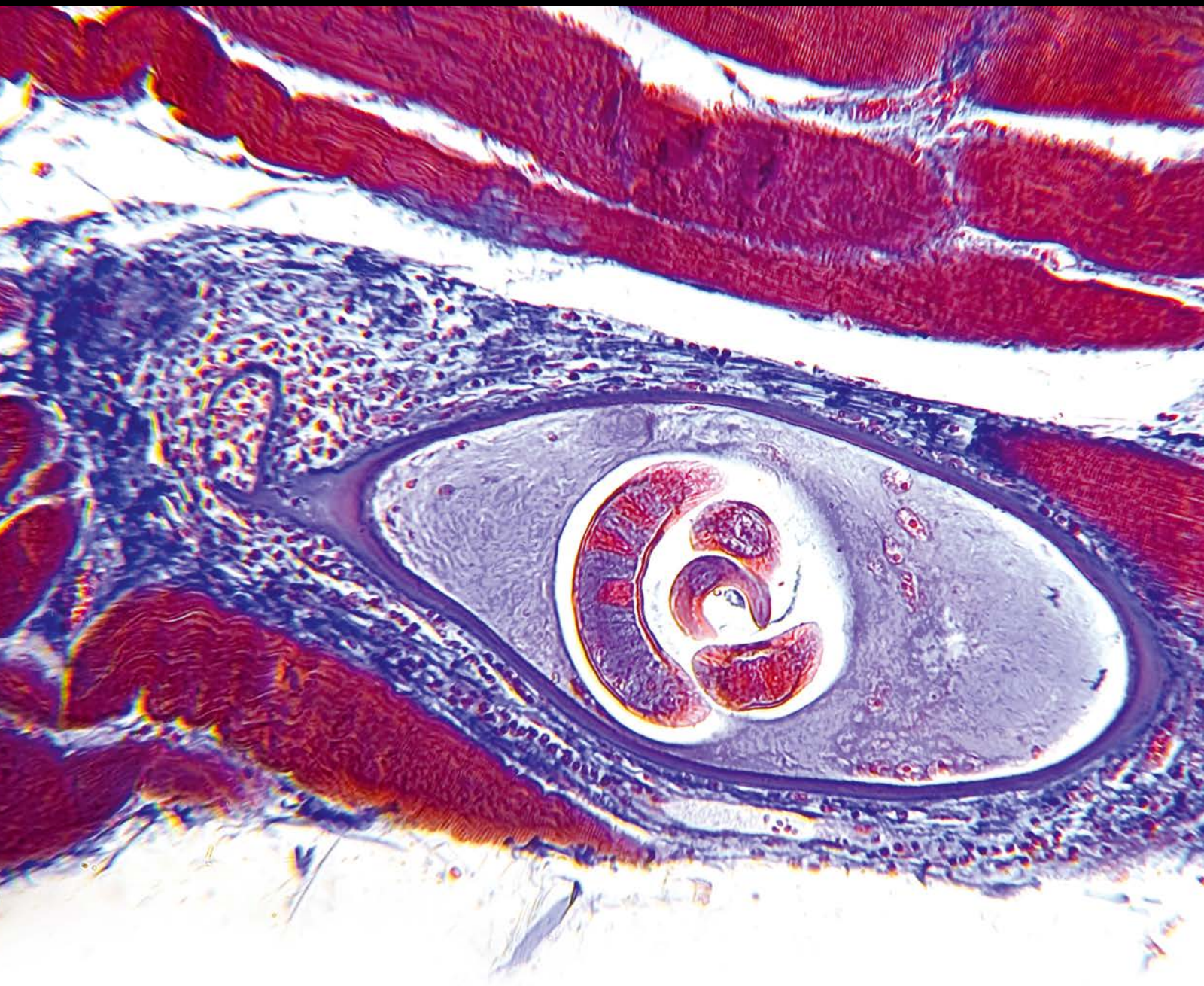
Gastroenterology Research and Practice

Gastrointestinal Inflammation and Repair: Role of Microbiome, Infection, and Nutrition

Guest Editors: Helieh S. Oz, Sung-Ling Yeh, and Manuela G. Neuman



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Editorial Board

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Editorial

Gastrointestinal Inflammation and Repair: Role of Microbiome, Infection, and Nutrition

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Gastrointestinal inflammation is a complex biological response to injury as a result of different stimuli such as pathogens, damaged cells, or irritants. Symbiotic microbiome in digestive tract is considered to protect gut by removing harmful stimuli and to enhance healing process. Alteration or absence of microbiome can lead to exacerbated type 2 immunity and allergic/infectious and inflammatory complications including parasitic diseases. Thus, the microbiota regulates type 2 responses and acts as a key element in harmonizing immune responses at mucosal surfaces. While the mechanism by which microbiota regulates type 2 immunity is unclear, it is known as a strong inducer of proinflammatory T helper 17 cells and regulatory T cells (Tregs) in the intestine. The signals at the sites of inflammation mediate rapid cell recruitment and differentiation in order to remove inflammatory inducers and promote tissue homeostasis restoration. However, persistent inflammatory stimuli or dysregulation of mechanisms of the restoration can lead to chronic inflammation. Different stressors can affect immune system and increase risk for infectious diseases, such as gastritis in postinfectious irritable bowel syndrome (IBS), and vice versa, as IBS patients have increased susceptibility to develop infectious gastroenteritis. Various viral (e.g., norovirus), microbial (e.g., *Campylobacter jejuni*, *Clostridium*, *Mycobacterium*), and parasitic agents (e.g., *Giardia*, helminthes) are known to be involved in the development of chronic inflammatory bowel diseases. Yet, the mechanisms of action are not well known and there is no available cure. Additionally, nutritional elements, such as n-3 polyunsaturated fatty acids, antioxidants, probiotics, and prebiotics directly and indirectly modulate GI immunity. Diets high in fat change the populations of innate microbiome in digestive tract and alter signaling to the brain and satiety, leading to obesity and inflammation.

H. pylori CagA and IL-1β. Gastric cancer is the 3rd cause of cancer mortality globally and infection by *Helicobacter* (*H. pylori*) infecting about 50% globally. It is a main risk factor for gastric cancer by the activity of virulence factor, CagA. *H. pylori*/CagA causes chronic inflammation and triggers gastric lesions leading to cancer. IL-1β is linked with tumor progression including gastric cancer. Inflammatory cytokines and IL-1β are associated with *H. pylori* infection but link between gastric cancer and IL-1β was reported before. H. Arevalo-Romero et al. report a link between CagA and IL-1β which triggers the instigation of the epithelia to mesenchymal cells by β-catenin nuclear translocation. In addition, it increases the expression of Snail1 and ZEB1 and downregulates morphological changes of nontransformed epithelial cells, MCF-10A, into acini formation. CagA by itself induced MMP9 activity and cell invasion. Authors present data to support the fact that IL-1β and CagA trigger the β-catenin pathway, leading to progression of aggressive tumors.

H. pylori and Metabolic Syndrome. *H. pylori* infects about 50% of global population with increased risk of atherosclerosis. Similarly, patients with metabolic syndrome are at increased risk of atherosclerosis. To assess the effects of *H. pylori* infection on serum lipids, body mass index, and metabolic syndrome in old Chinese people, in a clinical trial W. Yang and C. Xuan studied association of *H. pylori* and metabolic

syndrome in those who had gastroscopy exam. *H. Pylori* (+) patients had higher BMI, fasting glucose concentration, and a higher incidence of metabolic syndrome. In addition, they demonstrated that *H. pylori* infection, total cholesterol, and diabetes mellitus are significantly associated with the risk of metabolic syndrome.

H. pylori and Nutrition. Diet and nutrition influence *H. pylori*-associated disease result. In this review K. P. Haley and J. A. Gaddy discuss *H. pylori* ability to modify the host-immune response and to compete for the essential micronutrients. For instance, iron as well as zinc has great effect on host defense and pathogens interaction. *H. pylori has* evolved to deal with zinc stressor against microorganisms growth in human stomach by encoding multiple proteins involved in zinc efflux through its genome. In addition, *H. pylori* can change gastric flora to promote carcinogenesis, by increasing nitrosylating bacterial strains which convert nitrogen compounds in gastric fluid to carcinogens such as N-nitrosamines or nitric oxide. Authors discuss several aspects including those nutritional factors which can protect against *H. pylori* infection.

Metagenomic and Gut Microbial Diversity. Gut microbiome maintains nutrients metabolism and homeostasis of immune system. Metagenomics have provided the tools to explore relation between microbiome, dysbiosis, and state of diseases. A reliable methodology to profile gastrointestinal microbiome can help to reveal pathogenesis of several chronic inflammatory and infectious diseases and further to develop new preventive and therapeutic modalities. F. Benoit et al. apply profiling technology to study fecal samples from mice, cat, and a human subject. They discuss interindividual variations between these samples. Authors suggest the technique may be useful for clinical diagnostics in diet follow-up and treatments when appropriate controls are applied.

Prealbumin/CRP Score for Predicting Metastasis. Patients with gastric cancer have a poor prognosis with variation in their duration of survival. An appropriate prognostic technology may improve clinical care in patients. Z. Ghoreishi et al. assessed nutritional status and systemic inflammatory response of the patients before chemotherapy and present a new score system to predict metastasis. They compared prealbumin/CRP based prognostic score to compare with Glasgow prognostic score for predicting metastasis and nutritional status in patients. Authors discuss in detail statistical difference levels of prealbumin and CRP between patients with metastatic and nonmetastatic gastric cancer compared to other current methods to predict survival in this population. Future studies with large sample size will reveal the usefulness of this prognostic scoring system.

Fish Oil, Microbiota, and Ethanol Induced Hepatic Injury. Chronic consumption of ethanol may lead to oxidative stress, hepatic injury, alcoholic liver disease, and eventually cirrhosis. S.-C. Yang et al. investigated fish oil protective effects on hepatic structure in ethanol-fed rats based on the intestinal permeability and microbiota compared to olive oil. Ethanol induces dysbiosis, to disrupt intestinal barrier and increase permeability and endotoxemia. As was expected, liver enzymes activities, hepatic inflammatory cytokines, and plasma endotoxin levels were significantly upregulated in ethanol group with increased intestinal permeability and decreased numbers of fecal bifidobacteria ($p < 0.05$). However, these pathological effects were significantly ameliorated in those that received fish oil. The fecal *E. coli* lowered, but fecal bifidobacteria numbers were significantly increased in fish oil groups compared to olive oil. Authors conclude that dietary fish oil in ethanol consumption may normalize increased intestinal permeability and fecal microbial composition and reduce endotoxins and inflammatory cytokines and liver injury.

Prebiotic Formula Improves GI Bacterial Flora. Y.-L. Chen and coworkers screened healthy toddlers (18 months to 3 years) to investigate the impact of prebiotic containing formula (inulin, fructooligosaccharides, and galactooligosaccharides) on their GI microflora given 3 times a day for 6 weeks. The control formula with no prebiotics was delivered one week before and after the treatment period. Fecal samples were examined at different time points to measure the anaerobic bacteria, *Bifidobacterium* spp., and *Clostridium perfringens*. In addition, organic acids such as lactate, acetate, propionate, and butyrate were measured in the feces using high-performance liquid chromatography. The population of probiotic *Bifidobacterium* spp. significantly increased, while the total anaerobic bacteria and harmful *C. perfringens* were suppressed after 6-week administration period. Compared to that in the control period, the ratio of *Bifidobacterium* spp. to the total anaerobic bacteria significantly increased, and the ratio of *C. perfringens* to total anaerobes was significantly reduced. Furthermore, the levels of organic acids in the fecal samples significantly increased after consumption of prebiotic formula. Authors concluded that supplementation with the 3-prebiotic toddler formula may be beneficial for children's gut health.

Xylooligosaccharides and Microbiota. Probiotics are known to feed on prebiotics to support their growth and gut health. In a pilot trial S.-H. Lin et al. investigated the effects of short chain fatty acid (prebiotic) xylooligosaccharides and enriched rice porridge (combination cocktail) consumption additives into daily diet on the intestinal tract of human subjects. Twenty healthy subjects participated in a 6-week trial, in which half of subjects received the cocktail while the other 1/2 received placebo (rice porridge alone). Fecal samples at different time points were examined to study microflora. Investigators reveal daily consumption of the prebiotic cocktail to induce significant increases in fecal microbial counts in *Lactobacillus* spp. and *Bifidobacterium* spp., yet to decrease undesired *Clostridium perfringens* compared to placebo rice porridge. However, some changes occurring in the counts of coliforms in both groups may relate to possible favored effects by rice porridge. Authors conclude that improvement in intestinal microbiota balance supports the possible addition of prebiotic plus rice porridge into daily diet.

Tea and Clostridium difficile Infection. The incidence of *C. diff* infection and its mortality rate are on the rise in those susceptible. The fecal transplant has shown some benefit in the patients. Tea and its polyphenols are known best for several beneficial actions including antioxidant, anti-inflammatory, and antimicrobial effects. Contrary to beneficial reports on tea M. O. Evans II et al. reason that the antimicrobial effect of tea on gut may act similarly to undesired antibiotics in suspected individuals prone to altered gut microbiota to favor infection and *C. diff* recurrence. In a retrospective cross-sectional dietary survey investigators recruit subjects who had been diagnosed with *C. diff* infection episodes in past. From 64 enrolled patients to list their daily diet 19 had experienced recurrent episodes. A statistically significant number of patients with recurrence compared to nonrecurrence patients had used antibiotics ($p = 0.003$) or recorded tea ($p = 0.002$), coffee ($p = 0.013$), and eggs ($p = 0.013$), on 24-hour food recall. Authors conclude a possible link between tea drinking and *C. diff* recurrence to negatively affect the gut microbiota to support growth of *C. diff*. Future clinical trials are required to confirm whether tea supports the growth of *C. diff* in these recurrent patients.

Matrix Metalloproteinases, Inhibitors, and IBD. While the etiology of chronic inflammatory bowel disease is still under debate a series of inflammatory mediators are known to ignite the complication. Metalloproteinase (MMPs) proteolytic zinc-dependent enzymes are involved in remodeling and degradation of extracellular matrix and wound healing, as well as rheumatoid arthritis, atherosclerosis, and tumor metastasis. K. Jakubowska et al. report expression of MMPs to be distinctive in Crohn's disease compared to ulcerative colitis. The overexpression of MMPs and significantly weaker expression of the inhibitors may determine the development of IBD. Ulcerative colitis patients in particular demonstrate a significant correlation between increased MMPs expression and histopathological markers with disease progression. Authors suggest MMPs (2, 7, and 9) as potential therapeutic target and their inhibitors to reduce ulcerative colitis progression.

Mucosal Barrier and Crohn's Disease. The genetics, immunity, and environment factors are tightly regulating the progression of Crohn's disease in patients. Gut microbiota protects intestinal mucosa against inflammations. As dysfunction of intestinal mucosal barriers due to immune disorders and infections initiate Crohn's development, additional studies are required to explain exact protective action of intestinal mucosal barrier's in Crohn's disease. K. Wang et al. recapitulate recent findings regarding the correlations between the disorders of intestinal mucosal defenses including mechanical, chemical, immune, and biological barriers and Crohn's disease.

Syndecan-1 and Intestinal Epithelial Inflammation. Syndecan-1 (SDC1) is heparan sulfate proteoglycans (HSPGs) mainly expressed on the surface of epithelial cells. SDC1 is known to inhibit migration of neutrophils by downregulating chemokines and cytokines expression. Y. Zhang et al. examine the role of SDC1 in intestinal inflammatory responses using a transfected intestinal epithelial cell model. Authors conclude that suppressing SDC1 release from epithelial cells ameliorates severity of intestinal inflammation by inactivating NF-κB pathway and downregulating TNFα expression and suggesting the use of SDC1 as a possible targeted therapy.

Rifaximin and Mutaflor Synergy against Colitis. A. Dembinski et al. investigated the effect of antibacterial agent, rifaximin, and/or Mutaflor, a nonpathogenic bacteria strain (*Escherichia coli* Nissle 1917), administration on the healing of acetic acid-induced colitis in rats. The authors found that rifaximin and Mutaflor had synergic anti-inflammatory and therapeutic effects in colitis. This finding implies the interaction between antibiotics coadministered with probiotics as a possible alternative choice in the treatment of colitis.

Effectiveness and Safety of Entecavir versus Lamivudine. This is a systematic review and meta-analysis of 1254 patients with hepatitis B viral (HBV) infections-associated acute-on-chronic liver failure. J. Yang et al. focus on 2 commonly used antiviral treatments, entecavir and lamivudine, for effectiveness and safety in HBV patients. Authors report entecavir to be significantly more effective and with higher survival rate than lamivudine in patients treated after 1 year. As for the safety, both drugs (entecavir and lamivudine) were found to be equally safe and well tolerated in patients. Further meta-analysis research needs to be conducted on adverse effects of entecavir in prolonged treatments.

Molecular Diversity and Sapovirus Infection. *Sapovirus*, a calicivirus, is an important pathogen involved in nonbacterial acute gastroenteritis. This study investigated the molecular diversity of *Sapovirus* in outpatients with acute gastroenteritis from the city of Nanjing in China from 2011 to 2013. H. Zhang et al. used real-time PCR with specific primers and probes targeted at conserved regions of the RNA polymerase to human *Sapovirus*. The results showed that, compared to the reference strains, different amino acid substitutions were found in the Nanjing strain. Whether these substitutions are involved in the antigenic changes or the virus fitness to hosts requires further investigation. However, this study provides some cues in the treatment of *Sapovirus*-induced enteritis in the future.

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Research Article

Selective Induced Altered Coccidians to Immunize and Prevent Enteritis

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Microbiomic flora in digestive tract is pivotal to the state of our health and disease. Antibiotics affect GI, control composition of microbiome, and shift equilibrium from health into disease status. Coccidiosis causes gastrointestinal inflammation. Antibiotic additives contaminate animal products and enter food chain, consumed by humans with possible allergic, antibiotic resistance and enigmatic side effects. Purposed study induced nonpathogenic, immunogenic organisms to protect against disease and abolish antibiotics' use in food animals and side effects in man. Diverse species of Coccidia were used as model. Immature organisms were treated with serial purification procedure prior to developmental stages to obtain altered strains. Chicks received oral gavage immunized with serial low doses of normal or altered organisms or sham treatment and were challenged with high infective normal organisms to compare pathogenicity and immunogenicity. Mature induced altered forms of *E. tenella* and *E. necatrix* lacked developmental stage of "sporocysts" and contained free sporozoites. In contrast, *E. maxima* progressed to normal forms or did not mature at all. Animals that received altered forms were considerably protected with higher weight gain and antibody titers against challenge infection compared to those that received normal organisms ($p < 0.05$). This is the first report to induce selected protective altered organisms for possible preventive measures to minimize antibiotic use in food animals.

1. Introduction

The microbiomic flora living in the digestive tract plays a pivotal role in the state of our health and disease. Recent investigations have been focused on medication use specifically antibiotics that affect digestive tract, which can control the microbiome composition and shift the equilibrium from health into disease status [1].

The recent decades are associated with the severe increases in the prevalence of IgE-mediated [2] and non-IgE-mediated food allergies and coincide with the rise in the antibiotic supplementation in poultry and livestock. For instance, the glioma development has been linked with elevated IgE and possible food allergies. Additionally, glioma patients have shown drastically higher rate of penicillin skin positive test with elevated blood eosinophil counts than the nonglioma control subjects [3]. These findings indicate an intricate association amongst glioma progression and possible state of combined food and antibiotic allergies in these patients.

The increase in antibiotic resistance has created a complex dilemma facing the public wellbeing. Indeed, the use of antibiotics against infections has created conundrum, as high doses may facilitate evolution of antibiotic resistance than curing infections. On the other hand, antibiotics' combination and extended period of treatments can promote severe injuries [4]. Further, suboptimal dose of antibiotics use as recently recommended may not act sufficiently enough to eradicate the pathogens.

Meanwhile, current consumers' awareness regarding the antibiotics residue contamination of poultry products and antibiotic resistant pathogenic microbial has caused concerns and recommendations to minimize the use of antibiotics as growth promoters in livestock in the USA [5] and worldwide.

Coccidiosis is a costly infectious disease which causes morbidity as well as fatality in livestock and poultry industry.

So far, there is no safe and protective vaccine or practical prevention available [6]. Coccidians are host defined apicomplexan organisms which exclusively cause gastrointestinal inflammatory complications and diarrhea in man and animals. In contrast, *Toxoplasma*, another related member of Apicomplexa with intraintestinal (coccidian forms) and extraintestinal stages, is cosmopolitan and infects animals and man as well as every single organ and cell in the body [7]. Coccidiosis is one of the major diseases related to the food animals. Losses are directly due to morbidity as coccidiosis results in reduction in weight gain and egg production as well as affecting the quality of meat by decreased feed conversion, malabsorption, and maldigestion and further leads to mortality [8]. *Eimeria* infection predisposes animals to clostridial infection and necrotic enteritis [9]. Common preventive practice includes the use of subtherapeutic doses of antibiotic additives into poultry and livestock diets which contaminate eggs, milk, and meat production. Antibiotics enter the food chain and are consumed by humans with possible allergic, antibiotic resistance [10] and other yet elusive side effects. For instance, the use of sulfonamide as anticoccidial has encountered the emergence of drug-resistant strains [11]. The estimated annual cost of coccidiosis in broiler production alone has been tripled ($350 million) in last decades in USA [12, 13] mainly due to anticoccidial drugs used to control the infection. Although continuous medication in feed has been effective against the disease outbreak, there are several disadvantages including sequential development of drug resistance and side effects in the consumers which necessitate constant search for new and effective compounds and vaccination. Animals that recover from the infection develop species specific immunity induced by the exposure to organisms or live vaccines. The viable vaccination with Coccivac, a mixture of diverse pathogenic species of birds' *Eimeria*, has been used for over 1/2 century in the United States [14]. This live vaccine causes (1) poor feed conversion, (2) a long duration required to evolve immunity against the disease, (3) possibility of introducing infection, (4) and difficulties of administering and managing the flocks. Other experimental vaccines are the attenuated strains including serial passages of *E. tenella* into chorioallantoic chick embryo [15] which were found to be ineffective. Since their discovery over a century ago [16], *Eimeria* have been described as organisms with four encapsulated sporocysts, each containing two infectious organisms called "sporozoites."

The purpose of this study was to introduce nonpathogenic but immunogenic organisms to protect against the disease and to abolish the use of antibiotics in food animals and the side effects in the consumers. By utilizing purification procedure, altered *E. tenella* and *E. necatrix* were provoked to evolve 8 free sporozoites with no sporocyst formation. As a proof of concept, these altered organisms were less pathogenic than normal organisms but similarly immunogenic to normal forms. This investigation reports the formation of altered organisms to be serendipitous and to depend on the species of the organisms while their immunogenicity was reported to be comparable to normal organisms with use of birds as model and to warrant future investigations.

2. Materials and Methods

2.1. Ethics. This investigation was conducted according to the guidelines and approved by the Institutional Biosafety and the Institutional Animal Care and Use Committee (IACUC).

2.2. Animals. One-day-old specific pathogen-free (SPF) inbred White Leghorn chicks were obtained from the Poultry Science and kept in Coccidia-free and pathogen-free rooms, in daily disinfected wire-floor cages, and provided feed and water *ad libitum*. Each bird was tagged with a leg band for identification and weighed prior to, during, and at the end of the study. Weight gain/loss was calculated by subtracting the initial weight before inoculation from the weight obtained/lost following challenge and before termination of the study.

2.3. Oocysts Preparation. Sporulated oocysts of *E. tenella, E. necatrix,* and *E. maxima* were originally obtained from Eli Lilly and Company (Indianapolis, IN). Fresh cultures were prepared from the ceca or intestines of donor animals one week after oral inoculation. The contents were homogenized and the unsporulated organisms (oocysts) were separated from debris by sieve and centrifuged at 400 ×g and sediment added into 2.5% aqueous potassium dichromate (Sigma, St. Louis, MO) to obtain normal sporulated oocysts. In order to obtain altered oocysts, the homogenate containing unsporulated oocysts was further cleaned in a solution of 2.5% sodium hypochlorite and rinsed with distilled water (2x). The homogenates were centrifuged for 10 min at 400 ×g in a saturated salt solution (9 : 1 v.v) and then rinsed in distilled water. Oocysts were cultured in 2.5% aqueous potassium dichromate solution to enhance sporulation. The organisms were enumerated using a hemocytometer and diluted in phosphate buffer saline (PBS) according to each experiment before oral gavage directly into animal's crop. Normal control animals received sham (PBS) treatment.

2.4. Infection. At 2–6 weeks of age, the animals were inoculated via crop with 240, 2400, and 24000 sporulated oocysts of either the normal or altered strain one up to three doses according to the experimental design with a two-week interval. Infected birds were kept in isolation cages in a separate room and provided with food and water *ad lib*. One week after the challenge inoculation, animals were weighed and humanely euthanized by cervical dislocation immediately followed by open chest and cardiac exposure. Different organs including segments of ceca and intestines were immediately removed and slides smears were prepared to detect the organisms. Cecal lesions were scored from 0 to +5 according to the severity of infection [8], briefly as follows:

0 = normal mucosa and negative cecal/intestinal smears.

1 = no detectable pathology but organisms detected in the smears.

2 = scattered petechia on mucosa, organisms present on the smears, and normal cecal/intestinal contents.

3 = focal inflammation and thickening of mucosa and isolated hemorrhage detected in lumen.

4 = multifocal inflammation and thickening of mucosa and extensive hemorrhage in the lumen, with little or no fecal contents.

5 = enlarged cecum/intestine with blood or sloughed off mucosa and moribund or dead animals.

2.5. Oocysts Sporulation. Organisms' growth and sporulation were determined via microscopic examination. Representative samples were examined and percentage of sporulated oocysts determined every 4 h. Sporulation was achieved when >90% of organisms had achieved sporulation.

2.6. Anti-Eimeria Antibody Detection by ELISA. The Enzyme Linked Immunosorbent Assay (ELISA) was performed using a soluble oocyst antigen of *E. tenella* according to [8, 17]. Blood was collected into syringe from the brachial vein from live animals and serum separated following 5 min centrifuge. Each serum sample was heat inactivated at 56°C for 30 min before use and diluted in twofold increments. Peroxides conjugated γ chain rabbit anti-chicken IgG (Cappel Laboratories, West Chester, PA) was dispensed into each microwell coated with 11 μg of antigen and incubated with o-phenylenediamine (Eastman Kodak Co, Rochester, NY). Optical densities (OD) were documented at 490 nm on ELISA reader (DYnatech Lab Inc., Alexandria, VA).

2.7. Pathogenicity and Immunogenicity. *E. tenella* organisms were used as a model to compare the immunogenicity and pathogenicity of normal versus altered organisms. Oral gavage into crop with 240 and then 2400 oocysts of normal or altered organisms with a two-week interval was performed on three-week-old inbred White Leghorn chicks. Then animals were challenged with high infective (24,000) dose of normal *E. tenella*. One week after each inoculation, 6 animals per group were weighed and appearance and health status documented. Then blood samples from brachial vein were collected and animals humanely euthanatized by cervical dislocation and cardiac exposure followed by tissue specimens harvest.

2.8. Frozen Sections. Oocysts were centrifuged in 20% (W/V) bovine serum albumin (BSA) and 15% (W/V) sucrose. The pellets were fixed with 2 drops/mL of 25% glutaraldehyde. They were quick-frozen in liquid nitrogen and the blocks were sectioned at the 18 μm setting on cryostat. Oocysts were embedded with mounting agent to protect the oocysts from shattering during the sectioning procedure. The sections were stained with Haematoxylin and Eosin.

2.9. Statistical Analysis. Results are expressed as mean ± SEM unless otherwise stated. Data were evaluated utilizing ANOVA followed by appropriate post hoc test. Statistical significance was set at $p < 0.05$.

Table 1: Six White Leghorn birds/group were inoculated with normal (N) or altered (A) oocysts of *E. tenella* and euthanatized 1 week after. Control (C) birds received sham inoculation (0). Body weight gain/g.

Group	Strain	Oocyst/	Weight gain	Lesion	Ab titer (OD)
1	A	240	104 ± 5	1	0
2	N	240	85 ± 4	1	0
3	C	0	142 ± 8	0	0

3. Results

3.1. In Vitro Sporulation and Oocysts Description. Normal oocysts of *E. tenella* began cell division and sporulation 16–20 h in the external environment to each form of four internal sporoblasts. After 28 h four sporocysts were matured and the sporozoites' development was completed in 38 h (Figures 1(a)–1(f)). In contrast, altered oocysts initiated division approximately 12 h later than normal oocysts (28 h). Paradoxically, organisms started to flourish into newly formed free sporozoites at 36 h to shape no prior sporocysts formation and sporulation was completed after 44 h (Figures 2(a)–2(d)), that is, total 6 h longer than expected with normal oocysts. Greater than 95% of processed organisms developed into the altered form. The altered organisms were round to oval in shape (25 to 35 μm by 22.5 to 27.5 μm) and each contained eight banana-shaped free sporozoites (12.5–14 μm by 2.5 μm). While the measurements were in consistency with those of normal form, the exception was lack of sporocysts.

3.2. Effect of Storage on the Oocysts Lifespan. The altered organisms when stored at 4°C for 3 months; the free sporozoites within the oocysts then started to degenerate (Figure 2(e)). In contrast, normal oocysts preserved the appearance of sporocysts and sporozoites with no visible signs of degeneration over 1 year after storage at 4°C. This establishes the specific protective entity of the sporocysts provided for the longevity and infectivity of the sporozoites.

3.3. Altered Oocysts of Other Species of Eimeria. Attempts were made to determine whether or not parallel developmental alteration would occur with other related coccidian organisms. Similarly, the altered oocysts of *E. necatrix,* another pathogenic Coccidia, were induced utilizing the same methodology as described for *E. tenella* organisms. Likewise, altered *E. necatrix* oocysts contained eight free forming sporozoites and lacked sporocystic structures (Figure 2(f)). In contrast, when another species, *E. maxima*, was treated with similar procedure, the organisms either did not complete sporulation process or simply transformed into normal sporulated oocysts (Figures 2(g) and 2(h)).

3.4. Oocysts and Immunity. In order to investigate the immunogenicity of the organisms, the inbred animals each were given escalating doses from low 240 (Table 1) and then 2400 (Table 2) of normal or altered organisms ones with a two-week interval. The animals which were treated with altered strain all profoundly gained more weight ($p <$

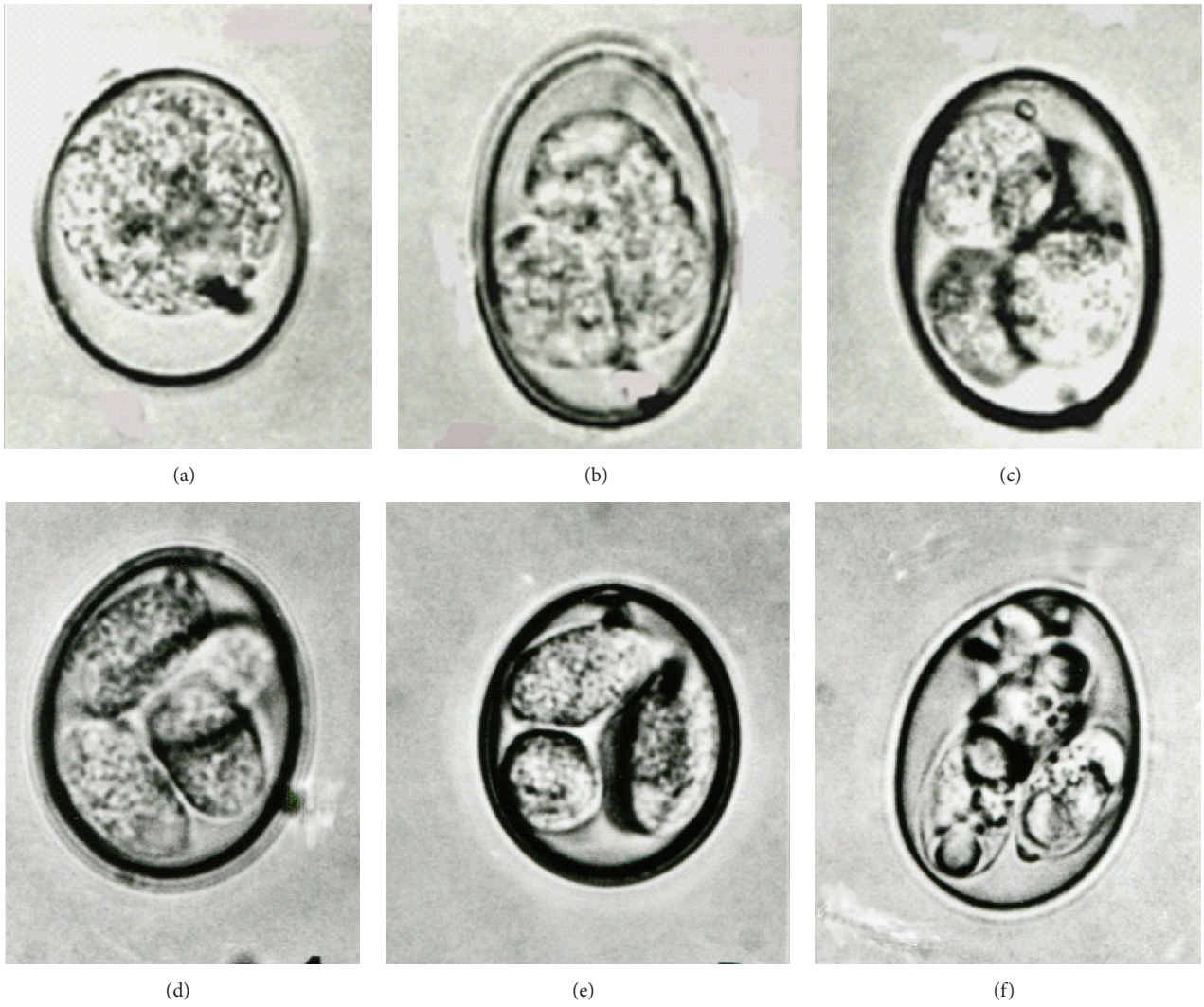

Figure 1: Normal oocysts of *Eimeria tenella* during *in vitro* growth and sporulation from 0 to 38 h (slide representative from over 100 organisms). (a) Immature organism (unsporulated oocyst) at 0 h × 1,000. (b) Normal organism start division at 16 h × 1,200. (c) Four spherical sporoblasts and a polar granule formation visible at 20 h × 1,100. (d) Ellipsoidal sporoblasts at 24 h × 1,000. (e) Sporocysts formation at 28 h × 1,000. (f) Sporocysts formation completed, each containing 2 mature sporozoites at 38 h × 1,000.

Table 2: Six White Leghorn birds/group were immunized with 240 followed by 2400 normal (N) or altered (A) oocysts of *E. tenella* with a two-week interval (week 3). Control (C) birds that received sham inoculation (0) gained most but with no titers. Body weight gain/g and sera antibody titer production in group A versus group N showed significant difference; $^{*}p < 0.05$.

Group	Strain	Oocyst/	Weight gain	Lesion	Ab titer (OD)
4	A	2400	$95 \pm 4^{*}$	1	42.0^{*}
5	N	2400	66 ± 6	1	25.0
6	C	0	180 ± 9	0	0

0.05) and considerably showed higher antibody titers in sera compared to those immunized with normal organisms ($p < 0.05$). Two weeks after the last immunizing dose (2400 normal or altered forms), remaining animals were all challenged with high dose of 24,000 normal organisms (Table 3). As a result, those animals that received only the challenge dose (24,000 normal oocysts) drastically lost weight ($p < 0.01$) and developed bloody diarrhea compared to immunized ones. The pathological lesions were scored as 3.5 ± 0.5 with moderately severe inflamed and thickened intestinal mucosal and hemorrhage detected in mucosa and lumen in contrast to those immunized animals ($p < 0.01$, Table 3).

All uninfected animals as expected were sera antibody negative. Both immunized groups with either altered or normal forms showed no pathological lesions and were antibody

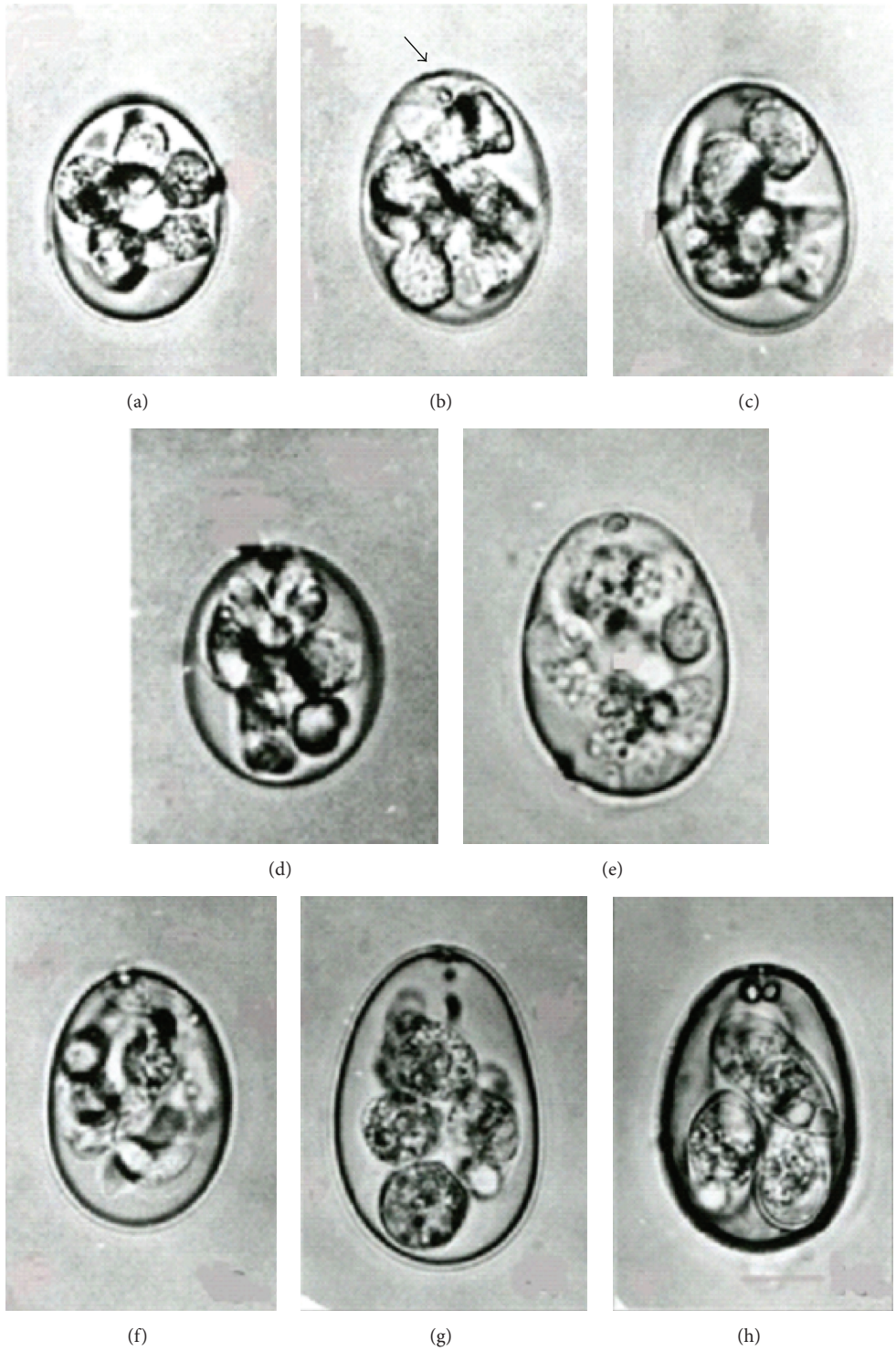

Figure 2: (a–e) Altered organisms (oocysts of *E. tenella*) during evolutional stages following procedure at 28 to 44 h (slide representative from over 100 organisms). (a) Altered organisms (oocysts) begin to divide at 28 h × 1,000. (b) Further development showing the polar granule (indicated by arrow) at 32 h × 1,200. (c) Free sporozoites forming from protoplasmic mass at 36 h × 1000. (d) Sporozoites formation complete at 44 h × 1,100. (e) Degenerating altered oocyst of *E. tenella* 3 months after storage at 4°C × 1,070. (f–h) *Eimeria necatrix* and *E. maxima* following the alteration procedure (slide representative from over 100 organisms). (f) *E. necatrix* proceeds to altered organism after the procedure to contain free sporozoites × 1,000. (g) *E. maxima* fail to complete sporulation after the altering procedure. × 800. (h) *E. maxima* form normal organisms containing 4 sporocysts, each with 2 sporozoites after the procedure. × 800.

Table 3: White Leghorns were immunized with 240 and 2400 normal (N) or altered (A) oocysts of *E. tenella* with a two-week interval. Infected control group (N^) received no previous immunization before final challenge. Remaining birds were challenged each with 24,000 normal oocysts. Controls (C) received (0) sham inoculation. N^ lost weight the most and developed moderately severe lesions. Body weight gain/g was significantly higher in uninfected animals and those immunized with altered compared with the normal organisms and there was weight loss in unimmunized animals. N = 6 animals/group. $^{\#}p < 0.01$, $^{*}p < 0.05$. ND = not done.

Group	Strain	Oocyst/	Weight gain	Lesion	Ab titer (OD)
7	A	24,000	$71 \pm 4^{*}$	0	50.0
8	N	24,000	21 ± 4	0	38.0
9	N^	24,000	$-50^{\#}$	$3.5 \pm 0.5^{\#}$	ND
10	C	0	171 ± 4	0	0

positive with higher titers developed in those immunized with altered compared to normal organisms. The major difference between inoculated groups was expressed with those animals immunized with altered organisms to gain considerably more weight and to be in better overall health status than those animals previously immunized with normal organisms (Table 3, $p < 0.05$).

4. Discussion

A major role for the vast population of intestinal microbiota is to prevent incursion and colonization of infectious microbial pathogens [1–18]. Antibiotics including the short term use can damage propagation of the commensal microbiome to favor susceptibility to dysbiosis. The commensal microbiota seems to be most important when established early in life [19]. Therefore, previous criteria such as cesarean versus natural birth and breastfeeding may no longer be considered as the main focal point. Therefore, the attention has been focused on the antibiotics which can control the microbiome composition [19] and the equilibrium between health and disease.

Noncommunicable and inflammatory complications such as allergic diseases affect over millions of people. The food allergies in westernized societies have been drastically increased mainly due to lifestyle and diet. Thus far, the pathogenesis of allergies specifically food related allergies has remained somehow elusive [20]. This coincides with the upsurge in the prevalence of IgE-mediated food allergies unsolved in children yet with possible environmental [2] and/or nutritional exposures involvement. Additionally, a profound relationship has been detected between the use of macrolide antibiotics and asthma development amongst European children (6–36 months of age) as in a multicenter prospective trial. There was a definite correlation between the use of macrolide in the first year of life and wheezing in 3-year-olds, independent of the respiratory infections [21]. Meanwhile, high prevalence of fluoroquinolone and macrolide resistance pathogens including *Campylobacter* in poultry and swine industry [22] due to misuse raises alarm about possible overexposure and ineffectiveness of these antibiotics in humans' therapy.

A link between exposures to environmental factors has been demonstrated in development of chronic inflammatory diseases such as Crohn's and ulcerative disease. These revelations comprise childhood exposure to different dietary elements [23]. Paradigm includes food protein induced enterocolitis syndrome (FPIES) as a non-IgE-mediated hypersensitivity in children that provokes symptoms of severe vomiting, diarrhea, and acidosis a few hours following consumption of dietary proteins, chicken, turkey, egg white, and cow's milk [24]. Indeed, eggs are reported as a frequent trigger of FPIES [25]. In sporadic cases in infants, the non-IgE-mediated hypersensitivity has been noted to shift to typical IgE-mediated cow's milk allergy [26]. The T cell activation may or may not be present in FPIES [27], while the mechanism(s) which predispose(s) one to FPIES has remained an enigma.

Antibiotic use has a rapid and major effect on the luminal bacterial community followed by long-lasting consequences for regulation of microbial population architecture and the host immunity [28]. Recent studies stress the mucosal-associated commensal bacterial to form a protective barrier to prevent food allergic sensitization. *In vivo* and *in vitro* studies support utilization of oral live biotherapies (e.g., prebiotics) to modulate luminal microbiome and to induce tolerance against food allergy in patients [28].

