longer-term studies, additional toxicant exposures (i.e., additional gavages) may be useful. In fact, circulating levels in plasma of

PCB 126 were undetectable in many of the mice examined at the end of this current study. In our previous study [181] circulating levels of PCB 126 at 8 weeks post first PCB exposure were detected in all mice analyzed with an average of 0.104 picomoles PCB 126/μL plasma.

We also observed some potentially adaptive responses to PCB 126. For example, in the colon, we observed a reduction in Tlr4. Tlr4 is involved in the inflammatory signaling response through its trigger of nuclear factor κ B. (NF-κB). However, the observed reduction in expression of Tlr4 in PCB treated mice may be in an attempt to attenuate the inflammatory responses already ongoing in the intestine. Indeed, it has been demonstrated that in murine macrophages, lipopolysaccharide stimulation actually resulted in the downregulation of Tlr4 [212]. Additionally, we observed a small increase in mRNA expression of tight junction proteins in the intestine of PCB-exposed mice. We hypothesize that this may also be a compensatory mechanism to repair following intestinal injury. Future research should look at direct measures of intestinal permeability upon pollutant exposure to better elucidate these effects. Additionally, our observations of an increase in the bacterial genera *Akkermansia* was unexpected and may also highlight potential compensatory mechanisms of pollutant exposure. Although it is well established that *Akkermansia* is beneficial, others have seen increases during proinflammatory events such as high fat feeding [197, 198, 213]. These findings highlight the diverse roles that specific bacterial populations may affect various disease states and the importance of follow up studies using additional model systems.

This is the first study to link PCB 126-induced disruption of the gut to changes in systemic metabolism. We observed that PCB treated mice had increased circulating levels of insulin, c-peptide, and glucose-dependent insulinotropic peptide (GIP) and reduced levels of glucagon-like peptide 1 (GLP-1). While we did not observe differences in glucose sensitivity during a glucose tolerance test (**Figure 3.7**), the increased levels of insulin and c-peptide in PCB exposed mice may indicate reduced insulin sensitivity (i.e., increased level of insulin required to stimulate cellular glucose uptake). Furthermore, GLP-1 and GIP are both insulinotropic incretins, but there is evidence that hyperinsulinemia and insulin resistance can cause impairments in GLP-1 secretion from intestinal L cells [214]. Because it is well understood that systemic inflammation can lead to insulin resistance, we believe that the high level of inflammation observed in PCB 126 exposed mice is responsible for the dysregulation of insulin signaling and subsequently further disruption of GLP-1 secretion from the intestine.

This disruption in metabolism was also observed in our hepatic metabolomic analyses where we found that mice exposed to PCB 126 displayed disruptions of glycolytic metabolism, evidenced by decreased levels of glucose 6-phosphate, 2-phosphoglycerate, 3-phosphoglycerate, and phosphoenolpyruvate. Furthermore, PCB 126 treated mice exhibited increases in glycerol, glycerol-3 phosphate, and the ketone body 3-hydroxybutryate, indicating a shift towards a dominance of fatty acid metabolism for energy resulting in ketone body formation. This shift away from carbohydrate metabolism to fatty acid metabolism could be due to the high circulating levels of insulin indicating a requirement for more insulin to

stimulate glucose uptake, thus causing fatty acid utilization and ketone body formation to be upregulated to meet energy demands more efficiently. Finally, we also show for the first time that exposure to a dioxin-like PCB can decrease fecal concentrations of a SCFA (formic acid). Interestingly, other SCFAs were not altered in this study which may implicate the disruption of specific microbe species by PCB 126. In future studies, higher resolution sequencing or the use of species-specific qPCR primers, may be useful to better tease out the impacts of lipophilic persistent organic pollutants on microbiota health.

Although this current study adds to a growing body of knowledge detailing the impacts of persistent organic pollutants on gut microbiota and host health, there are multiple limitations that may be more thoroughly addressed in follow-up studies. First off, only an IP glucose tolerance test (GTT) was completed. As more insulin may be released during an oral bolus of glucose, and with the observed changes to incretins due to PCB 126, it is plausible that our glucose tolerance observations may differ slightly depending on the administration method. Secondly, since all mice received a high cholesterol diet to promote cardiometabolic disease it is not possible to tease out how the interaction between cholesterol and toxicant exposure is modulating gut microbe and host health. A future, larger study, with a 2x2 factorial design (2-way ANOVA) would be useful to tease out the effects of diet, toxicant exposure, and their interaction on the observed changes. Finally, this study focused solely on male mice which is an important consideration since the gut microbiota may differ between sexes [215].

Our current study demonstrates that exposure to PCB 126 in a compromised model of cardiometabolic disease disrupts gut microbial populations, contributes to intestinal inflammation, systemic inflammation, and metabolic disruption. We hypothesize that the effects on systemic inflammation, intestinal inflammation, and gut dysbiosis with PCB 126 exposure may occur simultaneously and exert a feed-forward effect on each other. More studies are needed to determine if toxicant-initiated changes in gut microbiota are a causative mechanism linking exposures to dioxin-like pollutants to increased risk of pro-inflammatory diseases in humans.

**Table 3.1.** Gut microbiota population changes over time

| Bacteria Category | Baseline | | 72h | | 4 weeks | | Sacrifice | |
|---|---|---|---|---|---|---|---|---|
| **Phyla** | **Ctrl** | **PCB** | **Ctrl** | **PCB** | **Ctrl** | **PCB** | **Ctrl** | **PCB** |
| Actinobacteria* | 211.5 | 103.5‡ | 59.50 | 32.5 | 438.5 | 588.0 | 1420† | 884.5† |
| Bacteroidetes* | 30184 | 26688 | 30027 | 23665 | 35548 | 22046‡ | 24216 | 13624† |
| Firmicutes | 27318 | 34694 | 21597 | 20495 | 30933 | 26453 | 36192 | 40252 |
| Tenericutes | 420.1 | 254.1 | 4.7 | 8.5 | 17.2† | 0† | 76.7† | 35.5† |
| Verrucomicrobia | 10968 | 20623 | 40078† | 40935 | 23726† | 30138 | 29664† | 27487 |
| Other* | 4548 | 1478 | 1940 | 2417 | 10088 | 14956† | 4420 | 588‡ |
| **Family, Genus** | | | | | | | | |
| *Clostridiaceae** | 527 | 829 | 382 | 666 | 247 | 184 | 1596 | 2113 |
| *Coriobacteriaceae** | 150 | 19.0 | 4.50 | 7.0 | 177 | 112 | 964 | 579† |
| *Erysipelotrichaceae** | 58 | 40 | 47 | 133 | 18 | 133 | 15 | 42 |
| *Lachnospiraceae* | 3874 | 4842 | 3102 | 3418 | 3398 | 3110 | 1582† | 1198† |
| *Ruminococcaceae* | 4399 | 5820 | 3676 | 3341 | 4275 | 3141† | 2060 | 1989† |
| *S24.7** | 30184 | 26688 | 30027 | 23665 | 35548 | 22046 | 24216 | 13624†‡ |
| *Allobaculum* | 12 | 15 | 10 | 9 | 31 | 676 | 16810† | 21520† |
| *Akkermansia* | 10970 | 20620 | 40080† | 40940† | 23730† | 30140 | 29660† | 27490 |
| *Anaerostipes* | 343.5 | 209 | 0.7† | 0.3† | 0.3† | 0.1† | 0.4† | 0.1† |
| *Bifidobacterium** | 124 | 47 | 48.5 | 25 | 234 | 458 | 469 | 369 |
| *Coprobacillus** | 47 | 56 | 317† | 89‡ | 83 | 178 | 37 | 86 |
| *Coprococcus* | 424 | 556 | 365 | 251 | 314 | 242† | 118† | 209† |
| *Dehalobacterium* | 65.6 | 77 | 45 | 42.5 | 43.5 | 41.5 | 99 | 74 |
| *Lactobacillus** | 310 | 194 | 53† | 29 | 227 | 316 | 179 | 43†‡ |
| *Lactococcus** | 608 | 931 | 1027† | 859 | 1204† | 1381† | 1146† | 1458 |
| *Oscillospira** | 3560 | 4256 | 2718 | 2336 | 4356 | 3193 | 1732 | 2220 |
| *Ruminococcus** | 622 | 902 | 619 | 748 | 1076 | 753 | 440 | 548 |
| *Turicibacter** | 325 | 44.5 | 1 | 1.5 | 0.5† | 0.0† | 1.0 | 0.0† |

Statistically significant differences determined by mixed-effects modeling of absolute counts. † denotes statistical significance compared to baseline. ‡ denotes statistical significance between groups at specific timepoint. *median instead of mean values are reported due to the skew in the distribution of the absolute count values.

**Table 3.2.** Hepatic metabolomics analysis

| Metabolite Category | Fold Change PCB vs. Vehicle | P-value | Pathway |
|---|---|---|---|
| Carbohydrate Metabolism | | | |
| Glucose 6-phosphate | 0.70 | 0.014 | Glycolysis, |
| 2-phosphoglycerate | 0.50 | <0.010 | Gluconeogenesis, and |
| 3-phosphoglycerate | 0.66 | 0.0111 | pyruvate metabolism |
| Lipid Metabolism | | | |
| Glycerol | 2.05 | <0.001 | Glycerolipid metabolism |
| Glycerol 3-Phosphate | 2.38 | <0.001 | Glycerolipid metabolism |
| 3-hydroxybutryate | 1.49 | 0.0298 | Ketone bodies |
| Gut Microbiota Influenced Metabolites | | | |
| 5-hydroxyindoleacetate | 0.55 | <0.01 | Tryptophan metabolism |
| Indolelactate | 1.14 | 0.31 | Tryptophan metabolism |
| Indole-3-carboxylic acid | 0.87 | 0.74 | Tryptophan metabolism |
| 3-indoxyl sulfate | 0.96 | 0.70 | Tryptophan metabolism |
| Retinol | 0.58 | 0.015 | Vitamin A metabolism |
| Retinal | 0.52 | <0.01 | Vitamin A metabolism |
| Hypotaurine | 0.47 | <0.01 | Methionine, cystine, SAM |
| 1-methylhistamine | 0.33 | 0.081 | Histidine metabolism |
| N-acetylarginine | 0.64 | <0.010 | Urea cycle |
| Hypoxanthine | 0.79 | 0.012 | Purine metabolism |
| N-acetylphenylalanine | 0.35 | <0.010 | Phenylalanine metabolism |
| Hippurate | 0.52 | 0.067 | Benzoate metabolism |
| Creatinine | 0.52 | 0.086 | Creatine metabolism |
| Argininate | 0.72 | 0.014 | Urea cycle |
| N-oleoyltaurine | 1.74 | 0.049 | Endocannabinoid |
| O-methyltyrosine | 1.54 | 0.050 | Tyrosine metabolism |
| Urate | 2.13 | <0.01 | Purine metabolism |
| Dimethylglycine | 1.41 | 0.022 | Glycine, serine, and threonine metabolism |
| Glutarate | 1.79 | 0.097 | Fatty acid, dicarboxylate |

Statisticaly significant differences determined by Welch's two-sample t-tests. n=6 per group.

**Table 3.3.** Fecal SCFA analysis

| SCFA Name | Average Concentration (ng/mg feces) | p-value |
|---|---|---|
| Acetic Acid | | |
| Vehicle | 1344.7 | 0.94 |
| PCB 126 | 1313.3 | |
| Butyric Acid | | |
| Vehicle | 68.4 | 0.92 |
| PCB 126 | 72.5 | |
| Formic Acid | | |
| Vehicle | **469.7** | **0.002** |
| PCB 126 | **261.1** | |
| Isovaleric Acid | | |
| Vehicle | 32.5 | 0.77 |
| PCB 126 | 36.3 | |
| Propionic Acid | | |
| Vehicle | 311.9 | 1 |
| PCB 126 | 312.5 | |

Bold text denotes statistically significant differences between groups as determined by student's t-test. n=10 per group.

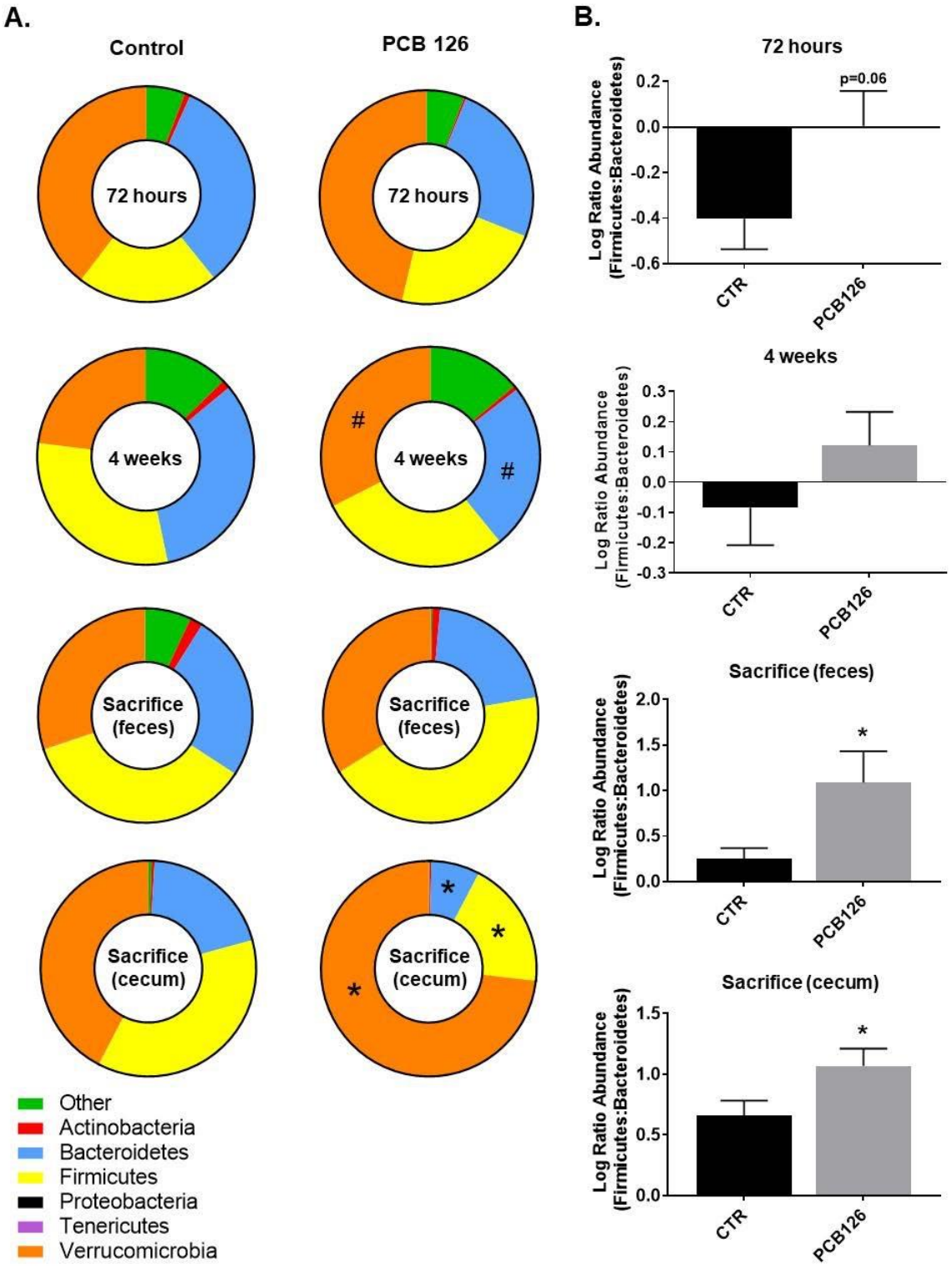
A.
Control
PCB 126
72 hours
72 hours
4 weeks
4 weeks
#
#
Sacrifice (feces)
Sacrifice (feces)
Sacrifice (cecum)
Sacrifice (cecum)
*
*
*
Other
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Tenericutes
Verrucomicrobia
B.
72 hours
p=0.06
Log Ratio Abundance (Firmicutes:Bacteroidetes)
0.2
0.0
-0.2
-0.4
-0.6
CTR
PCB126
4 weeks
Log Ratio Abundance (Firmicutes:Bacteroidetes)
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
CTR
PCB126
Sacrifice (feces)
Log Ratio Abundance (Firmicutes:Bacteroidetes)
2.0
1.5
1.0
0.5
0.0
*
CTR
PCB126
Sacrifice (cecum)
Log Ratio Abundance (Firmicutes:Bacteroidetes)
1.5
1.0
0.5
0.0
*
CTR
PCB126

**Figure 3.1. Exposure to PCB 126 drives phyla level alterations in bacterial populations over time.** Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet for 14 weeks and exposed to PCB 126 (1µmol/kg) at weeks 2 and 4. Fecal samples were collected at the start of the study, 72 hours, 4 weeks, and at sacrifice (12 weeks) post first PCB 126 exposure. Cecum contents were also collected at the conclusion of the study. 16S rRNA sequencing was conducted and data was analyzed using QIIME. **A.** Changes in the taxonomic composition of the gut microbiota over time at the phylum level. Significant differences were observed in the cecum contents at sacrifice. Cecum contents displayed a significant increase in Verrucomicrobia and decreases in Firmicutes and Bacteroidetes. Data are presented as relative abundances. **B.** Firmicutes / Bacteroidetes ratio changes over time. PCB 126 increased the ratio of Firmicutes / Bacteroidetes in both the feces and cecum contents at sacrifice. Data are presented as mean $\pm$ S.E.M (n=10 per group; Student's t-test). Statistical significance is denoted by * ($p<0.05$). # represents ($p<0.1$).

