The Role of the Microbiome in Sex and Obesity Effects on Ozone-Induced Airway Hyperresponsiveness

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The Role of the Microbiome in Sex and Obesity Effects on Ozone-Induced Airway Hyperresponsiveness

A dissertation presented

by

YoungJi Cho

to

The Committee on Higher Degrees in Biological Sciences in Public Health

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The Role of the Microbiome in Sex and Obesity Effects on Ozone-Induced Airway Hyperresponsiveness

ABSTRACT

Obesity is a risk factor for asthma, but standard asthma therapeutics have reduced efficacy in the obese. The obese asthmatic population is predominantly female, and often suffers from non-atopic asthma. Obesity alters the gastrointestinal microbial community structure. This change in structure contributes to some obesity-related conditions, including systemic inflammation, and could also be contributing to obesity-related asthma. Understanding the role of microbiota in obesity-related non-atopic asthma could lead to novel treatments for these patients.

In this thesis, I first demonstrated that airway hyperresponsiveness induced by exposure to ozone, a common non-atopic asthma trigger, was attenuated in germ free mice, and in mice after antibiotic treatment. I identified two short chain fatty acid producing bacteria, *Ruminococcus* and *Coprococcus*, whose relative abundance decreased with antibiotic treatment in correlation with the attenuation of airway responsiveness. Exogenous short chain fatty acid administration, on the other hand, increased the ozone-induced airway hyperresponsiveness. The data suggest that the microbiome contributes to ozone-induced airway hyperresponsiveness, likely via its ability to produce short chain fatty acids.
I next examined the impact of sex on responses to ozone. 16S sequencing of mouse fecal samples indicated differences in the gut microbial community structures of female and male lean mice. Compared to female mice, ozone-induced airway hyperresponsiveness was greater in male mice, and antibiotic treatment abolished this difference. In addition, compared to female weanlings housed in cages previously occupied by females, female weanlings housed in cages previously occupied by males developed greater ozone-induced airway hyperresponsiveness. Together, our data indicate that sex-related differences in the microbiome contribute to sex-related differences in ozone-induced airway hyperresponsiveness.

Finally, I examined the role of the microbiome in obesity-related increases in ozone-induced airway hyperresponsiveness. There were obesity-related differences in the gut microbiome that differed by sex. Compared to lean mice, obese mice of both sexes had augmented ozone-induced airway hyperresponsiveness. In obese female mice, antibiotics attenuated ozone-induced airway hyperresponsiveness, whereas antibiotics caused no change in obese male mice. Our data indicate that the microbiome contributes to the obesity-related increases in ozone-induced airway hyperresponsiveness in females, possibly via obesity-related changes in IL-6 and serum short chain fatty acids.

Together, our studies show that the microbiome contributes to the sex- and obesity-dependent effects on ozone-induced airway hyperresponsiveness. Understanding the role of the microbiome in obesity-related asthma may allow for the advancement of personalized therapeutics for obese asthmatic population.
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CHAPTER 1: Introduction

Adapted from:


1.1. Obesity and ozone are major public health concerns

Obesity is a global epidemic. Worldwide, the prevalence of obesity has more than doubled since 1980. The World Health Organization estimates that in 2014 more than one half billion adults were obese and another 1.9 billion were overweight. Statistics from the United States Center for Disease Control indicate that more than one third of the U.S. adult population is obese and another third is overweight. Obesity is a known risk factor for type 2 diabetes, hypertension, and atherosclerosis. Obesity has also emerged as a risk factor for asthma (1, 2). Both the prevalence and incidence of asthma are increased in the obese population (3-5). Weight loss causes substantial reductions in asthma symptoms in obese asthmatic patients, and reduces airway hyperresponsiveness (AHR) (6, 7). However, the mechanistic basis for this relationship has yet to be fully elucidated.

Ozone (O$_3$) is a known non-atopic asthma trigger (8, 9). O$_3$ causes airway epithelial cell injury, induces production of inflammatory cytokines and chemokines, and causes neutrophil recruitment (10). O$_3$-induced AHR may be particularly relevant in the obese, since O$_3$ exposure causes damage via oxidative stress, and obesity itself causes systemic oxidative stress (11, 12). In humans, obesity worsens the decrements in lung function that occur following exposure to O$_3$ (8, 9). In mice, O$_3$-exposure causes AHR and neutrophilic inflammation which models the non-atopic asthma of obese patients (13, 14). Furthermore, compared to lean mice, O$_3$–induced AHR and inflammation is augmented in the obese mice (15-19).
1.2. Obesity and asthma

Obesity increases the prevalence and the incidence of asthma, both in children and adults. (20-22). Obesity also appears to be common in severe asthma. Indeed, within the TENOR cohort, a US cohort of severe asthmatics, 30.7% of the children and 69.3% of the adults were obese versus obesity rates of approximately 20% for children and 35% for adults in the general US population (23). Similarly, in the British Thoracic Society Difficult Asthma Registry, a cohort of severe adult asthmatics in the UK, 48.3% were obese, nearly double the obesity prevalence observed in the general adult UK population (25%) (24). The observations that in obese asthmatics, weight loss causes substantial reductions in asthma symptoms, improves asthma control, and reduces airway hyperresponsiveness (AHR) (6, 7) indicate that at least for some obese asthmatics, obesity is not just a co-morbidity but a causal factor. Data from obese mice also support the ability of obesity to promote asthma: obese mice display AHR even in the absence of any other inciting stimulus (25-29).

Many obese asthmatics have difficulty controlling their asthma (30, 31). Indeed, steroids are less effective in obese than lean asthmatics (32). It is possible that aspects of the obese state, for example the low grade systemic inflammation characteristic of obesity, reduce steroid efficacy by interfering with corticosteroid signaling pathways (32). However, it is also possible that obese asthmatics have a phenotype that is not responsive to steroids: steroids target the immune responses typical of allergic asthma, but many obese asthmatics are non-atopic (33, 34). Understanding the mechanistic basis for obese asthma may allow for the development of other therapeutic options that have greater efficacy in this population.
There are sex differences in the prevalence and severity of asthma. Epidemiological studies have shown that asthma prevalence, severity, hospitalizations and mortality are higher among women than men (35, 36). There are also sex differences in obesity-associated asthma. Compared to obese adolescent males, obese adolescent females are at higher risk of developing asthma (37). Obese women also suffer more severe asthma than obese men (38, 39). Compared to non-severe asthmatic women, severe asthmatic women have higher body mass index (BMI), but there is no such difference in BMI between severe and non-severe asthmatic men (40, 41). In addition, there are sex differences in the effect of obesity on responses to O₃ exposure. O₃-induced decrements in lung function correlate with BMI in women, but not in men (42). Thus, current understanding strongly suggests a sex difference in the interaction between obesity and asthma, but it remains unclear which sex-specific factors are responsible for such interactions.

1.3. The gut microbiome in human health

Estimates are that more than 100 trillion bacteria from over a thousand different species colonize the human gastrointestinal (GI) tract. The collective genome of these bacteria (the microbiome) includes at least 150 fold more genes than are present in the human genome and has functional capacities humans lack (43). For example, gut bacteria are capable of metabolizing polysaccharides and dietary starches that are otherwise indigestible. This metabolism results in the production of short chain fatty acids (SCFA), such as butyrate, propionate, and acetate (44, 45) that can then be used by host cells, especially enterocytes and hepatocytes, for ATP synthesis or conversion to triglycerides or glucose. SCFAs also act as signaling molecules resulting in
effects both within and outside the GI tract. Gut microbiota participate in the synthesis and absorption of some vitamins and minerals, the conversion of primary to secondary bile acids, the detoxification of some xenobiotics, and contribute to proper intestinal epithelial functioning (46-54).

After birth, there are marked changes in the human gut bacterial community structure, that vary depending on Caesarian versus vaginal birth, formula versus breast feeding, host genetics, and especially with the introduction of solid food (55). The community structure begins to stabilize by the age of 2-3 years, but can still be substantially impacted by diet, antibiotic use, changes in geography, age, and environmental exposures (55). Consequently, there are marked inter-individual differences in the gut microbiota - differences that are less marked between related individuals but nevertheless greatly surpass intra-individual differences assessed across time (56).

There is increasing evidence that the microbiome plays an important role in asthma (57-59). Prenatal and post-natal uses of antibiotics in humans have been associated with increased risk of asthma development (60, 61), and oral ingestion of various strains of Lactobacillus and ingestion of bacterial products have also been shown to impact allergic pulmonary inflammation (62). In mice, disruption of the microbiota by antibiotic treatment leads to abnormal allergic airway responses (63, 64). Similarly, germ free (GF) mice, mice that are born and raised without exposure to microbes, have augmented allergic airway responses compared to mice raised in conventional specific-pathogen-free (SPF) facilities (65). These data support the hypothesis that
the microbiota shape allergic airway responses. However, the impact of the microbiome on non-allergic forms of asthma, which have different triggers and etiologies, is unknown.

1.4. Sex differences in the gut microbiota

Several groups have reported sex differences in the composition of the gut microbiome. For example, in mice, males have a greater abundance of bacteria of the Actinobacteria and Tenericutes phyla than females (66). Sex differences have also been observed in other organisms, including fish, howler monkeys, and humans (67-69). Sex differences in the gut microbiome can have important functional consequences. Markle et al., utilizing Non-Obese Diabetic (NOD) mice which spontaneously develop type 1 Diabetes (T1D), showed that the incidence of T1D is almost doubled in females compared to males (70, 71). In contrast, sex differences in the incidence of T1D are not observed in GF NOD mice. Furthermore, weanling females given male microbiota by gavage displayed elevated testosterone levels and lower incidence of T1D (72). Microbiota also contribute to lupus, a sex-specific disease affecting more women than men (73). The gut microbiome of lupus-prone mice is different between sexes (74). Lupus-prone males are not significantly different than controls in their gut microbial composition. On the other hand, compared to age- and sex-matched healthy controls, young female lupus-prone mice have fewer Lactobacillaceae and increased numbers of Lachnospiraceae. In addition, dietary intervention restoring Lactobacillaceae in the gut correlates with improved symptoms, and dietary intervention increasing Lachnospiraceae worsens the Lupus symptoms in female mice (74).
1.5. Impact of obesity on gut microbiota

Obesity alters the distal gut microbiota. In mice, both genetic obesity and obesity caused by high fat diet (HFD) feeding increase the ratio of bacteria of the Firmicutes to Bacteroidetes phyla (75-79), the two most abundant phyla in the mammalian gastrointestinal tract. The composition of the diet itself has a profound effect on gut community structure. In mice fed a HFD but food restricted in order to reduce body weight, the gut bacterial community structure is more similar to obese HFD-fed mice with unrestricted access to food than to mice fed a low fat diet ad libitum, even though the latter have body weights more similar to the HFD-fed food-restricted mice (78). The nature of the dietary fat also matters since changes in gut microbiota induced in mice by high fat feeding differ depending on whether the fat is derived from lard versus fish oil (80).

The marked inter-individual variability in the microbial composition of the human gut makes it more difficult to identify obesity-related effects on gut bacterial community structure in humans than in genetically inbred mice (45). Nevertheless, obesity has been shown to reduce the diversity of gut bacteria in human subjects (56, 81). Studies of diet-induced weight loss also indicate that the Firmicutes to Bacteroidetes ratio declines with weight loss in humans (82, 83) consistent with the increased Firmicutes to Bacteroidetes ratio observed in obese versus lean mice. Compared to obese subjects matched for initial weight who did not undergo surgery, obese subjects who have undergone Roux-en-Y gastric bypass surgery for weight loss also exhibit reductions in three species of Firmicutes (Clostridium difficile, Clostridium hiranonis, and Gemella sanguinis) along with increases in gammaproteobacteria (83). Importantly, it may
be the functional capacities of gut bacteria rather than their phylogenetic or taxonomic composition that is important: compositional differences at the taxonomic level do not necessarily result in differences at the functional level (84). Furthermore, key bacteria can have major health effects even when those bacteria are present at such low abundance that even substantial alterations in their prevalence have only a minimal impact on overall phylogenetic ratios (85). In this respect, it is important to note that the obese and lean human gut microbiomes do differ with respect to genes involved in carbohydrate, lipid and amino acid metabolism (56). Similarly, diet induced weight loss in children with genetic obesity (Prader Willi syndrome) also results in changes in microbial genes related to metabolism (86).

1.6. Functional effects of obesity-related changes in gut microbiota

Obesity-related changes in the gut microbiota have important functional consequences for weight gain. Germ free (GF) mice, weigh less than age-, strain-, and gender-matched conventionally raised SPF mice despite greater food consumption and reduced metabolism (87), likely because of reduced microbiota-associated energy harvest from the diet (77, 88). In addition, GF mice gain more weight after transplant with fecal contents from SPF obese versus SPF lean mice (77) or after transplant with fecal contents of obese human subjects versus their lean monozygotic twins (89, 90). In contrast, supplementation of feces from obese subjects with a member of the bacterial family, Christensenellaceae, a family enriched in feces from lean human subjects, results in reduced weight when transplanted into GF mice compared to feces from obese subjects alone (91). Finally, probiotics reduce body mass and body fat in mice with diet-induced obesity (92).
There is less evidence for a role of the gut microbiota in regulation of body weight in human subjects. However, a recent large scale analysis of glycemic responses to a variety of foods in 800 individuals indicated an association between obesity and the gut community structure (93). In addition, a study by Cani et al. (94) noted that administration of a prebiotic (a non-digestible food ingredient that stimulates growth of certain gut bacteria conferring a health benefit to the host) caused increases in several species in the *Bifidobacterium, Lactobacillus, Roseburia,* and *Faecalibacterium* genera, and also caused a significant increase in meal-induced satiety along with increases in the gut derived hormones (GLP-1 and PYY) that are known to cause satiety in humans (94). Similarly, a report from the Nurses’ Health Study indicates that among the various foods associated with weight loss, yogurt has the most profound effect (95). Yogurt is a probiotic, containing live microorganisms that provide health benefits to the host.

While differences in energy harvest from the diet account for at least part of the impact of microbiota on body weight (77, 88), the study of Cani et al. described above (94) indicates that microbes can also impact host eating behavior via effects on satiety hormones. However, some satiety hormones can also impact energy harvest. For example, Samuel et al. (88) noted reductions in the levels of PYY in GF mice versus SPF mice. PYY inhibits gut motility, and reductions in PYY in GF mice were associated with an increased rate of food transit through the intestines and hence less time for energy harvest. With respect to gut bacteria and satiety hormones, it is notable that colonization of GF mice induces central nervous system resistance to leptin (96), a satiety-inducing hormone, while prebiotics that reduce the Firmicutes to Bacteroidetes ratio increase leptin sensitivity (97). The production of metabolites that activate
cannabinoid receptors (which impact eating) is also affected by gut bacteria (98). In addition, taste receptors for fat are altered in the tongues and intestines of GF versus SPF mice (99), indicating that microbes also impact food preferences.

Obesity-related changes in gut bacteria also impact other aspects of the obese phenotype. For example, in mice, antibiotics reverse the insulin resistance caused by high fat feeding (100), suggesting that obesity-related changes in the gut bacterial community structure may contribute to Type 2 Diabetes. Indeed, a small study performed in human subjects, indicates that transfer of gut microbiota from non-diabetic into diabetic individuals results in improved insulin sensitivity (101). Furthermore, individual glycemic responses to a large variety can be predicted by components of the gut microbiome assessed in fecal samples (93). Consistent with these observations, probiotic treatments also impact the insulin resistance associated with obesity in mice (102).

Chronic low grade systemic inflammation is another consequence of obesity. The adipose tissue of obese mice and obese human subjects is infiltrated with activated macrophages producing a variety of pro-inflammatory cytokines and chemokines that spill over into the systemic circulation. Increasing evidence points to a role for the microbiota in these events. For example, HFD feeding results in an increase in the proportion of bacteria containing lipopolysaccharide (LPS, endotoxin) in the gut and alters the permeability of the intestinal epithelium resulting in systemic endotoxemia that contributes to the adipose tissue inflammation. Thus, pro-inflammatory cytokine levels are elevated by HFD in wildtype but not TLR4-deficient or MyD88-deficient mice that lack effective LPS signaling capacities (80, 103, 104). In addition,
treatment with *Akkermansia muciniphila*, bacteria that are typically attenuated in the obese, reverses both endotoxemia and adipose tissue inflammation, in HFD fed mice (105). Other probiotic treatments also affect the systemic inflammation associated with obesity in mice (106). Given that gut microbiome can regulate these obesity-related conditions, it is unsurprising that gut microbiome may also regulate obesity-related effects on the lungs.

1.7. **Obesity-related changes in gut microbial metabolites**

In obesity, there are also profound changes in the metabolome, the set of small molecule metabolites present in a given biological fluid, cell type, or tissue. Importantly, accumulating evidence suggests a key role for the microbiome in such metabolic changes. Whether and how such changes might contribute to obese asthma remains to be established. Both in humans and in rodents, the metabolomes of the liver, serum, urine, and adipose tissue are altered by obesity (107-116). Given that insulin resistance is common in obesity, it is perhaps not surprising that glucose, lactate, glycerol, fatty acids and β-hydroxybutyrate are increased in the blood of obese versus lean subjects. Blood metabolomics also consistently indicate obesity-related alterations in branched-chain amino acid metabolites (116-118). Since receptors for many fatty acids (GPR40, GPR41, GPR49, GPR84, and GPR120), for lactate (HCA₁/GPR81), and for β-hydroxybutyrate (HCA₂/GPR109A) exist (119, 120), and since such small metabolites have the capacity to diffuse across pulmonary capillaries, obesity-related changes in these moieties could impact lung function directly via activation of these receptors.
Gut microbiota metabolize dietary foodstuffs to produce a huge variety of small metabolites that can diffuse across the gut into the circulation, where some are further metabolized by host enzymes resulting in bacterial-mammalian co-metabolites. Thus, gut bacterial derived metabolites can affect not only the GI tract, but also other target organs, leading to the description of the gut microbiota as an endocrine organ (121). For example, trimethylamine, a microbial dependent metabolite derived from dietary choline, is oxidized in the liver to produce trimethylamine N-oxide (TMAO). Serum concentrations of TMAO are linked to atherosclerosis and cardiovascular disease risk (122, 123). Similarly, when the cecal contents of atherosclerosis-prone mice are transplanted into antibiotic-treated mice, the mice develop enhanced choline-diet induced atherosclerosis and TMAO (124). Even the brain is impacted by bacterial metabolites. Serum concentrations of the bacterial dependent metabolite, 4-ethylphenylsulfate (4EPS), are markedly elevated in mouse models of autism. Importantly, treatment with *B. fragilis* in the food reverses these elevations in 4EPS and also improves autism-like behavior (125).

