Medicinal lavender modulates the enteric microflora to protect against *Citrobacter rodentium*-induced colitis


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Abstract

Inflammatory Bowel Disease, inclusive of Crohn’s Disease and Ulcerative Colitis, are immunologically mediated disorders involving the microbiota in the gastrointestinal tract. Lavender oil is a traditional medicine used to relieve many gastrointestinal disorders. The goal of this study was to examine the therapeutic effects of the essential oil obtained from a novel lavender cultivar, *Lavandula x intermedia* cv Okanagan Lavender (OLEO) in a mouse model of acute colitis caused by *Citrobacter rodentium*. Colitic mice orally gavaged with OLEO resulted in less severe disease including decreased morbidity and mortality, reduced intestinal tissue damage, and decreased infiltration of neutrophils and macrophages with reduced levels of TNF-α, IFN-γ, IL-22, MIP-2α and iNOS expression. This was associated with increased levels of regulatory T cell populations compared to untreated colitic mice. Recently, we demonstrated that the composition of the enteric microbiota affects susceptibility to *C. rodentium*-induced colitis. Here, we found that oral administration of OLEO induced a microflora enriched with members of the phyla Firmicutes including Segmented Filamentous Bacteria known to protect against the damaging effects of *C. rodentium*. Additionally, during infection, OLEO treatment promoted the maintenance of microflora loads with specific increases of bacteria from the phyla Firmicutes and decreases in γ-Proteobacteria. We observed that the Firmicutes was intimately associated with the apical region of the intestinal epithelial cells during infection suggesting their protective effect was through contact of the gut wall. Finally, we show that OLEO inhibited *C. rodentium* growth and adherence to Caco-2 cells primarily through the activities of 1,8-cineole and borneol. These results indicate that while OLEO promoted Firmicute populations it also controlled pathogen load through anti-microbial activity. Overall, our results reveal that OLEO can protect against colitis through the microbial and immunity nexus and reports that a pharmacological agent, in this case OLEO, alters the normal enteric microflora.

Key words: enteric microbiota, *C. rodentium*-induced colitis, colonic inflammation, monoterpenes, inflammatory bowel disease
Introduction

Inflammatory Bowel Disease (IBD), inclusive of Crohn’s Disease and Ulcerative Colitis, are immunologically mediated disorders involving the microbiota in the gastrointestinal tract. IBD are a major health burden in the West (11, 35) and Canada has the highest prevalence and incidence of IBD in the world (8) with more than 200,000 people living with IBD with over 9,200 new cases diagnosed each year (11). As permanent cure from IBD is still not possible, most patients require constant medication and / or surgery to keep the disease under remission (11). One of the few therapeutic options is to target TNF-α because it plays a role in causing damage in the GI tract through its pro-inflammatory responses. However, TNF-α is required for protection of the mucosa against noxious stimuli (49, 66), which makes constant blockage of this cytokine deleterious for long-term therapy (12). Indeed, anti-TNF-α therapy increases the risk of infection and lymphoma (9, 42, 43). Thus, with current therapeutic strategies either risky or ineffective for long-term use, new therapies or methods for prevention of IBD remains a priority for reducing disease burden in North America.

Lavender (Lavandula spp.) essential oils (LEO) have been used for centuries to treat numerous ailments including acute and chronic intestinal symptoms (10, 15, 26, 27) due to its antimicrobial (10, 48) anti-inflammatory (29, 63) and anti-allergy (38) properties. The therapeutic strength of LEO is determined by the properties of their constituents, primarily the monoterpenes like 1, 8-cineole, borneol and linalool / linalool acetate which are anti-inflammatory in nature (33, 53, 57). While these constituents have been studied and shown to have beneficial properties in isolation, their synergistic effects on colitis were previously undetermined. We have produced a novel lavender cultivar, Lavandula x intermedia cv Okanagan Lavender which produces a unique blend of essential oil (OLEO: Okanagan Lavender Essential Oil) enriched with two of the major therapeutic components: 1,8-cineole and borneol (14). We tested the efficacy of OLEO in a murine model of acute colitis.

A robust and commonly used model of acute colitis, appropriate for investigations of bacterial-induced inflammation, exploits the attaching/effacing (A/E) murine bacterial pathogen, Citrobacter rodentium. C. rodentium is used to model infections by the human specific enteric bacterial pathogens, enteropathogenic Escherichia coli (EPEC) and enterohemorrhagic E. coli (EHEC). C. rodentium infects the apical region of the colonic epithelium of mice, interacting
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directly with the innate immune system and is characterized by Th1 and Th17 responses with neutrophil and macrophage infiltration, crypt cell hyperplasia, mucodepletion and barrier disruption resulting in acute colitis (6, 21-24, 37, 40). Recently, we demonstrated that the composition of the enteric microbiota affects susceptibility to \textit{C. rodentium}-induced colitis (20). In this study, we examined the effect of OLEO on the enteric microflora and intestinal immune responses during \textit{C. rodentium}-induced colitis. We found that colitic mice orally gavaged with OLEO resulted in less severe disease including decreased morbidity and mortality, reduced intestinal tissue damage, decreased infiltration of neutrophils and macrophages, reduced levels of cytokines, chemokines and inflammatory mediators and increased levels of regulatory T cell populations compared to untreated colitic mice. We show that oral administration of OLEO induced a microflora that was enriched with members of the phyla Firmicutes including Segmented Filamentous Bacteria (SFB) known to protect against \textit{C. rodentium}-induced colitis. Additionally, during infection, OLEO treatment promoted the maintenance of microflora loads with enriched populations of Firmicutes closely associated with the gut cell wall which corresponded with less systemic pathogen translocation measured \textit{in vivo}. OLEO helped control pathogen load through inhibition of \textit{C. rodentium} growth shown \textit{in vitro} microbial growth assays primarily through the effects of 1,8-cineole, and decreased adherence to intestinal epithelial cells shown with Caco-2 cell cultures primarily through the effects of 1,8-cineole and borneol. These results suggest that OLEO decreases damaging intestinal inflammatory responses through pro-commensal and anti-pathogen mechanisms and may be a potential therapeutic agent for colonic inflammation. These finding reveal that a pharmacological agent, like OLEO can alter enteric microflora which can affect enteric disease susceptibility.

