DISSERTATION

THE EFFECT OF HOP EXTRACT SUPPLEMENTATION ON GUT MICROBIOTA AND METABOLIC FUNCTION IN OVARIECTOMIZED MICE

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ABSTRACT

THE EFFECT OF HOP EXTRACT SUPPLEMENTATION ON GUT MICROBIOTA AND METABOLIC FUNCTION IN OVARIECTOMIZED MICE

Estrogen decline with aging, or menopause, is associated with increased risk for cardiometabolic diseases primarily due to altered metabolism and weight gain. Standard treatment has traditionally been with 17β-estradiol (E2) prescription, although its use has declined over the last decade due to associated increase in breast and ovarian cancer risk. As a result, use of phytoestrogenic herbal supplements has increased, due to their perceived safety and effectiveness in treatment of menopausal side effects. The gut microbiota may also be important in terms of mitigating disease risk and hormone exposure during the menopause transition, as our gut microbiota are important modulators of local and systemic inflammation. Gut microbes also can metabolize hormones and dietary flavonoids, altering their bioactivity and bioavailability. In this study, we supplemented ovariectomized (OVX) or control sham-operated C57BL/6 mice, with oral E2, a flavonoid-rich extract from hops (*Humulus lupulus*), or placebo carrier oil, and observed differences in adiposity, inflammation, and gut bacteria composition. Hops extract (HE) did not protect against ovariectomy-associated weight gain or increased visceral adiposity, while E2-treated animals had similar body weights and fat depot sizes as Sham-operated animals. However, HE was protective against liver triglyceride accumulation, to levels similar to Sham control and OVX E2 groups. We found no evidence of OVX having a significant impact on the overall gut bacterial community structure in any of our treatment groups. We did find differences in abundance of two bacteria; *Akkermansia muciniphila* was lower with HE treatment

in the Sham group, and *Ruminococcus gnavus* was higher with OVX compared to Sham control. Possible mechanisms of the interplay between gut bacteria, loss of estrogen, and hormone replacement will be discussed.

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HOP EXTRACT AS A SOURCE OF NOVEL PRENYLFLAVONOIDS FOR HUMAN HEALTH

Summary

The inflorescence of the hop plant, *Humulus lupulus L.,* contains unique compounds used primarily for brewing beer, yet also contain compounds that have bioactive properties for improving human health. Hops bittering soft resins and aromatic oils derived from their lupulin glands are essential for beer. A third component in lupulin are the hard resins that are undesirable in beer, yet contain bioactive polyphenols shown to lower disease risk. Extracts of hop hard resins contain in antioxidant and estrogenic prenylflavonoids, and are used today as health supplements, especially for the alleviation of menopausal discomforts. However, bioavailability of prenylflavonoids is affected by microbial metabolism in the gut. This review focuses on the use of prenylflavonoid-rich hop extracts by women for alleviation of menopausal complaints, with the added benefit of lowering disease risk including metabolic syndrome and cancer. The role of gut microbiota and bioavailability of the prenylflavonoids will also be discussed.

Introduction

Hops, or the inflorescence of *Humulus lupulus L.* (Cannabaceae), are an essential ingredient in beer providing bitterness, flavor, aroma and antimicrobial activity. Hop resins include the bitter "soft resin" acids that are more desirable, and the less bitter "hard resin" polyphenols. The most important soft resin in hops are the α-acid homologs, which isomerize during the boiling process into the bitter compound iso-α-acids. The vast majority of hops used worldwide are pelletized whole hops, however, extracts of iso-α-acids in their native or reduced forms have been gaining popularity in both large and craft breweries for their bitterness, shelf stability, and foam retention properties [1]. The soft resin extraction process involves supercritical CO_2, leaving 'spent hops' containing the hard resins as a byproduct [2]. A methanol extraction of spent hops yields 22 compounds including 12 prenylated chalcones and 5 prenylflavanones, including the estrogenic 8-prenylnaringenin (8-PN) or 'hopein', the biologically active xanthohumol (XN), and its isomer isoxanthohumol (IX) [3]. These compounds have been studied for their bioactive health properties such as mitigation of menopausal complaints, carcinogenesis, inflammation, and adiposity [4]. Therefore, it is of interest to major hop companies to utilize this hop extract agricultural by-product for pharmaceutical purposes.

Reports on the medicinal uses of hops date to before the Middle Ages. They were important in many cultures including European, Indian, Native American and Arabic communities. Infusions of hops were drunk for their sedative effects and hops-filled pillows were thought to help induce sleep [5]. Hops medicines were also used in the treatment of coughs, bladder and liver ailments, and digestive diseases. However, hop medicines were particularly valued by females and were included in baths for treatment of gynecological

disorders or as hops-infusions that were drunk to reduce hot flushes during menopause [6]. Prior to the advent of mechanical hops-picking, the estrogenic activity of hops also caused disruption of the menstrual cycle of female hops pickers [7, 8].

Table 1.1. Review of studies performed using prenylflavonoid-rich extracts (including XN, IX and 8-PN) from spent hops from the supercritical CO_2 extraction process of soft resins for the brewing industry

Effect	Type of Study	Model	Reference
Alleviation of menopausal discomforts and vasomotor symptoms	In vivo randomized, double-blind	Postmenopausal women	[42]
Alleviation of menopausal discomforts	In vivo randomized, double-blind, crossover	Postmenopausal women	[43]
Chemical characterization of spent hops extract	Chemical analysis	HPLC/MS chromatography	[3]
Chemical characterization of spent hops extract	In vitro, chemical analysis	Ishikawa cells, HPLC/MS	[65]
Chemopreventative through attenuation of estrogen oxidative metabolism	In vitro	Human mammary epithelial cells	[66]
Regulates detoxification enzymes in liver and mammary glands	In vivo	Rats	[67]

The identification of potent phytoestrogens, plant secondary metabolites that are able to act as estrogen receptor agonists or antagonists, isolated from hop extracts provides a scientific basis for these traditional uses. Today, hop extract-based pharmaceuticals are on the market for use as a natural estrogen replacement to alleviate symptoms of menopause (Table 1.1). Loss of estrogen during menopause is associated with increased risk for metabolic diseases such as obesity and heart disease. Traditional synthetic estrogen treatment has declined recently due to its associated increased risk in breast and endometrial cancer [9], and women are looking for a

more natural alternative. In this review, we discuss the use of hop extracts during menopause due to their estrogenic and health-promoting polyphenols, as well as the role of the gut microbiota on hop extract bioavailability and overall disease prevention.

Bioactive Compounds in Hops

Over 100 different compounds from hops have been identified and studied for their potential bioactivities [10]. The α-acid component, collectively known as humulones, have been shown to reduce proliferation and induce apoptosis in leukemia cells and are COX-2 inhibitors that have more anti-inflammatory activity than nonsteroidal indomethacin [11]. The β-acids, or lupulones, are antimicrobial against gram positive bacteria including common beer spoiling organisms [12]. Myrcene, a monoterpenoid from hop oil acts as an analgesic. Since hops analgesic effects can be counteracted by naloxone in mice, it has been hypothesized that it acts by stimulating endogenous opioid release via the α2-adrenoreceptor [13].

Prenylated flavonoids are the phytoestrogenic compounds found in hops extracts and include 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN), desmethylxanthohumol (DMX), isoxanthohumol (IX) and xanthohumol (XN). While flavonoids are present in almost all plants, prenylated flavonoids are limited to a few families including *Cannabaceae, Guttiferae, Leguminosae, Moraceae, Rutaceae* and *Umbelliferae* [14]. There are >1000 plant-derived prenylflavonoids characterized to date [15]. Hop prenylflavonoids 8-PN, XN and IX are unique to hops, and XN especially has been studied for its role in disease prevention and treatment, including obesity [16], diabetes [17], and cancer [18]. Therefore, the abundance of XN in hop extracts may help prevent metabolic diseases associated with menopause. Furthermore, in vitro activity of XN is increased as a hop extract form rather than in its pure form, perhaps due to the

presence of several other polyphenolic substances [19]. 8-PN and 6-PN bind to estrogen receptors, although both are found in relatively low abundance in hop extract, and 6-PN is a very weak estrogen agonist [20]. Isoxanthohumol is considered a pro-estrogen due to its ability to be converted to 8-PN by intestinal bacteria *in vivo* [21]. Research on hop prenylflavonoids for health has increased significantly in the past decade, and their reported bioactivities have recently been reviewed [4, 22-25].

Hop Prenylflavonoids as an Estrogen Replacement Therapy

Early studies on hop estrogenic activity, beginning in the 1950s, showed conflicting results due to the chemical variation of extracts used [8, 26, 27]. Xanthohumol, probably because of its high abundance in the polyphenol fraction, was originally identified as the molecule with estrogenic properties [28]. It wasn't until 1999 that Milligan and colleagues identified 8-prenylnaringenin (8-PN) as the responsible estrogenic compound in hops [29], and 8-PN remains the most potent phytoestrogen known to date [7, 30-32]. Other phytoestrogens studied, such as those found in soy and red clover extracts, preferentially bind to ERβ, which is the most abundant estrogen receptor in the digestive tract where it regulates intestinal permeability [33, 34]. Hops 8-PN show an equal [7], to two-fold higher [35] preferential binding to ERα compared to ERβ, suggesting that it may have broader estrogenic activity. Phytoestrogens including 8-PN are selective estrogen receptor modulators (SERMS), and can be receptor agonists or antagonists depending on the tissue site of action [36].

Prenylflavonoid phytoestrogens exert estrogenic effects through two mechanisms: direct binding to estrogen receptors, and disruption of aromatase enzyme activity. Aromatase is an enzyme responsible for synthesizing estradiol from androgens. Hop prenylflavonoids (XN, IX,

and 8-PN) have been shown to inhibit aromatase activity, thus lowering overall circulating estradiol levels [37]. This is of importance since breast tumors express abnormally high levels of aromatase, and its suppression mediates carcinogenesis [38]. Hop prenylflavonoids, especially XN, have been widely studied for their broad-spectrum chemopreventive activities, including growth inhibition of early-stage tumors, induction of carcinogen-detoxifying enzymes, and inhibiting procarcinogen activation [4, 39]. Since synthetic estradiol hormones pose a higher risk of breast cancer [40, 41], and hop extracts containing XN, IX and 8-PN have been shown to reduce menopausal complaints and frequency of hot flashes in post-menopausal women [42, 43], hop extracts may be a more suitable drug for hormone replacement therapies due to their estrogen receptor activity and inhibition of aromatase.

Just prior to the discovery of 8-PN, a similar molecule 8-isopentylnaringenin was extracted from a Thai menopausal drug derived from the *Anaxagorea luzonensis* tree. This was the first prenylflavonoid to show estrogenic activity, due to the 8-isopentenyl (prenyl) side chain's high affinity for estrogen receptors [44]. Flavonoids with a prenyl side chain are more lipophilic, resulting in greater affinity to cell membranes and proteins [45]. However, prenylation of the widely researched soy phytoestrogen genestein, surprisingly resulted in a loss of estrogenicity [30]. The relationship between minute changes in chemical structure and estrogenicity is complex and varying bioactivities of prenylated flavonoids may be due to differences in bioavailability and gut microbial metabolism.

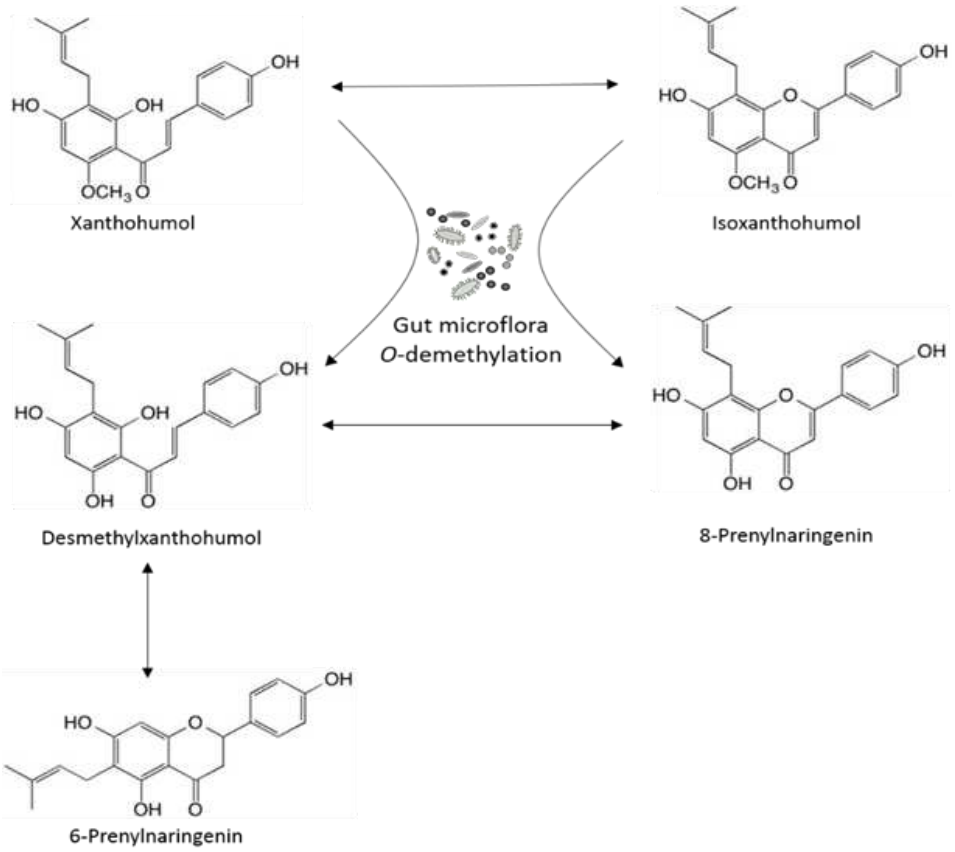

Fig 1.1: In vitro transformation of hop prenylflavonoids spontaneously, or by microbial metabolism

8-PN and Gut Microbiota

Plant polyphenols, including the prenylated flavonoids from hops, have been studied for their health benefits which include antioxidant activity, reduction of DNA damage, modulator of cell proliferation and metastasis, inhibition of pathogens, and modulation of immunity [46-48]. However, the bioavailability of these compounds in vivo is typically low, with limited accumulation and short-half life in the blood- stream and tissues, calling into question the mechanism by which these compounds exert their beneficial effects. Only recently have such benefits been correlated with the fermentation of polyphenols by gut microbiota [49]. Approximately 90-95% of dietary polyphenols accumulate in the colon where they are subjected

to microbial fermentation [50-52], where resulting metabolites may exert direct beneficial effects on intestinal inflammation and epithelial barrier integrity.

The bioactivity of hops extracts is, in part, mediated by gut bacterial metabolism of IX. Although the estrogenic effects of pure 8-PN are well-documented, its concentration in hop extracts and food sources, such as beer, is limited, with XN and IX being the dominant prenylflavonoids detected [53]. Recent studies have shown that the concentration of 8-PN varies widely between individuals, and differences in 8-PN exposure is primarily due to differences in liver CYP450 enzymes and gut bacteria composition. The human liver microsome CYP1A2 is able to produce 8-PN by *O*-demethylation of IX in vitro, and thus concentration and bioactivity of 8-PN may depend on liver microsome status [54].

However, exposure to 8-PN is probably more dependent on the composition of gut bacteria (Fig 1.1). It was first discovered in 2005 that gut bacteria increased exposure to 8-PN through metabolism of IX, specifically by the bacteria *Eubacterium limosum* [21]. Germ-free rats were unable to produce 8-PN from IX, unless *E. limosum* was co-administered as a probiotic [55]. While *E. limosum* is the only specific bacteria identified to date that can convert IX to 8-PN, there are likely several other species capable of *O*-demethylation of IX to 8-PN. It is interesting to note that *E. limosum* also produces butyrate, a short chain fatty acid that is beneficial for colon health [56].