Microbiota affect the metabolomes of the intestines, urine, liver, brain, and kidney (52, 126-133). The blood metabolome is also affected. For example, studies using GF mice and studies using antibiotic treatment indicate effects of gut microbiota on serum levels of many bacterial derived metabolites, including SCFA, pipecolate, choline, phenol sulfate, and hippurate (126, 134, 135). Thus, the altered gut microbiome of obesity may affect metabolites that circulate to the lungs and affect airway function. Below we discuss the role of the microbiota in the generation of two groups of metabolites: SCFAs and bile acids as well as the potential for these metabolites to impact obese asthma.
1.8. Short chain fatty acids

In the GI tract, SCFAs are produced from otherwise indigestible plant-derived polysaccharides via bacterial metabolism. Indeed, compared to SPF mice, GF mice have reduced intestinal SCFAs and excrete significantly more calories in their feces in the form of indigestible polysaccharides (45). Consistent with these observations, colonizing GF mice with *Bacteroides thetaiotaomicron* increases production of SCFAs in GF rodents (136). Similarly, mice fed a high fiber diet have increased gut Bifidobacterium, a bacterium that ferments dietary fiber to form SCFAs, and increased circulating SCFAs (135). Most SCFA production occurs in the distal colon and caecum and both the makeup of the microbiota present and the transit time through the colon impact the amount of SCFAs produced (137).

Data from both humans and mice indicate a relationship between the microbiome, SCFAs, and obesity. SCFAs are higher in the cecal contents of genetically obese mice which have a reduced ratio of Bacteroidetes to Firmicutes and increased fecal SCFAs are also observed in human obesity (137). In twins discordant for obesity, the microbiome of the obese twin is enriched for genes involved in carbohydrate fermentation to SCFAs (56). Given the ability of SCFAs to provide an energy source to the host, one might expect that elevated SCFA production in obesity would contribute to weight gain. Nevertheless, exogenously administered SCFAs actually reduce weight gain in mice (138). The ability of SCFAs to increase the production of satiety hormones, including GLP-1, PYY, and leptin (139, 140), and to increase energy expenditure (141), likely explains this apparent conundrum.
SCFAs have multiple effects that could impact obesity and asthma. First, SCFAs play a role in the regulation of T cells both in the GI tract and in peripheral tissues, by promoting the development of Tregs (142). Reductions of Tregs in adipose tissue are thought to contribute to the systemic inflammation of obesity (143). Second, SCFAs stimulate intestinal epithelial proliferation and differentiation (144) and could also contribute to repair of the epithelial cell damage that is typical of asthma. SCFA-mediated changes in epithelial barrier function could also impact the systemic endotoxemia of obesity. Anti-inflammatory effects of SCFAs could counter the effects of this endotoxemia, since SCFAs inhibit LPS-induced NF-κB activation and increases in TNFα in neutrophils and macrophages (137, 145). Notably, SCFAs produced by the gut microbiota are absorbed into the systemic circulation to be able to reach smooth muscle lining the vasculature, as well as the airways (146).

Although there are as yet no studies of the role of SCFAs in obesity-related asthma, data do suggest a role for the gut microbiome and for SCFAs in particular in modulating allergic asthma. GF mice, which have a reduced ability to produce SCFAs (45), develop greater allergic airways responses than SPF mice (147, 148). Furthermore, both elevations in circulating SCFAs induced by high fiber feeding and exogenously administered propionate protect against house dust mite-induced allergic airways inflammation in mice, and this protective effect is lost in GPR41-deficient mice (135). Similarly, low fiber-fed mice with circulating levels of SCFAs have increased allergic airways responses (135). These effects of SCFAs appear to occur at the level of the bone marrow and dendritic cell (DC) precursors that impact the immune response to allergen. In contrast, DCs are not involved in mediating the effects of non-allergic airway triggers like O₃ (149), and studies on non-atopic diseases involving innate as opposed to adaptive
immune response have shown that SCFAs contribute to worsening of symptom severity: Oral administration of SCFAs in GF mice activated microglia cells and promoted neuroinflammation and motor deficits in a model of Parkinson’s disease (150). Similarly, in a model of antibody-induced arthritis, which does not require adaptive immune response, SCFA treatment resulted in greater cellular infiltration, cartilage and bone destruction, and significant upregulation in inflammatory cytokine TNFα (151).

1.9. Bile acids

Primary bile acids, such as cholate and chenodeoxycholate, are synthesized from cholesterol and conjugated with either glycine (humans) or taurine (mice) in the liver, secreted into bile, and released into the duodenum in response to hormonal signals initiated by eating. In the intestines, bile acids contribute to the digestion of dietary fat by acting as emulsifiers. In the lower GI tract, most of the bile acids are absorbed back into the circulation and returned to the liver where they are taken up and re-excreted (the enterohepatic circulation), though some escape re-uptake and circulate in the systemic blood. The enterohepatic circulation thus prevents loss of cholesterol-containing moieties in the feces.

Gut microbes both modify and are modified by bile acids (83, 152, 153). Gut bacteria deconjugate and dehydroxylate bile acids resulting in the formation of secondary bile acids. Consequently, there is an increase in the ratio of conjugated to unconjugated bile acids in GF and antibiotic-treated mice (154). Bile acids themselves have bactericidal properties, via their detergent properties on bacterial membranes, though certain bacteria (e.g. *Bilophila wadsworthia*)
actually thrive in environments rich in bile acids (155). Thus changes in the release of bile acids, for example in response to high fat diets, can alter the community structure of the gut microbiome. Indeed there are substantial changes in the gut microbiome in rats fed a diet containing high levels of cholate (156).

In addition to their bacteriostatic and emulsifying actions, bile acids also have a signaling role (Fig. 1.1). Bile acid binding to two receptors, farnesoid X receptor (FXR), a nuclear receptor, and to a cell surface G-protein coupled receptor, TGR5, mediate these effects. FXR- and TGR5-mediated signaling events contribute to beneficial effects of bile acids against obesity and obesity-related conditions (157): bile acids increase energy expenditure through TGR5-mediated changes in thyroid hormone synthesis. Bile acids also cause TGR5-dependent secretion of the gut derived hormone PYY, which causes satiety (158). In the liver, bile acid-induced activation of FXR results in reduced fatty acid synthesis and decreases circulating triglycerides (157). FXR activation also inhibits hepatic gluconeogenesis. Indeed, FXR-deficient mice are insulin resistant (159). A major role for a bile/microbiome axis in obesity was also revealed by a study showing that GF mice receiving the fecal microbiota from an obese twin displayed not only a greater fat mass than mice receiving gut microbes from the lean twin but also reduced levels of several bile acids and reduced FXR-dependent gene transcription in the ileum and the liver (89).
Figure 1.1. Bile acid modification in obesity and obesity-related asthma. Schematic representation of ways in which bile acid modification in obesity may impact obesity-related asthma. FXR: farnesoid X receptor; TGR5: Takeda G protein-coupled receptor 5.
Bariatric surgery results in increased circulating bile acids (152) and in mice with HFD-induced obesity, these bile acids appear to mediate reduced eating, subsequent weight loss, and improved glucose tolerance, since these effects are reduced in FXR-deficient mice (160). Bariatric surgery alters gut microbial communities and there were also differential effects of bariatric surgery on the gut microbiome in FXR versus wildtype mice (160). Bariatric surgery also improves obesity-associated asthma (7), but whether or not bile acids- and/or microbiome-mediated changes are involved in these events remains to be determined. However, it is interesting to note that a recent metabolomic profiling of plasma from asthmatics versus healthy human subjects identified bile acids (taurocholate and glycodeoxycholate) among the metabolites that were affected by asthma (161).

Bile acids also have anti-inflammatory effects that may be relevant for asthma (162). For example, in macrophages, bile acid-induced TGR5 activation causes elevations in cAMP that inhibit NF-κB -mediated induction of pro-inflammatory cytokines by LPS (163). The observation that TGR5 agonists attenuate atherosclerosis in TGR5-sufficient, but not TGR5-deficient mice (164), indicates that there are important functional consequences of such anti-inflammatory effects of bile acids.

Finally, bile acids, via TGR5 signaling, promote relaxation of gastric smooth muscle (165). TGR5 dependent activation of GPCRs and consequent increases in cAMP mediate this relaxation. Since airway smooth muscle also relaxes in response to elevations in cAMP, it is conceivable that changes in bile acids could also impact the bronchoconstriction of asthma.
1.10. Conclusion

Obesity alters the microbiota in the gastrointestinal tract. Such changes could play a role in obesity-related asthma via alterations in production of metabolites with effects in the lung such as SCFAs, by contributing to the systemic inflammation of obesity, via changes in the host metabolism, insulin sensitivity, and even feeding behavior, and by changes in the immune system, especially IL-17A+ immune cells. It is also conceivable that obesity alters both the gut and the lung microenvironment to impact asthma development and or therapeutic responses (Fig. 1.2)
Figure 1.2. Possible role of the obese microbiota in asthma development. Schematic representation of ways in which altered gut (and possibly lung) microbiota in obesity might lead to augmented ozone response and asthma in the obese.

The hypothesis that obesity-related alterations in gut and/or lung microbiota contributes to obesity-related asthma has several important therapeutic implications. It may be that host immune system development can be shaped by factors affecting the developing gut microbial
communities, including early life choices and exposure to environmental bacteria. It is also feasible that altering the gut microbiota could ameliorate obesity-related asthma. Probiotic and prebiotic treatments have been shown to be effective against other obesity-related conditions (166-168). Transplantation of microbiota from healthy to diseased individuals is increasingly used for treatment of some intestinal illnesses, especially Clostridium difficile infection (169), (170), and has even been shown to increase insulin sensitivity in patients with metabolic syndrome (101). Understanding the role of gut and airway microbiota in obesity-related asthma could pave the way for development of microbiota-based treatments for this difficult to treat group.

This thesis aims to examine the hypothesis that obesity-related changes in the gut microbiome may contribute to the etiology of obese asthma. Because of the largely non-atopic nature of obese asthma, we used a mouse model in which changes in airway responsiveness, a canonical feature of asthma, were induced by a non-atopic asthma trigger, O₃. In chapter 2, we establish that in lean male mice the microbiome contributes to O₃-induced AHR, likely via its ability to produce short chain fatty acids. In chapter 3, we explore the sex differences in the microbiome and how they contribute to the differences in O₃-induced AHR between lean male and female mice. In chapter 4, we examine the role of the obese microbiome, and how the intersection between obesity, sex, and the microbiome contributes to the augmented O₃ response observed in obese mice. Lastly, in chapter 5, we summarize these findings and draw conclusions on the role of the microbiome at the nexus of obesity, sex, and asthma. We also discuss the implications of our findings for the development of personalized therapeutics.
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CHAPTER 2: The microbiome regulates pulmonary responses to ozone in mice

This work is currently in submission to *Environmental Health Perspectives*.

Youngji Cho performed all laboratory work and created figures 2.1-2.3, 2.5, and 2.6.

Galeb Abu-Ali performed the 16S analysis and created figure 2.4.
The microbiome regulates pulmonary responses to ozone in mice

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2.1. **ABSTRACT**

Previous reports indicate that gut microbiota impact airway responses in mouse models of atopic asthma. The purpose of this study was to examine the role of gut microbiota in a mouse model of non-atopic asthma induced by acute ozone exposure. We depleted the microbiota of conventional mice with either a single antibiotic (ampicillin, metronidazole, neomycin, or vancomycin) or a cocktail of all four antibiotics given via the drinking water, and assessed ozone-induced airway hyperresponsiveness (a canonical feature of asthma) and inflammatory markers. In air-exposed mice, airway responsiveness was not different in antibiotic- versus water-treated mice. In ozone-exposed mice, airway hyperresponsiveness and increases in bronchoalveolar lavage neutrophils observed in water-treated mice were attenuated with antibiotic cocktail treatment. Ozone-exposed germ-free mice also had attenuated airway responsiveness and neutrophil recruitment compared to ozone-exposed conventional mice. Mice treated with single antibiotics had attenuations similar to the cocktail-treated mice except for mice treated with neomycin, which were not different from water-treated mice. Analysis of 16S rRNA gene sequence data from fecal DNA indicated two bacterial genera that were decreased in those mice with reduced ozone-induced airway hyperresponsiveness after antibiotic treatment: *Ruminococcus* and *Coprococcus*. Both are short chain fatty acid producers. Serum analysis indicated reduced concentrations of the short chain fatty acid, propionate, in cocktail antibiotic-treated but not neomycin-treated mice. Furthermore, propionate supplementation of the drinking water resulted in augmented ozone-induced airway hyperresponsiveness in conventional mice. Our data indicate that the microbiome contributes to ozone-induced airway hyperresponsiveness, likely via its ability to produce short chain fatty acids.
2.2. INTRODUCTION

The air pollutant, ozone (O\textsubscript{3}), is a major public health concern worldwide. O\textsubscript{3} exposures cause dyspnea, cough, decreased lung function, susceptibility to lung infections, pulmonary inflammation, and increased asthma exacerbations (1-3). Indeed, the number of hospital admissions for asthma increases on days after high ambient O\textsubscript{3} concentrations (4). O\textsubscript{3} causes airway epithelial cell injury, induces production of inflammatory cytokines and chemokines, and causes neutrophil recruitment (5). Importantly, O\textsubscript{3} also causes airway hyperresponsiveness (AHR) (6), a canonical feature of asthma.

There is increasing evidence that the microbiome plays an important role in asthma (7-9). Asthmatic patients harbor different microbes in their lungs and sputum compared to healthy humans (9, 10). In addition, children whose guts are colonized with Helicobacter pylori are 40\% less likely to have childhood-onset asthma compared to children who are not colonized (11). Prenatal and post-natal uses of antibiotics in humans have been associated with increased risk of asthma development (12, 13), and oral ingestion of various strains of Lactobacillus and ingestion of bacterial products have also been shown to impact allergic pulmonary inflammation (14). In mice, disruption of the microbiota by antibiotic treatment leads to abnormal allergic airway responses (15, 16). Similarly, germ free (GF) mice have augmented allergic airway responses compared to mice raised in conventional specific-pathogen-free (SPF) facilities (17). Taken together, these data support the hypothesis that the microbiota shape allergic airway responses. However, the impact of the microbiome on non-allergic forms of asthma, which have different triggers and etiologies, is unknown.
The purpose of this study was to examine the hypothesis that the microbiome contributes to the asthma-like phenotype of mice exposed to O₃. To do so, we compared O₃-induced AHR in SPF versus GF mice and in control mice versus mice treated with either individual antibiotics (ampicillin, metronidazole, neomycin, and vancomycin) or the combination of these antibiotics. O₃-induced AHR was attenuated in germ free mice, in mice treated with the antibiotic cocktail, and in mice treated with all individual antibiotics except neomycin. 16S sequencing of fecal DNA identified two genera that were associated with O₃-induced AHR, *Ruminococcus* and *Coprococcus*, both known short chain fatty acid (SCFA) producers. SCFAs are majorly produced by anaerobic gut bacterial fermentation of fiber, and have been implicated in the role of the microbiome in various diseases including allergic asthma, insulin sensitivity, and colitis (18-21). Serum analysis indicated reduced concentrations of the SCFA, propionate, in mice with reduced O₃-induced AHR. Furthermore, propionate supplementation of the drinking water resulted in augmented O₃-induced AHR. Our data suggest that the microbiome is required for O₃-induced AHR, likely via its ability to produce SCFAs.

2.3. METHODS

*Animals.* All protocols were approved by the Harvard Medical Area Standing Committee on animals. For experiments with antibiotic or propionate treatment, seven week old male C57BL/6 mice were purchased from Taconic Farms (New York, NY) and acclimated to the Harvard T. H. Chan School of Public Health SPF (specific pathogen free) facility for at least one week prior to the initiation of treatment. For comparison of GF versus conventionally raised SPF mice, we
used male C57BL/6J GF mice raised in gnotobiotic facilities at the Harvard Digestive Diseases Center Gnotobiotics, Microbiology, and Metagenomics core (Boston, MA) and compared them to C57BL/6J mice from The Jackson Laboratories (Bar Harbor, ME). Mice were 8 weeks old at the start of antibiotic administration and 10 weeks old at the time of O₃ exposure.

**Protocol.** Four cohorts of mice were used. In the first cohort, mice were given a cocktail of antibiotics (AMNV: Ampicillin 1g/L, Metronidazole 1g/L, Neomycin 1g/L, Vancomycin 0.5 g/L) by addition to the drinking water for two weeks. Sucralose (8 g/L) was added for taste. Ampicillin is a β-lactam class drug which targets mostly gram positive and some gram negative bacteria. Metronidazole is a nitroimidazole which targets anaerobic bacteria. Vancomycin is a narrow-spectrum glycopeptide which targets gram positive bacteria such as methicillin-resistant *Staphylococcus aureus*. Neomycin is an aminoglycoside drug which targets gram negative aerobic bacteria. This type of antibiotic treatment protocol has been reported to result in an extremely marked reduction in the gut bacteria load as assessed DNA concentration in fecal pellets (22). Control mice were given regular drinking water with sucralose. After two weeks of treatment, mice were exposed to room air or to O₃ (2 ppm for 3 h). Mice were anesthetized for the measurement of pulmonary mechanics and airway responsiveness to inhaled aerosolized methacholine (1 to 100 mg/mL) 24 hours after exposure. Following these measurements, mice were euthanized, blood was collected for the preparation of serum, and bronchoalveolar lavage (BAL) was performed. In the second cohort, GF mice were administered the same cocktail of antibiotics or water for two weeks inside the isolator cages. After two weeks, they were taken out of the isolators, immediately exposed to O₃, and evaluated 24 h later as described above. In the third cohort, mice were treated either with the AMNV cocktail or with each antibiotic given
individually for two weeks, exposed to air or O₃, and evaluated as described above. Again, control mice were treated with drinking water with sucralose supplementation only. Fecal samples were collected from each mouse one day prior to exposure. In the fourth cohort, mice were given sodium propionate (200 mM) in the drinking water for 3 days. Control mice were given saline (50 mM) in the drinking water, as described by others (18).

**O₃ exposure.** During exposure, mice were placed in individual wire mesh cages within a stainless steel and Plexiglas chamber and exposed to either room air or O₃ (2 ppm) for 3 hours as previously described (23). Food and water were withdrawn during exposure and replaced immediately afterwards.