\textbf{Materials and Methods}

\textit{Lavender essential oil and purified constituents}

Wild type \textit{Lavandula x intermedia} was used to develop \textit{Lavandula x intermedia} cultivar Okanagan lavender by somatic mutagenesis as previously reported (14). The essential oil was extracted from either the wild type (LEO) or the mutant (OLEO) by steam distillation, using a modified a Likens-Nickerson type apparatus. Briefly, 500 g of flowers were boiled in de-ionized water for 45 minutes, and the vaporized essential oil constituents were condensed using a standard water-cooled condenser into a round bottom flask. Essential oil samples were kept in
dark glass bottles at 4 °C until used as a 1 mg/ml solution. Essential oil composition was established by gas-chromatograph mass spectrophotometry analysis described previously (14) and reported in table 1. Purified constituents 1,8-cineole (Sigma; cat# C80601), camphor (Fluka; cat# RA10260) and borneol (Fluka; cat# 13580) were stored in dark glass bottles at 4 °C until used as 1 M solutions.

*Mice*

Six- to eight-week-old C57BL/6 and C3H/HeOuJ mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in sterilized, filter-topped cages, handled in tissue culture hoods and fed autoclaved food and water under specific pathogen-free conditions in animal facilities at the Child and Family Research Institute in Vancouver. Sentinel animals were routinely tested for common pathogens. The protocols used were approved by the University of British Columbia’s Animal Care Committee and in direct accordance with guidelines drafted by the Canadian Council on the Use of Laboratory Animals.

*Bacterial strains, infection of mice and OLEO treatment*

Mice were infected by oral gavage using ~2.5 X 10^8 colony forming units (CFU) of *C. rodentium* DBS100 wild-type for 5-10 days. Modified from previously published literature orally administering lavender oil extracts (5), we gave a 50 mg/kg/day dose starting at 30 min post-infection throughout the infection. In the absence of infection for microbial analysis in C57BL/6 mice, OLEO was gavaged at 50 mg/kg/day for a total of 5 days. OLEO was diluted in mineral oil or mineral oil was gavaged alone as the control group. Mice were euthanized at indicated time points throughout infection and tissues were prepared for the analyses described below.

*Survival and body weight measurement*

Mice were monitored for morbidity throughout the infection and any that showed terminal symptoms such as dehydration and a hunched posture along with limited movement, piloerection and shaking, and/or severe weight loss (more than 20% of pre-infection body weight) were euthanized. Mice were weighed immediately prior to infection and at specified time intervals until day 10 p.i. Body weight data are presented as the mean percentage of the starting weight of each mouse at each time point, whereas survival data are presented as the percentage of the initial mice still surviving at each time point.
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**Tissue collection and bacterial counts**

Tissue collection and bacterial counts were performed as previously described (37). Briefly, mice were euthanized over the course of infection as indicated, dissected and their large intestines including the cecum were collected in 10% neutral buffered formalin (Fisher) for histological analyses, or processed for tissue pathology assays. For viable cell counts, the spleen and mesenteric lymph nodes were collected from C3H/HeOuJ mice, and homogenized in PBS pH 7.4, with dilutions plated onto LB agar plates.

**Histopathological scoring**

To assess tissue pathology, paraffin-embedded intestinal tissue sections (3 µm) that had been stained with haematoxylin and eosin were examined by two blinded observers and scores were averaged. Sections from six mice per group were assessed for submucosal edema (0 = no change; 1 = mild; 2 = moderate; 3 = profound), goblet cell depletion (scored based on numbers of goblet cells/high-power field averaged from five fields at 400X magnification where 0 = > 50; 1 = 25–50; 2 = 10–25; 3 = < 10), epithelial hyperplasia (scored based on percentage above the height of the control where 0 = no change; 1 = 1–50%; 2 = 51–100%; 3 = > 100%), epithelial integrity (0 = no change; 1 = < 10 epithelial cells shedding per lesion; 2 = 11–20 epithelial cells shedding per lesion; 3 = epithelial ulceration; 4 = epithelial ulceration with severe crypt destruction) and white blood cell infiltration (0 = < 5 WBC/high-power field; 1 = 5 to 20 WBC/high-power field; 2 = 21 to 60/high-power field; 3 = 61 to 100/high-power field; and 4 = >100/high-power field). The maximum score that could result from this scoring was 17, with scores ranging between 6 and 8 reflecting typical responses to *C. rodentium* infection, while scores > 8 reflect exaggerated tissue damage.