In a retrospective case-control trial (2004–2013), patients with glioma ($n = 913$) had profoundly higher rate of penicillin skin positive (IgE) test and elevated blood eosinophil counts than matched controls ($n = 1091$) with a possible link between glioma and food allergies [3]. Yet, β-lactam antibiotics, penicillin (e.g., amoxicillin and dicloxacillin), and cephalosporins (e.g., cefazolin and cefotaxime) are regularly used in animals. Abuse and illegal use of these antibiotics [29] as well as macrolides have been detected in food animal products which can result in bacterial resistance and allergy. For instance, tetracycline, one of the most common antibiotics used in poultry, is deposited and remains in bones and is passed into food chain with potential human health risk regardless of enforced monitoring of proper withdrawal times [30]. Diclazuril is another antimicrobial routinely used in poultry industry to protect against coccidial infection. We have recently reported diclazuril monotherapy or combined with atovaquone to be effective against congenital and maternal toxoplasmosis [7, 31, 32]. The combination antibiotic therapy most effectively protected pups against severe multiorgan inflammatory reaction and fatality and to candidate diclazuril mono or combined therapy as an attractive antitoxoplasmosis in humans.

The antibiotic resistant pathogens are caused by the extensive utilization of subtherapeutic antibiotics feed to poultry [5] and livestock. Recent consumer awareness regarding the antibiotics residual contamination in poultry products and the antibiotic resistant pathogenic microbial has created great concerns and recommendations to minimize the use of antibiotics as growth promoters in livestock in

the USA [5]. Alternative preventive measures are sought, desirable to eliminate or minimize the use of antibiotics in livestock and poultry industry.

In this study, the altered organisms differed from normal oocysts in that they lacked sporocysts, even though each contained eight free sporozoites. The altered organisms produced considerably less pathogenicity in immunodeficient animals compared to normal oocysts (unpublished data), while the protection induced by these organisms in immunocompetent inbred animals was no less than those induced by normal forms. Indeed, animals inoculated with altered organisms gained more weight and demonstrated minimal pathological symptoms due to infection and induced higher antibody titers compared to those that received normal forms. One century after the original discovery of eimerians, a reproducible method of altering infective forms to noninfective ones has been introduced here. Previous studies have used irradiation to alter the pathogenicity of the organisms [33, 34] or to induce resistance by the genetic selection of the avian host [35]. The intramuscular injection of the plasmid pcDNA3-1E of *E. acervulina* was reported to be safe in chicken [36]. Also, *E. tenella* EtMic2 (a microneme protein) displayed on the cell surface of *S. cerevisiae* used orally as a live vaccine was reported to protect against challenge infection by lowering lesion scores and fecal oocysts in birds and stimulated humeral innate immunity [37]. Our interrogation indicated that the process of mechanochemical alteration in developmental stages prior to sporulation can rationally lead to formation of altered organisms (*E. tenella* and *E. necatrix*) with ability to immunize against severe infections and to protect against luminal inflammation.

Normal organisms are reported to preserve internal structure for up to 25 years in 2.5% potassium dichromate [38]. The current study proves that sporocysts have a protective effect on preserving infectivity and longevity, as altered organisms which lack sporocysts and their structural entity degenerate after only 3 months. The three diverse species of *Eimeria* tested in this investigation (*E. tenella*, *E. necatrix*, and *E. maxima*) all did not react in a similar manner to the altering procedure. While *E. tenella* and *E. necatrix* both similarly followed procedure for formation of abnormal organisms, *E. maxima* failed to be processed to altered formation with no obvious explanation for this variability. There are some discrepancies in the life cycles of these species. *E. maxima* morphologically are somewhat larger than either of the two species. However, this may possibly have little or no effect on the developmental behavior divergence observed here.

Overall, this report may inform the medical community of the vast unwanted antibiotics use and residues in animal products and a possible advancement in prevention of coccidiosis by other approaches than the use of antibiotics, therefore lessening the chances in consumption of antibiotics contaminated animal products and changes in luminal microbiota and prevalence of the food allergies consequences in humans.

5. Conclusion

This is the first report to induce selected protective altered coccidian organisms for possible preventive measures to minimize antibiotic use in food animals. The altered organisms and the subsequent protective immunity they produce against severe infection require further investigation.

Competing Interests

The author declares that there are no competing interests.

Acknowledgments

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Review Article

Nutrition and *Helicobacter pylori*: Host Diet and Nutritional Immunity Influence Bacterial Virulence and Disease Outcome

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Helicobacter pylori colonizes the stomachs of greater than 50% of the world's human population making it arguably one of the most successful bacterial pathogens. Chronic *H. pylori* colonization results in gastritis in nearly all patients; however in a subset of people, persistent infection with *H. pylori* is associated with an increased risk for more severe disease outcomes including B-cell lymphoma of mucosal-associated lymphoid tissue (MALT lymphoma) and invasive adenocarcinoma. Research aimed at elucidating determinants that mediate disease progression has revealed genetic differences in both humans and *H. pylori* which increase the risk for developing gastric cancer. Furthermore, host diet and nutrition status have been shown to influence *H. pylori*-associated disease outcomes. In this review we will discuss how *H. pylori* is able to create a replicative niche within the hostile host environment by subverting and modifying the host-generated immune response as well as successfully competing for limited nutrients such as transition metals by deploying an arsenal of metal acquisition proteins and virulence factors. Lastly, we will discuss how micronutrient availability or alterations in the gastric microbiome may exacerbate negative disease outcomes associated with *H. pylori* colonization.

1. *H. pylori* Infects the Human Stomach

Helicobacter pylori is a Gram-negative member of the Epsilonproteobacteria class. Over 50% of the global human population is colonized with *H. pylori*, which inhabits the gastric niche of human hosts and is commonly acquired early in life. Furthermore, evidence indicates that *H. pylori* has colonized human hosts and coevolved for at least a thousand centuries [1–4]. The human stomach provides numerous nutritional opportunities and challenges for an invading prokaryote. To colonize the stomach successfully, *H. pylori* must survive the acidic pH in the lumen of the stomach, move through the mucus lining of the gastric tissue via chemotactic flagellar-mediated motility, attach to gastric epithelial cells using a repertoire of adhesins, and deploy cytotoxins to alter the gastric environment and create a hospitable niche for bacterial proliferation [3]. These bacterial toxins promote necrosis, autophagy, and proinflammatory signaling cascades [4, 5]. However, *H. pylori* persists in the stomach despite a robust inflammatory response, indicating that this organism has evolved elaborate mechanisms to circumnavigate the onslaught of host immunity [4–6].

2. *H. pylori* Infection and Disease Outcomes

Virtually all hosts infected with *H. pylori* experience gastritis while a smaller subset of these patients develop more serious outcomes such as peptic or duodenal ulcer, MALT lymphoma, or gastric adenocarcinoma. Nearly 75% of all gastric cancer and 5.5% of all malignancies worldwide can be attributed to *H. pylori* [4]. *H. pylori* infection is the strongest risk factor for developing gastric cancer [5]. It is proposed that the profound proinflammatory signaling initiated by *H. pylori* infection leads to atrophic gastritis, intestinal metaplasia, dysplasia, and finally gastric cancer [6]. This process, termed the "Correa pathway" is predicated on the chronic inflammation of the gastric mucosa which fosters a cascade of genotypic perturbations that ultimately

lead to carcinogenesis [6–9]. It is increasingly appreciated that carcinogenesis is established due to a constellation of factors including host genetics, environment, and bacterial strain differences [6–10]. A better understanding of how these factors intersect to promote disease progression could yield novel preventative or therapeutic strategies to ameliorate the global disease burden, which costs hundreds of thousands of human lives each year [10]. In this review we consider how nutrition, or the process by which an organism derives cofactors and metabolic precursors, impacts the progression of *H. pylori*-associated disease outcomes and gastric homeostasis. Furthermore, we discuss how host micronutrients can alter bacterial growth and virulence and ultimately influence pathogenesis.

H. pylori has an ancient association with human beings [1]. Although *H. pylori* strains exhibit remarkable genetic diversity, phylogenetic analyses have revealed that strains can be classified into distinct phylogeographic clades indicative of their origin [2, 3]. These results indicate that *H. pylori* strains have coevolved with their hosts, observations which are supported by results indicating that *H. pylori* has undergone reductive evolution during its association with man [11]. However, prolonged coevolution is commonly associated with commensal adaptation and concurrent loss of virulence [12, 13]. Because *H. pylori* exhibits strain-specific virulence and potential to cause disease, this supports a model in which the coevolution of *H. pylori* and its cognate human host has been perturbed [2, 3].

In some geographical settings, such as Asia, *H. pylori* infection and gastric cancer rates are correlative. However, in other areas, such as Africa, Malaysia, India, and Costa Rica, infection rates are high and gastric cancer rates are low [14–17]. These are collectively referred to as "enigmas" because the protective mechanisms in these populations are obscure. It is proposed that *H. pylori* potentially coevolves with its host to dampen pathogenic effects and promote immunological tolerance which facilitates protection against numerous autoimmune diseases including allergic airway disease [18, 19]. However, the role of geography, nutrition, and host genetics remains ill-defined in this model. Furthermore, regions within a single country, such as Colombia, experience differential disease outcomes [20]. Recent assessments of genetic variations in both host and *H. pylori* strain by multilocus sequence typing analyses (MLST) were performed to ascertain how the coevolutionary relationships between hosts and pathogens were shaping development of gastric cancer [2]. This work demonstrated that low-risk coastal Colombians exhibit phylogenetic variations consistent with an admixture of Amerindian, European, and African populations. Similarly, *H. pylori* strains recovered from these individuals primarily represented an African lineage of *H. pylori* that was concordant with the host genetic background [2, 3]. Conversely, mountain-dwelling Colombians exhibit phylogenetic variations consistent with Amerindian heritage and their *H. pylori* strains predominantly were associated with a European phylogenetic clade [2, 3]. The authors conclude that infection with a strain of *H. pylori* that is discordant with host phylogenetic background is predictive for increased risk of gastric cancer [2].

3. *H. pylori* Virulence Factors

Besides phylogenetic differences between host and pathogen, there are specific strain differences that have been associated with increased risk of gastric disease. *H. pylori* strains that harbor a 40 kb genomic island termed the "*cag*-pathogenicity island" (*cag*-PAI) have been associated with increased risk of gastric disease outcome [21]. The *cag*-PAI encodes a type IV secretion system (*cag*-T4SS) which is a macromolecular nanomachine that spans both the inner and outer membrane of *H. pylori*. The *cag*-T4SS functions to transport substrates, such as peptidoglycan, and effector molecules, such as the oncogenic cytotoxin CagA, from the bacterial cytoplasm into the host epithelial cell. The activity of the T4SS has multiple effects on the host including nuclear factor κB activation, IL-8 chemokine secretion, host cytoskeletal rearrangement, and recruitment of innate immune cells to the site of infection [22–25]. In addition to the *cag*-T4SS cytotoxin secretion, *H. pylori* also secretes a pore-forming cytotoxin, VacA [26]. VacA is an 88-kDa protein that is secreted through type V, or autotransporter secretion pathway [27]. It causes a variety of alterations in target cells including vacuolation, depolarization of membrane potential, permeabilization, disruption of endosomal and lysosomal trafficking, autophagy, programmed necrosis, and immune modulation including inhibition of T cell activation and proliferation. Interestingly, VacA and CagA appear to have antagonistic properties: CagA is highly proinflammatory, while VacA is immunosuppressive, and VacA induces CagA degradation via autophagic pathways [22, 27, 28]. Interestingly, both VacA and CagA are often coregulated in response to nutritional signals, indicating that *H. pylori* has evolved to utilize both of these toxins in concert under certain nutritional stresses [29]. Together, these two cytotoxins promote *H. pylori*-dependent pathogenesis.

Additionally, *H. pylori* utilizes a repertoire of outer membrane proteins to facilitate host-pathogen interactions. The adhesin BabA binds mucosal ABO/Lewis-B blood group carbohydrates and consequently facilitates adhesion to gastric surfaces. Adherence to the gastric mucosa and/or epithelial surface is a critical first step in colonization and ultimately aids bacterial virulence by promoting the interaction of the *cag*-T4SS with host cells [30, 31]. Another adhesin, SabA, binds to laminin and sialyl-dimaric-Lewis $\times$ glycosphingolipid receptor and is a member of the BabA protein family [32]. Upon binding to the receptor, SabA promotes hemagglutination via sialyl-Lex binding, a process that is critical for survival within the hostile gastric environment [33]. Additionally, *H. pylori* outer membrane protein and Hop-family proteins such as outer membrane inflammatory protein A (OipA, encoded by *hopH*) or HopZ protein are both required for gastric epithelial cell binding [33]. Although the host receptors for these proteins have not yet been identified, both proteins have been implicated in inflammation and/or carcinogenesis [34, 35]. Interestingly, there is a high degree of variation in the sequence of CagA, VacA, BabA, SabA, OipA, and HopZ, indicating that *H. pylori* adapts to its host by modifying the repertoire of virulence factors to accommodate niche-specific challenges [36].

4. *H. pylori* and Nutrition

In addition to host or strain genetic differences, environmental factors, such as host diet, are emerging as important components of the ecology within the gastric environment. It is likely that the gastric environment is highly influenced by host nutrient intake. Epidemiological studies have revealed that dietary habits such as high intake of green tea, fruits, or vegetables are protective against gastric cancer risk [37–39]. Conversely, case-controlled and cohort studies reveal that high intake of red meat and/or processed meat (which are high in transition metals) and preserved foods (pickled, dried, smoked, or salted) which are often high in salt is associated with increased risk of noncardia gastric cancer [40, 41]. Furthermore, the advent of refrigeration has radically changed the manner in which food is prepared for storage. Case-controlled population studies have demonstrated that access to refrigeration is protective against gastric cancer [42]. This is attributed to the fact that refrigeration leads to prolonged access to fresh foods such as fruits and vegetables, which would otherwise be unavailable. It is hypothesized that carotenoids, folate, vitamin C, and phytochemicals from fruits and vegetables have a protective role against carcinogenesis. Conversely, salt and the availability of some transition metals can alter *H. pylori* virulence and accelerate carcinogenesis [43, 44]. The contribution of these individual micronutrients to *H. pylori*-dependent diseases will be reviewed in detail below.

4.1. Salt. Gastric cancer is the third leading cause of death from cancer worldwide. While large geographic and ethnic differences in gastric cancer incidence exist, a common risk factor for gastric cancer development is high levels of dietary salt intake. A meta-analysis of studies analysing the association between diets rich in salt and gastric cancer risk concluded that salt consumption is directly associated with the risk of gastric cancer [45]. Furthermore, the risk of developing cancer increases with increased salt ingestion in a dose-dependent manner [46]. Studies included in this meta-analysis looked at the association between high salt diets and gastric cancer across a spectrum of countries and ethnicities. For example, the meta-analysis included studies which found a correlation between consumption of salty foods, such as miso soup, pickled vegetables, and salted fish within Japanese people, and a study conducted in Norway evaluating the risk of total salt intake and gastric carcinoma. Also included in this meta-analysis are studies which show no correlation between excessively salted foods and cancer; however the strain of *H. pylori* endemic to these regions lacks *cagA* and is associated with a decreased risk of gastric cancer as compared to strains harboring *cagA*. Additional studies indicated that the association between salt consumption and gastric cancer risk was highest amongst individuals who were habitual consumers of high salt foods [45]. The rationale for this association between heavy salt intake and gastric cancer is multifaceted and includes that salt perturbs the integrity and viscosity of gastric mucosa and promotes colonization by *H. pylori* both of which ultimately contribute to increased inflammation and subsequent gastric cell proliferation and endogenous DNA mutations [47–49]. One such study compared gastric tissue morphology of mice maintained on a standard diet compared to mice sustained on a high salt diet and found that animals within the high salt cohort had increased gastric epithelial cell hyperplasia and concomitant loss of parietal cells [49].

While high levels of salt consumption in the absence of *H. pylori* infection are associated with gastric cancer, the alterations to the gastric tissue mediated by high salt intake are further exacerbated by *H. pylori* colonization and drive disease progression. Studies aimed at elucidating the molecular mechanisms responsible for this increased susceptibility to cancer development have revealed a complex relationship whereby increased salt ingestion potentiates *H. pylori* carcinogenesis. In addition to promoting *H. pylori* colonization of the gastric mucosa high dietary salt exacerbates *H. pylori* induced inflammation. Studies performed in a Mongolian gerbil model determined that *H. pylori* infected animals maintained on a high salt diet had increased inflammation when compared to infected animals maintained on a normal diet [50]. The increase in inflammation was assessed using both histological examination of gastric tissue and comparing levels of the proinflammatory cytokine, IL-1β. Importantly, this increase in inflammation mediated by high salt levels was CagA dependent, and animals infected with a *cagA* deficient strain of *H. pylori* had significantly less inflammation, even in the context of high salt [50]. Studies investigating the regulation of *cagA* have found that its expression is increased in response to multiple environmental changes including increases in environmental salt concentrations [51]. In fact, this increase in *cagA* expression was detected *in vivo*, using RT-PCR on gastric tissue samples from infected animals. Accompanying the increase in inflammation, infected animals on a high salt chow were found to have augmented dysplasia and invasive gastric adenocarcinoma [50]. Concomitant with this disruption in tissue architecture and inflammation is an increase in *H. pylori* induced hypochlorydia in animals fed excessive salt [50]. Alterations in salt concentration also enhance production of several *H. pylori* outer membrane proteins notably, including HopQ, which is upregulated in response to high salt stress, and VacA which is upregulated in low salt conditions [52]. Together these studies indicate that increases in salt consumption result in alterations to both the host and *H. pylori* and this constellation of changes stimulates carcinogenesis.

4.2. Iron. Iron is an essential nutrient for nearly every living organism including *H. pylori* [53]. Iron is frequently used as an enzymatic cofactor and plays a critical role in respiration and electron transport [54]. To prevent bacterial growth, the human body exploits this need for iron by limiting bacterial access to this vital metal and sequestering iron intracellularly in a process referred to as nutritional immunity [55]. The majority of iron within the human body is localized within erythrocytes in the form of heme, a tetrapyrrole ring with a coordinated iron center. Heme is then further complexed within hemoglobin [56]. Any extracellular iron is rapidly removed by high-affinity iron binding proteins such as lactoferrin and transferrin [57]. Nutritional immunity

is a dynamic process capable of responding to pathogenic assaults on the host. Iron absorption and distribution are regulated through the hepatic peptide hormone, hepcidin. During the infectious process, inflammation can mediate increases in hepcidin leading to a hypoferremic response that depletes even further the available iron present within the host [58]. Together lactoferrin, transferrin, hepcidin, and numerous other proteins ensure that the human body has an inhospitably low level of iron available to invading bacteria.

While the human stomach is a unique organ in that it experiences large influxes of iron during digestion, the specific niche occupied by *H. pylori* is within the gastric mucosa, an area predicted to have little available iron [59]. *H. pylori* has evolved sophisticated mechanisms to circumvent the host's sequestration of iron and responds to the scarcity of this metal with a coordinated upregulation of iron acquisition systems and virulence factors [59–66]. One way that *H. pylori* mediates gene regulation in response to low environmental iron levels is through the global ferric uptake regulator (Fur), a transcriptional regulator [61–63]. *H. pylori* Fur is unique in that it can bind DNA sequences both when complexed to ferric iron and in its apo form [60]. Consequently, *H. pylori* Fur can regulate gene expression in response to conditions of both high and low iron. Many of the Fur regulated genes that are transcriptionally upregulated upon iron starvation facilitate the acquisition and trafficking of iron within the bacterial cell [60–62]. For example, when iron availability is low *H. pylori* increases the transcription of the high-affinity iron transporters *feA1, fecA2, frpB1*, and *feoB* facilitating an influx of iron into the cytoplasm [60–65]. Additionally, *H. pylori* increases binding of the host chelating proteins, lactoferrin and transferrin, upon iron starvation, both of which can be used as a source of nutrient iron. This increase in lactoferrin and transferrin binding is presumably through increasing transcription of the receptors for these proteins [59]. Together this coordinated upregulation of iron acquisition genes allows *H. pylori* to respond to and survive the iron deplete environment of the human host.

Many pathogenic bacteria coordinate the expression of virulence factors to the detection of changes in iron availability and *H. pylori* is no exception. Two of the most important virulence factors expressed by *H. pylori*, VacA and CagA toxin, are transcriptionally regulated in part by iron [63, 64]. Similar to VacA, once inside the cytoplasm, CagA mediates a cascade of changes within the cell including changes to cell morphology and immune signaling. Importantly, the activity of CagA in concert with VacA has been shown to initiate a perturbation in the inner leaflet of the cell membrane which results in the rerouting of transferrin receptors to the apical surface, ostensibly making all bound transferrin available to the bacterium [67]. Similarly, the human antimicrobial protein lactoferrin, which serves as an iron source for *H. pylori*, has been shown to repress the expression of both *cagA* and *vacA*, indicating that the human antimicrobial response can directly alter *H. pylori* virulence by altering the micronutrient gradient available to this bacterial pathogen [59, 68]. Recently, our work has indicated that the biogenesis and activity of the *cag*-T4SS increase upon iron starvation [65, 66]. Together these findings indicate that regulation of *H. pylori* toxin secretion is mediated by iron availability and that both toxins play a critical role in iron homeostasis.

Iron availability not only modulates expression and deployment of both *vacA* and *cagA*-T4SS *in vitro* but recent research utilizing a Mongolian gerbil infection model indicates that dietary iron levels augment disease progression and cancer development *in vivo*. In this infection model gerbils were maintained on iron replete and iron deplete diets beginning two weeks prior to infection and were maintained on these diets throughout the duration of the infection. Analysis of animals treated with a low iron diet revealed that they had markedly less hepatic iron present, as well as significantly less iron binding proteins, ferritin and hemoglobin, within their serum [66]. Iron levels within gastric tissue were also measured using Inductively Coupled-Plasma Mass-Spectrometry (ICP-MS) which demonstrated that iron concentrations within the replicative niche of *H. pylori* were drastically reduced upon subjection to an iron deplete diet [66]. Together these results confirm that an iron poor diet results in a global decrease in iron stores throughout the body, including the stomach. Within the same study, when comparing the disease outcome of animals fed an iron deficient diet to that of animals maintained on an iron sufficient diet, it was clear that the animals with decreased iron had greater immune cell infiltrate to the site of infection, a more rapid onset of gastritis, and a higher rate of cancer development, compared to the animals maintained on an iron rich diet [66]. The mechanisms driving these differences in inflammation and cancer development were found to be similar *in vivo* as they are *in vitro*, in the fact that the increased inflammation and disease severity are attributable to the deployment of the *cag*T4SS pili [65, 66]. The number of pili found in animals maintained on low iron diets versus high iron diets was determined using SEM to visualize and subsequently enumerate the amount of pili formation under both conditions. Consistent with this finding, strains harvested from iron deplete animals translocated a greater amount of CagA into host cells than strains from animals fed an iron rich diet. Together these data demonstrate that the diet of the host, specifically iron intake, has a large impact on the availability of nutrients for invading pathogens and consequently influences disease outcomes.

The correlation between reduced dietary iron and increased disease severity demonstrated in an animal model is mirrored within the human population. Individuals with low serum levels of the iron binding protein, ferritin, have more severe disease outcomes in the context of an *H. pylori* infection than individuals with adequate ferritin serum levels [69]. The mechanisms by which iron deficiency can arise are varied and include not only diets lacking necessary iron but also blood loss. Some strains of *H. pylori* are associated with hemorrhagic gastritis which may contribute to blood loss and successive iron deficiency. Furthermore, chronic *H. pylori* infection is associated with hypochlorhydria, an increased stomach pH, which may impede iron absorption as iron is more soluble at lower pHs. Importantly, case control studies have shown an inverse relationship between dietary iron intake and gastric cancer suggesting that iron deficiency

arising from both dietary factors and blood loss contributes to cancer progression during an *H. pylori* infection [70, 71].

4.3. Zinc. Similar to iron, zinc is gaining appreciation as a micronutrient that exerts great influence at the host-pathogen interface. Zinc is required for cellular processes in all domains of life, and the mammalian host exploits this requirement by chelating nutrient zinc within host innate immune S100A-family proteins, including EN-RAGE (calgranulin C, S100A12) or calprotectin (MRP-8, S100A8/A9) [72, 73]. This process, termed "nutritional immunity," tightly regulates zinc availability in response to infection and essentially starves the invading prokaryote. Both EN-RAGE and calprotectin are significantly elevated in *H. pylori* infected gastric tissues compared to uninfected tissues, and these proteins primarily localize to polymorphonuclear cells (neutrophils) recruited to the site of infection [72, 73]. The severity of inflammation, specifically the infiltration of neutrophils in response to *H. pylori* infection, was inversely proportional to mucosal zinc levels [74]. The authors conclude that low zinc levels could enhance inflammation, but it is equally plausible that the S100A-family proteins deposited by neutrophils at the site of infection could be contributing to the chelation and subsequent removal of zinc from the gastric mucosa.

H. pylori has a strict nutritional requirement for zinc to grow and both calprotectin and EN-RAGE have been shown to inhibit *H. pylori* growth and viability via zinc sequestration activity [72, 73]. In response to zinc sequestration (by calprotectin or synthetic chelators), *H. pylori* forms tenacious biofilms and alters its lipid A structure [75]. The alterations in lipid A structure under conditions of zinc starvation indicate that LpxF, LpxL, and LpxR enzyme functions are diminished [75]. This results in the presence of a lipid A structure which is penta-acylated and contains both a phosphoethanolamine residue at $1'$-position and a $4'$-phosphate which decorates the outer membrane [75]. These alterations in lipid A structure confer decreased cell surface hydrophobicity which enhances bacterial fitness in the presence of calprotectin. These results indicate that *H. pylori* modifies its lipopolysaccharide endotoxin production in response to nutrient zinc availability to circumnavigate the host immune response [75].

Interestingly, *H. pylori* exposure to EN-RAGE or calprotectin prior to coculture with gastric epithelial cells also results in diminished *cag*-T4SS activity including CagA translocation into host cells and proinflammatory IL-8 chemokine secretion. Additionally, the downregulation of *cag*-T4SS activity is associated with abrogation of *cag*-T4SS pilus deployment, results that were reversed by the addition of an exogenous source of nutrient zinc [65, 72, 73]. Together, these results indicate that *H. pylori* senses nutrient zinc in the gastric environment and has evolved to deploy the *cag*-T4SS in response to the presence of this transition metal. Epidemiological data supports a model in which zinc enhances the carcinogenic *cag*-T4SS activity, as high zinc intake has been associated with gastric noncardia adenocarcinoma [76]. Concordantly, high serum zinc levels and high zinc intake have been associated with *H. pylori* infection and antibody response, respectively [77]. Similar studies in pediatric patients have revealed no association between *H. pylori* and iron or zinc nutritional status but significant association between *H. pylori* infection status and copper nutritional status as determined by serum metal concentrations [78, 79].

Besides regulating endotoxin and cytotoxin secretion, zinc has also been implicated as an important cofactor for urease and nickel-iron hydrogenase (Ni, Fe-hydrogenase), enzymes that are critical for *H. pylori* survival in the low pH of the stomach [80–82]. Zinc is required for dimerization of the chaperone UreG, which participates in nickel trafficking to promote urease activation. The accessory protein UreE utilizes either nickel or zinc as a cofactor which is critical for activity [83]. The metallochaperone, HypA, binds zinc for appropriate structural stabilization and interacts with HypB to deliver nickel to both urease and Ni, Fe-hydrogenase, indicating that zinc is critical for bacterial physiology *in vivo* [84–86].

H. pylori has clearly evolved to experience zinc stress in the gastrointestinal environment due to evidence that this pathogen encodes multiple proteins involved in zinc efflux in its genome. CadA, CznABC, CrdB, and CzcAB proteins protect *H. pylori* from zinc toxicity [87–89]. CznABC efflux function is required for colonization in a gerbil model of *H. pylori* infection. These studies underscore the critical role that detoxification strategies play in bacterial metal homeostasis during pathogenesis. It is likely that *H. pylori* encounters transition metals including zinc in the micromolar range from the host diet [89]. Studies on short term supplementation with zinc sulfate reveal that cohorts maintained on zinc supplementation exhibit less gastritis than cohorts without zinc supplementation. However, bacterial burden was not altered by this addition of zinc, as would be expected with increased zinc toxicity within the bacterial cell [90]. The numerous epidemiological studies of dietary zinc intake and zinc supplementation have yielded heterogeneous results, and a recent meta-analysis concluded that no firm conclusions about dietary zinc could be reached at this time [91]. It is interesting to note that, of the studies which have shown a correlation between zinc intake and *H. pylori*-dependent disease progression, most have been on populations in Asia, which are commonly associated with *cag*-PAI-positive strains of *H. pylori* [16].

4.4. Nickel. In addition to zinc and iron, *H. pylori* requires the transition metal nickel for full virulence. *H. pylori* exploits nickel-containing metalloenzymes such as NiFe-hydrogenase and urease to circumnavigate the low pH environment of the human stomach [92]. Urease, one of the most abundant enzymes in *H. pylori* proteome requires 24 nickel atoms for activity [93]. However, excess nickel in the bacterial cell results in mismetallation of cellular enzymes, which can abrogate physiological activity. Consequently, *H. pylori* manages its cellular nickel economy by tightly controlling both nickel import and export functions [94]. Nickel is transported into the bacterial cell in *Helicobacter* spp. via a NixA permease, and the FrpB4 outer membrane protein in a TonB-dependent fashion [95–98]. Nickel efflux is achieved by the promiscuous CznABC transporter, which promotes nickel resistance in *H. pylori* cells [89]. It is likely that *H. pylori* will encounter micromolar concentrations of transition

metals such as nickel from host diet, which could ultimately influence the activity of these critical enzymes and nickel homeostasis functions [89]. Recent work by Campanale et al. indicates maintenance of patients on a nickel-free diet enhanced *H. pylori* eradication rate, supporting the essentiality of nickel for *H. pylori* pathogenesis [92].

5. Microbiome

Chronic *H. pylori* colonization leads to dramatic changes within the gastric environment including a reduction in parietal cells and subsequent increases in stomach pH and altered nutrient availability and local immune responses. Together, these *H. pylori* mediated changes in gastric physiology and immunology likely induces perturbations in the microbiome composition. While *H. pylori*-associated changes in microbiome structure are not fully understood, recent advances in both DNA sequencing and computational analysis have revealed an exceptionally complex microbiota in the human stomach. *H. pylori* colonization in specific pathogen-free female BALB/c mice leads to a decrease in the quantity of *Lactobacillus* species within the gastric microbiota when compared to noninfected mice [99]. In contrast *H. pylori* infection did not significantly alter the overall stomach microbiota composition within female C57BL/6N mice. Infection models using Mongolian gerbils found that *H. pylori* colonization altered both the number and localization of indigenous gastric microbiota ultimately leading to more severe gastritis [100]. Analysis of the microflora following a 12-week infection found dramatic differences in composition including the appearance of *S. aureus* and Enterococci and a decrease in number of Lactobacilli as well as an increase in number of *Bacteroides* [100]. Furthermore, gerbil studies have shown that *H. pylori* infected animals had alterations in the distribution of Bifidobacteria which was greater in the corpus than the antrum when compared to uninfected animals. Similarly, Aebischer et al. found that the stomachs from *H. pylori*-positive animals were colonized by bacterial species typically confined to the lower gastrointestinal tract [99]. Underscoring the complex relationship that exists between *H. pylori* and the indigenous microflora are studies indicating that some resident microbes may inhibit *H. pylori* growth, specifically Lactobacilli spp. [101, 102]. Discrepancies between studies may be because the ability of *H. pylori* to alter the stomach microbiome is influenced by the species of animal used, genetic background of the animal, specific strain of *H. pylori*, and length of infection. Together these findings indicate that *H. pylori* mediates changes to the host both directly as discussed previously and indirectly by altering the composition and distribution of its natural microbiota.

There are a limited number of studies investigating what effect *H. pylori* has on the microbiome within the human host. One analysis found that the microbial profiles of patients infected with *H. pylori* had increased numbers of non-*Helicobacter* Proteobacteria, Spirochetes, and Acidobacteria as compared to *H. pylori*-negative patients [103]. However, another study examining the effect of *H. pylori* colonization on the gastric microflora showed that *H. pylori* infection causes a shift in the microbiome such that there is an enrichment of Proteobacteria and a decrease in Actinobacteria [104]. Discrepancies in findings may be attributable to variations in bacteria surveillance techniques. As sequencing and analysis technologies improve and become more accessible the perturbation of host flora caused by *H. pylori* colonization will become more clearly defined.

In addition to defining how *H. pylori* alters the composition of the resident microbiome another issue to be resolved is elucidating what impact *H. pylori* mediated dysbiosis has on disease outcome. Specifically, it remains unclear if the gastric microbiota induces a more virulent *H. pylori* or if *H. pylori* induced changes in gastric flora promote carcinogenesis. In the transgenic insulin-gastrin (INS-GAS) mouse model of spontaneous gastric cancer, *H. pylori* drove disease progression and the development of intraepithelial neoplasia. Mice given antibiotics 8 weeks after infection to eradicate *H. pylori* had neoplasia significantly less than mice who received the antibiotics at 12 and 22 weeks after infection. Interestingly, *H. pylori*-free mice given similar antibiotics also displayed a decrease in the development of neoplasia. Taken together these observations indicate that gastric atrophy mediated by *H. pylori* or other factors predisposes to gastric carcinogenesis. The finding that earlier antibiotic treatment was more protective against gastric cancer both in the presence and in the absence of *H. pylori* may be attributable to the eradication of additional, unidentified cancer-potentiating microbes [105]. These findings are further supported by research which showed that germ-free INS-GAS mice had delayed onset of both gastritis and neoplasia compared to specific pathogen-free INS-GAS mice. In the same study *H. pylori*-monocolonization was found to accelerate disease progression resulting in early onset of neoplasia as compared to germ-free mice; however the gastritis was delayed and less severe than *H. pylori* infected mice that maintained a diverse microbiota [106]. The mechanism by which *H. pylori*-associated dysbiosis induces disease progression remains poorly defined. One rationale is that changes in the microbe community include increases of nitrosylating bacterial species which convert nitrogen compounds in gastric fluid to carcinogens such as N-nitrosamines or nitric oxide. Additionally the overgrowth of some bacteria may result in increases in DNA-damaging reactive oxygen species and reactive nitrogen species, which are potent mutagens and can contribute to gastric cancer. Lastly, the dysbiosis created by *H. pylori* may promote host inflammatory responses and accelerate metaplasia, atrophy, and cancer.

6. Conclusions

The intersection of host genetics, immune response, bacterial virulence expression, diet, micronutrient availability, and microbiome structure and composition undoubtedly influence the disease outcomes associated with chronic *H. pylori* infection. However, the complex relationship that each of these variables has with each other remains poorly defined. Future studies will seek to determine how these dynamic factors influence each other and can be exploited to

ameliorate disease risk and promote gastric health as the age of antibiotics begins to wane.

Disclosure

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Molecular Diversity of Sapovirus Infection in Outpatients Living in Nanjing, China (2011–2013)

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Aim. To gain insight into the molecular diversity of sapovirus in outpatients with acute gastroenteritis in Nanjing, China. *Methods*. The specimens from outpatients clinically diagnosed as acute gastroenteritis were detected by real-time PCR; RT-PCR was then performed to amplify part of VP1 sequences. The PCR products were cloned into pGEM-T Easy vector and bidirectionally sequenced. All sequences were edited and analyzed. A phylogenetic tree was drawn with the MEGA 5.0 software. *Results*. Between 2011 and 2013, 16 sapovirus positive cases were confirmed by real-time PCR. The infected cases increased from two in 2011 and six in 2012 to eight in 2013. The majority was children and the elderly (15, 93.75%) and single infections (15, 93.75%). Of the 16 real-time positive specimens, 14 specimens had PCR products and the analysis data of the 14 nucleic sequences showed that there was one GI genogroup with four genotypes, two GI.2 in 2011, three GI.2, and one GI.1 in 2012 and one GI.2, three GI.1, two GI.3, and two GI.5 in 2013. *Conclusion*. Our data confirmed continuous existing of GI genogroup and GI.2 genotype from 2011 to 2013 in Nanjing and the successive appearance of different genotypes from outpatients with gastroenteritis.

1. Introduction

Human calicivirus (HuCV) consists of noroviruses (NoVs) and sapovirus (SaV); they are important pathogens that are involved in nonbacterial acute gastroenteritis [1–3]. In a previous study our lab showed that HuCV has become the main pathogen of nonbacterial acute gastroenteritis in Nanjing after 2012 and replaced rotavirus as the predominant pathogen in 2013 [4].

HuCV, members of the family Caliciviridae, have 7.5-kb to 7.7-kb single-stranded genome of positive-sense RNA, which contains two or three open reading frames (ORFs). NoVs have three ORFs; ORF2 codes for the major capsid protein (VP1) with the highest degree of sequence variability in the genome. SaV has two ORFs and ORF1 codes for VP1, which also has a high degree of sequence variability. VP1 is the most important protein with diversity. We have analyzed the strain diversity of 75 strains of NoVs, genogroup II from 2010 to 2013 in Nanjing, and have found evolutionary evidence for the emergence of new GII.4 subclusters (2012 Sydney/AU) that gradually displaced previous GII.4 viruses in the population (2006b). Other scholars have also studied the strain diversity of sapovirus and found continuous existence of a single genotype in one region and successive appearance of genomically diverse sapovirus strains from patients with gastroenteritis in other countries or regions [5, 6]. However, very little is known about the circulating genotype or genomical diversity of sapovirus in Nanjing, China.

To understand the genomical diversity of sapovirus among sporadic cases in Nanjing, we analyzed sporadic sapovirus specimens collected by Nanjing Municipal Center for Disease Control and Prevention from April 2011 to October 2013.

2. Materials and Methods

2.1. Real-Time RT-PCR. Viral RNA was extracted as described in a previous paper [4]. For detecting human

sapovirus, the primers and probe targeted a conserved region of the RNA polymerase [7], which was used in 25 μL reaction volume with the Invitrogen Superscript III One-Step q-RT-PCR System in ABI 7500 FSAT SDS as described in a previous report [4]; the amplification results were determined, as in the same report [4].

2.2. RT-PCR and Sequencing of Sapovirus. A 358 to 364-nucleotide (nt) region of the 5′ end of the VP1 gene of 14 strains was amplified with primer set S1 and S2 using the Invitrogen SuperScript III One-Step RT-PCR System (Invitrogen Inc., Carlsbad, CA., USA). The forward primer S1 (5′-TA GTG TTT GAR ATG GAG GGY-3′) and reverse primer S2 (5′-CGG RCY TCA AAV STA CCB CC-3′) targeted positions 5159 to 5516 of reference strain X86560.1, Sapovirus-Manchester. The reaction was conducted with an initial RT step at 50°C for 30 mins, followed by PCR activation at 94°C for 2 mins and then 35 cycles of amplification (15 s at 94°C, 30 s at 48°C, and 30 s at 68°C) and a final extension step for 5 mins at 68°C in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The RT-PCR products were purified, cloned, and sequenced as described in a previous report [4]; one direction sequencing entirely covered another direction.

2.3. Sequence Edit and Analysis. All sequences generated in this study were edited and analyzed as noted in a previous report [4]. A phylogenetic tree was drawn with the software MEGA 5.0 [8]. For the MEGA analysis, the neighbor-joining method [9] was used as the statistical method; 10,000 replicates were tested for bootstrap analysis [10]. The evolutionary distances were computed using the Kimura 2-parameter method [11] and are in units of the number of base substitutions per site. The translated amino acid sequences were aligned using the Clustal W method algorithm in the MEGA 5.0 (available at: http://mega.software.informer.com/5.0/). Phylogenetic trees were displayed with Tree-View software (available at http://softadvice.informer.com/Treeview_32_Free_Download.html).

2.4. GenBank BLAST Search for Additional SaV GI, GII, GIV, and GV Sequences. To compare GI.1 sequences, GI.2 sequences, and GI.3 sequences from our study with reference sequences that have been detected globally, a GenBank BLAST search was conducted with parts of VP1 sequences that were generated in this study (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) [12, 13]. Reference sequences from the NCBI website (US National Library of Medicine National Institutes of Health; http://www.ncbi.nlm.nih.gov/) were selected for drawing the phylogenetic tree; they are Sapovirus-Manchester (X86560), Sapovirus Parkville (U73124), and others as shown in the phylogenetic tree.

2.5. Nucleotide Sequence Accession Numbers. The VP1 sequences identified in this study were submitted to GenBank and have been assigned accession numbers, KM282587–KM282600.

3. Results

3.1. Single Genogroup and Successive Appearance of Different Genotypes of SaVs among Outpatients in Nanjing 2011–2013. Between 2011 and 2013, the Nanjing Municipal Center for Disease Control and Prevention confirmed 2, 6, and 8 sapovirus positive cases by real-time RT-PCR, respectively. For these positive cases, the majority (15, 93.75%) was single infections and only 1 case (6.25%) was coinfected with rotavirus. There were 15 cases (93.75%) of children and the elderly, including 13 children younger than 5 years old and 2 adults older than 60 years old; there was 1 case (6.25%) of an adult between 18 to 60 years old. Of the 16 real-time positive specimens, PCR products were obtained from 14 specimens and nucleic sequences were determined. Among the 14 strains, there was one genogroup GI with four genotypes, including four GI.1, six GI.2, two GI.3, and two GI.5. There was only one genotype, two GI.2 in 2011. There were two genotypes, three GI.2, and one GI.1 in 2012. In 2013, there were four genotypes, one GI.2, three GI.1, two GI.3, and two GI.5.

3.2. Phylogenetic Relationships among SaV Strains. The phylogenetic analysis using the 358 bp to 364 bp nucleic acid sequences of 14 strains of HSVs showed different genotype diversity during different years and wide distribution of the same genogroup GI. For example, the 5 GI.2 strains from 2011 to 2013 in Nanjing had nucleotide identity level of 98%–100% between each other, 94%–100% compared to those of 15 GI.2 reference strains, and 94%–96% compared to two reference strains U95644.1|Sapporo virus-Houston/90 and U73124|Sapporo virus-Parkville. However, 4 GI.2 strains of 2011 and 2012 had nucleotide identity level of 99%-100% compared to outbreak strains from Japan, AB518056.1|Sapovirus Hu/Oshima1/2009/JP, AB894245.1|Sapovirus Hu/Ishigaki/35/2012, and AB894247.1|Sapovirus Hu/Ishigaki/37/2012, outbreak strains from Taiwan, EU124657.1|Sapporo virus Hu/SaV/9-5/Taipei/07/TW, and sporadic strains from Brazil, AB614356.1|Sapovirus Hu/G1.2/BR-DF01/BRA/2009 and KF924388.1|Sapovirus Hu/G1.2/VIG-AM-111209/BRA/2010; and 1 GI.2 strain of 2013 had nucleotide identity level of 99% compared to outbreak strains from Hungary, FJ844411.5|Sapovirus Hu/G1.2/Kecskemet/HUN3739/2008/HUN. The 4 GI.1 strains of 2012 and 2013 had nucleotide identity level of 96%–99% compared to those of 17 GI.1 reference strains and 98-99% compared to three strains, AJ251991.1|Human calicivirus strain Hu/SLV/Lyon/30388/98/F (98%), HM195198.1|Sapovirus Hu/GII.1/Jimei/Xiamen/2010/CHN (99%), and AY646854.2|Sapovirus Chanthaburi-74/Thailand (98%). Another example, the GI.3 strains of 2013 had nucleotide identity level of 90%–99% compared to 3 GI.3 reference strains, 99% compared to outbreak strain AB518057.1|Sapovirus Hu/Oshima8/2009/JP, and 90%-91% compared to other three reference strains. For the 2 GI.5 strains of 2013, they had nucleotide identity level of 99% between each other, and 98% compared to strain AB253740.2|Sapovirus Hu/Yokote1/06/JP, and 97% compared to strain DQ366345.1|Sapovirus Hu/Ehime643/March 2000 (Figure 1).