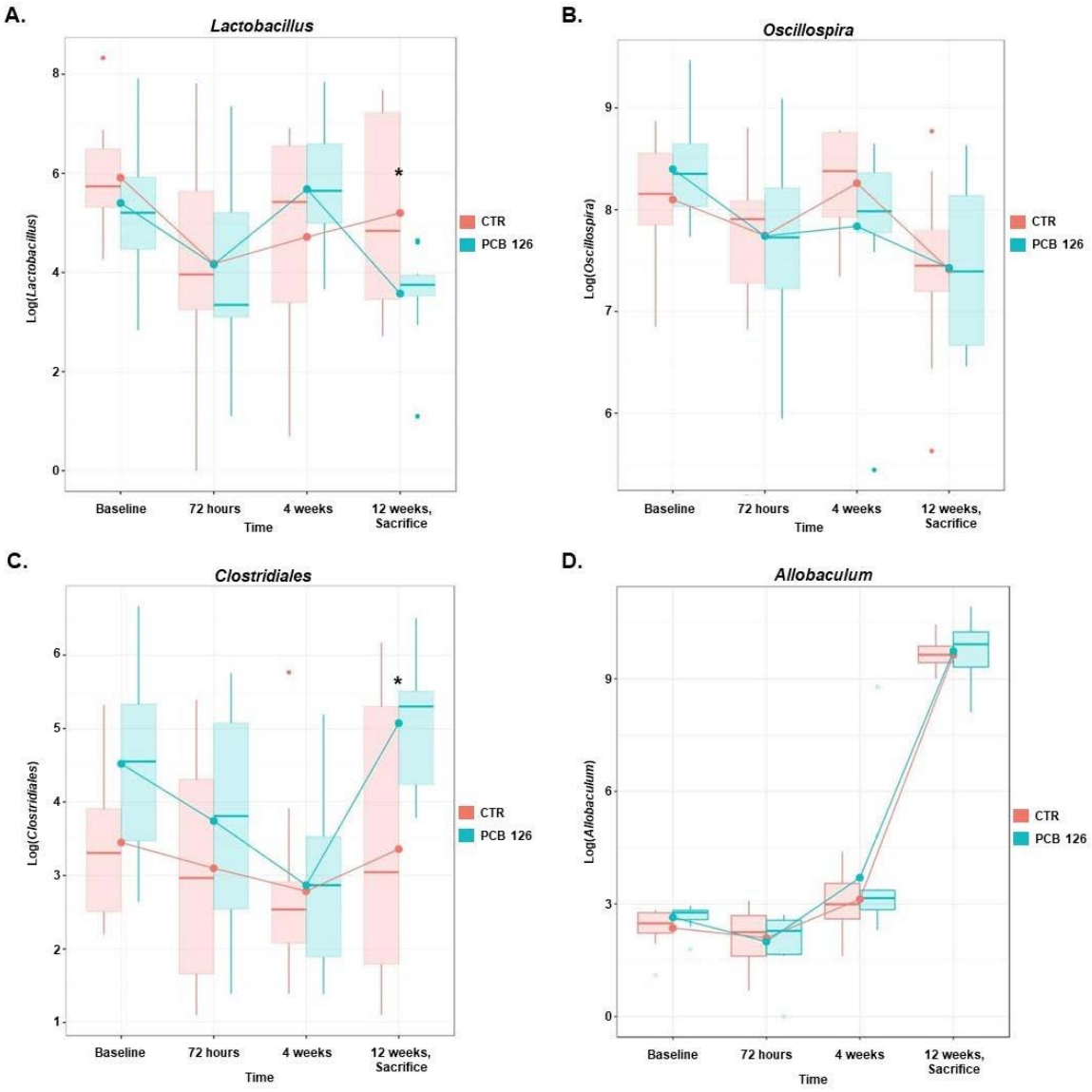


**Figure 3.2. PCB 126 modulates gut microbiota populations at the genra level over time**. Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet for 14 weeks and exposed to PCB 126 (1µmol/kg) at weeks 2 and 4. Fecal samples were collected at the start of the study, 72 hours, 4 weeks, and at sacrifice (12 weeks) post first PCB 126 exposure. A representative example of each of the 4 observed trends is depicted. For all generas, please see Table 3-1. A linear mixed-effects model

was utilized with Tukey's pair-wise multiple comparison procedure to assess the significance of change in bacterial genera at each time point. Statistical significance is denoted by * ($p<0.05$). (n=10 per group; Student's t-test).

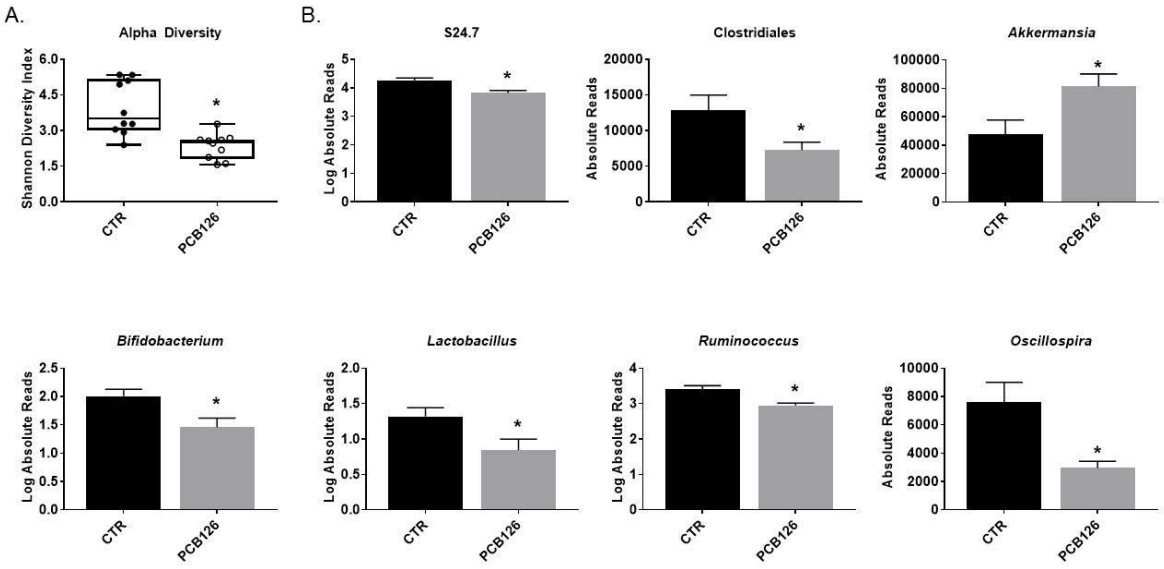


**Figure 3.3. Exposure to PCB 126 alters cecal bacterial genera and alpha diversity.** Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet for 14 weeks and exposed to PCB 126 (1µmol/kg) at weeks 2 and 4. Cecum contents were collected at the conclusion of the study. **(A)** Alpha diversity of cecum contents quantified using the Shannon Diversity Index. PCB 126 exposure decreased alpha diversity in cecum contents. **(B)** Order and genera level changes in cecal gut microbiota. PCB 126 decreased abundance of S24.7, Clostridiales, *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, and *Oscillospira* and increased *Akkermansia* abundance. Data are presented as mean ± S.E.M (n=10 per group; Student's t-test). Statistical significance is denoted by * ($p<0.05$).

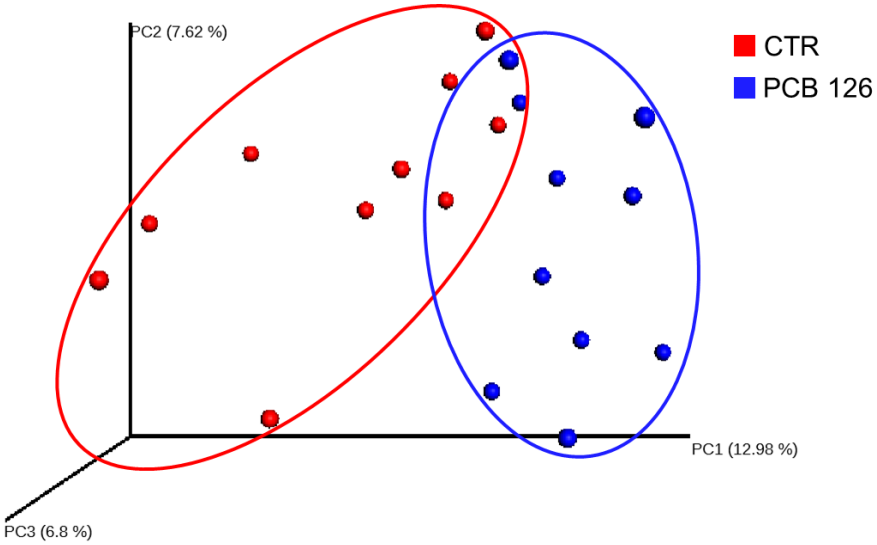


**Figure 3.4. Principal coordinates analysis of unweighted UniFrac distances the microbial communities in cecum contents.** PCB 126- exposed mice exhibited a significant difference in cecum microbial community structures compared to vehicle treated mice (R=0.2924, p=0.01). Analysis of similarity (ANOSIM) was used to identify microbial community differences.

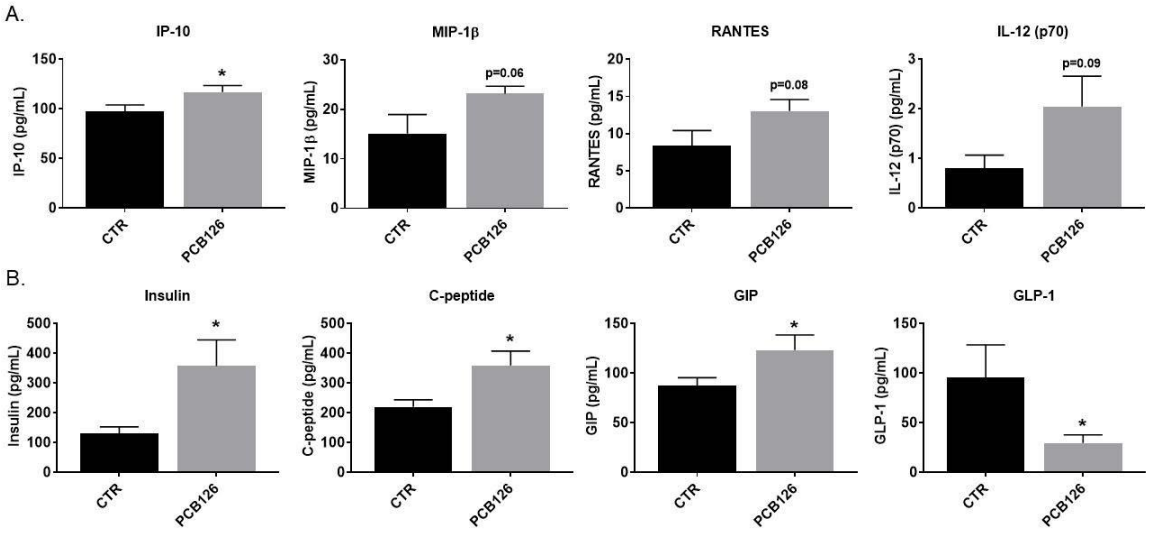


**Figure 3.5. Analysis of circulating cytokines and biomarkers of energy metabolism revealed that PCB 126 increases inflammation and alters insulin and related markers.** Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet for 14 weeks and exposed to PCB 126 (1μmol/kg) at weeks 2 and 4. Fecal samples were collected at the start of the study, 72 hours, 4 weeks, and at sacrifice (12 weeks) post first PCB 126 exposure. **(A)** Quantification of circulating cytokines (MAGPIX technology). PCB 126 increased Interferon gamma-induced protein (IP-10) plasma levels. **(B)** Quantification of circulating markers of energy metabolism (MAGPIX technology). PCB 126 increased plasma levels of insulin, c-peptide, and glucose-dependent insulinotropic peptide (GIP). PCB 126 reduced plasma levels of glucagon-like peptide 1 (GLP-1) Data are presented as mean ± S.E.M (n=9-10 per group; Student's t-test). Statistical significance is denoted by * ($p<0.05$).

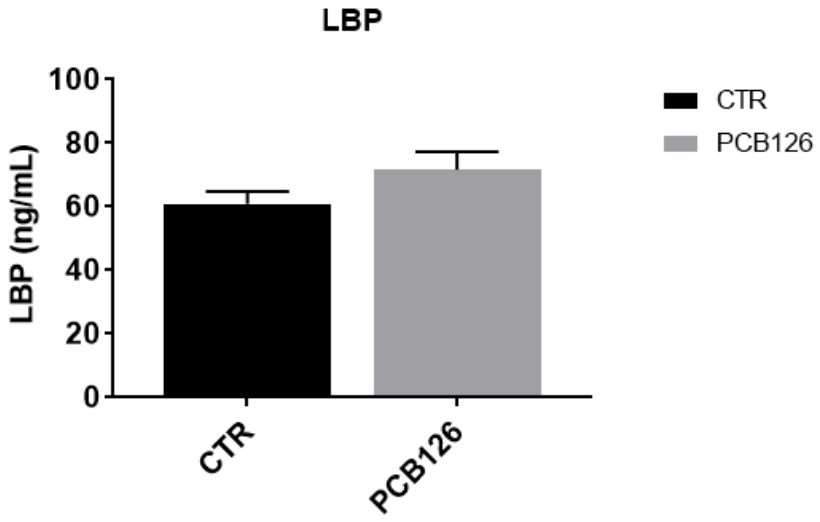


**Figure 3.6**. **Plasma concentrations of lipopolysaccharide binding protein (LBP) as determined by ELISA.** PCB exposed mice exhibited a trend towards increased circulating levels of LBP at the conclusion of the study ($p=0.142$; $n=8$). For group comparison, an unpaired t-test was used and significance was set at $p<0.05$.

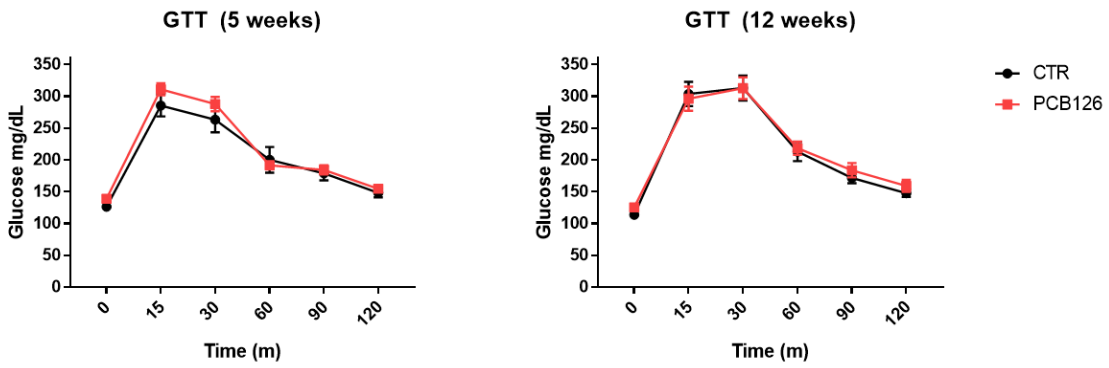


**Figure 3.7. No differences in glucose tolerance was observed between vehicle and PCB 126-exposed mice at 5 or 12 weeks post exposure.** Mice were fasted for 6h (7am-1pm)., and fasting blood glucose levels were measured with a hand-held glucometer (Accu-check Avivia, Roche, Basel, Switzerland) using 1-2uL of blood collected through the tail vein. Glucose was given via IP injection (2mg/g body weight, sterile saline) and blood glucose levels were measured at 15, 30, 60, 90, and 120 minutes post injection. For group comparison, an unpaired t-test was used for each timepoint and significance was set at $p<0.05$

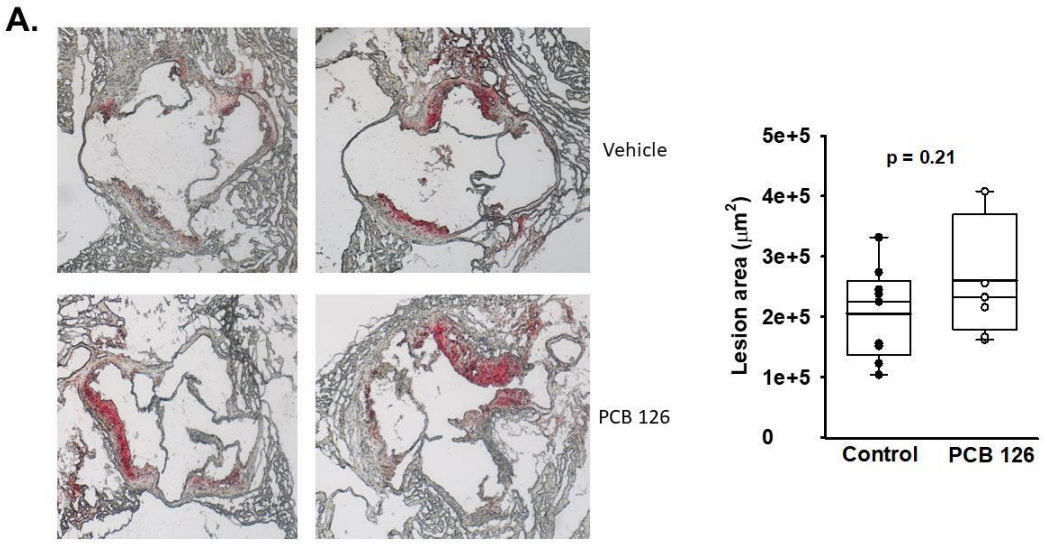


**Figure 3.8. PCB 126 exerts a modest increase in atherosclerosis development.** Male *Ldlr* -/- mice were fed a low fat, 0.15% cholesterol diet for 14 weeks and administered 1 μmol/kg PCB 126 at weeks 2 and 4. Aortic roots were frozen and serially sectioned from the emergence of the 3 valves. On average, the mean of 6 sections per mouse was utilized for quantitation. **A.** Shown are Oil Red O stained aortic root sections of mice exposed to vehicle control or PCB 126 with associated lesion area quantification (n=9 control, n=8 PCB; p=0.21; Student's t-test).