**Immunofluorescence**

Paraffin-embedded tissue sections were deparaffinized and rehydrated using standard techniques. For macrophage and neutrophil analysis, antigen retrieval of rehydrated tissues was performed using a 1 mg trypsin (Sigma) tablet dissolved in 1 mL of water for 20-30 minutes at room temperature. The tissue was washed and non-specific sites were blocked with 5% Bovine Serum Albumin (BSA; Sigma) followed by incubation with primary rat monoclonal antibody made against F4/80 for macrophages (Cedarlane Laboratories), rabbit polyclonal antibody made against myeloperoxidase for neutrophils (Neomarkers Thermofisher) or rabbit polyclonal
antibody made against FOXP3 (Santa Cruz) for regulatory T cells followed by secondary antibody (Goat-anti-Rat, labeled with green fluorescent Fluor 488) or (Goat-anti-Rabbit IgG, Dylite Fluor 594 labeled-Red). The tissue sections were mounted with Prolong® Gold antifade reagent containing 4’,6’-diamidino-2-phenylindole (DAPI; Invitrogen) and visualized with a Nikon Eclipse E800 microscope equipped with a Nikon digital camera DXM1200 operating through Nikon ACT-1 Version 2.20 software.

**RNA and DNA extractions and quantitative real-time PCR**

For RNA extractions, following euthanization of mice, colonic tissues were immediately transferred to RNA-later (Qiagen) and stored at -20°C. Total RNA was purified using Qiagen RNEasy kits (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad) using 1 μg RNA. For DNA extractions, following euthanization of mice, colonic tissues were homogenized and the bacterial genomic DNA was extracted using a Qiagen stool kit (Qiagen) according to the manufacturer’s instructions. Quantification of DNA was performed on a Bio-Rad CFX Manager 2.0 machine using Sso Fast Eva Green Supermix (Bio-Rad). All qPCR reactions were performed in duplicate in a volume of 10 µl, using high-profile white tubes and ultra clear sealing tapes (Bio-Rad). Using cDNA or 50 ng/µl of bacterial DNA, PCR was conducted with cycling conditions of 95°C for 30 s and 39 cycles of 95°C for 5 s followed by 55-60°C for 5 s and ending with 95°C for 10 s followed by a melt curve (65°C to 95°C, in increments of 0.5°C for 5 s). All primers were synthesized by Integrated DNA Technology (IDT), Canada. Primer efficiencies were verified according to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines. GAPDH, iNOS, TNF-α, mMIP-2α, mMCP-1, IFN-γ (22, 37), IL-10 (1) and IL-22 primers (72) used in this study were previously described. The specificity of bacterial primers used (Table 2) was verified using the insillico PCR database (http://insilico.ehu.es/) and the 16S RNA Ribosomal Database (http://rdp.cme.msu.edu/index.jsp). For Segmented Filamentous Bacteria (SFB), we used NCBI primer blast tool against Candidatus Arthomitus sp. SFB-mouse-Japan, complete genome (GenBank-AP012202.1) to design primers to amplify the region from 186099 to 186298 of the 16S rRNA gene. Expression of GAPDH was used as a reference for gene expression analysis whereas relative values for bacterial groups were normalized to total bacteria present amplified using a Eubacterial probe. Quantification of gene expression was
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carried out using CFX manager software version 1.6.541.1028 (Bio-Rad) where PCR efficiencies for each of the primer sets were incorporated into the final calculations.

**Fluorescent in situ hybridization and SYBR green DNA staining**

Fluorescent *in situ* hybridization (FISH) and SYBR analysis was performed as previously described (20, 41). Briefly, colonic luminal contents were weighed, homogenized and stored in 3.7% formalin at 4°C until use. For SYBR Green DNA staining, samples were filtered onto Anodisc 25 filters (Whatman International Ltd) with a pore size of 0.2 µm and 2.5 cm diameter. After complete drying, each sample was stained with SYBR green I nucleic acid gel stain (Invitrogen). For FISH, samples were filtered onto a polycarbonate membrane filter (Nucleopore Track-Etch Membrane, Whatman International Ltd), dehydrated and incubated overnight at 37°C in hybridization solution (0.9 M NaCl, 0.1 M TRIS pH 7.2, 30% Formamide, 0.1% SDS) containing a combination of 3 Texas-Red conjugated probes covering several members of the Firmicutes phyla [LGC354a: 5’-TGGAAAGATTCCCTACTGC-3’; LGC354b: 5’CGGAAGATTCCCTACTGC 3’; LGC354c: 5’CGGAAGATTCCCTACTGC 3’] (46, 54), Texas-Red conjugated Bacteriodes probe (CFB286: 5’-TCCTCTCAGAACCCCTAC-3’), FITC-labeled γ-Proteobacteria probe (GAM42a: 5’-GCCTTCCCACATCGTTT-3’), in combination with FITC or Texas-Red labeled Eubacterial probe (EUB338: 5’-GCTGCCCTCCCCTAGGAGT-3’). Sequence specificity of CFB and GAM (69), as well as EUB (2) and LGC (30) probes was verified through the 16S RNA Ribosomal Database or the probeCheck database (http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home). After incubation, the filters were washed in hybridization solution followed by wash buffer (0.9 M NaCl, 0.1 M TRIS pH 7.2). Filters were dried and mounted on glass slides using ProLong Gold® Antifade (Invitrogen) and viewed as above where 4-6 fields per disc were randomly chosen and the number of cells counted and averaged. Total microbiota load was calculated by averaging the number of SYBR-positive cells / g of tissue weight accounting for total filter area of 2.01 cm², picture dimensions of 5.622 x 10² and 100 X objective magnification. Percentages of Firmicutes, Bacteroidetes, and γ-Proteobacteria were calculated based on the number of positively-probed bacteria divided by the number of positive DAPI stained bacterial nuclei and averaged over several fields of view. Similar to Bergstrom *et al.*, (7) FISH was performed on tissue sections by deparaffinizing and rehydrating paraffin-embedded tissue sections and incubating overnight at 37°C in the dark with
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the FIRM and EUB probes. Sections were washed with hybridization solution, wash buffer and dH₂O and mounted and viewed as described above.

