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Original Article

The Effect of Hops (*Humulus lupulus* L.) Extract Supplementation on Weight Gain, Adiposity and Intestinal Function in Ovariectomized Mice

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Abstract: Estrogen decline during menopause is associated with altered metabolism, weight gain and increased risk for cardiometabolic diseases. The gut microbiota also plays a role in the development of cardiometabolic dysfunction and is also subject to changes associated with age-related hormone changes. Phytoestrogens are plant-based estrogen mimics that have gained popularity as dietary supplements for treatment or prevention of menopause-related symptoms. These compounds have the potential to both modulate and to be metabolized by the gut microbiota. Hops (*Humulus lupulus* L.) contain potent phytoestrogen precursors, which rely on microbial biotransformation in the gut to estrogenic forms. We supplemented ovariectomized (OVX) or sham-operated (SHAM) C57BL/6 mice, with oral estradiol (E2), a flavonoid-rich extract from hops, or a placebo carrier oil to observe effects on adiposity, inflammation, and gut bacteria composition. Hops extract and E2 protected against increased visceral adiposity and liver triglyceride accumulation in OVX animals. Surprisingly, we found no evidence of OVX having a significant impact on the overall gut bacterial community structure. We did find differences in abundance of *Akkermansia muciniphila,* which was lower with HE treatment relative to the OVX E2 treatment and to placebo in the SHAM group.

Keywords: adiposity; dysbiosis; hops; menopause; microbiota; 8-prenylnaringenin; obesity; ovariectomy

1. Introduction

The onset of menopause is marked by declining ovarian estrogen production and is associated with consequences for long-term health in women. The 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 (Kannel, Hjortland et al. 1976, Wajchenberg 2000, Abu-Taha, Rius et al. 2009). Accumulating evidence suggests that estrogen may be an important regulator of the intestinal microbiota (Gorbach 1984, Menon, Watson et al. 2013). 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 (Keenan, Janes et al. 2013, Cox-York, Sheflin et al. 2015, Liu, Park et al. 2015, Melvin 2016, Moreno-Indias, Sánchez-Alcoholado et al. 2016). 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" (Plottel and Blaser 2011). 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 for chronic disease risk, menopause is associated with numerous acute physical symptoms including hot flashes, mood changes, and insomnia. 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 (Chlebowski, Anderson et al. 2010) and the demand for HT has declined in the past decade due to its association with increased breast cancer and cardiovascular disease risk (Rossouw, Anderson et al. 2002, Santen, Allred et al. 2010, Siegel, Miller et al. 2015). Subsequent analysis of these studies demonstrated important nuances related to time and duration of HT (Roberts and Hickey 2016) but consumers remain cautious and often opt for alternative therapies to 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 can alleviate uncomfortable menopausal symptoms and may interact with the gut microbiota to mitigate the risk of chronic diseases (Jungbauer and Medjakovic 2014). 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 to date (Milligan, Kalita et al. 1999, Milligan, Kalita et al. 2002). 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 (Bowe, Li et al. 2006, Paoletti, Fallarini et al. 2009, Erkkola, Vervarcke et al. 2010, van Breemen, Yuan et al. 2014). The estrogenic activity of hop flavonoids is dependent on gut bacterial metabolism of the flavonoids xanthohumol (XN) and isoxanthohumol (IX) to the phytoestrogen 8-prenylnaringenin (8PN) (Possemiers, Heyerick et al. 2005). Commercial hops-based dietary supplements contain a mixture of XN, IX and 8PN. Human gut bacteria including *Eubacterium limosum*, (Possemiers, Rabot et al. 2008) and *Eubacterium ramulus* (Paraiso, Plagmann et al. 2019) are able to convert IX to 8PN, providing evidence for a mechanism by which the gut microbiota can influence resultant 8PN exposure.

In the present study, an ovariectomized (OVX) mouse model was used to investigate the effects of 17-β-estradiol (E2) and prenylflavonoid-rich hops extracts (HE) on metabolic parameters associated with menopause, while monitoring changes in the gut microbiota and intestinal function of female mice under an estrogen-depleted state. Ovariectomy (OVX) or sham (SHAM) surgeries in 7-month old retired breeder C57BL/6 mice were conducted. All animals were fed a purified, phytoestrogen-free diet and randomized to treatment groups given either a commercial supplement made from HE, E2, or placebo carrier oil. Based on data from a previous study conducted in rats, we hypothesized that the gut microbiota of OVX mice would be characterized by increased bacterial diversity and a higher proportion of Bacteroidetes phyla than animals undergoing sham surgeries. We further hypothesized that E2 and HE would mitigate effects of estrogen loss on the gut microbiota.