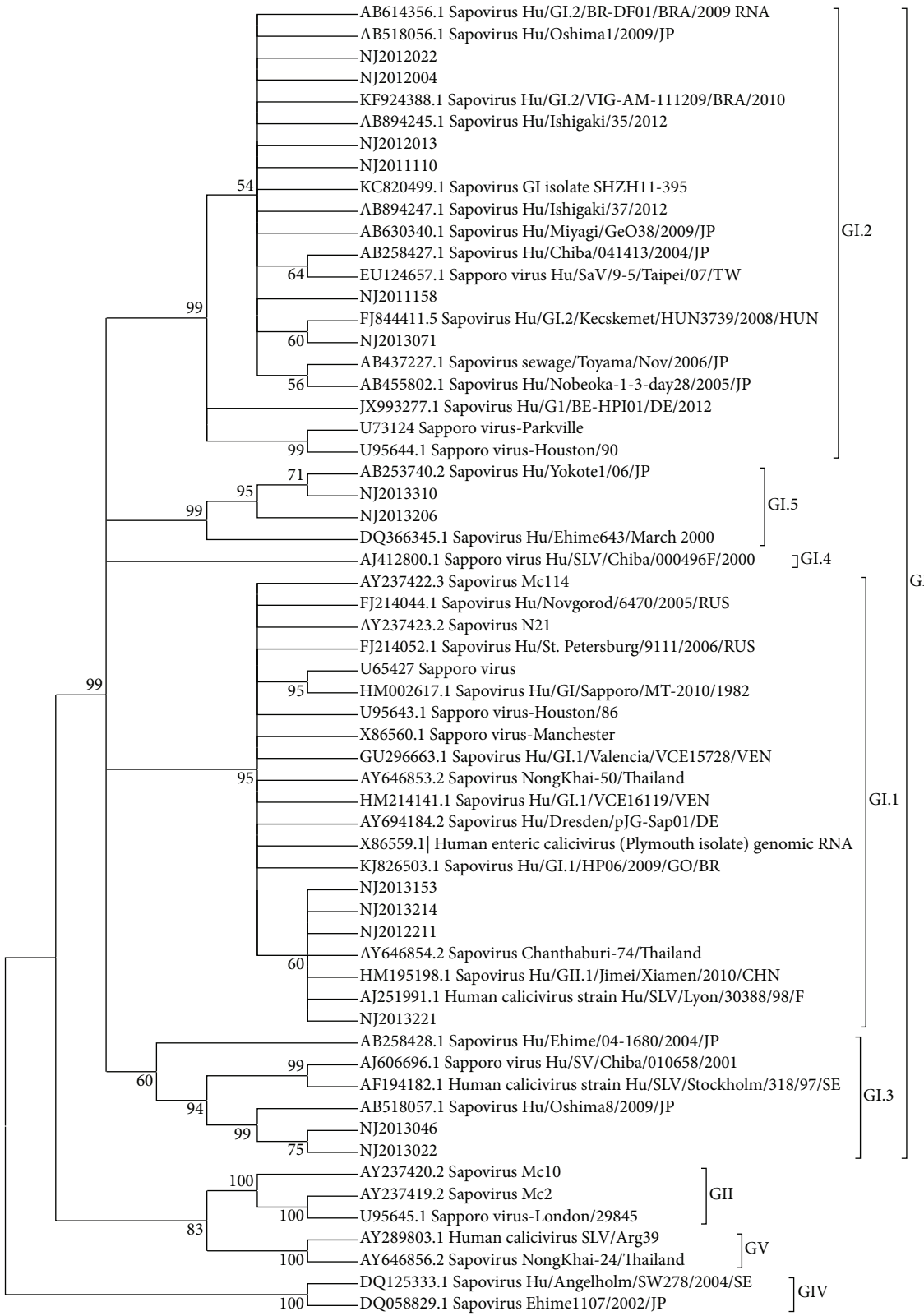


Figure 1: Phylogenetic analysis of the partial N terminal capsid gene (358 bp to 364 bp) of SaV strains identified in outpatients in Nanjing, China, between April 2011 and October 2013. The tree was constructed on the basis of the Kimura 2-parameter and neighbor-joining methods with MEGA5 software (http://www.megasoftware.net/) as described in Methods. The analysis involved 60 nucleotide sequences, 14 strains found in Nanjing presented as the year of detection and strain number, and 46 additional worldwide sequences presented with the NCBI accession number and details. GI, GII, GVI, and GV were genogroups, and GI.1, GI.2, GI.3, GI.4, and GI.5 were genotypes.

3.3. Molecular Diversity of SaVs GI Genotypes in Nanjing. Sapovirus GI.2 genotype was continuous existing throughout 2011, 2012, and 2013. The substitution specific for Nanjing strains was located at S406T (Figure 2, in red), according to the location of the reference sequence of Sapporo virus-Houston/90. It seemed that there was an accumulated variation for the GI.2 genotype in 2013, with at least one more substitution compared to other reference strains (Figure 2(a)). In 2013, more genotypes merged and there were more substitutions. In addition, except for ten substitutions existing in other strains, the Nanjing GI.3 strains had two more substitutions located at S113T (Figure 2(b), in red) and V116A (Figure 2(b), right bottom, in pink), according to the location of the sequence of reference strain Hu/SLV/Stockholm/318/97/SE. The Nanjing GI.1 genotype had no specific substitution; however four substitutions P1817S (Figure 2(c), in pink), S1819A (Figure 2(c), in blue), S1831T (Figure 2(c), in red), and V1834A (Figure 2(c), in green) apparently existed in other strains, like an accumulation, according to the location of the genome for Sapovirus-Manchester. The Nanjing GI.5 genotype had a substitution located at S1831T (Figure 2(d), in red), according to the location of the reference sequence of Sapovirus Hu/Ehime643/March/2000. There seemed to be more diversity among GI.3 genotype than among other genotypes. In short, of the 14 sapovirus Nanjing strains, there was one specific substitution of S to T located at the fourth amino acid from bottom (Figure 2, in red).

3.4. Conserved or Variable Sites of HSVs. Were there conserved or variable sites among different human sapovirus genogroups? To answer this question, we further compared the 14 sequences together with other sequences of four genogroups, GI, GII, GIV, and GV. The conserved two groups of sites were marked as boxes. First group was conserved for all HSVs, including four sites longer than two amino acids, which were "VFEMEG," "ATG," "NPYT," and "AGWGG" as marked in the box. Another group was conserved in the same genogroup. For genotype GI, they were "IQSN," "RTFAWNDRMP," and "SLHPNI" as shown in the box. The variable sites were marked by colors. There is a most variable site between 10 and 30 for all genogroups, since they were not only different within GI genogroup, but also different among GIV, GV, or GII genogroups, as showed in Figure 3.

4. Discussion

Our previous studies [4] showed that rotavirus and HuCV accounted for the majority of pathogens for outpatients, about ninety percent each year from 2010 to 2013 in Nanjing; HuCV replaced rotavirus as the predominant pathogen in 2013. For cases induced by the HuCV, the majority was caused by NoVs of genogroup II; the minority was caused by SaV. Our results also showed evolutionary evidence for the emergence of new GII.4 subclusters (2012 Sydney/AU) that gradually displaced the previous GII.4 viruses in the population (2006b) [4]. In the present study, we further analyzed SaV infection and found that the majority of cases were a single infection in children and the elderly. These results were similar to other countries or regions [5–7]. Only one genogroup of GI was found in Nanjing; however, the genotype changed from the unique GI.2 in 2011 to two types GI.2 and GI.1 in 2012 to four types GI.2, GI.1, GI.3, and GI.5 in 2013. This finding implies continuous existence of GI genogroup and GI.2 genotype in Nanjing, consistent with other reports from Japan that certain genotypes can last for long time in one region and that there is successive appearance of genomically diverse SaV strains from outpatients with gastroenteritis [5, 6].

Our results are consistent with those from similar studies. Although the infection of the SaVs was much less common than that of NoVs, the SaVs were distributed broadly. The infection cases of SaVs in Nanjing outpatients were totally 16 from 2011 to 2013, including 2 from 358 cases during 2011, 6 from 310 cases during 2011, and 8 from 375 cases during 2013. The 14 strains of HSVs analyzed in this paper in Nanjing had higher nucleotide identity to strains from Japan, Brazil, Hungary, and other cities in China compared to other references strains. An investigation from 2006 to 2007 in nine provinces in China found 10 SaVs strains in six regions, one GI.1 in Guangxi, one GI.1 in Hainan, one GI.1 in Shanxi, two GI.1 and one GI.3 in Hebei, one GI.1 and one GI.3 in Jilin, and one GI.1 and one GII.3 in Shanghai [14]. Other investigations showed that there were GI.1, GI.2, and GII.1 in Hangzhou during 2009 and 2010 [15]; GI.2 and GII in Shenzhen during 2011 [16]; GI.1, GI.2, and GII.3 in Shanghai during 2011 and 2013 [17]. Further studies are needed to better understand the geographical distribution of SaVs [18].

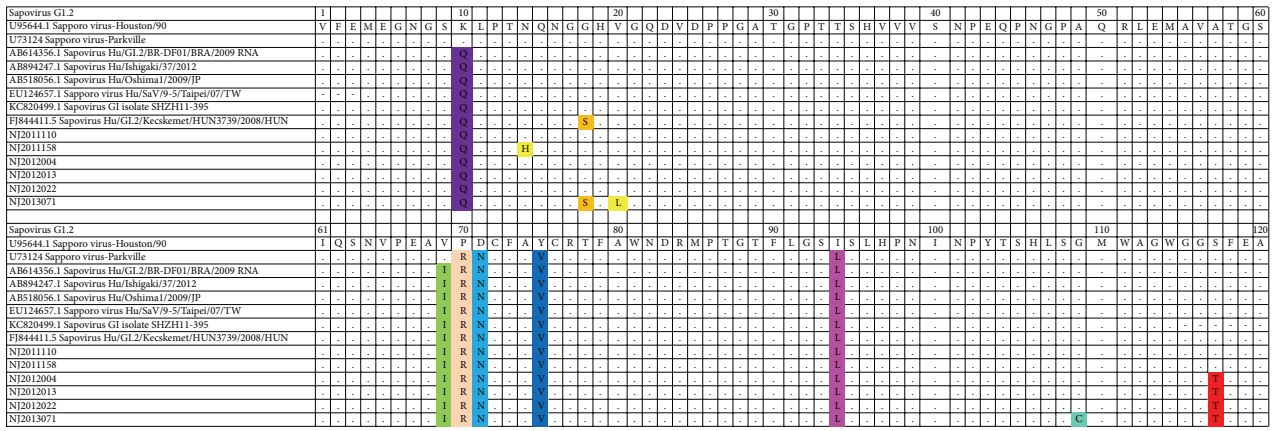
Despite the limited studies on SaV compared to NoVs, there are some significant advances achieved by use of genotyping [19] and the epidemiologic differences between inpatients and outpatients [20]. SaV capsid sequences have been analyzed for identification of the cleavage sites or proteolytic processing [21, 22]. To understand the amino acid changes of SaV strains in Nanjing, the translated amino acid sequences of 119–121 amino acids were compared to the reference strains and different amino acid substitutions were found in the Nanjing strains. In addition, conserved and varied sites were analyzed within GI genogroup or among four genogroups, GI, GII, GIV, and GV. Whether these substitutions are involved in the antigenic changes or the virus fitness to hosts and whether the virus escaped from host recognition require further investigation. Moreover, whether the conserved or varied sites are devoted to antigenic specificity also requires further study.

Competing Interests

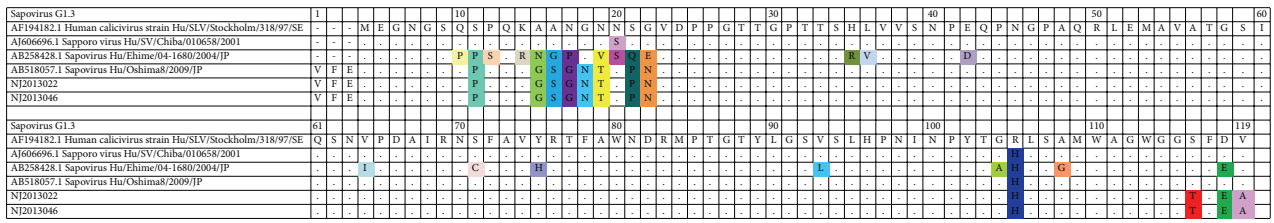
The authors declare that they have no competing interests.

Authors' Contributions

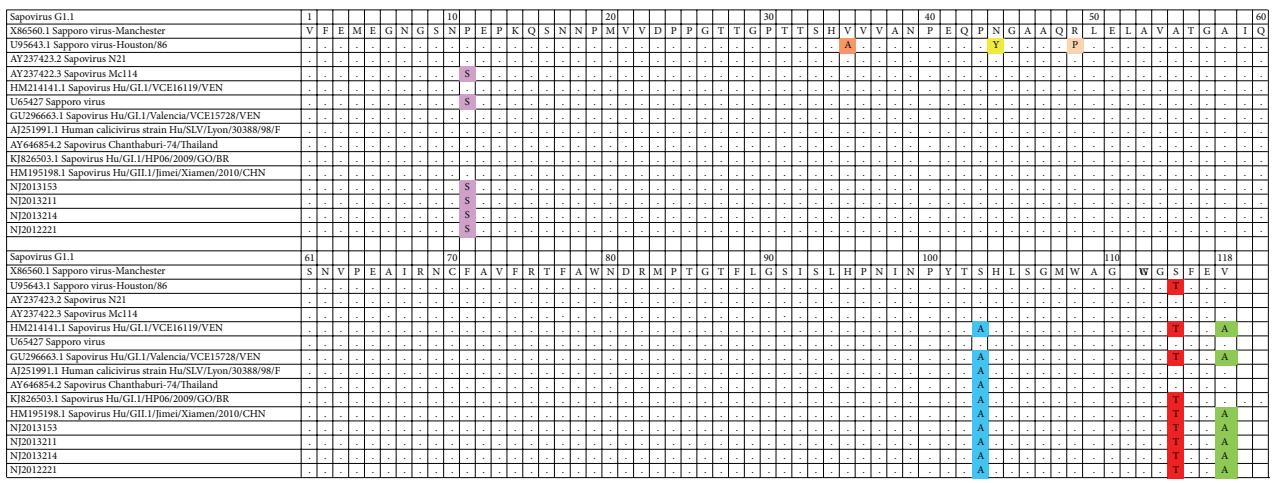
Hong-ying Zhang participated in designation, sequencing, analysis, and writing of the paper; Meng-kai Qiao contributed to real-time PCR; Xuan Wang contributed to real-time PCR and sample handling; Min He contributed to RT-PCR and



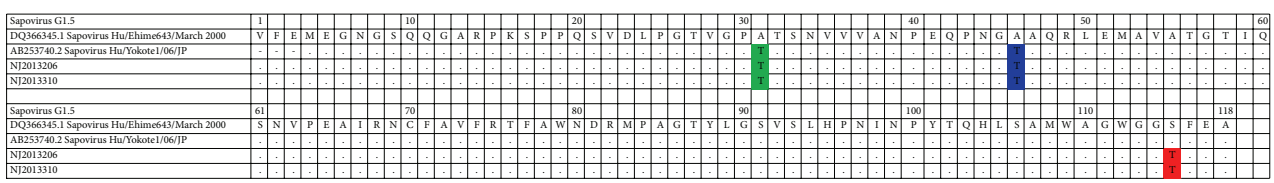
(a)



(b)



(c)



(d)

Figure 2: Amino acid substitutions of Nanjing strains compared to reference strains of (a) Sapovirus G1.2, (b) Sapovirus G1.3, (c) Sapovirus G1.1, or (d) Sapovirus G1.5, respectively. GI.1, GI.2, GI.3, and GI.5 were genotypes. Different colors were used for marking different substitutions in each genotype.

nucleic acid isolation; Li-min Shi contributed to RT-PCR and nucleic acid isolation; Guo-xiang Xie participated in designation and analysis; Hei-ying Jin participated in designation and analysis.

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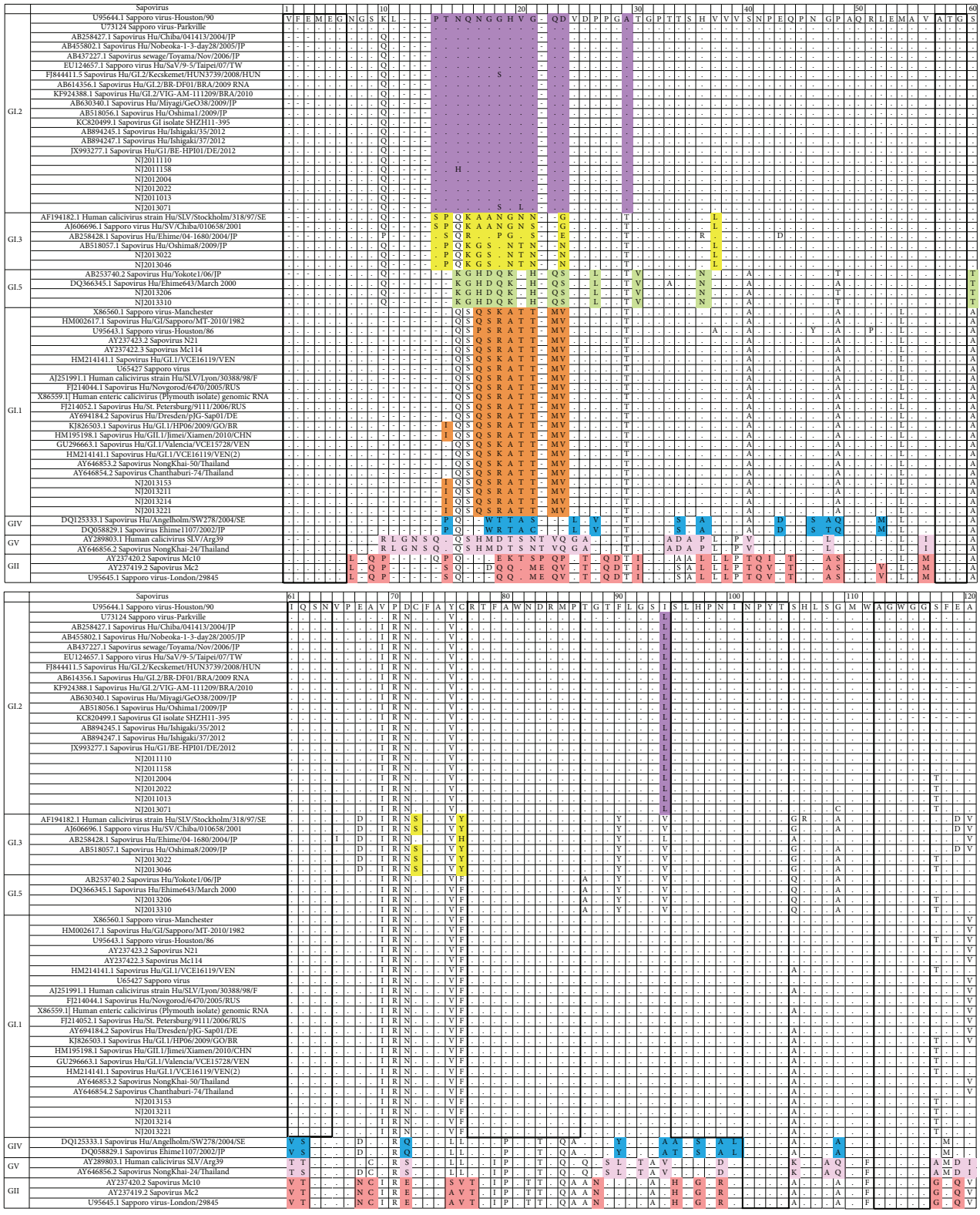

Figure 3: Conserved or variable sites of HSVs and Nanjing strains. The boxes were the conserved sites longer than two amino acids; some were conserved for all HSVs, including "VFEMEG," "ATG," "NPYT," and "AGWGG"; others were conserved sites for genotype GI, including "IQSN," "RTFAWNDRMP," and "SLHPNI." Colors were marked for the variable sites between 10 and 30 amino acids. GI, GII, GVI, and GV were genogroups, and GI.1, GI.2, GI.3, and GI.5 were genotypes.

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Review Article

Research Advance in Intestinal Mucosal Barrier and Pathogenesis of Crohn's Disease

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To date, the etiology and pathogenesis of Crohn's disease (CD) have not been fully elucidated. It is widely accepted that genetic, immune, and environment factors are closely related to the development of CD. As an important defensive line for human body against the environment, intestinal mucosa is able to protect the homeostasis of gut bacteria and alleviate the intestinal inflammatory and immune response. It is evident that the dysfunction of intestinal mucosa barriers plays a crucial role in CD initiation and development. Yet researches are insufficient on intestinal mucosal barrier's action in the prevention of CD onset. This article summarizes the research advances about the correlations between the disorders of intestinal mucosal barriers and CD.

1. Introduction

CD and ulcerative colitis (UC) are inflammatory bowel disease (IBD). As a chronic, nonspecific, and granulomatous bowel disease, CD often occurs in the whole layer of intestinal wall, and, mostly, its lesions are segmentally and asymmetrically distributed. It may appear in any part of the gastrointestinal tract, especially in terminal ileum and adjacent colon [1]. CD has a long course as well as poor prognosis. Moreover, it occurs refractorily and repeatedly. According to the epidemiological investigation [2–7], the incidence of CD is higher in some developed countries in Europe and the United States and is increasing in Asia areas (especially in China). Nowadays, the etiology and pathogenesis of CD have not yet been fully recognized. Various genetic, immunologic, and environmental factors have been proved to be associated with the occurrence and development of CD, among which the immunologic factor is considered to be one of the most important factors [8–11]. The intestinal mucosal barrier dysfunction caused by immune abnormalities and infection is critical in the pathogenesis of CD. In this article, we mainly summarized the research advances about the correlations between the disorders of intestinal mucosal barriers and CD, including mechanical, chemical, immune, and biological barriers.

2. The Structure and Function of Intestinal Mucosal Barrier

Intestinal mucosal barrier is composed of mechanical barrier, chemical barrier, immune barrier and biological barrier, constituting a defensive barrier between the human body and the surrounding environment. The mechanical barrier mainly consists of intestinal epithelial cells and epithelial tight junctions. Tight junction (TJ) is the main connection form between intestinal mucosal epithelial cells, and it also plays an important role in maintaining the integrity of structure and normal function of intestinal mucosal barrier. Chemical barrier is made up of many chemicals such as digestive acid secreted by gastrointestinal, digestive enzymes, lysozyme, mucopolysaccharides, glycoproteins, and glycolipids. Therefore, it is involved in the process of bacteriolysis to inhibit the invasion of pathogenic bacteria. Gut-associated lymphoid tissue (GALT) and secretory immunoglobulin A (SIgA) as well as some special cells (such as macrophages, natural killer cells, and intraepithelial lymphocytes) constitute the immune barrier, which is an important guarantee for the intestinal immunity homeostasis via identifying the autoantigens and exogenous antigens to regulate the immune response. Actually, biological barrier is a mutually dependent and interrelated microecosystem. It is mainly composed of

the resident intestinal flora, among which obligate anaerobe is the dominant bacterial community. Intestinal mucosal barrier is a barrier constituted between the organism and the surrounding environment. Those four barriers have distinguished structures and regulatory mechanism and each plays a different role in biological function. Intestinal mucosal barrier can effectively maintain the balance between pro- and anti-inflammatory factors and prevent pathogenic microorganism from entering into the tissues to keep the body healthy [12–15]. An important component of intestinal homeostasis and inflammation is the integrity of the intestinal barrier and the dysfunction of intestinal mucosal barrier is key to the occurrence of CD; therefore, maintaining the integrity of the intestinal mucosal barrier is of great significance in clinical CD prevention and treatment.

3. CD and Mechanical Barrier

The intestinal epithelial tight junction (TJ) is an important part of the intestinal mechanical barrier, and it is indeed the most essential structure to maintain the function of mechanical barrier. TJ is mainly composed of occludin, claudin, junction adhesion molecules (JAMs), and ZOs [16–18], among which claudin is the main frame protein, as the transmembrane protein in the claudin protein family, claudin-1, always plays a significant role in maintaining the integrity of intestinal epithelial TJ and the normal function of intestinal mechanical barrier [19, 20]. TJ possesses many protein complexes which are able to regulate the paracellular permeability. The intestine infection may be followed by TJ impairment, leading to intestinal epithelial permeability increase and intestinal mucosal barrier damage. This has been recognized as the key process to initiate the intestinal inflammation as well as the immune reaction. IFN-gamma can affect the expression of claudin-2 and occludin proteins through different mechanisms, like inducing the apoptosis of intestinal epithelial cells and destroying the integrity of intestinal epithelial TJ, eventually leading to IBD [21–23]. The aberrant increase of TNF-alpha level in the colonic mucosa of CD significantly reduced expression of occluding, claudin-1, and ZO-1 protein and mRNA and finally resulted in the structure impairment and TJ dysfunction. A new study [24] has also suggested that the inhibition of p38MAPK/p53 signaling pathway can increase the expression of TJ proteins (ZOs, protein-1, and occludin) and alleviate injury to the intestinal mucosal barrier.

4. CD and Chemical Barrier

The mucus secreted by gastrointestinal tract together with various other substances forms the intestinal mucosal chemical barrier, which is the key component of the body's natural immune system. Among all these substances, mucus is the most effective one in protecting the surface of intestinal mucosa. Intestinal mucous layer consisting of goblet cells and mucin (MUC) secreted by intestinal epithelial cells is the first defensive line to resist against extraneous pathogen through protecting and lubricating intestine. The intestinal mucous layer can be divided into external mucous layer which provided a suitable symbiotic environment for the gut microbiota and the internal mucous layer which protected the integrity of intestinal mucosal barrier by preventing microorganism from invading intestinal epithelium. Normally, only when the body is in a disease state caused by some abnormal factors could the bacteria penetrate the internal mucous layer and destroy the intestinal epithelium subsequently. MUC is not only the main component of the intestinal mucous layer but also the most important functional unit in mucus [25]. The mucin in the colorectum can be mainly divided into MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC13, and MUC17, among which MUC2 is the most important one [26, 27]. It has been proven that the allelic polymorphism of MUC1 and MUC2 is closely associated with CD. Moreover, a large number of inflammatory cytokines (such as IL-4, IL-6, IL-13, TNF-alpha, and IFN-gamma) can promote the secretion of MUC in epithelial cells cultured *in vitro* [28, 29]. Studies [30, 31] have shown that MUC2 has direct antibacterial effect by forming the antiprotease substrates to defend the bacterial invasion. In Th1 and Th2 colitis rats model, MUC1 could regulate Th17 immune response and inhibit inflammatory response as Th17 cytokines stimulated MUC1 generation whose negative feedback regulated Th17 generation, so as to downregulate T17 mediated immune response, finally inhibiting the inflammatory reaction [32].

5. CD and Immune Barrier

The immunological factor has been considered to be the key factor in the occurrence and development of CD. Intestinal mucosal immune barrier is essential for maintaining intestinal immune homeostasis. GALT is made up of lymphoid nodule, free lymphoid tissue, plasma cells, and the intestine-related tissue composed of lymphocyte in the epithelium. GALT is an important immune organ to maintain the integrity of intestinal mucosal barrier. SIgA secreted immune globulin with diverse functions and is a main antibody that plays an important role in effects of anti-infection and immunomodulation in defense system of mucosa. A related study [33] found that the level of SIgA expression in patients with CD decreased obviously compared to the normal controls, and its level was negatively correlated with the severity of CD. It can be concluded that the intestinal mucosal immune system will lose the immune tolerance ability when the pathogenic bacteria and its antigen intrude into body; then the pathogen invades the intestinal epithelium and destroys the intestinal mucosal barrier. Paneth cells (PC), which are the typical cells of small intestine, are vitally important components of intestinal mucosal barrier and the main effector cells of small intestinal mucosal barrier. PC contain a variety of antibacterial material such as defensins, lysozyme, and SIgA [34–36], in which both defensins and lysozyme have the spectrum antimicrobial activity and can promote the innate immune response by killing the bacteria and keeping the steady state of intestinal flora [37]. Antibacterial peptide is alkaline peptide and maintains the balance of intestinal flora and the integrity of intestinal mucosal

barrier via interacting with the bacteria in mucosal surface to keep endothelial cells away from being invaded [38–40]. Lysozyme can hydrolyze the peptidoglycan in pathogenic bacteria and change the osmotic pressure between intracellular and extracellular states. Recent researches [41, 42] indicate the therapeutic potential of lysozyme on various systemic inflammatory diseases. The functional lysozyme can also be used as a tracking reagent for microbial population in antibacterial tests. Besides, the nucleotide-binding oligomerization domain 2 (NOD2) expressed in PC could identify the bacterial peptidoglycan and kill the pathogens through the generation of antimicrobial peptide and induction of bacteria autophagy in the cell as well as the modulation of immunity [43, 44]. Researches [45, 46] have shown that the NOD2 gene mutation in CD may increase the susceptibility of the disease through influencing the interaction between ileal microbes and intestinal mucosal immunity. T cell immunoglobulin and mucin domain-3 (TIM-3), the newly discovered T cell immunoglobulin and mucin domain, is expressed specifically and merely on surface of the mature and active T cells. TIM-3 may be involved in the process of regulating T cells proliferation and activation and inhibiting the immune response mediated by Th1 cells [47–49]. TIM-3 plays an important role in chronic inflammatory and autoimmune diseases in humans [50, 51] and is a possible candidate for the treatment of disease in clinic. Simultaneously, TIM-3 also plays a critical role in regulating the activities of macrophages, dendritic cells, monocytes, natural killer cells, mast cells, and endothelial cells. The level of TIM-3 expression in Th1 cells of the intestinal mucosa in CD patients increased more obviously than in healthy persons, as decreasing the expression level of TIM-3 in Th1 cells may provide a new cure for a number of chronic inflammatory diseases in clinical practice [49]. Furthermore, regulating the levels of Th17 and Treg cells in intestinal mucosa could alleviate the intestinal inflammatory response and improve the integrity of intestinal epithelium mucosal barrier via increasing the expression of TJ proteins and mRNA and inhibiting the apoptosis of intestinal epithelial cells [52–55]. The severity of colitis is closely related to the level of IL-18 in intestinal epithelial cells, and, as a microbial modulator, the NOD-like receptor protein 6 (NLRP6) inflammasome can drive the microbial community stability [56–58]. Both IL-18 and NLRP6 inflammasome have key roles in maintaining homeostasis and intestinal barrier function.

6. CD and Biological Barrier

The biological barrier is constituted by normal flora and deposited in intestinal mucosa to maintain the integrity of the intestinal mucosal barrier. Normally, the microecological environment in intestine maintains homeostasis through the interdependence and mutual restrictions between probiotics and pathogenic bacteria. IBD is accompanied with alteration of intestinal flora, which could induce intestinal infection when body is affected by abnormal factors [59–61]. Both the *Bifidobacterium* and *Lactobacillus* are the probiotics. On one hand, they could restrict the pathogenic bacteria; on the other hand, they could repair the damaged mucosal barrier by adjusting the level of inflammatory cytokines. A study had demonstrated that lactic acid bacteria could decrease the levels of IL-6, TNF-alpha, toll-like receptor 4 (TLR4), and NF- kappaB mRNA and increase the level of IL-10 mRNA observably at the same time [62]. In the feces of patients with CD, the amount of *bacteroid*, *Bacillus,* and *Streptococcus* were increased, while the amount of *Bifidobacterium* was decreased [63]. Prebiotics would protect the integrity of intestinal epithelium barrier by promoting the expression of ZO-1 and occludin protein [64, 65]. Studies [66–69] have shown that the normal gut microbiota could prevent bacteria from contacting with the intestinal epithelium, and probiotics could balance the intestinal flora in experimental colitis model of rats through regulating the intestinal mucosal barrier and the levels of related immune cells. Therefore, probiotics may repair the damaged mucosa and maintain the integrity of intestinal mucosal barrier.

7. Conclusion

The mechanical, chemical, immune, and biological barriers play important role in protecting the gut against bacteria homeostasis, regulating the intestinal immune response and reducing the inflammatory response. Yet the comprehensive and systematic researches are insufficient on intestinal mucosal barrier's action in the prevention of CD onset. Therefore, it is of great significance to conduct more thorough studies and randomized controlled trials with largescale, multicentre, and high-quality. In addition, interventions by which to maintain the structural integrity and proper function of intestinal mucosal barrier are expected to be a rational and reliable approach in the prevention of CD in the future.

Competing Interests

There is no potential conflict of interests.

Authors' Contributions

Kuan Wang, Lu-yi Wu, Chuan-zi Dou, and Xin Guan contributed equally to this manuscript with the literature retrieval, sorting, and analysis; Kuan Wang wrote the manuscript; Huan-gan Wu and Hui-rong Liu conducted and revised the manuscript.

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Research Article

Tea and Recurrent *Clostridium difficile* Infection

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Background and Aims. Studies have shown effects of diet on gut microbiota. We aimed to identify foods associated with recurrent *Clostridium difficile* infection (CDI). *Methods*. In this cross-sectional survey, consecutive patients diagnosed with CDI were identified by electronic medical records. Colitis symptoms and positive *Clostridium difficile* assay were confirmed. Health-care onset-health-care facility associated CDI was excluded. Food surveys were mailed to 411 patients. Survey responses served as the primary outcome measure. Spearman's rank correlation identified risk factors for CDI recurrence. *Results*. Surveys were returned by 68 patients. Nineteen patients experienced CDI recurrence. Compared to patients without CDI recurrence, patients with CDI recurrence had more antibiotics prescribed preceding their infection ($p = 0.003$). Greater numbers of the latter also listed tea ($p = 0.002$), coffee ($p = 0.013$), and eggs ($p = 0.013$), on their 24-hour food recall. Logistic regression identified tea as the only food risk factor for CDI recurrence (adjusted OR: 5.71; 95% CI: 1.26–25.89). *Conclusion*. The present results indicate a possible association between tea and CDI recurrence. Additional studies are needed to characterize and confirm this association.

1. Introduction

The incidence of *Clostridium difficile* infection (CDI) is on the rise and has increased by a factor of three over the last decade [1]. In addition, the 30-day mortality rate for CDI is high [2] and the reported recurrence rate currently ranges from 15 to 20% [3]. However, in a recent study, the recurrence rate for CDI decreased after a duodenal infusion of feces was performed [4]. It is hypothesized that a fecal transplant reestablishes equilibrium in a microbiota by restoring specific bacterial species that are lacking. Recent nutrition studies have also demonstrated that diet reproducibly and predictably affects microbiota [5, 6]. Therefore, the aim of this study was to identify foods that may be associated with recurrent CDI in humans.

2. Methods

2.1. Study Design. Institutional review board approval (number 404471) was obtained to review medical records and mail questionnaires to appropriate patients according to a Health Insurance Portability and Accountability Act (HIPAA) waiver.

In this cross-sectional survey, electronic medical records from military clinics and corresponding regional hospitals were reviewed. Records that included an International Classification of Diseases (ICD) code-9 for CDI and a corresponding Current Procedural Terminology code for *C. difficile* assay were used to identify patients with CDI. The latter included an Xpert *C. difficile*/Epi Assay or an Xpert *C. difficile* Assay. Between October 2008 and December 2014, 986 consecutive

Table 1: Characteristics of the study population.

Characteristics of the study population[a]	Recurrence ($N = 19$)	No recurrence ($N = 49$)	p value
Male, N (%)	9 (47)	21 (43)	0.61
Age (years), median (IQR)	62 (55, 72)	63 (51, 74)	0.73
BMI (kg/m^2), median (IQR)	27.8 (25, 30.8)	26.8 (23.6, 30.9)	0.32
IFG or diabetes, N (%)	2 (11)	9 (18)	0.44
WBC[b] ($\times 10^3$/mm^3), median (IQR)	10.3 (7.1, 12.5)	11.30 (6.6, 12.8)	0.95
Creatinine[b] (mg/dL), median (IQR)	0.90 (0.80, 1.01)	0.97 (0.78, 1.38)	0.43
Albumin (g/dL), median (IQR)	3.45 (2.95, 4.40)	3.50 (3.10, 4.10)	0.92
Antibiotic prescribed within 8 weeks prior to CDI, N (%)	18 (95)	35 (71)	**0.038**
Antibiotic prescribed within 4 weeks prior to CDI, N (%)	17 (89)	25 (51)	**0.003**
Prescribed proton pump inhibitor, N (%)	2 (11)	10 (20)	0.35
Prescribed H2 blocker, N (%)	1 (5.3)	4 (8.2)	0.69
Admitted to hospital, N (%)	12 (63)	25 (51)	0.38
Days of hospitalization, median (IQR)	3.5 (3.50, 5.25)	4.0 (3.0, 4.0)	0.59
Initial treatment with metronidazole, N (%)	16 (84)	41 (84)	0.50
Initial treatment with vancomycin, N (%)	2 (11)	8 (16)	0.33
Initial treatment with metronidazole and vancomycin, N (%)	1 (5.3)	0 (0)	0.054

[a]Missing values: WBC = 14, creatinine = 12, and albumin = 15.
[b]The highest value if more than one value present.

patients were found to be treated for CDI. Each medical chart was reviewed individually to confirm colitis symptoms, positive assay, and baseline medical information. Patients with health-care facility-onset health-care facility associated (HO-HCFA) CDI, patients younger than 18 years of age, and patients who had died were subsequently excluded. Patients with HO-HCFA CDI were excluded because these patients were hospitalized for more than 72 h prior to CDI diagnosis and their in-hospital diet would not represent their at-home diet habits.

Therefore, 411 patients were mailed a food frequency and 24-hour food recall questionnaire. The food frequency questionnaire (FFQ) surveyed 19 food items patterned after the Diet History Questionnaire, a FFQ developed by the National Cancer Institute. This was the same format used by National Health and Nutrition Examination Survey III. The survey responses served as the primary outcome measure. The 24-hour food recall was performed only once. The FFQ asked participants to consider the past 12 months of food consumption. Subjects were asked to consider current diet habits. These surveys were mailed several months to years after the patients completed treatment or laboratory evaluation. The diet habits reflected would therefore be after any infection or recurrence of *C. difficile*.

The patients that returned the questionnaire were divided into two groups based on recurrent CDI. Patients with recurrent CDI were defined as those that had a resolution of their CDI symptoms prior to a recurrence of symptoms and also had a second positive *C. difficile* assay > 10 days and < 60 days after their initial positive assay.

3. Statistical Analysis

The Department of Clinical Investigation at San Antonio Military Medical Center analyzed the data. Baseline characteristics and questionnaire responses were analyzed. The method of Kraemer and Thiemann estimated that the sample size was adequate for a power of 80%. Spearman's rank correlation analysis was used to determine significant relationships. Baseline characteristics and food items with p values < 0.05 and correlation coefficient of 0.50 were then included in logistic regression performed with the Hosmer-Lemeshow test.

4. Results

Of the 411 patients contacted, 68 returned the survey provided and 19/68 (28%) had experienced CDI recurrence. Among the baseline characteristics included in our analysis (Table 1), prescription of non-*C. difficile* antibiotic 4 or 8 weeks prior to CDI diagnosis was found to be significantly associated with CDI recurrence. In particular, patients with CDI recurrence tended to have antibiotics prescribed within the 4 weeks preceding their infection compared with the patients without CDI recurrence (89% versus 51%, resp.; $p = 0.003$). Correspondingly, an analysis of the antibiotic prescription data by logistic regression indicated an odds ratio (OR) of 7.81 (95% confidence interval (CI), 1.30–46.8).

A comparison of the 24-hour food recall list for patients with and without CDI recurrence showed that consumption of tea (47% versus 14%, resp.; $p = 0.002$), coffee (53% versus 24%, resp.; $p = 0.013$), and eggs (47% versus 20%, resp.; $p = 0.013$) differed between the two groups (Table 2). However, the logistic regression performed identified mentioning tea as the only food risk factor for recurrent CDI (adjusted OR: 5.71; 95% CI, 1.26–25.89). In the logistic regression analysis performed mentioning coffee on 24-hour recall was not able to predict CDI recurrence (adjusted OR 4.154; 95% CI, 0.969–17.819). Additionally, mentioning egg was not able to predict recurrence (adjusted OR 4.380; 95% CI, 0.986–18.609). After two steps of backward elimination with the Hosmer-Lemeshow test, tea remained significant. This

Table 2: Foods mentioned in 24-hour recall listed by prevalence.

Food mentioned	Number of participants mentioning food		p value
	Recurrence group (%)	No recurrence group (%)	
Fried items	7 (37)	24 (49)	0.816
Chicken	8 (42)	17 (35)	0.285
Cheese	7 (37)	18 (37)	0.497
Breads	9 (47)	16 (33)	0.129
Coffee	10 (53)	12 (24)	**0.013**
Eggs	9 (47)	10 (20)	**0.013**
Yogurt	6 (32)	12 (24)	0.276
Tea	9 (47)	7 (14)	**0.002**
Tomatoes	6 (32)	9 (18)	0.119
Water	4 (21)	11 (22)	0.550
Fish	3 (16)	8 (16)	0.522
Salad	7 (37)	9 (18)	0.054
Potatoes	4 (21)	12 (24)	0.618
Broccoli	6 (32)	7 (14)	0.052
Milk	2 (11)	11 (22)	0.869
Sandwich	4 (21)	11 (22)	0.550
Banana	5 (26)	7 (14)	0.121

indicates that the observed distribution matched the expected distribution of our prediction model. Tea was the only food item able to predict CDI recurrence independent of antibiotic presence in our small study.

The FFQ responses also supported the results of the 24-hour recall responses. In the CDI recurrence group, 19 of 19 drank tea in the past year, compared with 41 of 48 in the group of patients without CDI recurrence (100% versus 85.4%, resp.; $p = 0.044$). The median frequency of tea consumption was also higher in the CDI with recurrence group (18 servings per month versus 6 servings per month; $p = 0.020$). The CDI with recurrence group showed a tendency to drink more coffee; 17 of 19 did so in the past year versus 38 of 49 in the group without recurrence (89.5% versus 77.6%, resp.; $p = 0.106$). The median consumption of coffee was higher in recurrence group (28 servings per month versus 22 servings per month; $p = 0.015$). Unfortunately egg consumption was not assessed on the FFQ.

5. Discussion

Of the 68 patients examined in the present study, those with CDI recurrence were more likely to list consumption of tea, coffee, and eggs in the 24-hour recall survey they returned. When a logistic regression model was applied, both antibiotic use four weeks prior to CDI development and consumption of tea (from 24-hour recall) were factors that predicted CDI recurrence. The results from the FFQ provided additional evidence of the association between tea consumption and CDI recurrence.

In the present study the power was limited to 80% due to sample size. This results in a large 20% risk of type two errors. It is certainly possible that other food items are identified in future studies.

FFQ and 24-hour surveys have been shown to correlate with serum markers of diet intake [7]. However, recall bias and misreporting in surveys are known phenomena [8]. The authors readily admit that the methodology in the present study is inferior to prospective studies in which food consumption is observed. Study design steps were taken to improve data quality. Firstly, the FFQ was patterned after a validated format developed by the National Cancer Institute [9]. Secondly, subjects were asked to recall current diet trends. Lastly, diet was assessed in two different manners, FFQ and 24-hour recall. These mitigations do not make this study preferable to a prospective trial with improved methodologies. The association between tea and CDI recurrence should be confirmed with studies of higher evidence. Additionally, future studies should be prospective, having more inclusive criteria and larger sample sizes.

In the present study both food measurement tools showed tea consumption differences between groups. Another recent study evaluated tea consumption. Their control group consisted of military veterans undergoing elective esophagogastroduodenoscopy or screening colonoscopy. They found 878/1728 (50.4%) were ever tea drinkers [10]. This is compared with our FFQ of the present study. Of those who had CDI without recurrence, 85.4% drank tea in the last year. Of those who had a CDI with recurrence, 100% drank tea in the last year.

It is hypothesized that the survey respondents consumed black tea more often than other types of tea since black tea is the type of tea most often consumed in the United States [11]. However, most respondents listed "tea" without specifying the type or temperature. While the production of black tea

involves the partial oxidation of green tea, many components are similar in both tea varieties [11].