**In vitro antimicrobial plate and culture assays**

For plate assays, *C. rodentium* culture (100 μl of 0.8 OD) was used to inoculate LB agar by spread plating. Fisherbrand filter paper (qualitative P8, porosity: course, flow rate: fast) was cut into disks with a 8-10 mm diameter, sterilized and inoculated with 20 μl of either LEO, OLEO, mineral oil, 1,8-cineole, borneol or camphor. Disks were place in the center of the plates and incubated @ 37°C for 18 hours. Zones of inhibition were measured and averaged. For culture assays, Caco-2 cells (ATCC) were grown at 37°C and 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/liter D-glucose, 1 x nonessential amino acids, L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (Sigma). The cells were seeded at high density in polystyrene T25cm² culture flasks or 24-well plates and used for experiments after becoming ~ 80% confluent. Wells were infected with *C. rodentium* at a MOI of 30 for 4 hrs in the presence of OLEO, LEO, 1,8-cineole, borneol, camphor or mineral oil and adherence assays were performed as previously reported (36). Briefly, cells were then washed with warm DMEM three times and scraped into warm phosphate-buffered saline (PBS) and mixed by pipetting. Serial dilutions were performed, and aliquots of the scraped cells were streaked on Lb agar plates and incubated at 37°C for 18 hrs. Bacterial colonies (CFU) were counted and averaged.

**Statistical analysis**

All the results are expressed as the mean value with standard error of the mean (SEM). Non-parametric Mann-Whitney *T*-tests were performed using GraphPad Prism version 4.00 for Windows. (GraphPad Software, San Diego, CA, USA, [http://www.graphpad.com](http://www.graphpad.com)).

**Results**

*OLEO decreases mortality and morbidity during C. rodentium-induced colitis*

Some studies have provided *in vitro* evidence for anti-microbial (10, 48) and anti-inflammatory (29, 63) properties of LEO while other studies have found that single components isolated from
LEO are anti-colitic in vivo (33, 57). We have produced a novel cultivar of lavender high in two of the therapeutic monoterpenes, 1,8-cineole and borneol (Table 1; (14). To determine if OLEO had therapeutic value against colitis, we tested its efficacy in a mouse model of infectious colitis using *C. rodentium*. As disease progression and prognosis in this model is highly dependent on the mouse strain used, we tested two strains of mice for this study. We orally gavaged either OLEO or mineral oil (vector only) in C57BL/6 mice which develop a self-resolving colitis with little infection-induced mortality, as well as highly susceptible C3H/HeOuJ mice which suffer 100% infection-induced morbidity (67). We found that OLEO could protect against infection induced morbidity and mortality in these mice (Figure 1). C3H/HeOuJ infected mice orally gavaged with OLEO suffered modest levels of mortality with 1/3 requiring euthanization by day 9 p.i. (Figure 1A). This was in contrast to infected mice gavaged with mineral oil exhibiting 100% infection-induced mortality requiring euthanization between day 6 and day 7 p.i. In C57BL/6 mice infected with *C. rodentium*, the body weight loss was significantly decreased when the mice were treated OLEO compared to mineral oil alone (Figure 1B). This suggested that OLEO rescued susceptible C3H/HeOuJ mice from lethal colitis and reduced infection-associated morbidity in resistant C57BL/6 mice.

**OLEO protects against severe cecal mucosal damage during *C. rodentium* infection**

Normally, C57BL/6 mice endure moderate inflammation and intestinal pathology characterized by increased crypt height, white blood cell infiltration, goblet cell depletion and the disruption of the epithelial integrity during *C. rodentium* infection (21). *C. rodentium* starts colonization of the cecal tissues and is disseminated from the cecum to the distal colon and both cecal and distal colonic tissues are equally colonized and damaged (21-23). To determine if OLEO could reduce the tissue damage associated with acute colitis, we examined both the caecal and distal tissues of mice taken from infected treated or infected control groups for assessment through histopathological scoring. As shown in Figure 2, the C57BL/6 mice orally gavaged with vector only (10 days p.i.) developed the above-described injuries in cecal tissues. In contrast, the C57BL/6 mice orally gavaged the OLEO developed little to no cecal damage and was similar to the uninfected healthy ceca. The averaged total scores for infected mice (untreated = 9.01 vs. OLEO treated = 2.98) suggest that cecal damage due to intestinal inflammation caused by *C. rodentium* is decreased when mice are treated with OLEO. Unexpectedly, we found that the...
distal colon was similarly damaged in treated and control mice (data not shown) suggesting that OLEO had a specific effect in the cecum but not the colon.

