Medicinal lavender modulates the enteric microbiota to protect against \textit{Citrobacter rodentium}-induced colitis


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Medicinal lavender modulates the enteric microbiota to protect against \textit{Citrobacter rodentium}-induced colitis. \textit{Am J Physiol Gastrointest Liver Physiol} 303: G825–G836, 2012. First published July 19, 2012; doi:10.1152/ajpgi.00327.2011.—Inflammatory bowel disease, inclusive of Crohn’s disease and ulcerative colitis, consists of 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, \textit{Lavandula \times intermedia} cultivar Okanagan lavender (OLEO), in a mouse model of acute colitis caused by \textit{Citrobacter rodentium}. In colitic mice, oral gavage 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, macrophage inflammatory protein-2α, and inducible nitric oxide synthase expression. This was associated with increased levels of regulatory T cell populations compared with untreated colitic mice. Recently, we demonstrated that the composition of the enteric microbiota affects susceptibility to \textit{C. rodentium}-induced colitis. Here, we found that oral administration of OLEO induced microbiota enriched with members of the phylum Firmicutes, including segmented filamentous bacteria, which are known to protect against the damaging effects of \textit{C. rodentium}. Additionally, during infection, OLEO treatment promoted the maintenance of microbiota loads, with specific increases in Firmicutes bacteria and decreases in \gamma-Proteobacteria. We observed that Firmicutes bacteria were intimately associated with the apical region of the intestinal epithelial cells during infection, suggesting that their protective effect was through contact with the gut wall. Finally, we show that OLEO inhibited \textit{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 promotes Firmicutes populations, it also controlled pathogen load through antimicrobial activity. Overall, our results reveal that OLEO can protect against colitis through the microbial-immunity nexus and that a pharmacological agent, in this case OLEO, alters the normal enteric microbiota.

enteric microflora; colonic inflammation; monoterpene; inflammatory bowel disease

INFLAMMATORY BOWEL DISEASE (IBD), inclusive of Crohn’s disease and ulcerative colitis, is an immunologically mediated disorder involving the microbiota in the gastrointestinal (GI) tract. IBD is a major health burden in the West (11, 35), and Canada has the highest prevalence and incidence of IBD in the world (8), with >200,000 people living with IBD and >9,200 new cases diagnosed each year (11). As a permanent cure from IBD is still not possible, most patients require constant medication and/or surgery to keep the disease in remission (11). One of the few therapeutic options is targeting of TNF-α, because it plays a role in causing damage in the GI tract through its proinflammatory 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, because current therapeutic strategies are risky or ineffective for long-term use, new therapies or methods for prevention of IBD remain a priority for reducing disease burden in North America.

Because of their antimicrobial (10, 48), anti-inflammatory (29, 63), and antiallergy (38) properties, lavender (\textit{Lavandula} spp.) essential oils (LEO) have been used for centuries to treat numerous ailments, including acute and chronic intestinal symptoms (10, 15, 26, 27). The therapeutic strength of LEO is determined by the properties of the constituents of LEO, primarily the monoterpenes, such as 1,8-cineole, borneol, and linalool/linalyl 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 have not been determined. We have produced a novel lavender cultivar, \textit{Lavandula \times intermedia} cultivar Okanagan lavender, which produces a unique blend of essential oil [Okanagan LEO (OLEO)] 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 bacteria-induced inflammation, exploits the attaching/effacing murine bacterial pathogen \textit{Citrobacter rodentium}. \textit{C. rodentium} is used to model infections by the human-specific enteric bacterial pathogens, enteropathogenic \textit{Escherichia coli} and enterohemorrhagic \textit{E. coli}. \textit{C. rodentium} infects the apical region of the colonic epithelium of mice, interacting directly with the innate immune system, and is characterized by T helper type 1 (Th1) and type 17 (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 microbiota and intestinal immune responses during \textit{C. rodentium}-induced colitis. We found that, in colitic mice orally gavaged with OLEO, disease was less severe: morbidity and mortality were decreased, intestinal tissue damage was reduced, infiltra-
tion of neutrophils and macrophages was decreased, levels of cytokines, chemokines, and inflammatory mediators were reduced, and levels of regulatory T cell populations were increased compared with untreated colitic mice. We show that oral administration of OLEO induced microbiota enriched with members of the phylum Firmicutes, including segmented filamentous bacteria (SFB), which are known to protect against C. rodentium-induced colitis. Additionally, during infection, OLEO treatment promoted the maintenance of microbiota loads with enriched Firmicutes populations closely associated with the gut cell wall, which corresponded with less systemic pathogen translocation measured in vivo. OLEO helped control the gut cell wall, which corresponded with less systemic growth with enriched Firmicutes populations closely associated with C. rodentium-induced colitis. Additionally, during infection, members of the phylum Firmicutes, including segmented fila-
tuded, and levels of regulatory T cell populations were in-
tation of neutrophils and macrophages was decreased, levels of 1,8-cineole and borneol. These results suggest that OLEO decreases damaging intestinal inflammatory responses through procommensal and antipathogen mechanisms and may be a potential therapeutic agent for colonic inflammation. These findings reveal that a pharmacological agent such as OLEO can alter enteric microflora, which can affect enteric disease susceptibility.