C. difficile is not considered a foodborne threat [12]. *C. difficile* is considered ubiquitous; therefore exposure to this bacterium seems inevitable. Small studies have found that 2.3–7.5% of vegetable samples and 0–62.5% of meat samples contain *C. difficile*[12]. Additionally, *Clostridium* spp. have been found in unpasteurized tea [13]. However, the incidence of CDI is far lower, occurring at an average rate of 147 per 100,000 person-years [14]. Thus, very few of those exposed to *C. difficile* develop CDI.

The population that develops CDI after *C. difficile* exposure is likely unique. For example, they have been shown to have decreased immune response to *C. difficile* toxin among other things [15]. Those who develop CDI and CDI recurrence also may have higher intake of tea. This is suggested in the present study. Tea may be associated with CDI and CDI recurrence not by infecting patients but by promoting an environment where *C. difficile* may colonize and recur.

In a meta-analysis, continued antibiotic use that was not associated with *C. difficile* treatment was found to be the most consequential risk factor for CDI recurrence [3] (OR, 4.2; 95% CI, 2.10–8.55). Tea has also been shown to have antimicrobial effects, and this may explain the identification of tea consumption as a risk factor for CDI recurrence in the present study.

Tea has many individual components that have been shown to be active against human oral flora [16], pathogenic microbes *in vitro* [16], and commensal flora *in vitro* [17]. Most studies describing the antimicrobial activity of tea have been performed *in vitro* or in animal models. One *in vivo* study was performed in 13 healthy human volunteers and showed a decrease of 1.4×10^{10} bacterial cells per gram of wet feces when black tea was consumed [18]. It is likely that this tea's antimicrobial action occurs in the colon based on the results of a study performed with ileostomy patients where 70% of tea flavonols (both parent compounds and metabolites) were detected in the ileal fluid [19]. These results suggest that a large number of tea compounds reach the colon. However, given the paucity of human *in vivo* studies that have examined the effect of tea on microbes [20], many questions remain unanswered.

There are various possible explanations of the current study. It is certainly possible that tea has a causal relationship with CDI recurrence. However that conclusion is beyond the scope of the present study. It is important to note that the food surveys were filled out months to years after patients recovered and were no longer under treatment or laboratory evaluation. As a result, it is possible that diet changes were made after the CDI or CDI recurrence. While a healthy diet has been encouraged in patients with CDI, there is no consensus recommendation regarding what foods to ingest or avoid [21, 22]. The Infectious Disease Society of America identified nutrition as a "research gap" in the colonization of *C. difficile* [23]. It is therefore unlikely that our participants were given consistent or specific advice. They were likely told to eat healthier and interpreted this in their own manner. Those with CDI recurrence may be more likely to begin consuming tea. That is a possible explanation of the present study. Additionally epigallocatechin gallate, an isolate of green tea, was recently shown to suppress virulence and was bactericidal in mice with CDI [24]. The association of tea and CDI recurrence could be causative or curative. Lastly, it should be noted that there was no asymptomatic control group in the present study. Certainly a great majority of persons consume tea without ever developing CDI because the absolute risk of CDI is so low. The present study cannot determine if tea consumption increases one's relative risk for CDI.

In the present study, consumption of tea was identified as a risk factor for predicting CDI recurrence in our logistic regression model. Previously identified risk factors for CDI recurrence have included continued use of non-*C. difficile* antibiotics, age > 75 years, renal failure, and antiulcer medications [3]. In the present study, 15 patients were older than 75 years, 8 patients had a creatinine level greater than 1.5, and 16 patients were on antiulcer medication. It is not clear why age, renal failure, and antiulcer medication were not identified as significant risk factors in the present study, although the exclusion of HO-HCFA CDI patients may have played a role. In addition, because the chart reviews were performed retrospectively, a high quality metric to assess whether the participants continued non-*C. difficile* antibiotics was not able to be established. Rather pharmacy records were reviewed to determine which patients had access to non-*C. difficile* antibiotics.

6. Conclusion

The results of the present study indicate a possible association between CDI recurrence and diet, particularly in regard to the consumption of tea, coffee, and eggs. These data are consistent with the growing body of evidence suggesting that diet affects the microbiota. However, further studies are needed to characterize and confirm the association of tea with CDI recurrence.

Abbreviations

CDI: *Clostridium difficile* infection
HO-HCFA: Health-care facility-onset health-care facility associated
FFQ: Food frequency questionnaire
CI: Confidence Interval.

Disclosure

The views expressed herein are those of the authors and do not reflect the official policy or position of Brooke Army Medical Center, the United States Army Medical Department, the United States Army Office of the Surgeon General, the Department of the Army, and Department of Defense or the United States Government. The corresponding author provided $146.66 (from his personal funds) to Write Science Right for Premium Editing. The scientific writer assigned to the paper was Dr. Annah Rolig. John Ward, Ph.D., Research Physiologist, Department of Clinical Investigation,

San Antonio Military Medical Center, Fort Sam Houston, Texas, performed statistical analysis.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Martin Oman Evans II was responsible for study concept and design and obtained grant funding. Brad Starley was responsible for study concept and design, important intellectual content, and study supervision. Jack Carl Galagan was responsible for acquisition of data and minor editing. Joseph Michael Yabes was responsible for acquisition of data. Joseph John Salama was responsible for final approval and drafting of the manuscript. Sara Evans was responsible for drafting of the manuscript.

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Research Article

Prebiotic Effects of Xylooligosaccharides on the Improvement of Microbiota Balance in Human Subjects

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It has been indicated that probiotics can be nourished by consuming prebiotics in order to function more efficiently, allowing the bacteria to stay within a healthy balance. In this study, we investigated the effects of xylooligosaccharides- (XOS-) enriched rice porridge consumption on the ecosystem in the intestinal tract of human subjects. Twenty healthy subjects participated in this 6-week trial, in which 10 subjects received XOS-enriched rice porridge while the others received placebo rice porridge. Fecal samples were collected at the end of weeks 0, 1, 3, 4, 6, and 7 for microorganism examination. The results showed that 6-week daily ingestion of the XOS-enriched rice porridge induced significant increases in fecal bacterial counts of *Lactobacillus* spp. and *Bifidobacterium* spp., as well as decreases in *Clostridium perfringens* without changing the total anaerobic bacterial counts, compared to that of placebo rice porridge. However, fluctuations in the counts of coliforms were observed in both groups during the 6-week intervention. In conclusion, the intestinal microbiota balance was improved after daily consumption of 150 g of rice porridge containing XOS for 6 weeks, demonstrating the prebiotic potential of XOS incorporated into foods. This also indicates the effectiveness of XOS as a functional ingredient in relation to its role as a prebiotic compound.

1. Introduction

It has been proposed that the dynamic and complex populations of gastrointestinal microorganisms play a pivotal role in human health [1, 2]. The intestinal microbiota not only exert metabolic activities but also participate in the defense against invading pathogens. It has been suggested that disruptions to intestinal microbial balance may lead to diseases including chronic intestinal diseases, colorectal cancer, type 2 diabetes, and obesity [1]. On the other hand, restoring the changed intestinal microbial balance to a more beneficial bacterial population may be beneficial in terms of supporting digestive or human health, which can be accomplished by administering probiotics and prebiotics or a combination of both (i.e., synbiotics) [2, 3].

Probiotics are bacteria that provide health-promoting properties for the host lining of the colon [2]. The most commonly used and/or studied probiotics are largely species of the genera *Bifidobacterium* and *Lactobacillus*, all of which can be found in the host's own microbiota and fermented foods [4]. Evidence suggests that these probiotic bacteria can alleviate lactose intolerance, inhibit the growth of harmful bacteria, prevent colon cancer, decrease cholesterol levels, improve digestion, reduce inflammation, and stimulate the immune system [4, 5]. Therefore, it has been considered that consuming probiotic rich foods (i.e., fermented foods) or supplements may replenish the beneficial bacterial populations, such as the genera *Bifidobacterium* and *Lactobacillus*, for the maintenance of gastrointestinal health or the prevention of diseases.

Prebiotics can nourish probiotics and encourage them to function more efficiently, allowing the bacteria to stay within a healthy balance [3]. They are nondigestible food ingredients, typically oligosaccharides that serve as the fuel for probiotics, allowing these beneficial microorganisms to thrive by going through the fermentation process [3]. Some of

the commonly known prebiotics are fructooligosaccharides, galactooligosaccharides, and lactulose [6–8]. In addition, other types of oligosaccharides, such as isomaltooligosaccharides (IMO) and XOS, are emerging as a potential novel source of prebiotics that can be used as functional ingredients in foods [9, 10]. Of the emerging prebiotic oligosaccharides, XOS have attracted increasing interest because of their health, physicochemical, and technological related properties. XOS are mixtures of oligosaccharides containing β-1,4-linked xylose residues which naturally occur in bamboo shoots, fruits, vegetables, milk, and honey [11]. XOS has been found to be predominantly utilized by members of the *Bifidobacterium* genus [12]. Furthermore, the consumption of XOS results in increased indigenous *Bifidobacterium* spp. levels in the gastrointestinal tract and fecal short-chain fatty acids in rats [13, 14]. However, studies investigating the prebiotic effects of XOS on gastrointestinal microbiota in human populations have been limited. Thus, the objective of the present study was to examine the prebiotic effect of XOS incorporated into rice porridge on the ecosystem in the intestinal tract of human subjects.

2. Materials and Methods

2.1. Subjects. The project was approved by the Taipei Medical University-Joint Institutional Review Board, number 201209023 (TMU-JIRB201209023). Subjects were recruited from Taipei Medical University by advertising on noticeboards on campus. The general health status of all volunteers was assessed by the use of a standard medical questionnaire. Exclusion criteria for participation in the study were a history of gastrointestinal disease and chronic diseases. The subjects were asked to avoid consumption of antibiotics or any food/supplements that may influence the microbiota one week prior to and during the study period. Written informed consent was obtained from every subject before participating in the study. Subjects were instructed to maintain their usual dietary habits and normal lifestyles during the study while being assessed for the restriction of prebiotic consumption during a two-week period prior to the intervention. Baseline fecal samples were taken before the treatment period began.

2.2. Study Design and Treatments. A randomized, placebo-controlled study design was carried out with 20 subjects. The study comprised three phases: a 1-week run-in phase, a 6-week intervention phase, and a 1-week washout phase. During the 1-week run-in phase, all subjects were instructed to maintain their usual diet but to avoid consumption of other prebiotic and probiotic products during the experiment. Twenty subjects were randomly divided into two groups: XOS and placebo. During the 6-week intervention period, the XOS group ($n = 10$) was instructed to consume rice porridge containing XOS (150 g per package containing ~1.2 g of XOS), while the placebo group ($n = 10$) was instructed to consume rice porridge without XOS. The products consumed in both the XOS and the placebo groups were identical in appearance, taste, and color. The experimental and placebo products were consumed with breakfast once daily for six weeks. Fecal specimens from each subject were collected at the end of the run-in phase (week 0), the intervention phase (weeks 1, 3, 4, and 6), and the washout phase (week 7). Subjects were asked to keep a three-day dietary record (2 weekdays and 1 weekend) and a stool frequency and consistency record once a week throughout the whole experiment as a way of examining their adherence to the diet. The experiment timetable is shown in Figure 1.

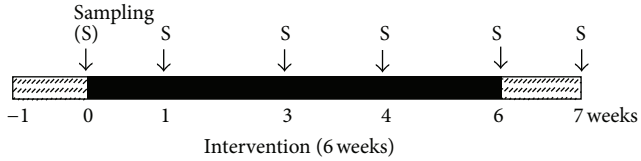

Figure 1: The experiment timetable.

2.3. Sample Collection and Microorganism Analyses. The middle section of a fecal sample from each subject was collected on the last day of weeks 0, 1, 3, 4, 6, and 7 and stored at −20°C for less than 24 h before analysis. Precisely 0.5 grams of the sample and 15 mL of anaerobic solution were thoroughly mixed to form a sample solution. Series of dilutions from 10^{-1} to 10^{-6} were prepared. Microorganism isolation and examination were performed using the methods previously developed [15]. In brief, *Bifidobacterium* spp. were incubated with bifidobacteria iodoacetate medium-25 for 48 hours (hr); *Lactobacillus* spp. were incubated with *Lactobacillus* anaerobic MRS with bromocresol green for 48 hr; *Clostridium perfringens* were incubated with tryptose-sulfite-D-cycloserine agar for 24-hr; coliform organisms were incubated with Endo agar plates for 24-hr; total anaerobic organisms were examined with CDC anaerobic blood agar. When counting colonies, plates with 30–300 colonies were included. The number of bacteria was presented as log CFU/g of wet weight of feces. The calculation formulae are listed as follows. For *Bifidobacterium* spp., *Lactobacillus* spp., coliform organisms, and total anaerobic organisms, the formula is CFU/plate × 20 (50 μL/plate) × dilution factor × 15 mL/sample (g), and, for *Clostridium perfringens*, the formula is CFU/plate × dilution factor × 15 mL/sample (g).

2.4. Statistical Analysis. Data are presented as the mean ± SD. Analysis of variance (ANOVA) with repeated measures and paired t-tests were performed using SAS version 9.1. p values smaller than 0.05 are considered statistically significant.

3. Results

All subjects successfully completed the experiment. The characteristics of the participants are shown in Table 1. No significant difference was observed in age (23.4 ± 1.6 versus 25.0 ± 1.7 years), BMI (19.7 ± 2.4 versus 20.7 ± 2.1 kg/m^2), or sex distribution (2 men and 8 women versus 2 men and 8 women) between the XOS and placebo groups.

There were no significant differences in the amounts of total anaerobic bacteria, *Lactobacillus* spp., *Bifidobacterium*

Table 1: Basic characteristics of subjects.

	n	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)
Placebo					
Male	2	24.5 ± 1.5	170.5 ± 2.7	68.5 ± 1.0	23.3 ± 0.6
Female	8	25.2 ± 1.8	160.8 ± 4.6	51.7 ± 5.5	20.0 ± 1.5
Total	10	25.0 ± 1.7	162.7 ± 5.8	55.1 ± 8.6	20.7 ± 2.1
XOS					
Male	2	24.5 ± 3.5	173.5 ± 2.1	67.0 ± 0.0	22.3 ± 0.5
Female	8	23.1 ± 1.0	159.6 ± 5.7	48.4 ± 4.1	19.1 ± 2.3
Total	10	23.4 ± 1.6	162.4 ± 7.7	52.1 ± 8.7	19.7 ± 2.4

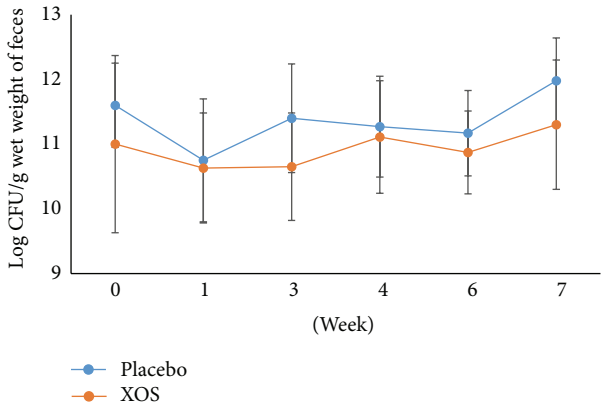

Figure 2: The changes in logarithm number (mean ± SD) of total anaerobic bacteria during the trial. Weeks 0-1: a run-in phase. Weeks 6-7: a 1-week washout phase.

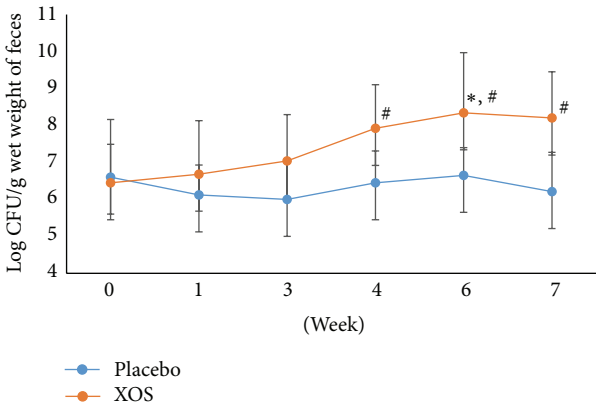

Figure 3: The changes in logarithm number (mean ± SD) of *Lactobacillus* spp. during the trial. Weeks 0-1: a run-in phase. Weeks 6-7: a 1-week washout phase. #Repeated measures ANOVA on different groups across time. *Paired t-test of week 6 versus week 0 within a group. $p < 0.05$.

spp., coliforms, and *Clostridium perfringens* in week 0 (the end of the run-in phase) between the two groups. After the 6-week intervention, as shown in Figure 2, the total anaerobic bacterial counts of the experimental and the placebo groups were not statistically different. In contrast, during the intervention period, the XOS group had significantly higher *Lactobacillus* spp. counts compared to the placebo group participants at weeks 4 and 6 ($p < 0.05$, Figure 3) and even after the 1-week washout period. In the XOS group, at week 6, the number of *Lactobacillus* spp. (Figure 3) was significantly higher than at week 0 ($p < 0.05$).

Bifidobacterium spp. counts remained similar for the XOS and placebo groups during the study except for a significant increase in the XOS group (Figure 4) as compared to the placebo group at week 6 ($p < 0.05$) and the baseline (Figure 4). However, the XOS group showed no significant differences in coliform populations (at weeks 4 and 6) as compared with week 0 (Figure 5). Both groups showed fluctuations throughout the study. In addition, the placebo group demonstrated a trend toward an elevation in coliform populations at week 4 as compared to weeks 1 and 3. Compared to the placebo group, the XOS group had lower populations of *Clostridium perfringens* at week 6 and one week after (Figure 6). However, at week 3, the placebo group showed similar counts for *Clostridium perfringens* to the XOS group at weeks 6 and 7. Yet, fluctuations and sudden increases occurred in the placebo group for *Clostridium perfringens* counts at weeks 6 and 7 compared to week 3 as well as to week 0 (Figure 6).

4. Discussion

In this study, we conducted a randomized, controlled study with the aim of evaluating the prebiotic effects of XOS on fecal microbiota in healthy human volunteers after a period of 6 weeks of daily consumption since the prebiotic evidence derived from human trials is still insufficient. More than 1,000 microbial species are known to inhabit the human GI tract, constituting a complex ecological community, which is also referred to as the intestinal microbiota [16]. This ecosystem contains approximately 10^{14} microorganisms which are predominantly represented by anaerobic bacterial species [17], and the vast majority of these reside in the colon with the populations reaching densities of up to 10^{12} counts per gram of content [16]. Among these microbiota, *Lactobacillus* and *Bifidobacterium* species are beneficial due to their fermentation characteristics, whereas *Clostridium perfringens* is detrimental to health because it is an opportunistic pathogen with the capability of causing food poisoning and necrotic

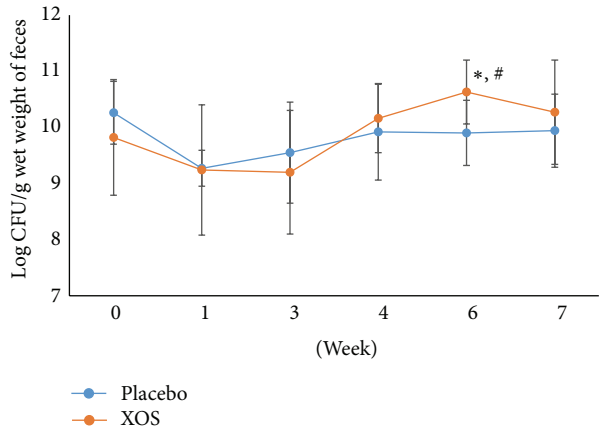

Figure 4: The changes in logarithm number (mean ± SD) of *Bifidobacterium* spp. during the trial. Weeks 0-1: a run-in phase. Weeks 6-7: a 1-week washout phase. #Repeated measures ANOVA on different groups across time. *Paired *t*-test of week 6 versus week 0 within a group. $p < 0.05$.

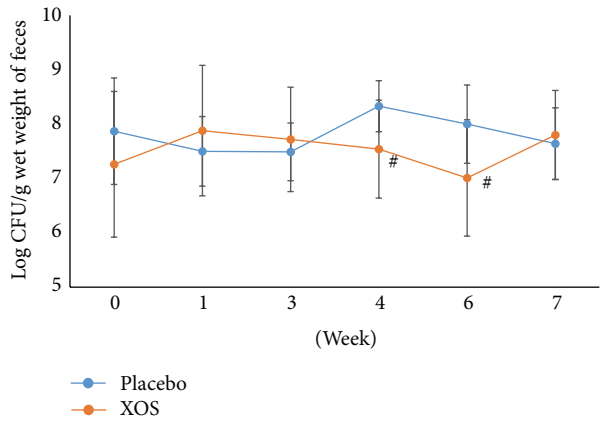

Figure 5: The changes in logarithm number (mean ± SD) of coliforms during the trial. Weeks 0-1: a run-in phase. Weeks 6-7: a 1-week washout phase. #Repeated measures ANOVA on different groups across time. $p < 0.05$.

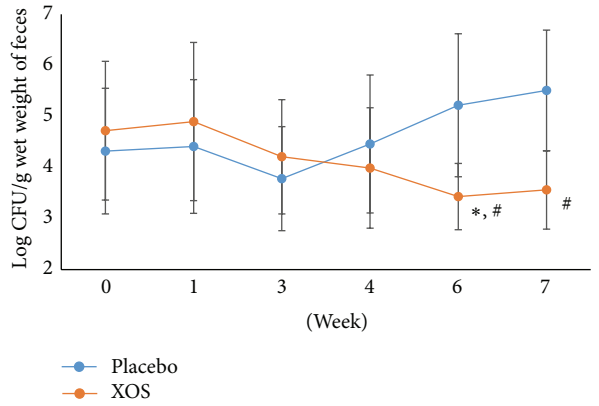

Figure 6: The changes in logarithm number (mean ± SD) of *Clostridium perfringens* during the trial. Weeks 0-1: a run-in phase; Weeks 6-7: a 1-week washout phase. #Repeated measures ANOVA on different groups across time. *Paired *t*-test of week 6 versus week 0 within a group. $p < 0.05$.

enteritis [18]. Studies have demonstrated that the intestinal microbiota are crucial to human health, and alterations in this ecosystem are linked to several diseases such as irritable bowel syndrome, type 2 diabetes, and obesity [1, 16]. Overall, the results suggest that changes in the bacterial growth were mediated by XOS incorporation into the food product. The addition of XOS to foodstuffs has prebiotic potential to alter the composition of the intestinal microbiota that could be relevant to intestinal health. In the present study, the counts of total anaerobic bacteria were unaffected by the addition of XOS to the rice porridge throughout the study, indicating that total anaerobic bacteria numbers remained steady instead of growing abnormally. Furthermore, we observed that consumption of rice porridge containing XOS resulted in a significant increase in the numbers of *Lactobacillus* spp. and *Bifidobacterium* spp. compared with the placebo, demonstrating the prebiotic potential of XOS as a food ingredient. It is well documented that both *Lactobacillus* spp. and *Bifidobacterium* spp. are preferentially able to ferment XOS, thereby utilizing them as an energy source for growth [11, 19, 20]. The prebiotic effect of XOS observed here, particularly the increased *Bifidobacterium* spp., is consistent with a previous intervention study, where capsule supplements containing XOS have been used [21]. However, regarding the observed changes in *Lactobacillus* spp., our result contradicted a previous study, which showed that the number of *Lactobacillus* spp. was unchanged after XOS supplementation [21]. These inconsistent results might reflect differences in the participants being studied or in the methodologies employed.

The increased populations of the health-promoting bacteria after prebiotic administration have been shown to eliminate the presence of pathogenic or potential pathogenic bacteria [7]. In the current study, the levels of fecal coliforms in response to treatments were also examined as this bacterial group is commonly used as an indicator of water contamination and the possible presence of pathogens [22]. However, fluctuating numbers of the fecal coliforms in both groups at weeks 4 and 6 were observed. It was difficult to determine whether these changes were attributed to the dietary intervention (e.g., the rice-based porridge) or other dietary factors, as all participants were allowed to consume their usual diets. Nevertheless, the observed lower number of fecal coliforms in the XOS group suggests that the XOS-containing diet seemed to be better tolerated. Furthermore, a similar response was observed, in which the abundance of pathogenic bacteria, *Clostridium perfringens*, was significantly lower in the fecal samples of the XOS group than in those of the control group. These data could be explained by the XOS suppressing the growth of *Clostridium perfringens*; the mechanisms underlying this effect are likely due to the production of short-chain fatty acids (SCFAs) via the fermentation of XOS in the colon [13, 23]. A decrease in intestinal pH has been reported as a consequence of the increased SCFA production which subsequently inhibits the overgrowth of pathogenic bacteria [24]. However, it should

be noted that the *Clostridium perfringens* counts exhibited a modest upward trend following the placebo treatment in the present study. From this finding, the possibility that rice porridge itself manipulates the growth of *Clostridium perfringens* cannot be ruled out. Previous studies have shown that polished rice ingestion appears to increase the number of fecal pathogenic bacteria, such as *Clostridium perfringens*, compared to unpolished rice [25]. In other words, this suggests that the presence of rice fiber may have the ability to depress the growth of certain intestinal pathogenic bacteria [25]. Additionally, there are some limitations to this study which should be noted. Firstly, the power of this investigation can be increased as the total number of subjects is increased. Secondly, fiber in the diet can have an impact on gut microbiota [26]; however, although we did not accurately measure the total intake of dietary fiber for the comparison between groups, we randomly assigned our subjects into the groups and reminded them not to change their dietary habits. Thus, the amount of fiber ingested should not be an issue in this study.

In conclusion, the intestinal bacterial phase was improved after the daily consumption of 150 g of rice porridge containing XOS for 6 weeks, suggesting the beneficial effects of XOS on intestinal functions and health. Further research on the incorporation of XOS into other kinds of food is warranted.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

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Research Article

Suppressing Syndecan-1 Shedding Ameliorates Intestinal Epithelial Inflammation through Inhibiting NF-κB Pathway and TNF-α

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Syndecan-1 (SDC1), with a long variable ectodomain carrying heparan sulfate chains, participates in many steps of inflammatory responses. But reports about the efforts of SDC1's unshedding ectodomain on intestinal epithelial inflammation and the precise underlying mechanism are limited. In our study, unshedding SDC1 from intestinal epithelial cell models was established by transfecting with unshedding SDC1 plasmid into the cell, respectively. And the role of unshedding SDC1 in intestinal inflammation was further investigated. We found that components of NF-κB pathway, including P65 and IκBα, and secretion of TNF-α were upregulated upon LPS stimulation in intestinal epithelial cells. SDC1, especially through its unshed ectodomain, significantly lessened the upregulation extent. It also functioned in inhibiting migration of neutrophils by downregulating secretion of CXCL-1. Taken together, we conclude that suppressing SDC1 shedding from intestinal epithelial cells relieves severity of intestinal inflammation by inactivating NF-κB pathway and downregulating TNF-α expression. These results indicate that the ectodomain of SDC1 might be the optional therapy for intestinal inflammation.

1. Introduction

Syndecan-1 (SDC1), a member of heparan sulfate proteoglycans (HSPGs) predominantly expressed on the surface of epithelial cells, is mainly composed of a short conserved cytoplasmic domain, a transmembrane domain, and a long variable ectodomain carrying heparan sulfate (HS) chains [1]. The majority of SDC1's functions are mediated by the ligand-binding HS moiety. Studies during the last several decades have shown that SDC1 regulates the activity of many inflammatory responses in a HS-dependent manner [2–4]. Through the HS chains, SDC1 can bind a variety of molecules, including cytokines, chemotactic factors, extracellular matrix components, and even heparin-binding proteins on the bacterial surface. Moreover, it also plays critical roles in leukocyte recruitment, resolution of inflammation, and matrix remodeling [5, 6].

The localization of SDC1 not only is restricted to the cell surface, but also functions as soluble HSPG, which can be proteolytically released from the cell surface by a process known as ectodomain shedding [7]. The ectodomain shedding is activated by several inflammatory factors and occurs as a part of host's responses regulation to inflammation, microbial pathogenesis, and wound healing [8–10]. For example, shed SDC1 facilitated the formation of CXC chemokine gradient to enhance transepithelial migration of neutrophils in a murine model of acute lung injury and inhibited host-derived antimicrobial peptides to promote *P. aeruginosa* infection in an intranasally induced lung infection mouse model [11, 12].

At present, it is appreciated that shed SDC1 plays important roles in the pathophysiology of many disease states; however, the precise underlying mechanism in intestinal inflammatory response is not yet clear. In multiple myeloma, high heparanase expression in osteoblast or stromal cells induced SDC1 shedding and stimulated signaling via HGF/c-met/IL-11 axis. Enhancing the expression of IL-11 and RANKL leads to raising osteolytic bone disease [13]. In allergic lung inflammation, the ectodomain of SDC1 can bind to the CC chemokines, including CCL11 and CCL17, and inhibit CC chemokine-mediated Th2 cell recruitment to the lung [14]. The soluble ectodomain also markedly activates fibroblast growth factor-2 mitogenicity during host response to tissue injury and is involved in morphogenesis and wound repair [15]. In the intestine, shed SDC1 ectodomains from enterocytes induced by TNF-α and IFN-γ was associated with decreased internalization of intestinal pathogenic bacteria [16]. Accompanied by the reduction of SDC1, glutamine can attenuate gut ischemia-reperfusion induced intestinal hyperpermeability, inflammation, and injury in SDC1 KO mice [17]. Existing data has implied the involvement of SDC1 in intestinal inflammation, but the detailed downstream process is sorely needed for better understanding.

In the present study, we constructed the plasmid which SDC1 is not shed induced by PMA, modeled intestinal inflammation *in vitro*, and attempted to elucidate the potential role of NF-κB pathway and cytokine secretion in initiating events of SDC1 shedding. Our data showed that activated SDC1 shedding contributed to intestinal inflammation, and suppressing its shedding from intestinal epithelial cells significantly relieved the severity of inflammation by inactivating NF-κB pathway, downregulating TNF-α expression, and inhibiting migration of neutrophils. The linkage of SDC1 and the NF-κB pathway in intestinal cells may unravel a novel mechanism in the contribution of SDC1 in intestinal inflammation. And the ectodomain of SDC1 might have particular therapeutic impact on intestinal inflammation.

2. Materials and Methods

2.1. Cell Culture and Treatments. Intestinal epithelial cells IEC-6, purchased from American Type Culture Collection, were grown routinely in DMEM medium (high glucose) supplemented with 10% fetal bovine serum (FBS; Gibco, CA, USA) and cultured in a 37°C humidified atmosphere containing 5% CO_2. Recombinant plasmid encoding wild type SDC1 (wt-SDC1) and unshedding SDC1 (mut-SDC1) were constructed using pcDNA 3.0 vector and transfected into cells with Lipofectamine 3000 (Invitrogen, CA, USA). In the treatment groups in all the experiments, cultured or transfected cells were treated with 1 μM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, MO, USA) for 15 min or 1 μg/mL LPS from *E. coli* (serotype 0111:B4; Sigma-Aldrich, MO, USA) for 24 h prior to the following experiments.

2.2. Reverse Transcription-PCR. Total RNA was extracted from cells using Trizol (Invitrogen, CA, USA). RNA samples were subjected to reverse transcription (RT) using a First Strand cDNA Synthesis Kit (Takara, Dalian, China). The PCR was initiated by 5 min incubation at 94°C and ended after a 10 min extension at 72°C, with 35 cycles for denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min using a PCR kit (SBS Genetech Co., Beijing, China). GAPDH mRNA was amplified simultaneously as an internal control.

2.3. Western Blot and Dot Blot Assay. Western blot was performed to detect the expression of SDC1 and NF-κB pathway. Cells were lysed in RIPA buffer with 1% PMSF, protease inhibitor cocktail, and phosphatase inhibitor. Protein was loaded onto a SDS-PAGE minigel and transferred onto PVDF membrane. After being probed with primary antibody at 4°C overnight, the blots were subsequently incubated with HRP-conjugated secondary antibody. Signals were visualized using ECL Substrates (Millipore, MA, USA). GAPDH was used as an endogenous protein for normalization.

Dot blot was performed to determine the shedding SDC1 ectodomain in cell culture supernatant. Supernatant was applied to ABS-Tween moistened Immobilon-Ny$^+$ membrane (Millipore, MA, USA) under a mild vacuum in dot blot apparatus (Whatman, NJ, USA). After three washes, the membranes were incubated in SDC1 antibody at 4°C overnight, followed by incubation of secondary antibody, and then exposed using ECL Substrates.

2.4. Immunofluorescence Assay. For immunofluorescent staining, cells were fixed in ice methanol for 10 min, blocked in PBS containing 5% BSA for 1 h at room temperature, and then incubated at 4°C overnight with SDC1 antibody (1 : 100; R&D, MN, USA). Cells without antibody incubation were used as control. After three washes, cells were labeled with PE-conjugated IgG for 1 h at room temperature. Nuclear status was determined after staining with DAPI for 10 min. Finally, cells were mounted on glass slides and examined with a fluorescence microscope.

2.5. Enzyme-Linked Immunosorbent Assay. Concentrations of TNF-α, IL-1β, and cytokine-induced neutrophil chemoattractant (CXCL-1) in the cell culture supernatant were determined by sandwich-type enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Boster, Wuhan, China). The absorbance was read at 450 nm and the concentration was determined by comparing their optical densities to the standard curve.

2.6. Isolation of Neutrophils and Migration Assay. Five milliliters of venous blood was drawn from healthy rats, into heparin-containing collection tubes, and processed within 2 h. Neutrophils were isolated as previously described [18]. For migration assay, 0.5×10^6 neutrophils in 10% FBS medium were added to the upper insert of the transwell polyester membrane filters (6.5 mm diameter inserts, 3.0 μm pore size; BD, NJ, USA) and 500 μL IEC-6 cell culture supernatant, collected at the indicated time point when secretion of CXCL-1 was highest, was added to the matched lower chamber. After 24 h incubation, nonmigrated cells were removed from

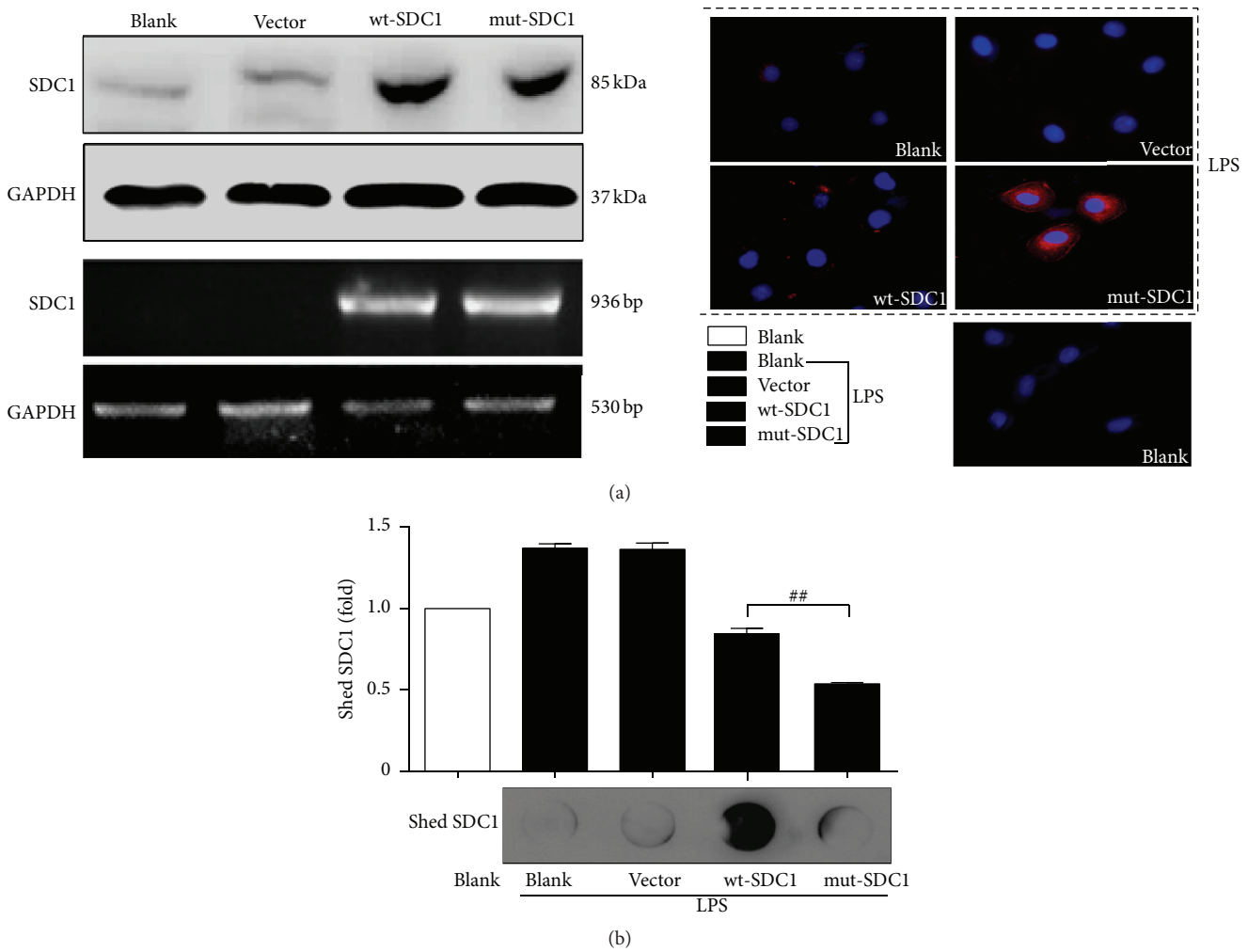

Figure 1: SDC1 shedding is blocked in mut-SDC1 transfected IEC-6 cells. PMA was used to induce SDC1 shedding in IEC-6 cells after being transfected with wt-SDC1 or mut-SDC1 plasmid. (a) Protein levels and mRNA levels of SDC1 in the cell were measured by western blot and RT-PCR; GAPDH was used as the loading control (left). Protein levels of cell surface SDC1 were measured by immunofluorescence. SDC1 (red) and cell nucleus (blue). Original magnifications: 400x. (b) Levels of shed SDC1 in the cell culture supernatant were detected by ELISA (upper) and dot blot (lower) ($^{\#\#}P < 0.01$).

the upper surface with a cotton swab. Migrated cells on the lower membrane surface were fixed in methanol and stained with 0.1% crystal violet, and cells in the lower supernatant were counted by trypan blue staining. Migration rate was calculated as a percentage of total neutrophils added to the upper insert.

2.7. Statistical Analysis. All data from 3 or greater independent experiments were expressed as mean ± SD and processed using SPSS 13.0 statistical software. Analysis of one-way ANOVA variance with Duncan's *post hoc* comparison was used for comparisons between groups. The level of significance was defined as $P < 0.05$.

3. Results

3.1. Induced Shedding of SDC1 by PMA Is Suppressed in mut-SDC1 Transfected Cells. SDC1 shedding in cells can be induced by PMA, resulting in the appearance of the extracellular domains in the medium [19]. Thus, to establish a cell model with unshedding SDC1, we first constructed and identified the mouse vector of wildtype-syndecan-1 (wt-SDC1) and unshedding-syndecan-1 (mut-SDC1); the sequencing results were in agreement with the GenBank records. Then we transfected mut-SDC1 or wt-SDC1 plasmid into intestinal epithelial cells and evaluated whether SDC1 shedding could be inhibited upon stimulation with PMA. At 24 h after transfection, IEC-6 cells exhibited dramatically increased expression of SDC1 at the mRNA and protein levels compared with the parental and vector-transfected cells (Figure 1(a), left). After stimulation with PMA for 15 min, SDC1 on wt-SDC1 transfected cells underwent shedding remarkably, while shedding of SDC1 from mut-SDC1 transfected cells was suppressed (Figure 1(a) right). Conditioned growth media from IEC-6 cells were collected to measure levels of SDC1 ectodomains (Figure 1(b), $P < 0.01$).

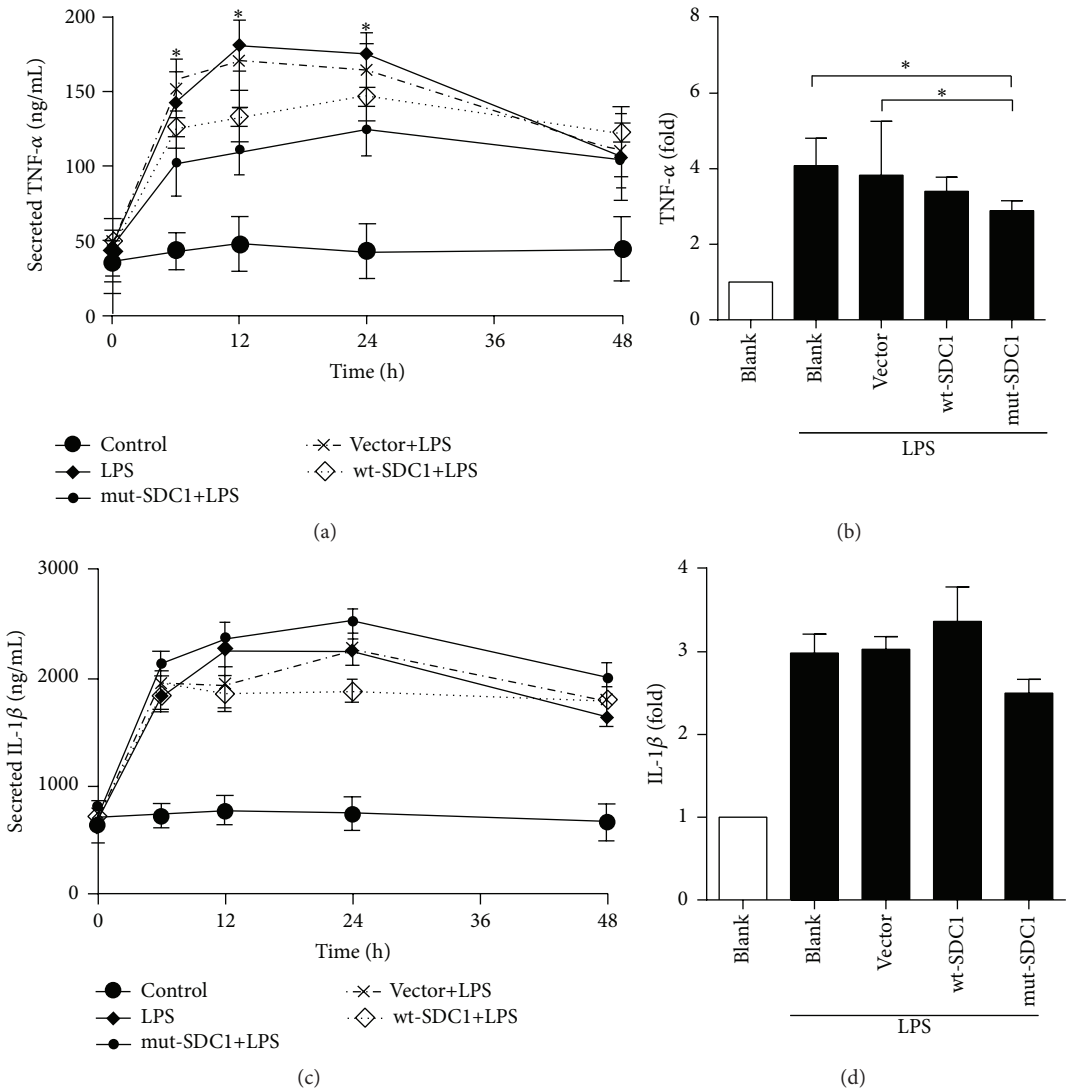

Figure 2: TNF-α and IL-1β secretions in IEC-6 cells were assessed by ELISA and q-PCR. LPS was used to induce secretions of TNF-α and IL-1β. (a), (b) SDC1 lessened secretions of TNF-α upon LPS stimulation. The decreases induced by unshedding SDC1 were more notable ($^*P < 0.05$). (c), (d) However, no significant change was observed in IL-1β.