2. Materials and Methods

2.1. Animal study.

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 7-month old retired breeder mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Retired breeders were chosen to more closely mimic the menopausal period of the mouse life cycle. 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 10.2% (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 entry ovariectomy, conducted by making 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 and removal

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of ovaries. Control groups underwent sham surgery, including exposure without ligation and removal of the ovaries. Muscle was sutured and the skin incision was closed with wound clips. Mice received analgesic (Meloxicam; 1.2 mg/kg) prior to surgery and for 24 hours post-surgery. Body weight and food intake were measured weekly for 12 weeks. The five study groups included: OVX Placebo (OVX; n=11), OVX plus hop extract (OVX HE; n=11), OVX plus 17β-estradiol (OVX E2; n=9), Sham Placebo (SHAM; n=10), and Sham HE (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 10.3% 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 HE and E2 were suspended in 20 L 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 (Ingberg, Theodorsson et al. 2012) and 400 mg/kg HE (Overk, Yao et al. 2005, van Breemen, Yuan et al. 2014) to the mice daily. This amount of HE was composed of 5.1 g/mg 8-prenylnaringenin (8PN), and 6.3 g/mg xanthohumol (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 (Table 1).

Table 1. Average daily caloric and macronutrient intake.

	Daily intake (g)	Total calories	% calories fat	% calories protein	% calories CHO
Diet	20.90	75.2	10.3%	13.8%	76.0%
Cookie	0.20	1.0	48.5%	5.3%	48.1%
Oil	0.03	0.3	100.0%	0.0%	0.0%
Total:	**21.13**	**76.6**	**11.1%**	**13.6%**	**75.4%**

2.2. 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 and confirming the absence of ovaries.

2.3. 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.

2.4. 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 3. 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.

Table 2. Primer Sequences used for gene expression.

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′

2.5. Quantification of Hop Prenylflavonoids in Hop Extract and 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 from the same cohort as study animals (identical to study animals in age, source, and environmental conditions). Mice 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 the mice licked it off 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 SHAM HE mice at termination. Mice were fasted for 4 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 4º C. The supernatant was transferred to a glass vial and analyzed by UHPLC-MS. A calibration curve was prepared in the same manner, by spiking known amounts of the synthetic standards XN and 8PN into serum prior to extraction.

UHPLC-MS was performed at the Proteomics and Metabolomics Core Facility at Colorado State University 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 5º C in the autosampler, and the analytical column was operated at room temperature, near 21º 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 3). 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 150º C and desolvation temperature 200º 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.

Table 3. UHPLC-MS quantitative and confirmatory transition for each analyte measured in serum. CV cone voltage, CE collision energy.

Molecule	Parent m/z	Fragment m/z	CV	CE
Xanthohumol	353.3	119	50	26
Xanthohumol	353.3	233.2	50	18
8-prenylnaringenin	339.3	219.2	60	22
8-prenylnaringenin	339.3	119	60	30
Naringenin	271.2	151.1	50	18
Naringenin	271.2	119	50	26
2,4-dihydroxychalcone	239.2	135.1	60	22
2,4-dihydroxychalcone	239.2	197.1	60	20
Xanthohumol-glucuronic acid	529.3	353.3	50	25
8-prenylnaringenin-glucuronic acid	515.3	339.3	50	25

2.6. Bile Acid Determination.

Fecal samples (25mg) from week 10 of the study, were homogenized in 500 L of NH_4OH, with 5 L 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 at the Colorado State University Proteomics and Metabolomics Core Facility on a Waters (Millford, MA, USA) Acquity UHPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer, as described previously (Sheflin, Borresen et al. 2017). 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 spectrometer 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.

2.7. Short Chain Fatty Acid Determination.

Fecal samples collected one week prior to study termination (11 weeks) were extracted for short chain fatty acids (SCFA) 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 at RT for 15 min. The supernatant was transferred to glass vials and stored at -80°C 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. SCFA were quantified using 5-point standard curves of commercially purchased standards (Sigma, St. Loius,MO, USA) and normalized to internal standard signal.

2.8. Intestinal Permeability and Inflammation.

One week prior to termination of the study (11 weeks), intestinal permeability was assessed in vivo as described previously (Cani, Bibiloni et al. 2008). 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 L 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 min. 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.