Though the in vivo estrogenicity resulting from xanthohumol or isoxanthohumol consumption still needs to be evaluated, human in vitro studies have shown a great deal of individual variability in the ability of gut bacteria to convert IX to 8-PN. In a study of 51 human in vitro fecal incubations with IX, individual samples were categorized as high, moderate, or low converters of IX to 8-PN. Interestingly, 20% of the samples showed no conversion, while 16%

produced very high levels of 8-PN [57]. Similar proportions were found in 50 post-menopausal women after orally consuming hop extract supplements. The ability to convert IX to 8-PN was negatively correlated with recent antibiotic use and alcohol consumption, and positively correlated with theobromine, which is found in chocolate [58]. This suggests that mixed results of the efficacy of hops extracts to treat menopausal symptoms in humans may be a result of variability in microbiota composition that contributes to determining whether individuals will be responsive or nonresponsive to the treatment.

Conclusions

Menopause and accompanying decline in host estradiol production is linked to increased risk for metabolic disease. The gut microbiota may be a key player during menopause, since it known to influence metabolic disease risk including cancer [59, 60]. Such risk may involve the interplay between the gut and host endocrine system [61]. Metabolism of steroid hormones and phytoestrogens by commensal flora is important in maintaining healthy colonic tissues. ERβ regulates intestinal permeability, and is the most abundant estrogen receptor in the digestive tract [33, 34]. Lowered levels of circulating host-produced estrogen or dietary phytoestrogens can therefore influence the growth and physiology of gut bacteria. Our lab recently found that ovariectomy and its associated loss of estrogen in female rats altered the gut community structure and increased species richness [62]. In another study, mice fed a diet containing isoflavone phytoestrogens showed protection against gut dysbiosis compared with a phytoestrogen-free diet, associated with a higher abundance of *Lactobacillales* and decrease in *Proteobacteria* [63]. In humans, it has been shown in post-menopausal women that supplementation with isoflavone phytoestrogens altered composition of dominant gut bacterial communities [64]. In conclusion,

loss of estrogen is known to alter the gut microbiota, which is likely to be linked with development of central adiposity and the increased risk for developing metabolic diseases in post-menopausal women.

Today, women are choosing not to take synthetic estradiol during menopause due to the increased risk of breast and uterine cancers [9], and instead looking for a more natural estrogen replacement. 8-PN is the most potent phytoestrogen known, and can mitigate the loss of estrogen during menopause and its associated changes in commensal gut flora. Hop extracts from spent hops not only contain 8-PN, but its precursor IX, the highly bioactive XN, and other polyphenols that can potentially prevent gut dysbiosis, as well as lower disease risk through their direct antioxidant, anti-inflammatory, and antiproliferation properties. Spent hop extracts are relatively easy and cheap to produce, and utilize a large amount of waste product from the brewing extract process that would otherwise be thrown out. Several companies already offer hop extract, often mixed with other compounds such as soy isoflavones, taken orally during and after menopause, and its safety and efficacy has been established in research outlined in Table 1. However, the role of commensal gut microbiota in efficacy of hop extract for alleviation of menopausal symptoms and lowered disease risk has not been directly investigated. We are currently addressing this knowledge gap in a rodent model, and have promising preliminary data showing changes in gut community structure and lowered metabolic syndrome risk with supplementation of spent hop extract.

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CHAPTER 2: PHYTOESTROGEN HOP EXTRACT AND 17B-ESTRADIOL TREATMENT FOR MENOPAUSE RELATED CARDIOMETABOLIC DISEASE RISK

Summary

Menopause is a significant life event in all women. It is associated with increased risk for cardiometabolic diseases primarily due to altered metabolism and weight gain, particularly increased abdominal adiposity. Standard treatment has traditionally been with 17β-estradiol (E2) prescription, although use has declined over the last decade due to associated increase in breast and ovarian cancer risk. As a result, use of phytoestrogenic herbal supplements has increased, due to their perceived safety and effectiveness in treatment of menopausal side effects. The most potent phytoestrogen known to date is the flavonoid 8-prenylnaringenin (8PN) from the hops plant (*Humulus lupulus)*. In this study, we used ovariectomized mice to study the in vivo metabolism of phytoestrogenic hop extract (HE) and its effect on cardiometabolic disease risk factors. We also measured levels of HE flavonoids and their metabolites in serum. HE extract did not protect against ovariectomy-associated weight gain or increased visceral adiposity, while E2-treated animals had similar body weights and fat depot sizes as Sham-operated animals. However, HE was protective against liver triglyceride accumulation, to levels similar to Sham control and OVX E2 groups. Importantly, HE did not contribute to uterine proliferation, while E2 treatment was associated with greater uterine weights. Results from this study will add to the body of literature supporting HE as a safe phytohormone treatment for menopause symptoms.

Introduction

After the age of 40, a woman's ovaries decline in estrogen production, marking the onset of menopause, with consequences for her long-term health. This loss of endogenous estrogen (17-β estradiol; E2) is associated with increased visceral adiposity and risk for metabolic disease such as cardiovascular disease (CVD) and fatty liver [1-3].

Menopause is also associated with uncomfortable symptoms such as hot flashes, insomnia, vaginal dryness, irritability and fatigue, which leads many women to seek treatment to alleviate these issues [4-6]. Historically, the first-line treatment was hormone therapy (HT), however the health consequences of HT came under fire with the Women's Health Initiative (WHI) Study [7]. It is unclear if HT affects cardiometabolic disease risk in women, and timing and dosage are important variables influencing outcomes [8]. Current evidence suggests that HT can be cardioprotective in women of low-risk for CVD if started within 10 years of menopause, but have the opposite effect if started after menopause has progressed [9, 10]. Despite potential for protection against cardiometabolic disease, research over the last two decades has associated HT with breast and uterine proliferation, increasing the risk of cancer in these tissues [11-15]. Therefore, many women, especially those with predisposed genetic risk for cancer, seek alternatives to HT for alleviating symptoms associated with the menopause transition.

As an alternative to HT, many women use dietary supplements containing naturally-occurring estrogen mimetics from plants, known as phytoestrogens. Common phytoestrogens found in herbal menopause relief supplements include polyphenolic compounds from soy, black cohosh, dong quai, red clover and hops. Today, it is estimated that almost half of all menopausal women take botanical phytoestrogens to alleviate symptoms [16, 17]. Several studies in human

and animal models show efficacy of phytoestrogens in relieving menopausal symptoms while possibly lowering risk of cardiometabolic diseases [18, 19].

In addition to their estrogenic activities, phytoestrogens are usually flavonoid compounds that are known to modulate antioxidant and anti-inflammatory pathways including PPAR, TNF-α, NF-kB, and MAPCK [20, 21]. Activation of these signaling pathways may help prevent diseases associated with metabolic syndrome. Several phytoestrogens have been shown to have anti-inflammatory effects, including resveratrol [22], genistein [23], and quercitin [24]. Beneficial health effects of phytoestrogens as flavonoids, aside from interaction with hormonal signaling, has been reviewed recently [25, 26]

Prenylflavonoids from hops (*Humulus lupulus*) have recently received attention for their beneficial role in health and disease, including menopause. Hops were first reported to have estrogenic properties in 1953 [27], yet anecdotal evidence from the early 1800's recounts female hop pickers experiencing menstrual aberrations during harvest [28]. Several prenylflavonoids from hops have been isolated and studied for their bioactive effects including estrogenicity. 8-prenylnaringen (8PN) is responsible for most of the estrogenic activity in hop extracts and is the most potent phytoestrogen known to date [29].

Purified 8PN and hop extracts containing mixtures of hop flavonoids have been studied in human, animal and in vitro models for their estrogenic activity, and alleviation of menopausal symptoms and associated disease risk [30, 31]. Previous ovariectomized murine models have shown that 8PN reduces hot flushes in mice [32] without inducing uterine growth and proliferation [33]. In menopausal women, oral administration of hop extract (HE) improved hot flushes and overall menopausal discomfort [34]. We used the same HE in our study, previously marketed as Lifenol®, from MetaGenics (Aliso Viejo, CA, USA).

Hop extracts contain not only 8PN, but also other prenylflavonoids including xanthohumol (XN) and its isomer isoxanthohumol (IX). XN and IX do not have appreciable estrogenic activity, yet are considered pro-estrogenic due to their conversion into 8PN through liver microsome and gut bacterial metabolism. XN in particular has received much attention for its effect on lowering cholesterol, blood sugar, and reducing weight gain [35-41].

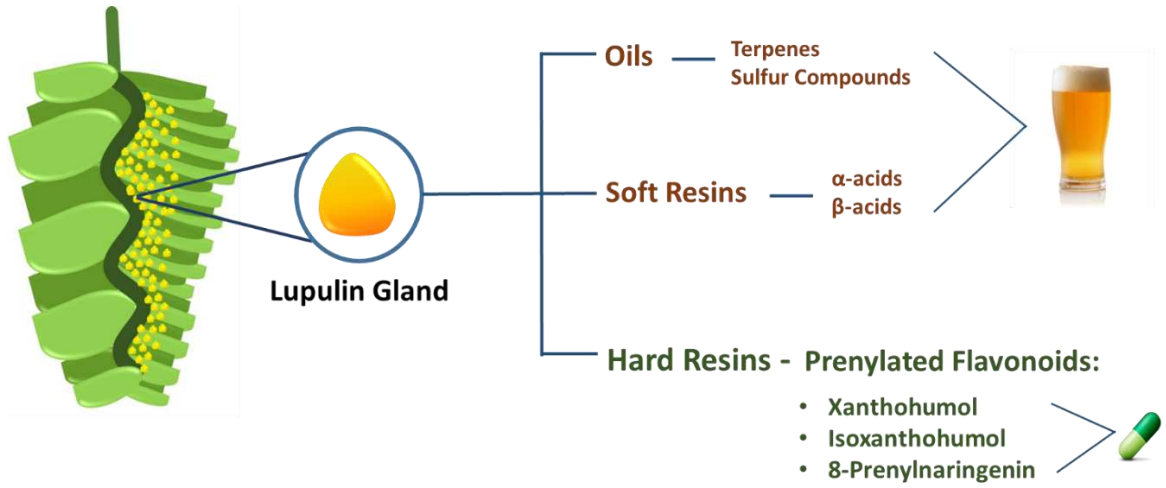

Fig 2.1: Important constituents of hops for brewing and pharmaceutical industries.

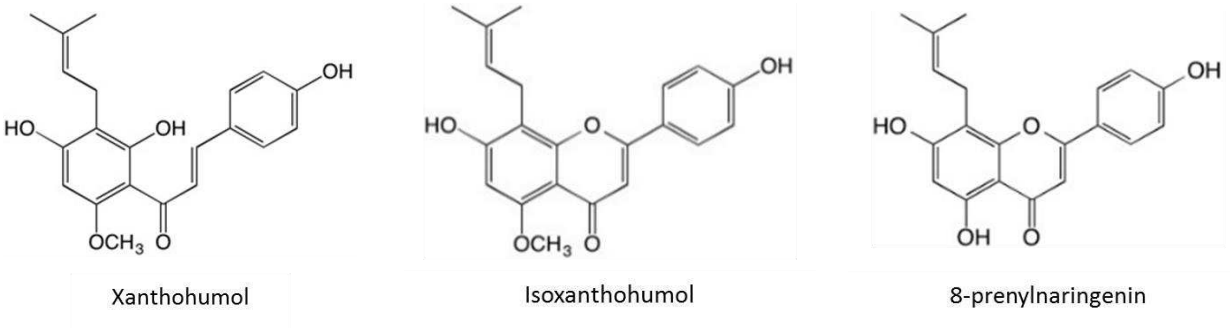

Fig 2.2: Major prenylated flavonoids in hop extract (HE).

In the present study, an ovariectomized (OVX) mouse model was used to investigate the effects of E2 and prenylflavonoid-rich HE on physiological parameters associated with menopause. Ovariectomy in murine models has been shown to induce deleterious metabolic outcomes including increased adiposity, similar to consuming a high fat diet [42]. The hypothesis tested was that OVX-induced changes in adiposity would be mitigated by hormone therapy via E2 or HE. Flavonoids in the HE formulation was quantified, their delivery in serum confirmed, and the in vivo kinetics of HE metabolism were quantified. Results showed that both E2 and HE protected against accumulation of liver triglycerides. Only E2 mitigated OVX-induced gain in visceral adipose tissue and overall body weight gain.

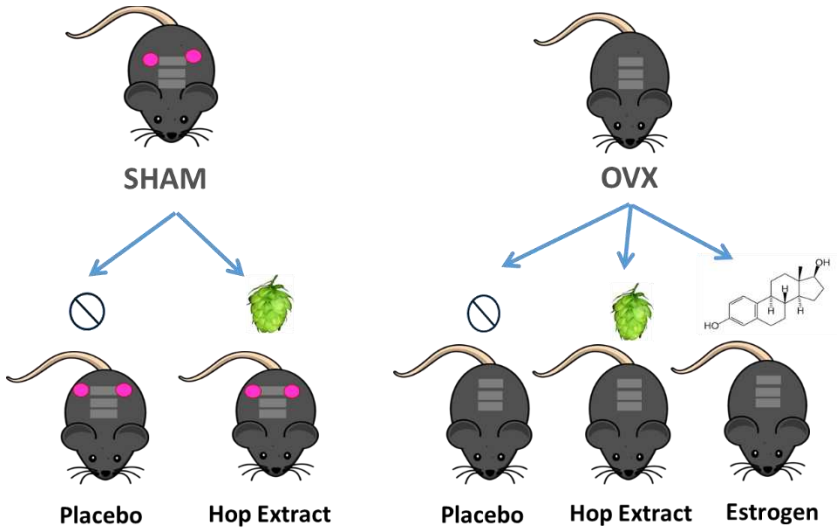

Fig 2.3 Study design. Age-matched female retired breeder C57BL/6J mice underwent ovariectomy (OVX) surgery to remove ovaries, or Sham surgery without removing ovaries.

Materials and Methods

Animals and Experimental Design

Animal conditions met the standards of the Animal Welfare Act regulations and Guide for the Care and Use of Laboratory Animals, and animal care and procedures were approved by the Colorado State University Institutional Animal Care and Use Committee.

Female C57BL/6 retired breeder mice were obtained from Charles River Laboratories (Wilmington, MA, USA). All mice were born on the same day and were 7 months old at the start of the study. Upon arrival, mice were housed individually in an environment controlled for temperature, humidity, and light cycle (12h light:dark). Mice were provided a phytoestrogen-free standardized purified low fat diet with 4% calories from fat (TD.08113 Harlan, Madison WI) and water ad libitum. After 2 weeks of acclimation, mice were individually housed and randomized into groups based on average weight and fasting blood glucose. Under isoflurane anesthesia, mice underwent dorsal ovariectomy (OVX) with an incision through skin and muscle just caudal to the last rib and about 1 cm ventral to the dorsal spinous process of the third lumbar vertebra, followed by ligation of ovaries. Control groups underwent sham surgery including exposure, but not ligation of the ovaries. Muscle was sutured and the skin incision was closed with wound clips. Mice received analgesic (Meloxicam) prior to surgery and for 24 hours post-surgery. Body weight and food intake were measured weekly for 12 weeks. Five study groups included: OVX Placebo (n=11), OVX plus hop extract (HE; n=11), OVX plus 17β-estradiol (E2, n=9), Sham Placebo (n=10), and Sham HE (n=8).