**Pulmonary Mechanics and Airway Responsiveness.** Mice were anesthetized and instrumented for pulmonary mechanics measurement as previously described (24). A positive end expiratory pressure of 3 cm H₂O was applied. Three volume excursions to total lung capacity (30 cm H₂O trans-respiratory system pressure), each separated by one minute, were first administered to establish a common volume history. The following sequence was then initiated. One minute after an excursion to total lung capacity, 10 breaths from an aerosol generated from PBS were administered. Total respiratory system resistance (Rₛ) was then measured by the forced oscillation technique every 15 seconds for the next 3 minutes (25). This sequence was then repeated with aerosolized methacholine chloride increasing in concentration from 1 to 100 mg/mL.
**Blood Collection and Bronchoalveolar Lavage.** Blood was collected from the heart via cardiac puncture. Serum was isolated and frozen at -80° C until analyzed for SCFAs. BAL was performed, and total BAL cells and differentials assessed as previously described (24). BAL supernatants were stored at -80°C until analyzed for total protein concentration and for IL-17A and osteopontin. BAL protein concentration was assessed using the Bradford Protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. BAL IL-17A and osteopontin were determined by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturers' instructions.

**16S Sequencing.** Fecal samples from mice were collected on the day before exposure. After a one-day incubation at 56° C with proteinase K, fecal DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Germantown, MD) using ~25 mg of feces DNA was adjusted to a concentration of 20 ng/μL using a NanoDrop (ThermoScientific, Wilmington, MA) and sent to the Forsyth Institute (Cambridge, MA) for 16S sequencing and analysis. DNA was PCR-amplified using the V3-V4 hypervariable region primers (~341F (forward primer) AATGATACGGCGACCACCGAGATCTACACTATGGTAAATTGTCTACTACGGGAGGCAGCAG ~806R (reverse primer) CAAGCAGAAGACGGCATACGAGATNNNNCCAGTGACGTCTACGGGACTACHVGGGTWTCTAAT) and 5 PrimeHotMaster Mix and purified using AMPure beads. 100 ng of each library was pooled, gel-purified, and quantified using a bioanalyzer and subsequently with PCR. 12 pM per sample of the sequencing library mixture (fragments) together with 20% Phix were sequenced on the MiSeq machine (Illumina, San Diego, CA). Reads were clustered into operational taxonomic units (OTUs) using the UPARSE protocol (26), and taxonomic
classification of OTUs was performed against the Greengenes version 13.8 16S rRNA gene database (27). Differential abundance and association with experimental variables was assessed using the Multivariate Association with Linear Models (MaAsLin) statistical framework (28). Association was considered significant if q-value (p-value corrected using the Benjamini-Hochberg correction method) < 0.25.

**Short Chain Fatty Acid Analysis.** 50 uL of serum was transferred to a glass tube (15x75mm) and diluted to 100 uL with 50 uL HPLC grade H$_2$O. 200 uL of a solution of 30 mM hydrochloric acid plus isotopically-labeled acetate (150uM), butyrate (10uM), and hexanoate (2uM) in water was added to each sample and the vortexed for 10 seconds. 300 uL of methyl tert-butyl ether (MTBE) was added to each sample, vortexed for 10 seconds to emulsify, held at 4 °C for 5 mins, then vortexed again for 10 seconds. Samples were centrifuged for 1 min to separate the solvent layers and the MTBE layer was then removed to an autosampler vial for GC-MS analysis. 10 uL of MTBE was removed from each sample and pooled in a separate autosampler vial for quality control. A series of calibration standards were prepared along with samples to quantify SCFAs. GC-MS analysis was performed on an Agilent 69890N GC -5973 MS detector (Santa Clara, CA). Data were processed using MassHunter Quantitative analysis version B.07.00 (Agilent, Santa Clara, CA). SCFAs were normalized to the nearest isotope labeled internal standard and quantitated using 2 replicated injections of 5 standards to create a linear calibration curve with accuracy better than 80% for each standard.

**Statistical Analysis.** Except for microbial community analysis (see above), the significance of differences between groups was assessed using ANOVA (for SCFAs) or factorial ANOVA
combined with LSD Fisher post-hoc analysis (Statistica Software, Tulsa, OK) using treatment and exposure as main effects. Student’s t-test was used for the GF mice and the exogenous propionate experiment. A p value < 0.05 (two-tailed) was considered significant. All values are expressed as mean ± standard error of mean.

2.4. RESULTS

2.4.1. Treatment with an antibiotic cocktail reduces responses to O₃.

To deplete their gut microbiome, mice were administered an antimicrobial cocktail (AMNV: Ampicillin, Metronidazole, Neomycin, Vancomycin) by addition to the drinking water for two weeks. Treatment with the AMNV cocktail did not affect body weight (Appendix Fig. 1A). After exposure to room air, airway responsiveness to methacholine was not different in mice given regular drinking water versus mice treated with the AMNV cocktail. In water-treated mice, O₃ challenge resulted in increased airway responsiveness, bronchoalveolar lavage (BAL) neutrophils, and BAL protein, a marker of damage to the alveolar/capillary barrier (29) (Fig. 2.1), which is consistent with previous reports (29-33). Compared to water-treated mice, O₃-exposed AMNV-treated mice had significantly reduced airway responsiveness (Fig. 2.1A), and lower BAL neutrophils (Fig. 2.1B), but AMNV treatment did not impact O₃-induced increases in BAL protein (Fig. 2.1C). The cytokines, IL-17A and osteopontin, have each been reported to play a role in O₃-induced AHR in mice (30, 31). However, while there was a significant increase in BAL concentrations of both cytokines in O₃- versus air-exposed mice, cytokine levels were not different in water-treated versus antibiotic-treated mice (Appendix Fig. 2). Thus, the
observed microbiome-dependent effects on O₃-induced AHR are not mediated by differences in these cytokines. Together, these findings implicated the gut microbiome in the development of AHR and neutrophilic inflammation in the lung after O₃ exposure, without affecting lung barrier injury.

Figure 2.1. An antibiotic cocktail attenuates O₃-induced AHR and neutrophil recruitment. Mice were treated with a cocktail of four antibiotics via their drinking water (ampicillin 1g/L, metronidazole 1g/L, neomycin 1g/L, vancomycin 0.5 g/L) [AMNV]. Sucralose (8 g/L) was added for taste. Control mice were given regular drinking water with sucralose. Shown are A) airway responsiveness to methacholine, B) BAL neutrophils and C) BAL protein, a marker of lung barrier injury, 24 hours after exposure to air or O₃ (2 ppm for 3 h). Rs: respiratory system resistance. Results are mean ± SE of data from n=6-8 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to water.

2.4.2. Germ-free mice have attenuated responses to O₃.

To confirm that it was indeed the microbial perturbation and not an off-target effect of the antibiotic treatment that dampened responses to O₃ (Fig. 2.1A, B), we next contrasted the responses of GF and SPF mice exposed to O₃. Body weights of SPF and GF mice were not different (Appendix Fig. 1B). Compared to SPF mice, GF mice had reduced airway responsiveness after O₃ exposure (Fig. 2.2A). BAL neutrophils, but not BAL protein, were also significantly reduced in O₃-exposed GF versus SPF mice (Fig. 2.2B, C). To further evaluate the
possibility of non-specific effects of the AMNV cocktail, we compared GF mice treated with drinking water to other GF mice that were given the AMNV antibiotic cocktail for two weeks before O₃ exposure. Body weight, airway responsiveness, BAL neutrophils, and BAL protein were not different in water-treated versus antibiotic-treated GF mice exposed to O₃ (Appendix Fig. 1C, Fig. 2.D-F). Together, these findings indicate that a decrease in the microbial burden, whether by antibiotic treatment or by gnotobiotic conditions, results in an attenuation of the response to O₃.

Figure 2.2. Germ-free (GF) mice have reduced responses to O₃ compared to specific-pathogen free (SPF) mice. A) Airway responsiveness, B) BAL neutrophils, and C) BAL protein in O₃-exposed GF mice and age- and sex-matched SPF mice treated with water. Also shown are D) airway responsiveness, E) BAL neutrophils, and F) BAL protein in O₃-exposed GF mice treated with water versus the antibiotic cocktail [AMNV]. Rrs: respiratory system resistance. Results are mean ± SE of data from n=8 per group. * denotes p<0.05 compared to GF.
2.4.3. **Ampicillin, metronidazole, and vancomycin, but not neomycin, attenuate O$_3$-induced AHR.**

As described above, AMNV-treated SPF mice had reduced airway responsiveness compared to water-treated mice after O$_3$ exposure. To narrow down possible microbial taxa involved in modulating airway responsiveness, we measured airway responsiveness in SPF mice treated for 2 weeks either with AMNV or with individual antibiotics. None of the antibiotics alone or in combination had any effect on body weight (Appendix Fig. 1D) or on airway responsiveness of air-exposed mice (Fig. 2.3A). Compared to water treatment, AMNV treatment reduced O$_3$-induced AHR (Fig. 2.3A), similar to results described above (Fig. 1). Treatment with ampicillin, metronidazole, or vancomycin also resulted in reduced O$_3$-induced AHR (p<0.02 in each case), whereas mice treated with neomycin responded similarly to the water-control group (p=0.83) (Fig. 2.3A). These findings eliminate microbial clades that are affected by neomycin as candidate modulators of non-allergic AHR.
Figure 2.3. Ampicillin, metronidazole, and vancomycin but not neomycin treatment attenuate O₃-induced AHR. Mice were treated with water, an antibiotic cocktail [AMNV], or individual antibiotics (ampicillin 1g/L, metronidazole 1g/L, neomycin 1g/L, vancomycin 0.5 g/L). Shown are A) Respiratory system resistance [Rrs] at 30 mg/mL methacholine and B) BAL neutrophils after air or O₃ exposure. Results are mean ± SE of data from n=6-8 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to water.
2.4.4. *Ruminococcus* and *Coprococcus* genera are depleted in mice with attenuated O$_3$-induced AHR.

To determine the effect of antimicrobials on the murine gut microbiome, we conducted microbial community 16S rRNA gene amplicon surveys from mouse stool collected with or without antimicrobial treatment (see Methods). Statistical association testing of taxonomic profiles and treatment variables demonstrated that both the cocktail and individual antibiotic treatments resulted in significant perturbations in gut microbial ecology (Table 2.1, Appendix Fig. 3). Consistent with other reports (34), several members of Firmicutes, Bacteroidetes, Actinobacteria, Deferribacteres, and Tenericutes phyla were depleted by the antibiotic treatments, with concurrent enrichment for $\gamma$-Proteobacteria clades (Table 2.1, Appendix Fig. 3). The phylum-level relative abundances of mice given water and neomycin were quite similar (Appendix Fig. 3B) but there were significant effects of the neomycin treatment at the genera-level (Table 2.1), including *Anaeroplasma* spp, *Anaerostipes* spp, and *Ruminococcus gnavus*, which indicated the overall antibiotic efficacy of the neomycin dose administered. Since our data showed that all antibiotic treatments except neomycin attenuated O$_3$-induced AHR (Fig. 2.3A), we therefore investigated taxa that were significantly affected by AMNV, ampicillin, metronidazole, and vancomycin treatments, but not by neomycin treatment. Relative to water-treated mice, *Ruminococcus* and *Coprococcus* genera of the Clostridiales order, were reduced in AMNV-, ampicillin-, metronidazole-, and vancomycin- treated but not neomycin-treated mice (Fig. 2.4, Table 2.1), suggesting that these two genera may be involved in the development of O$_3$-induced AHR.
Table 2.1. Bacterial taxa significantly altered by one or more antibiotic treatments.
Operational taxonomic units (OTU) significantly altered by combined or individual antibiotic treatments compared to water. Columns contain the regression coefficients from the linear model derived from multivariate statistical framework (MaAsLin), which calculates significant associations of each individual taxon between antibiotic treatments. If the coefficient is negative then the abundance of microbiota following treatment decreased compared to water treatment. Association was considered significant if q-value (p-value corrected using the Benjamini-Hochberg correction method) < 0.25.

<table>
<thead>
<tr>
<th>OTU</th>
<th>AMNV</th>
<th>Significant Associations (versus Water)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinobacteria (phyla) Actinobacteria (class) Actinomyccetales (order):</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micrococcaceae (family) <em>Rothia (genus) dentocariosa (species)</em></td>
<td>-0.011</td>
<td>-0.0128</td>
</tr>
<tr>
<td><em>Bacteroides (phyla) Bacteroidia (class) Bacteroidiales (order):</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroidaceae (family) <em>Bacteroides (genus)</em></td>
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<td>-0.174</td>
</tr>
<tr>
<td>Bacteroidaceae (family) <em>Bacteroides (genus) uniformis (species)</em></td>
<td>0.0667</td>
<td>0.0534</td>
</tr>
<tr>
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<td></td>
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<tr>
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<td>0.00494</td>
</tr>
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2.4.5. Role of short chain fatty acids.

*Ruminococcus* and *Coprococcus* genera are notable fermenters of non-digestible carbohydrates, a process which yields SCFA end products, primarily acetate, propionate, and butyrate (35).

SCFAs have previously been implicated in allergen-induced AHR (18). Hence, we compared serum SCFA in water-, AMNV-, and neomycin-treated mice. Serum propionate was reduced in...
AMNV-treated but not neomycin-treated mice (Fig. 2.5A). A similar trend was observed for serum acetate, but not butyrate (Appendix Fig. 4). The results suggested that microbiota-dependent changes in SCFAs, particularly propionate, might be contributing to the observed effects of antibiotics on O₃-induced AHR. To determine whether propionate has the capacity to impact responses to O₃, we administered exogenous propionate to SPF mice for 3 days via their drinking water followed by O₃ exposure. Propionate treatment did not affect body weight (Appendix Fig. 1E). Airway responsiveness was significantly greater in propionate-treated versus saline treated (control) mice exposed to O₃ (Fig. 2.5B), whereas BAL neutrophils were not affected by propionate treatment (Fig. 2.5C). Together, our results suggest that SCFA produced by the microbiota, are involved in the development of O₃-induced AHR in mice.

**Figure 2.5. Role of propionate in O₃-induced AHR.** A) Serum propionate levels in water-, AMNV-, and neomycin-treated mice after air exposure. * denotes p<0.05 compared to water. # denotes p<0.05 compared to Neomycin. B) Airway responsiveness and C) BAL neutrophils of saline-treated versus propionate-treated mice exposed to O₃. Results are mean ± SE of data from n=5-7 per group. * denotes p<0.05 compared to water.
2.5. DISCUSSION

Our data indicate that both GF mice (Fig. 2.2) and mice treated with certain types of antibiotics (Fig. 2.1, 2.3) have reduced O₃-induced AHR and inflammation. These data indicate that microbiota play a significant role in the pulmonary response to acute O₃ exposure. Importantly, only antibiotics that caused a significant reduction in two SCFA-producing genera, Ruminococcus and Coprococcus (Fig. 2.4), were effective in reducing responses to O₃ (Fig. 2.3). Taken in conjunction with our observations that antibiotic treatment reduced serum SCFA and that exogenous administration of the SCFA propionate, augmented responses to O₃ (Fig. 2.5), our data suggest that the role of microbiota is mediated through their ability to produce SCFAs.

16S sequencing of fecal DNA indicated that the antibiotic treatment doses used were effective in reducing their target bacteria (Table 2.1). Gram-positive bacteria such as Coprococcus genus and Ruminococcus gnarus were significantly reduced with ampicillin and vancomycin treatments. Neomycin reduced gram-negative bacteria such as the Anaeroplasma genus in the Tenericutes phyla, while metronidazole treatment reduced many of the taxa in the anaerobic Clostridiales order.

It is possible that the ability of antibiotics to attenuate responses to O₃ was due to the antibiotics affecting bacteria in the lung rather than the gut. Such an explanation could account for the lack of efficacy of the neomycin treatment on O₃-induced AHR (Fig. 2.3A), since after oral administration, both ampicillin and metronidazole are able to cross the gut epithelium, circulate systematically, and hence impact lung microbiota, whereas neomycin is not (36). However,
vancomycin does not cross the gut epithelium after oral administration either (36), but was as effective as ampicillin or metronidazole in reducing O₃-induced AHR (Fig. 2.3A). Instead, the lack of a significant effect of neomycin versus the other antibiotics likely stems from differences in the bacteria targeted by these antibiotics. Of the bacterial taxa affected by antibiotics (Table 1), only two, Ruminococcus and Coprococcus, were significantly reduced by each of the antibiotic treatments that reduced O₃-induced AHR (AMNV, ampicillin, metronidazole, and vancomycin) treatments but not by neomycin treatment (Fig. 2.4). A similar trend was observed with a third SCFA-producing genus, Clostridium (Appendix Fig. 2.5F), a genus within the same family as Ruminococcus and Coprococcus, whereas other known SCFA-producing OTUs did not show a correlation between antibiotic treatments and attenuation of O₃-induced AHR (Appendix Fig. 5).

Within the mouse gut microbiome, Ruminococcus and Coprococcus are two of the most abundant genera in the Clostridiales order (35). They are well-known plant degraders, fermenting complex carbohydrates into SCFAs such as acetate, propionate, and butyrate (35). Indeed, our data suggest that microbiota-derived SCFAs may be contributing to the observed role of the microbiome in responses to O₃: serum propionate was reduced in AMNV- but not neomycin-treated mice, similar to the impact of these treatments on O₃-induced AHR (Fig. 2.5A). Consistent with this hypothesis, propionate-treated mice had augmented AHR after O₃ exposure (Fig. 2.5B).

Whereas SCFAs appear to augment AHR-induced by O₃ (Fig. 2.5), others have reported that increased circulating levels of SCFAs (secondary to a high fiber diet) attenuate AHR induced by
allergen (18, 20). The reason for these disparate effects of SCFAs likely lies in the different factors driving O$_3$-induced versus allergen-induced AHR. Trompette et al (18) showed that the ability of SCFAs to attenuate allergen-induced AHR was the result of reduced dendritic cell (DC) hematopoiesis leading to a diminished type 2 T helper cell response. In contrast, DCs do not appear to be involved in mediating the effects of O$_3$ (37).

We do not know exactly how SCFAs affect O$_3$-induced AHR. The role of SCFAs in other health outcomes is thought to be mediated by several mechanisms. SCFAs in the gut release intestinal hormones like GLP1 and PYY (38) and activate vagal afferents (39) either of which could affect the lungs. SCFAs also activate the sympathetic nervous system (40), and sympathetic activation has been shown to cause metabolic changes that augment O$_3$-induced inflammation and injury (41) Finally, Aisenberg et al. has recently shown that SCFAs affect airway smooth muscle cytoskeletal remodeling (42), but whether such effects could promote AHR remains to be established.