3.2. Suppressing SDC1 Shedding Downregulates TNF-α, but Has No Effect on IL-1β. In IEC-6 cells, LPS stimulation led to significant upregulation of TNF-α and IL-1β in the transcript level. Transfection with wt-SDC1 and mut-SDC1 led to downregulation of these cytokines, but the decrease was more notable in the mut-SDC1 transfected cells; however, no difference of IL-1β was observed ($P < 0.05$, Figures 2(b) and 2(d)). We further investigated the contribution of inflammatory cytokine to SDC1's inhibitory effects on inflammatory responses by detecting expressions of TNF-α and IL-1β in the cell culture supernatant. From 0 to 24 h after LPS stimulation, a steady growth of TNF-α was maintained in IEC-6 cells. At 6, 12, and 24 h, contents of TNF-α were significantly reduced in wt-SDC1 and mut-SDC1 transfected cells ($P < 0.05$, Figure 2(a)), while at 48 h the difference was not significant. The decreased extent was more notable in mut-SDC1 transfected cells. However, no difference of IL-1β was observed (Figure 2(c)).

3.3. Suppressing SDC1 Shedding Inactivates NF-κB Pathway. We examined effects of SDC1 shedding on the NF-κB pathway in IEC-6 cell model of intestinal inflammation induced by LPS. Upon LPS stimulation, levels of total P65 and IκBα (Figure 2(a)), cytoplasmic P65 (Figure 2(b)), and nuclear P65 (Figure 2(c)) were all significantly upregulated. The upregulation extent was lessened in wt-SDC1 and mut-SDC1 transfected cells, especially in the latter. These data suggested that SDC1 can downregulate expression of P65 and IκBα to inactivate NF-κB pathway, and the downregulatory effect may primarily depend on suppressing the ectodomain shedding (Figure 3).

3.4. Suppressing SDC1 Shedding Inhibits CXCL-1 Secretion and Neutrophil Migration. Neutrophils are known as a vital component of the innate immune system and recruited to the inflammatory site as the first type of leukocytes during

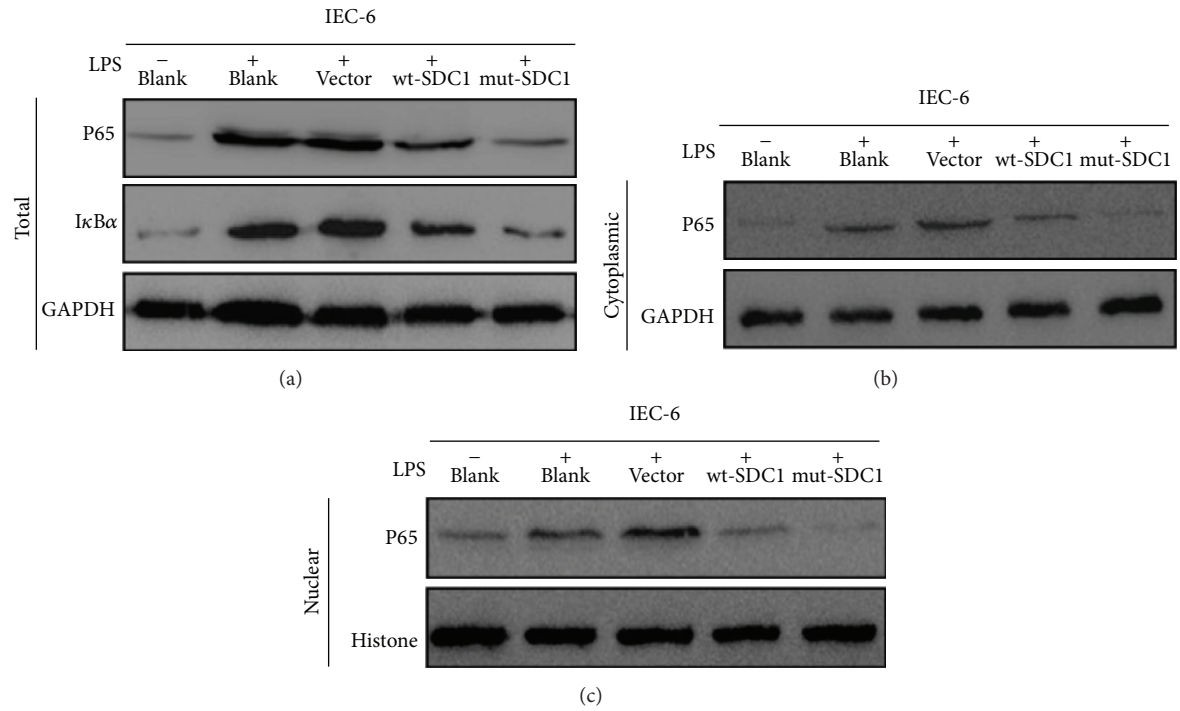

Figure 3: Activities of NF-κB pathway in IEC-6 cells were assessed by western blot. LPS was used to induce activation of NF-κB pathway. SDC1 downregulated total P65 and IκBα (a), cytoplasmic P65 (b), and nuclear P65 (c). The reductive extent induced by the unshedding SDC1 was more notable. GAPDH and histone expression were used as the loading control for total and cytoplasmic proteins and nuclear proteins, respectively.

inflammation [20], whose migration is always preceded by generation of the neutrophil chemoattractant CXCL-1 [21]. Thus, we assessed that maybe levels of SDC1 have impact on CXCL-1 and transepithelial migration of neutrophils. In IEC-6 cells, level of CXCL-1 started increasing in a time-dependent manner after LPS stimulation. wt-SDC1 and mut-SDC1 transfected cells both inhibited secretion of CXCL-1. The inhibitory effect was even greater in the latter ($P < 0.05$, Figure 4(a)).

Level of CXCL-1 was highest at 24 h after LPS stimulation, so we collected conditioned growth media of these cells at the above time point. Moreover, IEC-6 cell line is derived from normal rat intestine [22]; thus, we used the collected media to treat neutrophils which were isolated from rat venous blood, respectively. Migrations of neutrophils treated with media from LPS stimulated cells were significantly higher than the untreated cells. But this effect was remarkably inhibited when neutrophils were treated with media from wt-SDC1 and mut-SDC1 transfected cells. The inhibitory effect was even greater when media from mut-SDC1 transfected cells were introduced ($P < 0.01$, Figures 4(b) and 4(c)).

4. Discussion

The intestine is a unique tissue where an elaborate and harmonious balance is maintained, and this harmony is always in a state of controlled inflammation. If the balance is lost, disease can manifest such as inflammatory bowel disease (IBD), celiac disease, or food allergy [23]. Recently, accumulating evidence highlights the importance of SDC1, whose pathologic destruction disrupts epithelial homeostasis and internal immunity [10–12, 24]. In our study, we confirmed the protective role of SDC1 in intestinal inflammatory responses. We successfully constructed the unshedding model of SDC1 upon PMA stimulation, and, as a result, further experiments could be facilitated. Our results presented significant correlation between SDC1 shedding and inflammatory regulation in intestinal epithelial cells. SDC1, especially by the unshedding ectodomain, could inactivate NF-κB pathway, downregulate TNF-α, and inhibit migration of neutrophils induced by CXCL-1. All the results sufficiently consolidated that suppressing shedding of SDC1 could alleviate the inflammatory response, partially via inhibiting NF-κB pathway and TNF-α, which are consistent with the decreased level of SDC1 in mucosa but increased level of its ectodomain in serum in patients with Crohn's disease [6].

Since its discovery, NF-κB has been recognized as a critical regulator of epithelial tissue homeostasis and pathogenesis of chronic inflammatory diseases. Upon stimulation, IκB protein degrades and enables NF-κB to translocate into the nucleus, where it can regulate transcription of target genes and result in exacerbated inflammatory responses [25]. It has been reported that mucosal inflammation in patients with IBD and experimental models of intestinal inflammation is accompanied by elevated levels of activated NF-κB, particularly P65, P50, and c-Rel [26, 27]. Our results demonstrated that both total and cytoplasmic levels of P65 increased upon

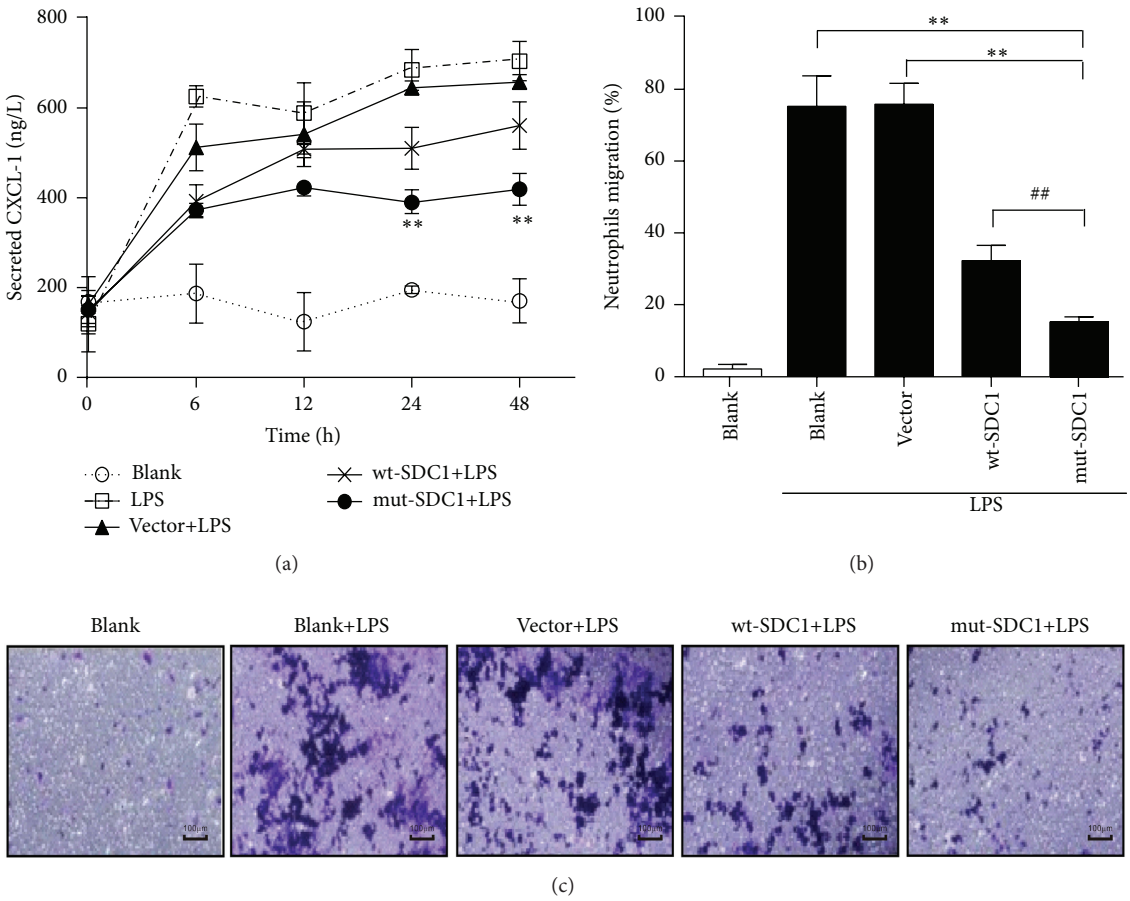

Figure 4: Effects of SDC1 on CXCL-1 secretion and migration of neutrophils. Expression of CXCL-1 was assessed by ELISA. LPS was used to induce secretions of CXCL-1. (a) The level of secreted CXCL-1 was downregulated by SDC1 ($^{**}P < 0.01$). (b, c) Cell culture supernatants containing high concentrations of CXCL-1 were used to induce migration of rat neutrophils. CXCL-1 secretion promoted migration of neutrophils; this promoting effect was diminished when cells were transfected with mut-SDC1. The transmigration was measured with use of transwell inserts; crystal violet staining was used to observe the cell quantity change ($^{**}P < 0.01$, $^{\#\#}P < 0.01$).

stimulation of LPS. The enhancement could be inhibited by SDC1, which is logically consistent with the previous findings. However, our results revealed that nuclear level of P65 decreased at the same time as downregulation of the total and cytoplasmic levels, suggesting it is still unclear whether SDC1 inactivates NF-κB pathway by suppressing P65 nuclear translocation or by completely destroying P65 gene expression. It also indicates the potential existence of some other novel regulatory proteins or nuclear partners, which control the activity of NF-κB, and further study is needed. But what can be certain now is that SDC1 on epithelial cell surfaces, especially through the unshedding ectodomain, can significantly downregulate P65 and IκBα. Taken together, these findings sufficiently consolidated that SDC1 played a suppressive role in intestinal inflammatory responses by inhibiting NF-κB pathway, and the efficient domain is the ectodomain.

Activation of NF-κB requires polyubiquitination and proteasomal degradation of IkBα, allowing NF-κB dimers to accumulate in the nucleus and activate gene transcription, accompanied by a striking increase of numerous cytokine including TNF-α and IL-1β [25]. In our study, following stimulation with LPS, P65 significantly accumulated, but IκBα failed to degrade. SDC1 inactivated NF-κB pathway via downregulating P65, and it failed to suppress IκBα degradation as well. We found that SDC1, especially through the intact unshedding ectodomain, can significantly reduce secretion of TNF-α, to alleviate inflammatory responses. IL-1β is also an important proinflammatory factor increased in intestinal inflammation [28]. It was found that degradation of IκBα induced by TNF-α and PMA in T lymphocytes and monocytes caused concomitant activation of NF-κB, followed by a dramatic increase in IκBα mRNA and protein synthesis [29]. The promoter of IκBα gene contains a κB site that is directly involved in its induction by the NF-κB complex, suggesting that the existence of an autoregulatory loop whereby IκBα regulates the activity of NF-κB was further demonstrated [30]. NF-κB can also regulate nuclear decay of IκBα/MAD-3 mRNA in monocytes without affecting its gene transcription [31]. Activation of NF-κB may parallel the increased level of IκBα and vice versa. Taken together, NF-κB/P65 forms a regulatory complex with IκBα, where they

can interact with each other, and rapid increase of IκBα after its degradation plays an important role in the reconstruction of the NF-κB/IκBα complex.

CXCL-1 is known as being able to induce inflammatory events via neutrophil migration and has been detected in lung inflammation, glomerulonephritis, and inflammatory exudates induced by LPS [32]. SDC1 contributes to neutrophil chemotaxis; shed and exogenous SDC1 ectodomain can induce neutrophil chemotaxis, inhibit epithelial wound healing, and promote fibrogenesis in mouse model of idiopathic pulmonary fibrosis [33]. However, the relationship between SDC1 and CXCL-1-dependent neutrophil migration was rarely studied. Our findings showed that change of CXCL-1 content caused by transfection with wt-SDC1 and mut-SDC1 intestinal cells was significantly downregulated, and migration of neutrophils induced by conditioned growth media of those transfected cells decreased as well. In intestinal inflammation, high level of CXCL-1 recruits neutrophil and aggravates intestinal injury. And this inflammatory response can be abolished by SDC1, especially by the unshedding ectodomain.

In conclusion, we found the protective role of SDC1, especially by its unshedding ectodomain, in inhibiting intestinal inflammation. It indicates the potential value and important insights of SDC1 in therapeutic improvement of intestinal inflammation. Suppressing shedding of SDC1 from intestinal epithelial cells plays an anti-inflammatory role in ameliorating colitis and thus is helpful for colitis treatment.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Yan Zhang and Zhongqiu Wang contributed equally.

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Research Article

Helicobacter pylori CagA and IL-1β Promote the Epithelial-to-Mesenchymal Transition in a Nontransformed Epithelial Cell Model

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Gastric cancer is the third cause of cancer death worldwide and infection by *Helicobacter pylori* (*H. pylori*) is considered the most important risk factor, mainly by the activity of its virulence factor CagA. *H. pylori*/CagA-induced chronic inflammation triggers a series of gastric lesions of increased severity, starting with gastritis and ending with cancer. IL-1β has been associated with tumor development and invasiveness in different types of cancer, including gastric cancer. Currently, it is not clear if there is an association between CagA and IL-1β at a cellular level. In this study, we analyzed the effects of IL-1β and CagA on MCF-10A nontransformed cells. We found evidence that both CagA and IL-1β trigger the initiation of the epithelial-to-mesenchymal transition characterized by β-catenin nuclear translocation, increased expression of *Snail1* and *ZEB1*, downregulation of *CDH1*, and morphological changes during MCF-10A acini formation. However, only CagA induced MMP9 activity and cell invasion. Our data support that IL-1β and CagA target the β-catenin pathway, with CagA leading to acquisition of a stage related to aggressive tumors.

1. Introduction

Gastric cancer (GC) is the fifth most frequently diagnosed malignancy in the world and the third cause of cancer death worldwide [1]. GC is strongly associated with infection by *Helicobacter pylori* (*H. pylori*), a microaerophilic Gram-negative bacterium that persistently colonizes the gastric mucosa of at least 50% of the world's population [2]. Due to its association with GC, *H. pylori* was classified as a class 1 carcinogen by the International Agency for Research on Cancer (IARC) [3–7].

H. pylori expresses several virulence and colonization factors [8–11]. The pathological effects of *H. pylori* in the gastric mucosa are associated with the presence of CagA (cytotoxin associated gene A), which is encoded in the cag pathogenicity island (cagPAI), a chromosomal DNA segment of about 40 kb encoding genes for a type IV secretion system (T4SS) [6, 12–14]. Different bacteria use T4SS to release effector molecules into host cells [15, 16]. Studies in GC cell lines showed that, after the adhesion of *H. pylori* to the epithelial cell, this secretion system is used to translocate the CagA protein [17]. Once CagA is inside the cell, it is phosphorylated in EPIYA motifs by members of the Src family of kinases and by the Abelson murine leukemia viral oncogene homolog 1 (c-Abl) kinase [18–23]. Phosphorylated and unphosphorylated CagA then activate a complex network of signaling molecules directly affecting cellular process related to cellular transformation, such as proliferation, cell survival, cell polarity, and the epithelial-to-mesenchymal transition (EMT) [24–31].

Chronic inflammation is an important driver of different types of cancer [32, 33]. Particularly, GC evolves through progressive inflammatory lesions, starting with superficial gastritis, followed by atrophic gastritis, intestinal metaplasia,

and dysplasia, to finally become a cancerous lesion [34, 35]. Precancerous gastric lesions are characterized by prominent infiltration of mononuclear and polymorphonuclear immune cells and the presence of inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin- (IL-) 1β, and IL-8 [36]. Epidemiological data also support an association between GC and polymorphisms in the genes encoding TNFα, IL-1β, and IL-8 [37–39]. A transgenic mouse ectopically expressing IL-1β develops progressive lesions that mirror the multistage process occurring during human gastric carcinogenesis. Interestingly, these pathological changes are accelerated when *H. pylori* infection is introduced providing evidence that IL-1β and *H. pylori* can cooperate during gastric carcinogenesis [40–44].

EMT is characterized by multiple transcriptional, biochemical, and morphological changes allowing a terminally differentiated epithelial cell to acquire a mesenchymal phenotype. Tumor cells become migratory after EMT, with increased capacity for degrading extracellular matrix (ECM) components and resistance to anoikis, a type of apoptosis of detached cells [45]. It is believed that progression from noninvasive to invasive tumors relies on activation of the EMT program characterized by activation of transcription factors Snail, Twist, Slug, and ZEB and loss of cell-to-cell junctional complexes induced by nuclear translocation of β-catenin, degradation of E-cadherin, and downregulation of the *CDH1* promoter. All together these processes associate with highly aggressive tumors [46].

We recently found that CagA induces anoikis resistance through activation of the AKT signaling pathway [47]. AKT is also an important mediator of EMT through inactivation of glycogen synthase kinase 3β (GSK3β), a critical negative regulator of β-catenin. Once GSK3β is inactive, β-catenin moves to the nucleus where it helps to turn on the EMT transcriptional program [48]. Recent studies have also supported a link between CagA, AKT, and EMT [49, 50]. In this study, using a nontransformed epithelial cell model we found that both CagA and IL-1β induced translocation of β-catenin to the nucleus and the onset of EMT, but only CagA led to enhanced MMP9 activity and cell invasion.

2. Material and Methods

2.1. H. pylori Strains and Culture. Two CagA positive *H. pylori* strains were used in this study: strain 11637 with a Western type CagA (EPIYA ABCCC) that was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA, number 43504) and strain NY02-149 with an East-Asian-type CagA (EPIYA ABD) that was kindly donated by Dr. Guillermo Perez-Perez from New York University. Two additional *H. pylori* CagA negative strains were used as controls: strain 365A3, which has a partial cagPAI lacking the *cagA* gene, and strain 254 that contains a nonfunctional cagPAI. All *H. pylori* strains were grown on blood agar (BD Biosciences, San Jose, CA, USA, number 211037) for 48 h at 5% CO_2 and 37°C.

2.2. MCF-10A Culture, Infection, and IL-1β Stimulation. MCF-10A cells are human mammary epithelial cells that were obtained from the American Type Culture Collection (ATCC CRL-10317, Manassas, VA, USA). MCF-10A three-dimensional (3D) or monolayer (2D) cultures were performed as previously reported by us and Debnath et al. [47, 51]. For infection assays, MCF-10A cells were seeded at 3000 cells/cm^2 and cultured for 48 hrs to reach 70% subconfluency and then switched to DMEM-F12 without fetal bovine serum (FBS). Afterwards, cells were infected with an *H. pylori* multiplicity of infection (MOI) of 100 and/or stimulated with 20 ng/mL of human recombinant IL-1β (Peprotech, Rocky Hill, NJ, USA) for 48 hrs. As a control for all experiments, cells were similarly handled but were not infected nor treated with IL-1β (mock infected/treated control cells). For 3D culture infection-stimulation assays, single cells suspensions were treated as mentioned above and then seeded in Matrigel (BD Biosciences, San Jose, CA, USA, number 354230). Cells were grown for 14 days changing medium every other day. In infected and/or IL-1β treated cells, media of days 2, 4, and 6 also contained bacteria and IL-1β to maintain the stimulus while the acini were growing and shaping.

2.3. Immunofluorescence. Cells grown under 3D conditions were fixed with paraformaldehyde (PFA Electron Microscopy Sciences Cat. 15713) at 3.7% for 20 minutes at room temperature, washed, and permeabilized with PBS-0.2% Triton X-100 for 20 minutes and then washed again and treated with PBS-0.02% Triton X-100 plus 10% goat-serum and 1% BSA (blocking buffer). Cells were then incubated overnight with anti-GM130 antibody (Genetex number GTX61445; 1 : 50 dilution in blocking buffer), washed, and incubated with anti-rabbit IgG labeled with Alexa-488 (Invitrogen, Carlsbad, CA, USA, number A11008, 1 : 100 dilution) for one hour. Cells were then stained with DAPI (Invitrogen, Carlsbad, CA, USA, number D1306). Finally, the preparations were washed and mounted in the 3D culture chambers adding 12 μL of VECTASHIELD (Vector Laboratories, Burlingame, CA, number H-1000) and observed in a confocal microscope Leica SP2 (Leica Microsystems, Wetzlar, Germany). Confocal images were analyzed with the Leica LAS AF-Lite 2.6.0 software.

For 2D assays, cells were grown on sterile glass coverslips in DMEM-F12 for 48 hours. Cells were then serum starved and IL-1β-stimulated or infected for 48 hours. Then, cells were subjected to the same staining process as described for the 3D cultures by using antibodies against β-catenin (Invitrogen, Carlsbad, CA, USA, number 138400), E-cadherin (BD Biosciences, San Jose, CA, number 610182), and ZO-1 (Genetex, Irvine, CA, number GTX108613). Finally, cell preparations were mounted on slides and observed with the inverted epifluorescence microscope Olympus IX50. Images were recorded with the DP72 digital camera and analyzed with Image-Pro Plus software (V7.0) averaged cybernetics (Silver Spring, MD, USA).

2.4. RNA Extraction and RT-PCR. Total RNA was obtained from IL-1β stimulated and *H. pylori* infected cells lysed with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA, number

15596018). Complementary DNA synthesis was performed using 2.5 μg of total RNA in a reaction mixture with SuperScript Kit VILO Master Mix (Invitrogen, Carlsbad, CA, USA, number 11755-050). *ZEB1* gene was amplified by using the oligonucleotide pairs, sense: GGG AAT GCT AAG AAC TGC TGG and antisense: GGT GTA ACT GCA CAG GGA GC. For *Snail1* gene the oligonucleotides were as follows: sense: TCG GAA GCC TAA CTA CAG CGA and antisense: AGA TGA GCA TTG GCA GCG AG; for *CDH1* sense: CCC ACC ACG TAC AAG GGT C and antisense: CTG GGG TAT TGG GGG GCA TC; for *RPLP0* sense: ATG GGG AAG CTG AAG GTC GG and antisense: GTG GCA GTG ATG GCA TGG ACT; for *GAPDH* sense: ATG GGG AAG GTG AAG GTC GG and antisense: GTG GCA GTG ATG GCA TGG ACT. The 20 μL PCR mixture contained 200 μM of dNTPs mix, 2.0 mM of $MgCl_2$, 200 nM of each primer, Taq Polymerase buffer, and 1.0 U of recombinant Taq Polymerase (Invitrogen, Carlsbad, CA, USA, number 11615-010). The reaction was performed with an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 5 min.

2.5. Zymography. MCF-10A cells were infected with *H. pylori* or treated with IL-1β for 48 h and culture supernatants were recovered and concentrated using 30 K cutoff Amicon Centricon filters (Millipore, Billerica, MA, USA, number UFC503024). Protease activity was revealed in 8% SDS-PAGE gels copolymerized with 1 mg/mL gelatin and activation buffer (50 mM Tris-HCl, pH 7.4, 4.5 mM $CaCl_2$). Gels were stained with Coomassie Blue and densitometric analyses were performed with the ImageJ software.

2.6. Invasion Assays. 1×10^3 IL-1β-stimulated or *H. pylori* infected MCF-10A cells were seeded in the inner chamber of Transwell units (Corning, NY, USA, number 3422) with Matrigel-coated polycarbonate filters as a substrate for degradation and filled up with DMEM-F12 free of serum. The outer chamber of the Transwell unit was loaded with DMEM-F12 supplemented with 10% of FBS as the chemoattractant. Cells were allowed to migrate for 36 h at 37°C. The porous membranes containing migrating cells were cut out from the inner chambers and fixed with 3.4% PFA for 30 min. Fixed cells were permeabilized with PBS-0.2% Triton X-100 and stained with DAPI.

2.7. SDS-PAGE and Western Blot. 30 μg of protein extracts was separated in 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and then transferred onto nitrocellulose membranes. Membranes were blotted with antibodies against E-cadherin, β-catenin, and β-actin as loading control (kindly provided by Dr. J. M. Hernandez; CINVESTAV-IPN, Mexico). HRP-conjugated secondary antibody was from Invitrogen (Carlsbad, CA, USA, number G21040). Positive bands were revealed by enhanced chemiluminescence.

2.8. Statistical Analysis. Statistical analyses were performed by one-way ANOVA test. Data are presented as mean ± standard deviation. p values ≤ 0.05 were considered significant.

3. Results

We addressed whether CagA alone and/or cooperating with the inflammatory cytokine IL-1β promotes β-catenin translocation, one of the first steps of EMT. In mock control cells β-catenin was localized to cellular membrane. In contrast, after IL-1β treatment or infection with *cagA* positive strains we observed that β-catenin was translocated to the nucleus (Figure 1(a)). We did not find evidence that the combination of the two stimuli was able to induce the translocation of β-catenin into the nuclei of cells with an additive or synergistic effect (Figure 1(b)). When cells were infected with *H. pylori* strains lacking *cagA* or with a defective cagPAI, the membrane localization of β-catenin was not altered (Figure 1(b)).

We analyzed the effect of CagA on other membrane proteins located at adherent junctions. We found that E-cadherin was distributed on the cell membrane homogeneously in uninfected cells, while in cells infected with the *cagA* positive strains there were some areas in which the fluorescent signal decreased (arrows) ((Figure 2(a)). CagA was also able to redistribute ZO-1 (arrows) (Figure 2(b)). We then evaluated the effect of CagA and IL-1β on MCF-10A 3D acini morphogenesis. In mock cells, GM130 localized towards the center of the acini in the apical face. However, when cells were stimulated with IL-1β or infected with *H. pylori cagA* positive strains a redistribution of GM130 protein was observed surrounding acini central cells (Figure 2(c) top panels and bottom enhanced images). These results show that CagA and IL-1β interfere with GM130 localization during acini morphogenesis.

When β-catenin and E-cadherin were analyzed by Western blot, we observed that both proteins remain stable after both CagA and IL-1β stimuli at early experimental timepoints (Figure 3(a)). On the other hand, the expression levels of transcription factors *ZEB1* and *Snail1*, two of the master regulators of EMT initiation, and of *CDH1*, the gene that encodes for E-cadherin, were significantly changed after both stimuli. We found an increased expression of *Snail1* and *ZEB1* after stimulation with IL-1β or infection (Figures 3(b) and 3(c)), but *Snail1* expression was significantly larger for the *H. pylori* with the East Asian EPIYA (ABD) than with the Western ABCCC strain, while *CDH1* expression was severely diminished by IL-1β treatment or infection with any of the *cagA* positive *H. pylori* strains (Figure 3(b)).

We then measured the activity of metalloproteases secreted in the supernatants of MCF-10A cells. Densitometric analysis of hydrolyzed bands in gelatin zymograms showed enzymatic activity with a molecular weight correlating with that of MMP9 (Figure 4(a)). This activity had a major peak when cells were subjected to *H. pylori* infection compared to that after IL-1β stimulus (Figure 4(b)). An invasion assay showed that CagA has the ability to confer migratory properties on *H. pylori* infected MCF-10A cells (Figures 4(c) and 4(d)). Interestingly, similar to the metalloproteinase assay, IL-1β was not as efficient to induce cell invasion. Finally, we found that the stimulation of invasion is dependent on AKT and Src kinase activities (Figures 4(c) and 4(d)). Src is the main kinase that phosphorylates CagA and we have

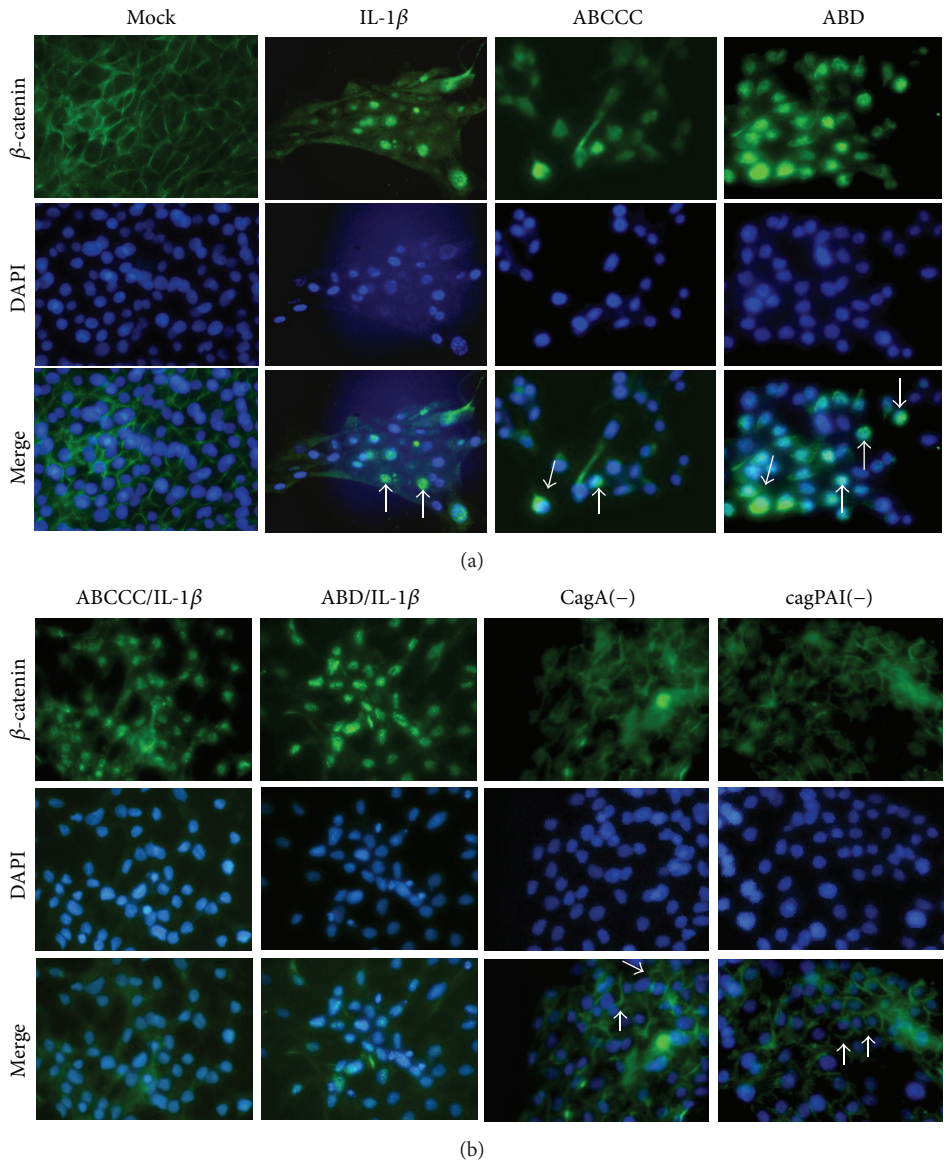

Figure 1: *H. pylori* CagA and IL-1β induce β-catenin nuclear translocation. (a) MCF-10A cells were infected with CagA positive strains (ABCCC or ABD) or stimulated with IL-1β. (b) MCF-10A cells were infected with CagA positive strains and stimulated with IL-1β or single infected with CagA negative variants CagA(−) and cagPAI(−). Immunofluorescence images show β-catenin (green) and nuclei (DAPI, blue). Arrows indicate nuclear staining (a) and membrane staining (b) of β-catenin. Figures are representative of three independent experiments performed in duplicate or triplicate.

previously shown that CagA-induced activation of AKT relies on Src activity [47]. Overall these results agree with a CagA and IL-1β-induced onset of EMT, with CagA promoting more aggressive cancer features.

4. Discussion

The nontransformed cell line MCF-10A recapitulates several traits of the architecture of glandular epithelium providing a system in which to ask questions about the mechanisms of cell growth, proliferation, growth factors independence, and cell polarity. This model has been used to study mechanisms of transformation triggered by viral and cellular oncogenes, including some that are not related to the development of breast carcinomas [51–54].

ZO-1, E-cadherin, and β-catenin are part of the epithelial apical junctional complexes that regulate cell polarity, proliferation, and differentiation [55–57]. *H. pylori* infection induces loss of polarity and relocation of ZO-1 protein in MDCK cells, which causes impairment of the epithelial barrier integrity [26, 30, 58]. E-cadherin is frequently lost in EMT-induced metastatic cancer cells [59]. We observed that although protein levels of E-cadherin remained unchanged, there was downregulation of the *CDH1* transcript after both IL-1β and CagA stimulus. E-cadherin degradation may be activated at later times than the ones analyzed in this study

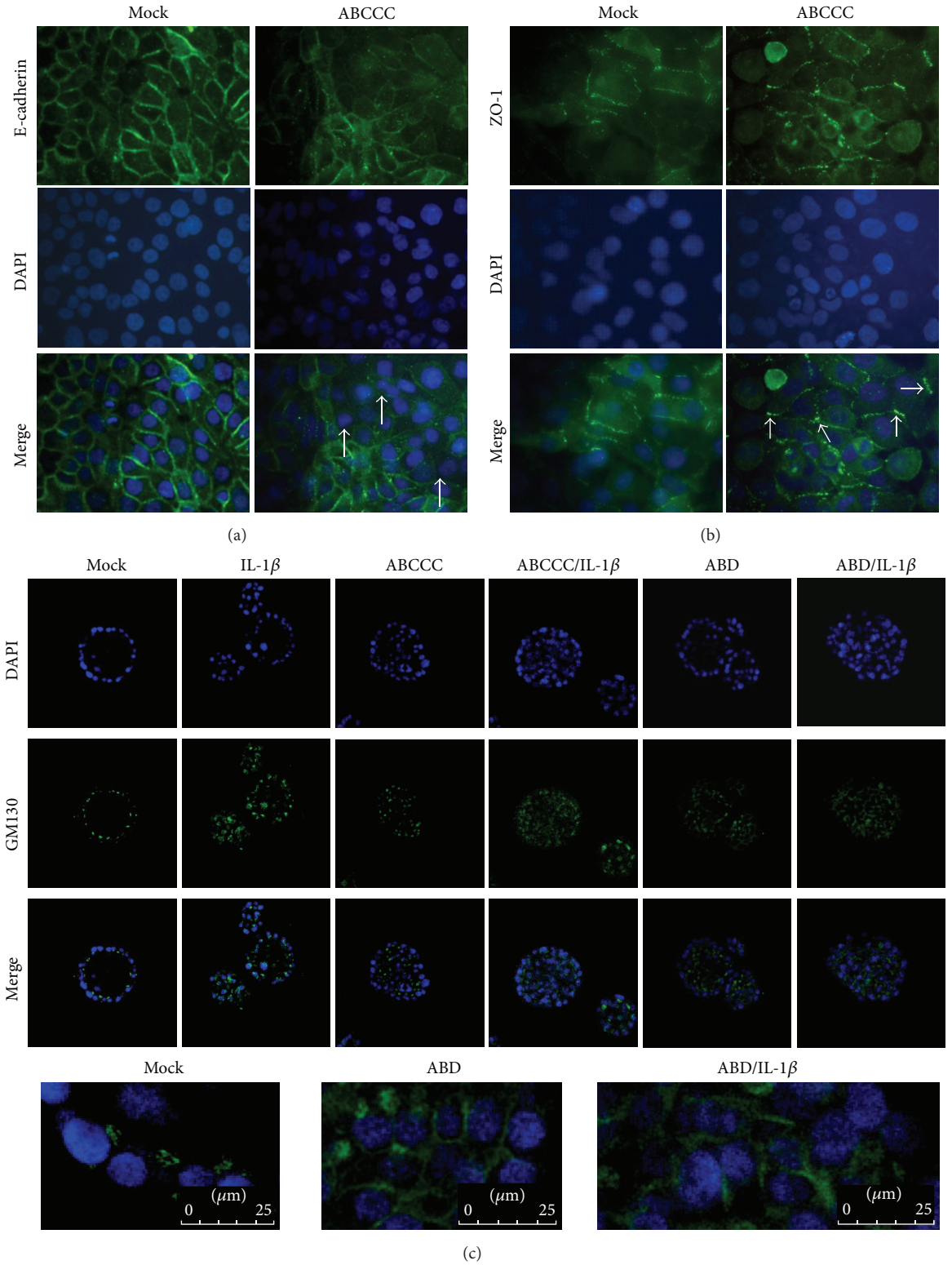

Figure 2: Localization of ZO-1 and GM130 is altered by *H. pylori* CagA. (a-b) Cells were infected with the CagA positive strain EPIYA ABCCC and were stained with anti-ZO-1 or anti-E-cadherin antibodies (green) and DAPI (blue). Arrows in panel (a) indicate the loss of signal of E-cadherin and in panel (b) show delocalization of ZO-1. (c) MCF-10A cells were infected with *H. pylori* positive strains ABCCC and ABD, stimulated with IL-1β, or both infected and stimulated during acini morphogenesis. The acini structures were stained with anti-GM130 antibody (green) and DAPI (blue) and confocal microscopy sections were made at 50% depth of the acini. The bottom panels show an enhanced fragment of the acini to see in more detail the GM130 distribution. Figures are representative of three independent experiments performed in duplicate or triplicate.

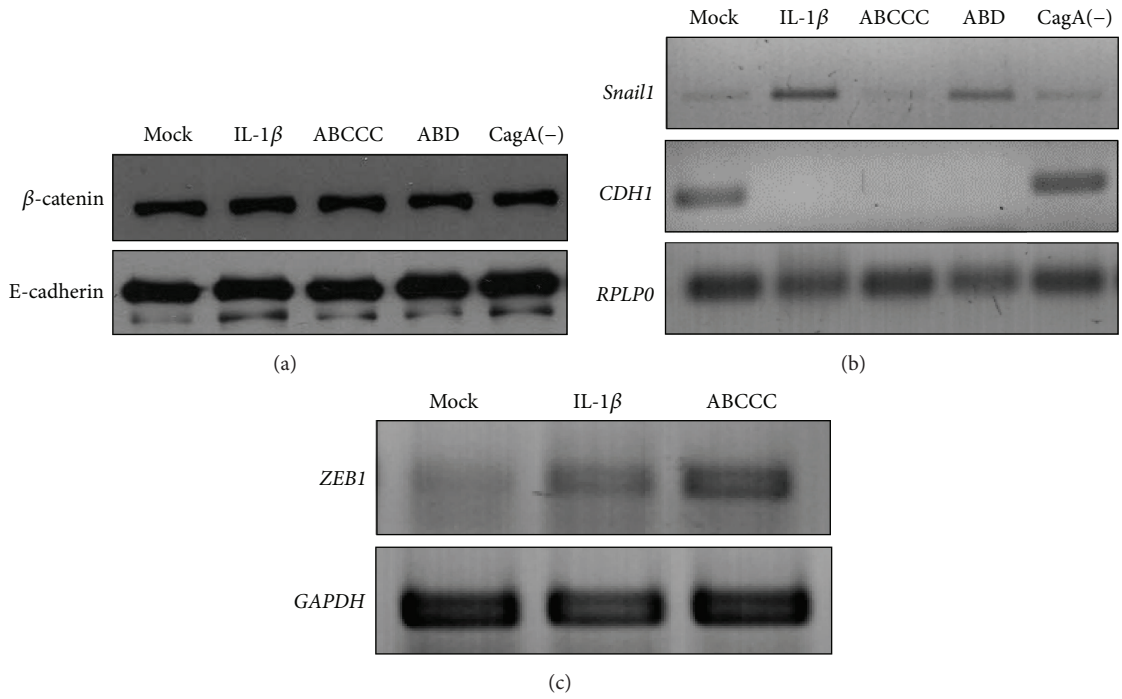

Figure 3: Nuclear translocation of β-catenin correlates with increased expression of *ZEB1* and *Snail1*. (a) Immunoblotting of whole cell lysates of infected or IL-1β-stimulated cells with anti-β-catenin and anti-E-cadherin antibodies. The relative expression of (b) *Snail1* and *CDH1* and (c) *ZEB1* genes was determined by semiquantitative RT-PCR. Expression of housekeeping genes *RPLP0* and *GAPDH* was used as internal control. Figures are representative of three independent experiments performed in duplicate or triplicate. Figures are representative of three independent experiments performed in duplicate or triplicate.

or EMT may happen without affecting levels of E-cadherin as it has been shown in other studies [60, 61].

A link between CagA and EMT has been shown in previous studies. In AGS and MKN74 cells *H. pylori* induced expression of mesenchymal markers *ZEB1*, *vimentin*, *Snail1*, *Snail3*, and *MMP9* with concomitant decreasing of the epithelial marker *keratin-7* [62]. EMT also generated cells with characteristics of cancer stem cells (CSC) and expression of CD44 [63]. However, AGS and MKN74 cells are transformed; hence the signaling pathways and biological processes associated with cancer are already altered before the expression of CagA [62, 63]. In the past two years, several primary human gastric organoid models have emerged to assess *H. pylori* infection or CagA activity. In one study, *H. pylori* was found to induce secretion of TNFα and IL-1β inflammatory cytokines, as well as several chemokines, through activation of NFκB [64]. In other studies, *H. pylori* altered the cell polarity through relocalization of claudin-7 by activation of β-catenin and Snail [65].

It is thought that resistance to anoikis is responsible for the survival of invading tumor cells upon undergoing EMT and detaching from the basal membrane [66]. We previously showed that CagA induced anoikis resistance via AKT phosphorylation and inactivation of the proapoptotic proteins BIM and BAD [47]. AKT is also an important inducer of β-catenin nuclear translocation through inactivation of GSK3β; nuclear β-catenin then initiates the EMT transcriptional program [67]. Other studies have shown CagA activity leading to β-catenin nuclear accumulation [31]. Our results show that translocation of β-catenin correlates with an increased expression of the *Snail1* and *ZEB1* EMT genes, which are involved in deregulation of adherens junctions by transcriptional repression of the *CDH1* promoter [68]. Furthermore, we also observed increased cell invasion and MMP9 protease activity promoted by CagA. Metalloproteinases degrade ECM components facilitating cell invasiveness. Interestingly, we only observed a CagA-mediated increased MMP9 activity and cell invasiveness, in spite of IL-1β efficiently promoting β-catenin translocation, transcriptional upregulation of *ZEB1* and *Snail1*, and downregulation of *CDH1*. This may be due to a more chronic requirement on IL-1β to achieve a similar activity. A recent report showed that stable EMT-related changes were only induced after >3 weeks of IL-1β treatment [69], while in our study treatment went only for 2 to 6 days. Also relevant is the lack of cooperation between CagA and IL-1β. This could be because infection by *H. pylori* may directly induce an inflammatory response in which IL-1β is present, thus already saturating the need for IL-1β activity.

