The Influence of Cherry Phenolic Compounds on Gut Mucosal Immunity.

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Introduction:

Estrogen is a steroid hormone having pleotropic, physiological effects, including the regulation of bone metabolism, reproduction, and immune function. It has been shown that when estrogen levels are reduced in the postmenopausal period, women are at increased risk of diseases such as osteoporosis, certain cancers, heart disease, and autoimmune diseases [1]. Most of these health issues have been linked to changes in immune cell function and inflammatory status that occur with estrogen deficiency [2]. Recently, interest has increased in understanding the influence of biologically active food components on aspects of the immune system located within the gut. The gut-associated lymphoid tissue makes up approximately 70% of the immune system and represents an important potential target to alter immune responses through the diet or dietary supplements. Certain foods are high in bioactive components known as phenolic compounds. These compounds have an aromatic ring structure and exhibit anti-inflammatory and antioxidant properties that could impact systemic immunity.

Tart cherries are a particularly rich source of phenolic compounds. Our research group is interested in studying the effects of tart cherry and bioactive phenolic compounds on the immune response. A series of *in-vivo* and *in-vitro* studies were designed and conducted to assess the potential of tart cherries to modulate the immune response in scenarios that mimic events occurring in estrogen deficiency. The goal of this project was to analyze the effects of tart cherry and its phenolic compounds on the gut mucosal immune response. The hypothesis that was tested was that supplementation with dietary tart cherry or its isolated phenolic compounds would alter the immune function. To test this hypothesis the following aims were developed.

- **Aim 1:** To examine the effects of dietary tart cherry supplementation on the inflammatory response within key gut-associated lymphoid tissue (GALT) in a mouse model of estrogen hormone deficiency.

- **Aim 2:** To examine how tart cherry polyphenolics affect cell proliferation and inflammatory status of epithelial and immune cells.

Aim 1:

Animals and Experimental Design. Five-month-old C57BL/6 female mice were randomly assigned to three groups. The mice were either Sham-operated where they underwent surgery, but the ovaries were left intact, or ovariectomized (OVX) where the ovaries are removed to create a model of ovarian hormone (i.e., estrogen) deficiency. The mice in the Sham and one OVX group were fed control (AIN-93M) rodent diet. The other OVX group was fed AIN-93M diet supplemented with 5% tart cherry powder (*See Figure 1)*. All diets were isocaloric, isonitrogenous and were adjusted for fiber, calcium, phosphorous, potassium, and vitamin K. Throughout the study animals were weighed on a weekly basis and provided with RO water. The treatment period lasted for 28 days after which the animals were anesthetized, exsanguinated and tissues were harvested. In particular, the small intestine was harvested and flushed with ice cold saline and one aspect of the gut associated lymph tissues, the Peyer's patches, was excised and snap frozen for future analyses.

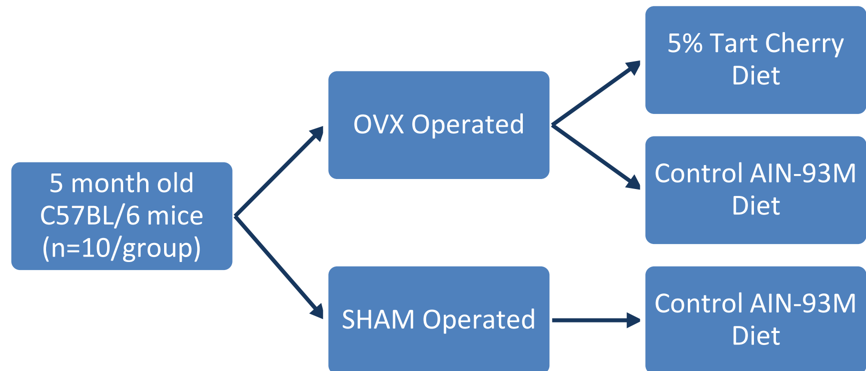

Figure 1. Experimental design for the animal study.