Antibiotics and germ free conditions attenuated not only O$_3$-induced AHR, but also O$_3$-induced increases in BAL neutrophils (Figs. 2.1-2.3). However, the mechanistic bases for the effects of the microbiome on AHR and neutrophils appear to differ. Whereas, microbiota-dependent changes in SCFAs, especially propionate, appear to account for the effects of microbiome on O$_3$-induced AHR (Fig. 2.5B), propionate had no effect of O$_3$-induced changes in BAL neutrophils (Fig. 2.5C). Others have reported that antibiotic treatment of mice causes a reduction in granulocytosis that is restored by administration of TLR ligands like LPS (43) and it is
conceivable that decreases in O₃-induced neutrophil recruitment caused by antibiotics (Figs. 2.1B, 2.3B) or germ free conditions (Fig. 2.2B) are similarly mediated.

In summary, our study indicates a role for the gut microbiome in pulmonary responses to acute O₃ exposure. Our findings also suggest that two genera, *Ruminococcus* and *Coprococcus*, contribute to these effects, likely via their capacity to produce SCFAs. Better understanding of the relationship between the microbiome and asthma could lead to new diagnostic biomarkers for asthma and novel therapeutics, including probiotics and prebiotics.
2.6. REFERENCES


CHAPTER 3: Sex differences in pulmonary responses to ozone in mice: role of the microbiome

This work is currently unpublished.

Youngji Cho performed all laboratory work and created figures 3.1, 3.2A, 3.4-3.15, and 3.18.

Galeb Abu-Ali performed the 16S analysis and created figure 3.2B, 3.3, 3.16, and 3.17.
3.1. ABSTRACT

There are sex differences in the prevalence of asthma. Pulmonary responses to ozone, a common asthma trigger, also differ by sex. However, the mechanistic basis for these sex differences remains largely unknown. In Chapter 2, we reported data indicating that the gut microbiome contributes to responses to ozone in male mice. There are sex differences in gut microbiota and ozone-induced airway hyperresponsiveness is greater in male than in female mice. The purpose of this study was to examine the role of the gut microbiome in these sex differences. To do so, we characterized the gut microbial ecology of male and female littermate mice by 16S sequencing of fecal DNA. We then depleted the gut microbiota with a cocktail of antibiotics, exposed the mice to ozone and assessed airway responsiveness. Responses to ozone were also assessed in female mice housed in cages conditioned by male or female mice. Compared to female mice, male mice had greater airway hyperresponsiveness to ozone. Antibiotic treatment attenuated airway response in males and increased airway response in females, abolishing sex-differences. We also placed female mouse pups, at weaning, into cages that were conditioned by adult male or female mice. After four weeks, female weanlings that were raised in cages previously occupied by adult males developed greater ozone-induced airway hyperresponsiveness than female weanlings raised in cages previously occupied by adult females. Thus, exposing female mice to a male microbiome reproduced the male pattern of greater airway response to ozone. Our data indicate that the mechanistic basis for ozone-induced airway hyperresponsiveness and inflammation may be different in males and females due to differences in their gut microbial composition.
3.2. INTRODUCTION

Ozone (O$_3$), a common air pollutant that causes powerful respiratory irritation, is a global public health issue. Exposure to O$_3$ causes coughing, shortness of breath, decreased lung function, susceptibility to lung infections, and pulmonary inflammation (1-3). O$_3$ also induces airway hyperresponsiveness (AHR), a canonical feature of asthma (4, 5). Indeed, O$_3$ is a trigger for asthma. Hospital admissions for asthma increase on days after high ambient O$_3$ concentrations (6) and in children, exposure to O$_3$ causes asthma symptoms even at concentrations below the EPA standard (7).

There are sex differences in the prevalence and severity of asthma. Epidemiological data show that asthma prevalence, severity, hospitalizations and mortality are higher among women than men; however, asthma-related emergency room visits and hospitalizations are higher among boys than girls before puberty (8-10). There are also sex differences in the pulmonary response to O$_3$ (11-13). In mice, after acute O$_3$ exposure, bronchoalveolar lavage (BAL) neutrophils and acute phase cytokines/chemokines are greater in females than males. In addition, in mice exposed to O$_3$, females have greater infection-induced lung inflammation, lower macrophage function, and higher mortality than males (14, 15). In contrast, there are no data on sex differences in O$_3$-induced AHR. However, sex differences in the innate airway responsiveness of male and female mice have been reported: males exhibit greater innate airway responsiveness than females (16).

To determine whether there were also sex differences in O$_3$-induced AHR, we exposed male and female C57BL/6 mice to room air or to O$_3$ (2 ppm for 3 hrs). Our data indicate that male mice have substantially greater O$_3$-induced AHR than female mice.
We have previously reported that antibiotic treatment reduces O₃-induced AHR in male mice (see Chapter 2). Consistent with these observations, O₃-induced AHR was also reduced in male germ-free (GF) versus conventional mice. Together, the data indicate a role for the microbiome in pulmonary responses to O₃, at least in lean male mice. Several groups have reported sex differences in the composition of the gut microbiome. For example, in mice, males have a greater abundance of bacteria of the Actinobacteria and Tenericutes phyla than females (17). Sex differences have also been observed in different types of fish, in howler monkeys, and in humans (18-20).

Sex differences in the gut microbiome can have important functional consequences. A landmark study by Markle et al. utilizing Non-Obese Diabetic (NOD) mice, which spontaneously develop type 1 Diabetes (T1D), showed that the incidence of T1D is almost doubled in females compared to males (21, 22). In contrast, sex differences in the incidence of T1D are not observed in GF NOD mice. Furthermore, weanling females given male microbiota by gavage displayed elevated testosterone levels and lower incidence of T1D (23). Microbiota also contribute to lupus, a sex-specific disease affecting more women than men (24). The gut microbiome of lupus-prone mice is different between sexes (25). Compared to age- and sex-matched healthy controls, young female lupus-prone mice have fewer Lactobacillaceae and increased numbers of Lachnospiraceae. Lupus-prone males, on the other hand, are not significantly different than controls in their gut microbial composition. In addition, dietary intervention restoring Lactobacillaceae in the gut correlates with improved symptoms, and dietary intervention increasing Lachnospiraceae worsens the Lupus symptoms in female mice (25).
The purpose of this study was to examine the hypothesis that sex-related differences in the gut microbiome contribute to sex differences in the asthma-like phenotype of mice exposed to O₃. To test this hypothesis, we perturbed the gut microorganisms of male and female mice using a cocktail of antibiotics (ampicillin, metronidazole, neomycin, and vancomycin) administered via the drinking water. Mice were subsequently exposed to air or to O₃. Antibiotic treatment abolished sex-differences in O₃-induced AHR. We also perturbed the microbiome by placing female mouse pups, at weaning, into cages that were conditioned by adult male or female mice. Once the mice reached adulthood, female pups that had been in cages previously occupied by adult males developed greater O₃-induced AHR than female pups in cages previously occupied by adult females. Thus, exposing female mice to a male microbiome reproduces the male pattern of O₃-induced AHR. Together, our data indicate that sex-related differences in the microbiome contribute to sex differences in the magnitude of O₃-induced AHR.

3.3. METHODS

Animals. All protocols were approved by the Harvard Medical Area Standing Committee on Animals. For experiments examining the impact of antibiotics on sex differences in the response to O₃, mice on C57BL/6J background from Jackson Laboratories (Bar Harbor, ME) were bred in the Harvard T. H. Chan School of Public Health (HSPH) specific pathogen free (SPF) facility to produce male and female littermates (i.e. with the same maternal microbiome). Mice were 8 weeks old at the start of antibiotic administration and 10 weeks old at the time of O₃ exposure. For cage conditioning experiments, mice conditioning the cages were littermate male and female
C57BL/6J mice bred in HSPH SPF facility. The conditioned mice were C57BL/6J female pups purchased with their mothers from Jackson Laboratories (Bar Harbor, ME). These mice were weaned at 3 weeks of age when we started the cage conditioning and 7 weeks old at the time of O₃ or air exposure. Female pups from each litter were distributed evenly between male-conditioned and female-conditioned cages.

**Protocol.** To examine the impact of antibiotics, a cocktail of antibiotics (ampicillin 1g/L, metronidazole 1g/L, neomycin 1g/L, vancomycin 0.5 g/L) was added to the drinking water of littermate male and female mice as previously described (Chapter 2). Sucralose (8 g/L) was added to the water for taste. Control mice were given regular drinking water with sucralose. After two weeks of treatment, mice were exposed to room air or to O₃ (2 ppm for 3 h). 24 hours after exposure, mice were anesthetized for the measurement of pulmonary mechanics and airway responsiveness to inhaled aerosolized methacholine. Following these measurements, mice were euthanized. Blood was collected for the preparation of serum, and bronchoalveolar lavage (BAL) was performed. To conduct a study in which male microbiota were transferred into female recipients, we adapted a “cage-conditioning” protocol from several co-housing studies (26-28). 8-week-old male and female mice (“donors”) were housed individually in cages for 3 days (Fig. 3.1A). After 3 days, the donors were moved to new cages. We placed 3 weanling female pups (“receivers”) into these male or female “conditioned” cages. This process was repeated every 3 days for 4 weeks. The receiver mice were always placed into cages conditioned by the same male or female donor mouse. At 7 weeks of age, the receivers were exposed to room air or to O₃, and evaluated as described above (Fig. 3.1B). Microbial transfer by co-housing or by placing donor feces in receiver cages, has been previously described (29-31) and likely occurs because
mice are coprophagic. We used the cage conditioning protocol rather than fecal gavage of male feces into females to reduce the stress from the repeated gavage procedure (32). In addition, the cage-conditioning protocol allowed us to mimic housing female mice with males, without the possibility of mating. We began the cage conditioning at weaning because the weaning period and the associated change in the diet of the mice (from mother’s milk to mouse chow) is a period of great perturbation in the microbiome (33, 34). We reasoned that weanlings would more easily receive and adopt the adult donor mouse microbiota than adult mice. For both the antibiotic treatment experiments and the cage conditioning experiments, fecal samples were collected from each mouse one day prior to O₃ or room air exposure.
Figure 3.1. Cage conditioning protocol and timeline. A) Visual representation of cage-conditioning protocol. Male and female mice were housed in individual cages. After three days, the mice (“donors”) were moved to a clean cage. Into the now “conditioned” cages, we placed three weanling female mice (“receivers”) which were thus exposed to the feces of the donor. This protocol was repeated every 3 days: the donor was moved to a clean cage, and the receivers were put in the newly conditioned cage. B) After four weeks of cage conditioning, the receivers were exposed to room air or to O₃, airway responsiveness was measured, and blood and bronchoalveolar lavage samples collected.
**O₃ exposure.** Mice were placed in individual wire mesh cages within a stainless steel and Plexiglas chamber, and exposed to either room air or O₃ (2 ppm for 3 hrs), as previously described (35). Food and water were withdrawn during exposure and replaced immediately after exposure.

**Pulmonary Mechanics and Airway Responsiveness.** Mice were anesthetized, intubated, ventilated, and instrumented for the measure of pulmonary mechanics as previously described (36). In the case of the mice treated with antibiotics and their controls, the chest wall was opened to expose the lungs to atmospheric pressure. In the case of the cage conditioned mice, the chest wall was left closed. A positive end expiratory pressure of 3 cm H₂O was applied. Three volume excursions to total lung capacity, each separated by one minute, were first administered to establish a common volume history. The following sequence was then initiated. One minute after an excursion to total lung capacity, 10 breaths from an aerosol generated from PBS were administered. Next, total lung resistance (Rₐ) for the open-chest mice, or total resistance of the respiratory system (RₐRS) for the closed-chest mice, was measured by the forced oscillation technique every 15 seconds for 3 minutes (37). This sequence was then repeated with aerosolized methacholine chloride in increasing concentrations from 1.0 mg/mL to 100.0 mg/mL. To construct dose-response curves to methacholine, we averaged the three highest values of Rₐ or RₐRS obtained after each dose.

**Blood Collection and Bronchoalveolar Lavage.** Whole blood was collected from the heart via cardiac puncture of the right ventricle. Serum was isolated using Microtainer tubes (Becton Dickinson, NJ) and stored at -80°C until further analysis. Serum bile acid levels were
determined by Mouse Total Bile Acids Assay (Chrystal Chem, Downers Grove, IL). Serum short chain fatty acids (SCFAs) were assayed as described below. Bronchoalveolar lavage was performed by lavaging the lungs twice with 1 ml ice cold PBS. The lavageates were pooled and centrifuged at 400 × g at 4°C for 10 min. BAL supernatants were stored at -80°C until further analysis. Cell pellets were resuspended in PBS and total number of cells assessed with a hemocytometer. Aliquots of cells were also centrifuged onto glass slides at 800 rpm for 10 min (Cytospin 3, Shandon, Sewickley, PA). The slides were air-dried overnight, and then stained with Wright-Giemsa (LeukoStat, Fisher Scientific, Pittsburgh, PA). Cell differentials were determined by counting at least 300 cells under 400X magnification. Total BAL protein concentration was assessed using the Bradford Protein assay (Bio-Rad, Hercules, CA). BAL IL-17A was determined by ELISA (R&D Systems, Minneapolis, MN). A portion of the BAL supernatants were concentrated approximately 5-fold using Amicon Ultra-0.5 centrifugal filters (Milipore, Billerica, MA) and various cytokines and chemokines were measured with a multiplex assay (EVE technologies, Alberta, Canada). The cytokine concentrations reported are those of the original BAL sample.

16S Sequencing. Fecal pellets were collected one day before exposure. After incubating the pellets for one-day at 56°C with proteinase K, fecal DNA was isolated with a QIAamp DNA Mini Kit (Qiagen, Germantown, MD) using ~25 mg of feces. After being adjusted to a concentration of 20 ng/μL using a NanoDrop (ThermoScientific, Wilmington, MA), fecal DNA was sent to the Forsyth Institute (Cambridge, MA) for 16S sequencing on Illumina MiSeq (Illumina, San Diego, CA) as previously described (Chapter 2). Microbial analysis was conducted at the Microbiome Analysis Core of the Harvard T. H. Chan School of Public Health.
Reads were ascribed operational taxonomic units (OTUs) using the UPARSE pipeline (38) and comparing to bacterial sequences in the GreenGenes database (39). Differential abundance and association with metadata was assessed using Multivariate Association with Linear Models (MaAsLin) statistical framework (40) (http://huttenhower.sph.harvard.edu/galaxy/).

**SCFA Analysis.** Serum isolated from whole blood was sent to University of Michigan Metabolomics Core (Detroit, MI) for short chain fatty acid (SCFA) analysis. 50 uL of serum was diluted to 100 uL with 50 uL HPLC grade H2O. 200 uL of a solution of 30 mM hydrochloric acid plus isotopically-labeled acetate (150uM), butyrate (10uM), and hexanoate (2uM) in water was added to each sample and vortexed for 10 seconds. 300 uL of MTBE was added to each sample, emulsified by vortex, held at 4 °C for 5 mins, then vortexed again for 10 seconds. Samples were centrifuged for 1 min and the MTBE layer was used for GC-MS analysis. A series of calibration standards were prepared along with samples to quantify SCFAs. GC-MS analysis was performed on an Agilent 69890N GC -5973 MS detector (Santa Clara, CA). Data were processed using MassHunter Quantitative analysis version B.07.00 (Agilent, Santa Clara, CA). SCFAs were normalized to the nearest isotope labeled internal standard and quantitated using 2 replicated injections of 5 standards to create a linear calibration curve with accuracy greater than 80%.

**Statistical Analysis.** Except for 16s sequencing analysis (see above), the significance of differences between groups was assessed using factorial ANOVA combined with LSD Fisher post-hoc analysis (Statistica Software, Tulsa, OK) using treatment, exposure, and sex as main
effects. A p value < 0.05 (two-tailed) was considered significant. All values are expressed as mean ± standard error of mean.

3.4. RESULTS

3.4.1. Sex differences in the composition of the fecal microbiome.

Fecal samples from male and female littermates treated with antibiotics or with regular drinking water were collected, and 16S ribosomal RNA gene libraries were prepared and sequenced to examine the gut microbial ecology of these mice. In both female and male water-treated mice, fecal microbial ecology was dominated by bacteria of the Bacteroidetes and Firmicutes phyla (Fig. 3.2A), consistent with other reports (28, 41). Female mice harbored significantly fewer Tenericutes and more Proteobacteria (Deltaproteobacteria class) than male mice (Fig. 3.2B). Further examination of the 16S data at the sub-phylum level indicated additional sex differences in the microbiome. In total, there were four operational taxonomic units (OTUs) that were significantly differentially present. Compared to females, males had less Turicibacter and Desulfovibrio genera, and more RF39 order and Candidatus Arthromitus genus (aka Segmented Filamentous Bacteria, or SFB) (Fig. 3.3). Antibiotic treatment had a profound effect on the fecal microbiome. Consistent with other reports (42), and with data from Chapter 2, many members of the Firmicutes, Bacteroidetes, Actinobacteria, Deferribacteres, and Tenericutes phyla were depleted by the antibiotic treatments, with concurrent enrichment for the Proteobacteria phylum (Gammaproteobacteria class), which now dominated (Fig. 3.4). Furthermore, the antibiotic treatment abolished sex differences in gut microbial ecology observed in the water-treated mice.
Figure 3.2. Sex differences in the microbiome: phylum-level analysis. A) Relative abundance of bacteria, at the phylum-level, in feces from water-treated male and female mice. Relative abundance of B) Tenericutes and C) Proteobacteria. Sequence reads was assigned to each phylum at 97% sequence similarity cut-off. Relative abundance was assessed using MaAsLin (40). The trapezoid boxes indicate the 25th and 75th percentile. The whiskers indicate the minimum and maximum values, and each dot denotes one mouse. The Tukey's notches on either side of the median line indicate within-sample variance. Red means higher abundance compared to females, and green means lower relative abundance compared to females. n=7-8 per group. q<0.25 as significant.
Figure 3.3. Sex differences in the microbiome: order and genus-level analysis. Relative abundance of A) *Turicibacter*, B) *Desulfovibrio*, C) RF_39, and D) *Candidatus Arthromitus* (aka *SFB*) in males and females. Relative abundance was assessed using MaAsLin (40) with q<0.25 as significant as described in Figure 3.2. n=7-8 per group.
3.4.2. Sex differences in O₃-induced AHR are abolished with antibiotic treatment.