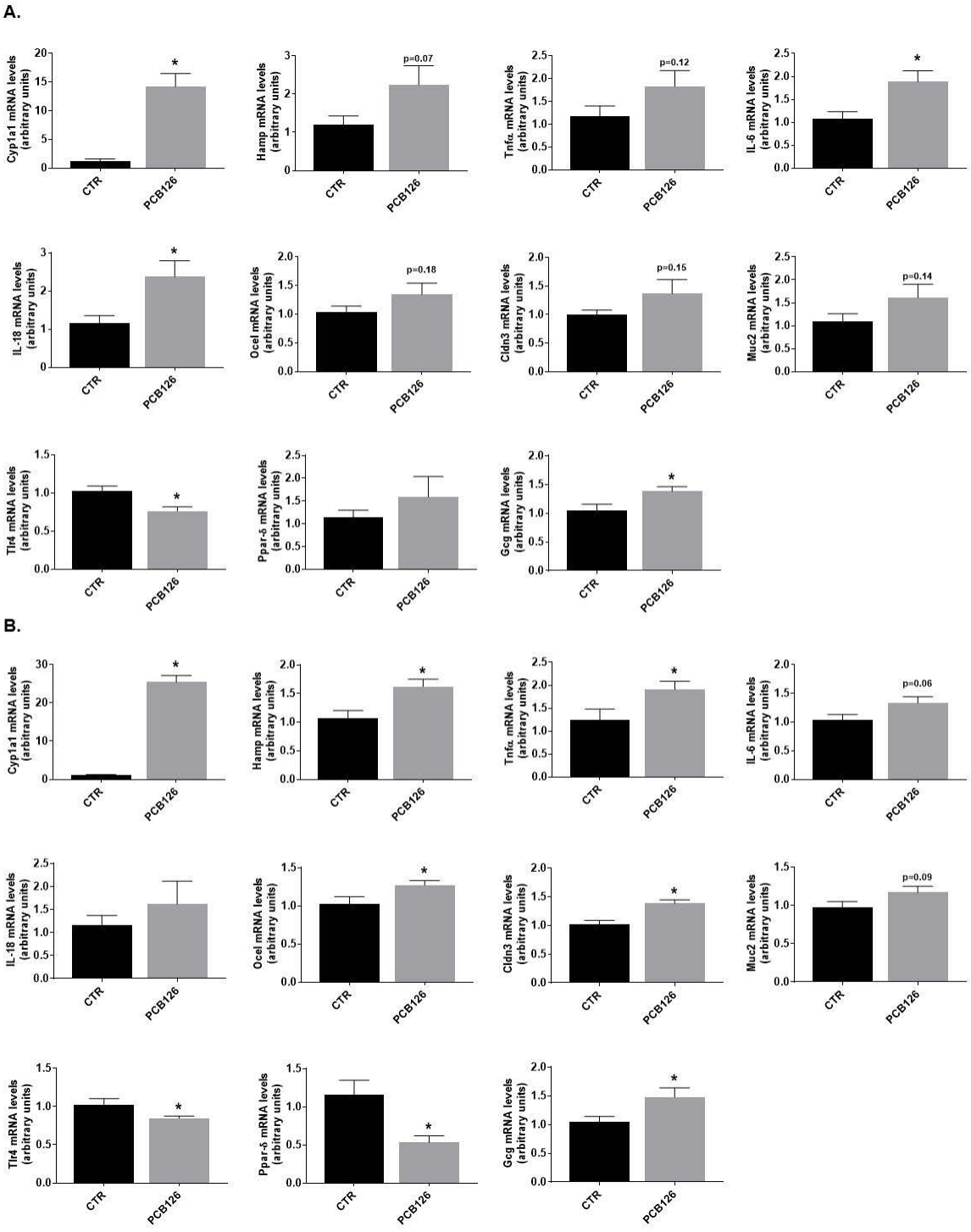


**Figure 3.9**. **PCB 126 exposure increases intestinal inflammation and alters markers of gut health.** Male Ldlr-/- mice were fed an atherogenic diet for 14

weeks and exposed to PCB 126 (1μmol/kg) at weeks 2 and 4. Intestinal samples were collected at the conclusion of the study. mRNA units were determined using the relative quantification method (ΔΔCT), normalized to control values. β-actin was used as the housekeeping gene for all jejunum and 18S was used for all colon gene expression quantifications. (A) Gene expression in the jejunum. PCB 126 increased expression of cytochrome p450 A1 (Cyp1a1), and markers of inflammation such as interleukin 18 (Il-18), (B) Gene expression in the colon. PCB 126 increased gene expression of Cyp1a1 and markers of inflammation such as hepcidin (Hamp), tumor necrosis factor (Tnfα), Interleukin 6 (Il-6). PCB 126 increased tight junction protein gene expression including occludin (Ocel) and claudin (Cldn3). Data are shown as mean + S.E.M. (n=10 per group; Student's t-test). Statistical significance is denoted by * ($p<0.05$).

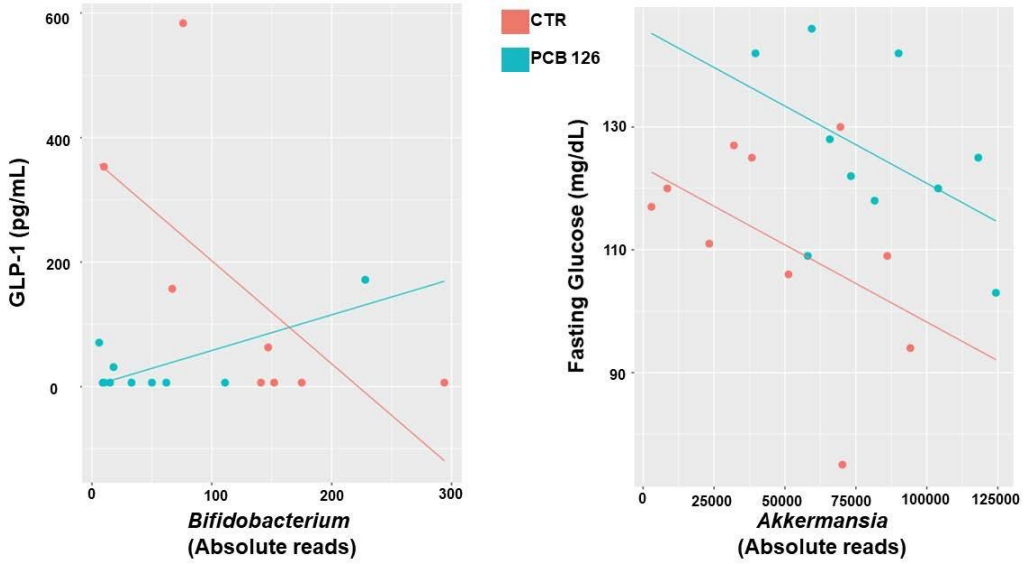


**Figure 3.10. Exposure to PCB 126 drives phyla level alterations in bacterial populations over time.** Male Ldlr-/- mice were fed an atherogenic diet for 14 weeks and exposed to PCB 126 (1μmol/kg) at weeks 2 and 4. Liver samples were collected at the conclusion of the study. Correlations between gut microbes and plasma markers were determined by linear regression modeling. GLP-1 levels were toxicant dependently associated with Bifidobacterium abundance (unadjusted p=0.0126). Fasting blood glucose levels were inversely associated with Akkermansia abundance, independent of toxicant exposure (unadjusted p=0.0191). Intersection of lines denote significant interaction.

# Chapter 4 Characterization of the protective effects of prebiotic consumption against PCB 126 toxicity

## 4.1 Synopsis

In chapter three it was demonstrated that exposure to PCB 126 increased inflammation and disrupted gut microbiota and metabolic homeostasis. It is well accepted that lifestyle modification (i.e. dietary changes and exercise) can contribute to benefits in host health as well as protection from pollutant toxicity. Due to our previously observed involvement and disruption of the gastrointestinal tract and gut microbiota during PCB exposure, employing nutritional interventions that have strong effects on these could be beneficial in attenuating PCB-health detriments. Inulin is a well-studied prebiotic dietary fiber that has been demonstrated to attenuate gut dysbiosis, improve glucose and lipid metabolism, and reduce cardiometabolic disease risk, making it a suitable nutritional intervention to combat the PCB-effects discussed in chapter three. Therefore, our objective in the present study was to elucidate the role of prebiotic nutritional intervention (i.e. inulin) on attenuating PCB-induced disruption of cardiometabolic health and gut microbiota populations. Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1μmol/kg) or vehicle at weeks 2 and 4. PCB exposure induced wasting and impaired glucose tolerance, which was attenuated by inulin consumption. Hepatic lipid accumulation, specifically microvesicularly, was observed with PCB exposure and lessened with inulin consumption. To examine potential underlying mechanisms contributing to these observed phenotypes, examination of microbial populations, metabolic regulatory parameters, and inflammatory

markers was conducted. 16S rRNA sequencing of gut microbial populations revealed an overall diet effect of lowering the Firmicutes: Bacteroidetes ratio, a common observation observed in metabolically healthy individuals [80]. Additionally, diet and exposure induced specific alterations in microbial populations at the genera level and are described within Table 4.2. It was also observed that PCB-induced disruption in glycolytic and gluconeogenic enzyme expression, which was improved by inulin feeding and may play a role in the improvements in glucose tolerance we observed in inulin fed mice. Finally, PCB exposure increased the hepatic levels of ceramides, a biomarker of cardiometabolic disease, and that inulin consumption reduced these levels, suggesting a protective role of inulin against cardiometabolic disease. Our current data of this ongoing study demonstrate that dietary intervention (i.e. inulin) is an effective means at attenuating PCB-induced metabolic disruption, inflammation, and gut microbial modulations, potentially reducing the risk of cardiometabolic disease.

## 4.2 Introduction

The burden of environmental pollutant exposure continues to increase worldwide, despite reductive efforts [2-4]. Exposure to dioxin-like pollutants poses numerous health risks for an individual including increased risk of cardiometabolic diseases such as atherosclerosis and diabetes [2, 3]. Due to the lipophilic nature of dioxin-like pollutants, humans are primarily exposed through consumption of contaminated of fatty foods such as fatty fish, beef, and dairy products [2, 38]. Importantly, this route of exposure allows for the

gastrointestinal tract to receive the highest levels of dioxin-like pollutants and therefore makes it a critical aspect to study. The gut and gut microbiota play an important role in overall host health and disruptions in these systems have been implicated in the development of several diseases including cardiometabolic diseases [80]. Our lab has previously demonstrated that exposure to the dioxin-like pollutant PCB 126 increases intestinal inflammation, disrupts gut microbiota, and alters host metabolism, and can accelerate development of cardiometabolic disease [143, 181]. Furthermore, the data discussed in chapter two demonstrated that PCBs can exert toxic effects on specific bacterial populations through membrane disruption, leading to disruption in microbial metabolite production that may negatively affect the host. Because of these findings demonstrating the toxicity of PCBs on the gut microbiota and gut health, utilization of dietary interventions by which to attenuate or reverse these negative effects may be a sensible means of reducing disease risks associated with PCB exposure.

Prebiotics are defined as “a selectively fermented ingredient that allows specific changes both in the consumption and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” [124]. The most widely understood and accepted prebiotics include certain types of dietary fiber [125]. Dietary recommendations for dietary fiber intakes are at least 28g and 35g for females and males, respectively. However, it has been documented that less than 3% of Americans are meeting these recommendations, highlighting an important avenue for improvement and intervention [125]. Not all dietary

fibers are prebiotic and modulate the microbiota. Fibers that have been documented to have functional prebiotic properties include fructooligosaccharides, galactooligosaccharides, some disaccharides, some nonstarch polysaccharides, and inulin [126]. The prebiotic inulin is an inulin type fructan that contains linear chains of fructosyl groups linked by β(2-1) glycosydic bonds, terminated with an α-D(1-2)-glucopyranoside ring group on the reducing end [128]. Inulin is found in vegetables like garlic, onions, and asparagus, but one of the most common forms of inulin commercially available is derived from chicory root [128] . Inulin is a potent prebiotic that has been demonstrated to have stimulatory effects on certain bacterial populations that have been associated with good health, including *Bifidobacterium* spp, and *Lactobacillus* spp [134]. Furthermore, inulin administration was shown to reduce deleterious bacteria, such as the sulfate-reducing genus *Bilophila* [134, 135]. Aside from modulation of the gut microbiota, consumption of chicory-derived inulin has been demonstrated to reduce adiposity, improve glucose sensitivity [132, 133]. Importantly, inulin consumption can reduce hepatic lipogenesis and plasma triglycerides as well as reduce atherosclerotic lesion formation, and thus may be protective against PCB-induced hepatic steatosis and acceleration of atherosclerosis [216, 217]. Thus, the objective of this study was to elucidate the role of prebiotic nutritional intervention (i.e. inulin) on attenuating PCB-induced disruption of cardiometabolic health and gut microbiota populations.

## 4.3 Materials and Methods

### 4.3.1 Animals, diets, and study design

Seven-week-old male *Ldlr -/-* mice were purchased from Jackson Laboratories and maintained on a 12-h light/ 12 h dark cycle at a temperature of 22$^{o}$C with 50% humidity. All mice received food and water ad libitum and measures of food intake and body weight were recorded weekly. The base diet and dosing schedule for this study have been previously shown to be an appropriate model of pollutant-accelerated cardiometabolic disease. Following one week of acclimation, mice were randomly divided into 4 groups (n=10). Mice were fed either a control high cholesterol diet containing 8% cellulose as the fiber source (2 groups; n=10 each) or a prebiotic containing high cholesterol diet with 8% of inulin as the fiber source (2 groups; n=10 each). The level of fiber in this study was chosen to represent a “high” fiber intake based on human dietary recommendations. The recommended intake of fiber is 14g per 1000 calories [218]. The traditional level of fiber in purified rodent diets is 5%, equating to 12g per 1000 calories. The 8% level of fiber equates to 21g per 1000 calories, which can be classified as a “high” fiber diet. Detailed diet compositions can be found in **Table 4.1**. At weeks 2 and 4, mice received either 1 µmol/kg of PCB 126 (AccuStandard, CT, USA) or safflower oil vehicle (Dyets, Bethlehem, PA, USA) via oral gavage. At the end of the study mice were fasted for 16 h, anesthetized and blood was collected via retro-orbital bleed. Liver, intestinal samples, and cecum samples were collected, snap frozen in liquid nitrogen, and stored at -80

°C until analysis. All experimental procedures were approved by the Institutional Animal Care at the University of Kentucky.

### 4.3.2 Glucose tolerance testing and body composition analysis

Intraperitoneal GTTs were conducted on weeks 5 and 8. Mice were fasted for 6 h and given an IP injection of glucose (i.e. 20% solution at 2 mg/g BW, sterile saline). Blood from the tail vein was collected at baseline, 15, 30, 60, 90, and 120 minutes post injection and blood glucose levels were quantified using a hand-held glucometer (Accu-check Avivia, Roche, Basel, Switzerland). Lean body mass and fat mass were analyzed at week 10 using EchoMRI (EchoMRI LLC, Houston, TX, USA).

### 4.3.3 DNA Extraction and 16S rRNA Sequencing

DNA extraction and 16S rRNA sequencing were conducted by the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory as previously discussed. Analysis was conducted using the program Quantitative Insights into Microbial Ecology (QIIME version 2.0) utilizing parameters discussed in Petriello et. al. 2018 [143].

### 4.3.4 Hepatic histology

Sections of liver were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin for histological examinations. Hepatic tissues sections were stained with hematoxylin-eosin (H&E) and examined by a pathologist via

light microscopy and visually scored based on macrovesicular fat percentages. Microvesicular fat accumulation was noted as “present” or “not present”. Photomicrographic images were captured at 20x magnification using a high-resolution digital scanner.

### 4.3.5 RNA Extraction and qPCR

Jejunum, colon, and liver samples were homogenized in TRIzol (Invitrogen, Carlsbad, CA) and mRNA was extracted according to manufacturer’s instructions. Quality and concentrations were quantitated using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). Complimentary DNA was generated utilizing qScript cDNA SuperMix (Quantabio, Beverly, MA) according to manufacturer’s protocols. Gene expression was determined via qPCR utilizing Taqman fast reagents (Thermo Scientific, Waltham, MA) in a CFX90 Real-Time PCR system (Bio-Rad, Hercules, CA). B-actin was used as the housekeeping gene for intestinal samples and 18S was used as the housekeeping gene for liver samples. The ΔΔCt relative quantification method was used to calculate fold differences in gene expression.

### 4.3.6 Analyses of circulating proteins related to metabolic function

Plasma metabolic hormones were quantified using the Milliplex Map Mouse Metabolic Hormone Magnetic Bead Panel- Metabolism Multiplex Assay (Millipore Corp, Billerica, MA, USA) following manufacturer’s protocols. Assays were measured on the Luminex Xmap MAGPIX system (Luminex Corp, Austin, TX,

USA) following manufactures instructions. For statistical analyses, values below the standard curve were represented as zero.

### 4.3.7 Quantification of hepatic metabolites related to cardiometabolic disease and metabolic function

To quantify levels of specific lipids, plasma was spiked with deuterium labeled internal standard mixture and extracted using methyl tert-butyl ether. The samples were analyzed using an Ultimate 3000 ultra high performance liquid chromatography system coupled to a Thermo Q-Exactive Orbitrap mass spectrometer equipped with a heated electrospray ion source (Thermo Scientific, CA, USA). Lipid extracts were separated on a Waters ACQUITY BEH C8 column (2.1 × 100 mm, 1.7 μm) with the temperature maintained at 40 °C. The flow rate was 250 μL/min, and the mobile phases were consisted of 60:40 water/acetonitrile (A), and 90:10 isopropanol/acetonitrile (B), both containing 10 mM ammonium formate and 0.1% formic acid. The samples were eluted with a linear gradient from 32 % B to 97 % B over 25 min, maintained at 97 % B for 4 min and re-equilibration with 32 % B for 6 mim.

### 4.3.8 Statistical analyses

Data were analyzed using GraphPad PRISM and are presented as mean $\pm$ SEM. Comparisons between groups were made by two-way ANOVA with post-hoc comparisons of the means. Statistical significance was set at a determined p value of $p<0.05$.

## 4.4 Results

### 4.4.1 Inulin consumption and PCB exposure exert differential effects on body composition

When examining body composition, it was observed that inulin consumption resulted in an overall lower weight gain irrespective of exposure **(Figure 4.1a)**. Furthermore, cellulose fed mice exposed to PCBs had significantly greater weight gain than exposed inulin fed mice. EchoMRI measurements of body composition revealed that PCB exposure resulted in reductions in percent body fat in cellulose fed mice, but not in inulin fed mice **(Figure 4.1b)**. Importantly, there were no differences in food intake between all of the groups **(Figure 4.1c)**.

