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Blend of organic acids and medium chain fatty acids prevents the inflammatory response and intestinal barrier dysfunction in mice challenged with enterohemorrhagic *Escherichia coli* O157:H7

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ABSTRACT

Impaired epithelial barrier function disrupts immune homeostasis and increases inflammation in intestines, leading to many intestinal diseases. The blend of organic acids (OAs) and medium chain fatty acids (MCFAs) has been shown to have synergistic bactericidal effect. In this study, we demonstrated that two blends of OAs and MCFAs (OM1 and OM2) could prevent the inflammatory response and intestinal barrier dysfunction in enterohemorrhagic *Escherichia coli* (EHEC)-infected mice. Treatments of OM1 and OM2 significantly reduced the body weight loss and production of IL-6 and TNF-α induced by EHEC. Mice treated with OM1 and OM2 showed decrease in serum D-lactic concentration, DAO activity and bacterial transfer to liver and spleen. Furthermore, OM1 and OM2 increased the expression of tight junction proteins occludin and ZO-1, mucus protein MUC-2, and host defense peptides mBD1, mBD2 and mBD3. Finally, OM1 and OM2 increased the population of *Lactobacillus* spp. and *Bifidobacterium* spp., but decreased that of *E. coli* in the cecum. These findings indicate that OM1 and OM2 may be used to develop a prophylactic agent for intestinal inflammation and injury in enteric pathogen infection.

1. Introduction

Intestinal barrier is a single layer of epithelial cells between the lumen and mucosal tissues, which contributes to nutrient absorption and protection against bacterial and toxin invasion [1,2]. The integrity of the epithelial barrier depends on tight junction (TJ) between adjacent epithelial cells [3,4]. TJ dysfunction is associated with increase in intestinal permeability, which eventually leads to bacterial and toxin invasion and influences intestinal health [5–7]. Additionally, the mucins and antimicrobial proteins that cover the surface of intestinal epithelium are the primary defense against pathogen attachment and invasion of epithelial cells [8]. Impaired epithelial barrier function disrupts immune homeostasis and increases inflammation in the intestine, which is associated with many intestinal diseases, such as inflammatory bowel disease (IBD) and enteric pathogen infection [4,9]. Thus, improving the functions of intestinal barrier may be a new target for disease prevention and therapy.

Medium chain fatty acids (MCFAs) are a family of saturated fatty acids, including caprylic acid (C8:0), capric acid (C10:0), and lauric acid (C12:0) [10]. They are naturally present in food, such as coconut oil, palm kernels, cow milk, and human breast milk [10–12]. MCFAs have been demonstrated to have antimicrobial properties against various pathogens such as *E. coli* O157:H7 [13]. MCFAs can relieve *P. acnes*-induced mouse ear swelling and granulomatous inflammation and protect IPEC-J2 against cyclophosphamide-induced barrier injury [14,15]. Organic acids (OAs), the natural compounds present in various foodstuffs and produced by some microorganisms [16], have bactericidal effects against *Escherichia coli* O157:H7 [13]. The combined use of MCFAs and OAs at low concentrations has a synergistic bactericidal effect [13]. The blend of OAs and MCFAs improves the growth performance and reduces diarrhea in weanling piglets orally challenged with enterotoxigenic *Escherichia coli* K88 [17]. Enterohemorrhagic *Escherichia coli* (EHEC) is an enteric pathogen that leads to non-bloody diarrhea mediated by intestinal epithelial barrier disruption during

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weaning period [4]. Therefore, the objective of the present study was to assess the effect of the blend of OAs and MCFAs on the inflammatory response and epithelial barrier function in the intestine of EHEC-infected mice.

2. Materials and methods

2.1. Reagents

OM1 was blends of organic acids and even-numbered medium-chain fatty acids. OM2 was blends of organic acids and odd-numbered medium-chain fatty acids. TRIzol reagent (Invitrogen, USA) and SYBR Green master mix (BIO-RAD, USA) were used to extract the RNA and for RT-qPCR analysis. Rabbit polyclonal Abs for zonula occludens (ZO)-1, occludin, MUC-1, MUC-2 and β-actin were from Affinity Bioreagents (Colorado, USA). The secondary Abs used for the immunostaining were goat anti-rabbit IgG labeled with FITC and Cy3, respectively (Abcam, USA). EHEC O157:H7 ATCC43889 was provided by Dr. WY. Zheng (Zhejiang University).

2.2. Animals

Three-week-old female C57BL/6 mice were obtained from the Hubei Research Center of Laboratory Animals (China). The mice were individually housed and maintained on a 12:12 h light-dark cycle under specific pathogen-free conditions. The mice from control groups and EHEC groups were fed basal diet with no supplementation, whereas the animals from Enro groups were fed basal diet supplemented with 0.01% enrofloxacin, and OM1 and OM2 groups were fed basal diet supplemented with 0.6% OM1 and 0.56% OM2 respectively after the 3-d equilibration period. After 3 days of feeding with corresponding diets, the mice were orally administered 0.1 mL PBS (control groups) or 0.1 mL PBS containing 1×10^8 CFU of EHEC O157:H7 once a day for 3 days. After the oral EHEC administration, the mice were euthanized and the tissues were collected.