All mice were maintained on a purified phytoestrogen-free diet (Harlan TD.08113) with 13.8% calories from protein, 76.0% from carbohydrates, and 10.2% from fat. Four to seven days post-surgery, mice began treatments of 17β-estradiol (E2; Sigma-Aldrich, St. Louis, MO), hop

extract (HE; MetaGenics, Aliso Viejo, CA) or placebo (sesame seed oil). The treatments HE and E2 were suspended in 20uL sesame oil and dissolved onto 0.2g of a hazelnut wafer cookie (Quadratini, Loacker®), while control placebo groups received only the cookie and sesame oil. All groups consumed cookies daily within 10 minutes of administration. Based on previous studies, we administered 56 mg/kg E2 [43] and 400 mg/kg HE [44, 45] to the mice daily. This amount of HE was composed of 5099.87ng/mg 8PN, and 6315.81ng/mg XN, as determined by UHPLC-MS of the powdered extract. Considering the diet plus cookie, mice obtained 11.1% of their total calories from fat, 13.6% from protein and 75.4% from carbohydrate (Fig A1).

Tissue Collection

Mice were fasted for 4 hours before termination, anesthetized with carbon dioxide and euthanized by exsanguination. Blood was collected and allowed to clot at room temperature for 30 minutes, then was incubated on ice for 90 minutes before centrifugation at 8,000 rpm for 10 minutes at 4°C. Serum was drawn off and stored at -80°C until further analysis. Liver, ileum, proximal colon and distal colon were excised, cleaned with saline and immediately frozen in liquid nitrogen. Adipose tissue, uterus and cecum tissues were weighed and recorded prior to freezing. Ovariectomy was confirmed by removing, weighing, and visually inspecting the uterus for absence of ovaries.

Quantification of Hop Prenylflavonoids in Serum

Hop extract powder was quantified for XN and 8PN. Eight (8) mg of powder was diluted with 500 µL of cold MeOH containing the internal standards 2,4-dihydroxychalcone and naringenin at 100 ng/mL. A further 1000-fold dilution was required to prevent signal saturation.

To quantify levels of XN, IX and 8PN in the blood after oral administration, we used 4 mice who were non-participants in the study, but were drawn from the same pool (identical to study animals in age, source, and environmental conditions.) Animals were fasted for 6 hours, and HE was suspended in sesame oil at the same mg/kg administered daily to study mice (400 mg/kg). The treatment was dropped onto the back fur, and licked within 5 minutes. Serum samples were collected over the next 30 hours from tail vein blood. To quantify levels of HE flavonoids and their metabolites in our study mice, serum was collected from OVX HE and Placebo HE mice at termination. Mice were fasted 6 hours, and had their last cookie with HE treatment 20 hours prior to serum collection.

Mouse serum samples were extracted using a protein precipitation protocol. To a 1.5 mL eppendorf tube, 19 µL of serum was added, followed by 80 µL of cold MeOH containing the internal standards 2,4-dihydroxychalcone and naringenin at 100 ng/mL. After vortexing 30 seconds, samples were centrifuged 10 minutes at 13,000 x g at 4^{o} C. The supernatant was transferred to a glass vial and analyzed by LC-MS. A calibration curve was prepared in the same manner, by spiking known amounts of the synthetic standards XN and 8-PNG into serum then extracting.

LC-MS was performed on a Waters Acquity M-class UPLC equipped with a trap valve manager coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on a Waters Atlantis dC18 stationary phase (300 µM x 150 mM, 3 µM). Mobile phases were acetonitrile with 0.1% formic acid (B) and water with 2 mM ammonium acetate (A). The analytical gradient was as follows: time = 0 min, 45% B; time = 2.5 min, 70% B; time = 5.5 min, 70% B; time = 6 min, 100% B; time = 7 min, 100% B; time = 7.5 min, 45% B; time = 12 min, 45% B. Trapping was performed using a Waters Symmetry C8

stationary phase (300 µM x 50 mM, 5 µM). Loading time was 2 minutes at 25% B. Flow rate was 15 µL for both trapping and analytical separation. Injection volume was 2 µL. Samples were held at 5° C in the autosampler, and the analytical column was operated at room temperature, near 21° C.

The mass spectrometer was operated in selected reaction monitoring (SRM) mode, where a parent ion is selected by the first quadrupole, fragmented in the collision cell, then a fragment ion selected for by the third quadrupole. Product ions, collision energies, and cone voltages were optimized for each analyte by direct injection of individual synthetic standards. A quantitative and confirmatory transition was developed for each analyte (Table 1). Interchannel delay was set to 3 ms. The instrument was operated in negative ionization mode and the capillary voltage was set to 2.1 kV. Source temperature was 150° C and desolvation temperature 200° C. Desolvation gas flow was 800 L/hr, cone gas flow was 150 L/hr, nebulizer gas flow was 7 Bar, and collision gas flow was 0.2 mL/min. Argon was used as the collision gas, otherwise nitrogen was used.

Liver Triglycerides

Liver tissue was digested in ethanolic potassium hydroxide, purified by two ethanol purification steps and precipitated with magnesium chloride. The supernatant was assayed using the Cayman (Ann Arbor, MI, USA) triglyceride colorimetric kit per manufacturer's instructions. Samples were measured against a standard curve (0-200 mg/dl), and measurements were normalized to liver weight.

Liver Gene Expression

Liver RNA was isolated with TRIzol reagent (Life Technology, Grand Island, NY, USA) based on manufacturer's instructions and quantified using a Q3000 UV spectrophotometer (Quawell Technology Inc, San Jose, CA, USA). Isolated RNA (425.8-1546.9 ng/mL) was synthesized into cDNA using iScript kit (Bio-Rad, Hercules, CA, USA). Samples were run in duplicate, and expression of ATP-binding cassette subfamily G (ABCG5 and ABCG8), steroid regulatory binding protein 1c (SREBP1c), fatty acid synthase (FASn), Hormone sensitive lipase (LIPE), and acetyl-CoA carboxylase (AcCoA) were compared among treatment groups. Primer sequences are listed in Table A2. Quantitative PCR was performed with BioRad SoAdvanced™ SYBR Green Supermix on CFX96™ thermal cycler (Bio-Rad, Hercules, CA, USA). Thermal cycling conditions were as follows: 3 min 95°, 40 cycles of 95°C for 10sec, 58°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 3 min. Results were normalized to the reference gene beta-2-microglobulin (β2M) and reported as relative expression change from cycle threshold.

Statistical Analysis.

All statistical analyses were done using GraphPad Prism version 6 (GraphPad Software, La Jolla California, USA). Multiple comparisons among treatment groups are reported as standard error of the mean, using one-way ANOVA and Tukey correction, with statistical significance set at $p < 0.05$. Outliers were identified using the Rout method with Q=1.0%.

Results

Weight gain and Adiposity

Ovariectomy resulted in increased body weight (Fig 2.4) despite no change in average food intake (Fig 2.5). Average starting body weight among treatment groups was not significantly different ($p>0.05$). 17β-estradiol (E2) treatment significantly ($p\leq0.05$) protected against weight gain due to ovariectomy, while hop extract (HE) had no effect. Body weight was significantly higher in OVX HE and OVX Placebo groups than the sham-operated groups by the second week and remained so throughout the study. In these two groups (OVX HE and OVX Placebo), ovariectomy significantly increased total visceral adipose tissue compared to Sham Placebo, while subcutaneous adipose tissue and brown adipose tissue were not significantly different (Fig 2.6). Uterine weight was significantly decreased in OVX Placebo and OVX HE groups, but was maintained in the OVX E2 group (Fig 2.7).

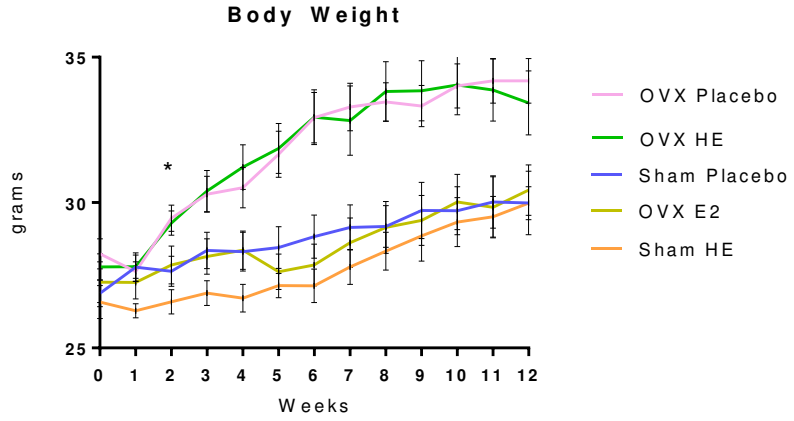

Fig 2.4: Total body weight gain during study. OVX Placebo and OVX HE showed significant weight gain starting at week 2 ($p<0.05$).

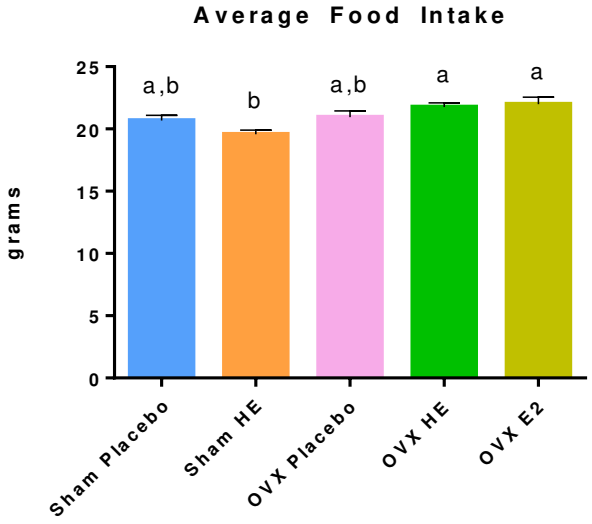

Fig2.5: Average food intake. Groups with different letters are significantly different ($p<0.05$)

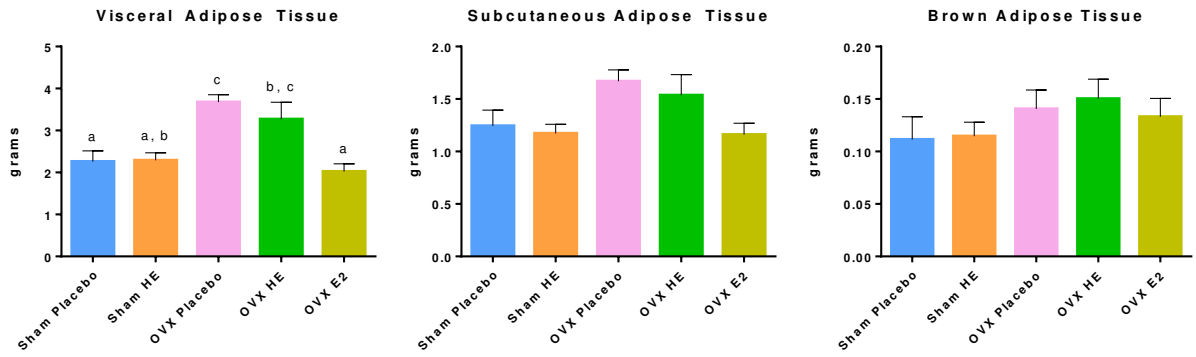

Fig 2.6: Total visceral, subcutaneous and brown adipose tissue weight. Groups with different letters are significantly different ($p<0.05$)

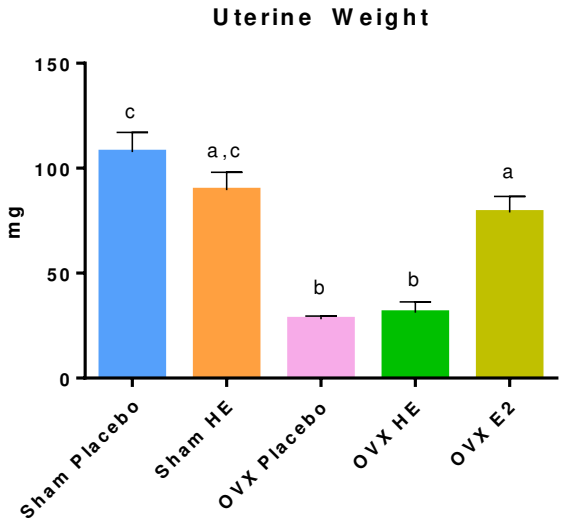

Fig 2.7: Uterine weight. Groups with different letters are significantly different ($p<0.05$)

Pharmacokinetics of Hop Extract

Circulating blood levels of HE were quantified by LC-MS. The pharmacokinetics of XN, IX and 8PN were quantified against known standards , while values of their glucuronidated species are relative peak area. Serum in non-study mice were measured after oral HE administration over a period of 30 hours. XN had a T_{max} of 1 hour with a C_{max} = 14.33 ng/mL. IX and 8PN both had a T_{max} of 3 hours, with C_{max} = 4.58 ng/mL ng/mL and 6.73 ng/mL respectively (Fig 2.8). Three glucuronidated species were detected (Fig 2.9). Measurements are reported as relative peak area, since known standards were not available to calibrate signal intensity. The glucuronidated compounds had a T_{max} of 4 hours for IX/XN glucuronic acid and 6PN/8PN glucuronic acid 2. 6PN/8PN glucuronic acid 1 had a T_{max} of 10 hours.