To determine whether the observed sex differences in the microbiome (Figs. 3.2, 3.3) have an impact on O₃-induced AHR, male and female littersmates were administered a cocktail of antibiotics via their drinking water for two weeks. After collecting fecal pellets for 16S sequencing, mice were exposed to room air or O₃. At the time of exposure, male mice weighed more than female mice, as expected, but antibiotic treatment did not affect body mass in either male or female mice (Fig. 3.5A). In water-treated and air-exposed mice, males had greater airway responsiveness than females (Fig. 3.5B), consistent with previous reports by others (16). In water-treated male mice, exposure to O₃ increased airway responsiveness, consistent with previous reports (5, 43-46). Compared to male water-treated mice, female water-treated mice had significantly smaller magnitude of O₃-induced AHR (Fig. 3.5C). Indeed, airway responsiveness was not significantly different in air- and O₃-exposed female mice given regular
drinking water. Antibiotic treatment attenuated O₃-induced AHR in male mice as previously described (Chapter 2), whereas in female mice the magnitude of O₃-induced AHR was slightly augmented following antibiotic treatment (Fig. 3.5C, D). The net effect of these sex differences in the impact of antibiotics was such that the sex differences in both innate airway responsiveness (the responsiveness of the air-exposed mice) and in O₃–induced AHR that were observed in water-treated mice (Fig. 3.5B, C) were abolished in antibiotic-treated mice (Fig. 3.5D). Together, these findings implicate the microbiota in sex differences in both innate and O₃-induced AHR.

Given the greater abundance of Candidatus Arthromitus (SFB) in male versus female mice (Fig. 3.3D), previous reports linking the presence of SFB to the development of IL-17A⁺ cells in the GI tract (47) and data indicating a role for IL-17A in O₃-induced AHR (45), we considered the hypothesis that the observed sex-differences in O₃-induced AHR (Fig. 3.5C) were the result of sex-differences in the production of IL-17A following O₃ exposure. Consequently, we measured IL-17A in the BAL fluid (Fig. 3.6). Exposure to O₃ increased BAL IL-17A in both male and female mice, with a significantly greater effect in the male mice (Fig. 3.6), consistent with the greater O₃-induced AHR in the male mice (Fig. 3.5C). However, antibiotic treatment had no effect on BAL IL-17A in either male or female mice and did not abolish sex differences in the impact of O₃ on BAL IL-17A (Fig. 3.6), whereas antibiotics did abolish sex differences in O₃-induced AHR (Fig. 3.5D). Thus, IL-17A does not account for microbiome-dependent sex differences in O₃-induced AHR.
Figure 3.5. Antibiotic cocktail abolishes male-female differences in O₃-induced AHR. Mice were treated with a cocktail of antibiotics via their drinking water. Control mice were given regular drinking water. Mice were exposed to room air or to ozone (O₃) (2 ppm) for 3 hours and studied 24 hours after exposure. A) Body weights of male and female water- and antibiotic-treated mice. B) Airway responsiveness of male and female mice treated with water or antibiotics and exposed to room air. C) Airway responsiveness of mice treated with water and exposed to O₃ or room air. D) Airway responsiveness of mice treated with antibiotics and exposed to O₃ or room air. R₁: resistance of the lung. Results are mean ± SE of data from n=9-11 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to female.
Figure 3.6. O$_3$-induced increases in bronchoalveolar lavage (BAL) IL-17A are not affected by antibiotics. BAL IL-17A in female and male mice treated with water or antibiotics and exposed to O$_3$ or room air. Results are mean ± SE of data from n=3-8 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to female mice.

In Chapter 2, we reported that in male mice, microbial generation of short chain fatty acids (SCFAs) may be responsible for the role of the microbiome in pulmonary responses to O$_3$. Trompette et al. have also established a role for SCFAs in modulating allergic inflammation and airway responsiveness (48). Consequently, to determine whether sex differences in microbial production of SCFAs might be driving the microbial-related sex difference in airway responsiveness (Fig. 3.5C), we measured serum SCFAs in male and female mice (Fig. 3.7). However, there was no discernible difference between male and female mice in any of the three most abundant SCFAs produced by microbiota (Fig. 3.7).
Bile acids have been implicated in microbiota-host interactions in other disease conditions (49, 50). Bile acids are produced and conjugated in the liver, and travel via the bile duct to the intestines where gut microbiota de-conjugate them and convert primary bile acids to secondary bile acids. The latter are absorbed back into the circulation and returned to the liver via the hepatic portal vein for reconjugation and recirculation to the bile (51-53), though some bile acids pass on to the systemic circulation. Circulating bile acids may have functional consequences given their ability to bind and send signals via two receptors, farnesoid X receptor (FXR), and a G-protein coupled receptor, TGR5 (50). In human airway epithelial cells, bile acids can suppress HIF-1α and modulate the immune response to bacterial infection and LPS treatment, resulting in increased production of inflammatory cytokines IL-6 and IL-8 (54). In macrophages, bile acid-induced TGR5 activation causes elevations in cAMP that inhibit NF-κB-mediated induction of pro-inflammatory cytokines by LPS (55). Org et al have also reported that gonadectomized mice have higher levels of several bile acids compared to sham control mice, and male gonadectomized mice have higher levels of bile acids compared to female gonadectomized mice (17). Hence, we examined the total serum bile acid levels in our male and female mice. In
water-treated mice, O₃ exposure caused an increase in serum bile acids in females but not males (Fig. 3.8). Antibiotic-treatment increased serum bile acids in air exposed female mice but had no effect in O₃ exposed female mice and did not impact serum bile acids in either air or O₃ exposed male mice (Fig. 3.8). Thus, sex differences in the impact of O₃ on serum bile acids do not appear to be related to the impact of the microbiome on bile acids.

**Figure 3.8. Serum bile acid levels are not different by sex.** Serum total bile acid levels in male and female mice after room air or O₃ exposure. Results are mean ± SE of data from n=7-11 per group. * denotes p<0.05 compared to air. % denotes p<0.05 compared to water.

3.4.3. O₃-induced inflammation

Exposure to O₃ increased BAL neutrophils in both male and female mice, but there was no sex difference in this response to O₃ (Fig. 3.9). Treatment with antibiotics reduced BAL neutrophils in male mice, consistent with our previous observations (Chapter 2), but had no effect in female mice (Fig. 3.9).
Several cytokines, including MIP-2, KC, IL-6, G-CSF, and IL-17A have been reported to play a role in O₃-induced neutrophilic inflammation in mice (56-58). Furthermore, sex differences in the effects of O₃ on IL-6 and TNFα expression have been described (11). As described above, we observed no effect of antibiotics on BAL IL-17A (Fig. 3.6A). To determine whether the other cytokines and chemokines might contribute to observed sex-differences in the impact of antibiotics on O₃-induced neutrophil recruitment to the lungs (Fig. 3.9), we conducted a multiplex assay of BAL fluid. Compared to air, O₃ caused significant increases in BAL G-CSF, KC, and IL-6 (Fig. 3.10A-C), whereas BAL TNFα and MIP-2 were not affected by O₃ (Fig. 3.10D, E). In water-treated mice, there were no significant sex differences in the impact of O₃ on BAL G-CSF, KC, or IL-6 (Fig. 3.10A-C). In antibiotic-treated mice, there was a trend towards reduced BAL IL-6 and KC in male versus female mice that reached significance for G-CSF.
However, as antibiotics tended to increase the concentrations of these cytokines (Fig. 3.10A-C), whereas antibiotics reduced BAL neutrophils in male mice (Fig. 3.9), changes in these cytokines do not appear to account for microbial effects on neutrophil recruitment.

Figure 3.10. BAL cytokine and chemokines implicated in O₃-induced neutrophil recruitment are not affected by antibiotic treatment. BAL A) G-CSF, B) KC, C) IL-6, D) TNFα, E) MIP-2 of female and male mice treated with antibiotics or water and exposed to O₃ or room air. BAL supernatants were concentrated approximately 5X before assay and the results were back-calculated to values present in the original BAL fluid. Results are mean ± SE of data from n=6-8 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to female mice. % denotes p<0.05 compared to water.
In water-treated mice, there were no sex differences in O₃-induced increases in BAL protein, a marker of damage to the alveolar/capillary barrier (43). O₃-induced increases in BAL protein were greater in males treated with antibiotics versus water (Fig. 3.11). In contrast, treatment with antibiotics did not affect BAL protein in female mice exposed to O₃. Thus, antibiotic-treated male mice had less neutrophil recruitment to the lung (Fig. 3.9) despite an augmented level of lung injury (Fig. 3.11).

Figure 3.11. BAL protein is augmented in antibiotic-treated male mice. BAL protein, a marker of lung alveolar/capillary barrier injury. Results are mean ± SE of data from n=7-11 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to female mice. % denotes p<0.05 compared to water.
3.4.4. Cage conditioning transfers male O₃-induced AHR to female mice.

To further evaluate the hypothesis that the microbiome contributes to sex-related differences in O₃-induced AHR, we placed weanling female mice in cages conditioned by adult male or female mice (Fig. 3.1A). Cage conditioning did not affect body mass (Fig. 3.12A). Compared to female mice living in cages conditioned by female mice, female mice living in cages conditioned by male mice had significantly greater airway responsiveness after O₃ exposure (Fig. 3.12B). In contrast, the cage conditioning protocol did not impact airway responsiveness in mice exposed to room air (Fig. 3.12B). Mice living in cages conditioned by male mice also had significantly greater increases in BAL neutrophils after O₃-exposure compared to mice living in cages conditioned by female mice (Fig. 3.12C), whereas O₃-induced increases in BAL protein were not affected (Fig. 3.12D). Together, these data are consistent with the hypothesis that the microbiota in the male-conditioned cages have the capacity to augment O₃-induced AHR and pulmonary neutrophilic inflammation in female mice without causing more injury. No effects of cage conditioning on BAL IL-17A were observed (Fig. 3.13) nor were there differences in serum SCFAs in mice conditioned in cages with male versus female mice (Fig. 3.14). There was a trend towards increased serum bile acids after O₃ in cage-conditioned mice, but there was no significant difference between females housed in cages conditioned by males and females housed in cages conditioned by females (Fig. 3.15). Thus, IL-17A, SCFAs and bile acids do not seem to account for the sex-differences in the O₃-response of cage-conditioned mice.
Figure 3.12. Females housed in cages conditioned by males develop greater O₃-induced AHR. Female weanlings were housed in cages conditioned by adult male mice. Control female weanling mice were housed in cages conditioned by adult female mice. A) Body weight of female mice housed in cages conditioned by male or female mice. B) Airway responsiveness of female mice conditioned in male or female cages and exposed to O₃ or room air. A) BAL neutrophils of female mice conditioned in male or female cages and exposed to O₃ or room air. B) BAL protein, a marker of lung alveolar/capillary barrier injury. $R_{RS}$: resistance of the respiratory system. Results are mean ± SE of data from n=11-12 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to female.
Figure 3.13. **IL-17A is unchanged by cage-conditioning.** BAL IL-17A in female mice conditioned with female or male cages and exposed to O₃ or room air. Results are mean ± SE of data from n=11-12 per group. * denotes p<0.05 compared to air.

Figure 3.14. **Cage conditioning does not affect SCFAs.** Serum A) acetate and B) propionate, and C) butyrate of water-treated female mice housed in cages conditioned by male or female donors after room air exposure. Results are mean ± SE of data from n=7-8 per group.
3.4.5. **16S analysis of fecal DNA from cage conditioned mice.**

To evaluate the impact of cage conditioning on the recipients' fecal microbiomes, we performed 16S sequencing on fecal samples of male and female donors, and on their respective female receivers. At a high taxonomic classification, we saw similar amounts of Bacteroidetes and Firmicutes, the two most common phyla in the gut, in the donor males and females, and in female receivers of male and female cages (Fig. 3.16).
Figure 3.16. Phylum level microbial abundance of cage-conditioned mice. A) Relative abundance of bacteria, at the phylum-level, in male and female donors used in cage-conditioning experiments. B) Relative abundance of various phyla in females housed in male-conditioned cages and females housed in female-conditioned cages. Sequence reads assigned to each phylum at 97% sequence similarity cut-off. Relative abundance was assessed using MaAsLin (40) with q<0.25 as significant. n=3-4 per donors, and 11-12 per receivers.

We then utilized a multivariate association statistical framework (MaAsLin) (40) to examine sub-phylum level microbial differences between females housed in male cages and females housed in female cages. Compared to females housed in female-conditioned cages, females housed in male-conditioned cages harbored significantly more Christensenellaceae family, and less Streptococcaceae family and Bacteroides acidifaciens species (Fig. 3.17). When we examined these taxa in our male versus female cohort (Figs. 3.2, 3.3), we found that although not significant, males had more Christensenellaceae family than females (p= 0.2122) similar to the mice housed in the male conditioned cages (Fig. 3.18). In contrast, Streptococcaceae family were not different in male versus female mice (p= 0.6218, data not shown) and Bacteroides acidifaciens species was not detected in the 16S sequencing of the male and female mice.
Figure 3.17. **Sub-phylum level microbial abundance of cage-conditioned mice.** Relative abundance of A) Christensenellaceae, B) Streptococcaceae, and C) *Bacteroides acidifaciens* in female mice “receivers” housed in cages conditioned by adult male or adult female mice. Relative abundance was assessed using MaAsLin (40) with <0.25 as significant. n=12 per group.

Figure 3.18. **Relative abundance of Christensenellaceae and Streptococcaceae in male and female mice.** Relative abundance of Christensenellaceae in male and female mice. The sex difference did not reach significance (p= 0.2122). n=12 per group.
3.5. DISCUSSION

Our data indicate that O₃-induced AHR is substantially greater in male than in female mice (Fig. 3.5C). Our data also confirm reports of others (27, 59) indicating that the gut microbial community structures of male and female mice are different (Figs. 3.2, 3.3), even when the mice are littermates and therefore exposed to the same microbiota at birth. Importantly, the sex-related difference in O₃-induced AHR was abrogated with antibiotic treatment (Fig. 3.5D), and the greater O₃-induced AHR of male mice was transferred to female weanling mice by placing them in cages conditioned by male adults (Fig. 3.12B). Overall, our data are consistent with the hypothesis that sex-differences in the microbiome contribute to sex differences in O₃-induced AHR.

O₃-induced AHR was greater in male than female mice (Fig. 3.5C). Importantly, these sex differences were abolished in antibiotic-treated mice (Fig. 3.5D). It is possible that the ability of antibiotics to abolish sex differences in responses to O₃ was due to an off-target effect of the antibiotics administration. However, in male mice, effects of antibiotics on responses to O₃ are mimicked by germ-free conditions and in germ-free mice, antibiotics have no impact on responses to O₃ (Chapter 2). Thus, the data are consistent with an important role for the microbiome in mediating sex differences in the response to O₃. Indeed, our data (Figs. 3.2, 3.3) indicate sex differences in the community structure of the gut microbiome. For example, male mice had a greater abundance of Tenericutes and fewer Deltaproteobacteria than female mice (Fig. 3.2B, C).
The observation that there are differences in the gut microbiomes of male and female mice (Figs. 3.2, 3.3) is consistent with reports by others (17, 60), though our results differ somewhat from those of others with respect to the particular bacteria that were impacted. For example, Org et al (17) and Liang et al (59) reported a greater abundance of Tenericutes in male versus female mice, consistent with our data (Fig. 3.2B). Liang et al observed a greater prevalence of Proteobacteria in female versus male mice, just as we did (Fig. 3.2B). By contrast, Org et al observed greater numbers of Proteobacteria and Actinobacteria in male mice, whereas we did not. The one unifying taxon that was more abundant in males across studies was the Tenericutes.

The differences across studies in sex-dependent differences in the microbiota could be due to several factors. Microbial composition and relative abundance are subject to rapid changes due to environmental conditions, diet, and even circadian rhythms (59, 60, 62-65). For example, the time of day at which fecal pellets were harvested could play a role since Liang et al reported that sex differences in the prevalence of Firmicutes and Bacteroidetes were observed at some times of the day but not others (59, 65). We consistently collected fecal pellets at the same time of day, approximately 2 PM.

Even in air-exposed mice, male mice had greater airway responsiveness than female mice (Fig. 3.5B). Others have also described greater innate airway responsiveness in male than female mice (16). There is evidence that sex hormones affect the sex differences in innate airway responsiveness (16, 66). Males are usually more responsive than females, but castrated male mice displayed airway responsiveness equivalent to that observed in intact females, and administration of exogenous testosterone to castrated males and intact females enhanced airway
responsiveness in both. Org et al (17) also reported that gonadectomy abolished the sex differences in the microbiome, and Yurkovetsky et al (23) reported that male castration did the same. Thus, sex hormones may be driving the sex differences in O₃-induced AHR observed in our mice via effects on gut microbiota.

Indeed, sex hormones are known to affect the microbiota (17, 22, 23), and some bacteria have receptors for mammalian sex hormones (67). Sex hormones also impact the intestinal mucosa in a way that permits greater survival of certain intestinal taxa over others (68, 69). Furthermore, there are sex differences in the production of metabolites which may impact the microbial community. For example, estrogen augments intestinal cholesterol absorption leading to an overproduction of bile (70, 71), which may impact the relative abundance of bile-loving taxa such as *Bilophila wadsworthia*. There is also evidence that the gavage of male microbiota to female mice changes the recipient’s own hormone levels (72), which could impact AHR (66, 73).

The observation that we could reproduce the augmented O₃-induced AHR of male mice by placing female weanling mice in cages conditioned by male mice (Fig. 3.12B) is also consistent with a role for the microbiome in sex differences in O₃-induced AHR. Microbial transfer by co-housing or by placing donor feces in receiver cages, has been previously described (29-31) and likely occurs because mice are coprophagic and display constant grooming behavior, allowing the microbiome from fecal pellets in cage nesting material to be consumed. Though the microbiota that distinguished females housed in male- versus female- conditioned cages (Figs. 3.16, 3.17) were different from the microbial alterations between male and female mice (Figs 3.2, 3.3), there were similar trends in Christensenellaceae (Fig. 3.18). That the cage conditioning
protocol did not reproduce the microbiomes of male and female mice with great fidelity is perhaps not surprising. For some microbiota, there is a strong degree of heritability resulting from the symbiotic relationship with the genotype of the host, and the host’s ability to secrete immunoglobulins, and antibacterial molecules (74, 75) or to metabolize bile acids that have microbicidal properties (76). Microbiota with high heritability are unlikely to be influenced much by external factors such as cage conditioning. Thus, cage conditioning is expected to result in transfer of certain bacteria, but not others, resulting in a microbial community in the recipient that is neither identical to the donor nor to its original self. Indeed, Markle et al. also observed that when male microbiota are gavaged into SPF-colonized female weanlings, the gavage does not lead to a male-like microbiome in the females. The recipient female mice did not retain their original microbiome nor did they adopt the donor male microbiome. Instead, a new microbiome distinct from either was established, a “third state” of the microbiome community structure (72). A “third state” of the microbial community structure has also been reported in several studies describing adoptive transfer experiments (72, 77).