MATERIALS AND METHODS

LEO and purified constituents. Wild-type Lavandula × intermedia was used to develop OLEO by somatic mutagenesis, as previously reported (14). The essential oil was extracted from the wild-type (LEO) or the mutant (OLEO) by steam distillation using a modified Likens-Nickerson-type apparatus. Briefly, 500 g of flowers were boiled in deionized water for 45 min, 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 they were used as a 1 mg/ml solution. Essential oil composition was established by gas chromatography-mass spectrophotometry, as described previously (14) and reported in Table 1. The purified constituents 1,8-cineole (catalog no. C80601, Sigma), camphor (catalog no. RA10260, Fluka), and borneol (catalog no. 13580, Fluka) were stored in dark glass bottles at 4°C until they were used as 1 M solutions.

Mice. Six- to 8-wk-old C57BL/6 and C3H/HeOuJ mice (Jackson Laboratories, Bar Harbor, ME) 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 (Vancouver, BC, Canada). Sentinel animals were routinely tested for common pathogens. The protocols were approved by the University of British Columbia Animal Care Committee and were carried out 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 × 10⁸ colony-forming units (cfu) of C. rodentium DBS100 wild-type for 5–10 days. Using a modification of a procedure for oral administration of lavender oil extracts described elsewhere (5), we administered a 50 mg·kg⁻¹·day⁻¹ dose starting at 30 min postinfection and continued throughout the infection. In the absence of infection for microbial analysis in C57BL/6 mice, OLEO was gavaged at 50 mg·kg⁻¹·day⁻¹ 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 analysis.

Survival and body weight measurement. Mice were monitored for morbidity throughout the infection, and animals that showed terminal symptoms, such as dehydration and a hunched posture, along with limited movement, piloerection and shaking, and/or severe weight loss (>20% of preinfection body weight), were euthanized. Mice were weighed immediately prior to infection and at specified intervals until 10 days postinfection. Body weight data are presented as mean percentage of the starting weight of each mouse at each time point, whereas survival data are presented as percentage of the initial number of mice surviving at each time point.

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 and dissected, and the large intestine, including the cecum, was collected in 10% neutral buffered formalin (Fisher) for histological analysis 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 Luria-Bertani (LB) agar plates.