**OLEO decreased C. rodentium-induced immune responses**

*C. rodentium* induced colitis is characterized by production of pro-inflammatory cytokines and chemokines (16). To evaluate the effect of OLEO on the production of inflammatory mediators during *C. rodentium* induced colitis, RNA extracted from cecal tissues of C57BL/6 mice was assessed by qPCR for the expression of genes known to be involved during infection-induced inflammation. We found that OLEO treated mice express significantly reduced levels of iNOS, IFN-γ, IL-22 and MIP-2α mRNA compared to infected control mice (Figure 3A). No significant difference was seen in expression of IL-10 between the groups. Correspondingly, there was decreased phagocytic cell infiltration in the OLEO-treated mice (Figure 3B). Immunofluorescent imaging showed decreased recruitment of macrophages and neutrophils in cecal tissues in *C. rodentium* infected mice treated with OLEO (Figure 3B). In contrast, many F4/80-positive macrophages and myeloperoxidase-positive neutrophils were recruited to the submucosa in infected C57BL/6 mice that were gavaged the vector only (10 days p.i.). To determine if regulatory T cell infiltration was also modulated with OLEO treatment during infection, we examined regulatory T cells via immunofluorescence using an antibody specific to FOXP3 (Figure 4C). We found that while *C. rodentium* infection inhibits the presence of infiltrating FOXP3+ T-regulatory cells, OLEO-treated mice maintained cecal FOXP3+ T-regulatory cells during infection at the level of uninfected mice. These results reveal that treatment with OLEO during acute colitis reduces damage-associated inflammation and maintains protective T-regulatory cells.

**OLEO modulates the enteric microflora**

The intestinal microflora is an important factor implicated in the susceptibility of colitis (19, 20, 52). We have previously shown that strain-dependent variation in susceptibility to *C. rodentium* is regulated by the intestinal microflora (20). To determine if OLEO modulated the ecology of the enteric microflora promoting resistance to acute colitis, we examined various bacterial groups previously implicated in gut health (Table 2) prior to infection in OLEO-treated and untreated mice. We orally gavaged either OLEO or mineral oil (vector only) in C57BL/6 mice and excised their cecal tissues, extracted bacterial DNA and used specific primers to amplify
relative levels of microbes via qPCR. Bacteroidetes and Firmicutes are the two predominating phyla in the gut with \( \gamma \)-Proteobacteria normally present in less than 5%. We found that OLEO modulated several enteric bacteria from the Firmicutes phyla (Figure 4). Segmented filamentous bacteria (SFB), \textit{Clostridia sp}, and \textit{Eubacterium rectale} were significantly increased in the cecums of OLEO treated mice whereas several other enteric microbes including \textit{Bacillus sp}, \textit{Lactobacillus sp}, and the \textit{Clostridium coccoides} group were unchanged. As well, we did not detect differences in the other dominant phyla in the gut such as members of the Bacteroidetes or Proteobacteria. Of particular importance, SFB has been shown to protect against \textit{C. rodentium}-induced colitis (32).

\textit{C. rodentium} decreases the total microbial load enriching \( \gamma \)-Proteobacteria and decreasing members from the phyla Bacteroidetes (41). To determine if OLEO treatment during \textit{C. rodentium} infection maintained total bacterial loads and altered the major phyla of colonic bacteria, we used SYBR green staining for total quantification and FISH since Lupp \textit{et al} 2007 (41) reported that during \textit{C. rodentium} infection, differences between samples were significant at the phylum level. Our results show that OLEO promotes the maintenance of the total microflora loads during infection (Figure 5A) and had significantly lower \( \gamma \)-Proteobacteria levels compared to the mineral oil control group (Figure 5B). While Bacteroidetes quantity was not different, the Firmicutes phyla were found in significantly higher quantity in OLEO treated mice compared to infected mice alone (Figure 5B). Since several members of the Firmicutes phyla are considered protective (50) and we observed that several Firmicutes were enriched prior to infection with OLEO treatment (Figure 4), we examined their proximal relationship with the intestinal epithelium during infection (Figure 5C). We observed that the Firmicutes had an intimate association with the apical region of the intestinal epithelial cells suggesting any protective effect was potentially through contact of the gut wall. This was different to untreated colitic mice whose gut wall had very little associated Firmicutes probe attached. This suggested that OLEO may selectively modulate the Firmicutes population and promote their association with the gut wall out-competing the \( \gamma \)-Proteobacteria, most of which is presumably \textit{C. rodentium}.

\textbf{OLEO inhibits systemic infection of \textit{C. rodentium}}

To determine if enteric microflora alterations and reduced colitis were associated with decreased \textit{C. rodentium} loads we assessed bacterial loads \textit{in vivo} in the C3H/HeOuJ mice orally gavaged
with the mineral oil and in the C3H/HeOuJ mice orally gavaged with OLEO. The spleen and mesenteric lymph nodes (MLN) were removed from infected mice immediately following euthanization for homogenization and plating of the tissue homogenates to determine colony forming units (CFU). The infected mice orally gavaged with the OLEO showed decreased systemic \textit{C. rodentium} (Figure 6). Bacterial translocation or systemic disease and total bacterial load is correlative in \textit{C. rodentium} colitis (6, 7, 20-24, 37, 67). This is likely because \textit{C. rodentium} is luminal and does not invade cells but rather attaches and effaces the intestinal epithelial cells and when these cells are eroded, like in ulcers, the pathogen crosses, becomes systemic and also proliferates forming biofilm-like structures with logs more bacteria in susceptible mice (21). Since OLEO inhibited systemic disease, our results suggest that OLEO may interfere with \textit{C. rodentium} adherence to the epithelium or growth.