Intestinal alkaline phosphatase (IAP) was measured in ileum tissue that was homogenized in a bullet blender and diluted in Bio-Plex Cell Lysis Kit buffer (BioRad, Hercules, CA). tissue by colorimetric assay against a standard curve, using SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec Inc, Fremont, CA) according to manufacturer's instructions. LPS-binding protein (LBP) and soluble CD14 (sCD14) in homogenized and diluted ileum tissue by an ELISA assay, using Boster CD14 PicoKine and LBP PicoKine kits (Bosterbio, Pleasanton, CA). Cytokines and chemokines IL-6, IL-10, IL-1β, MCP-1, MIP-1α, MIP-1β, IFNγ were measured in the proximal colon, homogenized and diluted as ileum tissue described above. Levels were measured by bead-based multiplex assay with Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Pannel kit (MilliporeSigma, Burlington, MA) according to manufacturers instructions.

2.9. DNA Extraction and 16S 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). Raw fastq data was processed using the dada2 pipeline using the software myPhyloDB version 2.0 (Manter, Korsa et al. 2016) and sequence reads were normalized by rarefaction to 5000 reads.

2.10. Statistical Analysis.

All statistical analyses were done using GraphPad Prism version 8.0.2 (GraphPad Software, La Jolla California, USA). Multiple comparisons among treatment groups are reported as standard error

of the mean, using one-way ANOVA with Tukey post hoc comparisons, with statistical significance set at p <0.05. Outliers were identified using the 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.

3. Results

3.1. Weight gain, Adiposity and Liver Triglycerides.

Compared to Sham, OVX resulted in weight gain, although terminal body weight differences did not reach significance (Table 4). However, the OVX Placebo group had significantly higher visceral adipose tissue (VAT) compared to SHAM Placebo. The OVX E2 group was protected from this VAT increase, and the OVX HE also showed some protection, although they were trending towards a significant increase in VAT. Subcutaneous adipose tissue (SAT) and brown adipose tissue (BAT) were not significantly different. Likewise there were no significant differences in cecal weights. Relative to SHAM Placebo, uterine weight was significantly decreased in OVX Placebo, OVX HE and OVX E2 groups (Table 4). The OVX Placebo group had significantly higher levels of liver triglycerides (TG) than all other groups (p<0.001, Fig 1), whereas liver TG in OVX HE, SHAM HE and OVX E2 were not significantly different from SHAM Placebo. Liver tissue was assessed for gene expression for several lipid transport and metabolism genes, including 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 (data not shown).

Table 4. Tissue weights at study termination. One-way ANOVA using Tukey post-hoc correction for multiple comparisons to Sham-Placebo group. All weights in milligrams except body weight is in grams. BW body weight; SAT; subcutaneous adipose tissue, BAT brown adipose tissue, VAT visceral adipose tissue, AT adipose tissue.

	Sham Placebo	**Sham HE**	**p-value**	**OVX Placebo**	**p-value**	**OVX HE**	**p-value**	**OVX E2**	**p-value**
BW (g)	31.0	30.1	0.96	34.3	0.09	33.9	0.16	31.0	0.99
Cecum	296.7	317.6	0.99	243.9	0.91	274.7	0.99	297.7	0.99
Uterus	107.7	89.5	0.32	28.0	<0.001*	31.2	<0.001*	78.9	0.023*
SAT	1.24	1.17	0.99	1.67	0.21	1.54	0.57	1.16	0.99
BAT	0.11	0.12	0.99	0.14	0.78	0.15	0.55	0.13	0.93
VAT	2.259	2.288	0.99	3.68	<0.001*	3.27	0.063+	2.02	0.97
VAT/ Total AT	0.627	0.638	0.98	0.671	0.09	0.655	0.48	0.608	0.89

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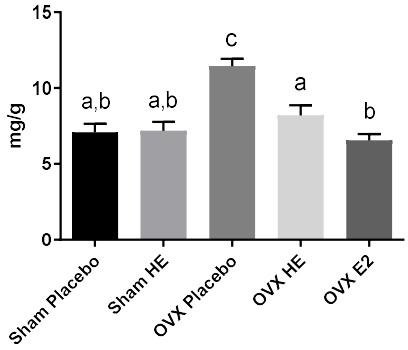

Figure 1. Liver triglycerides.