Peyer's patches represent small regions of immune tissue between the absorptive gut enterocytes (i.e., epithelial cells) containing high concentrations M-cells, B-cells, and T-cells. Due to the Peyer's patches potential to facilitate exposures of bioactive food components in the lumen to the immune cells, we examined alterations in gene expression of indicators of immune cell differentiation and activation.

Alterations in Gene Expression. RNA was extracted from the Peyer's Patches using the Trizol method [3](Life Technology). The integrity of the RNA was assessed using spectrometry and gel electrophoresis. cDNA was then synthesized, and qRT-PCR was performed to examine key genes

of interest related to pro-inflammatory, anti-inflammatory, and T-cell differentiation functions in this tissue. Gene expression of the anti-inflammatory mediator, interleukin (IL)-10, was upregulated in the cherry supplemented group compared to the Sham (P<0.0286) and tended to be increased compared to the OVX mice (**Figure 2A**). No statistically significant differences in IL-4 were observed due to OVX or diet (*data not shown*). In contrast, OVX increased transforming growth factor (TGF)-β expression, a key regulator of T-cell differentiation, but with tart cherry supplementation TGF-β was decreased (**Figure 2B**). This reduction in TGF-β expression partially normalized to the Sham mice. Signal transducer and activator of transcription (STAT3), which is downstream of TGF-β and involved in T-cell regulation, was upregulated with OVX compared to the Sham mice (**Figure 2C**). Tart cherry supplementation further upregulated STAT3 expression (p=0.0347). However, STAT5β, which is also downstream of TGF-β, was not significantly altered by either OVX or dietary treatment (*data not shown*).

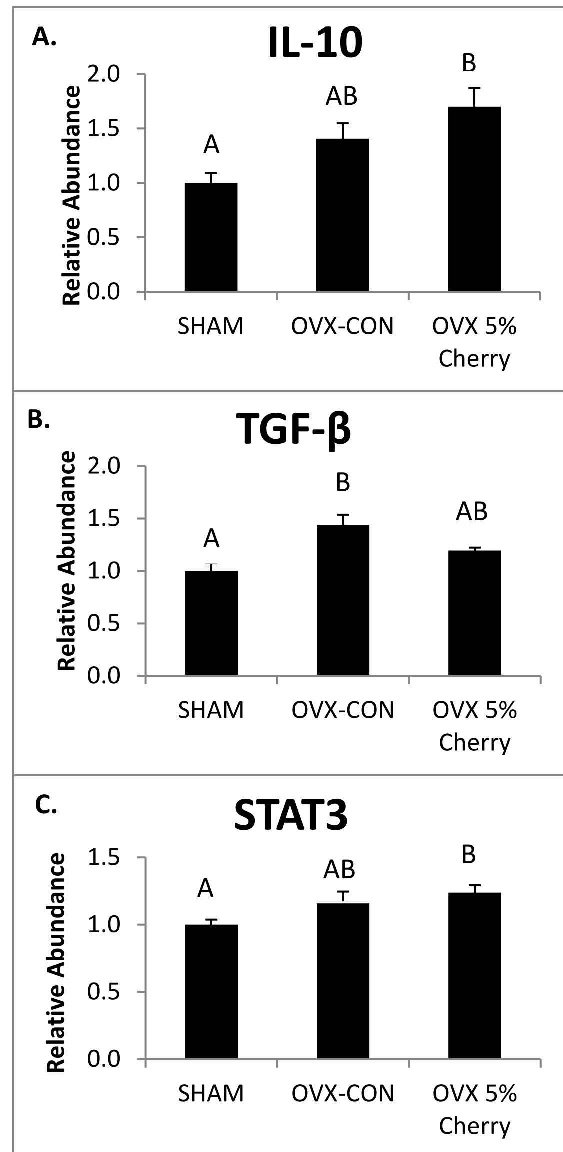

Figure 2. Alterations in gene expression in (A) IL-10, (B) TGF-β, and (C) STAT3 in the Peyer's patches tissues. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other (*p<0.05)*.