Despite the somewhat different microbial ecology that was transferred by cage conditioning, we were able to transfer the augmented male AHR response to female receivers (Fig. 3.12B). It may be that bacterial taxa that were different (Fig. 3.17) were key to this transfer. However, we cannot rule out the possibility that factors other than just the microbiota were transferred via this protocol. As mentioned above, sex hormones have been implicated in airway responsiveness and in impacting the sex-differences in the microbiome. Hence, it is possible that placing female mice into cages conditioned by male mice increased their own testosterone levels, perhaps in response to male pheromones. However, it is also possible that the functional characteristics of
the bacteria, rather than the specific bacteria present, are more important in transfer of the male phenotype into female receivers. Indeed, Burke et al noted that despite the high phylogenetic variability in microbial species composition on algae (only 15% similarity between samples), they shared a high similarity in functional composition (over 70% similarity) (78). In addition, research in biofilm or quorum sensing suggests that bacteria function differently in community structures compared to when they are alone (79). Thus, the dynamics of the different bacterial community in males and females may not be adequately captured when simply examining differences in specific taxa.

Although our data indicate an important role for the microbiome in the observed sex differences in O₃-induced AHR (Fig. 3.5C, D), the precise function of these microbiota remains to be established. In chapter 2, we attributed the antibiotic-attenuation of O₃-induced AHR in male mice to two genera, *Ruminococcus* and *Coprococcus*, both potent producers of SCFAs. Administering exogenous propionate to the mice in their drinking water led to an increase in airway responsiveness to O₃, confirming the capacity for SCFA-producing microbiota to play a role in the development of O₃-induced AHR. However, in chapter 2 we only utilized male mice. When we examined the microbiomes of the male/female cohort, we did not observe any significant difference in the abundances of *Ruminococcus* or *Coprococcus* (data not shown), nor in the levels of serum SCFAs (Fig. 3.14). Instead, we observed other sex-related microbial differences (Figs. 3.2, 3.3, 3.16-3.18) that may permit the differential responses to O₃ observed in male and female mice and in mice housed in male- versus female-conditioned cages. Thus, there is more than one kind of bacterial community structure that impacts responses to O₃. Exactly which microbial metabolites that are enriched in mice with these community structures affect
responses to O₃ remains to be elucidated. We did not find a difference in bile acids in either male versus female mice (Fig. 3.15) or in mice conditioned in male versus female cages. However, there remain many unexplored products of mammalian-bacterial interactions that could be driving the sex differences in O₃ response.

We did not observe sex differences in O₃-induced neutrophil recruitment to the lungs in water-treated male and female mice (Fig. 3.9). However, there was an effect of antibiotics on neutrophil recruitment in the male mice (consistent with our data in Chapter 2) but not female mice. There was also an increase in BAL neutrophils in O₃-exposed mice that had been housed in cages conditioned by male versus female mice (Fig. 3.12C). Our data suggest that there may be a role for the male microbiome in the neutrophil response to O₃. In contrast, Cabello et al. reported greater neutrophilic inflammation in female versus male mice (11). They used the same O₃ exposure regimen as we did and also used C57BL/6J mice. The reason for these disparate effects likely lies in the environmental conditions extant in the two mouse facilities and the impact of those conditions on the gut microbiomes of those mice. Several studies have reported a shift in microbial ecology structure of mice with same genetic background but reared in different facilities (80, 81). External factors such as housing, animal technician, and chow vendor may contribute to the difference seen in the total BAL cell count and neutrophil count.

In summary, our study indicates a role for the gut microbiome in sex differences in the pulmonary response to acute O₃ exposures, although the precise role of these microbiota remains to be established. Asthma prevalence, severity, hospitalizations and mortality are higher among women than men but there remains a gap of knowledge about the mechanistic basis for this sex
difference. Better understanding of the relationship between male and female microbiota and sex hormones could lead to more precise biomarkers or personalized therapeutics to address asthma in male and female patients, especially asthma triggered by air pollution.
3.6. REFERENCES


CHAPTER 4: The role of the microbiome in obesity-related increases in the pulmonary responses to ozone

This work is currently unpublished.

Youngji Cho performed all laboratory work and created figures 4.1, 4.2, 4.5-4.16.

Galeb Abu-Ali performed the 16S analysis and created figures 4.3 and 4.4.
4.1. ABSTRACT

Obesity is a risk factor for asthma, especially in women. Exposure to ozone, a common asthma trigger, causes airway hyperresponsiveness, a defining feature of asthma, and obesity augments pulmonary responses to ozone. We have previously reported a role for the gut microbiome in ozone-induced airway hyperresponsiveness in lean mice. Obesity and sex both impact the gut microbiome. Consequently, the purpose of this study was to examine the role of the gut microbiome in obesity-related increases in ozone-induced airway hyperresponsiveness and to determine whether this role differed by sex. 16S sequencing of fecal DNA from lean wildtype and obese db/db littermate mice indicated that sex differences in gut microbial ecology typically observed in lean mice were markedly attenuated in obese mice. Ozone-induced airway hyperresponsiveness was greater in obese than lean mice, regardless of sex, and obesity abolished sex differences in the magnitude of ozone-induced airway hyperresponsiveness that were observed in lean mice. Antibiotic treatment reduced ozone-induced airway hyperresponsiveness in obese female mice and markedly attenuated differences between lean and obese mice. In contrast, in obese male mice, antibiotics had little or no effect. Obesity augmented serum levels of short chain fatty acids, especially in female mice, and obesity also increased the abundance of Bacteroides, short chain fatty acid producers, in female but not male mice. Our data indicate that the microbiome contributes to obesity-related increases in ozone-induced airway hyperresponsiveness in female but not male mice, perhaps because of obesity-related changes in the prevalence of the short chain fatty acid producing bacteria. Understanding the role of gut microbiota in obesity-related asthma could pave the way for development of novel microbiota-based treatments for this difficult-to-treat group.
4.2. INTRODUCTION

Obesity is an important public health problem worldwide. The United States Center for Disease Control has reported that more than one third of the U.S. adult population is obese and another third is overweight (1). Obesity is a risk factor for type 2 diabetes, heart disease, and hypertension. Recent evidence indicates that obesity is also a risk factor for asthma (2, 3). Both the prevalence and incidence of asthma, especially non-atopic asthma, are increased in the obese population (4-6). Weight loss causes substantial reductions in asthma symptoms in obese asthmatic patients, and reduces airway hyperresponsiveness (AHR), a defining feature of asthma (7, 8). Obesity also reduces the effectiveness of established asthma therapeutics including corticosteroids, and makes it difficult to control asthma symptoms (9-11). Better understanding of the mechanistic basis for obesity-related asthma could lead to more effective therapeutics for this population.

Ozone (O₃) is a known non-atopic asthma trigger (12, 13). O₃ causes airway epithelial cell injury, induces production of inflammatory cytokines and chemokines, and causes neutrophil recruitment (14). O₃ exposure causes AHR, and O₃-induced AHR may be particularly relevant in the obese, since O₃ exposure causes epithelial damage via oxidative stress, and obesity itself causes systemic oxidative stress (15, 16). In humans, obesity worsens the decrements in lung function that occur following exposure to O₃ (12, 13). In mice, O₃-exposure causes AHR and neutrophilic inflammation, features similar to the non-atopic asthma of obese patients (17, 18). Furthermore, compared to lean mice, O₃–induced AHR and inflammation is augmented in the obese mice (19-25).
The gut microbiome is altered in obesity (26-30). Compared to lean mice, both genetically obese mice and mice with diet induced obesity have an altered gut microbial community structure. Compared to lean mice obese mice typically have relatively fewer bacteria of the Bacteroidetes phyla, and more bacteria of the Firmicutes phyla (26, 28, 31). Consistent with these observations, there is a relative reduction in Firmicutes and an increase in Bacteroidetes phyla in humans after weight loss (29). These obesity-related changes in the microbiome have several functional consequences for the host. In mice, the obese microbiome contributes to the adipose tissue inflammation of obesity and to other obesity-related conditions, including type 2 diabetes, metabolic syndrome, and non-alcoholic fatty liver disease (32-36). Antibiotic treatment also reduces metabolic endotoxemia in both diet-induced and genetically obese mice (32). We have reported that the gut microbiota play a role in the development of O₃-induced AHR in male mice (Chapter 2), and in the sex-differences observed between male and female lean mice (Chapter 3). However, the effect of the obese microbiome on O₃-induced AHR has not been evaluated.

Epidemiological data indicate that there are sex differences in asthma incidence, prevalence and severity. Compared to men, women have greater asthma prevalence, severity, exacerbation rates, hospitalizations and mortality (37, 38). There are also sex differences in obesity-associated asthma. Higher body mass index is associated with increased wheezing in girls but not in boys, and obese adolescent females but not males are at higher risk of developing asthma (39). Obese women also suffer more severe asthma than obese men (40, 41). Compared to non-severe asthmatic women, severe asthmatic women have higher body mass index (BMI), but there is no such difference in BMI between severe and non-severe asthmatic men (42, 43). In addition,
there are sex differences in the effect of obesity on pulmonary responses to O₃ exposure. In women, the correlation between increasing body mass index and O₃-induced decrements in lung function is stronger than in men (13). (Since Schwartz and co. do see an effect of obesity in men, likely the reason for lack of significant correlation in the Bennett study is a small “n”). Thus, current understanding strongly suggests a sex difference in the interaction between obesity and asthma, but it remains unclear which sex-specific factors are responsible for such interactions.

The purpose of this study was to examine the hypothesis that the microbiome contributes to the augmented responses to O₃ observed in obese mice. To do so, we bred db⁺⁻/⁻ parents to obtain obese db/db mice and lean wildtype (WT) littermates. Db/db mice lack the longform of the receptor for the satiety hormone, leptin, and gain about twice as much body weight as WT mice by 8 weeks of age. We utilized both female and male mice because we found sex differences in the O₃ response of mice in Chapter 3. The gut microbiome was perturbed with a cocktail of antibiotics (ampicillin, metronidazole, neomycin, and vancomycin) administered via the drinking water. In water-treated mice, O₃ caused a larger increase in airway responsiveness in db/db versus WT mice, whether the mice were male or female. Antibiotic treatment had a profound effect on obesity-related changes in the magnitude of O₃-induced AHR that differed by sex. In female db/db mice, antibiotic treatment attenuated O₃-induced AHR, almost to the levels of the WT mice. In contrast, in the male mice, antibiotic treatment slightly increased O₃-induced AHR. Hence, antibiotic treatment augmented the sex differences in the obese response to O₃. Our data indicate that sex-and obesity-related differences in the microbiome contribute to the impact of obesity on O₃-induced AHR in mice.
4.3. METHODS

Animals. All protocols were approved by the Harvard Medical Area Standing Committee on animals. Heterozygous $db^{+/-}$ breeders on a C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the Harvard T. H. Chan School of Public Health (HSPH) specific pathogen free (SPF) facility to obtain age- and sex-matched obese ($db/db$) and lean (WT) littermates (Fig. 4.1). At weaning, mice were separated by sex and placed in cages with other mice with matching genotypes, and thus allowed to develop their genotype-specific obese or lean microbiome. Mice were 8 weeks old at the start of antibiotic administration and 10 weeks old at the time of O$_3$ exposure (Fig. 4.1).

![Antibiotic protocol and timeline](image)

**Figure 4.1. Antibiotic protocol and timeline.** Obese ($db/db$) and lean (WT) littermates were bred from heterozygous $db^{+/-}$ breeders in the Harvard T.H. Chan School of Public Health SPF facility. At p21, mice were weaned into genotype- and sex-specific cages (up to 4 WT mice per cage, up to 3 $db/db$ mice per cage). At 8-10 weeks of age, mice were treated with a cocktail of four antibiotics via their drinking water (ampicillin 1g/L, metronidazole 1g/L, neomycin 1g/L, vancomycin 0.5 g/L) for two weeks. Sucralose (8 g/L) was added for taste. Control mice were given regular drinking water with sucralose. 1 day before exposure, fecal pellets were collected for microbial DNA extraction and 16S sequencing. Mice were then exposed to room air or ozone (O$_3$) (2 ppm for 3 hrs), allowed to rest for 24 hours, and then instrumented for the measurement of airway responsiveness. Blood was collected and bronchoalveolar lavage (BAL) performed.
**Protocol.** Mice were given a cocktail of antibiotics (ampicillin 1g/L, metronidazole 1g/L, neomycin 1g/L, vancomycin 0.5 g/L) by addition to the drinking water as previously described (Chapter 2). Sucralose (8 g/L) was added for taste. This type of antibiotic treatment protocol has been reported to result in an extremely marked reduction in the gut bacteria load as assessed by DNA concentration in fecal pellets (44). Control mice were given regular drinking water with sucralose. After two weeks of treatment, fecal pellets were collected one day before mice were exposed to room air or to O₃ (2 ppm for 3 h). Mice were anesthetized for the measurement of pulmonary mechanics and airway responsiveness to inhaled aerosolized methacholine (1 to 100 mg/mL) 24 hours after exposure. Following these measurements, mice were euthanized, blood was collected, and bronchoalveolar lavage (BAL) was performed.

**O₃ exposure.** During exposure, mice were placed in individual wire mesh cages within a stainless steel and Plexiglas chamber and exposed to either room air or O₃ (2 ppm) for 3 hours as previously described (22). Food and water were withdrawn for the 3 hours of exposure and replaced immediately after exposure.

**Pulmonary Mechanics and Airway Responsiveness.** Mice were anesthetized, intubated, and instrumented for pulmonary measurement as previously described (45). The chest wall cavity was opened to expose the lungs to atmospheric pressure. A positive end expiratory pressure of 3 cm H₂O was applied and three volume excursions to total lung capacity (30 cm H₂O trans-respiratory system pressure) were administered to establish a common volume history. One minute after an excursion to total lung capacity, 10 breaths of aerosolized PBS were administered. Total lung resistance (Rₐ) was then measured by the forced oscillation technique.
every 15 seconds for 3 minutes (46). This sequence was then repeated with aerosolized methacholine chloride increasing in concentration from 1 to 100 mg/mL. The three highest values of R_L at each dose were averaged to construct dose-response curves to methacholine.

**Blood Collection and Bronchoalveolar Lavage.** Blood was collected from the right ventricle via cardiac puncture with a 12-gauge needle. Serum was isolated and frozen at -80°C until further analysis. Serum bile acid levels were determined by Mouse Total Bile Acids Assay (Chrystal Chem, Downers Grove, IL) and serum short chain fatty acids (SCFAs) were assessed as described below. BAL was performed with two 1 mL instillations of PBS, which were pooled, and total BAL cells and differentials assessed as previously described (45). BAL supernatants were stored at -80°C until further analysis. BAL concentrations of IL-17A and IL-33 were determined by ELISA (R&D Systems, Minneapolis, MN, and eBioSciences, San Diego, CA, respectively). BAL protein concentration was assessed using the Bradford Protein assay (Bio-Rad, Hercules, CA) per manufacturer's instructions. A portion of the BAL supernatants were concentrated approximately 5-fold using Amicon Ultra-0.5 centrifugal filters (Milipore, Billerica, MA) and various BAL cytokines and chemokines were measured with a multiplex assay (EVE technologies, Alberta, Canada).

**16S Sequencing.** Fecal pellets were collected from each mouse on the day before exposure by picking up the animal and collecting the pellets directly into a microcentrifuge tube. After a one-day incubation at 56°C with proteinase K, fecal DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Germantown, MD) using ~25 mg of feces DNA. Fecal DNA was sent to the Forsyth Institute (Cambridge, MA) for 16S sequencing on MiSeq (Illumina, San Diego, CA), as
Microbial analysis was conducted at the Microbiome Analysis Core of the Harvard T. H. Chan School of Public Health. Reads were clustered into operational taxonomic units (OTUs) using the UPARSE protocol (47), and taxonomic classification of OTUs was performed against the Greengenes version 13.8 16S rRNA gene database (48). Differential abundance and association with experimental variables was assessed using the Multivariate Association with Linear Models (MaAsLin) statistical framework (49). Association was considered significant if q-value (p-value corrected using the Benjamini-Hochberg correction method) < 0.25.

**Short Chain Fatty Acid Analysis.** Serum isolated from whole blood was sent to University of Michigan Metabolomics Core (Detroit, MI) for short chain fatty acid (SCFA) analysis, as previously described (Chapter 2). 50 uL of serum was prepared for GC-MA analysis. A series of calibration standards were prepared along with samples to quantify SCFAs. GC-MS analysis was performed on an Agilent 69890N GC-5973 MS detector (Santa Clara, CA). Data were processed using MassHunter Quantitative analysis version B.07.00 (Agilent, Santa Clara, CA). SCFAs were normalized to the nearest isotope labeled internal standard and quantitated using 2 replicated injections of 5 standards to create a linear calibration curve with accuracy better than 80% for each standard.

**Statistical Analysis.** Except for 16S sequencing analysis (see above), the significance of differences between groups was assessed using factorial ANOVA combined with LSD Fisher post-hoc analysis (Statistica Software, Tulsa, OK) using treatment, exposure, sex, and genotype
as main effects. A p value < 0.05 (two-tailed) was considered significant. All values are expressed as mean ± standard error of mean (SEM).