3.2. Pharmacokinetics of Hop Prenylvlavonoids in Serum.

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 based on relative peak area. Serum in non-study mice was measured after oral HE administration over a period of 30 hours. XN had a detected T_{max} of 1 hour with a detected C_{max} = 14.33 ng/mL. IX and 8PN both had a T_{max} of 3 hours, with a detected C_{max} = 4.58 ng/mL and 6.73 ng/mL respectively (Fig 2A). Three glucuronidated species were also identified (Fig 2B). 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, while another chemically distinct form of 6PN/8PN-glucuronic acid detected had a T_{max} of 10 hours.

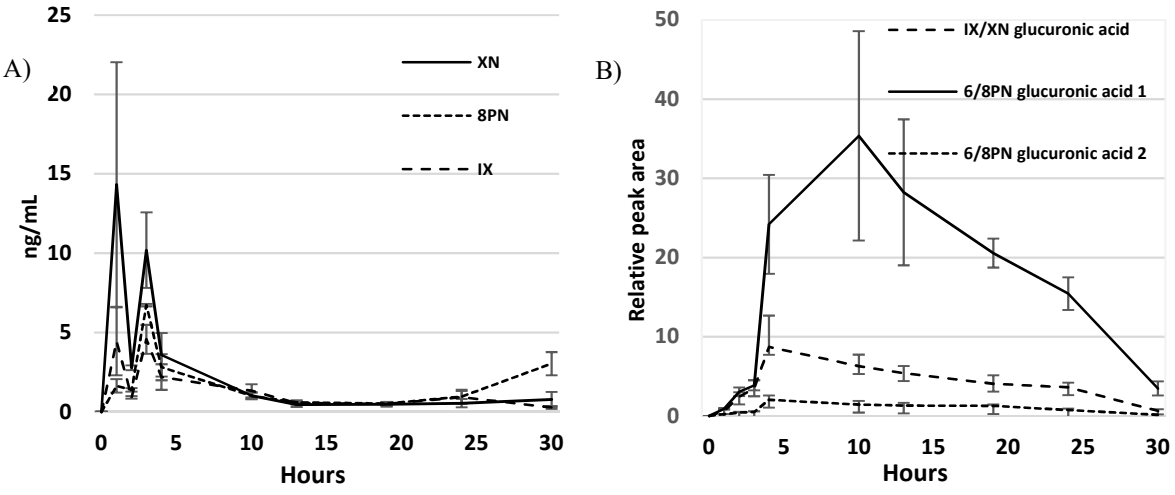

Figure 2. A) Pharmacokinetics of XN, IX and 8PN in serum after oral administration of hop extract. B) Pharmacokinetics of glucuronidated compounds of XN, IX, 8PN and 6PN. Values are relative peak area.

3.3. Bile Acid and Short Chain Fatty Acid Quantification

There were no statistically significant differences in fecal bile acids amongst groups (n = 5/group). One mouse in the OVX Placebo was a consistent outlier in levels of cholic, deoxycholic, ursodeoxycholic, chenodeoxycholic and glycocholic acids, and was not included in the analysis. Cholic acid, deoxycholic acid, ursodeoxycholic acid and chenodeoxycholic acid were highest in the OVX HE group and below detection in OVX E2 group (Fig S1). There were also no significant differences in fecal short chain fatty acid (SCFA) levels amongst groups (Fig S2). 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 (Lopetuso, Scaldaferri et al. 2013).

3.4. Intestinal Function

Measurement of intestinal barrier function with 40kD FITC-Dextran assay revealed no differences between groups, with most samples having fluorescent measurements consistent with background readings (data not shown). LPS-binding protein (LBP) and soluble CD14 (sCD14) dimerize to bind to circulating LPS, and increased levels in the plasma are indicative of higher circulating LPS levels and endotoxemia (Stein, Morris et al. 2003), making these viable proxy measures for LPS. Post mortem measurement of plasma LBP and sCD14 were not significantly different among groups (Fig S3). The levels of intestinal alkaline phosphatase (IAP), 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. However, IAP was significantly higher in the Sham HE compared to Sham Placebo group ($p=0.04$; Fig 3). Intestinal inflammation was assessed by examining colonic cytokines and chemokines. IL-6 and IL-10 levels were highest in the OVX HE group, though levels of IL-6, IL-10, IL-1β and MCP-1 did not reach statistical significance due to high variability within the group (Fig S4). Levels of IFN-γ, MIP-1α, MIP-1β were below detection in most samples (data not shown).