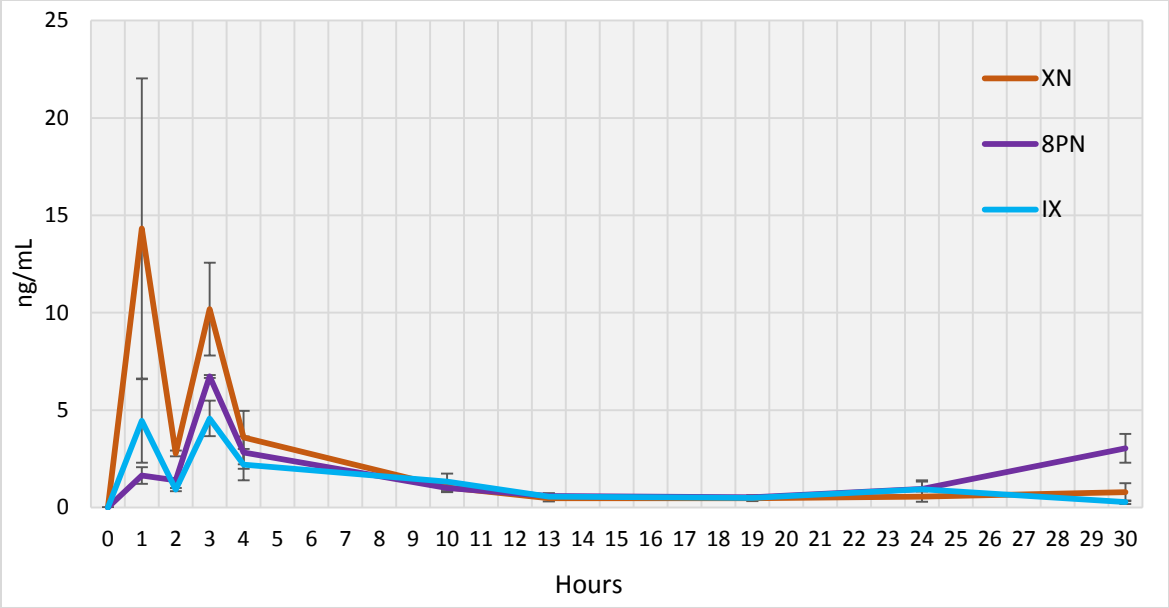

Fig 2.8: Pharmacokinetics of XN, IX and 8PN in serum after oral administration of hop extract

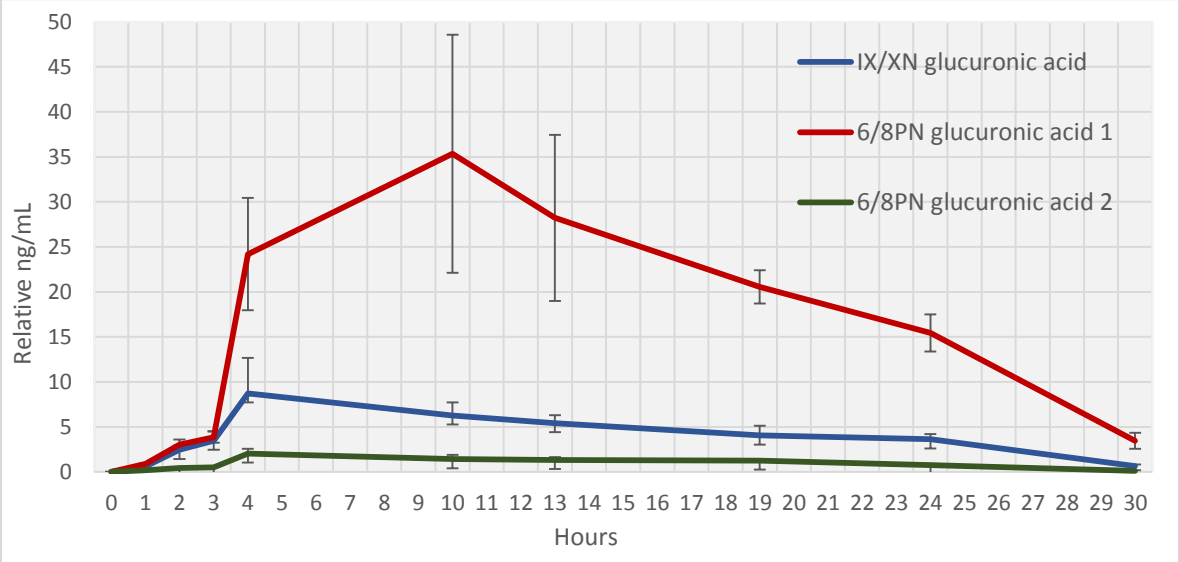

Fig 2.9: Pharmacokinetics of glucoronidated compounds of XN, IX, 8PN and 6PN. Values are relative peak area.

Serum metabolites were measured in serum of study mice at termination, 20 hours after oral HE administration with cookie and oil carrier (Table 2.1). Average levels of hop flavonoids and metabolites were not significantly different amongs sham vs OVX groups receiving HE.

Table 2.1: Levels of HE and their metabolites detected in serum 20 hours after oral administration.

Sample Name	Group	8PN	6PN	XN	IX	XN-Glucoronidate (Peak Area)	8PN-Glucoronidate (Peak Area)
53	Sham	5.60	11.03	5.10	2.03	9109	48031
42	Sham	5.48	13.22	3.67	2.12	3626	104722
35	Sham	8.64	10.87	8.52	3.15	ND	18479
4	Sham	ND	ND	ND	ND	ND	ND
8	Sham	ND	ND	3.89	ND	1407	ND
20	Sham	9.23	10.85	5.34	7.19	1578	13255
36	Sham	7.10	*NQ**	4.40	10.22	2294	11159
33	Sham	ND	ND	ND	ND	ND	ND
Average		**7.21**	**11.49**	**5.15**	**4.94**	**3602.80**	**39129.20**
3	OVX	8.11	66.80	6.64	0.68	ND	ND
28	OVX	9.57	11.86	6.15	5.72	6927	166674
9	OVX	ND	ND	ND	ND	ND	ND
6	OVX	4.60	12.70	4.04	0.82	3255	57789
30	OVX	7.90	11.72	6.05	2.80	3106	95049
56	OVX	13.22	14.33	6.53	4.31	4551	127438
14	OVX	7.06	10.99	7.47	9.79	5642	56873
49	OVX	8.20	11.06	6.31	3.45	6669	73248
45	OVX	4.95	*NQ**	ND	ND	ND	ND
43	OVX	3.81	10.45	3.34	0.54	7376	71022
Average		**7.11**	**11.88**	**5.62**	**3.62**	**5099.83**	**80236.50**
Limit of Detection		2.89	0.43	0.47	0.80	n/a	n/a
Limit of Quantification		9.62	1.42	1.56	2.66	n/a	n/a

Liver Adiposity

The OVX Placebo group had significantly higher levels of liver triglycerides (TG) than all other groups (Fig 2.10), whereas liver TG in OVX and Sham HE and E2 OVX were not significantly different from Sham Placebo. Gene expression from liver tissue was measured for the lipid transport and metabolism genes ATP-binding cassette subfamily G (ABCG5 and ABCG8), steroid regulatory binding protein 1c (SREBP1c), fatty acid synthase (FASn), hormone sensitive lipase (HSL), and acetyl-CoA carboxylase (AcCoA). The OVX HE group had lower

FASn and AcCoA carboxylase expression though differences amongst groups did not reach significance (Fig 2.11).

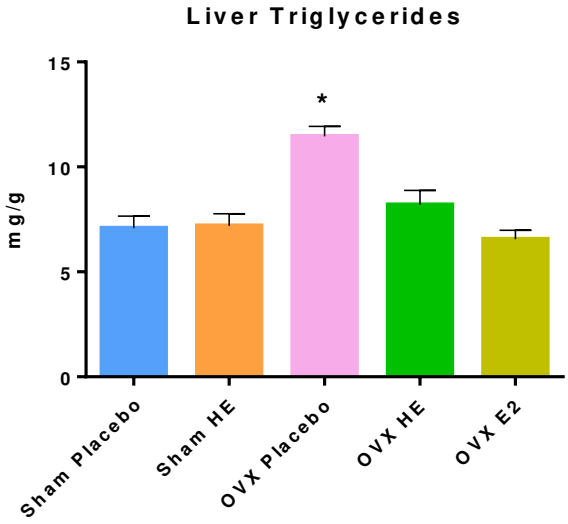

Fig 2.10: OVX placebo group had significantly higher liver triglycerides ($p<0.05$)

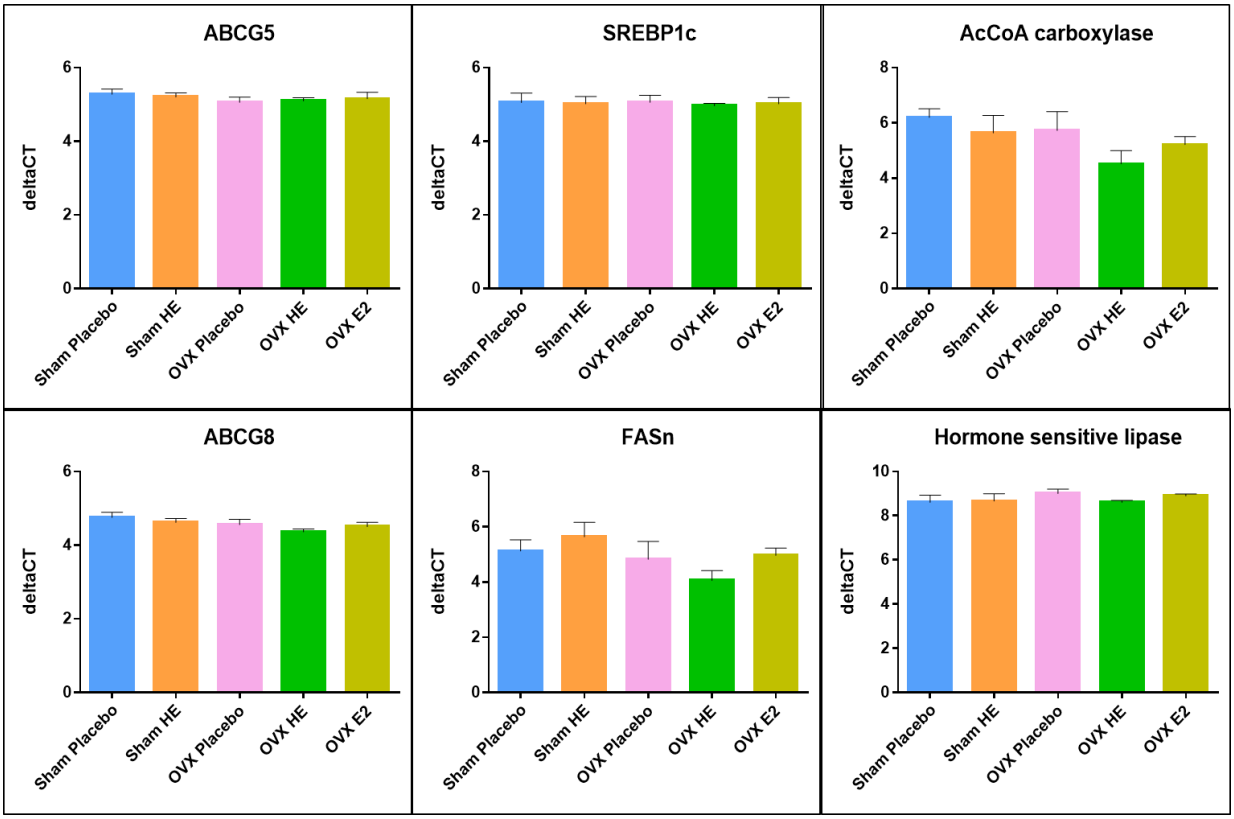

Fig 2.11: Liver gene expression of ABCG5, SREBP1c, AcCoA carboxylase, ABCG8, FASN, and Hormone Sensitive Lipase. There were no significant differences amongst groups for any gene.

Discussion

Menopause is often marked by an increase in weight, without change in diet or exercise habits. In our study, body weight increased with OVX, as previously reported [42, 46, 47]. Supplementation with E2 protected against OVX-induced weight gain, while HE treatment did not. Moreover, E2 elicited full protection from increased visceral adipose tissue (VAT), while HE offered no significant protection. Other flavonoids have been shown to mitigate increase in visceral adipose tissue, such as extracts from green tea (*Camelia sinensis)* [48, 49], red wine grapes [50], and licorice root [51], however, many of these studies used diets high in saturated fat, while the mice in the present study were on a low-fat diet. VAT is associated with increased

risk for several cardiometabolic diseases and cancers [52-54], so any decrease in its accumulation would likely be beneficial. It is not surprising that E2 treatment would decrease adiposity, since estrogen is involved in leptin signaling and other metabolic regulators of metabolic homeostasis and adiposity [55, 56]. There was protection against weight gain with HE treatment. It is possible that dosage did not have enough 8PN to exert significant estrogenic effect because the treatment was applied as a total extract from hops. It is difficult to compare the amount of 8PN administered with those previously reported. The fact that the measured concentration of each compound differed considerably from that reported by the analysis provided by Metagenics, suggests that even within a particular product, the proportion of the various phytochemicals may have differed appreciably.

Hop flavonoids, especially XN, is protective against a high fat diet induced liver triglyceride accumulation through regulation of genes involved in fatty acid and cholesterol metabolism [57-59]. We did not detect gene expression differences (Fig 2.11), although differences may have been masked since multiple samples were pooled. Possible alternative mechanisms for the decrease in liver TG include indirect effects through interactions of hop flavonoids with estrogen receptors, and/or a direct effect of flavonoid interaction with other signaling pathways. A similar flavonoid to 8PN, naringenin, was found to activate both PPARα and PPARγ while inhibiting LXRα in rat hepatocytes, regulating downstream fatty acid oxidation genes [57].

The levels of XN peaked at 1 hour after HE ingestion, while IX and 8PN peaked at 3 hours. This suggest spontaneous conversion of XN to IX, followed by enterohepatic circulation and conversion of IX to 8PN by liver microsome CYP1A2 [60] or microbial metabolism by colonic bacteria [61-63]. Results are consistent with van Breemen et al, who measured the

pharmacokinetcs of oral HE in women [45], and provide evidence that current study mice were exposed to levels of the various flavonoids in the HE, that could exert physiological effects including protection from gain in liver adiposity. These protective effects may be via estrogen receptor signaling, or interaction with several metabolic pathways involving adiposity and cardiometabolic disease risk.

Conclusions

Results confirmed E2 protection against OVX-induced adiposity, and demonstrated for the first time HE protection against liver triglyceride accumulation with OVX, albeit via unknown mechanisms. While HE dosage may have been too low to produce significant physiological effects, HE may be a suitable therapy for menopausal symptoms, with some added cardiometabolic benefits. These outcomes may be derived from HE flavonoid interactions with estrogen receptors in the face of endogenous estrogen loss or via direct anti-inflammatory, antioxidant and antilipogenic effects. The lack of effect of HE on uterine weight suggests an improved safety profile of HE as a therapy for menopausal symptoms as compared to E2.

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CHAPTER 3: GUT BACTERIA RESPONSE TO OVARIECTOMY, 17B-ESTRADIOL AND PHYTOESTROGEN HOP EXTRACT

Summary

Bacteria that reside in our gut are important moderators of several biological processes, including inflammation and cardiometabolic disease risk and progression. Menopausal estrogen decline is associated with increased risk for cardiometabolic and inflammatory-related diseases. This study, tested hypothesis that loss of ovarian estrogen in adult mice will result in pro-inflammatory alterations in the gut microbiota and intestinal environment. The study tested the hypothesis that supplementation with oral 17β-estradiol or hops- *(Humulus lupulus)* derived phytoestrogen will stabilize the gut microbiota and reduce intestinal inflammation. Ovariectomized (OVX) or control sham-operated C57BL/6 mice were supplemented with oral 17β-estradiol, phytoestrogen-rich extract from hops, or placebo (carrier oil), and cecal bacterial abundance was determined using the V3-V4 regions of the 16S rRNA gene using an Illumina MiSeq platform. OVX had no significant impact on the overall gut bacterial community structure in any treatment groups. However, *Ruminococcus gnavus* (Clostridiales), bacteria, enriched in individuals with inflammatory bowel disease, was increased with OVX and was positively correlated with inflammatory cytokines measured in the colon. *Akkermansia muciniphila* was less abundant with HE supplementation, but only within the Sham groups.. Overall, we detect no impact of OVX or phytoestrogenic hop extract on gut microbiota, suggesting that these products cannot be used to alleviate menopause-associated symptoms without disrupting the gut microbial community. Further research on the interplay between estrogen and the gut microbiota is necessary to establish any relationships.

Introduction

Human gastrointestinal (GI) tract is host to over 300 bacterial species, with a density of up to 10^{11} bacterial cells per gram of luminal content [1]. These bacteria, combined with resident viruses, yeasts, and other microorganisms make up the gut microbiota. These organisms have co-evolved with humans and provide critical contributions to host digestion, metabolism, and immunity, and are emerging as important modulators of human health [1-5]. Imbalance in the microbial community structure, or dysbiosis, has been associated with detrimental health outcomes and is implicated in the etiology of inflammatory intestinal diseases and various cancers [6-8]. Alterations in the gut microbiota are also implicated in regulating systemic immune responses via microbial effector molecules that can generate inflammatory responses in peripheral tissues and organs [4]. This microbial-induced inflammatory state can lead to metabolic dysregulation associated with non-alcoholic fatty liver disease (NAFLD), type II diabetes, cancer and cardiovascular disease [6, 9] Natural ageing and the associated shift in hormonal balance can change the composition of the gut microbiota over time, and may contribute to the increased risk of developing these diseases [10].