5. Conclusions

Our findings support that CagA oncoprotein from *H. pylori* and the inflammatory stimulus of IL-1β guide the onset of EMT in nontransformed cells using a model of acini morphogenesis. CagA but not IL-1β was found to induce cell

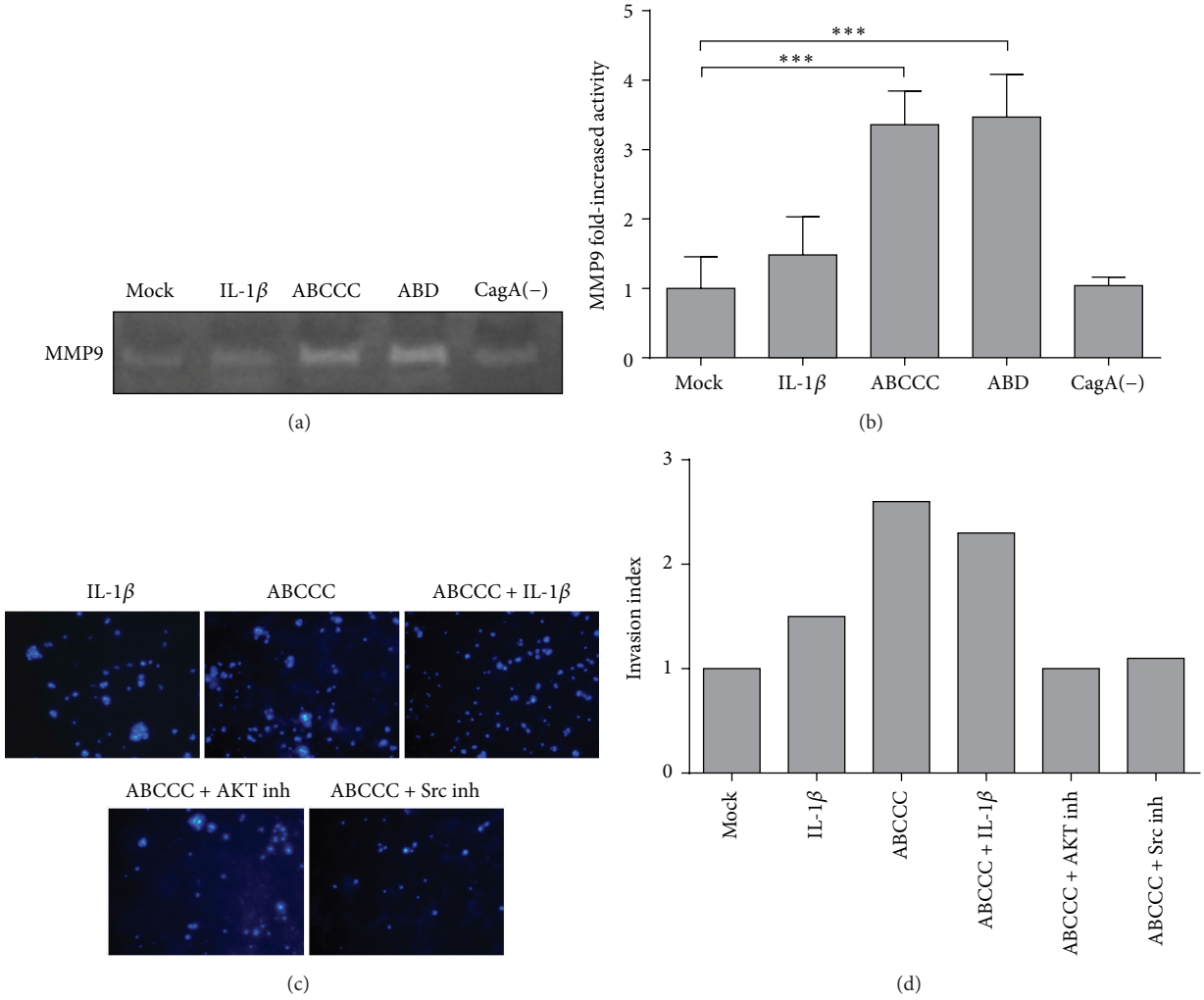

Figure 4: CagA but not IL-1β promotes MMP9 activity and invasion. (a) Representative gelatin zymogram showing metalloproteinase 9 (MMP-9) hydrolytic activity in culture media recovered from MCF-10A cells stimulated with IL-1β or infected with *cagA* positive and negative *H. pylori* strains. (b) Densitometric analysis of three independent experiments. (c-d) Invasion assays of cells stimulated with IL-1β or infected. AKT and Src kinase inhibitors (inh) were used to inhibit the epithelial-to-mesenchymal transition (EMT) and CagA activity, respectively. (c) shows a representative insert from which the invading cells were counted and (d) shows a graphical representation of the quantification of all assays. The bars are the mean fold changes normalized to mock cells. Experiments for (a) and (b) were done three times in duplicate and for (c) and (d) once in triplicate. Statistical analyses were performed by one-way ANOVA ($^{***}p \leq 0.001$) and mock cells were used as the baseline value.

invasion and formation of an aggressive phenotype related to cancers.

Competing Interests

The authors state that they do not have any competing interests.

Acknowledgments

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Research Article

Synergic Interaction of Rifaximin and Mutaflor (*Escherichia coli* Nissle 1917) in the Treatment of Acetic Acid-Induced Colitis in Rats

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Background. Inflammatory bowel disease results from the dysregulation of immune response to environmental and microbial agents in genetically susceptible individuals. The aim of the present study was to examine the effect of rifaximin and/or Mutaflor (*Escherichia coli* Nissle 1917, EcN) administration on the healing of acetic acid-induced colitis. *Methods*. Colitis was induced in male Wistar rats by rectal enema with 3.5% acetic acid solution. Rifaximin (50 mg/kg/dose) and/or Mutaflor (10^9 CFU/dose) were given intragastrically once a day. The severity of colitis was assessed at the 8th day after induction of inflammation. *Results*. Treatment with rifaximin significantly accelerated the healing of colonic damage. This effect was associated with significant reversion of the acetic acid-evoked decrease in mucosal blood flow and DNA synthesis. Moreover, administration of rifaximin significantly reduced concentration of proinflammatory TNF-α and activity of myeloperoxidase in colonic mucosa. Mutaflor given alone was without significant effect on activity of colitis. In contrast, Mutaflor given in combination with rifaximin significantly enhanced therapeutic effect of rifaximin. Moreover, Mutaflor led to settle of the colon by EcN and this effect was augmented by pretreatment with rifaximin. *Conclusion*. Rifaximin and Mutaflor exhibit synergic anti-inflammatory and therapeutic effect in acetic acid-induced colitis in rats.

1. Introduction

Genetic and environmental factors are involved in pathogenesis of inflammatory bowel disease (IBD). IBD results from the dysregulation of immune response to environmental and microbial agents in genetically susceptible individuals [1, 2]. Intestinal microflora plays a role in promoting and maintaining inflammatory process in this disease [3]. The intestinal flora contains various pathogens such as *Clostridium perfringens, Enterococcus*, Enterobacteriaceae, and *Bacteroides*. These bacteria are present in the large intestine of every healthy person in high concentrations, but, in the normal condition, they are separated from the colonic wall by an impenetrable mucus layer and are tolerated by the

host. In patients with IBD, this separation is disturbed; bacteria adhere to the mucosa and invade epithelial cells with concomitant inflammatory response [4]. Moreover, the concentration of mucosal bacteria is higher in patients with IBD than in healthy persons and this concentration is proportional to the severity of the disease [4, 5].

Rifaximin is a locally acting antibacterial agent that is practically unabsorbed after oral administration (absorption less than 0.4%) and for this reason this medicine is risk-free of systemic side effects. Rifaximin exhibits a broad-spectrum activity against enteric Gram-positive and Gram-negative bacteria. Rifaximin has been found to be useful in the treatment of traveler's diarrhea and irritable bowel syndrome and in preventing of the stress-induced gut inflammation [6–9]. There are studies showing that rifaximin is effective in the treatment of IBD. Clinical trials have indicated that administration of 800 mg rifaximin twice daily for 12 weeks induces remission with few adverse events in patients with moderately active Crohn's disease (CD) [10], and remission previously obtained with standard treatment can be sustained in patients with moderately active CD by administration of rifaximin as well [11]. Rifaximin has been also found to be effective in the treatment of ulcerative colitis [12–14].

Living microorganisms that enter gastrointestinal tract and exert a beneficial effect on the host are called probiotics. There are studies showing the therapeutic effect of probiotics in the prevention or treatment of the gastrointestinal tract diseases [15, 16].

Escherichia coli Nissle 1917 (EcN) is a nonpathogenic strain of the Enterobacteriaceae family. It was originally isolated by a physician Alfred Nissle during the First World War on the Balkan Peninsula from the feces of a soldier, who in contrast to his comrades, did not develop enterocolitis [17]. Study performed by Altenhoefer et al. [18] has tested the interference of EcN with *Salmonella* invasion of human embryonic intestinal epithelial INT407 cells. Simultaneous administration of EcN 1917 and *Salmonella enterica* serovar Typhimurium strain C17 resulted in up to 70% reduction of *Salmonella* invasion efficiency. Furthermore, invasion of *Yersinia enterocolitica*, *Shigella flexneri*, *Legionella pneumophila*, and even *Listeria monocytogenes* was inhibited by EcN without affecting the viability of the invasive bacteria. There are also studies indicating the therapeutic effect of the EcN in patients with IBD. Maintaining remission in ulcerative colitis by the treatment with EcN has been shown to be as effective as treatment with "the gold standard" mesalazine [19–22]. On the other hand, there is only one clinical study showing beneficial effect of EcN in maintaining remission in patients with colonic Crohn's disease [23]. Application of EcN has reduced the risk for relapse and minimized the need for glucocorticoids.

The above observations suggest that rifaximin and Mutaflor can influence the course of IBD. The aim of the present study was to compare the effect of treatment with rifaximin and Mutaflor on the healing of acetic acid-induced colitis in rats. In addition, we investigated whether administration of the combination of rifaximin plus Mutaflor leads to any synergic interaction of their therapeutic effects in this model of IBD.

2. Materials and Methods

2.1. Animals and Treatment. Studies were performed on 64 male Wistar rats weighing 250–270 g and were conducted following the experimental protocol approved by the First Local Commission of Ethics for the Care and Use of Laboratory Animals in Cracow (Permit Number 2/2013 released on January 16, 2013). Animals were housed in cages in room temperature and a 12 h light-dark cycle. Rats were fasted with free access to water for 18 h before induction of colitis. Later food and tap water were available *ad libitum*.

Animals were randomly divided into eight equal experimental groups: (1) control rats without induction of colitis and treated intragastrically (i.g.) with saline; (2) rats without induction of colitis and treated i.g. with *Escherichia coli* Nissle 1917 (EcN); (3) rats without induction of colitis and treated i.g. with rifaximin; (4) rats without induction of colitis and treated i.g. with the combination of rifaximin plus EcN; (5) rats treated i.g. with saline after induction of colitis; (6) rats treated i.g. with EcN after induction of colitis; (7) rats treated i.g. with rifaximin after induction of colitis; and (8) rats treated i.g. with the combination of rifaximin plus EcN after induction of colitis.

Colitis was induced by a rectal enema with 1 mL of 3.5% (v/v) acetic acid aqueous solution in rats anesthetized with ketamine (50 mg/kg i.p., Bioketan, Vetoquinol Biowet, Gorzów Wielkopolski, Poland). Acetic acid solution was administered through a polyethylene catheter inserted into the rectum. There are different models of acetic acid-induced colitis and the tip of catheter can be positioned from 1.2 [24] to 8 cm [25] proximal to the anus verge. For this reason we have chosen an intermediate depth of catheter insertion, 4.5 cm from the anus. Rats without induction of colitis obtained rectal enema with an aqueous saline solution administered in the same manner as a solution of acetic acid in animals with induction of colitis.

Rifaximin (50 mg/kg/dose; Xifaxan, Norgine B.V., Amsterdam, Netherlands) and/or the probiotic strain *Escherichia coli* Nissle 1917 (approx. 10^9 CFU/dose, Mutaflor; Ardeypharm GmbH, Herdecke, Germany) were given i.g. once a day for 7 days, starting at the day of colitis induction. Each dose of Mutaflor was given 2 h after treatment with rifaximin. In rats treated with saline, each dose of saline was given at the same time as in animals treated with Mutaflor. The last administration of saline, rifaximin, Mutaflor, or the combination of rifaximin plus Mutaflor was carried out 24 h before the end of experiment.

At the 8th day of study, animals were anesthetized and the research was terminated. This single observation period was chosen because the protocol of our research has been prepared in accordance with the policy of 3Rs (Replacement, Reduction and Refinement). Previous studies have shown that the potential therapeutic effects of factors being tested are clearly visible on the 8th day of study after the seven-day treatment [26].

2.2. Measurement of Colonic Blood Flow and Colonic Damage. Seven days after rectal enema with saline or induction of colitis, rats were anesthetized again with ketamine. After

opening the abdominal cavity and exposure of the colon, the rate of colonic blood flow was measured using laser Doppler flowmeter (PeriFlux 4001 Master monitor, Perimed AB, Järfälla, Sweden), as described previously [27]. The measurement of mucosal blood flow was performed every time in five parts of the descending and sigmoid colon and the main value of five records was expressed as the percentage of the value obtained in the animals from the control group. After the measurement of colonic blood flow, anesthetized animals were euthanized by exsanguination from the abdominal aorta. Then, the area of mucosal damage was measured, using a computerized planimeter (Morphomat, Carl Zeiss, Berlin, Germany), as described previously [28].

2.3. Biochemical Analysis. After measurement of the area of mucosal damage, biopsy samples of colonic wall or colonic mucosa were taken for histological examination and determination of mucosal DNA synthesis (an index of mucosal cell vitality and proliferation), mucosal interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α) concentration, and mucosal activity of myeloperoxidase. DNA synthesis was determined by measurement of [^{3}H]thymidine incorporation ([6-^{3}H]-thymidine, 20–30 Ci/mmol, Institute for Research, Production and Application of Radioisotopes, Prague, Czech Republic) into mucosal DNA as described previously [29]. The incorporation of labeled thymidine into DNA was determined by counting 0.5 mL DNA-containing supernatant in a liquid scintillation system. DNA synthesis was expressed as tritium disintegrations per minute per microgram of DNA (dpm/μg DNA).

Samples of the colonic mucosa, in which the concentration of IL-1β and TNF-α was measured, were homogenized in phosphate buffer at 4°C. Then the homogenate was centrifuged and the concentration of IL-1β and TNF-α was determined in the supernatant using the Rat IL-1β Platinum ELISA (Bender MedSystem GmbH, Vienna, Austria) or Rat TNF-α ELISA Kit (Koma Biotech, Seoul, South Korea), respectively. The concentration of IL-1β and TNF-α in the colonic mucosa was expressed in nanograms per 1 gram of tissue.

Biopsy samples for measurement of mucosal myeloperoxidase activity were homogenized in ice-cold potassium phosphate and, until the marking was done, stored at the temperature of −60°C. Marking myeloperoxidase activity was performed with the use of a modification of the method described by Bradley et al. [30]. Results were expressed in units per gram of tissue.

2.4. Histological Examination of the Colon. Samples of the colon were fixed in 10% buffered formaldehyde and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin and examined by the pathologist uninformed about treatment given. The histological grading of colonic damage such as ulceration, inflammation, depth of the lesion, and fibrosis was determined using a scale of Vilaseca et al. [31] as described in detail previously [32].

2.5. Microbiological Analysis. Feces samples were taken and transported in deep-freeze conditions to the microbiological laboratory where DNA was extracted according to the previously described procedure [33].

Escherichia coli and *Enterococcus* species in the fecal samples were quantified by quantitative real time PCR (qPCR) according to the method described by Pilarczyk-Zurek et al. [34] and Ryu et al. [35], respectively. To detect specific DNA sequences, ready-to-use JumpStart Taq ReadyMix kit (Sigma-Aldrich, Saint Louis, USA), fluorescently FAM dye labeled probe (F) GGGAGTAAAGTTAATACCTTTGC, (R) CTCAAGCTTGCCAGTATCAG, FAM-CGCGATCACTCC-GTGCCAGCAGCCGCGGATCGCG-BHQ1 (GenoMed) for *E. coli*, and (ECST748F) AGAAATTCCAAACGAACTTG and (ENC854R) CAGTGCTCTACCTCCATCATT for *Enterococcus* spp. (SYBR Green dye) were used. A standard curve was prepared. DNA from given numbers of *E. coli* ATCC25922 and separately *E. faecalis* ATCC19433 was added in serial dilutions from 10^1 to 10^7 cells to a series of qPCRs. The reactions were carried out in a CFX96 thermocycler (BioRad). Detection and quantitation were linear over the range of DNA concentrations examined. To determine the number of both bacterial species cells, the fluorescent signals detected from DNA feces samples (in duplicate) in the linear range of the assay were averaged and compared to a standard curve.

EcN DNA in the fecal samples were detected by triplex PCR method described by Blum-Oehler et al. [36] using three primers pairs: Muta 5/6; Muta 7/8; and Muta 9/10. The reactions were carried out on the CFX96 thermocycler (BioRad). All PCR products were analyzed by electrophoresis through 3% agarose gels (Prona).

2.6. Statistical Analysis. Statistical analysis of the data was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant when P was less than 0.05.

3. Results

Macroscopic and microscopic evaluation of the colon showed no damage in control saline-treated animals without induction of colitis (Figure 1, Table 1, and Figure 2(a)). No colonic damage was also seen in animals without induction of colitis and treated with Mutaflor, rifaximin, or a combination thereof (Figure 1, Table 1). Rectal enema with acetic acid solution caused induction of colitis in all rats subjected to this procedure. In saline-treated rats, 7 days after the induction of colitis, the area of mucosal damage reached a value of $11.5 \pm 0.6\,\text{mm}^2$. Microscopic examination of the colon showed the presence of large lesions reaching the level of muscular membrane or even serous membrane (Table 1, Figure 2(b)). This alteration was associated with moderate or heavy inflammatory infiltration and the presence of mild fibrosis.

Macroscopic examination showed that treatment with Mutaflor given alone after induction of colitis tended to reduce the area of mucosal damage in the colon; however this

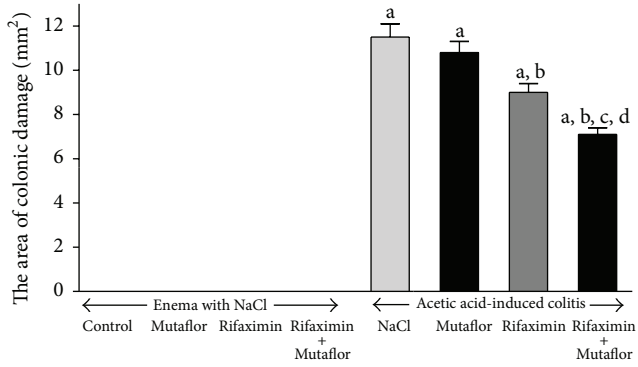

Figure 1: Influence of *E. coli* Nissle 1917 (Mutaflor) and rifaximin on the area of colonic lesions in rats without or with acetic acid-induced colitis. Mean value ± SEM. $N = 8$ animals in each experimental group. [a]$P < 0.05$ compared to control saline-treated rats without induction of colitis; [b]$P < 0.05$ compared to colitis + NaCl; [c]$P < 0.05$ compared to colitis + Mutaflor; and [d]$P < 0.05$ compared to colitis + rifaximin.

Table 1: Influence of *Escherichia coli* Nissle 1917 (Mutaflor) and rifaximin on morphological signs of colonic damage in rats without or with acetic acid-induced colitis observed 8 days after rectal administration of saline or acetic acid solution (colitis).

	Morphological changes			
	Grading of colonic damage (0–2)	Inflammatory infiltration (0–3)	Depth of damage (0–3)	Fibrosis (0–3)
Saline (control)	0	0	0	0
Mutaflor	0	0	0	0
Rifaximin	0	0	0	0
Rifaximin + Mutaflor	0	0	0	0
Colitis + NaCl	3	2-3	2	1-2
Colitis + Mutaflor	3	2-3	1-2	1
Colitis + rifaximin	2-3	1-2	1-2	1
Colitis + rifaximin + Mutaflor	2	1	1	0-1

Numbers represent the predominant histological grading in each group.

effect was statistically insignificant. Microscopic examination of the colon showed no effect of Mutaflor given alone on a size of damage and inflammatory infiltration in rats with colitis. Only a depth of colonic damage and a grade of fibrosis were slightly reduced in some animals (Table 1, Figure 2(c)).

In contrast, treatment with rifaximin given alone significantly reduced the area of mucosal damage by 22% when compared to animals with colitis treated with saline (Figure 1). Microscopic examination showed that the administration of rifaximin reduces the extent of colonic damage, inflammatory infiltration, and development of fibrosis (Table 1, Figure 2(d)).

Maximal reduction of the area of mucosal damage in macroscopic examination was observed in rats with colitis treated with the combination of rifaximin plus Mutaflor. The area of damage in those group of rats was significantly lower than in animals treated with saline, rifaximin, or Mutaflor given alone (Figure 1). Also microscopic evaluation showed that treatment with the combination of rifaximin plus Mutaflor maximally reduced the colonic damage in rats with colitis (Table 1, Figure 2(e)).

In the rats without induction of colitis, administration of Mutaflor and rifaximin given alone or in their combination failed to affect mucosal blood flow in the colon (Figure 3). Induction of colitis significantly reduced blood flow in colonic mucosa by around 35% in comparison to a value observed in control saline-treated rats. In rats with colitis, administration of Mutaflor given alone tended to improve colonic blood flow, but this effect was statistically insignificant. In contrast, the administration of rifaximin significantly improved blood flow through the colonic mucosa. The greatest improvement in blood flow in colonic mucosa of rats with colitis was observed after treatment with the combination of rifaximin plus Mutaflor (Figure 3).

In rats without induction of colitis, administration of Mutaflor or rifaximin given alone or in their combination was without any effect on DNA synthesis in colonic mucosa (Figure 4). Induction of colitis by the enema with acetic acid led to reduction in DNA synthesis in colonic mucosa by 38%. Administration of Mutaflor or rifaximin given alone did not significantly affect DNA synthesis in colonic mucosa in rats with colitis. Treatment with the combination of rifaximin plus Mutaflor partly, but significantly reversed the colitis-evoked reduction in mucosal DNA synthesis in the colon (Figure 4).

In rats without induction of colitis, intragastric administration of Mutaflor or rifaximin for 7 days failed to affect mucosal concentration of interleukin-1β (IL-1β) or Tumor Necrosis Factor-α (TNF-α) in the colon (Figures 5 and 6, resp.). Induction of colitis caused more than 8-fold increase in concentration of IL-1β and more than 6-fold increase in concentration of TNF-α in colonic mucosa. Administration of Mutaflor after induction of colitis was without significant

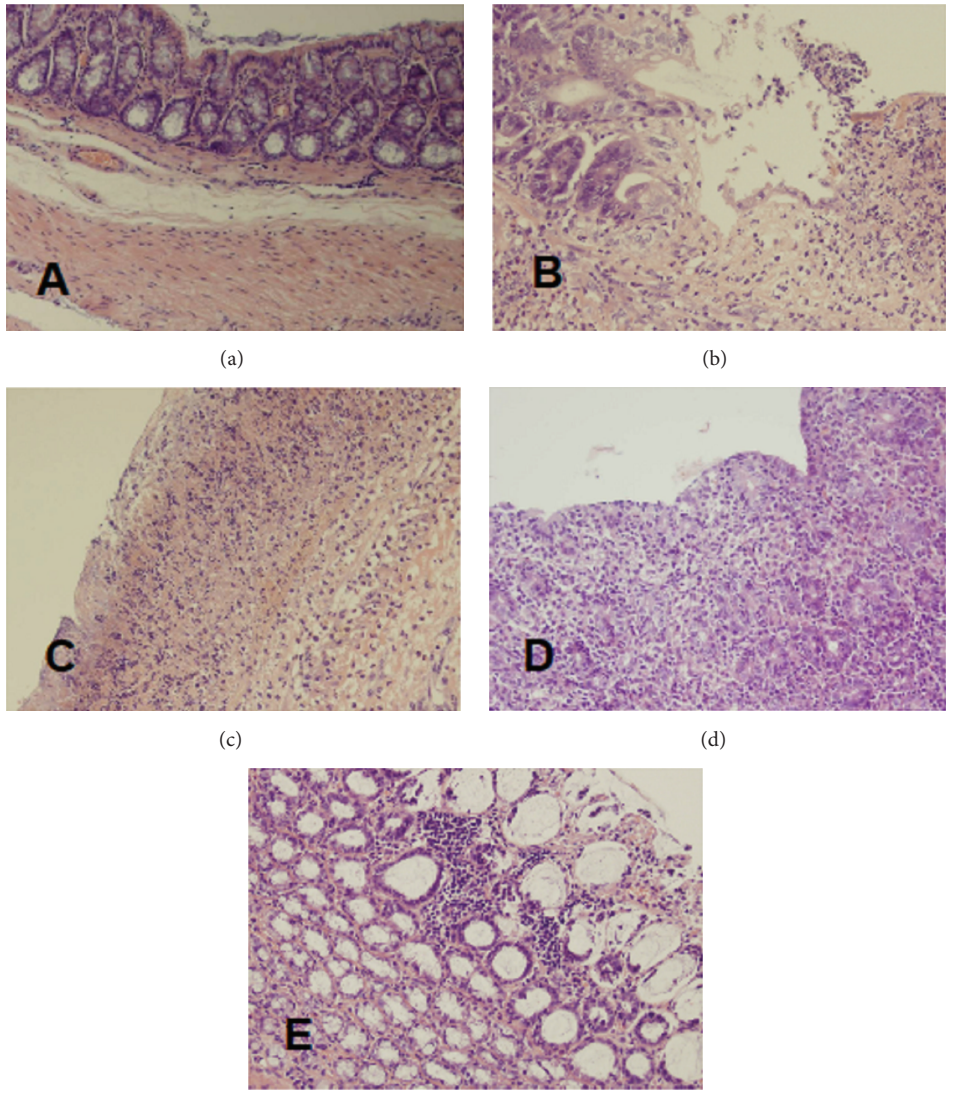

Figure 2: Histological features of the rat colonic mucosa stained by haematoxylin and eosin (original magnification 400x). (a) Control rats without induction of colitis and treated with saline for 7 days; (b) rats with colitis treated with saline for 7 days; (c) rats with colitis treated with Mutaflor for 7 days; (d) rats with colitis treated with rifaximin for 7 days; and (e) rats with colitis treated with the combination of rifaximin plus Mutaflor for 7 days.

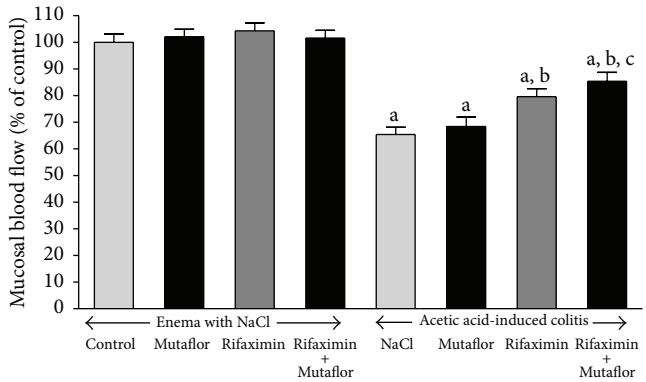

Figure 3: Influence of *E. coli* Nissle 1917 (Mutaflor) and rifaximin on mucosal blood flow in the colon in rats without or with acetic acid-induced colitis. Mean value ± SEM. $N = 8$ animals in each experimental group. [a]$P < 0.05$ compared to control saline-treated rats without induction of colitis; [b]$P < 0.05$ compared to colitis + NaCl; and [c]$P < 0.05$ compared to colitis + Mutaflor.

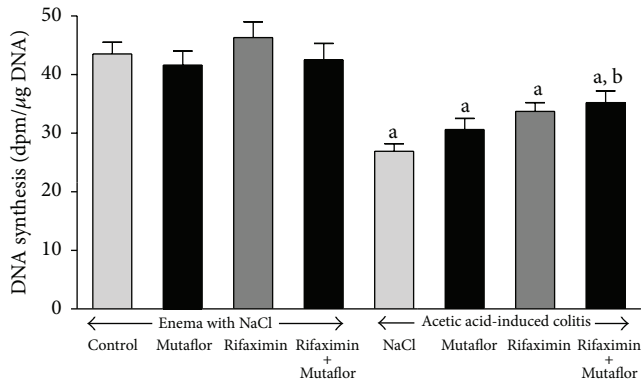

Figure 4: Influence of *E. coli* Nissle 1917 (Mutaflor) and rifaximin on DNA synthesis in colonic mucosa in rats without or with acetic acid-induced colitis. Mean value ± SEM. $N = 8$ animals in each experimental group. [a]$P < 0.05$ compared to control saline-treated rats without induction of colitis; [b]$P < 0.05$ compared to colitis + NaCl.

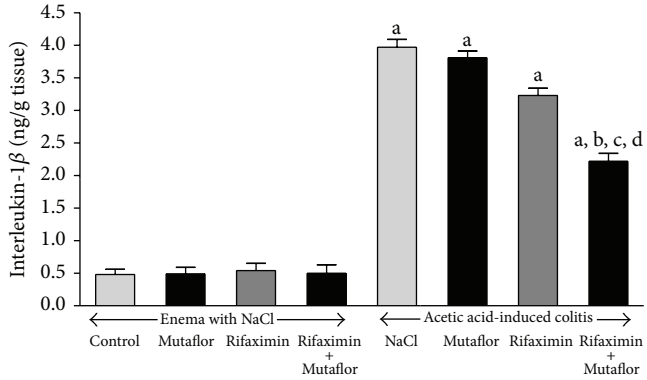

Figure 5: Influence of *E. coli* Nissle 1917 (Mutaflor) and rifaximin on of interleukin-1β concentration in colonic mucosa in rats without or with acetic acid-induced colitis. Mean value ± SEM. $N = 8$ animals in each experimental group. [a]$P < 0.05$ compared to control saline-treated rats without induction of colitis; [b]$P < 0.05$ compared to colitis + NaCl; [c]$P < 0.05$ compared to colitis + Mutaflor; and [d]$P < 0.05$ compared to colitis + rifaximin.

effect on mucosal concentration of IL-1β or TNF-α in the colon. Rifaximin administered alone caused a partial decrease in the level of IL-1β and TNF-α in colonic mucosa of animals with colitis. However, only the reduction of TNF-α concentration was statistically significant when compared to level observed in rats with colitis treated with saline. Maximal reduction in concentration of IL-1β and TNF-α in colonic mucosa was observed after administration of combination of rifaximin plus Mutaflor (Figures 5 and 6).

Administration of Mutaflor or rifaximin given alone as well as treatment with the combination of rifaximin plus Mutaflor was without any significant effect on mucosal myeloperoxidase activity in the colon in the rats without colitis induction (Figure 7). Induction of colitis caused more than a 3-fold increase in myeloperoxidase activity in colonic mucosa. Treatment with Mutaflor tended to reduce the colitis-evoked increase in myeloperoxidase activity in the colonic mucosa, but this effect was statistically insignificant. In contrast, administration of rifaximin resulted in a significant reduction in mucosal activity of myeloperoxidase in rats with colitis. Maximal reduction of the colitis-induced increase in mucosal myeloperoxidase activity as observed after treatment with combination of rifaximin and Mutaflor (Figure 7).

Table 2: Influence of treatment with *Escherichia coli* Nissle 1917 (Mutaflor) and/or rifaximin and induction of colitis on the total number of *Escherichia coli* identified by qPCR in feces samples.

Experimental groups	The number of bacteria (CFU/g)
Control	$2.41 \times 10^5 \pm 7.59 \times 10^4$
Mutaflor	$6.42 \times 10^6 \pm 3.21 \times 10^6$
Rifaximin	$2.95 \times 10^2 \pm 1.69 \times 10^2$
Rifaximin + Mutaflor	$5.54 \times 10^4 \pm 2.28 \times 10^4$
Colitis + NaCl	$7.57 \times 10^8 \pm 4.38 \times 10^{8a}$
Colitis + Mutaflor	$1.11 \times 10^7 \pm 4.22 \times 10^{6b}$
Colitis + rifaximin	$1.27 \times 10^3 \pm 6.68 \times 10^{2b}$
Colitis + rifaximin + Mutaflor	$4.52 \times 10^5 \pm 1.68 \times 10^{5b}$

Mean value ± SEM. $N = 8$ observations in each experimental group. [a]$P < 0.05$ compared to control; [b]$P < 0.05$ compared to colitis + NaCl.

In control rats without induction of colitis and treated i.g. with saline, the concentration of *E. coli* was 2.41×10^5 colony forming units (CFU) per gram of feces (Table 2). In rats without induction of colitis, treatment with Mutaflor tended to increase the concentration of *E. coli* in feces, whereas administration of rifaximin tended to reduce the number of

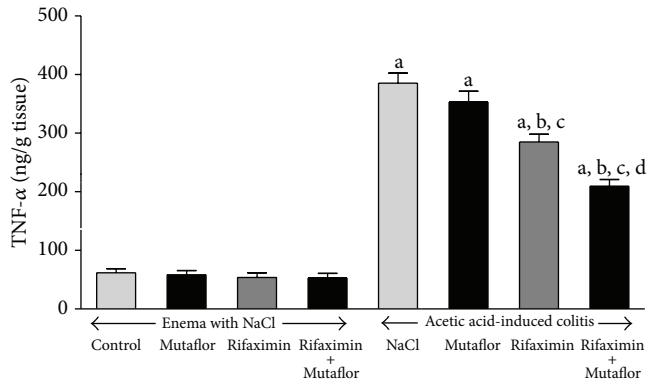

Figure 6: Influence of *E. coli* Nissle 1917 (Mutaflor) and rifaximin on of Tumor Necrosis Factor-α concentration in colonic mucosa in rats without or with acetic acid-induced colitis. Mean value ± SEM. $N = 8$ animals in each experimental group. [a]$P < 0.05$ compared to control saline-treated rats without induction of colitis; [b]$P < 0.05$ compared to colitis + NaCl; [c]$P < 0.05$ compared to colitis + Mutaflor; and [d]$P < 0.05$ compared to colitis + rifaximin.

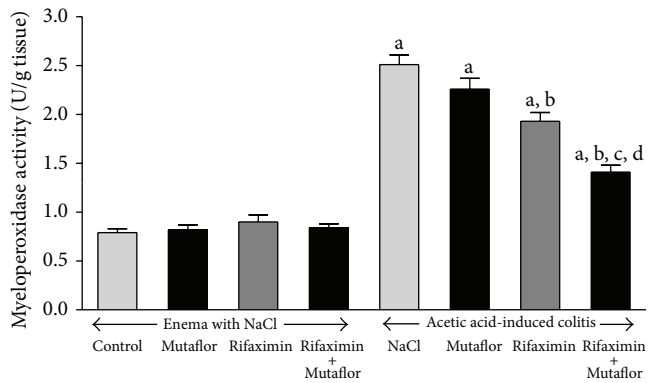

Figure 7: Influence of *E. coli* Nissle 1917 (Mutaflor) and rifaximin on myeloperoxidase activity in colonic mucosa in rats without or with acetic acid-induced colitis. Mean value ± SEM. $N = 8$ animals in each experimental group and each time of observation. [a]$P < 0.05$ compared to control saline-treated rats without induction of colitis; [b]$P < 0.05$ compared to colitis + NaCl; [c]$P < 0.05$ compared to colitis + Mutaflor; and [d]$P < 0.05$ compared to colitis + rifaximin.

those bacteria in the stool. However, both of those results were statistically insignificant. Induction of colitis caused statistically significant increase in concentration of *E. coli* in the stool. This effect of colitis on the number of *E. coli* in feces was significantly reversed by treatment with Mutaflor and rifaximin given alone or in their combination (Table 2).

In rats without induction of colitis and treated with saline, the concentration of *Enterococcus* spp. was 3.54×10^7 colony forming units (CFU) per gram of feces (Table 3). In rats without induction of colitis, treatment with Mutaflor or rifaximin given alone or in their combination significantly reduced the concentration of *E.* spp. in feces. Induction of colitis significantly increased the concentration of *E.* spp. in the stool. Treatment with Mutaflor and rifaximin given alone or in their combination significantly reversed the colitis-evoked increase in the number of *E.* spp. in feces (Table 3).

In all rats without treatment with Mutaflor, the presence of *E. coli* Nissle 1917 (EcN) was not detected (Figure 8). Administration of Mutaflor resulted in colonization of the large intestine by EcN in all rats without induction of colitis. This effect was increased in animals pretreated with rifaximin before Mutaflor administration. In animals with colitis, the colonization of the large intestine by EcN following administration of Mutaflor was less effective. Administration

Table 3: Influence of Mutaflor (*Escherichia coli* Nissle 1917), rifaximin, and colitis applied alone or in their combination on the number of *Enterococcus* spp. identified by qPCR in feces samples.

Experimental groups	The number of bacteria (CFU/g)
Control	$3.54 \times 10^7 \pm 5.90 \times 10^4$
Mutaflor	$3.23 \times 10^5 \pm 5.73 \times 10^{3\,a}$
Rifaximin	$6.88 \times 10^1 \pm 3.78 \times 10^{1\,a}$
Rifaximin + Mutaflor	$3.59 \times 10^2 \pm 7.69 \times 10^{1\,a}$
Colitis + NaCl	$4.34 \times 10^8 \pm 5.68 \times 10^{6\,a}$
Colitis + Mutaflor	$2.45 \times 10^7 \pm 3.22 \times 10^{5\,a,b}$
Colitis + rifaximin	$1.45 \times 10^2 \pm 7.26 \times 10^{1\,a,b,c}$
Colitis + rifaximin + Mutaflor	$6.34 \times 10^4 \pm 7.05 \times 10^{3\,a,b,c}$

Mean value ± SEM. $N = 8$ observations in each experimental group. [a]$P < 0.05$ compared to control; [b]$P < 0.05$ compared to colitis + NaCl; and [c]$P < 0.05$ compared to colitis + Mutaflor.

Figure 8: Determination of Muta 5/6 (361 bp), Muta 7/8 (427 bp), and Muta 9/10 (313 bp) amplicons by PCR method as indices of the presence of *E. coli* Nissle 1917 (EcN). Representative findings in (line 1) control rats without induction of colitis and treated intragastrically (i.g.) with saline; (line 2) rats without induction of colitis and treated i.g. with Mutaflor; (line 3) rats without induction of colitis and treated i.g. with rifaximin; (line 4) rats without induction of colitis and treated i.g. with the combination of rifaximin plus Mutaflor; (line 5) rats treated i.g. with saline after induction of colitis; (line 6) rats treated i.g. with Mutaflor after induction of colitis; (line 7) rats treated i.g. with rifaximin after induction of colitis; and (line 8) rats treated i.g. with the combination of rifaximin plus Mutaflor after induction of colitis. Line M—DNA mass ruler.

of Mutaflor given alone in rats with colitis led to the presence of primers specific to the EcN only in two cases on 8 observations. The addition of rifaximin before each dose of Mutaflor improved colonization of the colon by EcN. The presence of the EcN was observed in all animals in this experimental group (Figure 8).

4. Discussion

Our present study has shown that treatment with rifaximin accelerates the healing of acetic acid-induced colitis. This effect has been found as a faster reduction in the area of colonic damage, as well as a decrease in mucosal level of myeloperoxidase (MPO), interleukin-1β (IL-1β), and Tumor Necrosis Factor-α (TNF-α).

MPO is an enzyme most abundantly present in azurophilic granules of neutrophil granulocytes. Antimicrobial function of neutrophils is related, among others, to activity of MPO and possibility to generate hypochlorous acids and reactive oxygen species (ROS) during the respiratory burst [37]. MPO is released by activated neutrophils and for this reason tissue activity of MPO reflects the degree of tissue infiltration by neutrophils and may be used as an indirect marker of tissue oxidative stress [37, 38]. In turn, IL-1β and TNF-α are important proinflammatory cytokines. IL-1β is a proinflammatory cytokine responsible for initiating the release of a cascade of proinflammatory factors during inflammation [39]. Administration of rifaximin after induction of colitis has decreased the activity of MPO and reduced concentration of IL-1β and TNF-α in colonic mucosa, reflecting the reduction in the local inflammatory reaction.

Treatment with rifaximin has also affected blood flow and DNA synthesis in colonic mucosa in rats with acetic acid-induced colitis. Induction of colitis has strongly reduced those parameters, whereas administration of rifaximin has significantly improved blood flow and DNA synthesis in rats with colitis. Mucosal blood flow plays an important role in the protection and healing of mucosa in the gut [40, 41]. Previous studies have shown that exposure of gastric mucosa to potentially noxious factors results in little or no damage, as long as adequate blood flow is maintained, whereas reduction in mucosal blood flow leads to severe gastric injury [41].

Rate of mucosal DNA synthesis can be recognized as an index of cell vitality and cell proliferation. Previous studies have shown that inhibition of mucosal cell proliferation or excessive apoptosis results in the development of ulcers [42, 43], whereas a stimulation of mucosal cell proliferation exhibits protective and healing in the gastrointestinal tract [28, 44–46].

Therapeutic effect of rifaximin in acetic acid-induced colitis shown in our present study is in harmony with previous experimental [47] and clinical studies [10–13].

In contrast to effects obtained after treatment with rifaximin, we have found that administration of Mutaflor tends to improve the healing of acetic acid-induced colitis, but this effect is weak and statistically insignificant. Moreover, the presence of *Escherichia coli* strain Nissle 1917 (EcN) has been found only in feces of two per eight rats treated with Mutaflor alone after induction of colitis. This observation explains why studies showing the therapeutic effect of EcN in colitis are so few and far between. There are some experimental studies performed on rodents showing preventive and/or therapeutic effect of pretreatment or treatment with EcN in colitis evoked by dextran sodium sulfate [48], trinitrobenzene sulfonic acid [49], or transfer of $CD4^+$ $CD62L^+$ T lymphocytes from BALB/c mice in SCID mice [48]. Moreover, there are three clinical studies performed in adult patients [19–21] and one in children and adolescents [50] showing that EcN (Mutaflor) given orally is useful in preventing relapses in inactive ulcerative colitis (UC) and its efficacy is comparable to effects of standard therapy with mesalazine. Moreover, studies performed by Rembacken et al. [21] have found that oral treatment with EcN leads to remission in the similar percentage of patients with active ulcerative colitis as treatment with mesalazine. Unfortunately, their data are difficult to interpret because all patients at the same time were

treated with hydrocortisone acetate enemas or prednisone given orally according to the severity of disease. Moreover, all patients received a 1-week course of oral gentamicin [21].

There is also clinical trial examining the potential therapeutic effect EcN in UC [22]. Patients with moderate distal activity in UC were assigned to treatment with either 40, 20, or 10 mL enemas containing 10^8 EcN/mL or placebo. Authors have found that according to an intent-to-treat-population analysis the number of responders was not significantly higher in EcN group than in the placebo group. On the other hand, they have also reported that the Jonckheere-Terpstra rank correlation for dose-dependent efficiency indicated a significant correlation of per-protocol responder rates. Time to remission was shortest in patients treated with EcN 40 mL. However, it must be pointed out that groups of patient were not equivalent.

In the case of Crohn's disease (CD), there is only one clinical study showing that administration of EcN can help in maintaining remission in this disease [23].

The most important finding of our present study is the observation that administration of combination of rifaximin plus EcN in the course of acetic acid-induced colitis generates a greater therapeutic effect than any of these agents given alone. It was manifested by a statistically significant acceleration of healing of colonic damage, as well as by a reduction in local inflammatory process found as a decrease in MPO activity and a reduction in concentration of IL-1β and TNF-α in colonic mucosa. Moreover, we have found that treatment with combination of rifaximin plus EcN maximally improves blood flow and DNA synthesis in mucosa of the colon in rats with colitis.