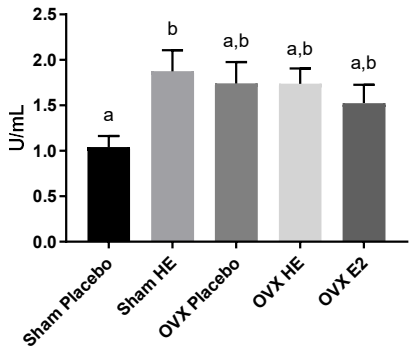

Figure 3. Intestinal alkaline phosphatase. .

3.5. Microbiota Analyses

There were no alterations in the gut microbial community structure (alpha and beta-diversity) as a result to OVX and E2 or HE supplementation in cecal contents collected upon study termination. Cecum weights were similar amongst all groups suggesting that bacterial abundances were also similar, although this was not confirmed by qPCR. There was one notable difference at the phyla level: Verrucomicrobia was lower in Sham HE compared to the Placebo and OVX E2 groups (Fig 4A). Verrucomicrobia contains only one identified species, Akkermansia muciniphila (Fig 4B), and therelative abundance of this species was also lower in Sham HE group compared to Sham Placebo ($p=0.03$)and OVX E2 ($p=0.04$).

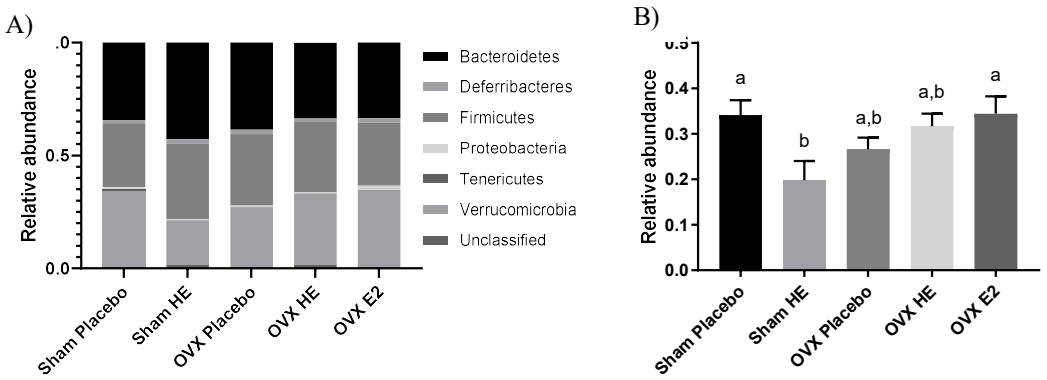

Figure 4. A) Relative abundance of bacterial phyla in the cecum B) Microbial relative abundance of *Akkermansia muciniphila* in the cecum.

4. Discussion

The onset of menopause is associated with increased risk of several chronic diseases of the cardiovascular system, bone and insulin-sensitive tissues. Microbial composition of the gut has been similarly identified as a modulator of these conditions. Given the interest in alternative treatments for menopause-associated side effects and the role of the gut microbiota in the metabolism estrogen and phytoestrogenic compounds such as those in hops, we sought to investigate these interactions in an ovariectomized mice. Menopause is often marked by an increase in body weight, without change in diet or exercise habits. Although it did not reach statistically significance relative to the SHAM group, there was a trend toward body weight increase with OVX, as previously reported (Arjmandi, Alekel et al. 1996, Davis, Castelo-Branco et al. 2012, Sánchez-Garrido, Ruiz-Pino et al. 2015). Supplementation with E2 and to a lesser effect HE protected against accumulation of visceral adipose tissue (VAT) in these animals. Other flavonoids have been shown to mitigate increases in visceral adipose tissue, such as green tea extracts (*Camellia sinensis)* (Murase, Nagasawa et al. 2002, Bose, Lambert et al. 2008), red wine grapes (Rivera, Morón et al. 2009), and licorice root (Madak-Erdogan, Gong et al. 2016). Many of these studies employed diets high in saturated fat, therefore it is interesting that HE exerted protective effects in the present study where mice were on a low-fat diet. VAT is pro-inflammatory and is associated with increased risk of several cardiometabolic diseases and cancers (Pouliot, Després et al. 1994, Calle and Kaaks 2004, Berg and Scherer 2005), so preventing or reducing its accumulation is beneficial. It is not surprising that E2 treatment decreased adiposity, since estrogen is involved in leptin signaling and other regulators of metabolic homeostasis (Mauvais-Jarvis, Clegg et al. 2013, Frank, Brown et al. 2014). We hypothesized that HE would have similar protective effects due to the estrogenic activity of 8PN. The reduced effect may be attributed to incomplete conversion of IX to 8PN. Our analyses did not detect bacterial species (*Eubacterium limosum* and *E. ramulus*) previously reported as converters of IX to 8PN, and our own pharmacokinetic data suggests a high degree of variability between animals with regard to bioavailability of the HE compounds.