Menopause is a life event all women undergo, marked by decline of estrogen production by the ovaries and a significant increase in the risk of developing cardiovascular disease, diabetes and certain cancers. Accumulating evidence suggests that estrogen may be an important regulator of the intestinal microbiota [11, 12]. Recent research in murine models has shown that loss of endogenous estrogen production through removal of the ovaries by ovariectomy (OVX) alters the composition of intestinal bacteria [13-17]. Gut bacteria are capable of metabolizing numerous exogenous and endogenous compounds, including estrogens, implicating the gut ecosystem in the disease risk associated with menopause. The ‘estrobolome’ is a newly defined

term describing “the aggregate of enteric bacterial genes whose products are capable of metabolizing estrogens” [18]. The actions of the estrobolome can increase or decrease bioavailability of endogenous and exogenous estrogens thus influencing a woman’s lifetime estrogen exposure. This reciprocal interaction between the gut microbiota and estrogens may have important implications in women’s health and determining the risk of developing age-associated diseases.

Aside from the implications on chronic disease risk, menopause is associated with numerous acute physical symptoms including hot flashes, mood changes, and insomnia. As a result, many women seek treatments such as hormone therapy (HT) to alleviate these symptoms. However, the demand for HT has declined in the past decade due to its association with increased breast cancer risk [19-21]. Thus, there is a need for alternative therapies to more safely address menopausal symptoms and help maintain the beneficial effects of estrogen on bone and cardiovascular health. One potential alternative is the use of botanical supplements that contain estrogen-like compounds, called phytoestrogens. By activation of estrogen receptors, phytoestrogens may alleviate uncomfortable menopausal symptoms and may potentially have the added benefit of balancing the gut microbiota and mitigating the risk of chronic diseases [22].

Estrogenic compounds have been reported in varying concentrations in soybean, flax and sesame seeds, kudzu root and wild yam. However, flavonoid compounds from hops, *Humulus lupulus*, are among the most potent phytoestrogens identified [23, 24]. Several studies in murine models and human trials have shown the effectiveness of hop flavonoids on reducing menopausal symptoms while lowering the risk of cardiometabolic diseases through direct flavonoid interaction with endogenous antioxidant and anti-inflammatory pathways [25-28].

The bioavailability of hop flavonoids, in particular 8-prenylnaringenin (8PN), is dependent on gut bacterial metabolism from its precursors xanthohumol (XN) and isoxanthohumol (IX) [29]. Commercial hops-based dietary supplements contain a mixture of XN, IX and 8PN. One human bacteria able to convert IX to 8PN, *Eubacterium limosum,* was recently identified [30], providing evidence for a mechanism by which a menopausal woman's gut microbiota can influence her exposure to 8PN.

The primary goal of this study was to assess changes in the gut microbiota and intestinal function of female mice under an estrogen-depleted state. The secondary goal was to determine if orally administered 17β-estradiol or phytoestrogenic hop extract would mitigate changes induced by estrogen loss. Ovariectomy or sham surgeries in 7-month old retired breeder C57BL/6 mice were conducted. All animals were fed a purified, phytoestrogen-free diet and randomized in treatment groups given either a commercial supplement made from hops extracts,17-β-estradiol, or placebo carrier oil. Based on data from a previous study conducted in rats, it was hypothesized that the gut microbiota of ovariectomized mice would be characterized by increased bacterial diversity and a higher proportion of Bacteroidetes phyla than animals undergoing sham surgeries. The hypothesis that estradiol and hops extracts will mitigate effects of estrogen loss on the gut microbiota was tested.

Materials and Methods

Animals and Experimental Design

All methods pertaining to animal care and experimental design and procedures are described in Chapter 2. All experimental procedures with animals were approved by the Colorado State University Institutional Animal Care and Use Committee, Protocol #: 14-5067A.

DNA Extraction and Sequencing

Cecal contents were collected with sterile cotton swabs at termination, flash frozen in liquid nitrogen, and stored at -80°C until analysis. Whole genomic DNA was extracted using MoBio Powersoil DNA extraction kit (MoBio, Carlsbad, CA, USA) per manufacturer's instructions. Extracted DNA was sent to Research Testing Laboratories (Lubbock, TX, USA) for library preparation by amplification of the V3-V4 ribosomal rRNA gene variable regions and sample indexing, and paired-end sequences were generated on an Illumina MiSeq platform (San Diego, CA, USA). Paired raw fastq files were assembled and processed using the default myPhyloDB ver. 1.2.0 [31] sequencing pipeline, which utilizes the mothur bioinformatics software for sequence processing [31]. Briefly, sequences were screened (maxhomop=0, maxambig=0), removed of chimeras (vsearch), and classified to the K-mer based nearest neighbor (KNN) in the GreenGenes ver. 13_5 reference database. After processing, data were normalized by sub-sampling without replacement to 13038 sequence reads per sample. One hundred independent normalization tests were conducted and the average abundance for all normalization tests was used in further analyses.

Bile Acid Determination

Sterilely-collected fecal samples (25mg) from week 10 of the study, were homogenized in 500uL of NH_4OH, with 5uL internal standards glycodeoxycholic acid d-4, deoxycholic acid d-4, and taurocholic acid d-5. The mixture was vortexed and incubated at 60°C for 1 hour followed by sonication for 30 minutes. One mL of HPLC grade water was added and incubated at -80°C overnight. Samples were then centrifuged at 4°C at 10,000 rcf for 30 minutes. The clear supernatant was transferred for UHPLC-MS analysis.

Analysis was performed on a Waters (Millford, MA, USA) Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer, as described previously [32]. Chromatographic separations were carried out on a Waters HSS T3 stationary phase (1 x 100mm, 1.8 µM). The mobile phases were methanol (M) and water with 0.1% formic acid, and 2 mM ammonium hydroxide (A). The samples were held at 4°C and column at 70°C. The analytical gradient was carried out as follows: At 0 min, 0.1% M; time 0.5min, 0.1% M; time 2min, 30% M; time 15min, 97% M; time 16min, 97%M; time 16.5min, 0.1% M; time 21min, 0.1% M. Flow rate was 210 µL/min and injection volume was 2 µL. The mass spectrometry was operated in selected reaction monitoring (SRM) mode. Inter-channel delay was set to 3 ms and the MS was operated in both negative and positive ionization modes with capillary voltage at 2.1 and 3.2 kV. Source temperature was 150°C and desolvation temperature was 500°C with a gas flow was 1000 L/hr, cone gas flow 150 L/hr, and collision gas flow 0.2 mL/min. Nebuliser pressure was 7 Bar and argon was used as the collision gas. Waters TargetLynx software was used for peak integration.

Short Chain Fatty Acid Determination

Fecal samples collected 1 week prior to study termination (11 weeks) were extracted for short chain fatty acids by homogenizing approximately 25g of frozen feces with acidified water (pH 2.5; adjusted with 12M HCl) containing 1 mM ethylbutyric acid as an internal standard. Samples were vortexed and then sonicated for 60 minutes. After sonication, they were centrifuged at 10,000 rpm for 15 minutes. The supernatant was transferred to glass vials and stored at -80C prior to GC-FID analysis.

Sample extracts were analyzed on an Agilent 6890 Series Gas Chromatograph equipped with flame ionization detection (GC-FID; Agilent Inc., Santa Clara, CA). Injection rate was 10:1 split ratio, and inlet temperature was 22°C and translet line temperature was held at 230°C. Separation was achieved on a 30m TG-WAX-A column (ThermoScientific, 0.25 mm ID, 0.25 um film thickness) at 100°C for 1 min and ramp rate of 8°C per minute to 180°C, held at 180C for 1 minute, ramped to 200°C at 20C/min and held for 5 minutes. Helium carrier flow was maintained at 1.2 mL per minute. Short chain fatty acids were quantified using 5-point standard curves of commercially purchased standards (Sigma, St. Loius,MO, USA) and normalized to internal standard signal.

Intestinal Permeability and Inflammation

One week prior to termination of the study (11 weeks), intestinal permeability was assessed in vivo as described previously [33]. In summary, mice were fasted for 6 hours and orally gavaged with 40 kD FITC-Dextran (Sigma, St. Louis, MO) dissolved in water at 400 mg/kg body weight. At 1 and 4-hour time points, approximately 200 uL of tail vein blood was collected, incubated in the dark at room temperature for 30 minutes. Samples were centrifuged at 5,000g at 4°C for 10 minutes. Serum was removed and diluted with equal parts PBS. Fluorescence was read at $485_{EX}/535_{EM}$ and concentration was calculated based on standard curve of serially diluted untreated serum spiked with FITC-dextran.

Statistical Analysis

Comparisons between treatment groups were determined in Prism software (GraphPad, version 6) using one-way ANOVA and Tukey with post-hoc for false discovery rate with a

significance of $p \leq 0.05$. Outliers were determined using ROUT method with Q=1.0%. Phylogenetic EdgeR differential abundance analysis was created in R, as a general linear model with treatment as main effect. The tree was constructed referencing full-length16S sequences from GreenGenes 13.5 database, and only significant OTUs are shown. Correlation matrices were created in MyPhyloDB using the R package corrplot. Correlations shown are significant ($p<0.05$). Intensity of color and degree each circle is filled in represents Pearson's R.

Results

Changes in Microbial Abundance

Data from 16s sequencing is useful to compare compositional differences in microbial communities, but is not a good indicator of the absolute size of a microbial population. While microbial abundance was not measured directly (e.g., qPCR, etc.), cecum weight at termination was determined as a proxy measure of microbial biomass. It is known that compared to a chow diet, a high fat diet increases cecum size in mice [32] and germ-free animals have exceptionally large ceca [33]. Cecal weights were not significantly different ($p=0.4768$) among samples, suggesting they had equivalent microbial population sizes (Figure 3.1).

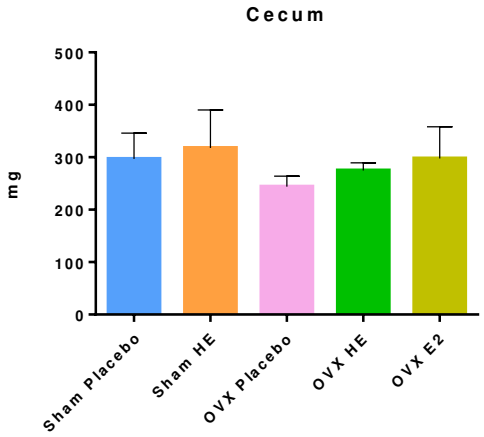

Figure 3.1: Cecum weights were not statistically different amongst groups

DNA extracted from cecal contents was sequenced using Illumina MiSeq platform, targeting the V3-V4 region of rRNA. At the phylum level, there was considerable variation in relative abundance amongst individual mice within treatment groups (Fig 3.2). We detected six different phyla of which Bacteroidetes, Firmicutes, and Verrucomicrobiota were the most abundant. There were no significant differences in Bacteroidetes, Firmicutes, or the proportion of these organisms between treatment groups. Within Verrucomicrobia, *Akkermansia muciniphila* (*A.* muciniphila) was the only species positively identified. Sham-operated animals receiving hops extracts (Sham HE) had the lowest levels of *A. muciniphila*, which were significantly lower than levels seen in Sham Placebo (p=0.0315) and OVX E2 (p=0.0501) (Figure 3.3).

Aside from *Akkermansia,* significant differences in abundance were found in the order Clostridiales (Firmicutes phyla) at the OTU level (Fig 3.4). Within the Lachnospiraceae family, *Ruminococcus gnavus,* abundance was significantly higher in OVX vs Sham in the Placebo groups (Figure 3.5; p=0.0447).

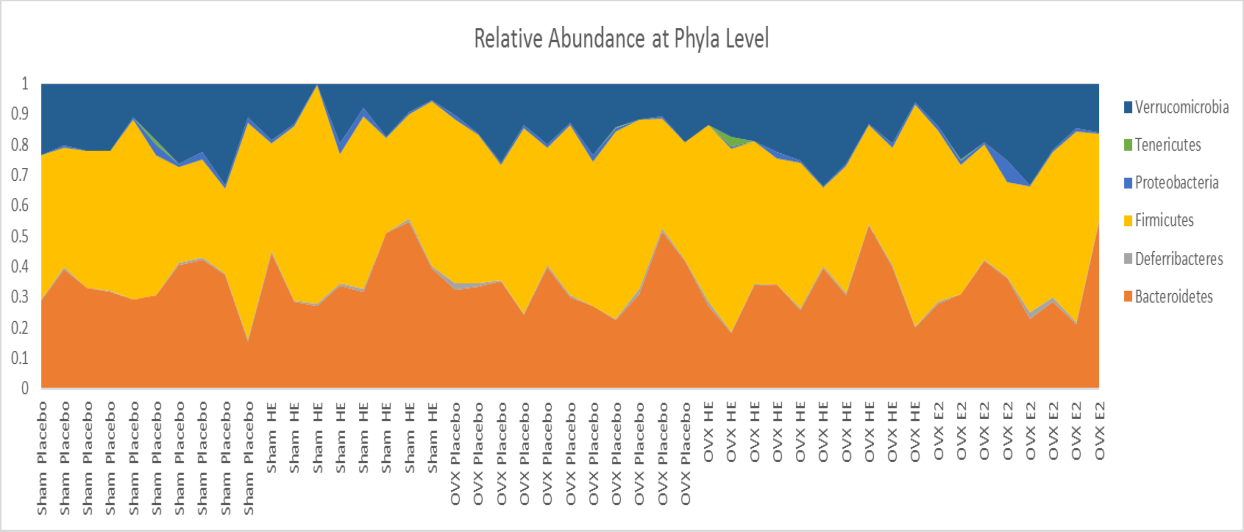

Fig: 3.2: Relative abundance at phyla level by individual mouse

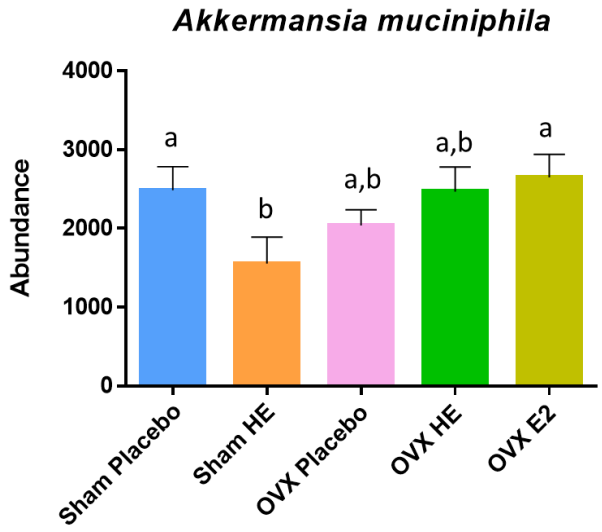

Fig 3.3: Abundance of Akkermansia muciniphila. Sham HE is significantly lower than Sham Placebo (p=0.0315) and OVX E2 (p=0.0501)