Histopathological scoring. For assessment of tissue pathology, paraﬃn-embedded intestinal tissue sections (3 μm) that had been stained with hematoxylin 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 (on the basis of the height of the control; 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 (WBC) infiltration (0 = <5 WBCs/high-power field, 1 = 5–20 WBCs/high-power field, 2 = 21–60 WBCs/high-power field, 3 = 61–100 WBCs/high-power field, 4 = >100 WBCs/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 and scores >8 reflecting 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 tablet (Sigma) dissolved in 1 ml of water for 20–30 min at room temperature. The tissue was washed, and nonspeciﬁc sites were blocked with 5% BSA (Sigma) and then incubated with primary rat monoclonal antibody made against F4/80 for macrophages (Cedarlane Laboratories), rabbit polyclonal antibody made against myeloperoxidase for neutrophils (Neomarkers, Thermo Fisher), or rabbit polyclonal antibody made against FOXP3 (Santa Cruz Biotechnology) for regulatory T cells and then with secondary antibody [goat anti-rat labeled with Fluor 488 (green) or goat anti-rabbit IgG labeled with Dylight Fluor 594 (red)]. Tissue sections were mounted with Prolong Gold Antifade reagent containing 4′,6′-di-
amidino-2-phenylindole (DAPI; Invitrogen) and visualized with a microscope (Nikon Eclipse E800) equipped with a digital camera.
RNA extractions, after the mice were euthanized, colonic tissues were homogenized, and the bacterial genomic DNA was extracted using a stool kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized with the iScript cDNA synthesis kit (Bio-Rad) using 1 ng of RNA. For DNA extractions, after the mice were euthanized, colonic tissues were homogenized, and the bacterial genomic DNA was extracted using a 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 quantitative PCRs (qPCRs) were performed in duplicate in a volume of 10 µl with use of high-profile white tubes and ultraclear sealing tapes (Bio-Rad). With use of 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, finally, 95°C for 10 s followed by a melt curve (from 65°C to 95°C in 0.5°C increments for 5 s). All primers were synthesized by Integrated DNA Technology. Primer efficiencies were verified according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. GAPDH, inducible nitric oxide synthase (iNOS), TNF-α, mouse macrophage inflammatory protein (MIP)-2α, mouse mast cell protease-1, IFN-γ (22, 37), IL-10 (1), and IL-22 (72) primers are described elsewhere. The specificity of bacterial primers (Table 2) was verified using the In Silico PCR database (http://insilico.ehu.es/) and the 16S rRNA database (http://rdp.cme.msu.edu/index.jsp). For SFB, we used National Center for Biotechnology Information primer blast tool against SFB, segmented filamentous bacteria. 

### Table 2. Bacterial primers

<table>
<thead>
<tr>
<th>Bacterial sp.</th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing Temperature, °C</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus sp.</td>
<td><strong>GCGGCGTCGCTAATAGATGC</strong></td>
<td><strong>CTTGATCTACGTCAGGCGGCT</strong></td>
<td>60</td>
<td>55</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td><strong>AYAGCCTTTTCGAAAAAAGAAG</strong></td>
<td><strong>CCGATCAACCTCAGCAATTTT</strong></td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>Bacteroides sp.</td>
<td><strong>GAGGAGAAGGTCCCGCCAG</strong></td>
<td><strong>GCGTACCTTGTCCGTTCAG</strong></td>
<td>60</td>
<td>55</td>
</tr>
<tr>
<td>Clostridia sp.</td>
<td><strong>GCTGCTAACTCCCCGTATGTC</strong></td>
<td><strong>GACAGCGGAGCTCATCAGA</strong></td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td>Clostridium cocoidei sp.</td>
<td><strong>AAAGCCCCTAATACGACTTCATA</strong></td>
<td><strong>CTTGGAGTTTCTTCCGCCAA</strong></td>
<td>60</td>
<td>45</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td><strong>CTCCACGAGCCGGGCTAA</strong></td>
<td><strong>GCGTACGGCAGCAACTCTCAAG</strong></td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td><strong>CCCTATTGTGATGTCATCATT</strong></td>
<td><strong>ACTGTTGTACTCCGAGT</strong></td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>Eubacterium rectale</td>
<td><strong>AATCTCAGGGAGGACG</strong></td>
<td><strong>AAGGCTGACATCACATTCG</strong></td>
<td>60</td>
<td>31,71</td>
</tr>
<tr>
<td>Eubacteria</td>
<td><strong>CCTTGGGACTGTGCAACTTTG</strong></td>
<td><strong>TTCAACGGCATCCATC</strong></td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>Lactobacillus sp.</td>
<td><strong>CGGAGCATGGAATCTTCCA</strong></td>
<td><strong>GCGGCTGACTTGAAGCTC</strong></td>
<td>55</td>
<td>Present study</td>
</tr>
</tbody>
</table>

SFB, segmented filamentous bacteria.
dilutions were performed, and aliquots of the scraped cells were streaked on LB agar plates and incubated at 37°C for 18 h. Bacterial colonies (cfu) were counted and averaged.

Statistical analysis. Values are means ± SE. Nonparametric Mann-Whitney t-tests were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA; http://www.graphpad.com).