\textit{OLEO inhibits adherence and growth of C. rodentium in vitro}

To assess whether OLEO or the purified constituents of OLEO could prevent \textit{C. rodentium} adherence, we used an \textit{in vitro} assay similar to Khan \textit{et al} (36) where cultured Caco-2 cells where infected with \textit{C. rodentium} and also incubated with OLEO, LEO and the individual constituents that are higher in OLEO and reported to have therapeutic activity (Table 1: borneol, 1,8-cineole), as well as camphor. The cells were infected with \textit{C. rodentium} and adherence was determined as previously described (36). We found that OLEO had significantly less adherent \textit{C. rodentium} than LEO (Figure 7A). We found that both 1,8-cineole and borneol but not camphor were important in blocking adherence since both of these purified constituents had significantly less adhered \textit{C. rodentium} compared to mineral oil and similar levels as to OLEO. Since \textit{C. rodentium} has been reported to not bind Caco-2 cells (65), we also performed this assay using Enteropathogenic \textit{Escherichia coli} (EPEC) which is human specific and binds Caco-2 cells and found similar trends (data not shown). These results suggest that the primary constituents involved in protecting intestinal epithelial cells against pathogen adherence are 1,8-cineole and borneol, the two constituents that are higher in OLEO compared to LEO (Table 1).

To assess whether OLEO had anti-microbial activity against \textit{C. rodentium}, plate-killing assays were carried out to determine the zones of inhibition. Plates inoculated with \textit{C. rodentium} and treated with disks infused with OLEO showed a zone of inhibition with a diameter of 24 ± 0.7 mm (Figure 7B). For comparisons, we examined LEO finding a statistically similar zone of
inhibition at 22 ± 1.2 and that 1,8-cineole was the only constituent that had anti-microbial activity. In isolation however, 1, 8-cineole was not as effective as the oil extracts at 2.7 fold less inhibition. In contrast, plates treated with disks alone or disks infused with mineral oil, camphor or borneol were completely overgrown with *C. rodentium* showing no zone of inhibition. We also found that OLEO inhibited the growth of *C. rodentium* using nutrient broth growth assays (data not shown). These results suggest that a combination of constituents in OLEO synergistically produce the greatest anti-microbial activity.

**Discussion**

Current treatment options to manage colitis include immunosuppressors, anti-TNF-α therapy and corticosteroids. These treatment options have significant side effects like increased susceptibility to infection and cancer (28). There is a great need for safer and more effective treatment options. In this study, we examined *Lavandula x intermedia* cultivar Okanagan Lavender essential oil (OLEO) for its potential protective effects during murine *C. rodentium*-induced colitis. Our study shows that oral gavage of OLEO results in an attenuated colitis as confirmed by its effects on morbidity, mortality, histology and inflammatory responses. The protective mechanism of OLEO treatment was associated with modulation of the enteric microflora selectively increasing several members of the Firmicutes phyla such as SFB and inhibition of *C. rodentium* growth and adherence to intestinal epithelial cells *in vitro* and systemic infection *in vivo*. This study reports that OLEO can protect against murine infectious colitis. If this holds true for humans as well, OLEO could be a potential therapeutic strategy against intestinal inflammation.

We have recently shown that the microbiota plays a critical role in disease susceptibility to acute infectious colitis (20). In this study, we found that therapeutic treatment with OLEO protected against infectious colitis and this was associated with selective increases of several members of the Firmicutes bacterial phyla including SFB, *Clostridium sp.*, and *Eubacteria rectale*. SFB has been shown to protect against *C. rodentium*-induced colitis through Th17 immune responses (32). Although further investigation is required, it is possible that OLEO’s enrichment of SFB is modulating Th17 immunity and contributing to the protection against *C. rodentium*-induced colitis. During infection, the OLEO treated group was able to maintain total bacterial loads which was enriched with Firmicutes and depleted of γ-Proteobacteria, presumably most of which was *C. rodentium*. In support of this, OLEO treatment resulted in less systemic pathogen load
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and OLEO was found to inhibit *C. rodentium* growth and adherence to intestinal epithelial cells *in vitro*. While we did not address the mechanism by which OLEO enriched bacteria from the Firmicutes phyla and simultaneously prevented growth and attachment of *C. rodentium*, it is feasible that OLEO through 1,8-cineole and borneol interfere with pathogen adherence by blocking or masking the pathogen adherence antigens or host binding sites. Additionally, the Firmicutes could be protecting against *C. rodentium* colonization which could lead to decreased intestinal injury due to decreased inflammatory responses. In fact, several studies have shown that changes and reduction in the proportion of specific phylogenetic groups of Firmicutes are lower in patients with active IBD compare to healthy subjects (18, 34, 44, 58, 61, 64). In support of this, we found that Firmicutes bacteria were closely associated with the gut mucosal surface during infection which could help promote colonization resistance. Some studies have shown that some Firmicutes protect against intestinal inflammation through the synthesis of butyrate which is an important energy source for epithelial cells and involved in epithelial cell differentiation and wound healing (56, 61). Another study found that the diversity of butyrate producers like *Clostridium coccoides* groups and *Eubacterium rectal* were lower in samples from Ulcerative Colitis patients corresponding to lower levels of butyryl-CoA transferase, responsible for butyrate production (68). While we didn’t find a significant difference between the major butyrate producers like the *Clostidium coccoides* group, we did find other butyrate producers *Eubacterium rectale* and other members of *Clostridia sp* were enriched with OLEO treatment. Further research is required to determine if OLEO-induced enteric microflora alters butyrate production. In summary, microbial modulation through OLEO treatment has a significant impact on intestinal immune responsiveness to colitis and could well represent the means through which other pharmacological agents effect gastrointestinal responsiveness, similarly seen with nonsteroidal anti-inflammatory drug-induced intestinal injury through dysbiosis (70).