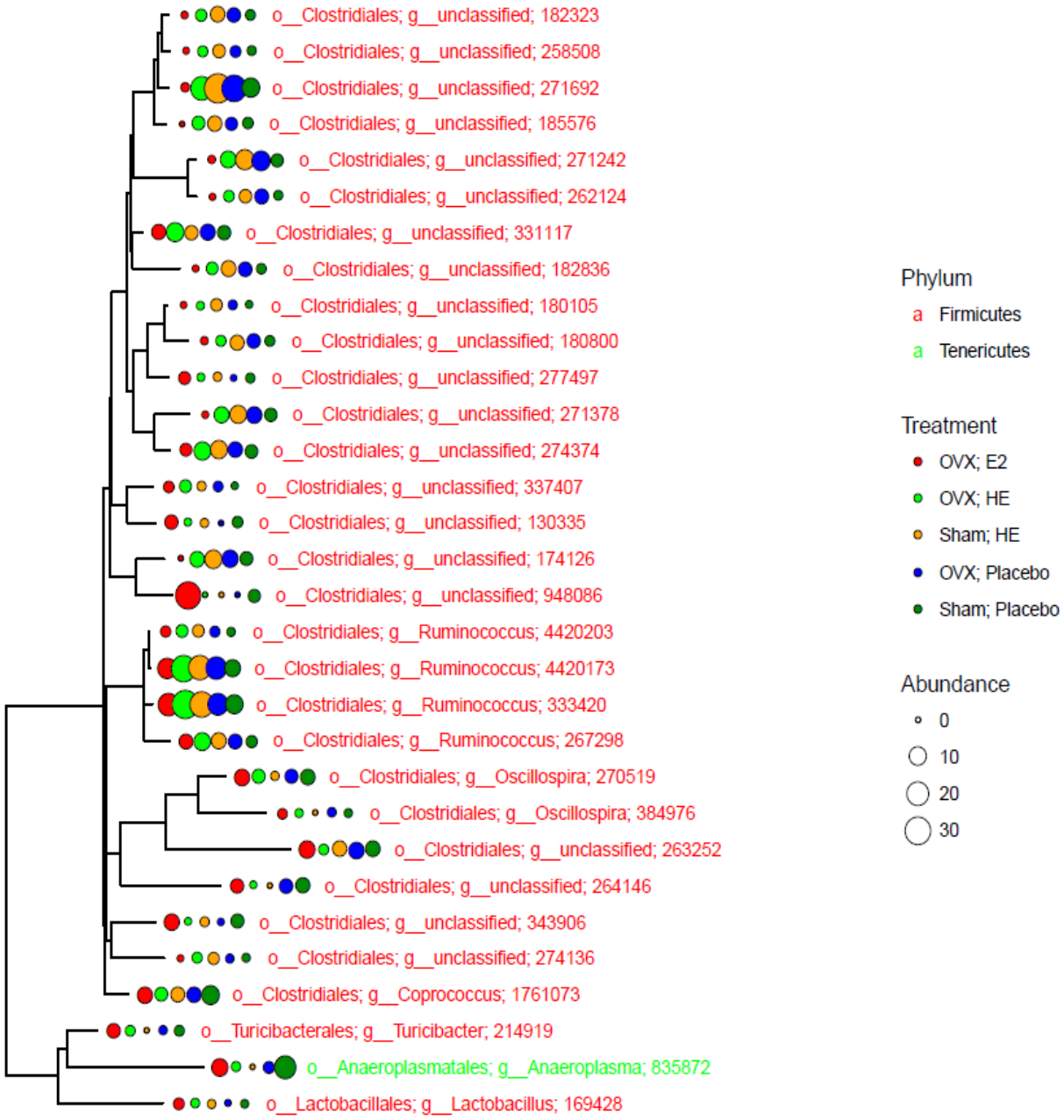

Fig 3.4: Phylogenetic EdgeR differential abundance analysis. Phylogenetic tree was created using the nearest neighbor full-length 16S sequences in the GreenGenes database. The tree shows significantly different abundances at OTU_99 level with p≤0.05, FDR=0.1. Names are the order, genus, and GreenGenes accession numbers (v.13_5)

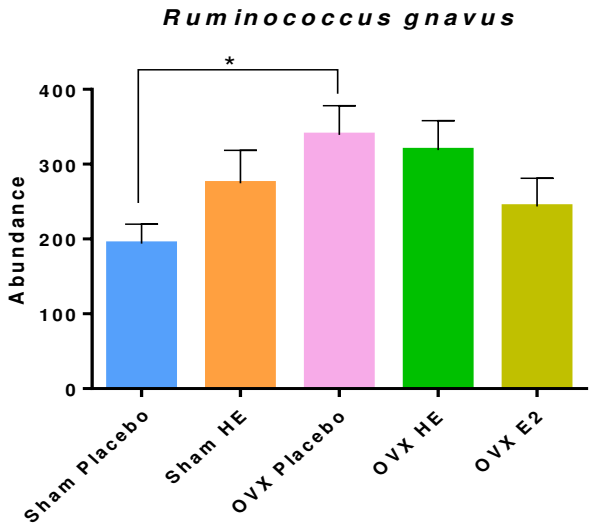

Fig 3.5: Abundance of Ruminococcus gnavus. Significantly higher abundance in OVX Placebo than Sham Placebo (p=0.0447)

Correlation Matrices of Bacterial Abundance

The relationship between different bacteria within a community may be equally as important as their abundance. In the current study, several correlations were observed between bacteria at several taxonomic levels. At the family level (Fig supplementary material ##), *Verrucomicrobiaceae* and *Lachnospiraceae* were negatively correlated (R= -0.399964, p=0.004956). *Verrucomicrobiaceae* also negatively correlated with *Peptococcaceae* (p=0.003729), and *S24-7* (p=0.041047). At the species level, *Akkermansia muciniphila* and *Rumminococcus gnavus* were negatively correlated (R= -0.492759, p=0.000344) (Fig 3.6). *Akkermansia muciniphila was* also positively correlated *with Mucisispirilum shaedleri* (R^2= 0.396811 and p=0.005352).

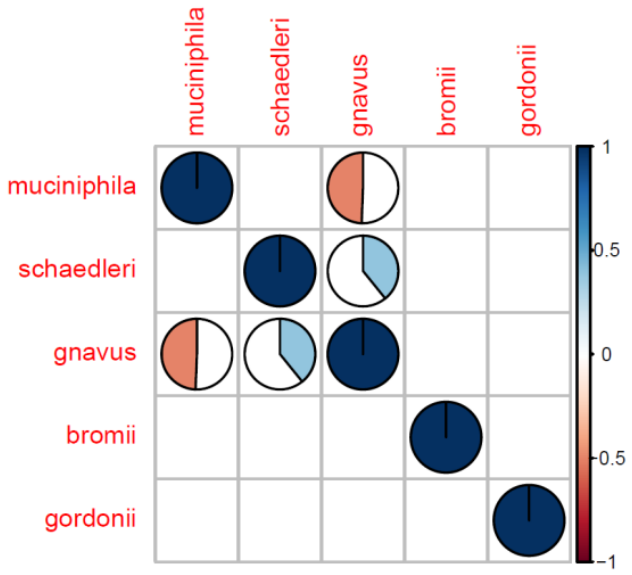

Fig 3.6: Correlation matrix of bacterial species abundance with significance of p<0.05. Blue represents positive correlation, and red negative correlations. Intensity of color and degree of circles filled represents Pearson's R value.

Although levels of phytoestrogen metabolites were not statistically different between OVX HE vs Sham HE treatment groups (data not shown), several surgery-independent correlations emerged between bacterial classes and serum HE metabolites (methods described in Chapter 2) (Fig 3.7). SMB53 in the Clostridiaceae family was negatively correlated with 8PN (R= -0.668571, p= 0.001748) and IX (R= -0.518258, p= 0.026221). Also in the clostridia group, *Ruminococcus gnavus* was positively correlated with XN (R= 0.589905, p= 0.008690).

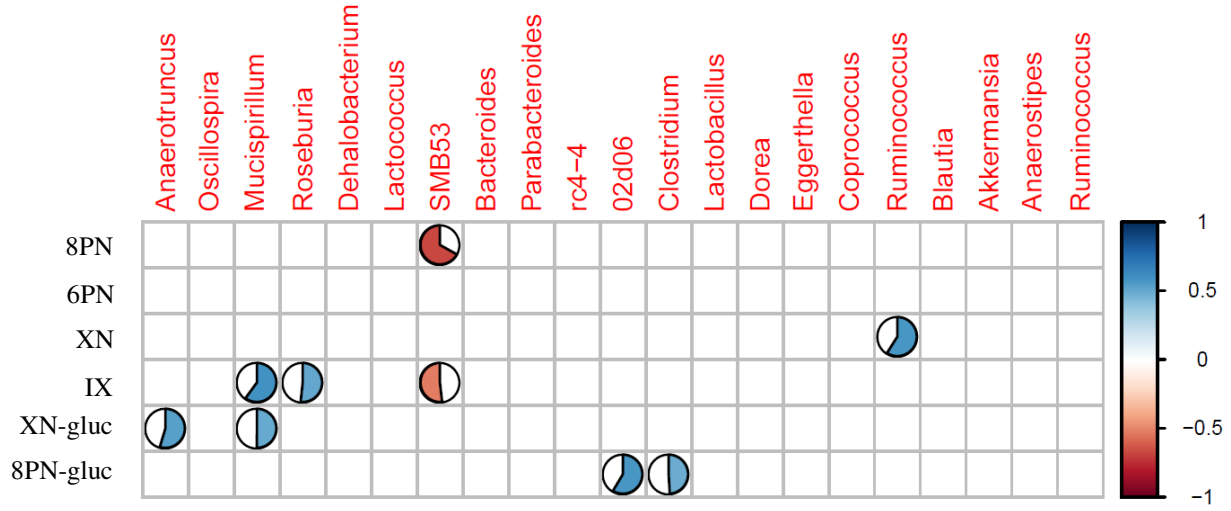

Fig 3.7: Correlation of hop extract flavonoids and their metabolites with bacterial genera with significance of p<0.05. Blue represents positive correlation, and red negative correlations. Intensity of color and degree of circles filled represents Pearson's R value.

Microbial Metabolism (Bile Acids, Short Chain Fatty Acids)

There were no significant differences in fecal bile acids amongst groups (n = 5). One mouse in the OVX Placebo was a consistent outlier in levels of cholic, deoxycholic, ursodeoxycholic, chenodeoxycholic and glycocholic acids, which could mask any differences. Cholic acid, deoxycholic acid, ursodeoxycholic acid and chenodeoxycholic acid were highest in the OVX HE group and below detection in OVX E2 group (Fig 3.8).

There were no significant differences in fecal short chain fatty acid (SCFA) levels amongst groups (Fig 3.9). Acetate was detected in the majority of the samples. However, many samples were below the level of detection for butyrate and proprionate. This may be because butyrate is absorbed by the colonic epithelial cells as a fuel source, and proprionate is shuttled to the liver, leaving feces relatively depleted [34].

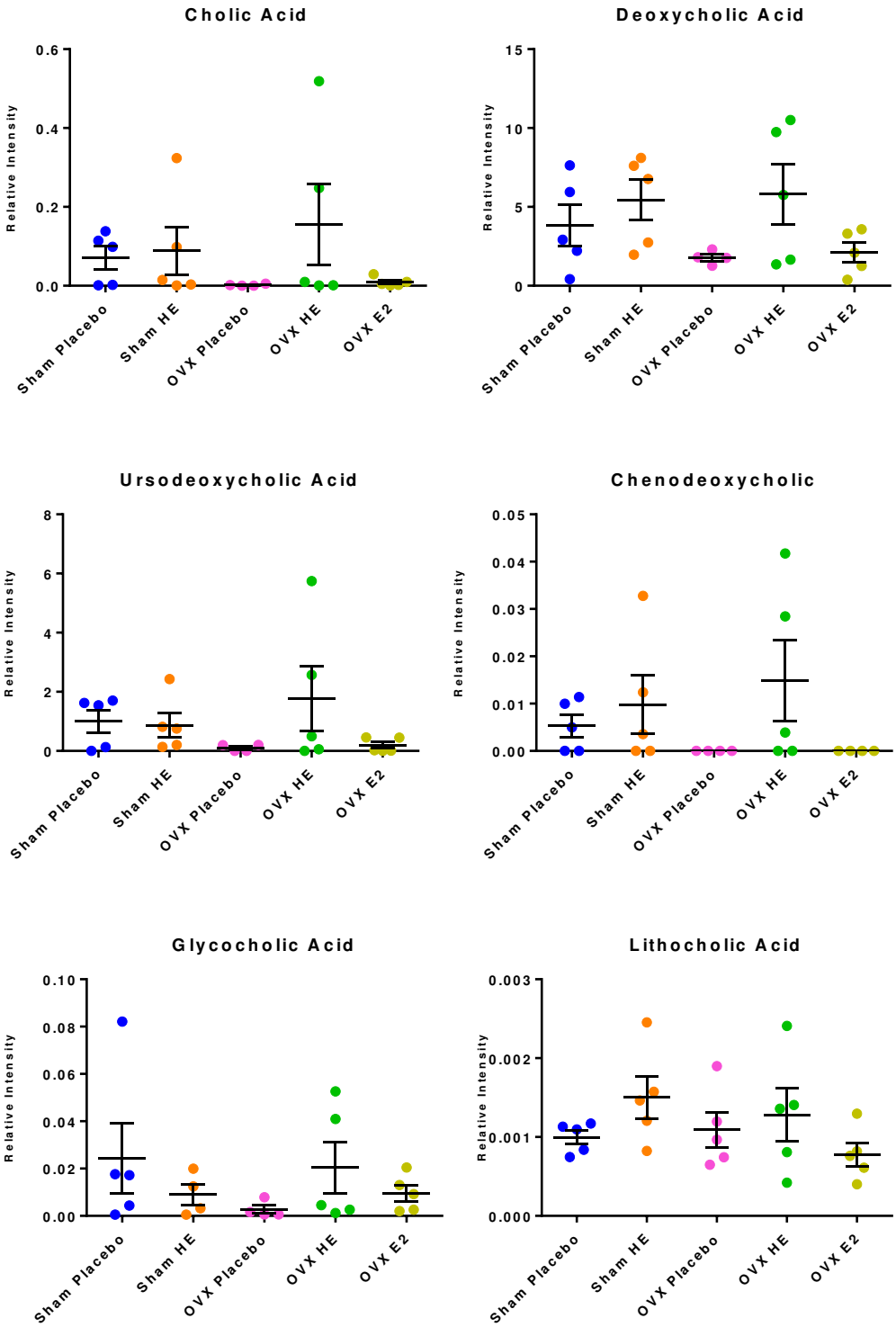

Fig 3.8: Bile acids in fecal samples. Outliers were removed using ROUT method, Q=1.0%

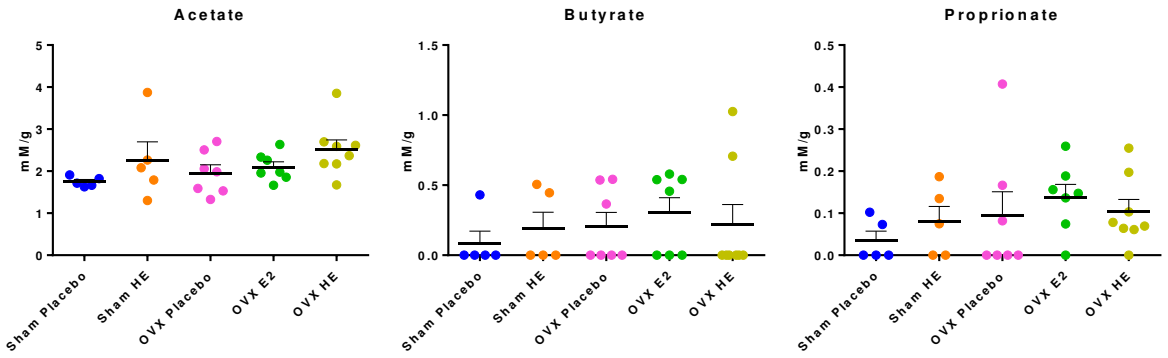

Fig 3.9: Levels of short chain fatty acids (SCFA) in fecal samples

Intestinal Function (FITC-dextran, CD14/LBP, IAP)

Measurement of intestinal barrier function with 40kD FITC-Dextran assay revealed no differences, and most measurements were below detection level (data not shown). LBP and CD14 dimerize to bind to circulating LPS, and increased levels in the plasma are indicative of higher circulating LPS levels and endotoxemia [35], making these viable proxy measures for LPS. Post mortem measurement of plasma LBP and CD14 were not significantly different among groups (Fig 3.10). Intestinal alkaline phosphatase levels, an enzyme that plays a role in neutralization of luminal endotoxin, were similar amongst the three OVX treatment groups and were not significantly different from Sham Placebo. Alkaline phosphatase was higher in the Sham HE compared to Sham Placebo group ($p=0.0390$, Fig 3.11).