### 4.4.2 Inulin protects against PCB 126 disruption of glucose tolerance.

To assess the effects of pollutant exposure and inulin consumption on glucose tolerance, an intraperitoneal glucose tolerance test (IGTT) was conducted at weeks 5 and weeks 8. At week 5, PCB-exposure increased fasting blood glucose levels in cellulose fed mice **(Figure 4.2a)**. Such an increase was not observed in inulin-fed mice. Furthermore, cellulose fed mice exposed to PCBs displayed a greater area under the curve (AUC) compared in exposed inulin fed mice. At week 8, no significant differences in fasting blood glucose were observed, but the effects of PCB exposure and inulin feeding on AUC observed at week 5 remained significant **(Figure 4.2b)**. Importantly, no differences in circulating insulin levels at the end of the study were observed (data not shown).

### 4.4.3 Inulin reduces PCB-induced hepatic steatosis

When examining liver composition, we observed that PCB exposure significantly increased liver weight in cellulose fed mice, which was attenuated with inulin consumption **(Figure 4.3a)**. Overall, inulin-fed mice exhibited lower liver weights compared to cellulose fed mice. A significant overall effect of PCB exposure on increasing liver weights was apparent. Interestingly, PCB exposure significantly reduced hepatic macrovesicular fat accumulation in cellulose fed mice, however increases in microvesicular fat accumulation was evident in this same group **(Figure 4.3b)**. Overall, inulin fed mice exhibited lower macrovesicular fat accumulation and an observation of lower microvesicular fat accumulation in exposed inulin fed mice compared to exposed cellulose fed mice was noted (Figure 4.3b).

### 4.4.4 Inulin and PCB 126 exert differential effects on gut microbial populations

Upon examination of gut microbial populations via 16S rRNA sequencing, we observed several effects and interactions between pollutant exposure and inulin consumption. At the phylum level, mice fed inulin had lower levels of Verrucomicrobia than cellulose fed mice, irrespective of exposure **(Figure 4.4a).** Furthermore, a significant increase in Actinobacteria in in all mice fed inulin compared mice fed cellulose was observed and a significant increase in Bacteroidetes was apparent in vehicle treated inulin fed mice compared to vehicle treated cellulose fed mice (Figure 4.4a). There were no significant effects of pollutant exposure on the Firmicutes to Bacteroidetes ratio, however a

significant diet effect was observed in which inulin fed mice had a lower ratio than cellulose fed mice **(Figure 4.4b)**. Alpha diversity, as calculated using the Shannon Diversity Index, revealed a significant exposure effect with PCB exposed mice having a reduced diversity compared to non-exposed mice **(Figure 4.4c)**.

At the genera level, a significant increase in *Coprococcus* in cellulose fed mice exposed to PCB 126 was found, which was attenuated by inulin feeding **(Figure 4.5)**. PCB exposed mice fed cellulose also exhibited a trend towards reductions in *Allobaculum* which was significantly increased by inulin feeding (Figure 4.5). Furthermore, inulin exposure significantly increased the abundance of *Bifidobacterium* and *Lactobacillus* and reduced the abundance of *Ruminococcus*. Inulin fed mice also exhibited lower levels of *Akkermansia*, irrespective of pollutant exposure (Figure 4.5).

### 4.4.5 Inulin attenuates PCB-induced hepatic inflammation and alters markers of xenobiotic metabolism

When examining hepatic gene expression of markers of inflammation, it was observed that PCB exposure increased expression of tumor necrosis factor alpha (*Tnfα*) and lipopolysaccharide binding protein (*Lbp*), which was attenuated by inulin consumption **(Figure 4.6a)**. Furthermore, exposed inulin fed mice also exhibited reduced levels of toll like receptor 4 compared to exposed cellulose fed mice. Furthermore, expression of hepcidin (*Hamp*) was lower in inulin fed mice, irrespective of exposure (Figure 4.6a). Quantification of markers of pollutant

exposure and detoxification revealed that PCB exposure induced expression of cytochrome p450 (*Cyp1a1*) in both diets, however mice fed inulin exhibited significantly lower levels compared to cellulose fed mice **(Figure 4.6b)**. Furthermore, exposed inulin fed mice exhibited lower levels of multidrug resistance protein (*Mrp2*) compared to exposed cellulose-fed mice (Figure 4.6b).

### 4.4.6 Inulin reduces metabolites associated with cardiometabolic disease

To examine the effect of both PCB exposure and inulin feeding on cardiometabolic disease risk, hepatic metabolites associated with cardiometabolic disease were quantified. Using lipidomic analyses, it was seen that in cellulose fed mice, PCB exposure increased hepatic levels of ceramide species including 18:1/20:0 and 18:1/24:1 as well as total ceramides **(Figure 4.7a)**. In exposed mice fed inulin, a significant reduction in ceramide species 18:1/20:0 was observed, with a trend towards reduction in species 18:1/24:1 as well as total ceramides. Importantly, quantification of hepatic expression of microsomal triglyceride transfer protein (*Mttp*), responsible for ceramide transport, was decreased in PCB exposed mice fed cellulose and was rescued with inulin feeding **(Figure 4.7b)** .

### 4.4.7 Inulin and PCBs differentially alter hepatic markers of glucose metabolism and circulating metabolic hormones.

To elucidate the effects of PCB exposure and inulin consumption on glucose metabolism, hepatic expression of key glycolytic and/or gluconeogenic enzymes

was examined **(Figure 4.8)**. PCB exposure significantly reduced expression of pyruvate kinase (*Pklr*) in cellulose fed mice, while the reduction in exposed inulin fed mice was not significant. Additionally, an overall reductive effect of PCB exposure on glucokinase (*Gck*) and phosphoenolpyruvate carboxykinase (*Pck1*) expression was observed, regardless of diet. However, an overall effect of inulin consumption on increasing *Pck1* expression was observed. Furthermore, glucose 6-phosphatase (*G6pc*) expression was significantly increased in inulin fed mice, regardless of exposure.

Magpix technology was utilized to quantify circulating metabolic hormone levels at the end of the study **(Figure 4.9)**. In cellulose-fed mice, PCB exposure significantly reduced circulating leptin levels while no differences in leptin levels were observed between the inulin-fed groups. Additionally, a significant interaction of diet and exposure on leptin levels was observed. Furthermore, inulin consumption had an overall significant effect of increasing circulating PYY levels, while a PCB exposure had an overall reductive effect (Figure 4.9).

## 4.5 Discussion

Exposure to environmental pollutants, specifically dioxin-like pollutants, poses numerous health threats to those exposed, including metabolic disruptions, hepatic lipid accumulation, and gut dysbiosis. A common route of exposure to dioxin-like pollutants is through contaminated foods and thus the gastrointestinal system is exposed to the highest amounts of these pollutants. Utilizing a mouse model of cardiometabolic disease, our lab has previously demonstrated that exposure to PCB 126 induces gut dysbiosis, increases systemic and intestinal

inflammation, accelerates atherosclerosis, and also induces metabolic dysfunction [143, 181]. It is well understood that nutritional modulation of pollutant toxicity effective means of reducing detrimental health effects of pollutant exposure [61]. Therefore, in this study we sought to examine the role of prebiotic nutritional intervention (i.e. inulin) on attenuating our previous observations of PCB-induced disruption of gut microbiota and cardiometabolic health. While analyses remain ongoing, the data currently demonstrate that inulin feeding attenuated PCB-induced disruption of gut microbiota, host metabolism, hepatic steatosis, and inflammation.

Dioxin-like pollutants are known for exerting various effects throughout the body; one such effect being induction of significant loss of body weight, known as wasting [219]. PCB exposure resulted in a significant reduction in body fat percentage, while inulin-fed mice were protected from this effect. Interestingly, throughout the study, inulin fed mice gained less weight than cellulose fed mice, highlighting the potential adipose specific wasting induced by toxicant exposure only observed in cellulose fed mice. Our lab has previously observed this wasting effect upon exposure to PCB 126 in a mouse model of non-alcoholic steatohepatitis (NASH) [42], indicating that the liver injury may play a role in this wasting effect. Other studies have noted this effect of dioxin-like pollutants on wasting but attributed it to reductions in food intake, which was observed in the present study [220, 221]. Our observations of wasting in conjunction with no changes in food intake are noteworthy, suggesting disruptions in energy metabolism and storage. It is important to note that no observations of wasting

were noted in our previous study (chapter three) using this model of cardiometabolic disease, which may highlight a potential unique interplay between the slightly elevated cellulose diet (i.e. 8% vs 5%) that altered the toxicity and effects of PCB exposure. The observation of this effect is something that calls for further examination in future studies.

When examining systemic host health, the liver is of utmost importance. The liver is a vital organ for responsible for metabolism of both dietary constituents and pollutants and is the second organ, following the gut, that comes in contact with polychlorinated biphenyls. The liver has a unique and important relationship with most organ systems of the body and recent research has substantiated evidence for the interdependent relationship of the gut and liver, termed the “gut-liver axis” as well as the liver and the heart, termed the “liver-heart axis” [222, 223]. In individuals with gut dysbiosis and/or cardiovascular disease, it is not uncommon to observe liver pathologies such as nonalcoholic steatohepatitis (NASH) or nonalcoholic fatty liver disease (NAFLD) [222, 223]. Importantly, it is well accepted that exposure to persistent organic pollutants contributes to the pathogenesis of NASH and NAFLD and thus has been recognized as its own entity, “toxicant induced steatohepatitis” (TASH) [24]. Dioxin-like pollutants have been demonstrated to not only increase hepatic lipid accumulation, the beginning stages of TASH, but also to exacerbate non-alcoholic fatty liver disease, leading to more advanced hepatic pathologies such as fibrosis [24, 42, 65]. Our lab has previously studied this effect of pollutant exposure utilizing a mouse model in which mice are fed a methionine-choline deficient (MCD) diet to induce NAFLD.

In aforementioned study, exposure to PCB 126 drastically exacerbated liver and metabolic dysfunction induced by MCD feeding as evidenced by wasting, increased systemic inflammatory markers, and presence of hepatic fibrosis [42].

Hepatic macrovesicular fat accumulation is what is most commonly observed in obesity and NASH/NAFLD and is characterized by lipid droplets that accumulate extrahepatocellularly. Microvesicular lipid accumulation is less common and is characterized intrahepatocellular lipid accumulation, giving them a foamy appearance and has been correlated with more advanced histology of NAFLD [224]. Macrovesicular lipid accumulation was apparent in unexposed cellulose fed mice, consistent with our previous studies and the progression of cardiometabolic disease that is induced using the present mouse model. However, in exposed cellulose fed mice, a shift from macrovesicular to microvesicular lipid accumulation was observed, indicating a disruption in energy metabolism and/or lipid transport. Importantly inulin fed mice were protected from both macrovesicular and microvesicular hepatic lipid accumulation. This reduction in hepatic lipid accumulation with inulin feeding is consistent across numerous studies [225-228]. Importantly, in humans, it was observed that inulin and inulin-propionate ester supplementation attenuate hepatic steatosis in patients with NAFLD [225]. To our knowledge, the present study is the first to examine inulin supplementation on dioxin-like pollutant exposure, but inulin feeding has been studied in context of other xenobiotics. For example, it was demonstrated that inulin supplementation reduced hepatic steatosis and xenobiotic-induced (i.e. phenobarbital) liver injury in rats fed a high fat, high

sucrose diet [228]. One mechanism by which inulin may attenuate hepatic steatosis is hypothesized to be through the production of short chain fatty acids, specifically propionate [229]. It has been observed that in hepatic steatosis, propionate levels were significantly decreased in portal plasma [228, 230]. Importantly, propionate has been reported to inhibit fatty acid synthesis and thus may provide one contributing mechanism by which inulin can attenuate hepatic steatosis [231, 232]. However, it is important to note that the rate of utilization of SCFA often make it difficult to truly capture levels present in portal or systemic circulation and thus more mechanistic research is needed to truly understand the role of inulin in attenuating hepatic steatosis.

Due to the liver being the central hub of metabolic processes, it is well understood that hepatic inflammation and lipid accumulation can alter overall metabolism, specially glucose metabolism [233, 234]. In the present study, PCB exposure in cellulose fed mice resulted in glucose intolerance at both weeks 5 and 8 of the study, as evidenced by an increased area under the curve. Importantly, inulin feeding was able to attenuate these disruptions in glucose tolerance. PCB impairment in glucose tolerance has been documented by other labs [162, 235]. To better understand the mechanisms behind these improvements in glucose tolerance observed with inulin feeding, gene expression of hepatic enzymes involved in glycolysis and gluconeogenesis were quantified. Examining the rate limiting enzymes of glycolysis revealed that PCB exposure significantly reduced pyruvate kinase (*Pklr*) and phosphofructokinase (*Pfkl*) gene expression in mice fed cellulose, but not in mice fed inulin. There was also an

insignificant decrease in glucokinase (*Gck*) expression in cellulose fed mice, which was not present in inulin fed mice. To our knowledge, these PCB-induced alterations in hepatic glycolytic enzymes have not been reported previously and could play a role in an inability to properly metabolize glucose. We hypothesize that these alterations were attenuated in inulin fed mice due to our observed improvements hepatic lipid accumulation and inflammation, as these factors have been demonstrated to contribute to metabolic dysfunction [233, 234].

The gastrointestinal tract is a key organ is tightly linked to various organ systems including the liver, cardiovascular system, and neurological system [222, 223, 236]. The gut microbiota, defined as the trillions of bacteria residing within the gastrointestinal tract can be strongly influenced by dietary and environmental factors and may play a role in the detrimental health effects observed with PCB exposure. A common measure of gut dysbiosis, or the abnormal distribution of bacterial taxa, is alpha diversity. Alpha diversity calculates the richness and evenness of a microbial sample and thus offers a means by which to broadly compare the microbial composition of samples. Interestingly, there were no significant effects of inulin feeding on alpha diversity. This finding has indeed been observed in the literature previously [135]. Some reports indicate that inulin feeding actually reduces alpha diversity, possibly due to the stimulation of a few specific taxa that then predominate the microbiome [135, 237]. Diversity is a good measure of looking at differences, but is not necessarily an indicator of health, like some researchers like to report [238]. Therefore, diversity should be treated as a starting point for further inquiry rather than as an outcome measure

of “health”. A similar measure employed by researchers is to examine the Firmicutes/Bacteroidetes ratio as this is observed to be increased in chronic inflammatory diseases and metabolic syndrome. Contradictory to our previous study, we observed no significant effect of exposure on this ratio, again highlighting a discrepancy in the level of cellulose. However, mice fed inulin had a lower Firmicutes/Bacteroidetes ratio, irrespective of exposure. This may suggest a “healthier” microbial composition in our inulin mice. It is important to note that with these comparisons being reliant on phyla level differences, it can overgeneralize microbial composition and thus deeper taxonomic examination should always be employed.

When looking at more specific microbial populations, interesting effects of both diet and exposure were apparent. The levels of *Akkermansia*/Verrucomicrobia observed in our study are notably elevated in all groups, compared to a typical murine microbial signature [149]. This observation was previously noted by our lab and we hypothesize that the high levels of cholesterol in this model are influencing the growth of these bacteria [143]. *Akkermansia muciniphila* is commonly recognized as a beneficial bacterium, consuming intestinal mucin and enhancing barrier function [88]. However, researchers have noted that an overabundance of *A. muciniphila* can actually exacerbate intestinal inflammation in mice [198]. Our consistent finding of strikingly elevated levels of *Akkermansia*/Verrucomicrobia in this high cholesterol model is a very important finding that should be pursued further to better elucidate the specific mechanisms driving the large bloom of these bacteria.

The genus *Allobaculum* appears to be sensitive to dietary interventions. A significant diet effect of inulin increasing the level of this genus, irrespective of exposure was observed. It has been demonstrated that *Allobaculum* decreases under high fat feeding and that prebiotic (oligofructose) feeding is able to increase the abundance of this genera [239]. This increase with prebiotic treatment has been reproduced several times in the scientific literature [239-241]. Importantly, the genus *Allobaculum* has been associated with improved intestinal barrier function and also resistance to the development of NAFLD [242]. Additionally, increases in *Allobaculum* have also been observed with metformin treatment, a clinically effective drug used for the management of diabetes [243]. Therefore, our findings of increased *Allobaculum* abundance in all groups fed inulin may be associated with our findings of reduced hepatic steatosis/inflammation and improved glucose tolerance and metabolism.

Interestingly, PCB exposure increased the level of *Coprococcus* in mice fed cellulose, but not in mice fed inulin. Microbial literature characterizing the genus *Coprococcus* found an inability of the strains tested to ferment inulin, potentially explaining why we saw a very low level of this genus in all mice fed inulin [244]. The increases observed in exposed mice fed cellulose could be due to a toxic effect on other bacterial groups, allowing *Coprococcus* to proliferate. The genus *Coprococcus* is a characterized butyrate producer, which is commonly regarded as being beneficial for intestinal health [245]. However, in chapter two, PCB exposure in fecal cell suspensions did not impede butyrate production, indicating that butyrate producing bacteria may be unsusceptible to PCB toxicity.

With regards to the common probiotic bacteria *Lactobacillus* and *Bifidobacterium*, inulin feeding significantly increased levels of these bacteria, irrespective of exposure. However, exposure to PCB 126 in inulin fed mice further increased the levels of these bacteria. This may be due to a toxic effect of PCBs on other bacterial populations, allowing the proliferation of *Bifidobacterium* and *Lactobacillus*. Inulin feeding is well accepted to stimulate *Bifidobacterium* and *Lactobacillus*. For example, in humans, inulin supplementation has been demonstrated to increase *Bifidobacterium* and *Lactobacillus* following 14 days of supplementation [246]. Furthermore, in a randomized, double-blind placebo-controlled crossover study, increases in *Bifidobacterium* and *Lactobacillus* were observed in healthy adults administered long-chain inulin for 14 days [247]. Additionally, studies found that these genera were not only increased following prebiotic supplementation, but that this increase was associated with improvements in metabolic health and glucose metabolism [203, 204]. *Bifidobacterium* and *Lactobacillus* exhibit very little proteolytic activity and are predominantly saccrolytic, which some argue, is beneficial [127]. Along these lines, in inulin fed mice reductions in circulating p-cresol were observed (data not shown), product of microbial proteolytic fermentation. This suggests a reduced capacity for proteolytic fermentation in inulin-fed mice, which may confer a health benefit to the host [127]. Overall, inulin exhibited pronounced effects on the microbiota which may contribute to our observations of improved systemic host health and protection against pollutant toxicity.