Another important finding of our present study was the observation that pretreatment with rifaximin before administration of EcN favors the colonization of the colon by EcN. The presence of EcN has been found in all rats treated with the combination of rifaximin plus Mutaflor. Currently, great attention is given to the role of commensal bacteria in the pathogenesis of IBD. There are studies showing an increase in colonic population of Enterobacteriaceae, including *E. coli* in patients with UC and CD versus a control group [51]. Kleessen et al. [51] have found a bacterial invasion of mucosa in colonic specimens of UC patients, as well as in ileal and colonic specimens obtained from CD patients. In contrast to that, no bacteria were detected in tissues of healthy humans [51]. Similar findings have been found by Mylonaki et al. [52]. They have detected higher number of epithelium-associated *E. coli* in active than inactive UC or controls. Epithelium-associated *E. coli* counts were also higher in CD. Moreover *E. coli* were also found as individual bacteria and in clusters in the lamina propria in UC and CD patients but in none of the controls.

In harmony with the above-mentioned observations are recent studies performed by Elliott et al. [53] and Vazeille et al. [54]. Elliott et al. [53] have found that intramacrophage *E. coli* are commonly observed in lamina propria macrophages in mucosal biopsies from CD patients, rarely in UC and not at all in healthy controls. Authors have concluded that persistence of *E. coli* within macrophages located in lamina propria may provide a stimulus for chronic inflammation. The role of *E. coli* in the pathogenesis of IBD has also been confirmed by findings of Vazeille et al. [54]. They have found that monocyte-derived macrophages from CD patients are impaired in the ability to control intracellular adherent-invasive *E. coli* and exhibit disordered cytokine profile. Moreover, currently performed meta-analysis has revealed that intestinal colonization with phylogenetic group B2 *E. coli* is associated with UC [55].

Data mentioned above and our results taken together suggest that substitution of other, potentially pathogenic strains of *E. coli* by nonpathogenic strain Nissle 1917 plays an important role in therapeutic effect of coadministration of rifaximin plus Mutaflor in acetic acid-induced colitis. Substitution of other *E. coli* strains by EcN is also important due to the rapid development of resistance of *E. coli* against rifaximin in the case of use of this antibiotic [56]. Moreover, our present study has shown treatment with Mutaflor and rifaximin given alone or in their combination significantly reversed the colitis-evoked increase in the colonic number of *Enterococcus* spp.

Finally, we conclude that rifaximin and Mutaflor exhibit synergic anti-inflammatory and therapeutic effect in acetic acid-induced colitis in rats. This observation suggests that rifaximin plus Mutaflor may be the optimal choice in the treatment of colitis by probiotics.

Competing Interests

The authors declare that they have no competing interests.

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Clinical Study

Influence of *Helicobacter pylori* Infection on Metabolic Syndrome in Old Chinese People

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Background. H. pylori infection is one of the most common chronic infectious inflammatory diseases worldwide and is also a risk factor for atherosclerosis. Patients with metabolic syndrome are known to be at increased risk for atherosclerosis. The aim of our study was to assess the effects of *H. pylori* infection on serum lipids, body mass index (BMI), and metabolic syndrome in old Chinese people. *Material and Method.* A total of 191 (133 males and 58 females, aged 73.19±11.03 years) people who had gastroscopy examination in our hospital were divided into *H. pylori*-positive group ($n = 80$) and *H. pylori*-negative group ($n = 111$). *H. pylori* infection was diagnosed by rapid urease test. *Results.* Patients with *H. pylori* infection had higher BMI and fasting glucose levels and incidence of metabolic syndrome ($p < 0.01$). It was found that BMI ($p < 0.01$, OR 74.469), *H. pylori* infection ($p < 0.01$, OR 5.427), total cholesterol ($p < 0.01$, OR 15.544), and diabetes mellitus ($p < 0.01$, OR 23.957) were significantly associated with the risk of metabolic syndrome by binary logistic regression analysis. *Conclusions.* Patients with *H. pylori* infection had higher BMI and fasting glucose levels and had incidence of metabolic syndrome.

1. Introduction

H. pylori, a Gram-negative bacterium that dwells on the gastric epithelium, infects over 50% of the human population, with a high rate in those living in developing countries [1]. *H. pylori* can cause many gastrointestinal diseases, including peptic ulcers, chronic gastritis, and gastric mucosa-associated lymphoid tissue lymphoma (MALToma). It is also considered a class I carcinogen that can induce chronic inflammation and gastric cancer [2, 3].

In recent years, several studies demonstrated that the outcome of *H. pylori* infection may not be confined to the digestive tract, and that the infection can be associated with extradigestive pathologies including atherosclerotic vascular diseases [4–6]. Atherosclerosis is a multifactorial disease. *H. pylori* may disturb lipid and glucose metabolism in a way that may increase the risk of atherosclerosis [7].

Metabolic syndrome has become a worldwide public health issue, and it is also a risk factor for atherosclerosis. According to the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III), metabolic syndrome is composed of the following major components: abdominal obesity, insulin resistance (IR), elevated BP, and dyslipidemia [8].

This study aimed to determine the prevalence of metabolic syndrome and its components in *H. pylori*-positive patients in middle-aged and elderly Chinese population.

2. Material and Method

2.1. Study Population and Data Collection. This study was conducted at Navy General Hospital. All qualified subjects who attended their annual health examination during the year of 2014 were initially enrolled. Inclusion criteria were complete, available medical records and having gastroscopy in our hospital; having not used proton pump inhibitors (PPIs), histamine type 2 receptor antagonists (H_2A), antibiotics, bismuth, or sucralfate for up to one month prior to the endoscopy; stopping using various anticoagulation and

antiplatelet drugs for more than a week; normal blood pressure (diastolic blood pressure (DBP) of less than 90 mmHg and systolic blood pressure (SBP) of less than 150 mmHg) or well-controlled hypertension (DBP of less than 90 mmHg and SBP of less than 150 mmHg). Exclusion criteria were the presence of hematological diseases; past history of cancer; major gastrointestinal surgery, including partial or total gastrectomy or colectomy; pulmonary disease; smoking; alcohol consumption; nephrotic syndrome or serum creatinine levels higher than 422 μmol/L; having been using the medicine which is known to be able to effect blood coagulation, such as aspirin and clopidogrel; platelet count below 100000/mL; previously being diagnosed with anemia. The definition of anemia in this study was serum hemoglobin < 120 g/L for males and <110 g/L for females. Finally, 191 eligible subjects were enrolled (133 males and 58 females, aged 73.19 ± 11.03 years). Written informed consent was obtained from each subject and was recorded by the physician who explained the study procedures. The study was reviewed and approved by the Ethics and Research Committee of the Navy General Hospital (Beijing, China) and the reported investigations were carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

2.2. Metabolic Syndrome Diagnosis. Metabolic syndrome was diagnosed following the China Adult Dyslipidemia Prevention Guide on metabolic syndrome [9]. The subject was in accordance with three or four of the following criteria: (1) overweight and/or obesity: body mass index (BMI) > 25.0 kg/m^2; (2) hyperglycemia: fasting blood glucose (FBG) ≥ 6.1 mmol/L or 2 h postmeal plasma glucose >7.8 mmol/L or having been diagnosed with diabetes mellitus; (3) hypertension: SBP ≥ 140 or DBP ≥ 90 mmHg or taking ≥1 antihypertensive agent; (4) dyslipidemia: fasting triglycerides (TGs) ≥ 1.7 mmol/L and/or high-density lipoprotein cholesterol (HDL-C) ≤ 0.9 mmol/L for males and ≤1.0 mmol/L for females.

2.3. Clinical Examination. All of the subjects were interviewed regarding current health status (diabetes mellitus, hypertension, and gastrointestinal diseases) and were asked not to do exercise for one day prior to the medical examination. Blood pressure was measured in the right arm using a mercury sphygmomanometer after 20 min of rest with the participants in a sitting position. The first and fifth Korotkoff sounds were used as systolic and diastolic blood pressure. Standing height, body weight, and waist circumference were recorded for all subjects. Waist circumference was measured with the measuring tape positioned midway between the lowest rib and the superior border of the iliac crest as the participants exhaled normally. BMI was calculated as weight divided by height squared.

2.4. Biochemical Analyses. Blood samples were taken into anticoagulated tubes from participants after an overnight fast of more than 12 h. Plasma separated by centrifugation at 3000 ×g for 10 minute at room temperature. The levels of creatinine, BUN, total cholesterol, triglyceride, and glucose were measured using a multichannel analyzer (Roche Hitachi 737; Boehringer Mannheim Diagnostics, USA).

2.5. Gastroscopy and H. pylori Examination. All subjects were required to refrain from intake of food and water on the morning of gastroscopy, and gastroscopy was performed routinely under light intravenous sedation and local anesthetic spray to the oropharynx. A diagnosis of *H. pylori* infection was made if *H. pylori* morphology was seen on histopathological examination and the rapid urease test during gastroscopy was positive. Patients with negative results in one or both examinations were considered to be *H. pylori*-negative.

The gastroscopic procedures were performed using an upper gastrointestinal video endoscope (Olympus EVIS EXERA III, CV-190). The whole stomach was examined first with conventional endoscopy. After the whole stomach mucosa was observed the sites were chosen for biopsy of the gastric mucosa. The biopsy forceps were taken from the distal helicobacter 1-2 cm mucous membrane and then put it in the urease test wells for *H. pylori* quick test (Biohit Plc., Helsinki, Finland). The exact time of the placement of the biopsies in the urease test wells was recorded and the wells were inspected for color change at 2 min, 30 min, 2 h, and 24 h. The test was assigned positive when there was a color change of at least 2 mm radius of red cloud around the biopsy specimen or complete color change of the yellow well to red or magenta; negative color stayed the same. At the same time, a piece of gastric mucous membrane specimen was taken for pathologic examination. The gastric tissue specimens were submitted to the pathologist for histological analysis. The hematoxylin-eosin and the Giemsa stainings were used for identification of *H. pylori*. To minimize the potential bias, the pathological analysis was made by one experienced pathologist at Pathological Laboratory of Navy General Hospital.

2.6. Statistical Analysis. Data were expressed as mean ± SD or counts. Statistical analysis was performed using SPSS version 16.0 (SPSS Inc., Chicago, IL), and the level of statistical significance was defined as $p < 0.05$. The independent samples t-test was used for the comparisons of continuous data, while the chi-square test was used for the comparisons of categorical variables. Binary logistic regression analysis was used to determine the factors that were associated with metabolic syndrome.

3. Results

3.1. Baseline Characteristics. Among the 191 enrolled patients (133 males and 58 females, aged 73.19 ± 11.03 years), 80 (59 males and 21 females) were diagnosed with *H. pylori* infection. The prevalence of *H. pylori* infection was 41.89% (males 44.36% and females 36.21%). The characteristics of the patients, classified being *H. pylori*-positive or *H. pylori*-negative, are presented in Table 1. Patients with *H. pylori* infection had higher BMI and fasting glucose levels and incidence of metabolic syndrome ($p < 0.01$).

Table 1: Characteristics of study subjects according to the *H. pylori* infection.

Variables	*H. pylori* negative ($n = 111$)	*H. pylori* positive ($n = 80$)	p value
Age	71.89 ± 11.07	75.00 ± 10.80	0.055
Male, n (%)	74 (66.67)	59 (73.75)	0.295
SBP (mmHg)	132.79 ± 13.33	131.58 ± 14.19	0.547
DBP (mmHg)	74.06 ± 8.23	75.74 ± 9.66	0.200
BMI (kg/m^2)	23.10 ± 2.74	24.31 ± 2.70	0.003
Metabolic syndrome, n (%)	42 (37.84)	43 (53.75)	0.001
Total cholesterol (mmol/L)	4.22 ± 1.15	4.36 ± 0.88	0.383
Triglycerides (mmol/L)	1.34 ± 0.81	1.21 ± 0.52	0.221
Fasting glucose (mmol/L)	5.66 ± 1.40	6.20 ± 1.80	0.022
Creatinine (μmol/L)	101.54 ± 34.79	100.65 ± 24.23	0.845
BUN (mmol/L)	6.03 ± 2.26	6.27 ± 1.90	0.443
Hypertension, n (%)	32 (28.83)	19 (23.75)	0.435
Diabetes mellitus, n (%)	19 (17.12)	21 (26.25)	0.135

Table 2: The results of binary logistic regression analysis on metabolic syndrome.

	Variable	SE	Beta	p value	OR	95.0% CI	
						Lower	Upper
Age	<65 = 0, ≥65 = 1	0.674	0.503	0.485	1.602	0.428	5.999
Male, n	Female = 0, male = 1	0.582	−0.066	0.837	0.887	0.283	2.776
SBP (mmHg)	<140 = 0, ≥140 = 1	0.623	0.602	0.216	2.161	0.637	7.324
DBP (mmHg)	<90 = 0, ≥90 = 1	0.909	0.831	0.290	2.619	0.441	15.551
BMI (kg/m^2)	<25 = 0, ≥25 = 1	0.683	4.117	0.001	74.469	19.507	284.29
H. pylori infection (n)	Absent = 0, present = 1	0.575	1.538	0.003	5.427	1.757	16.76
Total cholesterol (mmol/L)	<5.69 = 0, ≥5.69 = 1	0.945	2.620	0.004	15.544	2.441	98.981
Triglycerides (mmol/L)	<1.7 = 0, ≥1.7 = 1	0.704	0.243	0.797	0.834	0.21	3.316
Fasting glucose (mmol/L)	<6.1 = 0, ≥6.1 = 1	0.959	1.392	0.091	5.053	0.772	33.068
Creatinine (μmol/L)	<115 = 0, ≥115 = 1	0.796	1.076	0.258	2.46	0.517	11.703
BUN (mmol/L)	<8.2 = 0, ≥8.2 = 1	0.91	0.237	0.873	0.865	0.145	5.151
Hypertension (n)	Absent = 0, present = 1	0.679	−0.111	0.910	0.926	0.245	3.504
Diabetes mellitus (n)	Absent = 0, present = 1	1.202	3.182	0.008	23.957	2.271	252.722

3.2. H. pylori Infection and Risk Factors for Metabolic Syndrome. Binary logistic regression analysis was used to evaluate the risk factors for metabolic syndrome. Metabolic syndrome was taken as the dependent variable and age, gender, SBP, DBP, BMI, *H. pylori* infection, total cholesterol, triglyceride, fasting glucose, creatinine, BUN, hypertension, and diabetes mellitus were taken as independent variables. It was found that BMI ($p < 0.01$, OR 74.469), *H. pylori* infection ($p < 0.01$, OR 5.427), total cholesterol ($p < 0.01$, OR 15.544), and diabetes mellitus ($p < 0.01$, OR 23.957) were significantly associated with the risk of metabolic syndrome (Table 2).

4. Discussion

This study showed a positive association between *H. pylori* infection and the prevalence of metabolic syndrome among a group of subjects from middle-aged to elderly Chinese population, which is in agreement with the previous studies [10, 11]. According to the multiple logistic regression analyses performed in this study, *H. pylori* infection was found to be associated with an increased risk of metabolic syndrome, indicating that *H. pylori* infection could be used as a risk factor of metabolic syndrome.

The mechanisms underlying the association between *H. pylori* infection and metabolic syndrome and its role in predicting metabolic syndrome in obese patients are unclear. There are three possible mechanisms that might explain our findings. First, *H. pylori* infection impairs secretion balance of proinflammatory cytokines and CRP, angiotensinogen, free fatty acids, and leptin hormone, and thus, reactive oxygen species begin to accumulate. Subclinical chronic inflammation induced by *H. pylori* infection occurs via impaired cytokine balance and stimulated macrophages [12, 13]. There are explanations that this leads to unresponsiveness to insulin in the peripheral tissue and subsequently to metabolic syndrome [14–17]. Second, Ghrelin, as a multifunctional polypeptide secreted from gastric mucosa, is involved in ingestion, appetite, and nutrition, especially lipid absorption and lipogenesis [18, 19]. The Ghrelin can also

modulate insulin sensitivity and stimulate insulin-induced glucose uptake [20], and the *H. pylori* infection can impair Ghrelin synthesis [21]. Third, the previous studies showed that infection with *H. pylori* had a positive association with high LDL, low HDL, and cardiovascular disease and successful *H. pylori* eradication decreased the risk of high LDL and low HDL [22].

In short, metabolic syndrome is significantly increased in patients with *H. pylori* infection, which will help to explore the pathogenesis of the metabolic syndrome. It is necessary to further research the relationship between *H. pylori* infection and metabolic syndrome. We believe that *H. pylori* eradication has the potential to be used in prevention and treatment of the metabolic syndrome.

A few limitations warrant consideration. First, we did not investigate the patients with *H. pylori* infection after treatment. Second, this was a single-center study and thus our relatively small sample size may have posed a limitation to this study. Therefore, our findings need to be confirmed in multicenter and prospectively designed studies. Third, we did not examine *H. pylori* infection by urea breath test or stool antigen test, which cannot exclude the past infection; that is, in case the patients had severe atrophy, *H. pylori* cannot be detected there. Finally, we did not investigate the Ghrelin or proinflammatory cytokines, which could confound the pathogenesis of the metabolic syndrome.

Competing Interests

The authors report no conflict of interests in the study design, collection, analysis, and interpretation of the data, and/or drafting of the paper.

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Research Article

A Prebiotic Formula Improves the Gastrointestinal Bacterial Flora in Toddlers

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We aimed to investigate the effect of enriched 3-prebiotic formula (including inulin, fructooligosaccharides, and galactooligosaccharides) on toddler gut health by measuring fecal microbiota. Our results revealed that the consumption of 3-prebiotic formula three times per day giving total intake of 1.8 g prebiotic ingredients significantly showed the increased number of probiotic *Bifidobacterium* spp. colonies and the reduced populations of both *C. perfringens* and total anaerobic bacteria on the fecal bacterial flora in toddlers at 18~36 months. In addition, total organic acids in the fecal samples significantly increased which improves the utilization of bifidus under acidic conditions after consumption of the 3-prebiotic formula. Therefore, using the formula enriched with prebiotic may maintain gut health in toddlers.

1. Introduction

The intestinal flora of newborn infants is an important physiologic factor in gut function and development of the immune system, which decreases as a child ages. Probiotics, such as lactobacilli and bifidobacteria, have been reported to increase populations of friendly bacteria and inhibit activities of harmful or pathogenic microbes in the human intestines and to be beneficial for maintaining individuals' health [1, 2]. Many studies have shown that infant formulas supplemented with probiotics, such as *Bifidobacterium* spp., can increase the population of bifidobacteria, enhance mucosal resistance against gastrointestinal infections, and reduce gut disorders [3–6].

Prebiotics are defined as indigestible food ingredients that are specifically fermented by bifidobacteria. Their presence in the intestines beneficially affects the host by selectively stimulating not only the growth and activity of bacteria and the formation of bacterial flora in the colon, but also the host's immune defenses, thus improving host health [7]. It was verified that inulin, lactulose, fructooligosaccharides, isomaltooligosaccharides, and galactooligosaccharides possess prebiotic properties [8–10]. Human milk oligosaccharides were reported to selectively stimulate the growth of bifidobacteria and lactobacilli in the intestines that may directly contribute to natural defenses against infection [11–13]. The composition and structure of human milk oligosaccharides cannot be reproduced by food processing; therefore, prebiotics are being considered for fortification of infant formulas. Cow milk-based infant formulas supplemented with a prebiotic mixture of galacto- and fructooligosaccharides can stimulate intestinal flora and the numbers of fecal bifidobacteria and lactobacilli similar to that of breast-fed infants [14, 15]. Inulin is composed of a group of fructose polymers that are only partially digested by upper-intestinal enzymes. Moreover, inulin was shown to consistently increase absorption and retention of several minerals and to improve bone mineralization [16–18].

The aim of the current study was to examine the effect of supplementing toddler formulas with three prebiotic ingredients (3-prebiotic formula), including inulin, fructooligosaccharides, and galactooligosaccharides, on the gastrointestinal bacteria flora and human physiology among 18~36-month-old children.

2. Materials and Methods

2.1. Subjects. Toddlers aged 1.5~3 years were screened by a pediatric physician. None had any evidence of gastrointestinal diseases or other health problems. For 2 weeks before the

experiment, none of the subjects took any antibiotics or any drugs or supplements that might affect the gastrointestinal bacteria. A detailed explanation of the study design was given to the parents of subjects before participating in this trial. Each parent of subjects was enrolled with written informed consent. The study was approved by Taipei Medical University Research Ethics Committee.

2.2. Treatment. The toddler formula (3-prebiotic formula) was supplemented with three prebiotic ingredients, including inulin, fructooligosaccharides, and galactooligosaccharides (600 mg per 240 mL), provided by Bristol-Myers Squibb (Taipei, Taiwan).

2.3. Experimental Design and Process. We conducted an 8-week dietary intervention trial with 30 1.5~3-year-old children. The study period for each toddler was 8 weeks, comprising an initial 1 week of consuming formula without prebiotics (control period), an administration period of 6 weeks of consuming 240 mL of the 3-prebiotic formula three times a day, and 1 week of follow-up with the original formula without prebiotics.

During the experimental period, parents daily filled out a questionnaire on the fecal samples frequency and consistency. Consistency scores were recorded as follows: 1 = watery; 2 = soft and formed; 3 = hard. Fecal samples were collected and analyzed for the fecal bacterial flora on days 1, 7, 21, 35, 49, and 56. The fecal bacterial flora assessed was *Bifidobacterium* spp., *Clostridium perfringens*, and total anaerobic bacteria. Organic acids, including lactate, acetate, propionate, and butyrate, were analyzed on days 7 and 49.

Bifidobacterium spp. in the fecal samples were used as an indicator of the probiotics in the intestinal tract and were incubated on bifidobacterium iodoacetate medium-25 (BIM-25), while *C. perfringens* was an indicator bacterial pathogen in the intestines and was incubated on tryptose-sulfite-D-cycloserine (TSC) agar with 100 mL of a D-cycloserine solution and 100 mL of 50% egg yolk emulsion. Total anaerobic bacteria in the feces were calculated on CDC Anaerobe Blood Agar plates and were used as the reference. Colonies were counted after 48 h of incubation to determine colony-forming units (cfu) per gram of wet weight of feces. The logarithm-values of the colonies of *Bifidobacterium* spp., *C. perfringens*, and total anaerobic bacteria in each gram of feces represented the gastrointestinal bacterial flora of subjects.

Organic acids such as lactic acid, acetic acid, propionic acid, and butyric acid in the feces were analyzed with high-performance liquid chromatography (HPLC). Briefly, precisely 4 g of feces was mixed with 25 mL acetonitrile thoroughly before being centrifuged at 20,000 ×g for 30 min. The supernatant was removed and requantified to 25 mL before being syringe-filtered through a Titan (pore size: 0.2 μm) filter. The final filtrate was injected into an HPLC system containing an Aminex HPX-87H column (300 × 7.8 mm), a solution of 3.75 mM sulfuric acid as the mobile phase, and a UV. The flow rate was set at 0.4 mL/min and the column temperature was set at 65°C.

Table 1: Subject characteristics.

	Subjects
N	38
Boys (*n*)/girls (*n*)	17/21
Age (years)[1]	2 ± 0.6
Height (percentile, th)	75
Weight (percentile, th)	50~75

[1]Values are expressed as the mean ± SD.

2.4. Statistical Analysis. Data are expressed as the mean ± SEM. A paired *t*-test was performed on each of the variables to assess mean differences across time with SPSS software (Chicago, IL, USA). Differences were considered significant for p values of <0.05.

3. Results

3.1. Physiological Effect of Supplementation with the 3-Prebiotic Formula. Overall, 38 subjects (17 boys and 21 girls) completed the entire experiment; three subjects withdrew from the study due to personal reasons. Baseline characteristics are shown in Table 1. The frequency of diarrhea among subjects significantly decreased between weeks 0 and 6 ($p < 0.05$) of the administration period according to descriptions in the parents' questionnaires (data not shown).

3.2. Effect of 3-Prebiotic Formula Administration on the Fecal Bacteria Flora. After ingestion of the 3-prebiotic formula, the number of *Bifidobacterium* spp. colonies significantly increased compared to that at week 0 ($p < 0.05$) and was significantly lower after the follow-up period (Table 2). Conversely, the population of *C. perfringens* significantly decreased between weeks 0 and 6 of the administration period, and the increase after the follow-up period was notably lower than that in the control period and at the baseline. A decrease with a time-response effect was noted in the number of *C. perfringens* colonies after subjects consumed the 3-prebiotic formula. During the administration period, total anaerobic bacteria were significantly reduced with time ($p < 0.05$), although total anaerobic bacteria in the follow-up period were lower than those in the control period ($p < 0.05$).

We made further observations of the growth of friendly and harmful bacteria in the gastrointestinal tract after ingestion of the 3-prebiotic formula. The population of *Bifidobacterium* spp. was compared to the total anaerobic bacteria, and an increase was observed during the experimental period ($p < 0.05$), which significantly differed from that of week 0, although the ratio was found to have decreased in the follow-up period ($p < 0.05$) and was still significantly higher than that in the control period (Figure 1(a)). Compared to the ratio of *C. perfringens* to the total anaerobic bacteria in each phase, the ratio gradually decreased during the experimental period ($p < 0.05$), and the decrease remained until the end of the study (Figure 1(b)). Moreover, the ratio of *Bifidobacterium* spp. to *C. perfringens* during ingestion of the 3-prebiotic formula significantly increased with a time-response effect

Table 2: Effects of 3-prebiotic formula administration on the fecal bacterial flora.

	Control period (1 week)	Administration period (6 weeks)				Follow-up period (1 week)
		0	2	4	6	
B	9.1 ± 0.5	9.2 ± 0.4	9.5 ± 0.4*	9.5 ± 0.2*	9.6 ± 0.3*	9.4 ± 0.2†
C	5.5 ± 0.7	5.3 ± 0.5	4.4 ± 0.5*	4.3 ± 0.7*	3.8 ± 0.9*	4.2 ± 0.7*†
T	10.0 ± 0.3	9.98 ± 0.2	9.5 ± 0.3*	9.3 ± 0.3*	9.2 ± 0.7*	9.5 ± 0.3*†

Data are expressed as colony-forming units (cfu) in log (numbers per gram of feces). B, *Bifidobacterium* spp.; C, *Clostridium perfringens*; T, total anaerobic bacteria.

*Values significantly differ compared to that at week 0 of the administration period ($p < 0.05$). †Values significantly differ compared to that of the control period ($p < 0.05$).

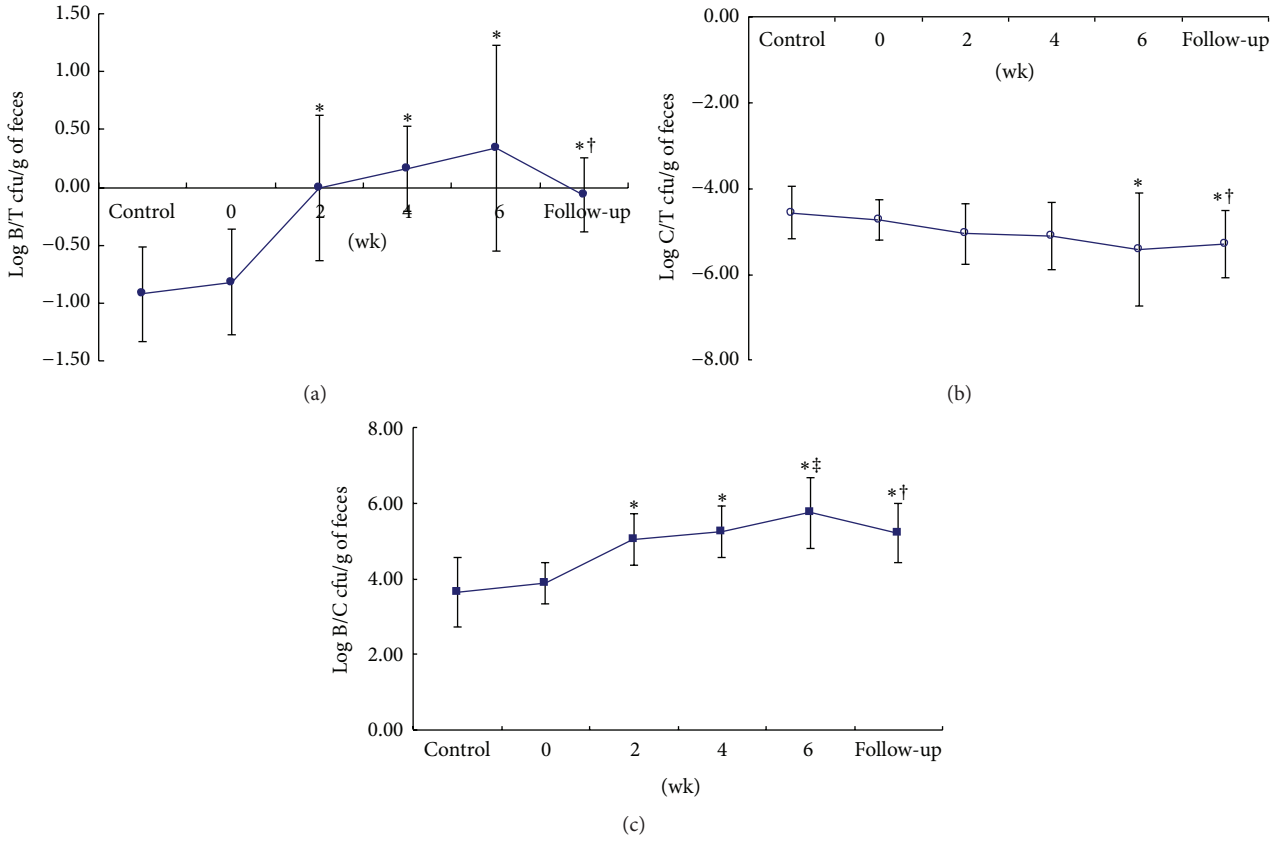


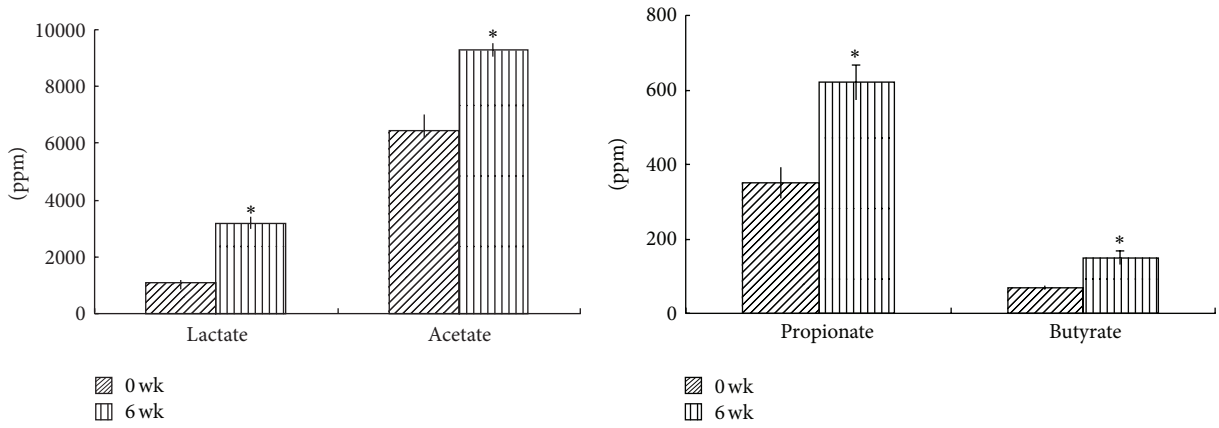
Figure 1: Effect of 3-prebiotic formula administration on the ratio of fecal bacterial flora in healthy subjects. Numbers of *Bifidobacterium* spp. (B), *Clostridium perfringens* (C), and total anaerobic bacteria (T) are expressed as log values of colony-forming units (cfu) per gram weight of feces. (a) Log_{10} B/T cfu/g wet weight of feces. (b) Log_{10} C/T cfu/g weight of feces. (c) Log_{10} B/C cfu/g wet weight of feces. In the control period and follow-up period, the control formula was used and not the 3-prebiotic formula. *Values significantly differ compared to that of week 0 of the administration period ($p < 0.05$). †Values significantly differ compared to that of the control period ($p < 0.05$). ‡Values significantly differ compared to that at week 2 of the administration period ($p < 0.05$).

compared to week 0 of the administration period ($p < 0.05$). Additionally, the ratio at week 6 was even higher than that at week 0 of the administration period ($p < 0.05$). The ratio in the follow-up period was lower but still showed an increase compared to week 0 of the administration and control periods ($p < 0.05$, Figure 1(c)).

3.3. Effect of the 3-Prebiotic Formula Administration on Organic Acids. The index of organic acids, including lactate, acetate, propionate, and butyrate, significantly increased after the administration period compared to levels measured before the administration period (Figure 2).

4. Discussion

Probiotics such as bifidobacteria and lactobacilli are reported to prevent gastrointestinal diseases and disorders [19, 20]. We found that administration of the 3-prebiotic formula



(a) Lactate and acetate

(b) Propionate and butyrate

Figure 2: Effect of 3-prebiotic formula administration on contents of fecal organic acids. (a) Products of lactate and acetate and (b) propionate and butyrate. Data are expressed as the mean ± SD. *Values significantly differ compared to that of week 0 of the administration period ($p < 0.05$).

increased the growth of *Bifidobacterium* spp., decreased the growth of *C. perfringens*, and improved the host gastrointestinal bacterial flora, thus decreasing diarrhea conditions in toddlers. There are abundant bifidobacteria in the feces of healthy breast-fed infants and they have lower incidence of infantile diarrhea. As infants gradually grow up and develop into toddlers, there are many causes that induce children's diarrhea. The increase in *C. perfringens* bacteria is an abnormal condition of the colonic microbial population, and the decrease in *Bifidobacterium* spp. shows that the body is in an unhealthy state [21]. When populations of pathogenic bacteria that produce exotoxins or result in abnormal colonic microbial populations in the upper part of the intestines increase, diarrhea may occur. Recent reports indicated that supplementation with *Bifidobacterium* spp. and *Lactobacilli acidophilus* may enhance innate mucosal immune defense to protect against some immune-based disorders, inhibit the growth of intestinal pathogenic bacteria, lower the instances of diarrhea and bacterial infections induced by antibiotics or rotaviruses, and promote the health of the host [22–24]. Because enzymes in mammals cannot hydrolyze the α-1,6 linkages of carbohydrates, ingestion of oligosaccharides can increase *Bifidobacterium* spp. in the feces due to increases in probiotics in the intestines [25]. Similar to the results of the present study, supplementation with three probiotics, inulin, fructooligosaccharides, and galactooligosaccharides, selectively stimulated the indigenous bifidobacteria and lactobacilli and proliferation of bifidobacteria and lactobacilli after 4 weeks of ingestion of a 3-prebiotic formula. Moreover, we found that the ratio of *Bifidobacterium* spp. to total anaerobic bacteria and the ratio of *C. perfringens* to the total anaerobic bacteria improved compared to those before ingesting the 3-prebiotic formula. This indicates that the 3-prebiotic formula might improve the gastrointestinal bacterial flora of toddlers.

Oligosaccharides stimulate the growth of bifidobacteria and lactobacilli. One study showed that these bacteria may reduce the survival of pathogens by producing organic acids [26]. Organic acids, such as lactate, acetate, propionate, and butyrate, make up 85%~95% of short-chain fatty acids (SCFAs) in the large intestines. SCFAs provide energy to colonocytes and are the main fermentation products of the microbial breakdown of carbohydrates in the large intestine. We found that the 3-prebiotic formula significantly increased lactic acid and SCFA production. Some researchers indicated that the presence of butyrate regulates the differentiation of normal colonocytes [27] and increases the sensitivity of immune reactions to cancer cells [28]. It was also shown that butyrate inhibited risk factors for colon cancer and adenomas [29–31]. Furthermore, the production of lactic acid and SCFAs after ingestion of a 3-prebiotic formula may indirectly result in a more-acidic environment, which extensively inhibits most gram-positive and gram-negative bacteria [8, 21, 24, 32]. Under acidic conditions, the growth bacterial pathogen is suppressed, and the utilization of bifidus in an infant's intestines is enhanced [33].

Organic acids that induce decreases in intestinal pH values in the human gut tract may increase the absorption of some specific minerals, such as iron and calcium [34, 35]. Several studies have demonstrated that both inulin and oligosaccharides are effective prebiotics and also benefit the bioavailability of minerals including calcium and magnesium [36–38] and gut health [39, 40]. For adults, doses of prebiotics more than 8 g/day increased the absorption and retention of Ca [38]. Thus, the 3-prebiotic formula containing 250 mg/100 mL might have a similar positive effect on the intestinal tract of toddlers.

Overall, the 3-prebiotic formula increased the growth of probiotics and continued to produce more organic acids. By increasing the production of lactic, acetic, and propionic acids by bifidobacteria, a lower pH value may enhance the activity of bifidus and utilization of minerals.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Snapshot on a Pilot Metagenomic Study for the Appraisal of Gut Microbial Diversity in Mice, Cat, and Man

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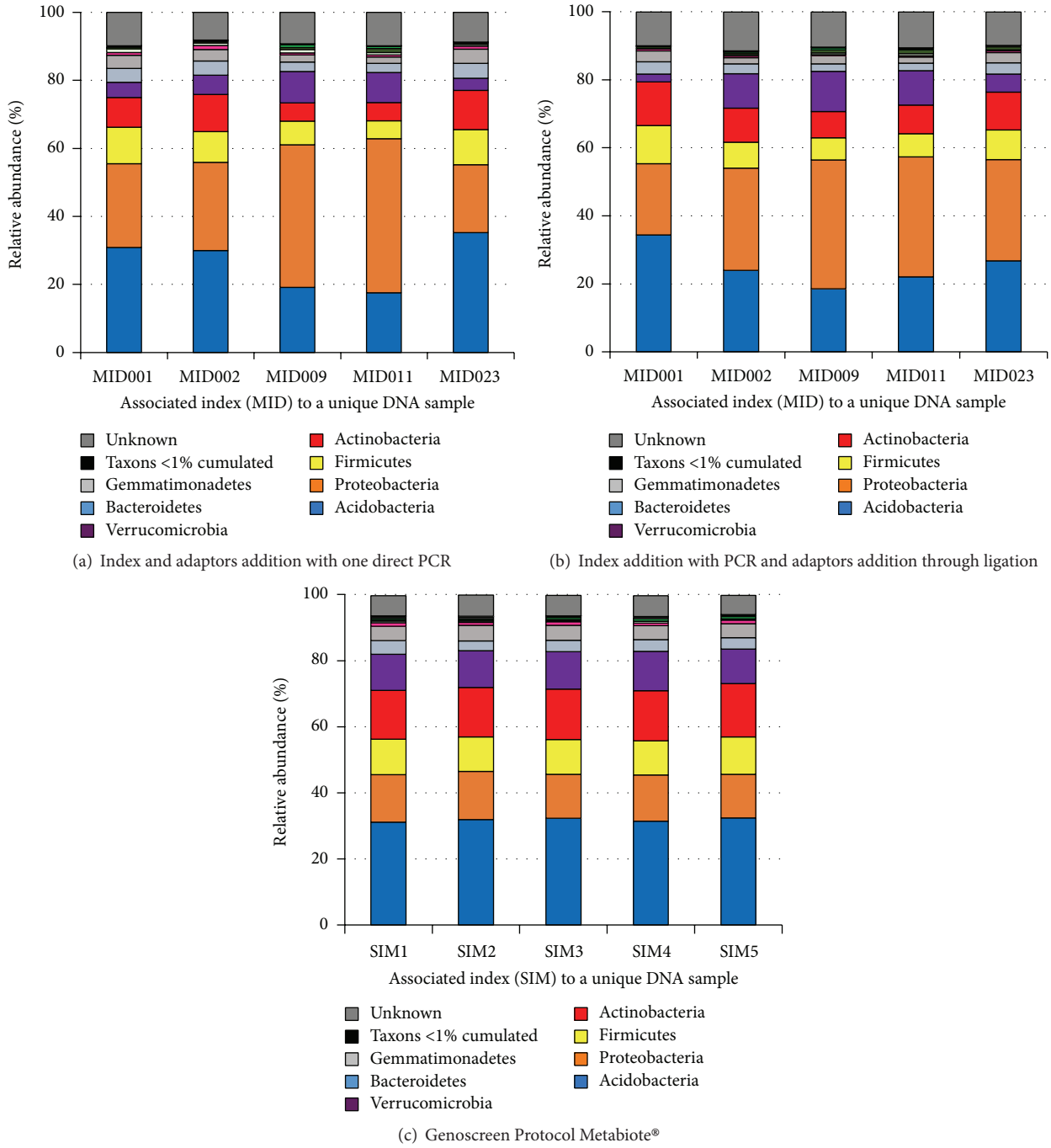
Gut microbiota plays a key role in the maintenance of homeostasis and host physiology, comprising development, metabolism, and immunity. Profiling the composition and the gastrointestinal microbiome with a reliable methodology is of substantial interest to yield new insights into the pathogenesis of many diseases as well as defining new prophylactic and therapeutic interventions. Here, we briefly present our methodology applied to fecal samples from mice and then further extended to the samples from a cat and a single human subject at 4 different time points as examples to illustrate the methodological strengths. Both interindividual and time-related variations are demonstrated and discussed.

1. Introduction

Recent developments in metagenomics have provided researchers with the tools needed to open the "black box" of microbiome science. These novel technologies have enabled the establishment of correlations between dysbiotic microbial communities and many diseases. Extended approaches and meticulous data interpretation will be important for resolution of these discrepancies. In this context, diagnostic tools and analytic solutions for research purposes are needed to support clinical studies in humans and preclinical developments using mice. The growing need to survey the tremendous microbial diversity in a culture independent manner has led to the development of molecular methods through sequence profiling of part of conserved genes such as 16S rDNA, in various scientific fields including ecology (plants, animals), agronomy, biotechnology, and of course human health. Next-generation sequencing technologies providing unprecedented throughput of data are now routinely used to assess bacterial community composition in complex samples. Depending on whether rough/basic bacterial signature or extensive resolution of taxonomic assignment of organisms is needed, the time and costs for 16S rRNA profiling *versus* full genome analysis or bacterial RNA sequencing may vary from 1 to 50.