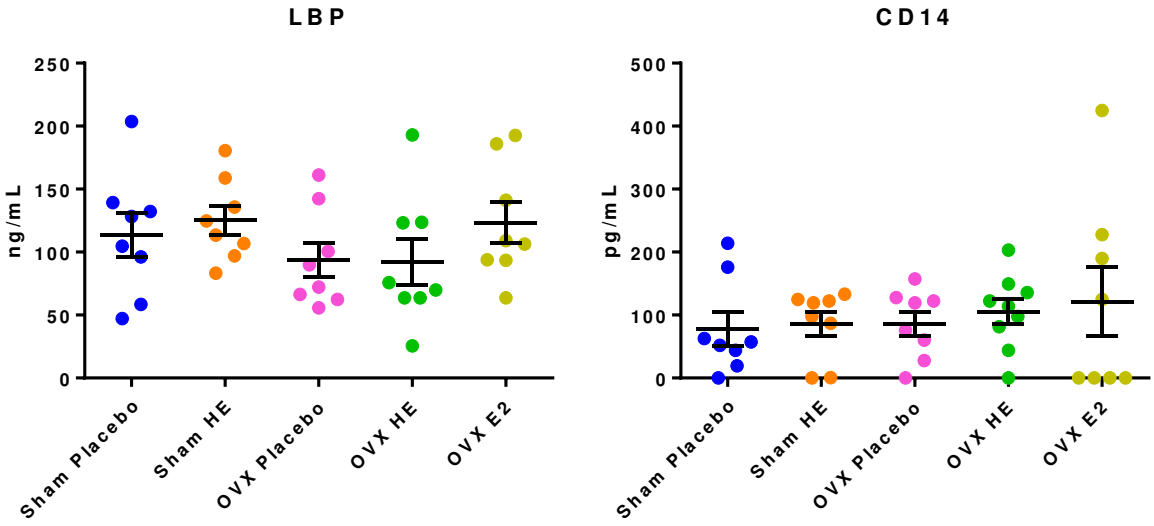

Fig 3.10: Blood measures of endotoxin binding proteins LBP, and CD14. No significant differences were observed.

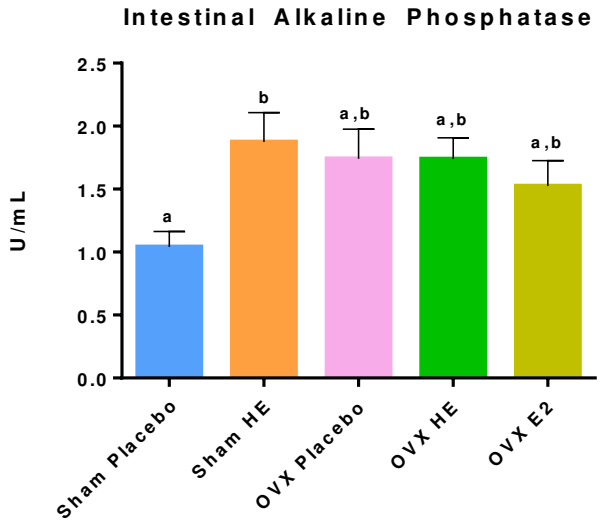

Fig 3.11: Intestinal alkaline phosphatase. Sham Placebo vs Sham HE p=0.0390

Further measurements of intestinal inflammation included the colonic cytokines and chemokines IL-1β, IL-6, IL-10, and MCP (Fig 3.12). IL-1β levels were more variable amongst the OVX groups, with OVX Placebo showing highest levels. There were three outliers

identified, one in each of the Sham Placebo, OVX Placebo and OVX HE groups (ROUT method Q=1.0%). Anti-inflammatory IL-10 levels were highest in the OVX HE group vs OVX Placebo (p=0.0075). IL-1β was highly variable within the OVX Placebo group and was slightly lowered with HE and E2 treatment. MCP-1 levels showed no significant difference. Levels of IFN-γ, MIP1a, MIP13β were below detection in most samples (data not shown).

Correlating bacteria at the genus level with cytokine levels (Fig 3.13), IL-10 positively correlated with *Roseburia* (R= 0.581951, p= 0.000878), *Dehalobacterium* (R=0.435539, p=0.019619), *Lactococcus* (R= -0.451663, p= 0.014936), *Ruminococcus* (R= 0.443784, p=0.017095), and *Blautia* (R=0.47804, p= 0.009481). IL-1β was positively correlated with *Anaerotruncus* (R= 0.408618, p= 0.030026). IL-6 was positively correlated with *Roseburia* (R= 0.581951, p=0.000878) and *Ruminococcus* (R= 0.443784, p= 0.017095). It is important to note *Ruminococcus* correlations in this data are members of the *Lachnospiraceae* family and include *R. gnavus* and an unclassified *Ruminococcus* species.

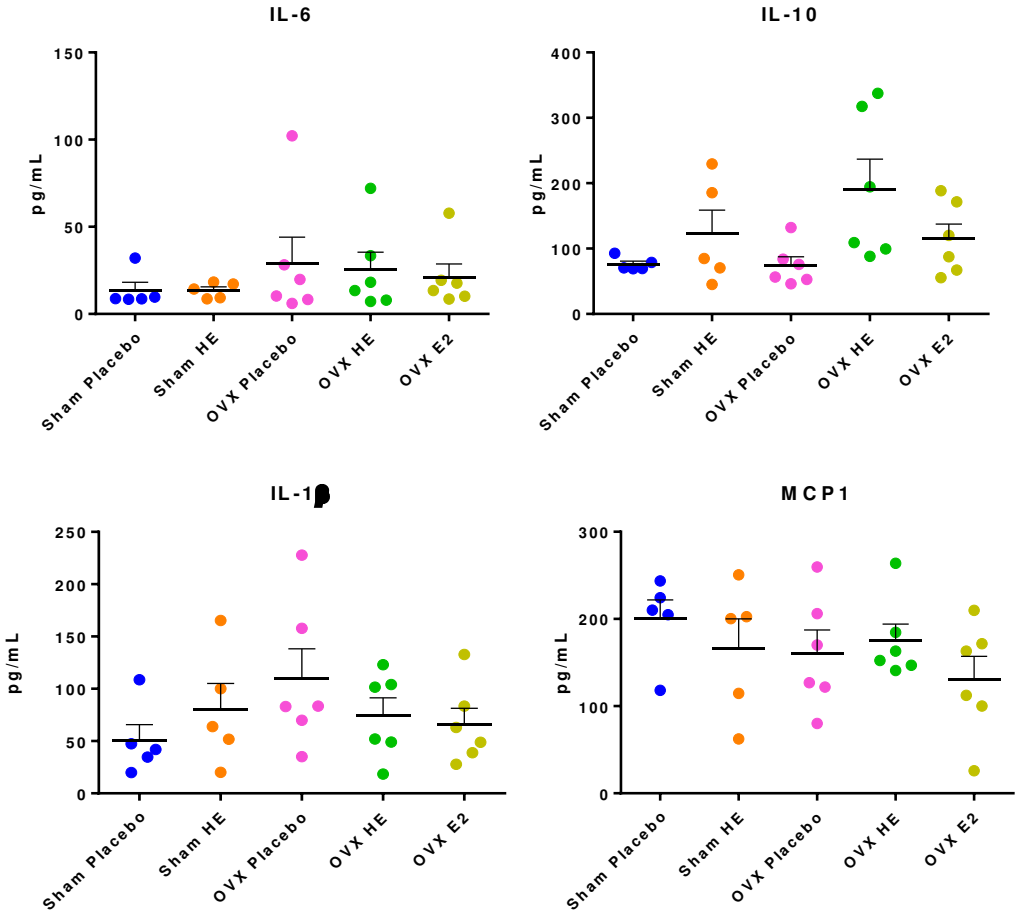

Fig 3.12: Cytokine levels in colon of IL-6, IL-10, IL-1β, and MCP1.

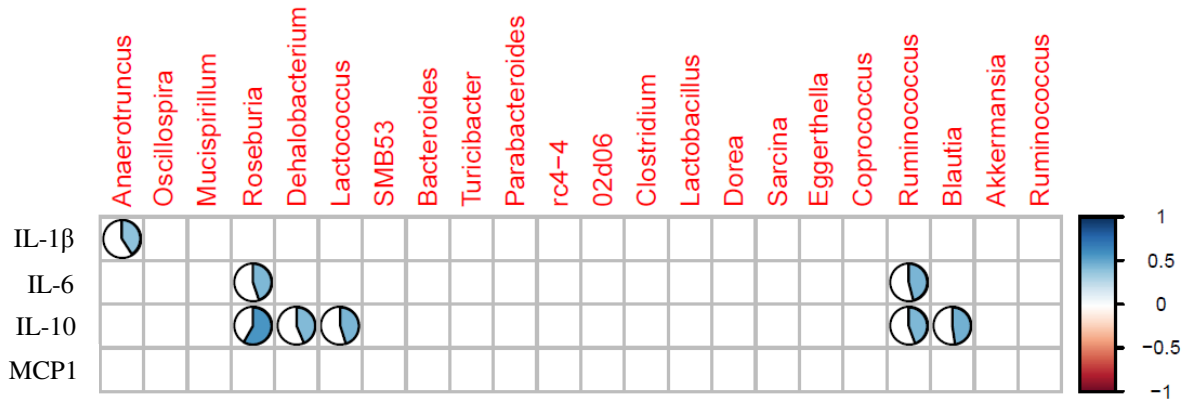

Fig 3.13. Correlation matrix of bacterial genera with cytokine levels with significance of $p<0.05$. Blue represents positive correlation, and red negative correlations. Intensity of color and degree of circles filled represents Pearson's R value.

Discussion

In this study, cecal weights indicate that total bacterial population sizes were similar among all treatment groups. There were no major shifts in the gut bacterial community composition or structure. However, differences in abundance of certain bacterial species, most notably *Ruminococcus gnavus* and *Akkermansia muciniphila,* were identified. Growth of these species may have been influenced by endogenous estrogen status and/or HE supplementation. The mechanism for these changes is unclear, but could involve direct interaction of HE with gut bacteria, indirect effects of estrogen receptor signaling, or other indirect effects due to changes in adiposity. Bacteria within the Clostridiales order showed the greatest changes in abundance amongst groups (Fig 3.4). This is of interest since Clostridia possess β-glucuronidase enzyme activity, which deconjugates estrogen metabolites. Deconjugation allows reabsorption of these metabolitesthrough the mucosa, resulting in increased estrogen bioavailability in the bloodstream [36, 37]. The abundance of bacteria in the Clostridiales order correlated with HE flavonoids, but were not significantly different between OVX HE and Sham HE groups, suggesting an interaction of HE flavonoids on microbial populations regardless of estrogen exposure or body weight.

The Clostridiales order also contains several families, genera, and species that are known to colonize spaces between mucosoal folds in the colon, and have direct influence on immune receptors in the endothelial layer. Many species in the Clostridiales order produce the short chain fatty acid (SCFA) butyrate, which supresses expression of proinflammatory cytokines from immune cells, while also serving as fuel for colonic epithelial cells [34]. In this study, there was no difference in fecal SCFA levels with OVX or estrogen/phytoestrogen treatment. However, it is possible that the analysis of fecal samples rather than cecal samples may have masked any

treatment-related differences since most SCFA are absorbed by colonic epithelial cells or are trafficked to the liver prior to fecal excretion (REF).

Within the order Clostridiales, *Ruminococcus gnavus* was significantly higher in abundance in OVX Placebo vs. Sham Placebo (Fig 3.5). *R. gnavus* is associated with inflammatory bowel disease (IBD), in particular as Crohn's disease manifestation [38, 39]. In twins discordant for Crohn's disease, *R. gnavus* was reportedly higher in abundance in the twin with Crohn's than in their healthy sibling [40]. However, another study showed that while *R. gnavus* was enriched in biopsies of Crohn's patients, it was also associated with macroscopically and histologically normal tissue [39]. Therefore, the role of this bacteria in inducing intestinal inflammation is still unclear, although it may represent an early marker of gut dysbiosis associated with estrogen loss.

As with *R. gnavus*, *Akkermansia mucinophila* resides in the mucosal layer of the inner folds of the gut, in close contact with epithelial cells. The abundances of these two bacteria were negatively correleated. This relationship is supported by a similar inverse relationship in the mucin layer of patients with ulcerative colitis, who have reduced *A. muciniphila* and increased *R. gnavus* [39, 41]. These two bacteria potentially compete for the same niche in the mucosal layer.

A. mucinophila utilizes intestinal mucin as its sole energy source [42]. Degredation of mucins stimulates regeneration of new mucins by epithelial cells, and releases mucin metabolites utilized by other bacterial species in the lumen. Higher levels of *A. muciniphila* are associated with lower body weight in humans and mice [43-45], and *Akkermansia* is positively associated with lower levels of blood glucose, LDL cholesterol and triglycerides [46]. Moreover, probiotic feeding of live, but not heat-killed *A. muciniphila* reversed high-fat diet induced metabolic

disorders and increased endocannabonioid levels in mice, suggesting an important role in the regulation of host metabolism, inflammation and gut barrier function [47].

There was a significant decline in *A. muciniphila* in the Sham HE group versus Sham Placebo. HE flavonoids are bactericidal against Gram-positive bacteria [48], but not against Gram-negative bacteria such as *Akkermansia mucinophila*, so it is unlikely that HE supplementation direclty inhibited *Akkermansia mucinophila* in this study. An indirect effect of HE on *A. muciniphila* is further supported by lack of correlation with HE flavonoids and their metabolites (Fig##). There was not a surgery effect of HE on *A. muciniphila*, as abundance was not different between the OVX HE vs OVX Placebo groups. Body weight may also be an important driver for *A. muciniphila* growth, as OVX HE mice were significantly heavier than Sham HE.

There was no significant evidence of increased intestinal inflammation or compromised gut barrier function with OVX, perhaps because no major changes in bacterial diversity or abundance were observed. However, there were some significant correlations between specific cytokines and bacterial abundance, suggesting there may have been bacterial-immune cell interactions. Most notably, Il-10 was positively correlated with *Ruminococcus gnavus* (of Lachnospiraceae family)*, Dehalobacterium,* and *Blautia* species, all within the Clostridiales order. Several species in the Clostridiales are known to activate intestinal Treg cells, inducing both pro- and anti-inflammatory cytokine signalling [49]. *Rumminococcus gnavus* was also correlated with increased pro-inflammatory IL-6 levels, along with *Roseburia*. Elevated levels of IL-6 is associated with inflammatory chronic diseases associated with menopause, such as rheumatoid arthritis, coronary artery disease, and cancer [50, 51]. Further research is warrented

to expound the role of Clostridiales bacteria in particular on inflammatory and anti-inflammatory pathways in the gut, with regard to estrogen status.