4.4. RESULTS

4.4.1. Sex- and obesity-related differences in the composition of the gut microbial community.

Data from the WT mice that were littermates of these obese mice were previously reported (Chapter 3) and are provided here to permit comparisons with the db/db mice. In both WT and db/db mice, fecal microbial ecology was dominated by bacteria of the Bacteroidetes and Firmicutes phyla (Fig. 4.2A, B), consistent with previous reports (28, 31). In WT mice, there were sex differences in both Proteobacteria and Tenericutes phyla (Chapter 3). In contrast, in db/db mice these sex differences were no longer observed (Fig. 4.2B). Our data suggest that the effect of obesity dominates over the effect of sex on microbial community structure.
We also examined the impact of obesity on the fecal microbial composition in male mice and in female mice. Compared to male WT mice, male \( \text{db/db} \) mice had a significantly greater abundance of bacteria in the Firmicutes phyla, consistent with other reports of the impact of obesity on the gut microbiome\(^{(31, 50)}\), but did not differ significantly in any other taxon (Fig. 4.3). Compared to female WT mice, female \( \text{db/db} \) mice had a significantly lower abundance of bacteria in the Proteobacteria phyla and an increase in Cyanobacteria (Fig. 4.4A, B). At a lower taxonomic classification, female \( \text{db/db} \) mice also had fewer \( \text{Allobaculum} \) and \( \text{Turicibacter} \), bacteria in the Firmicutes phyla, and more \( \text{Candidatus Arthromitus} \) (aka \( \text{Segmented Filamentous Bacteria} \), or \( \text{SFB} \)) and \( \text{Bacteroides} \) (Fig. 4.4C-F). Interestingly, the increase in \( \text{SFB} \) and decrease in Proteobacteria and \( \text{Turicibacter} \) (Fig. 4.4A, D, E) in the female \( \text{db/db} \) mice mirrored the differences between female and male WT mice (Chapter 3), and these changes contributed to the abrogation of sex differences in the obese mice.
Figure 4.3. Significant differences in microbial composition of male lean and obese mice. Relative abundance of Firmicutes phyla, the only OTU (Operational Taxonomic Unit) that was significantly different between male WT and db/db mice. Relative abundance was assessed using MaAsLin (49). The trapezoid boxes indicate the 25th and 75th percentile. The whiskers indicate the minimum and maximum values, and each dot denotes one mouse. The Tukey's Notches on either side of the median line indicate high within-sample variance. Red means higher abundance compared to WT, and green means lower relative abundance compared to db/db. q-value (false discovery rate corrected using the Benjamini-Hochberg correction method) < 0.25 as significant. & denotes p<0.05 compared to WT. n=5-8 per group.
Figure 4.4. Significant differences in microbial composition of female lean and obese mice. Relative abundance of A) Proteobacteria, B) Cyanobacteria, C) Allobaculum, D) Candidatus Arthromitus, E) Turicibacter, and F) Bacteroides in female WT and db/db mice. Relative abundance was assessed using MaAsLin (49) with q<0.25 as significant. & denotes p<0.05 compared to WT. n=5-8 per group.

Antibiotic treatment had a profound effect on the fecal microbiome. Consistent with other reports (51), and with data from Chapter 2 and 3, several members of the Firmicutes, Bacteroidetes, Actinobacteria, Deferrribacteres, and Tenericutes phyla were depleted by the antibiotic treatments, while the Proteobacteria phylum was enriched (Fig. 4.5). Furthermore, the
antibiotic treatment abolished obesity-related differences in gut microbial ecology observed in the water-treated mice (Figs 4.2, 4.4).

![Diagram of bacterial phyla abundance](image)

**Figure 4.5. Antibiotics abrogate sex- and obesity-related differences in the microbiome.** Relative abundance of bacterial phyla in antibiotic-treatment A) WT female and male, and B) db/db female and male mice. n=5-8 per group.

### 4.4.2. Impact of antibiotic treatment on O$_3$-induced AHR in obese male and female mice.

At the time of exposure, $db/db$ mice weighed about twice as much as the WT mice, and antibiotic treatment did not affect body mass in either WT or $db/db$ mice (Fig. 4.6). In mice exposed to room air, whether the mice were male or female, airway responsiveness was greater in obese than in lean mice, and antibiotic treatment did not significantly alter this innate AHR in obese mice (Fig. 4.7).
Figure 4.6. **Antibiotic treatment does not affect body weight.** Body weights of female and male WT and db/db mice treated with water or antibiotics. Results are mean ± SE of data from n=8-11 per group. & denotes p<0.05 compared to WT. # denotes p<0.05 compared to female mice.

Figure 4.7. **Obesity causes innate airway hyperresponsiveness in male and female mice and antibiotic treatment does not this responsiveness.** Airway responsiveness of air-exposed A) female and B) male mice treated with water or antibiotics. $R_L$: resistance of the lung. Results are mean ± SE of data from n=5-10 per group. % denotes p<0.05 compared to water. & denotes p<0.05 compared to WT.
In water-treated female mice, O$_3$ exposure caused a significant increase in baseline R$_L$ (PBS dose in Fig. 4.8A) in db/db but not WT mice (Fig. 4.8A). O$_3$ also increased airway responsiveness to a significantly greater extent in db/db than WT mice (Fig. 4.8A), as previously reported (22, 52). Treatment of db/db mice with antibiotics abolished the O$_3$-induced increase in baseline R$_L$ observed in water treated mice and significantly attenuated O$_3$-induced AHR (Fig. 4.8B). Indeed, treatment of female mice with antibiotics virtually abolished obesity-related differences in O$_3$-induced AHR (Fig 4.8B).

In water-treated male mice, O$_3$ exposure increased baseline R$_L$ in db/db but not WT mice (Fig. 4.8C), though the effect of O$_3$ was smaller in magnitude than in the female db/db mice (Fig. 4.8A) (Fig. 4.8C). O$_3$ also increased airway responsiveness to a significantly greater extent in db/db than WT mice (Fig. 4.8C), similar to the results obtained in female db/db mice. Indeed, in obese mice, there was no sex-difference in the magnitude of O$_3$-induced AHR (compare the db/db O$_3$-exposed mice in Figs. 4.8A and 4.8C), whereas in lean mice sex differences were observed (compare the WT O$_3$-exposed mice in Figs. 4.8A and 4.8C). At the lower doses of methacholine, R$_L$ was actually significantly greater in antibiotic- than water-treated db/db mice exposed to O$_3$, whereas no differences were observed at the higher doses of methacholine (Fig. 4.8D). Because the antibiotic-treated female db/db mice had attenuated O$_3$-induced AHR, and antibiotic-treated male db/db mice had similar or even increased AHR compared to their water-treated counterpart, antibiotic treatment augmented the sex differences in the response to O$_3$ in the obese mice.
Figure 4.8. Differing effects of antibiotics on O$_3$-induced AHR in male and female obese mice. Airway responsiveness of A) water-treated female mice, B) antibiotic-treated female mice, C) water-treated male mice, and D) antibiotic-treated male mice exposed to room air or O$_3$. $R_L$: resistance of the lung. Results are mean ± SE of data from n=5-10 per group. % denotes $p<0.05$ compared to water. & denotes $p<0.05$ compared to WT.
We have previously reported that in female mice, IL-17A is required for obesity-related increases in O₃-induced AHR (53, 54): an IL-17A neutralizing antibody attenuates O₃-induced AHR in db/db but not in WT mice. Furthermore, gut microbiota have been showed to regulate the expression of IL-17A producing cells (18). To determine whether the impact of antibiotics on O₃-induced AHR observed in obese mice (Fig. 4.8B, D) was the result of effects of antibiotics on O₃-induced IL-17A production, we measured BAL IL-17A in these mice. Exposure to O₃ increased BAL IL-17A in all groups of mice (Fig. 4.9A). In female water-treated mice, O₃ exposure caused greater increases in BAL IL-17A in db/db than WT mice, consistent with previous reports (54). In contrast, in male water-treated mice O₃-induced increases in BAL IL-17A were not different in db/db versus WT mice. This discrepancy appeared to be the result of significantly higher BAL IL-17A in the male WT mice versus female WT mice, rather than a lower response in male db/db mice versus female db/db mice. Antibiotic treatment had no effect on BAL IL-17A in either male or female, WT or db/db mice (Fig. 4.9A), whereas antibiotics did attenuate O₃-induced AHR in db/db female mice (Fig. 4.8B). Thus, IL-17A does not account for microbiome-dependent effects of obesity on O₃-induced AHR.

IL-33 has also been reported to contribute to the augmented O₃-induced AHR observed in obese female mice (55): ozone causes greater increases in BAL IL-33 in obese than lean mice, and an IL-33 receptor blocking antibody reduced O₃-induced AHR in obese but not lean mice. To determine whether microbiome-dependent differences in the release of IL-33 into the airways by O₃ might explain the differential impact of antibiotics on obesity-related increases in O₃-induced AHR in male and female mice (Fig. 4.9B), we measured BAL IL-33. In water-treated mice, O₃-induced increases in BAL IL-33 were greater in female db/db than WT mice, consistent with
previous reports (55), whereas there was no impact of O3 on BAL IL-33 in male mice. However, antibiotic treatment had no effect on O3-induced increases in IL-33 in female db/db mice, even though antibiotics did attenuate the O3-induced AHR in db/db female mice (Fig. 4.8B). Thus, effects on IL-33 release are unlikely to account for microbiome-dependent effects on O3-induced AHR in female db/db mice.

IL-33 mediates its effects on airway responsiveness in part via its ability to promote type 2 cytokine release from innate lymphoid cells and γδ T cells within the airways (55-58). Thus, it is possible that the effect of antibiotic treatment on O3-induced AHR is not mediated at the level of IL-33 release, but rather at the level of the cytokine/chemokines downstream of IL-33. To examine this hypothesis, we examined IL-5 and IL-9, two of the type 2 cytokines downstream of IL-33 (59) (Fig. 4.9C, D). Like BAL IL-33, the magnitude of obesity-associated increases in BAL IL-5 and BAL IL-9 was greater in female versus male db/db mice. However, antibiotic treatment did not abrogate the effect of obesity on O3-induced increases in these cytokines in the female mice. Our data suggest that microbiome effects on type 2 cytokines (Fig. 4.9B-D) are unlikely to account for the microbiome effects on O3-induced AHR in the female mice (Fig. 4.8B).
Figure 4.9. Cytokines implicated in O₃-induced AHR are impacted by sex and obesity. BAL A) IL-17A, B) IL-33, C) IL-5, and D) IL-9 in female and male WT and db/db mice treated with antibiotics or water and exposed to room air or O₃. IL-17A and IL-33 was assayed by ELISA at 1X concentration. BAL supernatants were concentrated approximately 5X before IL-5 and IL-9 assay and the results were back-calculated to values present in the original BAL fluid. Results are mean ± SE of data from n=3-8 per group. * denotes p<0.05 compared to air. % denotes p<0.05 compared to water. # denotes p<0.05 compared to female mice. & denotes p<0.05 compared to WT.

In addition to type 2 cytokines, IL-6 also participates in IL-33-induced increases in AHR in mice (58). IL-6 may be particularly relevant to obesity-related AHR because recent evidence implicates IL-6 in the development of obesity-associated asthma (60): in asthmatic patients, high serum IL-6 correlates with high BMI, worse lung function, and more asthma exacerbations. In
addition, gut microbiota drive IL-6 production in joints of mice with antigen-induced arthritis (61). We measured BAL IL-6 to determine whether microbiome-dependent differences in IL-6 might explain the differential impact of antibiotics on obesity-related increases in O3-induced AHR in male and female mice (Fig. 4.10). In water-treated mice, O3-induced increases in BAL IL-6 were greater in db/db than WT mice, consistent with previous reports (55). Antibiotic treatment significantly decreased O3-induced increases in IL-6 in female db/db mice, but had no effect on male db/db mice. Thus, effects of antibiotics on O3-induced BAL IL-6 in the db/db mice correlated with effects of antibiotics on O3-induced AHR (Fig. 4.8B, D). The results suggest that IL-6 may contribute to microbiome-dependent impacts on O3-induced AHR in obese mice.

**Figure 4.10. BAL IL-6 is impacted by antibiotic treatment in obese female mice.** BAL IL-6 in female and male WT and db/db mice treated with antibiotics or water and exposed to room air or O3. BAL supernatants were concentrated approximately 5X before assay and the results were back-calculated to values present in the original BAL fluid. Results are mean ± SE of data from n=6-8 per group. * denotes p<0.05 compared to air. % denotes p<0.05 compared to water. # denotes p<0.05 compared to female mice. & denotes p<0.05 compared to WT.
Bile acids have been implicated in microbiota-host interactions in other disease conditions (62-64). Circulating bile acids may act as signaling molecules by binding to the farnesoid X receptor (FXR), or to a G-protein coupled receptor, TGR5 (63) (Chapter 3). Since the primary stimulus for release of bile acids from the gall bladder is eating, especially fatty foods, bile acids might sit at the nexus of microbiome and obesity. Furthermore, recent reports have shown that the bile acid release during a meal activates intestinal FXR (65). Therefore, to examine a possible role for the bile acids in the microbiome dependent effects of obesity on O₃-induced AHR, we measured serum bile acids (Fig. 4.11). Compared to lean mice, total serum bile acids were greater in obese mice, whether the mice were male or female. However, antibiotic treatment did not affect the levels of bile acid in either WT or obese, male or female mice. Thus, the microbiome-dependent effects of O₃ on AHR in obese mice do not appear to be related to effects of the microbiome on circulating bile acids, though we cannot rule out the possibility that there were differences in particular bile acid metabolites that contributed.
Figure 4.11. Serum bile acid levels are higher in the obese than lean mice but not affected by sex or antibiotic treatment. Serum total bile acid levels in female and male WT and db/db mice after room air or O3 exposure. Results are mean ± SE of data from n=7-11 per group. * denotes p<0.05 compared to air. % denotes p<0.05 compared to water. & denotes p<0.05 compared to WT.

In chapter 2, we reported that compared to water-treated mice, antibiotic-treated mice had reductions in the short chain fatty acids (SCFA)-producers, *Ruminococcus* and *Coprococcus*, and corresponding reductions in serum propionate. Moreover, exogenous administration of propionate augmented O3-induced AHR (Chapter 2). The results suggested that microbiota-dependent changes in SCFAs, particularly propionate, might be contributing to the effects of antibiotics on O3-induced AHR, at least in lean male WT mice. Therefore, we examined serum SCFAs in water-treated WT and db/db mice (Fig. 4.12). Serum concentrations of acetate, propionate, and butyrate were each increased in obese versus lean mice, consistent with previous reports in human subjects (66). Interestingly, serum propionate was significant greater in obese
female than obese male mice (Fig. 4.12 B). A similar trend was observed for acetate and butyrate (Fig. 4.12A, C), but did not reach statistical significance. Consistent with these observations, there was a significant increase in the propionate producer *Bacteroides* in female *db/db* mice versus female WT mice (Fig. 4.4F), whereas no significant difference was detected in male mice. Our data suggest that the greater abundance of *Bacteroides* and production of SCFAs by these bacteria may explain the augmented O$_3$-induced AHR of female *db/db* mice versus female WT mice, and subsequent attenuation of O$_3$-induced AHR in the female *db/db* mice following antibiotic treatment.

**Figure 4.12. Impact of obesity and sex on serum short chain fatty acids (SCFA).** Serum A) acetate, B) propionate, and C) butyrate of water-treated female and male WT and *db/db* mice. Results are mean ± SE of data from n=7-8 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to female mice.

**4.4.3. Impact of obesity on O$_3$-induced inflammation and injury: effect of antibiotic treatment**

In female water-treated mice, exposure to O$_3$ increased BAL neutrophils to a greater extent in obese *db/db* than in lean WT mice, consistent with previous reports (22, 67, 68), whereas no
effect of obesity on BAL neutrophils was observed in male mice (Fig. 4.13). No effect of antibiotic treatment on BAL neutrophils was observed in female mice, whereas in male mice, treatment with antibiotics reduced BAL neutrophils, in both obese and lean mice.

![Graph showing BAL neutrophils](image)

**Figure 4.13.** O₃-induced increases in BAL neutrophils: effect of sex and antibiotics. BAL neutrophils of male and female WT and db/db mice treated with antibiotics or regular drinking water and exposed to room air or O₃. Results are mean ± SE of data from n=5-10 per group. * denotes p<0.05 compared to air. % denotes p<0.05 compared to water. # denotes p<0.05 compared to female mice. & denotes p<0.05 compared to WT.

Numerous cytokines and chemokines have been implicated in O₃-induced neutrophil recruitment to the lungs (55, 68-70). Among these, our data suggest an important role for IL-17A and for IL-33 in the ability of obesity to augment O₃-induced neutrophil recruitment in female mice. Others have reported that the effects of IL-33 on neutrophil recruitment to the lungs are mediated at least in part by IL-6 and by chemokines that utilize the CXCR2 receptor (58), such as CXCL1. CXCL1 along with G-CSF also contributes to effects of IL-17A on neutrophil recruitment.
Consequently, we assayed BAL CXCL1 (KC) and G-CSF in these mice. Similar results were obtained with both cytokines. Compared to WT mice, db/db mice had greater O3-induced increases in these cytokines (Fig. 4.14). In addition, compared to male db/db mice, female db/db mice had greater O3-induced increases in G-CSF. Antibiotics did not have an effect on either cytokine in male or female, WT or db/db mice. Together the data suggests that KC and G-CSF do not account for the role of the microbiome in attenuating O3-induced neutrophil recruitment in male mice.

Figure 4.14. Neutrophil chemoattractant and survival factors are higher in obese than lean mice but not different by sex or antibiotic treatment. BAL KC and G-CSF of female and male WT and db/db mice treated with water or antibiotics and exposed to room air or O3. BAL supernatants were concentrated approximately 5X before assay and the results were back-calculated to values present in the original BAL fluid. Results are mean ± SE of data from n=6-8 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to female mice. & denotes p<0.05 compared to WT.
Regardless of sex, O₃-induced increases in BAL protein, a marker of lung epithelial injury, were greater in obese versus lean mice (Fig. 4.15), as previously described (22). Treatment with antibiotics did not affect BAL protein in either male or female obese mice exposed to O₃ (Fig. 4.16). We also observed obesity- and sex-related differences in BAL concentrations of other cytokines and chemokines in O₃-exposed mice (Fig. 4.16). However, antibiotic treatment did not affect any of these cytokines or chemokines, except for an increase in MIP-2 in male db/db mice exposed to O₃.

**Figure 4.15. BAL protein is higher in obese than lean mice but not different by sex or antibiotic treatment.** BAL protein, a marker of lung alveolar/capillary barrier injury. Results are mean ± SE of data from n=7-11 per group. * denotes p<0.05 compared to air. % denotes p<0.05 compared to water. # denotes p<0.05 compared to female mice. & denotes p<0.05 compared to WT.
Figure 4.16. BAL cytokines/chemokines are increased with obesity but not impacted by antibiotic treatment. BAL A) Eotaxin, B) IL-10, C) IL-2, D) IL-9, E) MCP-1, F) IP-10, G) LIF, and H) MIP-2 of female and male WT and db/db mice treated with water or antibiotics and exposed to room air or O₃. BAL supernatants were concentrated approximately 5X before assay and the results were back-calculated to values present in the original BAL fluid. Results are mean ± SE of data from n=6-8 per group. * denotes p<0.05 compared to air. % denotes p<0.05 compared to water. # denotes p<0.05 compared to female mice. & denotes p<0.05 compared to WT.
4.5. DISCUSSION

Our data indicate that O$_3$-induced AHR is substantially greater in obese than in lean mice, that the microbiome contributes to this effect of obesity, and that there are sex differences in the role of the microbiome (Fig. 4.8). Our data also indicate that the gut microbial community structures of obese and lean mice are different (Figs. 4.2-4.4), consistent with other reports (31, 50, 71, 72), even when the mice are littermates and therefore exposed to the same microbiome at birth. Importantly, sex differences in the gut microbial structures of lean mice (Chapter 3) were abrogated in obese mice (Fig. 4.2), indicating that obesity dominates sex in its effect on the gut microbiome. In addition, there were sex differences in the levels of serum propionate between obese female and male mice, which correlated with sex differences in the SCFA-producing Bacteroides genus (Fig. 4.4F, 4.10B). Propionate was previously implicated in the development of O$_3$-induced AHR in lean male mice (Chapter 2). The greater amount of Bacteroides and serum propionate in female db/db mice versus male db/db mice may account for the greater effect of obesity and for the impact of antibiotic in the attenuation of O$_3$-induced AHR in the female db/db mice. Finally, we found sex differences in the impact of obesity on the neutrophilic inflammation caused by O$_3$, differences that were magnified in antibiotic-treated mice (Fig. 4.13). Overall, our data are consistent with the hypothesis that obesity- and sex-associated differences in the microbiome contribute to the differential response to O$_3$ observed in lean and obese mice.