We were able to detect the major HE flavonoids, as well as several glycosylation products, in mouse serum. The bi-modal peaks in XN and IX suggest these compounds are absorbed in both the small intestine and colon. The levels of XN were highest at 1 hour after HE ingestion, while IX and 8PN were higher at 3 hours post-consumption. This spontaneous conversion of XN to IX, followed by enterohepatic circulation and conversion of IX to 8PN by liver microsome CYP1A2 (Guo, Nikolic et al. 2006) or microbial metabolism by colonic bacteria (Possemiers, Heyerick et al. 2005, Nikolic, Li et al. 2006, Possemiers, Rabot et al. 2008). These results are consistent with van Breemen et al, who measured the pharmacokinetics of oral HE in women (van Breemen, Yuan et al. 2014), and provide evidence that the mice in the current study were exposed to flavonoid levels capable of exerting physiological effects. Hop flavonoids, especially XN, have been shown to be protective against high fat diet-induced accumulation of liver triglycerides, modulated via regulation of genes involved in fatty acid and cholesterol metabolism (Goldwasser, Cohen et al. 2010, Legette, Luna et al. 2013,

Sumiyoshi and Kimura 2013). We did observe a reduction in liver triglycerides in HE-treated animals, however, we could not confirm that these were due to altered expression of genes involved in lipid metabolism. It is possible the reduction in liver triglycerides was mediated through other pathways. Naringenin, a flavonoid structurally similar to 8PN, was found to activate both PPARα and PPARγ while inhibiting LXRα in rat hepatocytes, regulating downstream fatty acid oxidation genes (Goldwasser, Cohen et al. 2010).

We have previously demonstrated in a rat model that OVX was accompanied by alterations in the gut bacteria (Cox-York, Sheflin et al. 2015). In this study, we hypothesized that OVX would alter the mouse gut microbiota and that HE and E2 would prevent or minimize these effects. However, we saw no significant differences in alpha or beta diversity across treatment groups and the community composition and size, inferred from a lack of differences in cecal weights, was similar, even at finer taxonomic scales. The lack of differences, particularly between OVX and SHAM groups was unexpected, particularly because OVX was accompanied by increased visceral adiposity and several previous studies have reported OVX-associated microbial shifts (Kaliannan, Robertson et al. 2018) . However, there are multiple environmental factors that can influence the microbiota, such as diet, age, and time post-OVX that may account for differences between our study and previous reports. Additionally, differences in sequencing depth and sequence processing pipelines could account for this discrepancy. As retired breeders, the mice in the current study were also multi-parous, whereas most OVX studies are in virgin animals. While we are not aware of any studies investigating the difference in gut microbiota between nulli- and multiparous subjects (human or animal), there is some evidence that the vaginal microbiome is associated with parity (Nasioudis, Forney et al. 2017).

The only notable microbiota difference we detected was a significant reduction of *Akkermansia muciniphila* in SHAM HE compared to SHAM placebo (Fig 4B). Growth of this species may have been influenced by interactions between endogenous estrogen and HE supplementation, as this reduction was not observed in OVX HE animals. This idea is supported by Chen et al who observed a decrease in *Akkermansia* in OVX mice supplemented with combined conjugated estrogens plus Bazedoxifene (a selective estrogen receptor modulator) along with a decrease in deconjugation enzymes (Chen, Liu et al. 2018). *Akkermansia mucinophila* utilizes intestinal mucin as its sole energy source (Derrien, Vaughan et al. 2004) and degredation of mucins stimulates regeneration of new mucin by epithelial cells and releases mucin metabolites that are utilized by other bacteria in the lumen. Furthermore, higher levels of *A. muciniphila* are associated with lower body weight in humans and mice (Angelakis, Armougom et al. 2012, Karlsson, Önnerfält et al. 2012, Shin, Lee et al. 2013), and *A. muciniphila* is positively associated with lower levels of blood glucose, LDL cholesterol and triglycerides (Brahe, Le Chatelier et al. 2015). 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 (Everard, Belzer et al. 2013). Thus, further investigation of the effects of HE on these bacteria may be warranted.