The intestinal environment, in addition to harboring important microbial populations, functions in hormonal regulation of appetite and metabolism [248]. Quantification of circulating metabolic hormone levels revealed differential effects of diet and exposure. Peptide YY (PYY) is an enteroendocrine peptide that is secreted by intestinal L cells and aids in the regulation of body weight [249]. PYY is known as an anorexigenic hormone, and signals to reduce appetite. Interestingly, PYY levels were overall higher in inulin fed mice compared to cellulose fed mice and exposure exhibited an overall reductive effect on PYY. It is well accepted that dietary fiber consumption increases PYY secretion and induces satiety [249]. Furthermore, it has been observed that short chain fatty acid (SCFA) production from microbial fermentation of fiber can contribute to the regulation of PYY secretion [250, 251]. The overall exposure-induced reductions in PYY levels we observe could suggest that SCFA levels are modified or that there is disruption in intestinal L cell health. We will further explore this potential mechanism through quantification of SCFA levels in the plasma and feces and examination gene expression coding for these metabolic hormones within the ileum.

Leptin, a well characterized adipokine, contributes to regulation of energy balance through inhibition of hunger and modulation of glucose and fatty acid metabolism [252]. Serum levels of leptin were significantly reduced in cellulose-fed exposed mice. This finding has been reported previously in the literature with exposure to persistent organic pollutants. For example, in the Yusho cohort, a Japanese population of dioxin-exposed individuals, a significant reduction in

serum leptin levels was observed [253]. Interestingly, there are several studies demonstrating that leptin exerts an insulin-like effect [252]. For example, leptin administration has been demonstrated to contribute to normoglycemia and improving insulin responses in response to streptozotocin-induced diabetes [254]. Therefore, detriments to leptin levels in cellulose-fed exposed mice could contribute to our observed disruptions in glucose homeostasis. Our observation of a reduced level of leptin is not surprising due to the reduced level of body fat observed in our cellulose-fed exposed mice, however this finding highlights a role of the adipose tissue in PCB toxicity and protection with inulin consumption that needs to be further explored.

Aside from microbial modulation and hormone modulation, another potential mechanism of inulin-induced improvements host health and metabolism is reduction in metabolites associated with cardiovascular disease. In the liver, a significant increase in ceramide species, including 18:1/20:0 and 18:1/24:1 was observed in exposed mice fed cellulose, while mice fed inulin were protected from this increase. It has recently been accepted that ceramides are a biomarker of cardiovascular disease risk, increasing inflammation, reactive oxygen species, and causing cellular dysfunction [255]. Ceramides are not only linked with cardiovascular disease but also with NAFLD [256]. For example, an increased serum level of ceramides was observed in obese children with NAFLD [257]. It has also been demonstrated that modulation of ceramide production in NAFLD helps reduce the progress of atherosclerosis, highlighting the strong tie between the hepatic and cardiovascular systems [258]. In support of our ceramide

concentrations in the liver, a PCB-induced decrease in *Mttp* was observed in cellulose fed mice and attenuated in inulin-fed mice. *Mttp* is a critical protein involved in the transfer of ceramides from the liver to the plasma [259]. Consistent with these data, PCB-induced increases in ceramides within the plasma were not observed. (data not shown). Our observations of increases in hepatic ceramides along with presence of hepatic steatosis and inflammation are noteworthy in that they are major risk factors of cardiometabolic disease. Furthermore, to our knowledge this is the first study demonstrating that exposure to PCBs increases ceramide production, highlighting a new mechanism of PCB toxicity to be explored in future studies.

The present study adds significantly to the growing body of literature demonstrating the beneficial effects of nutrition on combatting pollutant toxicity, however there are a few limitations to note that should be addressed in subsequent experiments. Firstly, it is recommended that humans consume 14g of fiber per 1000 calories [218]. The traditional level of fiber in purified diets and our previous study is 5%, equating to 12g per 1000 calories. The diets in the current study both had fiber present at the 8% level, equating to 21g per 1000 calories. Therefore, the cellulose and inulin diets used in the present study can be considered high fiber diets and thus future studies should examine all levels of fiber intake (i.e. low, adequate, high) to fully understand the interactions between fiber consumption and pollutant exposure. It is also important to note that the present study only utilizes male mice. Future studies should employ male and female mice as there are various differences including that of specific aspects of

metabolism and gut microbiota populations. Finally, there are still measurements to be made to further elucidate the mechanisms driving the observed phenotypes in this study. To better understand the role of the microbiota in the inulin-driven protection against PCB toxicity we will quantify SCFA levels in the feces and plasma. As discussed in chapter one, SCFA levels are sensed by specific G-protein coupled receptors (GPRs), GPR41 and GPR43, which are involved in numerous systemic processes including the regulation of glucose and lipid metabolism [86, 91]. Therefore, quantification of SCFA is important to follow up on due to our present observed effects of PCBs on glucose metabolism and lipid accumulation as well as the PCB effects on SCFA production in observed both chapter two and chapter three. Additionally, quantification of fecal, plasma, and hepatic levels of PCBs will be conducted to examine if cellulose or inulin drive different deposition profiles of these pollutants, thereby influencing their toxicity. Inulin has been well demonstrated to reduce cholesterol and improve lipid profiles in humans [127]. Therefore, quantification of cholesterol and triglycerides both in the liver and in plasma will also be conducted to observe if a reduction in these could be a potential mechanism by which inulin attenuates inflammation and hepatic steatosis. Finally and importantly, atherosclerotic lesions will be quantified to examine if the observed protective effects of inulin translate to an attenuated atherosclerotic risk.

Overall, the data presented demonstrates that consumption of the prebiotic inulin is capable of attenuating PCB 126-induced disruption of gut microbial population, host metabolism and systemic inflammation. Importantly, the

elevated level of fiber in the control group compared to our previous study (8% cellulose vs. 5% cellulose) may confer metabolic alterations and may also impact the toxicity of PCB 126, which is a parameter that needs further examination. We hypothesize that through alterations in gut microbial populations and microbial metabolite production, that inulin is able to reduce cardiometabolic disease risk. This study is important in that increasing dietary inulin consumption is an easily attainable means for all human populations and poses little to no risk for health. More research is needed to elucidate the direct mechanisms of attenuations in PCB toxicity induced by inulin feeding.

**Table 4.1.** Diet Formulation

| | 8% Cellulose | | 8% Inulin | |
|---|---|---|---|---|
| | gm | kcal | gm | kcal |
| *Protein* | 18.6 | 20.0 | 19.2 | 20.0 |
| *Carbohydrate* | 64.9 | 70.0 | 64.0 | 70.0 |
| *Fat* | 4.1 | 10.0 | 4.2 | 10.0 |
| *Total* | | 100.0 | | 100.0 |
| *Kcal/gm* | 3.71 | | 3.83 | |
| | | | | |
| ***Ingredient*** | | | | |
| *Casein, Lactic* | 200 | 800 | 200 | 800 |
| *L-Cystine* | 3 | 12 | 3 | 12 |
| | | | | |
| *Corn Starch* | 375 | 1500 | 343.1 | 1372.4 |
| *Maltodextrin 10* | 125 | 500 | 125 | 500 |
| *Sucrose* | 200 | 800 | 200 | 800 |
| | | | | |
| *Cellulose* | 87.5 | 0 | 0 | 0 |
| *Inulin* | 0 | 0 | 84.8 | 127.2 |
| | | | | |
| *Soybean Oil* | 25 | 225 | 25 | 225 |
| *Cocoa Butter* | 20 | 180 | 20 | 180 |
| | | | | |
| *Mineral Mix S10021* | 10 | 0 | 10 | 0 |
| *Dicalcium Phosphate* | 13 | 0 | 13 | 0 |
| *Calcium Carbonate* | 5.5 | 0 | 5.5 | 0 |
| *Potassium Citrate* | 16.5 | 0 | 16.5 | 0 |
| | | | | |
| *Vitamin Mix V10001* | 10 | 40 | 10 | 40 |
| *Choline Bitartrate* | 2 | 0 | 2 | 0 |
| | | | | |
| *Cholesterol* | 1.6 | 0 | 1.6 | 0 |
| | | | | |
| *Total* | 1094.10 | 4057 | 1059.50 | 1094.10 |

**Table 4.2.** Genera level differences in gut microbial populations

| | Cellulose + Vehicle | Cellulose + PCB 126 | Inulin + Vehicle | Inulin + PCB 126 |
|---|---|---|---|---|
| ***Other*** | 371.9 ± 67.0 | 229.8 ± 25.8 | 527.4 ± 25.6* | 709.4 ± 73.1*‡ |
| ***Bifidobacterium*** | 12.1 ± 3.0 | 24.1 ± 9.7 | 597.2 ± 136.7* | 1432.5 ± 196.6*‡ |
| ***Lactobacillus*** | 19.8 ± 6.7 | 13.1 ± 4.8 | 152.1 ± 41.9* | 403.1 ± 95.9*‡ |
| ***Lactococcus*** | 219.6 ± 42.7 | 212.1 ± 72.4 | 123.7 ± 19.5 | 161.3 ± 31.3 |
| ***Turicibacter*** | 38.5 ± 10.4 | 11.7 ± 3.0‡ | 3.6 ± 0.8* | 3.9 ± 1.3 |
| ***Clostridium*** | 18.4 ± 4.3 | 19.6 ± 2.9 | 4.2 ± 1.4* | 2.5 ± 0.7* |
| ***Dehalobacterium*** | 74.2 ± 8.8 | 43.9 ± 7.7 | 88.3 ± 23.2 | 46.3 ± 7.9 |
| ***Coprococcus*** | 302.0 ± 38.0 | 863.6 ± 234.4‡ | 61.9 ± 21.0* | 108.7 ± 23.5 |
| ***Dorea*** | 126.0 ± 14.7 | 41.2 ± 13.4‡ | 21.3 ± 8.2* | 16.4 ± 4.7* |
| ***[Ruminococcus]*** | 462.0 ± 58.0 | 560.4 ± 35.1 | 129.0 ± 16.3 | 908.0 ± 193.5‡ |
| ***rc4-4*** | 1405.0 ± 409.1 | 0.8 ± 0.5‡ | 211.0 ± 96.1* | 249.0 ± 44.9* |
| ***Oscillospira*** | 2755.2 ± 352.2 | 1784.1 ± 214.2‡ | 1251.0 ± 215.7* | 1115.0 ± 118.4 |
| ***Ruminococcus*** | 727.4 ± 63.9 | 861.8 ± 85.1 | 545.6 ± 75.5 | 340.8 ± 58.4* |
| ***Allobaculum*** | 5064.8 ± 1593.3 | 2007.5 ± 565.8 | 12019.1 ± 2319.5* | 14419.7 ± 1895.7* |
| ***Coprobacillus*** | 66.9 ± 14.2 | 82.8 ± 16.1 | 1.0 ± 0.5* | 2.8 ± 1.0* |
| ***Sutterella*** | 772.2 ± 295.0 | 1092.1 ± 178.2 | 1585.3 ± 135.0* | 993.7 ± 133.1 |
| ***Desulfovibrio*** | 2129.3 ± 811.1 | 6.2 ± 1.4‡ | 2.6 ± 0.8* | 7.6 ± 3.6 |
| ***Akkermansia*** | 37403.2 ± 2784.1 | 43081.5 ± 3849.6 | 21070.1 ± 2235.5* | 31416.8 ± 2044.5* |

*indicates a significant difference compared to cellulose diet; ‡ indicates a significant difference compared to vehicle of same diet (n=10). Data analyzed by two-way ANOVA with post hoc comparisons of the means.

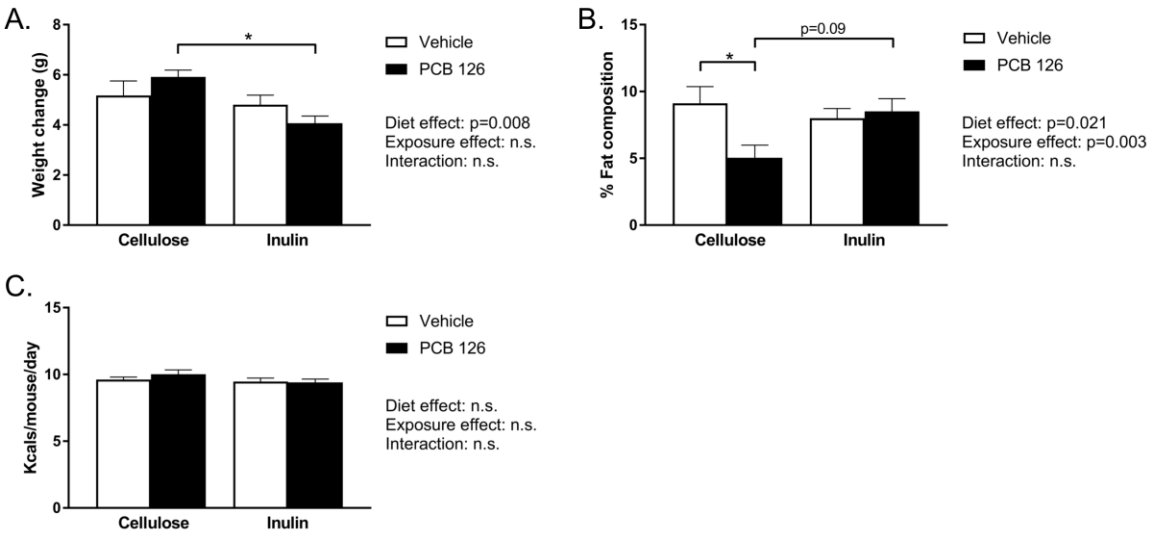


**Figure 4.1. Inulin consumption protects against PCB-induced wasting.**

Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1μmol/kg) or vehicle at weeks 2 and 4. **A.** Weight change (g) from baseline to the end of the study. Cellulose fed mice exposed to PCBs had significantly greater weight gain than exposed inulin mice. **B.** Fat composition quantified using EchoMRI. PCB exposure reduced body fat percentage in the cellulose-fed group, which was attenuated by inulin consumption. **C.** Average energy intake (kcals) per day. Data are presented as mean ± S.E.M (n=10 per group). Statistical significance is denoted by * ($p<0.05$).

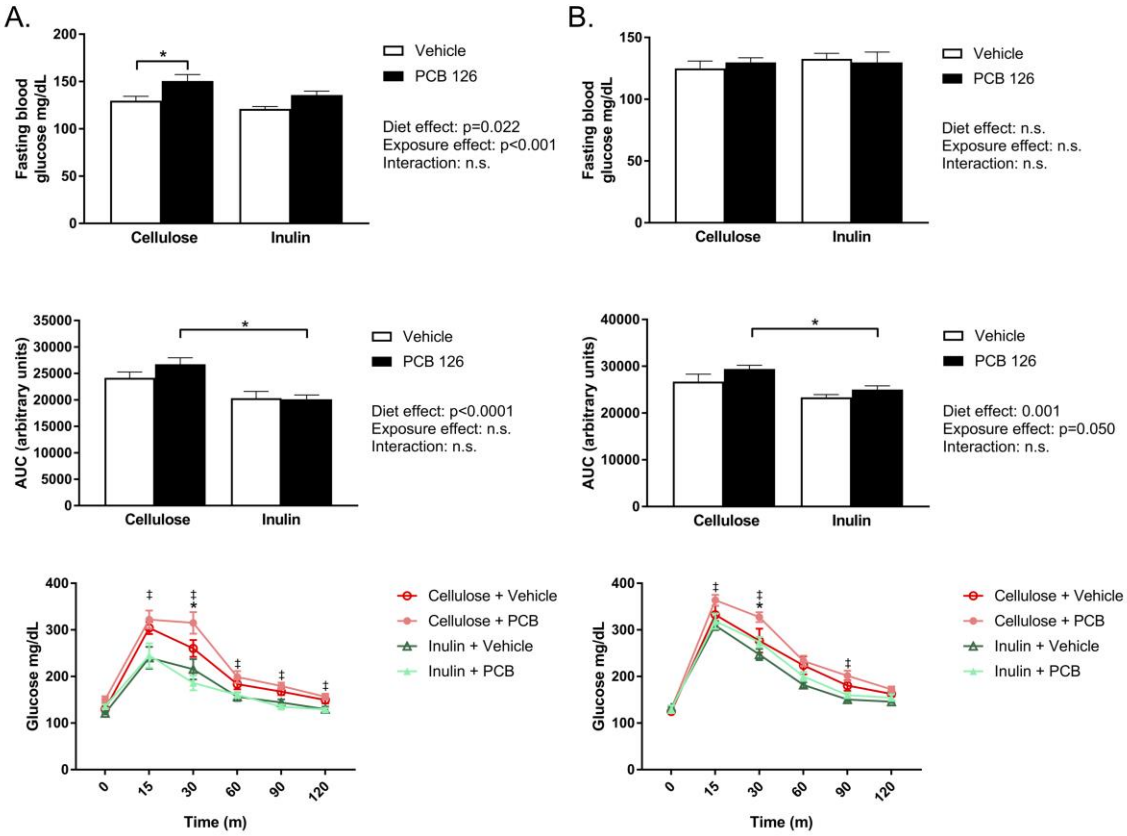


**Figure 4.2. Inulin attenuates PCB-disruption in glucose tolerance.** Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1μmol/kg) or vehicle at weeks 2 and 4. **A.** Fasting blood glucose and glucose tolerance testing at week 5. PCB exposure increased fasting blood glucose levels in cellulose fed mice and not in inulin fed mice. Cellulose fed mice exposed to PCBs displayed a greater area under the curve (AUC) compared to exposed inulin fed mice. **B.** Fasting blood glucose and glucose tolerance testing at week 8. No differences were observed in fasting blood glucose. The effects of PCBs and diet on AUC remained significant at 8 weeks. Data are presented as mean ± S.E.M (n=10 per group). Statistical

significance is denoted by * ($p<0.05$). For glucose curves, ‡ indicates a significant difference between “Cellulose + PCB” and “Inulin + PCB” and * indicates a significant difference between “Cellulose + Vehicle” and “Cellulose + PCB”.