We have previously shown that obesity augments O$_3$-induced AHR in female mice (19, 22, 55, 67, 73). Only female mice were utilized in those studies because females dominate the obese
asthma population (2, 10, 74). In this study, we show that obesity also augments O₃-induced AHR in male mice (Fig. 4.8C), though the effect of obesity was not as great in males as in females, in large part because of greater responses to O₃ of the lean male mice. Interestingly, the higher O₃-induced AHR in male versus female WT mice (Chapter 3) was abolished in obese mice (Fig. 4.8A, C). Indeed, in obese mice, the only apparent sex difference in O₃-induced changes in lung mechanics was the magnitude of the effect of O₃ on baseline Rₐ, which was greater in female versus male mice. Interestingly, the sex differences observed in the microbial community structure of WT mice were also abolished in db/db mice, indicating that the impact of obesity dominates over the impact of sex in the composition of the microbiome and in airway responses to O₃.

It is possible that the ability of antibiotics to attenuate O₃-induced AHR in the female db/db mice was due to a non-specific effect of the antibiotics rather than effects on gut microbiota. However, in male mice, effects of the antibiotic treatment on responses to O₃ were reproduced in germ-free (GF) mice, implicating the depletion of certain microbiota rather than off target effects of the antibiotic administration in the attenuation of O₃-induced AHR (Chapter 2). Thus, the data indicate a complex interaction between obesity, sex, and the microbiome in regulating O₃-induced AHR.

There were substantial sex differences in the impact of obesity on O₃-induced inflammatory cytokine production (Figs. 4.8-4.10). In water-treated mice, obesity augmented O₃-induced increases in BAL IL-17A and IL-33 (Fig. 4.8, 4.9) in female but not male mice and there were correspondingly greater increases in BAL IL-5, IL-9, and IL-6 (Fig. 4.9, 4.10), cytokines known
to be regulated by IL-33 (55, 58). Microbiota have been implicated in the induction of these cytokines within the gut. For example, IL-17A is induced by the presence of segmented filamentous bacteria (75), and significant decreases in IL-33 and type 2 cytokines are observed in the intestines of GF versus conventionally-raised mice with inflammatory bowel disease (76). However, for the most part, we did not observe any changes in BAL cytokines in antibiotic treated versus water-treated mice (Figs. 4.9, 4.10), suggesting a more dominant role for O$_3$ and for obesity than for the microbiome in the expression of these cytokines in the lung.

IL-6 was a notable exception. Antibiotic treatment attenuated the O$_3$-induced increase in BAL IL-6 in obese female mice, which correlated with the antibiotic attenuation of O$_3$-induced AHR in the obese females. In contrast, antibiotic treatment did not impact male BAL IL-6 or their O$_3$-induced AHR. Previous studies utilizing IL-6 knockout mice have shown that IL-6 is required for allergic AHR (77). Furthermore, a recent study has implicated IL-6 in the development of obesity-associated asthma (60): in asthmatic patients, high serum IL-6 correlated with high BMI, worse lung function, and more asthma exacerbations. Furthermore, obesity increases serum IL-6, likely because adipose tissue is a major source of this IL-6 (78). Importantly, antibiotic treatment reduces IL-6 mRNA expression in adipose tissue (32). Together, these data suggest that the microbial effects on IL-6 may be important in the O$_3$-induced AHR of female mice.

In addition to the sex- and obesity-associated differences in inflammatory cytokines, there was also a sex difference in the impact of obesity on serum concentrations of the SCFA propionate. The majority of circulating SCFAs come from microbial fermentation of dietary fiber (79).
Importantly, obesity increased serum propionate to a significantly greater extent in female than male mice (Fig. 4.12B) and similar trends were observed for the SCFAs acetate and butyrate (Fig. 4.12A, C). Obesity also caused significant increases in the relative abundance of the propionate-producing bacteria, *Bacteroides*, in female but not male mice (Fig. 4.4F). We have previously reported that exogenous administration of propionate augments the magnitude of O₃-induced AHR (Chapter 2). Hence, it is conceivable that antibiotic treatment decreased the magnitude of O₃-induced AHR in female mice because their AHR is being driven by SCFA-producing bacteria. In contrast, in obese male mice, other non-microbiota-dependent factors must be driving their enhanced O₃-induced AHR.

There were sex differences in the ability of obesity to promote neutrophil recruitment after O₃ exposure. Compared to female WT mice, female *db/db* mice had greater O₃-induced increases in BAL neutrophils, consistent with previous reports in obese female mice (55, 73, 80), whereas this was not true in male mice (Fig. 4.13). As described above, BAL concentrations of IL-33 and IL-6, which have each been implicated in obesity-related increases in the ability of O₃ to recruit neutrophils to the lungs (55, 67), were both greater in obese female than obese male mice exposed to O₃ (Figs. 4.9B, 4.10) and may explain the sex-dependent effects of the impact of obesity on BAL neutrophils (Fig. 4.13). Antibiotic treatment attenuated O₃-induced neutrophil recruitment in males, but not in females (Fig. 4.13). IL-33 or IL-6 cannot be contributing to this attenuation because neither cytokine were attenuated by antibiotic treatment in male mice (Fig. 4.9B, 4.10). Antibiotic treatment also did not affect other neutrophil chemotactic factors we measured, including KC, IP-10, and actually increased MIP-2 in male mice, suggesting that they are not responsible for the attenuation of neutrophils in the male mice (Figs. 4.14A, 4.16). Thus,
the antibiotic reduction of neutrophils in male mice must be attributed to other factors that are affected by the microbiome in the male mice. Others in our lab have reported that treatment with an antibody to gastrin-releasing peptide (GRP) attenuates O₃-induced neutrophil recruitment in \textit{db/db} mice (81), but it remains to be established whether the release or activity of GRP is impacted by the microbiome.

We observed sex differences in the impact of the microbiome on obesity-related increases in the response to O₃ (Fig. 4.8). Obesity augments responses to O₃ in humans (12), and the magnitude of this impact is greater in women versus men (82). There are also sex differences in the prevalence of obesity-associated asthma, and the magnitude of risk is greater in women (40, 74). Clinical asthma phenotyping data also indicate that obese asthma occurs predominantly in older females, and weight loss improved AHR only in those with little or no evidence for an allergic (IgE-mediated) asthma (83). Our data indicate that sex differences in the impact of obesity on the gut microbiome may play a role in the sex-biased nature of this asthma phenotype.

Obesity increases the risk, magnitude, and symptoms associated with asthma, especially in older women, yet obesity also reduces the effectiveness of established asthma therapeutics. Microbiome-based therapies such as dietary changes, probiotics and prebiotics, may provide an alternative therapeutic strategy for obese asthmatic patients. Importantly, these therapies may need to be tailored differently for men and women, because of sex-associated and obesity-associated differences in microbial ecology.
4.6. REFERENCES


CHAPTER 5: Conclusion
5.1. The role of the microbiome in obesity-associated asthma

The goal of this research was to examine the hypothesis that obesity-related changes in the gut microbiome may contribute to the etiology of obese asthma. Because obese asthma is more commonly non-atopic than atopic, we utilized a mouse model of non-atopic airway hyperresponsiveness, a canonical feature of asthma, in which airway hyperresponsiveness was induced by O$_3$ exposure.

In chapter 2, we compared O$_3$-induced AHR in conventionally-raised versus GF mice and in control mice versus mice treated with either individual antibiotics (ampicillin, metronidazole, neomycin, and vancomycin) or the combination of these antibiotics. O$_3$-induced AHR was attenuated in GF mice, in mice treated with the antibiotic cocktail, and in mice treated with all individual antibiotics except neomycin. 16S sequencing of fecal DNA identified two genera that were associated with O$_3$-induced AHR, *Ruminococcus* and *Coprococcus*, both known SCFA producers. Serum analysis indicated reduced concentrations of the SCFA, propionate, in mice with reduced O$_3$-induced AHR after antibiotics. Furthermore, supplementation of the drinking water with propionate resulted in augmented O$_3$-induced AHR. In summary, we established that the microbiome contributes to O$_3$-induced AHR, likely via its ability to produce short chain fatty acids.

In chapter 3, we explored sex differences in the microbiome and how they contribute to the differences in O$_3$-induced AHR observed in lean male versus lean female mice. Our interest in sex differences was motivated by the predominantly female bias of the obese asthma population.
We perturbed the gut microbiomes of male and female mice using an antibiotic cocktail given via the drinking water. Mice were subsequently exposed to air or to O₃. Antibiotic treatment abolished sex-differences in O₃-induced AHR. We also placed female mouse pups, at weaning, into cages that were conditioned by adult male or female mice. Once the mice reached adulthood, female pups that had been in cages previously occupied by adult males developed greater O₃-induced AHR than female pups in cages previously occupied by adult females. Thus, exposing female mice to a male microbiome reproduced the male phenotype of greater O₃-induced AHR. Together, our data indicate that sex-related differences in the microbiome contribute to sex differences in the magnitude of O₃-induced AHR.

Finally, in chapter 4, we examined the role of the obese microbiome in the augmented O₃-induced AHR observed in obese mice, and the role of sex in the interaction between obesity and the microbiome. To do so, we bred heterozygote db⁺⁻ mice to obtain male and female obese db/db mice and lean WT littermates. The gut microbiome was perturbed with the same cocktail of antibiotics administered via the drinking water that was used in Chapters 2 and 3. We found that sex differences in the microbiome of lean mice were largely abrogated in the obese mice. Similarly, sex-differences in the magnitude of O₃-induced AHR observed in lean mice were abrogated in obese mice. Compared to lean mice, both male and female obese mice exhibited greater O₃-induced AHR. Furthermore, whereas lean male mice had greater responses to O₃ than lean female mice, that was no longer true in obese male and female mice. Antibiotic treatment had a profound effect on obesity-related changes in the magnitude of O₃-induced AHR that differed by sex. In female db/db mice, antibiotic treatment attenuated O₃-induced AHR, almost to the levels of the WT mice. In contrast, in the male mice, antibiotic treatment slightly
increased O₃-induced AHR. Hence, antibiotic treatment augmented the sex differences in the obese response to O₃. We found higher serum SCFA in female versus male db/db mice and obesity-related increases in the prevalence of the SCFA producer, Bacteroides in female but not male mice. Thus microbiome-dependent changes in SCFA may explain the impact of antibiotics in attenuating O₃-induced AHR in female db/db mice but not in male db/db mice. Our data indicate that sex-and obesity-related differences in the microbiome contribute to the impact of obesity on O₃-induced AHR.

5.2. Sex differences

There is increasing evidence for disparate progression and mechanisms of disease in male and female subjects. There is often a noted sex bias in experimental design and analyses in cell and animal research (1), and the National Institute of Health has recently called for the inclusion of both sexes in any research funded by public funds (2, 3). The utilization of one sex in research obscures key sex differences that could guide clinical studies and may contribute to incorrect drug dosing or lack of efficacy in treatment in a subset of population, such as the late-onset female non-atopic obese asthmatics. Indeed, we found sex differences in the microbiome, and sex differences in the response to O₃ in mice (Chapter 3). In addition, the phenotype of greater O₃-induced AHR in male mice was transferred to female mice that were housed in cages previously occupied by male mice. Our data suggest that sex differences in the microbiota of male and female asthmatic patients could contribute to sex differences in the prevalence and severity of asthma.
Over two decades have passed since obesity-associated changes in the microbiome were first noted (4, 5), yet there has been surprisingly little research on how this obesity-associated microbiome impacts non-gastrointestinal disease. In addition, there is a gap in knowledge on how obesity-associated changes in the microbiome differ between male and female subjects. We found evidence that the impact of obesity on the microbiome dominates over the impact of sex, although there were still some differences in the microbial community structure of obese males and obese females (Chapter 4). Obese male and female mice treated with water responded similarly to O$_3$, but antibiotic treatment had a profound effect on obesity-related changes in the magnitude of O$_3$-induced AHR that differed by sex. Our data indicate the importance of examining the nexus of obesity, sex, and the microbiome in patients’ response to O$_3$ and other non-atopic factors that trigger the innate immune response.

5.3. **Therapeutic implications**

Obesity is a risk factor for asthma (6, 7). Both the prevalence and incidence of asthma are increased in the obese population (8-10). Weight loss causes substantial reductions in asthma symptoms in obese asthmatic patients, and reduces airway hyperresponsiveness (AHR) (11, 12). Additionally, many obese asthmatics have difficulty controlling their asthma (13, 14). Indeed, steroids are less effective in obese than lean asthmatics (15). It is possible that aspects of the obese state, for example the low grade systemic inflammation characteristic of obesity, reduce steroid efficacy by interfering with corticosteroid signaling pathways (15). However, it is also possible that obese asthmatics have a phenotype that is not responsive to steroids: steroids target the immune responses typical of allergic asthma, but many obese asthmatics are non-atopic (16,
17). Understanding the mechanistic basis for obese asthma may allow for the development of other therapeutic options that have greater efficacy in this population.

Our data in Chapter 2 indicate that one of the SCFAs, propionate, may contribute to the development of O₃-induced AHR. Furthermore, compared to lean mice, serum SCFAs were greater in obese mice, consistent with their augmented O₃-induced AHR (Chapter 4). The pulmonary response to acute O₃ exposure involves activation of the innate immune system (18). Others have reported that the role SCFAs in disease states differs depending on whether the innate or adaptive immune system is involved (19). For example, in models where the adaptive immune system is important, such as allergic airways disease and experimental autoimmune encephalomyelitis, SCFA treatment is found to be beneficial (19). On the other hand, in models in which only the innate immune system is involved, such as the initial-stages of antigen-induced arthritis and Parkinson’s model, SCFAs exacerbate inflammation and symptom severity (19). Thus, a careful consideration of microbial-associated therapeutic recommendations is necessary depending on the specific disorder. Interventions such as high fiber diets that promote the survival of SCFA-producing bacteria are often recommended in obesity. However, our data indicate that such dietary changes might actually exacerbate symptoms in an obese non atopic asthmatic, as it might augment airway hyperresponsiveness. The different microbial community structures of males and females, and of lean and obese subjects, may result in heterogeneity of response to the increase in circulating SCFAs caused by high fiber diets. Further studies are needed to examine SCFAs and their role, whether beneficial or harmful, in human health.
A role for the microbiome in obesity-related asthma has both public health and therapeutic implications. The gut microbiome is shaped by early life events, including mode of delivery, breastfeeding, diet, and antibiotic use. Understanding the impact of these factors on the development of both obesity and asthma could alter early life decisions that impact long-term disease development. Greater understanding of the role of microbiota in obesity-related asthma could also pave the way for the development of novel microbiota-based treatments for this difficult-to-treat group. For example, it is conceivable that altering the gut microbiota with probiotics, prebiotics, or even fecal transplants could ameliorate obesity-related asthma or improve the ability of obese asthmatics to respond to standard asthma therapeutics. Indeed, such interventions are effective against other obesity-related conditions (20-23). Microbiota-based therapies that impact weight might also prove effective, given the efficacy of weight loss in obese asthmatics (12). Since diet is a major determinant of the gut microbial community structure, it is also conceivable that incorporating more of certain foods into the diet could improve obese asthma even in the absence of major weight loss. Finally, it may be that host immune system development can be shaped by factors affecting the developing gut microbial communities, including early life choices and exposure to environmental bacteria. Understanding the role of gut microbiota in obesity-related asthma could pave the way for development of novel microbiota-based treatments for this difficult-to-treat group.
5.4. REFERENCES


Appendix
Appendix Figure 1: Body weight is unchanged in germ-free mice, and with antibiotic and propionate treatments. Body weight of A) control water [W] versus antibiotic cocktail [AMNV] treated mice, B) specific pathogen free [SPF] versus germ-free [GF] mice, C) GF mice given AMNV versus control water, D) control water [W] versus antibiotic cocktail [AMNV], ampicillin [A], metronidazole [M], neomycin [N], and vancomycin [V] treated mice, and E) water versus propionate treated mice. Results are mean ± SE of data from n=6-8 per group.
Appendix Figure 2: O₃-induced increases in BAL cytokines are not affected by antibiotic treatment. A) BAL IL-17A and B) BAL osteopontin assessed via ELISA. Results are mean ± SE of data from n=6-8 per group. * denotes p<0.05 compared to air. # denotes p<0.05 compared to water treatment.
Appendix Figure 3: Microbial abundances of mice treated with water, AMNV, and individual antibiotics. A) alpha-diversity calculated by inverse of the Simpson diversity index, and B) relative abundance of bacteria, at the phylum-level, in feces of mice treated with water, cocktail [AMNV] or individual antibiotics. Each dot in (A) denotes one mouse. Sequence reads assigned to each phylum at 97% sequence similarity cut-off. n=8 per treatment group.
Appendix Figure 4: Serum acetate and butyrate in water, neomycin, and AMNV-treated mice. Serum A) acetate and B) butyrate levels in water-, AMNV-, and neomycin-treated mice after air exposure. Results are mean ± SE of data from n=7 per group. # denotes p<0.05 compared to Neomycin.
Appendix Figure 5: Relative abundance of known short chain fatty acid microbes after antibiotic treatments. Relative abundance of A) Akkermansia, B) Anaerostipes, C) Bacteroides, D) Bifidobacterium, E) Blautia, F) Clostridium, G) Dialister, H) Prevotella, I) Streptococcus, and J) Veillonella after control water, antibiotic cocktail (AMNV), ampicillin, metronidazole, neomycin, and vancomycin treatments. Results are mean ± SE of data from n=8 per group.