Conclusions

Menopause is associated with a marked increase in overall body weight, visceral adiposity, and triglyceride accumulation in the liver and circulating blood (Haffner, Katz et al. 1991, Tankó, Bagger et al. 2005, Ahn, Schatzkin et al. 2007), known risk factors for cardiometabolic disease and cancer (Colditz, Willett et al. 1987, Hamm and Weir 2015). Accumulating evidence suggests that estrogen-mediated impacts on the gut bacteria may contribute to these physiologic changes. However, there were no significant OVX-related effects on the gut microbiota in our study, suggesting that short-term estrogen loss per se is not detrimental to the gut microbiota, but may be dependent on other physiological and environmental parameters such as parity and diet. Independent of the gut microbiota, HE and E2 provided protection against menopause-associated visceral adiposity and liver triglyceride accumulation. Flavonoids in HE, most notably XN, are known modulators of lipid metabolism (Mendes, Monteiro et al. 2008, Costa, Rodrigues et al. 2017, Takahashi and Osada 2017) and inflammatory cytokines (Cho, Kim et al. 2008, Lupinacci, Meijerink et al. 2009, Dorn, Kraus et al.

2010). Since menopause-associated adiposity 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 (Heyerick, Vervarcke et al. 2006, Erkkola, Vervarcke et al. 2010). 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 needed to delineate the effects of HE on menopausal side-effects as well as obesity-related disease risk.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Data

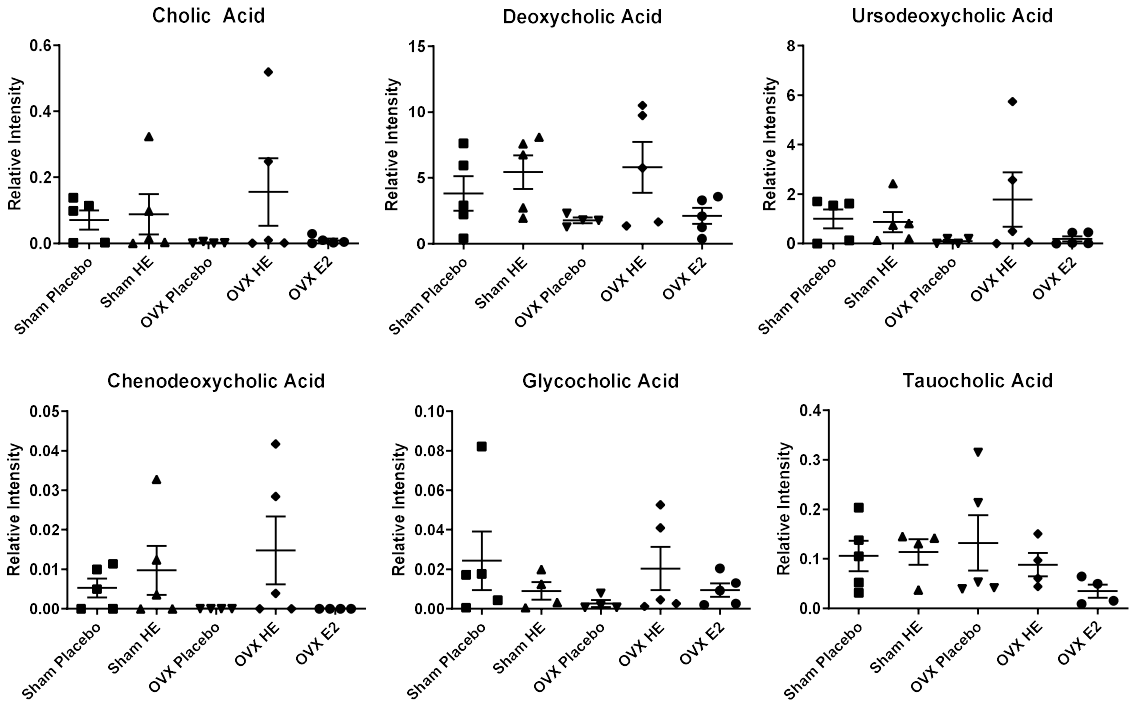


Figure S1. Bile acids in fecal samples. .