2.3. Intestinal morphology

The middle jejunum of the mice was collected and fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5-mm thickness were deparaffinized in xylene and stained with H&E. Images were obtained using a DM3000 microscope (Leica Microsystems, Wetzlar, Germany). The villous height and crypt depth of the jejunum were measured with Image-Pro software (Media Cybernetics, Rockville, MD).

2.4. Relative quantification in real-time PCR

Total RNA was extracted from the jejunum tissue samples using Trizol reagent (Invitrogen, USA). The RNA was transcribed into cDNA by the first strand cDNA synthesis kit (TOYOBO, Japan). Real-time PCR was performed using the Bio-Rad CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA). Primers for real-time PCR in this study were shown in Table 1. Each reaction system included 5 μL of Fast Start Universal SYBR green master mix (BIO-RAD, USA), 0.3 μL of the forward primer (10 μM), 0.3 μL of the reverse primer (10 μM) and 4.4 μL of cDNA (diluted 20-fold). After denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 10 s and 60 °C for 35 s were performed. The melting curves were analyzed to evaluate the specificity of the PCR products. All samples were analyzed in triplicate, and the mean value was used. The *β-actin* gene was used as housekeeping gene. The 2-ΔΔCt method was used to determine the relative mRNA expression levels.

2.5. Inflammation and gut permeability assessment

To analyze the levels of IL-6, TNF-α, D-lactic (D-LA) and diamine oxidase (DAO) in serum, the corresponding IL-6, TNF-α, D-LA and DAO ELISA kits (Bio Value, U.K.) were used according to the manufacturer's instructions.

Table 1
Primer sequences for RT-qPCR.

Gene	Primer sequence	Annealing temperature (°C)
zo-1	F:TCATCCCAAATAAGAACAGAGC R:GAAGAACAACCCTTTCATAAGC	52
occludin	F:AAGCAAGTGAAGGGATCTGC R:GGGGTTATGGTCCAAAGTCA	54
mucin-1	F:TGGATTGTTTCTGCAGATTTT R:CCTGACCTGAACTTGATGCT	58
mucin-2	F:CCCAGAAGGGACTGTGTATG R:TGCAGACACACTGCTCACA	65
mBD1	F:TGCCTTCAACATGGAGGATTCTG R:CCATCGCTCGTCCTTTATGCTCA	56
mBD2	F:GAAGCAGAACTTGACCACT R:TCGAACAGGGGTTCTTCTCT	59
mBD3	F:TTGTTTGAGGAAAGGAGGCA R:GCTAGGGAGCACTTGTTTGC	60
IL-6	F:CCAGAGATACAAAGAAATGATGG R:ACTCCAGAAGACCAGAGGAAAT	60
TNF-α	F:CCAATGGCAGAGTGGGTATG R:TGAAGAGGACCTGGGAGTAG	58
β-actin	F:GGCACCACACCTTCTACAATG R:GGGGTGTTGAAGGTCTCAAAC	56

2.6. Immunofluorescence

Paraformaldehyde-fixed and paraffin-embedded sections of the jejunum were used for immunofluorescence analysis. Briefly, sections of 5-mm thickness were deparaffinized and rehydrated and then processed for Ag retrieval. The sections were then incubated in 3% hydrogen dioxide for 20 min in dark. Then, the sections were incubated with primary Abs (1:200 dilution) specific for ZO-1 and MUC-2. Fluorescently labeled Abs were used as secondary Abs (1:50 dilution). Nuclei were stained with DAPI. Images of immunofluorescent sections were captured using an Eclipse Ti-SR microscope with a DS-U3 Image-Pro system (Nikon).

2.7. Western blotting

Total protein from jejunum tissues was extracted with RIPA Lysis Buffer (Boster, China), and the quantification of protein was conducted via a BCA kit (Keygen, China). Samples were loaded onto a 8% separating gel. Subsequently, proteins were transferred onto PVDF membrane (Millipore Corp., Bedford, MA). The membrane was blocked for nonspecific binding for 3 h (5% skimmed milk in TBST) and then incubated overnight at 4 °C with Abs for ZO-1, occludin, MUC-1, MUC-2 and β-actin. Then, the membranes were washed 4 times, followed by incubation with secondary Abs for 1 h at 37 °C. After another 4 times of washing, the membrane was exposed by using the Supersignal chemiluminescence detection kit (Thermo Scientific, USA) in imaging system Westernbright™ peroxide (Advansta, California, USA). The protein amount was normalized with the amount of β-actin as internal control.

2.8. Bacterial transfer during EHEC infection

After the mice had been treated as described in Section 2.2, eight mice from the control, EHEC, EHEC + Enro, EHEC + OM1 and EHEC + OM2 groups were randomly selected, respectively. The mice were euthanized, and their liver and spleen were collected and homogenized in cold PBS. The numbers of CFUs were determined by plating serial dilutions on Luria-Bertani agar plates.