Summary of Findings Related to Aim 1: These findings indicate that cherry supplementation is capable of altering inflammatory gene expression in an estrogen deficient model by increasing anti-inflammatory gene expression as well as normalizing genes involved in regulating T-cell differentiation. The data support the hypothesis that supplementing the diet with foods rich in phenolic compounds such as tart cherry can favorable alter the gut mucosal immune response.

Aim 2:

Extraction of Tart Cherry Phenolics. Total polyphenolics were extracted from dried Montmorency tart cherries with a series of extractions in 80% ethanol combined with

sonication. The crude extract was then purified on a C-18 cartridge to remove excess carbohydrates. The purified sample was then roto-evaporated and re-suspended in Millipore water. The final extract was then filtered in a 0.22 µm filter prior to cell culture. The Folin-Ciocalteu assay [4] was employed to quantify the total phenolics and to ensure phenolic content was not lost during the purification process.

Cell Culture Experiments. Caco-2 cells (ATCC), derived from a human colon adenocarcinoma, were used as a model of gut epithelial cells. For each of the following experiments, these cells were cultured using Eagle's Minimum Essential Medium (EMEM; ATCC) supplemented with 20% fetal bovine serum (FBS)(Invitrogen) and 1% penicillin/streptomycin antibiototic (Sigma Aldrich) solution. Cells were grown at 37°C with 5% CO2/95% air-humidified atmosphere. Sub-culturing was accomplished prior to cells reaching 80% confluence, using 0.25% trypsin–EDTA (Sigma Aldrich).

Experiment 1--Gut Epithelial Cell Proliferation. Preliminary MTT assays were conducted on Caco-2 cells to determine the effects of phenolic compounds extracted from tart cherry powder and lipopolysaccharide (LPS; *E. coli,* Sigma Aldrich) on cell growth and proliferation. Cells were plated at 20,0000 cells/well and cultured for 24, 48 or 72 hours. At each time point, the medium was aspirated and cells were washed twice (250ul PBS). Next, 200ul incomplete EMEM was added along with 50ul of MTT to each well. Following a 4 hour incubation at 37°C the media were aspirated, 200 uL DMSO and 25 uL glycine buffer were added and the plate read immediately at 570nm. Doses of phenolic compounds (**Figure 3**) were selected based on the literature and final doses to be used in these experiments were determined based on MTT results (i.e., no observed negative effect on cell proliferation). Final doses of phenolics used in subsequent experiments ranged from 0-10 µg/mL.