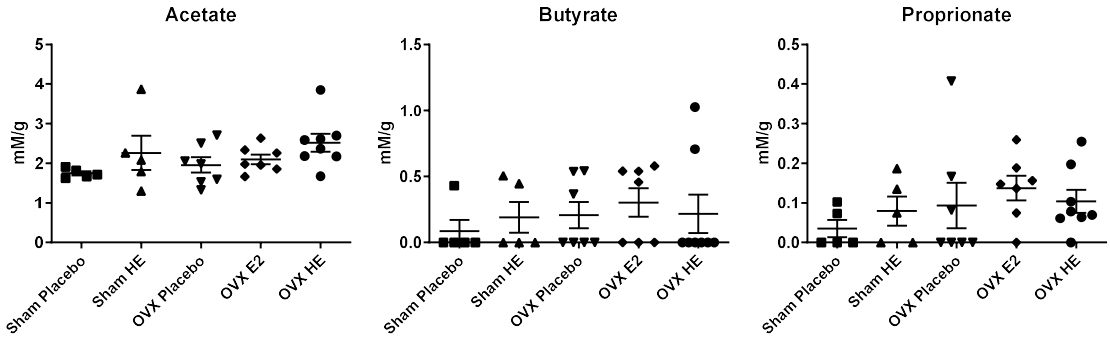
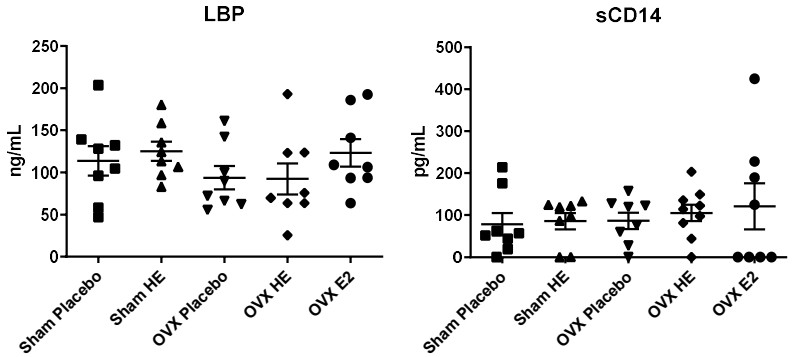


Figure S2. Levels of short chain fatty acids (SCFA) in fecal samples.



605 **Figure S3.** Blood measures of endotoxin binding proteins LBP and soluble CD14.

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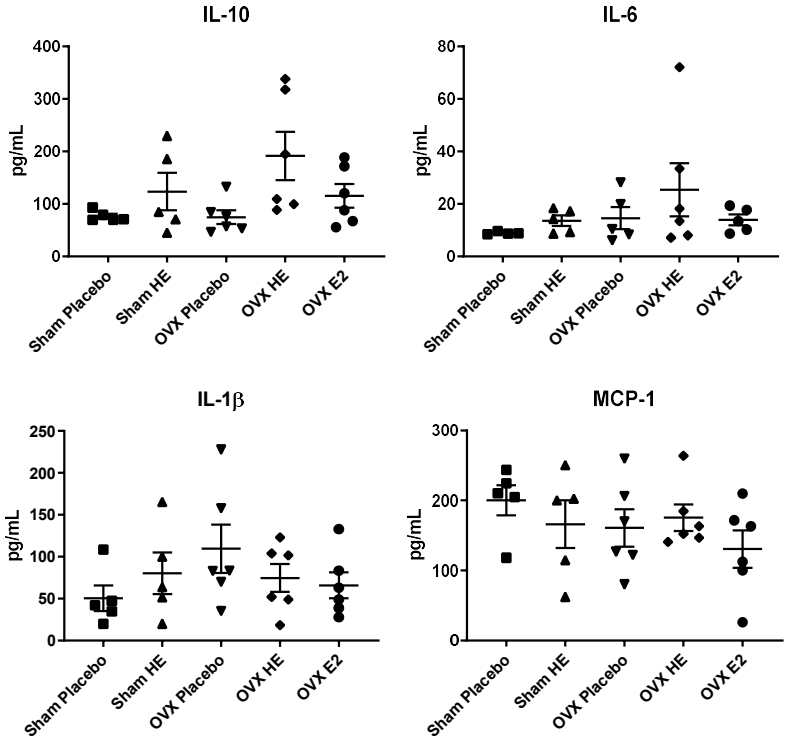

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608 **Figure S4.** Cytokine and chemokine levels in proximal colon.