Conclusions

In this study, there were no major changes in gut microbiota community structure, in response to OVX. There were no major bacterial changes with E2 or HE supplementation. These results suggest that at the macro level, the gut microbiota is resilient to loss of estrogen. However, there were some minor changes, most notably an increase in *Rumminococcus gnavus* with OVX, and correlations with inflammatory pathways in the colon. There was significant decrease in *Akkermansia muciniphila* in the Sham HE group that was not seen in the OVX HE group. This discrepancy suggests estrogen-dependent effects of this flavonoid. Lack of major microbial changes associated with HE supplementation suggests these treatments do not negatively affect gut ecology. Further research is necessary to elucidate the relationship between hormonal status, dietary flavonoids and phytoestrogens, and the gut microbiota.

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CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of this research was to investigate potential changes in gut microbiota and disease risk associated with menopause. Ovariectomized (OVX) mice were supplemented with 17β-estradiol (E2) or phytoestrogen-rich hop extract (HE) for 12 weeks, and Illumina MiSeq platform was used to sequence the cecal gut bacterial community. Several physiological parameters including intestinal inflammation and adiposity were measured. Results showed that OVX alone, or supplementation with HE, does not significantly alter the gut microbial community structure, or systemic and intestinal inflammation. Supplementation with HE did result in significant protection against liver triglyceride accumulation and did not result in proliferation of uterine weight. Further research is warranted on the use of HE as therapy for reduction of menopausal symptoms and contribution to overall health benefits.

Initial hypotheses were derived from results of a previous study in which ovariectomized rats bred for either low aerobic capacity (Low Capacity Runners;LCR) or high aerobic capacity (High Capacity Runners;HCR) to determine the effect of exercise on gut microbiota in a menopausal model [1]. In the LCR group, a sedentary model, OVX resulted in higher abundance of Bacteroidetes phylum, increased microbial diversity, and decreased levels of short chain fatty acids (SCFA). These results were not seen in the HCR group, suggesting physical activity modulates OVX-associated changes in the gut microbiota. Results from the current study were more similar to those reported for the HCR rats. This may be due to the inherently high activity level of mice coinciding with the increased activity associated with the HCR rats. It is possible that more pronounced changes in the microbiota would have occurred if the study had been prolonged. Interestingly, the OVX group did experience significant weight gain relative to

controls. In diet-induced models of obesity, microbiota alterations often precede this weight gain [2, 3]. Transplant studies with germ-free mice have shown introduction of microbiota from an obese individual leads to the development of adiposity [4]. The microbiota-independent weight gain in OVX animals suggests that mechanisms other than energy harvest may be driving increased adiposity in this model.

Diet is clearly an important factor in shaping the gut microbiota and diet-estrogen-microbiota interactions may be important in the overall progression of menopause related disease risk. In both the current study and the LCR/HCR rat study, animals were provided with low fat phytoestrogen-free purified diets in order to isolate the effects of OVX from confounding influence of a HFD. Numerous studies in murine and human models have shown that high-fat diet-induced obesity is associated with increased Firmicutes and decreased Bacteroidetes, the two most dominant bacterial phyla in our gut [5-7]. However, it is unclear if changes are due to diet or weight status alone. Hildebrandt et al found that in transgenic mice, a high-fat diet drove changes in bacterial community structure, notably an increase in Firmicutes and Proteobacteria phylum, regardless of obesity status [8]. In humans, this cause and effect is difficult to unravel, since obesity is associated with long-term, often a lifetime, of eating a Western diet. A recent study has demonstrated the compound effects of high fat diet and OVX in rats, where diet-induced obesity was slightly increased with OVX [9].

To explore how a combination of endogenous estrogen reduction and a high fat, western-style diet interacts to influence gut microbiota populations, a small study was conducted to determine the influence of diet and loss of estrogen on the gut microbiota in ovariectomized mice (n=3-5). All methods of animal care, surgery, sample collection and processing, are described in chapters 2 and 3 of this dissertation. In this study, mice were older (12 months vs 7 months) and

fed either a low-fat chow diet (LFD; 6.2% calories from fat, 44.2% from CHO, 18.6% from protein. Teklad 2018, Madison, WI, USA) or HFD (45% calories from fat, 35% from CHO, 20% from protein. Research Diets D12451, New Brunswick, NJ, USA) for 9 weeks. Fecal DNA was sequenced and bacterial abundance, richness and diversity were compared amongst groups.

Although there were no significant differences in bacterial diversity or species richness amongst treatment groups, there was a significant increase in Firmicutes phylum with a HFD versus LFD in both sham and OVX operated mice (Fig 4.1). Pairwise comparison of treatments within the HFD group revealed that OVX animals had significant more abundant Firmicutes than sham-operated mice (Fig 4.2). This is of interest since increased Firmicutes and decreased Bacteroidetes is associated with obesity and cardiometabolic disease risk [7, 10-12]. Consistent with previous results, OVX animals consuming a low-fat diet did not have significantly altered gut microbiota compared to the sham operated animals. Principal component analysis (PCA) of abundance at the OTU level and Phyla levels showed that diet was a greater source of variability amongst groups than OVX status (Fig 4.3). Interestingly, overall body weight was not significantly different amongst groups (data not shown), supporting a larger body of literature that suggests that dietary components drive microbial changes, which then contribute to weight alterations [7, 13-15].

In our current study of this dissertation, we found that OVX alone resulted in changes within the Clostridiales order (Fig 3.4), which influence inflammation status in the gut through modulating colonic immune cells [16]. One bacterial species, *Rumminococcus gnavus* was significantly increased with OVX versus sham control (Fig 3.5). This was indicated in a follow-up study (Fig 4.4), perhaps because there was considerable inter-individual variability and low number of subjects (n=3-5).

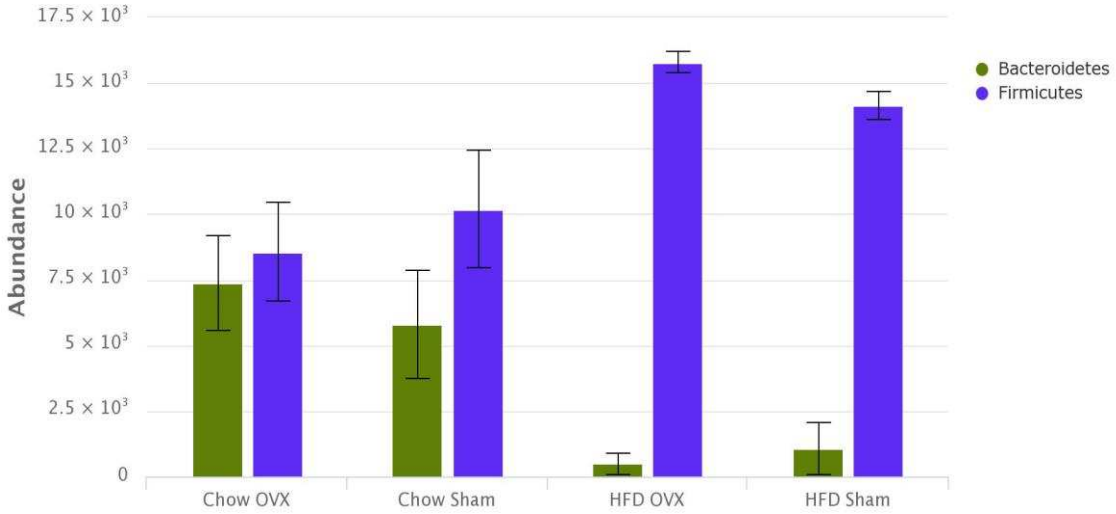

Fig 4.1: Significantly different bacterial abundance at the phyla level. One-way ANOVA with Tukey post-hoc correction. Bacteriodetes p=0.0000492, Firmicutes p=0.0000622, Tenericutes p=0.00195. Error bars represent standard deviation from the mean.

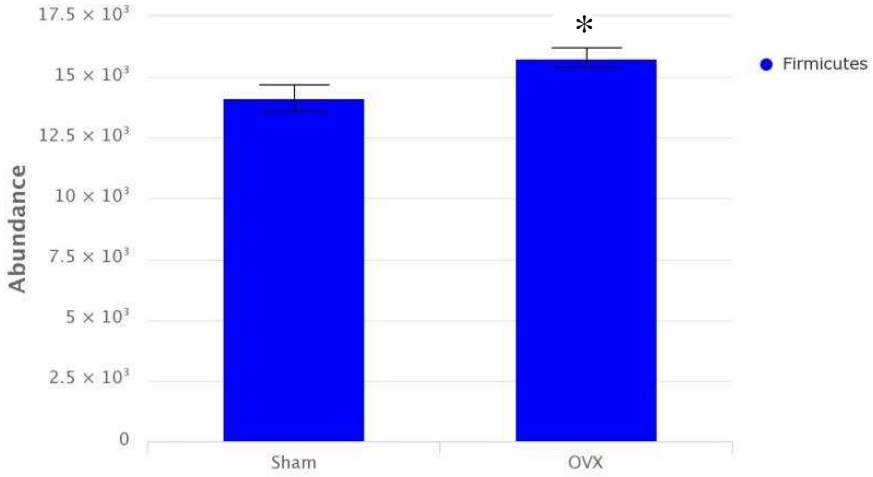

Fig 4.2 Significantly different abundance at phyla within HFD group, OVX resulted in increased Firmicutes (p=0.00272). There were no significant differences in abundance within the chow group. Error bars represent standard deviation from the mean.

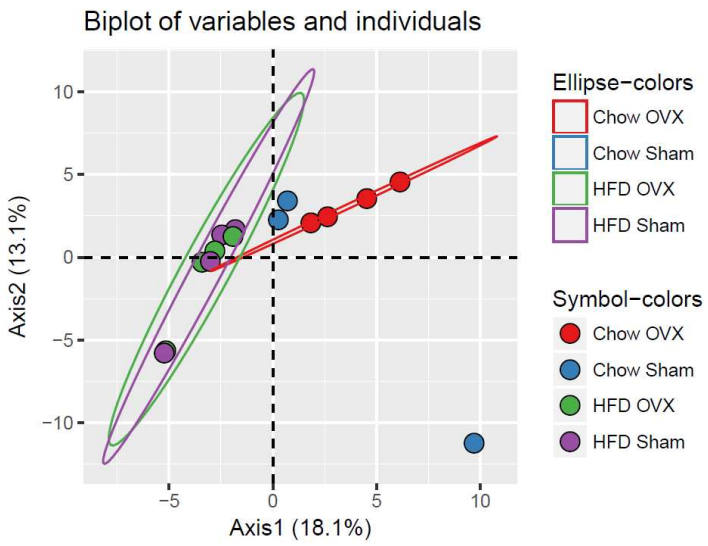

Fig 4.3: Principal Components of Analysis plot amongst groups at the OTU level. Confidence level for ellipse set at 0.95.

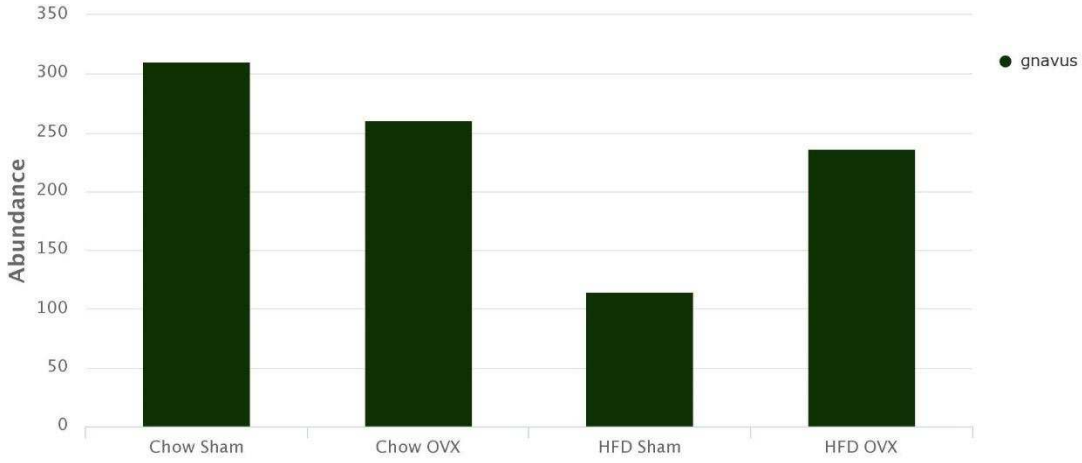

Fig 4.4: Abundance of Ruminococcus gnavus was not significantly different amongst groups (p=0.562)

Summary and Future Directions

Menopause is associated with a marked increase in overall body weight, visceral adiposity, and triglyceride accumulation in the liver and circulating blood [17-19], known risk factors for cardiometabolic disease and cancer [20, 21]. Evidence is accumulating that gut

bacteria are moderators of adiposity and inflammation. There was no significant evidence in our studies that gut bacteria are disturbed with short-term estrogen loss per se. However, estrogen loss compounded with high fat diet may precipitate microbial dysbiosis and contribute to metabolic derangement.

Seemingly independent of the gut microbiota, HE and E2 provided protection against menopause-associated liver triglyceride accumulation. Flavonoids in HE, most notably xanthohumol (XN), are known modulators of lipid metabolism [22-24] and inflammatory cytokines [25-27]. Since menopause-associated weight gain and liver triglyceride accumulation poses a significant risk for the development of cardiometabolic diseases, HE could be an appropriate treatment for women undergoing menopause. It has been demonstrated in human models that HE is effective at relieving uncomfortable side effects of menopause including hot flashes, insomnia and mood swings [28, 29]. However, there has not been a study in menopausal women on the effectiveness of HE on reducing or preventing menopause-associated adiposity. Future studies in human and murine models are warranted to delineate the effects of HE on menopausal side-effects as well as adiposity-related disease risk.

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APPENDIX

Table A1: Nutritional composition of diet (Harlan TD.08113) and treatment received daily

	Daily intake (g)	Total fat (g)	Saturated fat (g)	Unsaturated fat (g)	Total calories	Total calories from fat (g)	% calories from fat	Total calories from protein (g)	% calories from protein	Total calories from CHO (g)	% calories from CHO
Diet	20.90	0.86	0.00	0.00	75.24	7.71	10%	10.37	14%	57.18	76%
Cookie	0.20	0.06	0.04	0.02	1.04	0.50	48%	0.06	5%	0.50	48%
Oil	0.03	0.03	0.00	0.03	0.27	0.27	100%	0	0%	0	0%
Total:		**0.94**	**0.04**	**0.04**	**76.55**	**8.49**	**11.09%**	**10.42**	**13.61%**	**57.68**	**75.35%**

Table A2: Primer sequences

Target gene	Sequence
ABCG5	For: 5'-CAGGGACCGAATTGTGATTG-3' Rev: 5'-GAACACCAACTCTCCGTAAG-3'
ABCG8	For: 5'-CTGGAATCCTGAGAGGATAG-3' Rev: 5'-TAGGTCGCCCTTTGTATTGG-3'
Fasn	For: 5'- AGACTACAGACGACAGCAACC-3' Rev: 5'-CTCTCAGACAGGCACTCAGC-3'
SREBP1c	For: 5'-TGGTGGGCACTGAAGCAAAG-3' Rev: 5'-CACTTCGTAGGGTCAGGTTCTC-3'
AcCoA carboxylase	For: 5'-CTTCGCCATAACCAAGTAGAG-3' Rev: 5'-GTTTCCGAGAGGATGAGTTTC-3'
Hormone sensitive lipase	For: 5'-CAGTCAATGGAGACACTTGG-3' Rev: 5'-GGGTCTCACTTCATCTTTGG-3'