RESULTS

OLEO decreases mortality and morbidity during C. rodentium-induced colitis. Some studies have provided in vitro evidence for antimicrobial (10, 48) and anti-inflammatory (29, 63) properties of LEO, while other studies have reported that single components isolated from LEO are antiocolitic in vivo (33, 57). We have produced a novel cultivar of lavender high in two of the therapeutic monoterpenes, 1,8-cineole and bornanol (Table 1) (14). To determine if OLEO had therapeutic value against colitis, we tested its efficacy in a mouse model of C. rodentium-induced infectious colitis. As disease progression and prognosis in this model are highly dependent on the mouse strain, we tested two strains of mice. We orally gavaged 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), with OLEO or mineral oil (vector only). We found that OLEO could protect against infection-induced morbidity and mortality in these mice (Fig. 1). Infected C3H/HeOuJ mice orally gavaged with OLEO suffered modest levels of mortality, with one-third of the animals requiring euthanization by 9 days postinfection (Fig. 1A), in contrast to infected mice gavaged with mineral oil, which exhibited 100% infection-induced mortality and required euthanization between 6 and 7 days postinfection. Body weight loss was significantly decreased in C. rodentium-infected C57BL/6 mice treated with OLEO compared with mineral oil alone (Fig. 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, WBC infiltration, goblet cell depletion, and disruption of 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 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 the cecal and distal tissues of infected treated or infected control mice for assessment by histopathological scoring. As shown in Fig. 2, the C57BL/6 mice orally gavaged with vector only (10 days postinfection) developed the above-described injuries in cecal tissues. In contrast, cecal tissue of the C57BL/6 mice orally gavaged with OLEO developed little to no damage and was similar to cecal tissue of the uninfected healthy mice. The averaged total scores for infected mice (9.01 and 2.98 for untreated and OLEO-treated mice, respectively) 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 proinflammatory 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. OLEO-treated mice express signifi-
OLEO modulates the enteric microbiota. The intestinal microflora is an important factor in the susceptibility of colitis (19, 20, 52). We previously showed 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 microbiota, 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 C57BL/6 mice with OLEO or mineral oil (vector only) and excised 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; γ-Proteobacteria is normally present at <5%. We found that OLEO modulated several Firmicutes enteric bacteria (Fig. 4). SFB, Clostridium spp., and Eubacterium rectale were significantly increased in the ceca of OLEO-treated mice, whereas several other enteric microbes, including Bacillus spp., Lactobacillus spp., and the Clostridium cococeus group, were unchanged. Nor did we detect differences in the other dominant phyla in the gut, such as members of Bacteroidetes or Proteobacteria. Of particular importance, SFB have been shown to protect against C. rodentium-induced colitis (32).

C. rodentium decreases the total microbial load, enriching γ-Proteobacteria and decreasing members from the phylum Bacteroidetes (41). To determine if OLEO treatment during 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 et al. (41) reported that, during C. rodentium infection, differences between samples were significant at the phylum level. OLEO promoted the maintenance of the total microflora loads during infection (Fig. 5A), and γ-Proteobacteria levels were significantly lower in the OLEO-treated than the mineral oil control group (Fig. 5B). While Bacteroidetes quantity was not different, the Firmicutes quantity was significantly greater in OLEO-treated than untreated infected mice (Fig. 5B). Since several Firmicutes are considered protective (50) and we observed that OLEO treatment enriched several Firmicutes prior to infection (Fig. 4), we examined their proximal relationship with the intestinal epithelium during infection (Fig. 5C).
observed an intimate association of Firmicutes with the apical region of the intestinal epithelial cells, suggesting that any protective effect was potentially through contact with the gut wall. This was different from untreated colitic mice, whose gut wall showed very little attachment of associated Firmicutes probe. This suggested that OLEO may selectively modulate the Firmicutes population and promote their association with the gut wall, outcompeting the \( \text{H9253} \)-Proteobacteria, most of which are presumably \( C. \text{rodentium} \).

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

OLEO inhibits adherence and growth of \( C. \text{rodentium} \) in vitro. To assess whether OLEO or the purified constituents of OLEO could prevent \( C. \text{rodentium} \) adherence, we used an in vitro assay similar to that used by Khan et al. (36). Cultured...
Caco-2 cells where infected with *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, and 1,8-cineole), as well as with camphor. The cells were infected with *C. rodentium*, and adherence was determined as previously described (36). We found significantly less adherent *C. rodentium* in OLEO- than LEO-treated cells (Fig. 7A). We found that 1,8-cineole and borneol, but not camphor, were important in blocking adherence, since *C. rodentium* adherence was significantly less (and similar to adherence in OLEO-treated cells) in cells treated with both of these purified constituents than in cells treated with mineral oil. Since it has been reported that *C. rodentium* does not bind Caco-2 cells (65), we also performed this assay using enteropathogenic *E. coli*, 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, which are present in higher levels in OLEO than LEO (Table 1).