Colitis is associated with neutrophil infiltration into intestinal crypts, attracted via the chemokine macrophage inflammatory protein 2 alpha (MIP-2α), which can lead to tissue damage due to the constant release of inflammatory cytokines, proteases and reactive oxygen species (47). Thus, blocking the activity of neutrophils or decreasing the expression of MIP-2α, is a potential therapeutic strategy against colitis (47). OLEO-treated infected mice had attenuated responses from pro-inflammatory mediators including MIP-2α with corresponding decreased neutrophil infiltration. Additionally, macrophages cause tissue damage by promoting pro-
inflammatory and cytotoxic activities due to the release of reactive oxygen and nitrogen species and inflammatory cytokines such as TNF-α (39). OLEO-treated infected mice had attenuated macrophage infiltration and responses from pro-inflammatory mediators including TNF-α, IFN-γ, and IL-22. Additionally, OLEO treatment resulted in a significant reduction in expression of nitric oxide synthase (iNOS), which can cause oxidative tissue damage. All of these responses have been shown to be important for host defense during *C. rodentium* infection (3, 25, 59, 60). These results are significant considering therapies for IBD that aim to inhibit the action of pro-inflammatory cytokines can reduce disease severity and/or disease progression (17). Finally, regulatory T cells play an important role in suppressing intestinal inflammatory responses and it has been shown that these cells suppress colitis and may be an efficacious therapy for IBD (51). While *C. rodentium* infection resulted in the loss of regulatory T cell populations, OLEO was able to maintain these populations during infection. Overall, OLEO treatment decreases damaging pro-inflammatory responses and promotes regulatory responses important for balanced GI immunity.

In conclusion, the large decrease in mortality seen in the susceptible mice as well as the significant decrease in morbidity, cecal tissue damage, pro-inflammatory cytokine production and immune cell infiltration seen in the more resistant mice, provides us with strong support that OLEO protects against infectious colitis. Protection was associated with modulation of the enteric microflora with selective increases in Firmicutes and anti-microbial and anti-adherence activities against *C. rodentium*. This corresponded with decreases in inflammatory and injurious host defensive responses and maintenance of T regulatory cells important for balancing gastrointestinal immunity. Our results reveal that OLEO has potential therapeutic effects against acute colitis through the microbial and immunity nexus. As demonstrated by these results and others (70), ingested drugs can modulate the enteric microflora, future studies should examine how other drugs could affect selective modulation of certain microbial populations that could either be detrimental or beneficial.

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Disclosures

None

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Figure 1. OLEO reduces mortality and morbidity during *C. rodentium* infection. A) *C. rodentium* infection in susceptible C3H/HeOuJ mice orally gavaged with vector only (mineral oil) induced greater mortality compared to the mice orally gavaged with OLEO. In this experiment, 100% of the mice orally gavaged with mineral oil required euthanization between days 6 and 7 p.i., whereas 1/3 mice orally gavaged with OLEO required euthanization on day 9 p.i in each of 3 separate experiments with one representative experiment shown here. Each point represents the percentage of surviving mice from an initial population of three mice. B) The body weights of resistant C57BL/6 mice orally gavaged either the vector or OLEO were followed over the first 10 days of a *C. rodentium* infection. Each datum point represents the average weight data pooled from at least 6 mice and is expressed as the percentage of the initial body weight with SEM. The body weight loss was significantly greater for the C57BL/6 mice orally gavaged the mineral oil compared with the mice orally gavaged with OLEO at 10 days p.i.. (*, *P* < 0.05)

Figure 2. OLEO treatment reduces cecal tissue damage during *C. rodentium* infection. C57BL/6 mice orally gavaged with either vector only (mineral oil) or OLEO were infected with *C. rodentium* and assessed for intestinal pathology at 10 days p.i.. A) Representative micrographs show that the cecal tissues isolated from infected mice orally gavaged the vector only were damaged in contrast to the ceca isolated from infected mice orally gavaged with OLEO (Magnification, 100X; staining, haematoxylin and eosin). B. Histopathological scoring of epithelial integrity, white blood cell infiltration, submucosal oedema, mucodepletion and hyperplasia in cecal tissues from C57BL/6 mice orally gavaged either the vector only or OLEO. The ceca from the mice orally gavaged the vector only scored significantly higher for damage than mice orally gavaged with OLEO. The mean scores are indicated by the bar graph with the
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SEM averaged from six mice. The asterisk denotes the significantly increased damage comparing the total scores from C57BL/6 mice orally gavaged the vector only with mice orally gavaged with OLEO at day 10 p.i. (*, \(P < 0.01\))

**Figure 3. OLEO treatment reduces intestinal pro-inflammatory responses while maintaining T-regulatory cell infiltration during *C. rodentium* infection.**