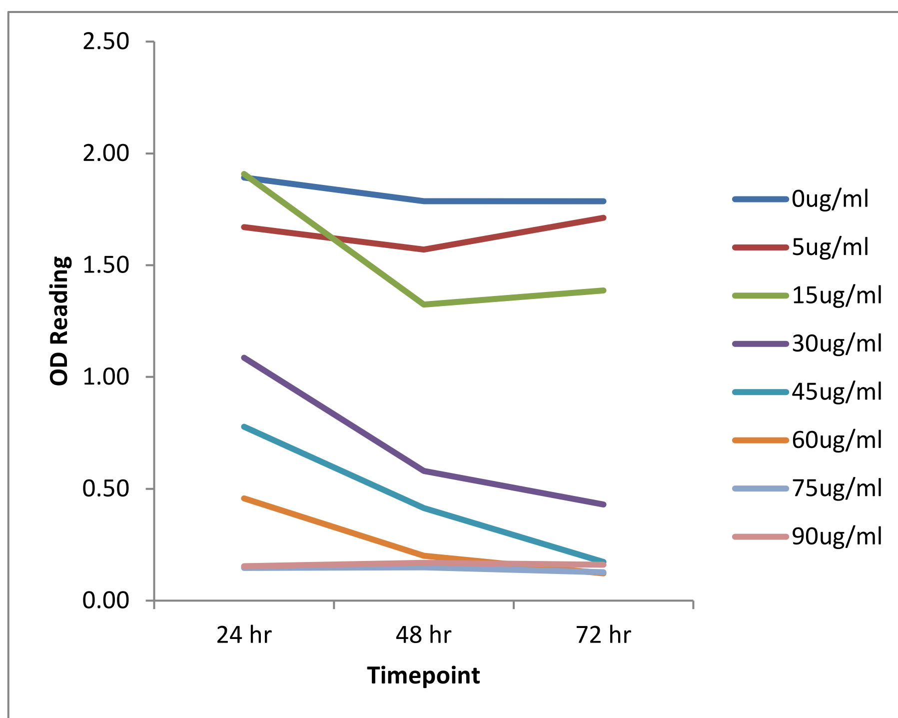

Figure 3. MTT results from Caco-2 cells treated with doses of tart cherry phenolics (0-90 µg/mL).

Experiment 2—Dose-response Study of Phenolic Compounds on Caco-2 Cells. To determine the effects of the cherry phenolic under inflammatory conditions (i.e., LPS) on Caco-2 cells, alterations in gene expression and protein abundance associated with pro-inflammatory and anti-inflammatory mediators as well as T-cell differentiation were quantified. Cells were plated at 400,000 cells/well into 24 well plates (n=24 wells/treatment group). Media were changed every two days. After approximately 9 days in culture, Caco-2 cells had formed a confluent monolayer and were then treated with the purified cherry phenolics (PP; 0, 2.5, 5, and 10 ug/ml) under normal or inflammatory conditions (LPS 0 or 1ug/ml).