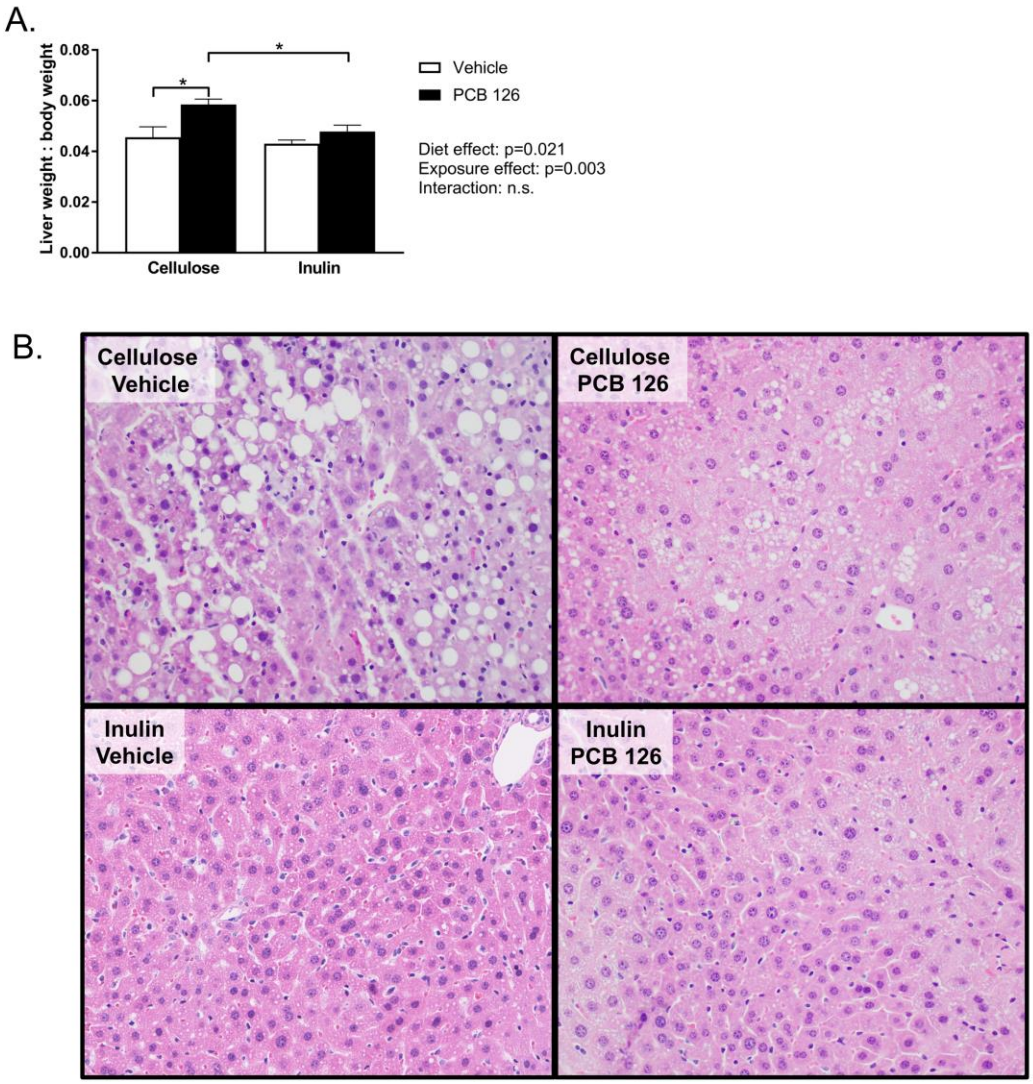


**Figure 4.3. Inulin consumption reduces PCB-induced hepatic lipid accumulation.** Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1µmol/kg) or vehicle at weeks 2 and 4. **A.** Liver weight/body weight ratio. PCB exposure increased liver weight in cellulose fed mice, which was attenuated by inulin feeding. **B.** Hematoxylin and eosin staining of hepatic tissue sections. Increases in microvescicular fat accumulation was evident in PCB exposed mice fed

cellulose, which appeared to be attenuated by inulin consumption. Inulin feeding also had an overall effect of reducing macrovesicular fat accumulation. Data are presented as mean ± S.E.M (n=10 per group). Statistical significance is denoted by * ($p<0.05$).

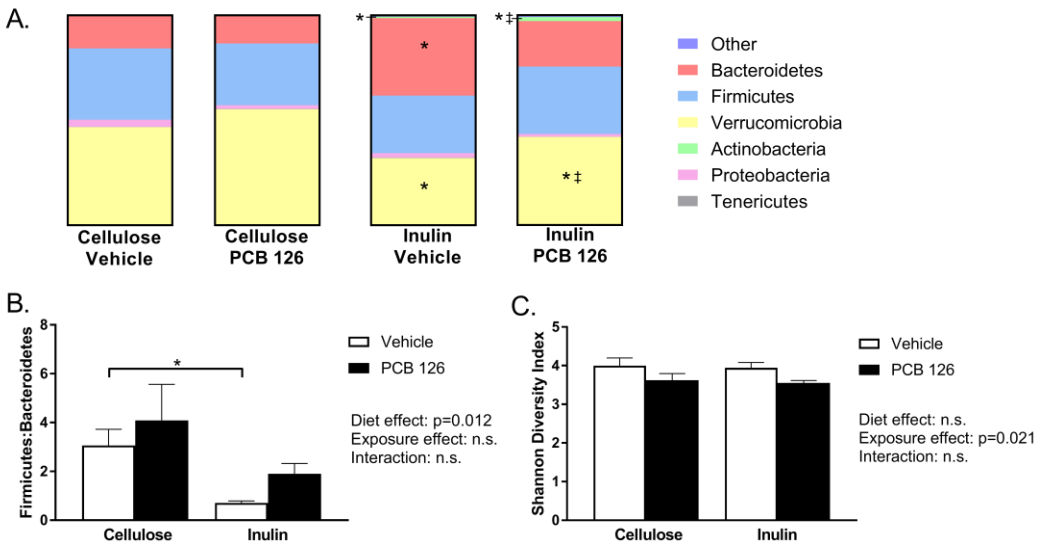


**Figure 4.4. Inulin feeding drives shifts in microbial composition at the phyla level.** Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1µmol/kg) or vehicle at weeks 2 and 4. Cecum contents were also collected at the conclusion of the study. 16S rRNA sequencing was conducted and data was analyzed using QIIME. **A.** Differences in the phyla level composition of the gut microbiota. Inulin feeding decreased Verrucomicrobia and increased Actinobacteria. *indicates a significant difference compared to cellulose diet and ‡ indicates a significant difference compared to vehicle of same diet. Data are presented as relative abundances. **B.** Firmicutes/Bacteroidetes ratio. Inulin feeding had a significant overall effect of lowering the Firmicutes/Bacteroidetes ratio. **C.** Alpha diversity (Shannon Diversity Index). PCB exposure had a significant overall effect of lowering alpha diversity. Data are presented as mean ± S.E.M (n=10 per group; two-way ANOVA with post hoc comparisons of the means). Statistical significance is denoted by * ($p<0.05$).

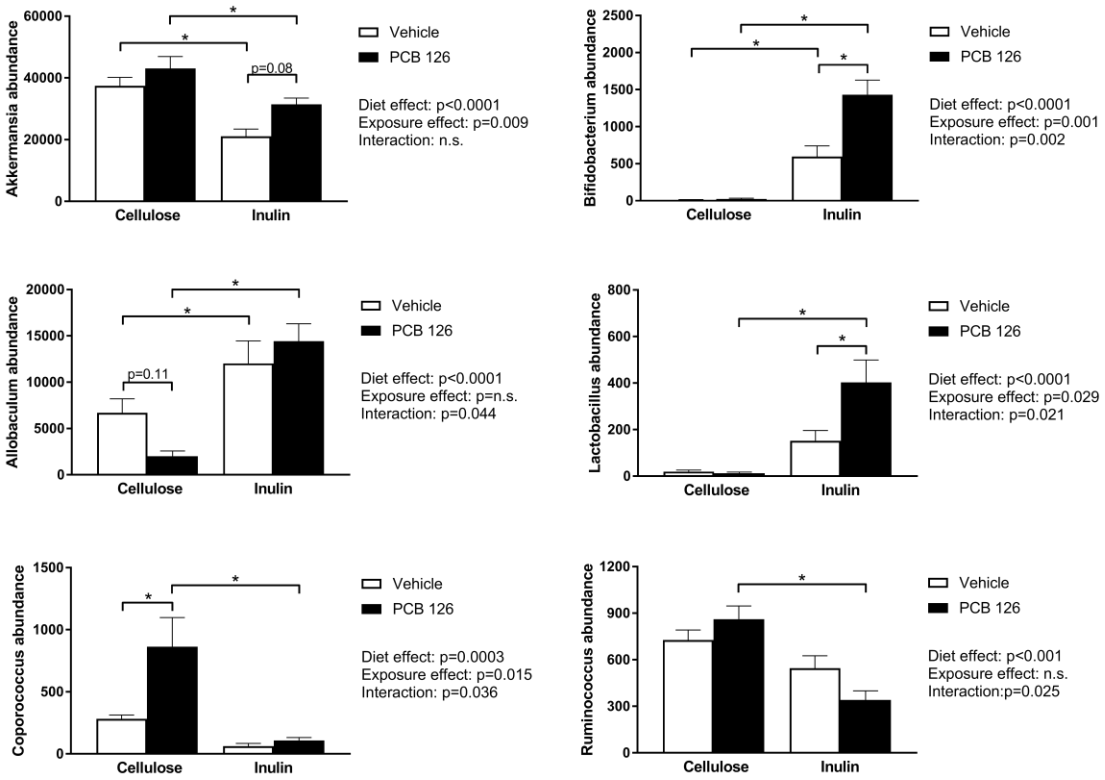


**Figure 4.5. Inulin and PCB exposure modulate gut microbial populations.**

Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1μmol/kg) or vehicle at weeks 2 and 4. Cecum contents were also collected at the conclusion of the study. 16S rRNA sequencing was conducted and data was analyzed using QIIME. PCB exposure increased *Coprococcus* abundance in cellulose fed mice, which was attenuated in mice fed inulin. PCB exposure increased the level of Akkermansia irrespective of diet. Irrespective of exposure, inulin feeding increased levels of *Bifidobacterium*, *Lactobacillus,* and *Allobaculum* compared to cellulose feeding. Data are presented as mean ± S.E.M (n=10 per group; two-way ANOVA with post hoc comparisons of the means). Statistical significance is denoted by * ($p<0.05$)

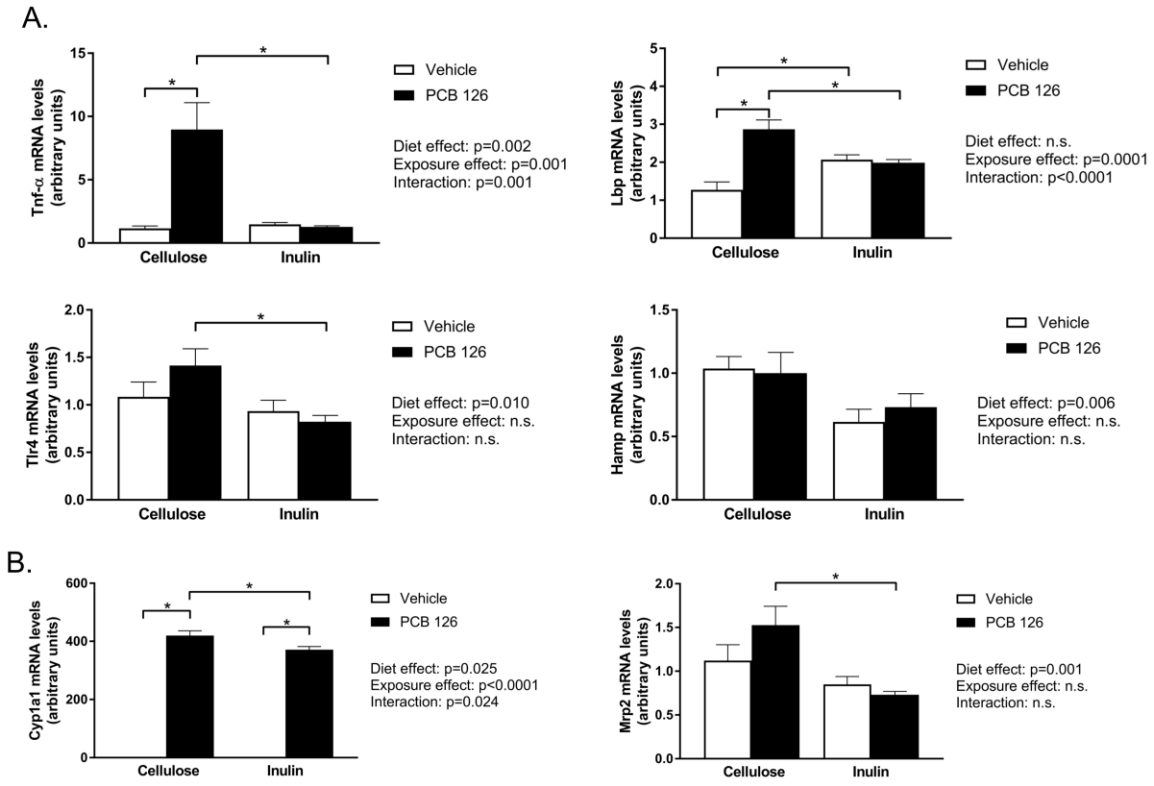


**Figure 4.6. Inulin attenuates PCB-induced changes in hepatic inflammatory and detoxification markers.** Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1μmol/kg) or vehicle at weeks 2 and 4. Liver samples were collected at the conclusion of the study. mRNA units were determined using the relative quantification method (ΔΔCT), normalized to control values. 18S was used as the housekeeping gene for all hepatic gene expression quantifications. **A.** mRNA quantification of hepatic markers of inflammation. PCB exposure increased expression of tumor necrosis factor alpha (Tnfα) and lipopolysaccharide binding protein (Lbp), which was attenuated by inulin consumption. **B.** mRNA quantification of hepatic markers of xenobiotic detoxification. PCB-induced

increases in cytochrome p450 (Cyp1a1) expression was lower in inulin fed mice compared to cellulose fed mice. Multidrug resistance protein (Mrp2) expression was lower in exposed inulin fed mice compared to exposed cellulose fed mice. Data are presented as mean $\pm$ S.E.M (n=10 per group). Statistical significance is denoted by * ($p<0.05$).

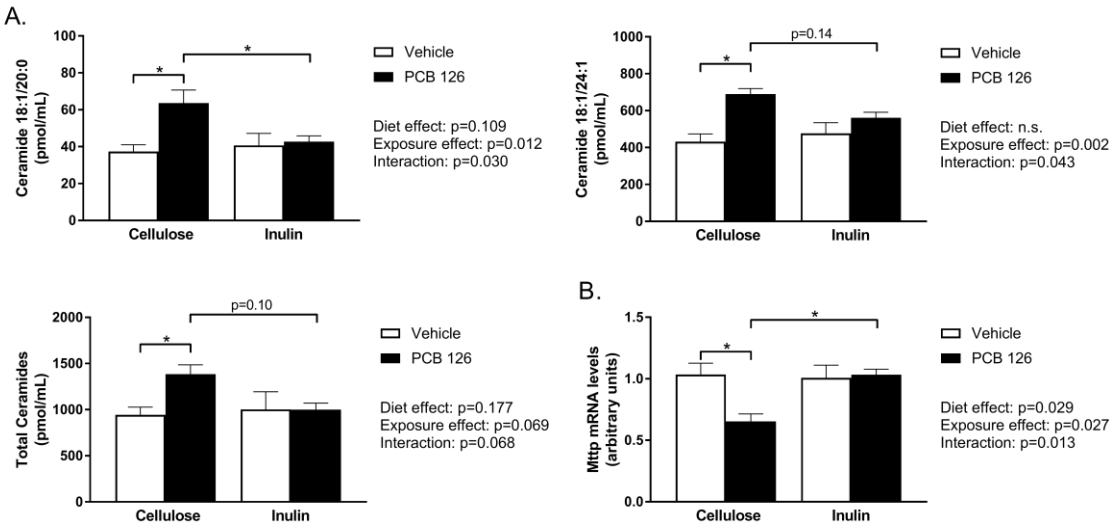


**Figure 4.8. Inulin attenuates PCB-induced increase in hepatic ceramides.** Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1µmol/kg) or vehicle at weeks 2 and 4. Liver samples were collected at the conclusion of the study. **A.** Hepatic quantification of ceramide levels via lipidomics. PCB exposure increased hepatic ceramide species 18:1/20:0 and 18:1/24:1 and total ceramides in cellulose fed mice but not inulin fed mice. **B.** mRNA quantification of microsomal triglyceride transport protein (*Mttp*). In mice fed cellulose, PCB exposure decreased expression of *Mttp*, which was attenuated in exposed mice fed inulin. mRNA units were determined using the relative quantification method (ΔΔCT), normalized to control values. 18S was used as the housekeeping gene for all hepatic gene expression quantifications. Data are presented as mean ± S.E.M (n=10 per group). Statistical significance is denoted by * ($p<0.05$).