A) RNA extracted from cecal tissues of C57BL/6 mice was assessed by qPCR for the expression of cytokine genes TNF-\(\alpha\), IFN-\(\gamma\), IL-22, IL-10, MIP-2\(\alpha\) and iNOS. Tissues were collected at 10 days p.i. Expression of the genes for TNF-\(\alpha\), IFN-\(\gamma\), IL-22, MIP-2\(\alpha\) and iNOS were decreased in the mice orally gavaged with OLEO compared to the mice orally gavaged the vector only whereas there was no change in IL-10. Bars represent the averaged fold expression from 6 mice with SEM. Asterisk’s denote significantly increased fold expression of mRNA from infected mice orally gavaged the vector only compared to OLEO treatment. B) OLEO-treated mice showed a decrease in infiltrating macrophages and neutrophils during *C. rodentium* infection. Cecal tissue from infected C57BL/6 mice orally gavaged with OLEO or vector only were stained for the presence of F4/80-positive macrophages (shown in green), MPO-positive neutrophils (shown in red) and DAPI-stained for host cell nuclei (shown in blue). (100X magnification). C) *C. rodentium* infection inhibits the presence of infiltrating FOXP3+ T-regulatory cells while OLEO-treated mice maintained FOXP3+ T-regulatory cells during *C. rodentium* infection at the level of uninfected mice. Cecal tissue from infected C57BL/6 mice orally gavaged with OLEO or vector only were stained for the presence of FOXP3-positive cells and DAPI-stained for host cell nuclei followed by quantification in whole tissues pieces at 100X magnification. *C. rodentium* infection
resulted in significantly decreased levels of FOXP3 positive cells / tissue while OLEO treatment prevented this. (*, \( P < 0.05 \); ***, \( P < 0.001 \))

**Figure 4. OLEO treatment enriches several members of the Firmicutes phyla of the intestinal microflora.** Oral administration of OLEO induces a microflora that is enriched with members of the phyla Firmicutes including Segmented Filamentous Bacteria (SFB), *Clostridia* sp, and *Eubacterium rectale*. C57BL/6 mice were gavaged with OLEO at 50 mg/Kg/day for 5 days, sacrificed, intestines excised and DNA extracted for qPCR using specific primers to the 16S rRNA gene of bacterial species in the microbiota. Quantitative values are shown as relative values normalized to total bacteria present amplified using a Eubacterial probe. (**, \( P < 0.001 \); *, \( P < 0.05 \))

**Figure 5. During *C. rodentium* infection, OLEO treatment maintains total bacterial loads, with enriched populations of Firmicutes and depleted \( \gamma \)-Proteobacteria.** Intestinal samples from infected mice with or without OLEO treatment were homogenized, filtered onto membranes and quantified using SYBR green nucleic acid dye (A) or hybridized with probes specific to Firmicutes, \( \gamma \)-Proteobacteria, Bacteroidetes and Eubacteria for FISH analysis. (B) Membranes were examined via fluorescent microscopy to determine total bacteria by counting SYBR-positive cells or percentages of bacterial phyla from 5-10 random fields of view. Bars represent mean bacterial numbers with SEM. In A, asterisks denote significantly increased total bacterial populations of OLEO treated infected mice intestines compared infected mice intestines alone. In B, asterisks denote significantly increased Firmicutes and significantly decreased \( \gamma \)-
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Proteobacteria in OLEO treated infected intestines compared to infected mice intestines alone.

C) FISH analysis on tissues sections using the Firmicutes specific probe revealed increased interaction of Firmicutes (red) with the intestinal epithelial cells (nuclei stained in blue with DAPI) during OLEO treatment in infected mice compared to infected mice alone. A Eubacterial probe was also co-stained (green; 400X magnification). (*, P < 0.05).

**Figure 6. OLEO inhibits C. rodentium systemic disease.** Oral gavage of OLEO reduces the number of colony forming units (CFU) recovered from the A) mesenteric lymph nodes (MLN) and the B) spleen in susceptible C3H/HeOuJ mice during C. rodentium infection. Bacteria were enumerated at 10 days p.i. from homogenized tissues and plated on agar. (*, P < 0.05)

**Figure 7. OLEO inhibits C. rodentium adherence and growth in vitro.** A) OLEO prevents the adherence of C. rodentium to Caco-2 cells. OLEO had significantly lower adherence compared to LEO while 1,8-cineole and borneal but not camphor were shown to inhibit adherence to Caco-2 cells similarly as to OLEO. Caco-2 cells were grown to confluency and inoculated with C. rodentium in the presence of oil extract or purified constituents for 4 hours. Adherent C. rodentium were plated on nutrient agar in serial dilutions and CFU units were averaged and plotted. B) Anti-microbial activity against C. rodentium is seen with OLEO and LEO and 1,8-cineole which was significantly lower that OLEO. No antimicrobial activity was seen for borneol, camphor or the control mineral oil. C. rodentium was used to inoculate nutrient agar plates and a filter disk infused with the oil extract or purified constituent was incubated for 18 hr
at 37°C. Zone of inhibition (in mm) was measured from disks to visible growth of bacteria after incubation and the averaged values were plotted (**, \( P < 0.001 \); *, \( P < 0.05 \)).

**Table 1**: Concentration (in %) of the major constituents in wild type Lavandula x intermedia (LEO) and Lavandula x intermedia cv Okanagan Lavender (OLEO) essential oils (14).

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<tr>
<th>Constituent</th>
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<td>LEO</td>
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<tr>
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<tr>
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<td>Borneol</td>
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**Table 2**: Bacterial primers used in this study

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<tr>
<td>Lactobacillus sp.</td>
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<td></td>
</tr>
<tr>
<td>SFB</td>
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</tbody>
</table>

Reference numbers are given in parentheses when available.
A

Uninfected  10 days p.i.  10 days p.i. + OLEO

B

Histopathological Scoring

Uninfected  day 10 p.i.  day 10 p.i. + OLEO
Table 1: Concentration (in %) of the major constituents in *wild type Lavandula x intermedia* (LEO) and *Lavandula x intermedia* cv Okanagan Lavender (OLEO) essential oils (14).

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