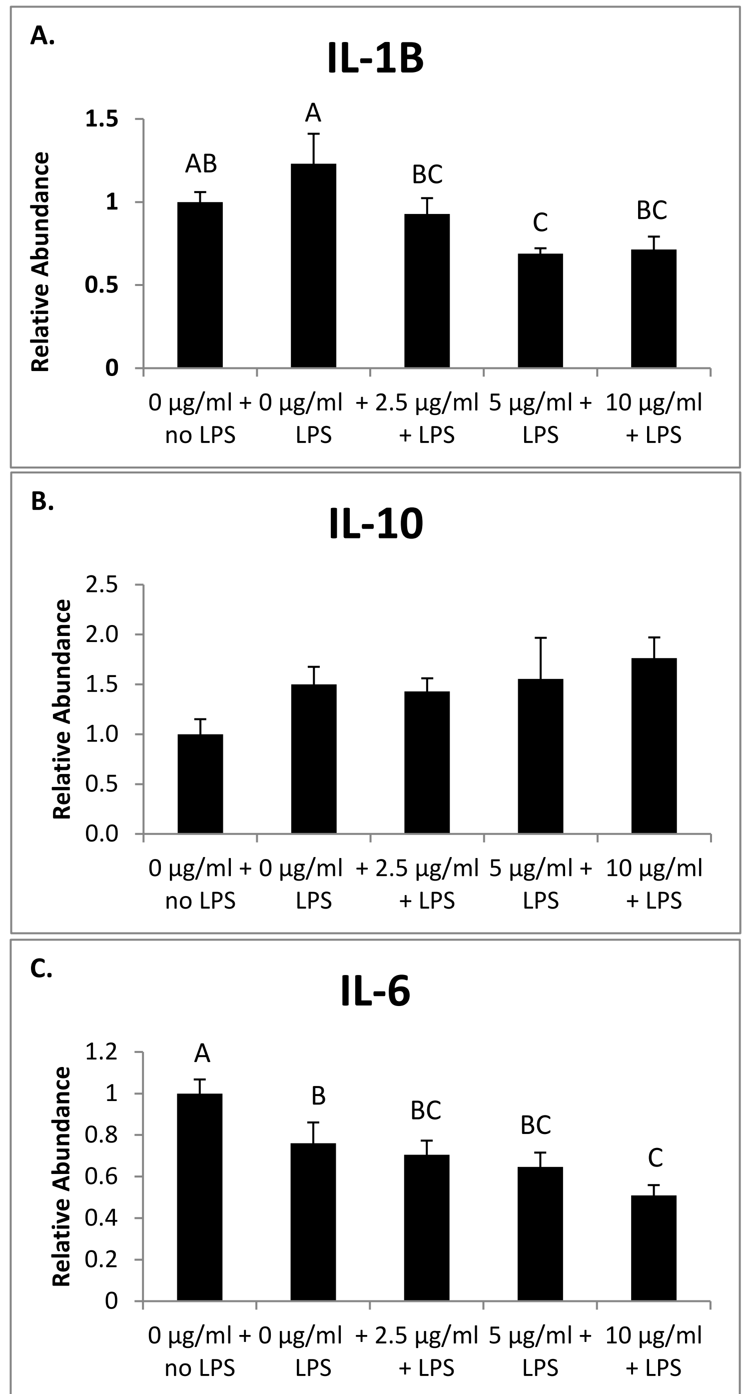

Figure 4. Effects of cherry phenolic compounds under inflammatory conditions (LPS=1 µg/mL) on the relative abundance of RNA for (A) IL-1β, (B) IL-10 and (C) IL-6. Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other (*p<0.05)*.

Alterations in Gene and Protein Expression. After 24 hours of treatment, RNA and protein were extracted. The RNA extraction with Trizol followed the same procedure described previously on the mouse tissues (Aim 1) and stored until cDNA synthesis and qRT-PCR were performed. Protein was extracted using RIPA buffer, and concentration was quantified by Bradford assay.

Phenolic compounds at all doses evaluated down-regulated IL-1β ($p < 0.01$), a pro-inflammatory cytokine, in the LPS-treated Caco-2 cells (**Figure 4A**). However, the anti-inflammatory cytokine, IL-10, was not altered by LPS or the phenolic compounds at the doses used in this experiment(**Figure 4B**). It should be noted that IL-10 abundance was low for all treatments. IL-6 which is known to have

both pro- and anti- inflammatory effects was suppressed with LPS. The highest dose of the phenolic extract (10 μg/mL) further reduced the relative abundance of IL-6 in Caco-2 cells under inflammatory conditions (**Figure 4C**).

Two important regulators of T-cell differentiation include transforming growth factor (T GF)-β and SMAD family member (SMAD2). The relative abundance TGF-β RNA was decreased with LPS treatment and the phenolic extract at all doses failed to alter this response (**Figure 5A**). SMAD2 , which is downstream of TGF-β, responded similarly to the LPS and phenolics (**Figure 5B**).