To assess whether OLEO had antimicrobial activity against *C. rodentium*, plate-killing assays were carried out to determine the zones of inhibition. Plates inoculated with *C. rodentium* and treated with disks infused with OLEO showed a 24 ± 0.7 mm diameter zone of inhibition (Fig. 7B). For comparison, in disks infused with LEO, we found a statistically similar 22 ± 1.2 mm diameter zone of inhibition, and we found that 1,8-cineole was the only constituent with antimicrobial 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. Using nutrient broth growth assays, we also found that OLEO inhibited the growth of *C. rodentium* (data not shown). These results suggest that a combination of constituents in OLEO synergistically produce the greatest antimicrobial activity.

**DISCUSSION**

Current treatment options to manage colitis include immunosuppressors, anti-TNF-α therapy, and corticosteroids. Because these treatment options have significant side effects, such as increased susceptibility to infection and cancer (28), there is a need for safer and more effective treatment options. In this study, we examined OLEO for its potential protective effects during murine *C. rodentium*-induced colitis. Our study shows that oral gavage with 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 microbiota, selectively increasing several Firmicutes members, 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 recently showed that the microbiota play 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 in several Firmicutes bacteria, including SFB, Clostridium spp., and E. 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 were 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, and OLEO inhibited C. rodentium growth and adherence to intestinal epithelial cells in vitro. While we did not address the mechanism by which OLEO enriched Firmicutes bacteria and simultaneously prevented growth and attachment of C. rodentium, it is feasible that OLEO through 1,8-cineole and borneol interferes with pathogen adherence by blocking or masking the pathogen adherence antigens or host binding sites. Additionally, Firmicutes could be protecting against C. rodentium colonization, which could lead to decreased intestinal injury due to decreased inflammatory responses. Several studies have shown that changes and reduction in the proportion of specific phylogenetic groups of Firmicutes are lower in patients with active IBD than healthy subjects (18, 34, 44, 58, 61, 64). In support of this, we found that Firmicutes bacteria were closely associated with the gut...

Fig. 5. During C. rodentium infection, OLEO treatment maintains total bacterial loads, with enriched Firmicutes and depleted γ-Proteobacteria populations. 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, γ-Proteobacteria, and Bacteroidetes for fluorescent in situ hybridization (FISH) analysis, with Eubacteria used to probe the total number of bacteria in each sample (B). Membranes were examined via fluorescent microscopy to determine total bacteria by counting SYBR Green-positive cells or percentages of bacterial phyla from 5–10 random fields of view. Values are means ± SE. *P < 0.05 vs. 10 days pi. C: FISH analysis of tissue sections using the Firmicutes-specific probe revealed increased interaction of Firmicutes (red) with intestinal epithelial cells (nuclei stained blue with DAPI) during OLEO treatment in infected mice compared with untreated infected mice. A Eubacterial probe was also costained (green). Original magnification ×400.
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 is involved in epithelial cell differentiation and wound healing (56, 61). Another study found less diversity of butyrate producers, such as *Clostridium coccoides* groups and *E. rectale*, in samples from ulcerative colitis patients, corresponding to lower levels of butyryl-CoA transferase, which is responsible for butyrate production (68). While we did not find a significant difference between the major butyrate producers, such as the *C. coccoides* group, we did find that other butyrate producers, such as *E. rectale* and other members of *Clostridia* spp., 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 affect GI responsiveness, as 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 MIP-2α, which can lead to tissue damage as a result of 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 proinflammatory mediators, including MIP-2α, with corresponding decreased neutrophil infiltration. Additionally, macrophages cause tissue damage by promoting proinflammatory and cytotoxic activities due to the release of reactive oxygen and nitrogen species and inflammatory cytokines such as TNF-α (39). In OLEO-treated infected mice, macrophage infiltration and responses from proinflammatory mediators, including TNF-α, IFN-γ, and IL-22, were attenuated. Additionally, OLEO treatment resulted in a significant...
reduction in expression of iNOS, which can cause oxidative tissue damage. All these responses have been shown to be important for host defense during C. rodentium infection (3, 25, 59, 60). These results are significant, inasmuch as therapies for IBD that aim to inhibit the action of proinflammatory 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 proinflammatory responses and promotes regulatory responses important for balanced GI immunity.

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

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