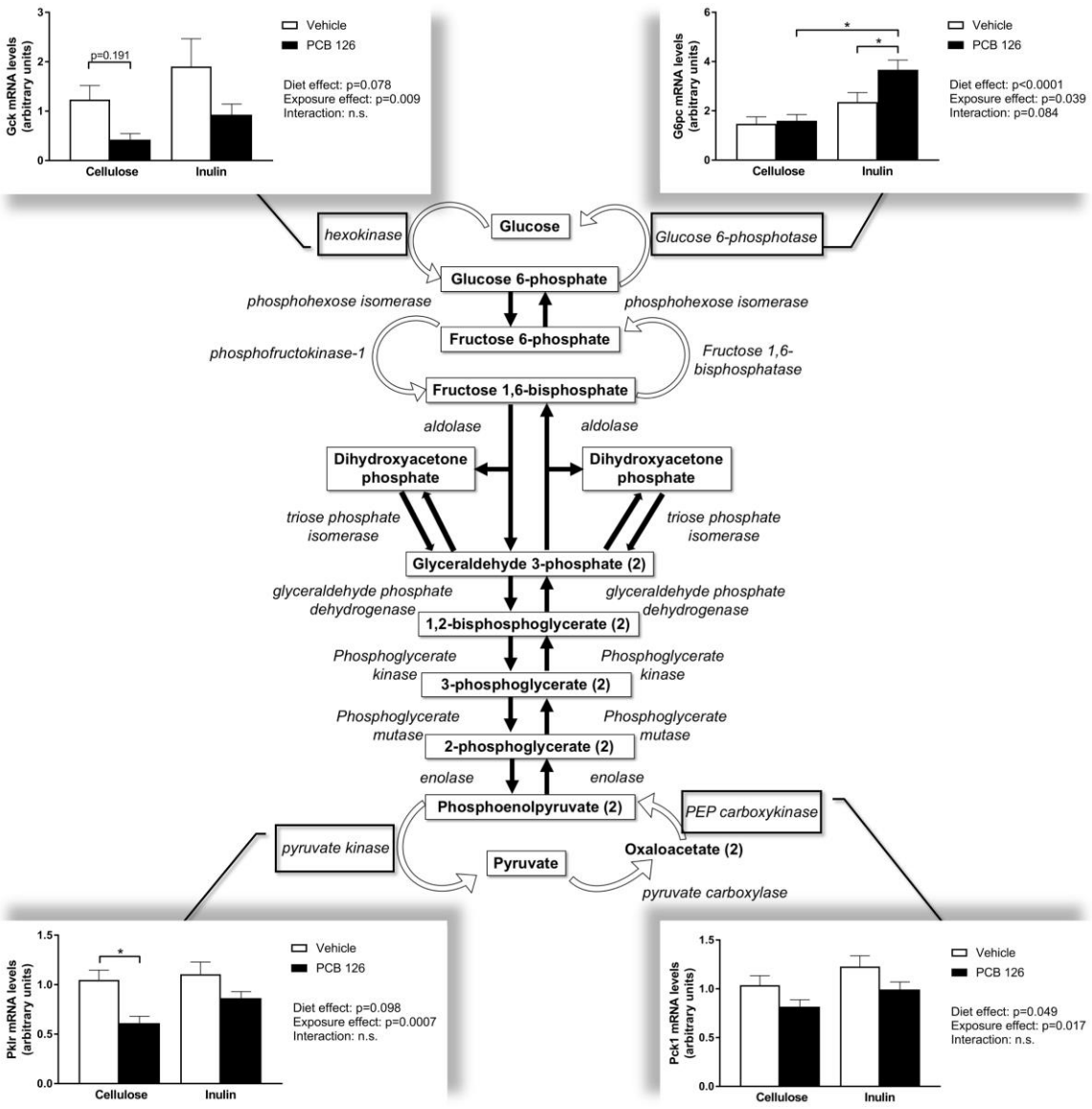


**Figure 4.8. PCB disruption of glucose metabolism is attenuated by inulin.** Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1µmol/kg) or vehicle at weeks 2 and 4. Liver samples were collected at the conclusion of the study. mRNA units were determined using the relative quantification method (ΔΔCT), normalized to control values. 18S was used as the housekeeping gene for all hepatic gene

expression quantifications. PCB exposure reduced expression of rate limiting glycolytic enzymes glucokinase/hexokinase (*Gck*) and pyruvate kinase (*Pklr*), which was not observed in inulin fed mice. Overall, inulin feeding increased gluconeogenic enzyme expression including PEP carboxykinase (Pck1) and glucose-6-phosphate (G6pc) irrespective of exposure. Data are presented as mean $\pm$ S.E.M (n=10 per group). Statistical significance is denoted by * ($p<0.05$).

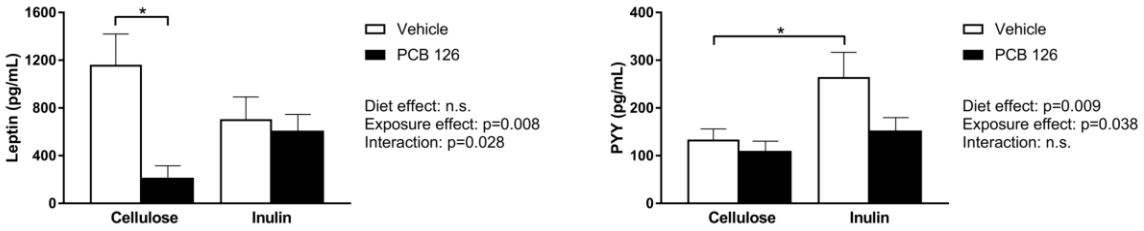


**Figure 4.9. PCB 126 and inulin alter circulating metabolic hormones**. Male *Ldlr*$^{-/-}$ mice were fed an atherogenic diet containing 8% cellulose or 8% inulin for 12 weeks and exposed to PCB 126 (1μmol/kg) or vehicle at weeks 2 and 4. Circulating hormones related to energy metabolism were quantified via Magpix technology. PCB exposure reduced leptin levels in cellulose fed mice but not in inulin fed mice. An overall effect of inulin consumption on increasing PYY levels was observed while a reductive effect of PCB exposure on PYY was noted. Data are presented as mean $\pm$ S.E.M (n=10 per group). Statistical significance is denoted by * ($p<0.05$).

# Chapter 5 Overall Discussion

## 5.1 Discussion

### 5.1.1 Summary

The research presented in this dissertation discusses novel observations into the effects of polychlorinated biphenyl (PCB) exposure on the gut microbiota and how this impacts overall host health. Our primary goal is to find means by which to decrease the negative health effects of pollutant exposure; thus, this dissertation provides insight into a novel avenue of nutritional mediation in pollutant exposures, the consumption of prebiotics (i.e. dietary fiber). The gut microbiota is influenced by a variety of factors including dietary intake as well as environmental exposures and has been implicated in the development of several diseases, including cardiovascular disease and diabetes. We demonstrated that PCB exposure detrimentally impacts the gut microbiota, specifically by decreasing diversity, increasing the inflammatory-associated Firmicutes to Bacteroidetes ratio, and decreasing specific beneficial bacterial genera. Furthermore, we elucidated that PCB exposure directly impacts certain bacteria through membrane disruption, leading to an overall change in fermentation acid production in the host and thus increasing the risk of metabolic diseases. Finally, we demonstrated the role of prebiotic (i.e. inulin) consumption in attenuating PCB-induced microbial and metabolic dysfunction.

### 5.1.2 PCB 126 disrupts microbial fermentation of a prebiotic substrate

There is evidence that exposure to environmental pollutants can impact gut microbial populations but the mechanisms and specific bacterial populations affected remains unknown. It has been shown that pollutants including 2,3,7,8 tetrochlorodibenzofuran, benzo[a]pyrene, and PCBs can alter gut microbial pollutions as well as induce intestinal inflammation [18, 19, 178, 185, 193]. However, these studies did not address the direct impact of these various pollutants on the bacteria themselves. Conversely, research on environmental bacterial populations and pollutants has substantiated that there is indeed an interplay between pollutants and bacterial populations [157, 158]. For example, research on the ability of particular bacterial populations to degrade pollutants such as polyaromatic hydrocarbons has been appreciably studied and has yielded promising results [260]. Due to the vast presence of PCBs in the environment, past research has examined their effects on soil bacterial populations and determined that there was no convincing evidence that PCBs exert genotoxicity but instead may have effects on the bacterial cell membrane [157, 158]. While these findings provide some insight into the interactions between pollutants and bacteria, it is important to note that the bacteria found in soil environments are primarily aerobes while the bacteria residing in the mammalian gut are primarily anaerobes, which may cause differences in metabolism and response to pollutant exposure. The work described in chapter two of this dissertation attempted to address this gap in the literature by studying the direct effects of PCB exposure on isolated gut microbial populations.

Using an inulin-fermenting isolate from murine feces, we determined that as concentrations of PCB 126 increased, there was a significant impediment on bacterial viability. To determine if PCBs were membrane disruptive in an anaerobic gut microbe, we quantified intracellular potassium and confirmed our observed reductions in viability to be due to perturbations in the bacterial cell membrane. This is important in that this is the first instance in which it has been demonstrated that PCBs can exert direct toxic effects on specific gut microbial populations. Furthermore, our findings that these disruptions translated to overall alterations in fermentation acid production from a mixed fecal microbial sample demonstrate the impact that PCB disruption of gut microbiota can have on the overall host. Importantly, upon increasing the prebiotic inulin substrate, the bacteria were able to overcome the disruptions in fermentation acid production. Fermentation acid or short chain fatty acid (SCFA) production has been more recently understood to influence numerous properties that affect host health [84, 91]. The primary SCFAs acetate, propionate, and butyrate all exert differential effects throughout the human body. Butyrate functions primarily within the intestine, serving as a preferential energy source for colonocytes and has been associated with improved intestinal health [245]. Acetate and propionate exert functions within the liver and peripherally, modulating glucose and lipid metabolism [85]. Therefore, PCB-induced disruptions in gut microbial production of SCFAs could be responsible for the observed disruptions in hepatic metabolism, glucose tolerance, and intestinal inflammation. Overall, our findings of PCB-induced disruption of gut microbial fermentation, alleviated by increased

prebiotic substrate, highlights that the bacteria that can proliferate at a higher level of inulin are likely not sensitive to PCB exposure. Therefore, focusing our research on elucidating and targeting the effected populations could be greatly beneficial for host health.

### 5.1.3 Exposure to PCB 126 disrupts gut microbial and metabolic homeostasis

Dioxin-like pollutants are found in many animal food products and thus human exposure is initiated upon consumption, with the first impact being on the gastrointestinal tract. Thus, in work described within chapter three of this dissertation we attempted to address the role of the gut in the PCB-toxicity. We hypothesized that PCB toxicity initiated in the gut and influenced the development of peripheral inflammation and disease.

It has been well established that cardiometabolic disease is strongly linked to gastrointestinal health and the gut microbiome. It has been demonstrated that individuals with cardiometabolic diseases possess unique microbial signatures, suggesting a potential interplay of the microbiota in the pathogenesis of these diseases. For example, individuals with atherosclerosis and type 2 diabetes exhibit increases in Firmicutes and reductions in Bacteroidetes [261]. Additionally, an epidemiological study investigating cardiovascular health indicated that stool populations of *Prevotella 2* and *Prevotella 7* were associated with an increased lifetime risk for cardiovascular disease [262]. Interestingly, treatment with antibiotics was able to lower circulating levels of TMAO, a biomarker that has been identified as a risk factor for cardiovascular disease.

Furthermore, these researchers demonstrated that antibiotic-induced reduction in TMAO correlated with a decrease in aortic plaque and a decreased number of macrophages within the plaque [104, 263]. These data highlight the critical interplay of the gut microbiota in the pathogenesis of disease.

It has also been well established that dioxin-like pollutant exposure can increase the risk of cardiovascular disease developments [38]. Recently, our lab demonstrated that exposure to PCB 126 increases systemic inflammation and accelerates the development of atherosclerosis in a mouse model of cardiometabolic disease [181]. To expand on this previous finding and to elucidate the effect of PCB exposure on the gut microbiota and how this may play a role in cardiometabolic disease, LDLr-\- mice were fed an atherogenic diet and exposed to a low dose of PCB 126 and parameters of metabolic and microbial health were analyzed. We found that PCB exposure induced disruption of gut microbial populations, primarily in the cecum contents. PCB exposure reduced gut microbial diversity and increased the Firmicutes to Bacteroidetes ratio, both of which are observed in chronic inflammatory conditions and cardiometabolic diseases. This finding is consistent with other studies demonstrating that pollutant exposure, specifically dioxin, results in increases in the Firmicutes to Bacteroidetes ratio, which correlated with liver and immune toxicity [193].

We also observed PCB-induced reduction of *Oscillospira*, *Bifidobacterium*, and *Lactobacillus,* all of which have been correlated with positive health outcomes for the host. Importantly, we observed a significant positive correlation

between circulating levels of glucagon like peptide-1 (GLP-1) and *Bifidobacterium* that was dependent on PCB exposure. This finding is important due to the role of GLP-1 on modulating glucose metabolism through control of insulin secretion as well as studies demonstrating that *Bifidobacterium* supplementation can improve insulin sensitivity [206]. Along with this, our observations of hyperinsulinemia in the absence of glucose intolerance in PCB-treated mice suggest a prediabetic phenotype. Other labs have observed impairments in glucose tolerance in response to PCB-exposure, but our finding is the first to highlight that these impairments may be linked to gut microbial disruption.

Additionally, we conducted hepatic metabolomics to examine the impact of PCB exposure on metabolism. We observed that PCB exposure reduced glycolytic intermediates, increased fatty acid metabolism intermediates, and induced alterations in metabolites that have been demonstrated to be influenced by host-microbial interactions (e.g. N-acetylphenylalanine, dimethylglycine). We hypothesized that our observed shift away from carbohydrate metabolism to fatty acid metabolism could be a consequence of hyperinsulinemia. Due to these findings of altered liver metabolites, the subsequent study (chapter four) focuses on following up on this finding and examining hepatic metabolism and health more thoroughly.

Overall, we hypothesize that our findings of increased inflammation, metabolic disruption, and gut dysbiosis in PCB-exposed mice may occur concurrently and have a feed-forward effect on each other. These findings reveal a novel

opportunity for intestinally-targeted nutritional intervention to attenuate the detrimental effects of PCBs on both gut dysbiosis and overall host health.

### 5.1.4 Inulin consumption reduces gut and systemic toxicity of PCB 126

It is well understood that a healthful diet, rich in fruits, vegetables, and foods high in bioactive compounds, can confer a benefit on cardiometabolic disease risk and reduction [218]. Our lab has shown previously that flavonoids and polyphenols can be protective against PCB-related endothelial cell dysfunction, a beginning pathological step in the development of atherosclerosis [264]. Furthermore, we have also demonstrated that consumption of bioactive components can reduce pollutant-induced oxidative stress, which is a known player in the development of metabolic dysfunction [73]. In work described within chapter three of this dissertation, we attempted to expand the scope of nutritional modulation of PCB toxicity to parameters that are well known to have an influence on gut health and the gut microbiota, specifically the prebiotic fiber inulin. Inulin is an inulin type fructan found in many vegetables (e.g. onions, leeks, and chicory root) and is composed of chains of fructosyl groups linked by $\beta$(2-1) glycosydic bonds, terminated with an $\alpha$-D(1-2)-glucopyranoside ring group on the reducing end [128, 134]. Inulin is added to many food products to increase fiber content and provide sweetness without adding significant caloric content. It has been found that in the US, most individuals fall below the recommendations of 25g or 38g of fiber per day for women and men, respectively [113, 125]. This provides an important avenue for intervention due

to fiber's known benefits on gastrointestinal health, cardiometabolic health, and weight management. Importantly, PCB exposure has been demonstrated to have detrimental impacts on all of these health conditions, and thus consumption of a diet rich in fiber is a feasible means by which to address and attenuate the negative effects of pollutant exposure. In the work described within chapter four of this dissertation we attempted to elucidate the role of inulin in modulating pollutant toxicity by supplementing mice with a diet high in inulin and subsequently exposing them to low levels of PCB 126.

We found that exposure to PCB 126 induced significant fat mass loss in cellulose fed mice. Interestingly, this wasting was attenuated in exposed mice fed inulin. The lipophilic nature of dioxin-like pollutants such as PCBs cause them to accumulate in adipose tissue where they can induce inflammation and cause disruption in adipocyte metabolism [158]. Furthermore, research has shown that if PCB exposed obese mice undergo weight loss, the PCBs are released into circulation where they can exacerbate inflammation and diabetic symptoms, indicating a somewhat protective role of adipose tissue in preventing pollutant toxicity in other organs [162]. We hypothesize that the wasting induced by PCB exposure resulted in greater circulating levels of these pollutants, which could contribute to disruptions in host health and metabolism. We intend to follow up on levels of PCBs both in circulation and within the feces to address this hypothesis.

While cellulose fed mice exposed to PCBs exhibited a lower percent body fat, their livers were actually heavier and harbored a greater amount of

microvesicular fat. It is well accepted and understood that hepatic steatosis can contribute to metabolic disruptions and inflammation [233, 234]. If our hypothesis of greater circulating levels of PCBs in cellulose fed mice is correct, their participation in enterohepatic circulation could exacerbate the effects on the hepatic and gastrointestinal systems. Consistent with our observations of hepatic steatosis, PCB-exposed mice exhibited higher levels of inflammatory markers and inulin feeding was able to attenuate these increases. Furthermore, we observed that PCB exposure altered expression of key glycolytic and gluconeogenic enzymes and that inulin feeding was able to blunt these effects, suggesting a potential mechanism for our observations in disrupted glucose tolerance with PCB exposure and amelioration by inulin.

In chapter three, we established that PCBs induced alterations in the gut microbiota and that some of these alterations correlated with disruptions in metabolic markers. Due to inulin's high prebiotic capacity, we hypothesized that inulin feeding would attenuate PCB-induced disruptions in the microbiota and that these alterations would lead to overall improved host health. Indeed, we observed several effects of inulin feeding on microbial populations. Inulin is known to be a bifidogenic compound, stimulating the growth of *Bifidobacterium* [133]. As expected, we observed significant increases in the levels of *Bifidobacterium* in inulin fed mice, irrespective of exposure. Interestingly, exposure to PCB 126 in inulin fed mice further increased the levels of these bacteria. We hypothesize that this finding is due to a toxic effect of PCBs on other bacterial populations, thereby allowing for the proliferation of

*Bifidobacterium*. Furthermore, we also observed that inulin fed mice exhibited a lower Firmicutes:Bacteroidetes ratio, irrespective of exposure, suggesting a "healthier" microbial signature. Inulin appeared to exert a stronger effect on influencing microbial populations than did PCB exposure, which could be expected due to inulin's known prebiotic capabilities.

Due to our more thorough exploration of hepatic effects of pollutant exposure in this study, we discovered an avenue that has not previously been explored by our lab. In chapter four, we identified that hepatic ceramide levels were increased with PCB exposure. This is important in that ceramides have been more recently accepted as a biomarker for cardiometabolic disease risk [255]. Furthermore, we observed that inulin feeding was able to attenuate this increase. We also determined that hepatic microsomal triglyceride transfer protein (*Mttp*) expression was reduced with PCB exposure, which has been demonstrated to function in ceramide transport out of the liver [259]. Consistent with our findings, the level of *Mttp* expression was restored with inulin feeding, indicating a potential mechanism that needs to be further explored. To our knowledge, this is the first instance in which ceramide levels were examined with PCB exposure and thus this finding opens up a new avenue of exploration into PCB-toxicity.