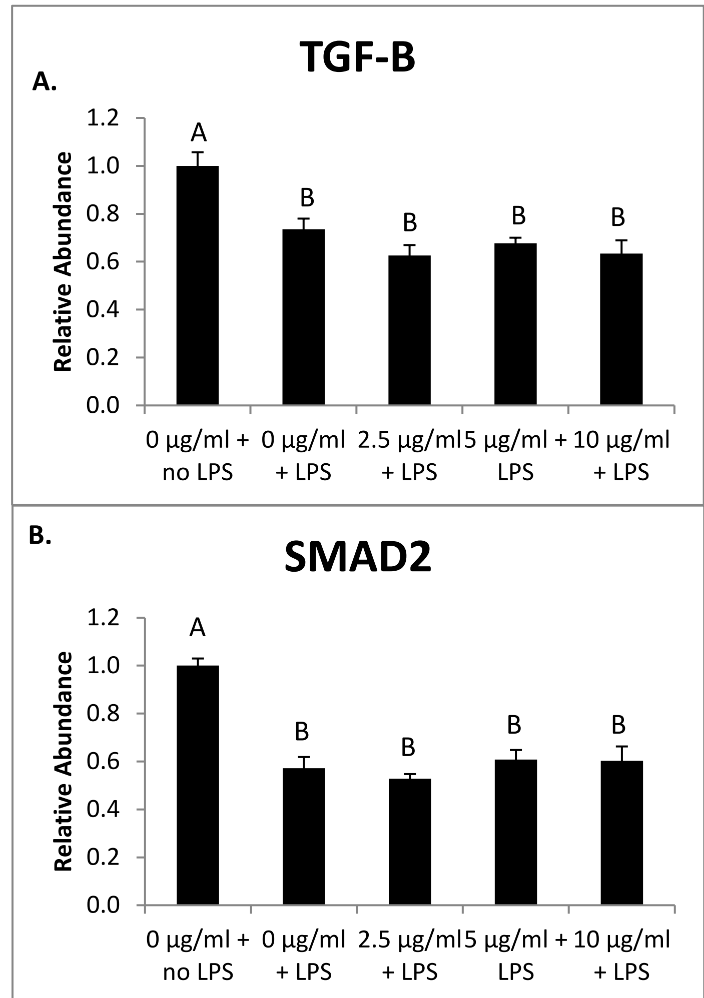

Figure 5. Influence of cherry phenolic compounds on the relative abundance of RNA for (A) TGF-β and (B) SMAD2 under inflammatory conditions (LPS=1 μg/mL). Bars represent the mean ± SE. Bars that do not share the same superscript letter are statistically different from each other (*$p<0.05$)*.

Experiment 3—Gut Epithelial Cell Response and Its Influence on T cells. CACO-2 cells were cultured as previously described and then treated with cherry extract (0, 2.5, 5, or 10 μg/mL) for 24 hrs. The medium was then removed and fresh medium was added. Conditioned medium was collected after 24 hours of treatment. Jurkat T-lymphocytes (ATCC) were plated at 500,000 cells/well into 12 well plates with RPMI 1640 media supplemented with 10% FBS and 1% pen/strep. The Jurkat cells were incubated overnight and then the next day they were treated with 15% or 30% conditioned media from the Caco-2 cells. After 6 hours, cells were activated using a PMA/Ionomycin cocktail in DMSO (10 ng/mL & / 0.5uM; Sigma Aldrich) or vehicle only (n=6wells/ treatment group). All DMSO final concentrations were kept below 0.1% per well. Cell culture supernatant was collected and aliquoted 24 hours post stimulation with conditioned media for future interferon-gamma (IFN-γ) ELISA (R&D Systems) analysis.

Summary of findings related to Aim 2. The *in-vitro* data show phenolic compounds extracted from tart cherry decreased the inflammatory response as indicated by reduced IL-1β gene expression by Caco-2 cells stimulated with LPS. No effect was observed due to the cherry

phenolic extract on the anti-inflammatory cytokine IL-10. However, these data only reflect changes in transcription, and should be confirmed with protein assays. Interestingly, the response of IL-6 was unanticipated. Due to this cytokines potential for pro- or anti-inflammatory effects, further experiments are needed to understand the role of IL-6 in this system. The transcriptional changes in TGF-β and SMAD2 raise the question of whether or not the epithelial cell response to the phenolics will alter or influence T-cell activation and differentiation. Additional studies will be required to confirm if dietary phenolic compounds' effects on gut epithelial cells alter the gut mucosal immune response.

Suggested Future Work:

Future work will include the evaluation of the protein response in the pro- and anti-inflammatory mediators as well as key regulators of T-cell differentiation in samples collected from both the animal and cell culture studies. These analyses will be used to confirm changes observed transcriptionally. Conditions for Experiment 3 need to be optimized and may require repeating the cell culture experiments. Prior to the completion of the overall study, PCR analysis will be performed on another gut associated lymphoid tissue(GALT), the lamina propria, and histological evaluation of the colon and small intestine will be completed.

Publication Plans:

These data, in part, were presented in a paper in the biomedical category during the 2015 OSU Research Symposium and at the 2016 Experimental Biology Meeting in San Diego, California. Additionally, the findings of this study will be combined with metagenomic data into a manuscript to be submitted to a nutrition or biomedical journal. The anticipated submission date for the manuscript is spring 2017.

References

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