When comparing the toxicity and effects of PCB exposure in the studies described in chapter three and chapter four, we observed discrepancies that should be noted. The only difference between the models used in these chapters is the level of fiber. In chapter three, the level of fiber in the control group was 5% from microcrystalline cellulose, whereas in chapter four, the level

of fiber in the control groups (cellulose) were 8% from microcrystalline cellulose. While a higher level of fiber consumption is believed to be more beneficial to host health, in chapter four we actually observed greater toxicity of PCB exposure despite a higher level of fiber. When comparing the exposed groups in both studies, we observed exacerbation of wasting and glucose tolerance in mice fed 8% cellulose but this was not observed in mice fed 5% cellulose. Interestingly, the microbial effects of PCB exposure in cellulose fed were not as apparent in the study described in chapter four compared to our previous findings in chapter three. Quantification of PCB levels in the feces and plasma is an ongoing analysis to better compare the body burden of PCBs between these two studies. Overall, we observed a discrepancy between the level of cellulose fiber (5% vs 8%) that may influence the toxicity of PCBs and will be followed up on in future studies.

## 5.2 Future directions and conclusions

The levels of environmental pollutants throughout the world continue to rise, despite numerous efforts towards reduction [2, 3]. Because pollutant exposure is mostly unavoidable, it is important to not only understand its impacts on human health, but also to elucidate means to provide protection against and/or ameliorate symptoms of exposure. One of the primary routes of exposure to lipophilic pollutants such as PCBs is through the ingestion of contaminated food products (e.g. fatty fish, meat, and dairy) and thus, focusing on the gastrointestinal tract is critical to understand the whole scope of PCB toxicity [2].

There are numerous future directions that can be pursued related to the work described within this dissertation. Firstly, to elucidate role of the microbiota in the influence of pollutant-induced disease, studies utilizing mouse models in which the microbiota has been manipulated could provide critical information. The research described in chapter two and three of this dissertation highlighted that PCB exposure can impact viability and fermentative properties of specific gut microbial populations and can do so through membrane disruptive mechanisms. However, it is still unknown what proportion of the impacts on the microbiota we observed are from direct disruption of specific bacteria or from indirect disruption through host inflammation and disease progression. Therefore, utilizing means of eliminating, reducing, or shifting gut microbial populations could be helpful in elucidating their role in PCB toxicity. One such means to do so would be the use of broad-spectrum antibiotic treated mice to eradicate a large proportion of the microbiota. Antibiotic treated mice have been used for studies investigating the role of eradicating specific groups of bacteria and for investigating the effect of disrupting the gut microbiome at various host life stages [265]. The use of antibiotic-treated mice is easily attainable and maintained with minimal costs but does not allow complete control of microbial population and elucidation of specific host-microbe interactions [265]. Furthermore, there is the potential of drug-pollutant interactions that could influence the actions of PCBs and/or antibiotics. Another, more rigorous, method of elucidating the role of the microbiota is to use germ free mouse models. The terminology germ free is defined as an animal free of bacteria, viruses, fungi, parasites, and protozoa from

birth [266]. The use of a germ-free mouse model to examine PCB-induced toxicity would allow us to understand the role that the microbiota in a mechanistic, more extreme sense. While this model will provide further insight into host-microbe interactions in PCB exposure, due to the extreme nature of this model, it can lack translatability. GF mice often need to be supplemented with vitamins, specifically K and B, and have a less developed and less functional small intestine, making energy absorption and utilization less efficient [266]. While utilizing GF models will provide us with critical information on the microbial influence of PCB toxicity, due to our labs interest on cardiometabolic disease and pollutant exposure, a logical next step would be the utilization of gnotobiotic mouse models. Gnotobiotic, derived from the Greek "*gnōtos*" (known) and "*biotic*" (life), simply means that GF mice are colonized with a specific microbial population, often from humans, to generate a humanized mouse model [267]. Humanized mouse models developed from GF mice have been used in a variety of areas of research including those focusing on cardiovascular diseases, neurological diseases, and metabolic diseases [267]. Thus, our lab could utilize the microbial population taken from individuals with cardiovascular/cardiometabolic disease and develop gnotobiotic mouse models to better understand the interactions between host physiology and a dysbiotic microbiota. Employing this method would provide us with the most translatable data, regarding the microbiota, to date and allow us to explore novel mechanisms of pollutant toxicity and develop interventions that better target these mechanisms. The use of the above discussed methods in a stepwise fashion

could allow us to garner critical data regarding host-microbe-pollutant interactions that examine a range potential mechanisms.

Another future direction for the laboratory could be to look more into the effects of PCBs and dietary fiber on gut microbial derived metabolites that reach peripheral circulation. One of the primary mechanisms that the gut microbiota influence host health outcomes is through the generation of metabolites that can exert differential effects on various host tissues [173]. The most understood metabolites produced by the gut microbiota are short chain fatty acids (SCFA), but there are numerous other metabolites that have not been explored or even discovered. Discovery of novel microbial derived metabolites from PCB and/or fiber exposure could open new avenues of exploration of the mechanisms by which PCBs exert their toxicity and the observed protective effects that we observed with fiber consumption.

Our lab has extensive expertise in examining the protective effects of polyphenols against pollutant-induced toxicity. Because dietary fiber and polyphenolic compounds are found predominantly in plant matter (i.e. fruits and vegetables), examining the potential of these compounds in combination could be greatly efficacious and highly translatable to the human population. The aryl hydrocarbon receptor (AhR) is critical in gut health and intestinal immunity [268]. However, as discussed throughout this dissertation, PCBs exert their effects through strong binding to AhR, leading to the upregulation of cytochrome p450 (Cyp1a1) and production of reactive oxygen species that lead to cellular dysfunction and inflammation [2]. It has been demonstrated that certain gut

microbial derived metabolites can act as AhR agonists and/or antagonists [269]. Importantly, it was found that the tryptophan metabolite indole functioned as an AhR antagonist and inhibited 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced Cyp1a1 expression [270]. Gut microbial derived tryptophan metabolites, as discussed in chapter one, of this dissertation have been demonstrated to exert protective effects on intestinal immunity and overall host health [94, 96, 98, 270]. High levels of tryptophan can be found in numerous food products, both animal and plant derived. One of the highest tryptophan content plant foods is broccoli, which also is high in dietary fiber [95]. Because of our discovery that fiber consumption can be protective against PCB-induced toxicity and the known effects of tryptophan metabolites, a combination of these could be highly therapeutic as well as translatable.

In conclusion, this dissertation demonstrates that exposure to PCB 126 exerts deleterious effects on gut microbiota both directly through membrane disruptive mechanisms and indirectly through inflammatory mechanisms within the gut and peripherally. Our lab has previously shown that nutritional interventions, specifically polyphenol consumption is able to modulate the toxicity of PCBs and thus, are a potential means by which to prevent toxicant-induced diseases [73, 264]. To expand the scope of nutritional modulation of pollutant toxicity beyond just polyphenols, in this dissertation we have shown that consumption of prebiotic dietary fiber is not only able to attenuate PCB-induced changes in the gut microbiota, but also able to prevent against peripheral inflammation and hepatic steatosis. Taken together, the data presented in this

dissertation support the growing body of literature indicating that use of dietary prevention and/or intervention measures may be a practicable approach to diminish disease risks associated with environmental pollutant exposure.

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# Vita

Jessie Baldwin Hoffman

## Education

| | |
|---|---|
| 2009-2013 | Newberry College, Newberry, SC<br>Bachelor of Science in Biology |
| 2013-2015 | The University of North Carolina at Greensboro, Greensboro, North Carolina<br>Master of Science in Nutrition |

## Professional Positions Held

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|---|---|
| 2011-2013 | Academic Tutor, Newberry College |
| 2013-2015 | Graduate Teaching Assistant, The University of North Carolina at Greensboro |
| 2013-2015 | Graduate Research Assistant, The University of North Carolina at Greensboro<br>Title: Impact of California Table Grapes on Systemic Inflammation, Insulin Resistance, and Hepatic Steatosis in Mice Fed an American-Type Diet<br>Mentor: Dr. Michael McIntosh, Department of Nutrition |

## Scholastic and Professional Honors

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|---|---|
| 2009-2012 | Summerland Honors Scholar, Newberry College |
| 2013-2015 | Greensboro Graduate Scholar Award, The University of North Carolina at Greensboro |
| 2014 | Jefferson Pilot Corporation Fellowship, The University of North Carolina at Greensboro |
| 2016 | Outstanding Graduate Student Poster Presentation: 3rd Place, 19th Annual Gill Heart Institute Cardiovascular Research Day, University of Kentucky |
| 2015-2017 | T32 Nutrition and Oxidative Stress Training Fellowship, University of Kentucky |
| 2017-2018 | Department of Pharmacology and Nutritional Sciences Student of the Year; University of Kentucky |
| 2018 | Emerging Leaders in Nutrition Science Poster Competition; 3rd Place, Nutrition 2018 (ASN); Boston, MA |
| 2018 | Outstanding Graduate Student Poster Presentation: 3rd Place, 8th Annual Barnstable Brown Diabetes and Obesity Research Day, University of Kentucky |

**Publications**

2015 Shen, W., **Baldwin, J.,** Collins, B., Hixson, L., Lee, K.T., Herberg, T., Starnes, J., Chuang, C.C., Reid, T., Gupta, S., McIntosh, M. 2015. Low level of trans-10, cis-12 conjugated linoleic acid decreases adiposity and increases browning independent of inflammatory signaling in overweight Sv129 mice. J. Nutr. Biochem. Jun;26(6):616-25. PMID: 25801353

2016 **Baldwin, J.,*** Collins, B.,* Wolf, P.G., Martinez, K., Shen, W., Chuang, C.C., Zhong, W., Cooney, P., Cockrell, C., Chang, E., Gaskins, H.R., McIntosh, M.K. 2016. Table grape consumption reduces adipostiy and markers of hepatic lipogenesis and alters gut microbiota in butter fat-fed mice. J. Nutr. Biochem. Jan; 27: 123-35. PMID: 26423887.

2016 Collins, B., **Baldwin, J.,** Martinez, K, Lila, MA., McIntosh, MK. 2016. Grapes and Gastrointestinal Health: Implications with Intestinal and Systemic Diseases. In: Pezzuto, JM, editor. Grapes and Health. Springer; p.119-138. NLB ID: 101686657

2016 Collins, B.,* **Hoffman, J.B.,*** Martinez, K., Grace, M., Lila, M.A., Cockrell, C., Nadimpalli, A., Chang, E., Chuang, C.C., Zhong, W., Cooney, P., Hopkins, R., McIntosh, M.K. 2016. An extractable polyphenol-rich fraction obtained from table grapes decreases adiposity, insulin resistance, and markers of inflammation in high-fat fed C57BL/6J mice. J. Nutr. Biochem. May; 31: 150-65. PMID: 27133434

2016 Petriello, M.C., **Hoffman, J.B.**, Sunkara, M., Wahlang, B., Perkins, J.T., Morris, A.J., Hennig, B. Dioxin-like pollutants increase hepatic flavin containing monooxygenase (FMO3) expression to promote synthesis of the pro-atherogenic nutrient biomarker trimethylamine N-oxide from dietary precursors. J. Nutr. Biochem. Apr 1;(33):145-153. PMID: 27155921

2016 Petriello, M.C., **Hoffman, J.B.**, Morris, A.J., Hennig, B. 2016. Emerging roles of xenobiotic detoxification enzymes in metabolic diseases. Rev Environ Health. Nov; doi: 10.1515/reveh-2016-0050 PMID: 27837601

2016 **Hoffman J.B.,** Petriello, M.C., Hennig, B. 2016. Impact of nutrition on pollutant toxicity: an update with new insights into epigenetic regulation. Rev Environ Health doi: 10.1515/reveh-2016-0041

2017 **Hoffman, J.B**., Hennig, B. 2017. Protective Influence of Healthful Nutrition on Mechanisms of Environmental Pollutant Toxicity and Disease Risks. Ann Ny Acad Sci doi: 10.1111/nyas.13365

2017 Wahlang, B., Perkins, J.T., Petriello, M.C., **Hoffman, J.B.**, Hennig, B. 2017. A compromised liver alters polychlorinated biphenyl-mediated toxicity; Toxicology doi: 10.1016/j.tox.2017.02.001.

2017 Wahlang, B., Barney, J., Thompson, B., Wang, C., Hamad, OM., **Hoffman, J.B**., Petriello, M.C., Hennig, B. 2017. PCB126 exposure increases risk for peripheral vascular diseases in a liver injury mouse model; Toxicol Sci. doi: 10.1093/toxsci/kfx180.

2017 Petriello, M.C., Brandon, J.A., **Hoffman, J.B**., Wang, C., Tripathi, H., Abdel-Latif, A., Ye, X., Li, X., Yang, L., Lee, E., Barney, J., Wahlang, B., Hennig, B., Morris, A.J. 2017. Dioxin-like PCB 126 increases systemic inflammation and accelerates atherosclerosis in lean LDL receptor deficient mice. Toxicol Sci. doi: 10.1093/toxsci/kfx27

2018 **Hoffman, J.B**.,* Petriello, M.C.,* Vsevolozhskaya, O., Morris, A.J., Hennig, B. 2018. Dioxin-like PCB 126 increases intestinal inflammation and disrupts gut microbiota and metabolic homeostasis. Environ Pollut. doi: 10.1016/j.envpol.2018.07.039.

2018 **Hoffman, J.B**., Flythe, M.,Hennig, B. 2018. Environmental pollutant-mediated disruption of gut microbial metabolism of the prebiotic inulin. Anaerobe. doi: 10.1016/j.anaerobe.2018.11.008.

* indicates equal contribution to manuscript (both primary authorship)

**Abstracts**

2015 **Baldwin, J.,** Collins, B., Wolfe, P., Shen, S., Chuang, C., Zhong, W., Cooney, P., Gaskins, H., McIntosh, M. 2014. California table grape consumption reduces adiposity, hepatic triglycerides, lipogenic gene expression, and abundance of sulfidogenic bacteria in mice fed butter fat. Experimental Biology 2015 in Boston, MA

2015 **Hoffman, J.,** Petriello, M., Collins, B., McIntosh, M., Hennig, B. Table grape feeding in high-fat fed mice attenuates body fat gain, hepatic steatosis, and systemic inflammation: a potential nutritional approach to PCB protection. Ohio Valley Society of Toxicology (OVSOT) 2015 Annual Meeting

2015 **Hoffman, J.,** Petriello, M., Collins, B., McIntosh, M., Hennig, B. Table grape consumption reduces body fat accumulation, hepatic steatosis, and inflammation in mice high fat fed mice: a potential nutritional approach for PCB protection. Superfund Research Program 2015 Annual Meeting in San Juan, Puerto Rico

2016 **Hoffman, J.,** Petriello, M., Hennig, B. Butryate modulates Cav-1 and its binding partner AhR, leading to differential Cyp1a1 and Cyp1b1 gene expression in vascular endothelial cells. Experimental Biology 2016 in San Diego, CA

2016 **Hoffman, J.,** Petriello, M., Hennig, B. Exposure to the Short Chain Fatty Acid Butryate modulates Cav-1 and AhR, affecting Cyp1a1 and Cyp1b1 gene expression in vascular endothelial cells. 11th Annual CCTS Spring Conference in Lexington, KY

2016 **Hoffman, J.,** Flythe, M., Hennig, B. Modulation of Gut Microbial Fermentation of Dietary Fiber by PCB126. 19th Annual Gill Heart Institute Cardiovascular Research Day in Lexington, KY

2016 **Hoffman, J.,** Flythe, M., Hennig, B. PCB126 Modulates Fecal Microbial Fermentation of the Dietary Fiber Inulin. Superfund Research Program 2016 Annual Meeting in Durham, NC.

2017 **Hoffman, J.,** Flythe, M., Hennig, B. PCB126 Exposure Modulates Gut Microbial Fermentation of the Dietary Fiber Inulin. Experimental Biology 2017 in Chicago, IL

2017 **Hoffman, J.,** Flythe, M., Hennig, B. PCB126 Exposure Modulates Gut Microbial Fermentation of the Dietary Fiber Inulin. 7th Annual Barnstable Brown Obesity and Diabetes Research Day in Lexington, KY.

2017 **Hoffman, J.,** Petriello, M., Hennig, B. PCB 126 increases intestinal inflammation and disrupts gut microbiota and metabolic homeostasis. 20th Annual Gill Heart Institute Cardiovascular Research Day in Lexington, KY.

2017 **Hoffman, J.,** Petriello, M., Hennig, B. PCB 126 disrupts gut microbiota and increases intestinal inflammation in a mouse model of atherosclerosis. Superfund Research Program 2017 Annual Meeting in Philadelphia, PA.

2018 **Hoffman, J.**, Petriello, M., Hennig, B. Dioxin-like PCB 126 disrupts gut microbiota, increases intestinal inflammation and alters host metabolism. Keystone Conference on Manipulation of the Gut Microbiota for Metabolic Health in Banff, Alberta.

2018 **Hoffman, J.B**., Petriello, M.C., Vsevolozhskaya, O., Morris, A.J., Hennig, B. Interactions between dietary cholesterol and environmental pollutant increase inflammation, disrupt gut microbiota, and modulate host metabolism. 8th Annual Barnstable Brown Obesity and Diabetes Research Day in Lexington, KY.

2018 **Hoffman, J.B**., Petriello, M.C., Vsevolozhskaya, O., Morris, A.J., Hennig, B. Interactions between diets high in cholesterol and dioxin-like pollutants increase inflammation,

disrupt gut microbiota, and modulate host metabolism. Nutrition 2018; American Society of Nutrition Annual conference in Boston, MA.

2018 **Hoffman, J.B**., Petriello, M.C., Barney, J., Deng, P., Flythe, M., Hennig, B. Prebiotic fiber (inulin) attenuated PCB 126-induced disruption of gut microbiota and host metabolism. Food & Nutrition Conference & Expo (FNCE) 2018; in Washington, DC.