2. Materials and Methods

The Roche 454 GS FLX and GS Junior Sequencing Systems have been employed by researchers worldwide to accurately characterize diverse microbial communities, as demonstrated in the over 1,000 metagenomics publications to date. However, several protocols for amplicon-based sequencing of 16S rRNA still exist and are widely used to perform these analyses whereas no study has looked at their respective impact on taxonomical description, relative abundance of taxa, and diversity and richness indexes. A comparison of two classical amplicon library preparations (Direct PCR and Ligation) has been performed by Genoscreen (Lille, France) which led them to develop an optimized and standardized solution for the analysis of microbiota named Metabiote. Indeed, starting with DNA extracted from one unique sample



(a) Index and adaptors addition with one direct PCR

(b) Index addition with PCR and adaptors addition through ligation

(c) Genoscreen Protocol Metabiote®

Figure 1: Impact of three different 16S-amplicon library preparation methods on the observed relative abundance of bacterial taxa. One unique sample of soil (known to have the highest microbial community complexity) was used to compare three distinct methods of molecular indexing such as MID (multiplex identifier) or Genoscreen-developed sample identifier multiplex (SIM). Five examples of final bacterial profiling are shown for each of the three methods used to illustrate the reproducibility of DNA extraction.

of soil (known to have the highest microbial community complexity), several identical libraries ($n = 48$) were prepared with distinct molecular tags as indexes namely MID (standing for multiplex identifier), following two classical protocols (resp. amplification and ligation) and the developed Metabiote® Protocol, using an own molecular index system, namely, sample identifier multiplex (SIM). As an example using 5 different MID (from the 48 items), Figure 1 clearly demonstrated the impact of indexing step on the observed relative abundance of taxa at the phylum level starting from one unique sample (Figures 1(a) and 1(b)). On the contrary, Metabiote protocol based on SIM shows a clear greater homogeneity in its results, with no impact of the indexing step (Figure 1(c)). Additionally, Metabiote

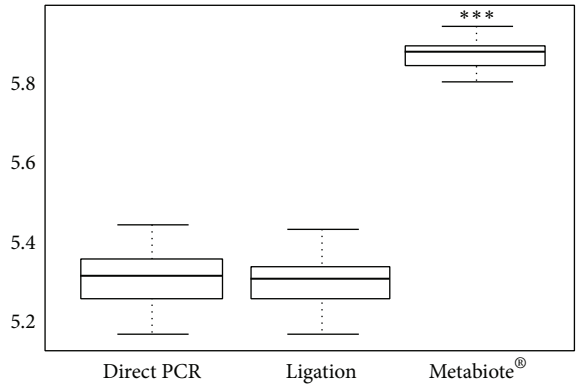


Figure 2: Shannon diversity index obtained with the three different protocols of 16S-amplicon library preparation. $N = 48$ distinct indexes were used to tag a unique sample (from soil) for each of the three methods of molecular indexing. The Metabiote indexing system reveals an extended diversity reached, $^{***}P < 0.001$, and Mann-Whitney test.

process gives access to higher bacterial diversity information compared to the two other classical protocols when estimated with the Shannon index [1], as shown for the 48 indexed samples (Figure 2), $p < 0.01$, using the Mann-Whitney statistical analysis. Ten female BALB/c mice (6 weeks old on arrival, Charles River Laboratories, Saint-Germain sur l'Arbresle, France) were housed in a controlled environment (with a temperature of 22°C, a 12 h/12 h light/dark cycle, and *ad libitum* access to food and water) for a minimal acclimatization period of 12 days. All animal experiments were performed according to the guidelines of the *Institut Pasteur de Lille* Animal Care and Use committee and in compliance with the Amsterdam Protocol on Animal Protection and Welfare and Directive 86/609/EEC on the Protections of Animals Used for Experimental and Other Scientific Purposes (updated in the Council of Europe's Appendix A). The animal work was also compliant with French Legislation (the French Act 87-848, dated 19-10-1987) and (the European Communities Amendment of Cruelty to Animals Act 1976). The study's objectives and procedures were approved by the Nord-Pas-de-Calais region's Ethic and Welfare Committee for Experiments on Animals (Lille, France; approval number: 19/2009R). The individual murine fecal samples were freshly collected during defecation, immediately frozen in liquid nitrogen and stored at −80°C until further process. Samples from human feces (1-2 grams in duplicate) from a single healthy volunteer (43 years old, male) were collected at 0, 24, 30, and 48 h time points, quickly frozen, and stored at −80°C. Finally, the single fecal sample of cat origin was taken from the freshly made kitty litter (Globule). All samples were blinded and processed for DNA extraction. Metabiote kit has been used for library preparation according to Genoscreen's recommendations (Genoscreen, Lille, France).

Final libraries each containing 12 different samples identified by a SIM were amplified by emPCR as described in the GS Titanium Amplification Method Manuel Lib-L (http://454.com/downloads/my454/documentation/gs-junior/method-manuals/GSJunior_emPCR_Lib-A_RevApril2011.pdf). Sequencing was performed on a GsFLX Instrument using version 2.9 software. Amplicon libraries were each sequenced on one separate eighth of PicoTiterPlate (PTP) resulting in between 84 000 and 115 000 Passed Filter reads. Read length histogram shows the typical achieved modal read lengths that is in agreement with the Metabiote V3V4 amplicon length. Metabiote OneLine Pipeline has been used to assess microbial population definition, diversity, and comparison. This pipeline comprises the following steps: preprocessing (SIM sorting, no mismatch in specific primer, read length selection, elimination of reads with ambiguous bases, signal quality filter, and homopolymers exclusion), chimera detection, OTU clustering, comparison to the database Greengenes, and taxonomic establishment based on the use of QIIME pipeline [2].

3. Results and Comments

We first report consistent analysis of samples from distinct origins: human subject, mouse, and cat. A representative example of the corresponding human, cat, and mouse microbial profiles, respectively, obtained at the phylum, family, and genus level is shown in Figure 3. Obviously, the methodology allows identifying highly specific signatures for material from each origin. According to the phylum level, both Firmicutes (over 70%) and Bacteroidetes (20–25%) are detected in mice and man in ranges in agreement with expected results, while Proteobacteria is restricted to a marginal group in mice; the latter is found substantial (10%) in the human subject. Tenericutes were only detected in mice samples. Surprisingly, Gram-negative species are negligibly detected in the cat fecal material where besides the major Firmicutes (85%) Actinobacteria are highly represented (15%). The latter is essentially assigned to *Bifidobacterium* species at the genus level, showing that extremely anaerobic strains are effectively identified. In line, near 50% of the cat bacterial community is made of *Clostridium* species while Clostridiales are part of 5% in human subject and 10% in mice. Data presented here show that methodology allows identifying highly specific signatures for material from each origin. Of note, mice fecal samples appear more similar to human feces than the cat, suggesting a possible use of murine for microbial-related studies and research purposes. However, genetically engineered animals to carry similar microbial profile as man would ideally be desirable. This would require the generation of microbiota-humanized mice with steady and long-term maintenance of the symbiotic communities. So far, no evidence of such complete tolerance has been achieved as some specific human-derived species are probably unable to durably colonize the mouse digestive tract.

We then report human intraindividual variations during a short time course sampling. Structure of the intestinal microbiota varies substantially between individuals [3]. Furthermore, the gut microbiota composition is dynamic and may endure slight variations following the daytime activity, including work habits, sleeping period, and obviously eating varied diets. We collected samples of fecal replicates (two duplicates) from a single human subject at 4 different time points of 0, 24 h, 30 h, and 48 h. As shown in Figure 4,

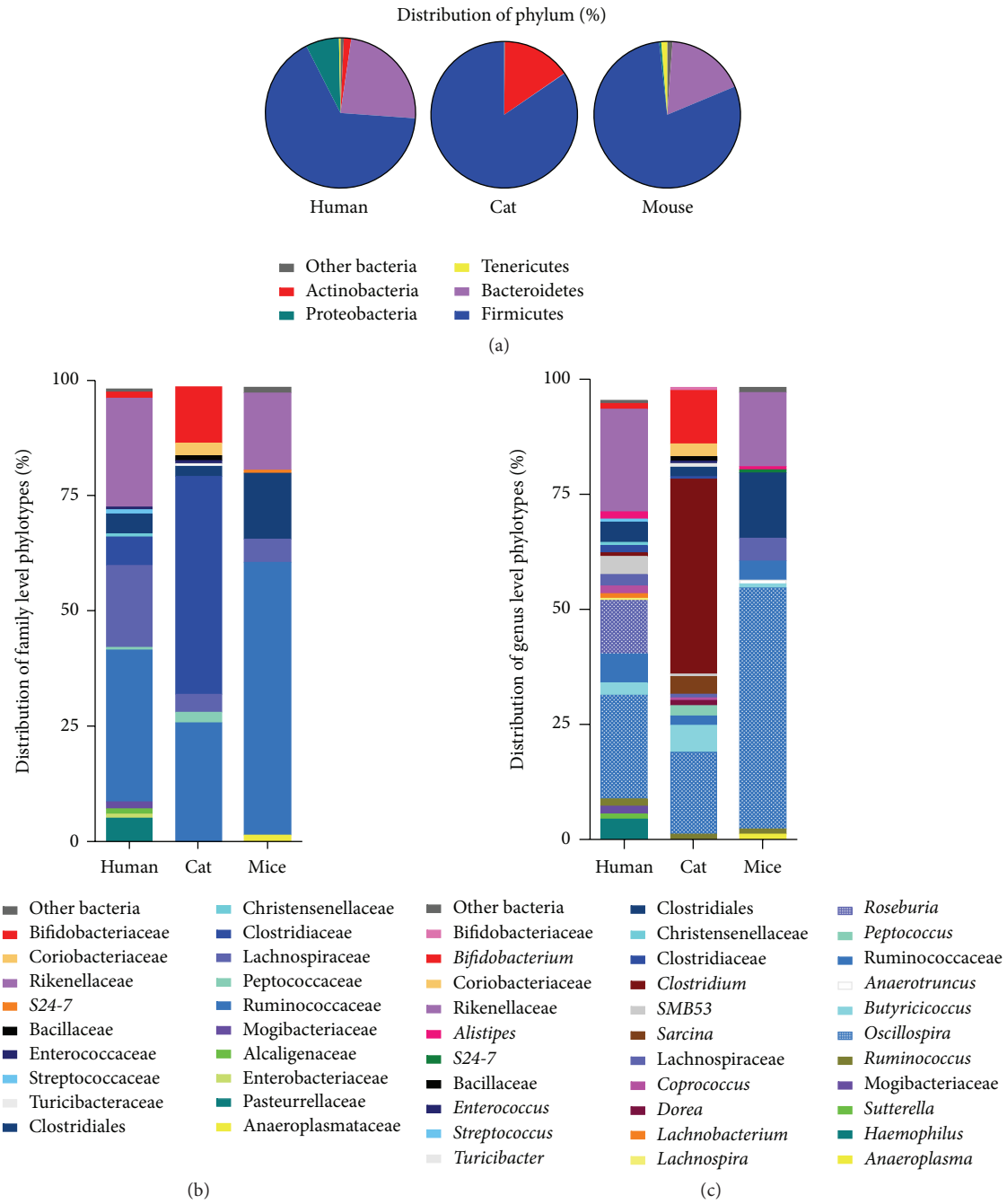


Figure 3: Bacterial community profiling of human, cat, and mouse fecal samples at the phylum (a), family (b), and genus (c) level phylotypes, illustrating possible simultaneous analysis of microbiota assessment of samples from distinct origins.

the composition of phylum, family, and genus at the same time point demonstrates minor changes showing that replicates are quite similar. In contrast, more important variations are seen with respect to time. For example, although the core bacterial community is preserved during day time (Rikenellaceae, *Roseburia*, and *Oscillospira*), the microbial profiling is clearly different after 24 h, revealing an increase in *Ruminococcus*. Likewise, analysis at the 48 h time point showed a higher proportion of the phylum Proteobacteria (corresponding to *Haemophilus* spp. from Pasteurellaceae), *Sutterella,* and the clone SMB53 (candidate genus of Clostridiaceae) concomitantly with a drop in Rikenellaceae. Interpreting the sources and consequences of these changes is elusive and mostly speculative here. However, the subtle fluctuations could reasonably be attributed to the direct or indirect impact of ingested food particle and other unidentified activities. Such dynamic has to be considered to conclude to a dysbiotic or stable microbiota and avoid misinterpretation. Indeed,

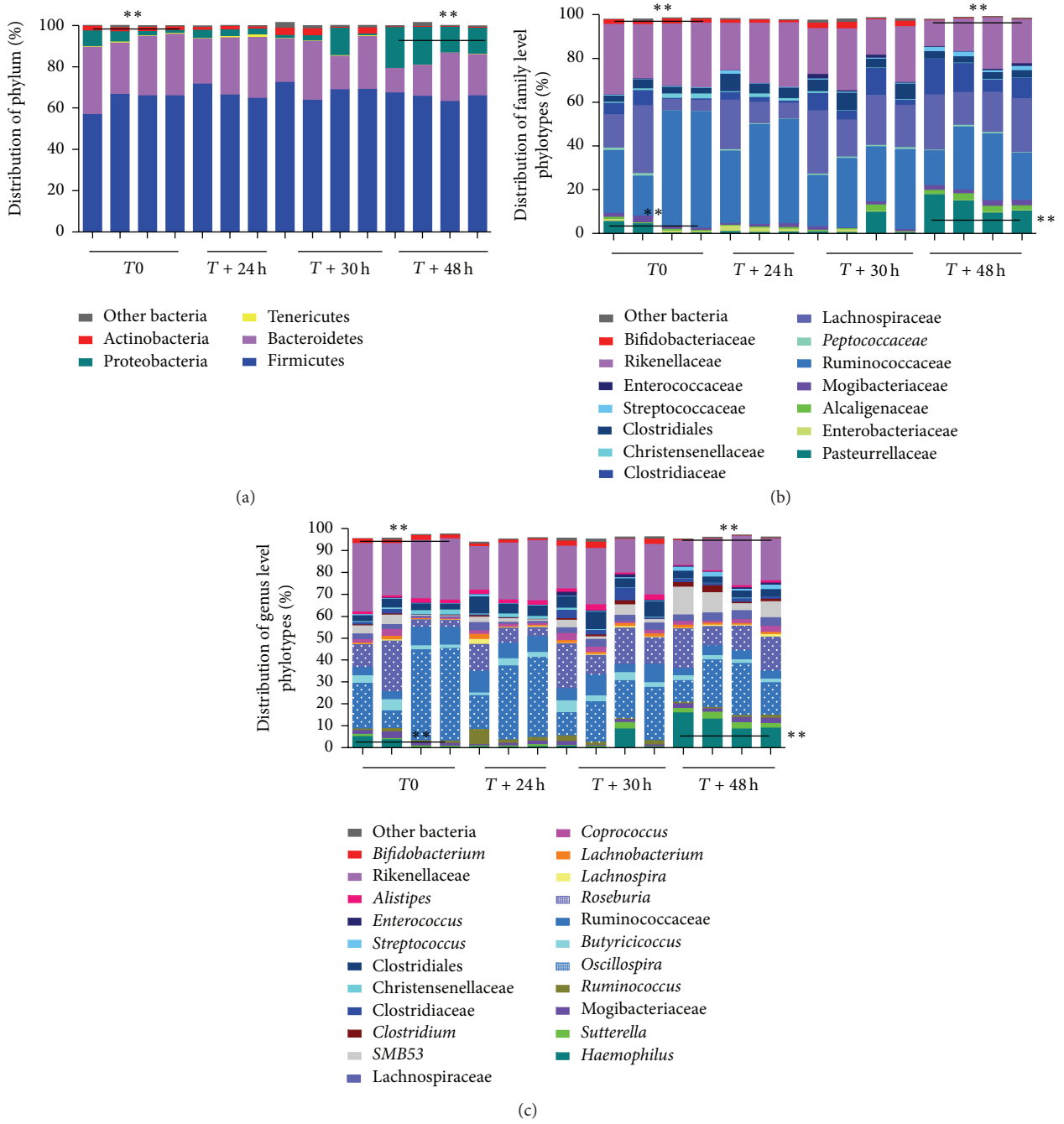


FIGURE 4: Short term changes in the bacterial community profiling at the phylum (a), family (b), and genus (c) level phylotypes of fecal samples obtained at different time points (0, 24, 30, and 48 h) from a single human subject. For example, a significant increase of Proteobacteria is demonstrated at 48 h compared with $T0$ ($^{**}P < 0.01$) whereas Actinobacteria are not detectable any more ($^{**}P < 0.01$) (a). These changes are confirmed at lower levels of detection, respectively, Pasteurellaceae (b) and *Haemophilus* (c) and *bifidobateriaceae* (b) and *bifidobacteria* (c) ($^{**}P < 0.01$). *Mann-Whitney test, $n = 4$ samples/time points.

it may allow further stratification of distinct responders both in modeling immune and infectious diseases and for personalized therapeutic interventions.

Finally, we addressed the interindividual variations in cohoused mice. A relative uniformity of biological responses is essential in murine experimental models worldwide. Individuality in gut microbiota composition is shaped by complex environmental and host genetic factors [4] and, consequently, variable bacterial communities correspond to specificity in immune and metabolic pathways [5, 6]. The composition (and activities) of intestinal symbiotic microbial consortia highly depends on the mice genetic backgrounds [7] but huge variations between isogenic adult mice reared in different research institutions and providers are observed too [8], as

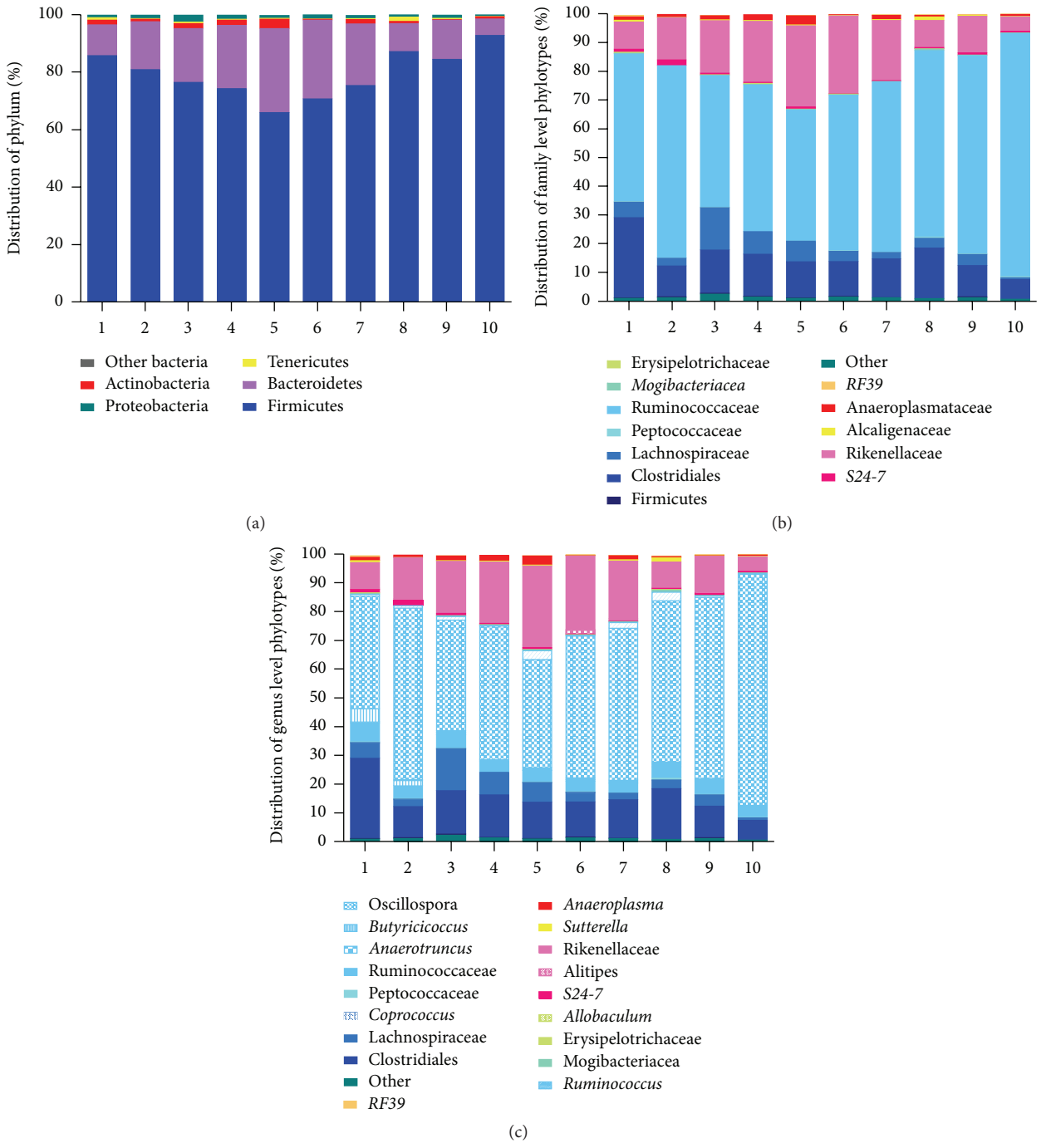


Figure 5: Bacterial community profiling at the phylum (a), family (b), and genus (c) level phylotypes of fecal samples from 10 mice cohoused in the same cage, showing the huge interindividual diversity.

well as important seasonal changes. Moreover, single specificity in mice microbiota profiles may also evoke concerns for research purpose. Here, we questioned the diversity among ten individual mice from the same conventional cage. Figure 5 illustrates a detailed overview of such individual profiles on the phylum, family, and genus level. Abundance in Firmicutes can represent 60% to 90% while the Bacteroidetes range from 10 to 35%. Less frequent phyla such as Tenericutes and Proteobacteria could or could not be detected. For example, *Ruminococcus* spp. are identified in only 6 mice from the group while four mice are *Alistipes* positive. Such diversity is constantly observed in cagemates from distinct providers upon the arrival and following various diets or treatments (data not shown). Neither coprophagy nor long-term cohousing seems to be able to standardize this fact.

While interindividual variations were previously demonstrated both in mice [9] and in humans, these observations are of great importance in research and should not to be neglected. As far as we can exclude technical bias, intra- and interindividual variations may mask interspecies variations. Our data suggest that minimum 10 mice are required to consider the interindividual variation in the baseline and to exclude possible discrepancies. In addition, it may clearly serve as corner stone for research purposes in microbiota-presumed diseases modeling in rodents, the latter being more realistic and thus fitting the 3Rs ethical rules (replacement, reduction, and refinement) [10]. Although the microbiome science needs a "healthy dose of skepticism" [11], it also requires reliable and consistent tools for gold standard metagenomic analysis.

Collectively, we briefly present a methodology (Metabiote) applied to the microbial profiling of fecal samples from mouse, man, and cat origin. We point out both inter- and intraindividual variations of gut microbial composition in a healthy subject. Knowing the composition of the microbial community alone does not necessarily lead to an understanding of its function. However, such analyses can be helpful to explain time-related changes and discrepancies among animals. Thus, this study suggests the procedure to be useful for diagnostics including dysbiotic states and follow-up of diet and treatments in clinical studies, considering the proper controls are included.

Competing Interests

Louise-Eva Vandenborght, Nathalie Adele-dit-Renseville, and Stéphanie Ferreira are employees of Genoscreen. The other authors declare no conflict of interests regarding the present study.

Authors' Contributions

Louise-Eva Vandenborght and Coline Plé contributed equally to this work while Stéphanie Ferreira and Foligné Benoît are both senior authors.

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Review Article

The Comparative Efficacy and Safety of Entecavir and Lamivudine in Patients with HBV-Associated Acute-on-Chronic Liver Failure: A Systematic Review and Meta-Analysis

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Background. Currently, both of entecavir and lamivudine are effective for patients with HBV-associated acute-on-chronic liver failure (ACLF). However, there is no consensus on the efficacy of entecavir versus lamivudine for patients with HBV-associated ACLF. The aim of the study was to compare the efficacy and safety of entecavir with that of lamivudine for HBV-associated ACLF patients. *Methods.* Publications on entecavir versus lamivudine in HBV-associated ACLF patients were comprehensively identified. Odds ratio and mean difference were used to measure the effect. *Results.* Ten studies, totaling 1254 patients, were eligible. No significant differences between the two drugs presented in the 1-, 2-, 3-, or 6-month survival rates. However, after 12 months of treatment, patients prescribed entecavir had a statistically higher survival rate ($p = 0.008$) and lower total bilirubin ($p < 0.0001$) and alanine aminotransferase ($p = 0.04$) levels compared to patients prescribed lamivudine. More patients achieved HBV negative levels when taking entecavir as measured at 1-, 3-, and 12-month time points and had a lower rate of HBV recurrence. *Conclusion.* While entecavir and lamivudine are both relatively safe and well tolerated, entecavir was more efficacious in terms of survival rate and clinical improvement in long-term treatment. Further prospective randomized controlled trials are needed to validate these results.

1. Introduction

Acute-on-chronic liver failure (ACLF), defined as a condition where acute hepatic insult occurs simultaneously with manifestation of jaundice and coagulopathy, complicated within 4 weeks by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease [1]. A major cause of ACLF in Asia is chronic hepatitis B virus (HBV) [2]. HBV-associated ACLF has an extremely poor prognosis [3]. There is no standard treatment for ACLF; rather treatment follows the paradigm of addressing the predisposing event, alleviating the inflammatory response and providing supporting care. Artificial liver support is in many cases used as a stabilizing measure for patients with ACLF. However, it is not reckoned to reduce the mortality of patients suffering from ACLF [4, 5]. Currently, liver transplantation is deemed the only really effective therapy for ACLF, but a shortage of suitable donors and the high cost of transplant surgery hinder its clinical application [6, 7]. Therefore, establishment of more effective noninvasive therapeutic strategies is urgently needed.

The mechanism of HBV-associated ACLF remains vague. Nevertheless, viral factors, host factors, and their interactions have great impact on the prognosis of ACLF [8–11]. Nucleos(t)ide analogues such as lamivudine, entecavir, telbivudine, and tenofovir disoproxil fumarate (TDF), which suppress the replication of HBV [12, 13], can improve liver function, reduce cirrhotic complications, and decrease the incidence of hepatocellular carcinoma in patients with chronic hepatitis B. More recent, encouraging studies have concluded that antiviral therapy can increase the overall survival rate and ameliorates liver function in patients with HBV-associated ACLF compared with subjects not treated with nucleos(t)ide analogues [14–16].

Entecavir is superior to lamivudine in the suppression of HBV replication with an extremely low mutation rate in

both HBeAg-positive and HBeAg-negative patients [17, 18]. The theoretical cause of entecavir's success in the long-term treatment of ACLF may lie in the latter's severe reactivation of HBV. However, the clinical data on the efficacy and safety of entecavir and lamivudine contain the inconsistencies arising from the paucity of larger sample sizes, contemporary controls, and long-term research. Studies conducted by Wen et al. [19], Yuen [20], and Zhang et al. [21] have suggested entecavir's relative efficacy compared to lamivudine, while one study by Cui et al. [22] found no significant differentials between patients with HBV-associated ACLF treated with entecavir and lamivudine. Therefore, this meta-analysis was performed to explore whether a more thorough analysis of extant study data could settle the vexed question of which was safer and more effective in treating HBV-associated ACLF patients—entecavir or lamivudine?

2. Materials and Methods

2.1. Methods. The research methods follow the preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) [23].

2.2. Selection and Exclusion Criteria. In this meta-analysis, randomized controlled trials and cohort studies were eligible with efficacy comparison of entecavir and lamivudine for patients suffering from hepatitis B-associated ACLF.

According to the criteria of acute-on-chronic liver failure (ACLF) from both the Chinese Medical Association and Asian Pacific Association [24], a set of baseline metrics were established for judging study data on patients with hepatitis B-associated ACLF. Studies were eligible when the subjects met the following criteria:

(1) The presence of serumal hepatitis B surface antigen (HBsAg) for at least 6 months.

(2) HBV DNA level $>10^5$ copies/mL.

(3) Serum total bilirubin (TBIL) concentration $>85\,\mu$mol/L and plasma prothrombin activity <40% or international standard ratio (INR) ≥1.5.

(4) No complications or comorbidities such as hepatic encephalopathy or abrupt and obvious increase of ascites or spontaneous bacterial peritonitis.

Studies conforming to any of the following criteria were excluded:

(1) Coinfection with hepatitis A, hepatitis C, hepatitis D, hepatitis E, cytomegalovirus, or human immunodeficiency virus (HIV).

(2) Other concomitant liver diseases, such as drug hepatitis, alcoholic liver disease, autoimmune hepatitis, or Wilson's disease.

(3) Patients suffering from serious medical disease or tumor.

(4) A previous course of any antiviral therapy during the preceding 6 months.

2.3. Data Collection Process. A comprehensive search was completed of the Cochrane Central Register of Controlled Trials, PubMed, Medline, Embase, China National Knowledge Infrastructure (CNKI), and the Chinese BioMedical Literature Database. In addition, reference items of the eligible studies and relevant reviews were checked for qualified studies. The following keywords were searched: "entecavir", "lamivudine", "nucleoside analogue", "nucleotide analogue", "liver failure", "hepatic failure", "acute on chronic liver failure", and "chronic hepatitis B". The search strategy used in PubMed is as follows: "(acute-on chronic liver failure [Title/Abstract]) AND (HBV) AND (lamivudine OR entecavir OR nucleoside analogues OR nucleotide analogues)".

Two researchers (Jiao Yang and Hang Sun) independently conducted the literature retrieval, study selection, and data extraction. Differences in assessment were resolved by consensus.

2.4. Assessment of Study Quality. For randomized controlled trials (RCTs), the Cochrane risk of bias tool including random sequence generation, allocation concealment, blinding of participants and personnel, blinding outcome assessment, incomplete outcome data, selective reporting, and other sources of bias was used to evaluate the quality of the included studies. Newcastle-Ottawa Scale (NOS) involving the selection of cohorts, comparability of cohorts, and assessment of the outcomes was applied to assess the quality of observational cohort studies. Studies with an overall score ≥7 were defined as high-quality.

2.5. Efficacy Measures. The primary efficacy endpoint was overall survival rate of different time points. Secondary efficacy endpoints were recurrence rate of HBV, incidence of HBV negative, TBIL, ALT, and PTA changes as measures of hepatic improvement. The safety of entecavir and lamivudine was also assessed in the meta-analysis.

2.6. Data Analysis. The analysis was conducted by the use of RevMan 5.3 (Nordic Cochrane Centre, Cochrane Collaboration). A p value of less than 0.05 was regarded as statistically significant. Heterogeneity was assessed using the χ^2 square test and I^2 statistic. $I^2 < 50\%$ or $p > 0.10$ was considered to indicate no significant heterogeneity between studies and the fixed-effects model was employed to analyze the data. Otherwise, the random-effects model was used. Publication bias was evaluated by a funnel plot. Odds ratio (OR), mean difference (MD), and 95% confidence interval (CI) were used as effect measurements.

3. Results

3.1. Study Selection. Of the 1687 manuscripts identified, 735 duplicates were removed. 10 studies [19, 21, 22, 25–31] were selected as eligible for the next phase of detailed analysis. 1254 patients (629 using entecavir and 625 for lamivudine) in total met the inclusion criteria for this meta-analysis (Figure 1). Of the included studies, four were randomized controlled trials, two were prospective cohort studies, and four were retrospective cohort studies. All the studies were performed

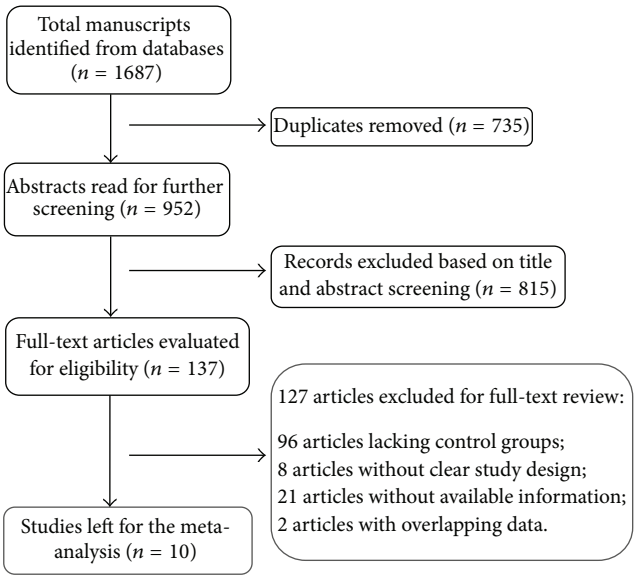


Figure 1: Flow diagram of literature selection process.

in China. The dosages of entecavir and lamivudine were unified in the eligible studies. All patients included were given routine comprehensive treatment, including intensive care monitoring, nutritional supplementation, and plasma, electrolyte, and acid-base equilibrium, and prophylaxis and treatment of complications. The baseline characteristics of the eligible studies are shown in Table 1.

3.2. Risk of Bias in Included Studies. The overall quality of the eligible studies in this meta-analysis was suboptimal. There were four RCTs in the meta-analysis. For the RCTs, selection bias, reporting bias, and other biases were not clear, performance bias was high, and attrition bias was low (Figure 3). The quality of cohort studies was shown in Table 2. No obvious publication bias was found (Figure 4).

3.3. Efficacy Comparison

3.3.1. Overall Survival Rates

(1) One-Month Survival Rate. Five studies reported the details of survival rates at one month, with a total of 504 patients (249 patients using entecavir and 255 taking lamivudine). No significant heterogeneity was observed between these studies (I^2 = 0% and p = 0.97) and the fixed effect model was used. Comparable survival rates at this time point between patients given entecavir and those on lamivudine (86.75% versus 81.96%; OR: 1.52; 95% CI: 0.92, 2.52; p: 0.1) are shown in Figure 2.

(2) Two-Month Survival Rate. Four studies involving 186 patients using entecavir and 184 using lamivudine reported the data regarding two-month survival rates. Patients using entecavir had no significant difference in two-month survival rate compared to those on lamivudine (72.58% versus 65.22%; OR: 1.48; 95% CI: 0.94, 2.32; p: 0.09). The assessment of heterogeneity acquired p = 0.87 in Cochran's Q test and I^2 = 0%, meaning no variability of the included studies (Figure 2).

(3) Three-Month Survival Rate. Six studies provided three-month survival rate data. We included 318 patients taking entecavir and 319 using lamivudine. I^2 = 0% and p = 0.87 indicated no significant heterogeneity in those studies and the fixed effect model was applied. Comparative data on improvements in the three-month survival rate between patients with entecavir and those using lamivudine (67.92% versus 67.08%; OR: 1.06; 95% CI: 0.75, 1.48; p: 0.75) are also shown in Figure 2.

(4) Six-Month Survival Rate. Data regarding overall six-month survival rates were presented in three studies with 193 patients in the entecavir group and 214 in that of lamivudine. We found that entecavir was no better than lamivudine in raising the six-month survival rate for patients with chronic hepatitis B-associated acute-on-chronic liver failure (74.09% versus 73.83%; OR: 0.98; 95% CI: 0.61, 1.57; p: 0.94). I^2 = 0% and p = 0.94 showed no obvious heterogeneity among those studies (Figure 2).

(5) Twelve-Month Survival Rate. Five studies had information on overall twelve-month survival rates, including 344 patients taking entecavir and 349 taking lamivudine. Patients on entecavir had a higher overall survival rate than those on lamivudine (84.30% versus 77.08%; OR: 1.79; 95% CI: 1.17, 2.75; p: 0.008). Due to the limited heterogeneity between the eligible studies (I^2 = 0% and p = 0.76), the fixed effect model was used (Figure 2).

3.3.2. HBV DNA Negative

(1) One-Month HBV DNA Negative. Six studies with 382 patients in the entecavir group and 353 in the lamivudine cohort reported the incidence of one-month HBV DNA negative changes. No significant heterogeneity was found (I^2 = 0% and p = 0.74). Therefore, the fixed effect model was used. Patients given entecavir presented a higher HBV DNA negative rate than subject on lamivudine at one month (65.71% versus 43.91%; OR: 2.85; 95% CI: 2.06, 3.94; p < 0.00001) (Table 3).

(2) Three-Month HBV DNA Negative. Data on the HBV DNA negative rate at three months were available in three studies. These studies included 236 patients taking entecavir and 229 taking lamivudine. More patients with entecavir achieved negative levels of HBV DNA than those with lamivudine (86.44% versus 64.63%; OR: 3.49; 95% CI: 2.20, 5.53; p < 0.00001). With I^2 = 0% and p = 0.83 the lack of significant heterogeneity led to the application of the fixed effect model was applied (Table 3).

(3) Twelve-Month HBV DNA Negative. Three studies comprising 215 patients on entecavir and 201 on lamivudine reported data on twelve-month HBV DNA negativity. No apparent heterogeneity was found between those studies (I^2 = 0% and p = 0.84). Entecavir largely enhanced rates of

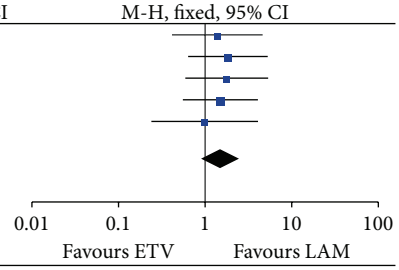
Table 1: Baseline characteristics of the included studies.

Study	Region	Study design	Number of patients		HBV DNA log 10 copies/mL		HBeAg-positive		PTA (%) or INR (means ± SD)		TBIL (means ± SD) (%)		ALB (means ± SD) (%)		ALT (means ± SD) (%)		Treatment duration (months)
			ETV	LAM	ETV	LAM	ETV	LAM	ETV	LAM	ETV	LAM	ETV	LAM	ETV	LAM	
Hu et al., 2010 [25]	China	cohort	90	90	8.2 ± 1	8.2 ± 1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	6
Gao et al., 2015 [26]	China	cohort	46	66	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	24
Cui et al., 2010 [22]	China	cohort	33	34	5.9 ± 1.5	5.9 ± 1.5	10	13	2.27 ± 0.55	2.61 ± 1.03	20.10 ± 11.24	19.91 ± 8.56	33.36 ± 4.43	31.64 ± 5.32	364 (47–2861)	226.5 (22–2314)	3
Chen et al., 2012 [27]	China	cohort	42	30	7.04 ± 1.6	7.25 ± 0.89	NA	NA	34.88 ± 12.27	32.18 ± 11.44	326.29 ± 201.35	332.65 ± 182.65	31.45 ± 5.79	29.59 ± 5.63	324.19 ± 310.04	287.61 ± 261.50	3
Lai et al., 2013 [28]	China	cohort	93	89	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Na	NA	3
Zhang et al., 2014 [21]	China	cohort	65	54	7 ± 1.4	7.2 ± 1.6	21	23	24.7 ± 6.0	25.1 ± 5.7	331.6 ± 74.8	320.1 ± 82.4	28.7 ± 6.9	29.4 ± 5.3	352.5 ± 77.2	345.2 ± 89.5	13
Wen et al., 2010 [19]	China	RCT	46	47	NA	NA	NA	NA	29.8 ± 8.5	30.6 ± 9.1	375.2 ± 200.3	389.4 ± 198.1	31.4 ± 4.6	32.7 ± 3.9	402.5 ± 292.7	395.8 ± 297.4	12
Cai and Shi, 2011 [29]	China	RCT	38	34	NA	NA	NA	NA	34.6 ± 8.1	34.8 ± 9.6	256.5 ± 137.4	257.6 ± 135.9	31.4 ± 4.6	32.8 ± 4.5	395.4 ± 235.3	387.1 ± 245.4	12
He, 2013 [30]	China	RCT	57	57	6.2 ± 2.1	6.1 ± 1.9	NA	NA	30 ± 8	30 ± 8	339 ± 135	342 ± 148	31 ± 6	31 ± 6	399 ± 245	404 ± 237	6
Yuan, 2015 [31]	China	RCT	149	148	NA	NA	NA	NA	33.89 ± 7.78	33.96 ± 7.80	260.41 ± 140.11	260.38 ± 140.10	31.88 ± 6.11	31.89 ± 6.10	396.51 ± 240.12	397.00 ± 240.22	12

ETV: entecavir; LAM: lamivudine; NA: not available; PTA: prothrombin activity; INR: international standard ratio; TBIL: total bilirubin; ALB: albumin; ALT: alanine aminotransferase; RCT: randomized controlled studies.

Study or subgroup	ETV Events	ETV Total	LAM Events	LAM Total	Weight	Odds ratio M-H, fixed, 95% CI
Cai and Shi 2011	32	38	27	34	18.3%	1.38 [0.41, 4.61]
Chen et al. 2012	33	42	20	30	20.3%	1.83 [0.64, 5.28]
Cui et al. 2010	26	33	23	34	19.5%	1.78 [0.59, 5.34]
Gao et al. 2015	39	46	52	66	26.4%	1.50 [0.55, 4.07]
Hu et al. 2010	86	90	87	91	15.6%	0.99 [0.24, 4.08]
Total (95% CI)		**249**		**255**	**100.0%**	**1.52 [0.92, 2.52]**
Total events	216		209			

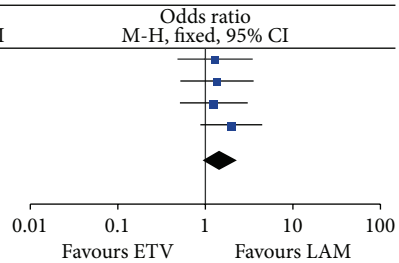
Heterogeneity: $\chi^2 = 0.58$, df = 4 ($p = 0.97$); $I^2 = 0\%$
Test for overall effect: $Z = 1.63$ ($p = 0.10$)



(a)

Study or subgroup	ETV Events	ETV Total	LAM Events	LAM Total	Weight	Odds ratio M-H, fixed, 95% CI
Chen et al. 2012	29	42	19	30	22.2%	1.29 [0.48, 3.47]
Cui et al. 2010	19	33	17	34	23.0%	1.36 [0.52, 3.56]
Gao et al. 2015	36	46	49	66	28.3%	1.25 [0.51, 3.05]
Zhang et al. 2014	51	65	35	54	26.6%	1.98 [0.88, 4.46]
Total (95% CI)		**186**		**184**	**100.0%**	**1.48 [0.94, 2.32]**
Total events	135		120			

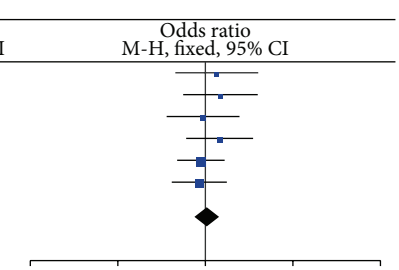
Heterogeneity: $\chi^2 = 0.73$, df = 3 ($p = 0.87$); $I^2 = 0\%$
Test for overall effect: $Z = 1.69$ ($p = 0.09$)



(b)

Study or subgroup	ETV Events	ETV Total	LAM Events	LAM Total	Weight	Odds ratio M-H, fixed, 95% CI
Cai and Shi 2011	30	38	25	34	8.4%	1.35 [0.45, 4.02]
Chen et al. 2012	29	42	18	30	9.9%	1.49 [0.56, 3.96]
Cui et al. 2010	16	33	17	34	13.1%	0.94 [0.36, 2.45]
Gao et al. 2015	36	46	47	66	12.7%	1.46 [0.60, 3.51]
Hu et al. 2010	60	90	63	91	31.7%	0.89 [0.48, 1.66]
Lai et al. 2013	45	69	44	64	24.1%	0.85 [0.41, 1.76]
Total (95% CI)		**318**		**319**	**100.0%**	**1.06 [0.75, 1.48]**
Total events	216		214			

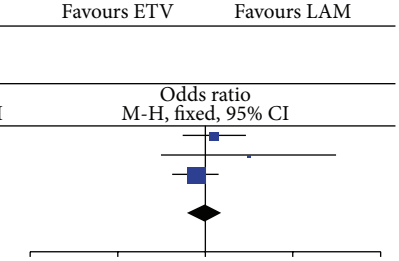
Heterogeneity: $\chi^2 = 1.86$, df = 5 ($p = 0.87$); $I^2 = 0\%$
Test for overall effect: $Z = 0.32$ ($p = 0.75$)



(c)

Study or subgroup	ETV Events	ETV Total	LAM Events	LAM Total	Weight	Odds ratio M-H, fixed, 95% CI
Gao et al. 2015	33	46	44	66	29.2%	1.27 [0.56, 2.88]
He 2013	56	57	54	57	2.7%	3.11 [0.31, 30.84]
Hu et al. 2010	54	90	60	91	68.1%	0.78 [0.42, 1.42]
Total (95% CI)		**193**		**214**	**100.0%**	**0.98 [0.61, 1.57]**
Total events	143		158			

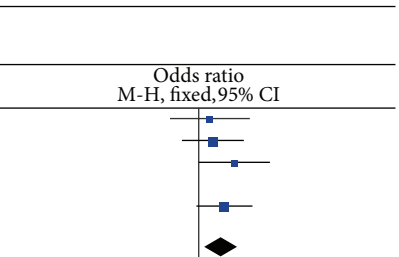
Heterogeneity: $\chi^2 = 1.93$, df = 2 ($p = 0.38$); $I^2 = 0\%$
Test for overall effect: $Z = 0.07$ ($p = 0.94$)



(d)

Study or subgroup	ETV Events	ETV Total	LAM Events	LAM Total	Weight	Odds ratio M-H, fixed,95% CI
Cai and Shi 2011	29	38	24	34	19.1%	1.34 [0.47, 3.84]
Gao et al. 2015	33	46	42	66	31.0%	1.45 [0.64, 3.28]
Wen et al. 2010	37	46	29	47	17.9%	2.55 [1.00, 6.51]
Yuan 2015	149	149	148	148		Not estimable
Zhang et al. 2014	42	65	26	54	32.0%	1.97 [0.94, 4.11]
Total (95% CI)		**344**		**349**	**100.0%**	**1.79 [1.17, 2.75]**
Total events	290		269			

Heterogeneity: $\chi^2 = 1.16$, df = 3 ($p = 0.76$); $I^2 = 0\%$
Test for overall effect: $Z = 2.66$ ($p = 0.008$)



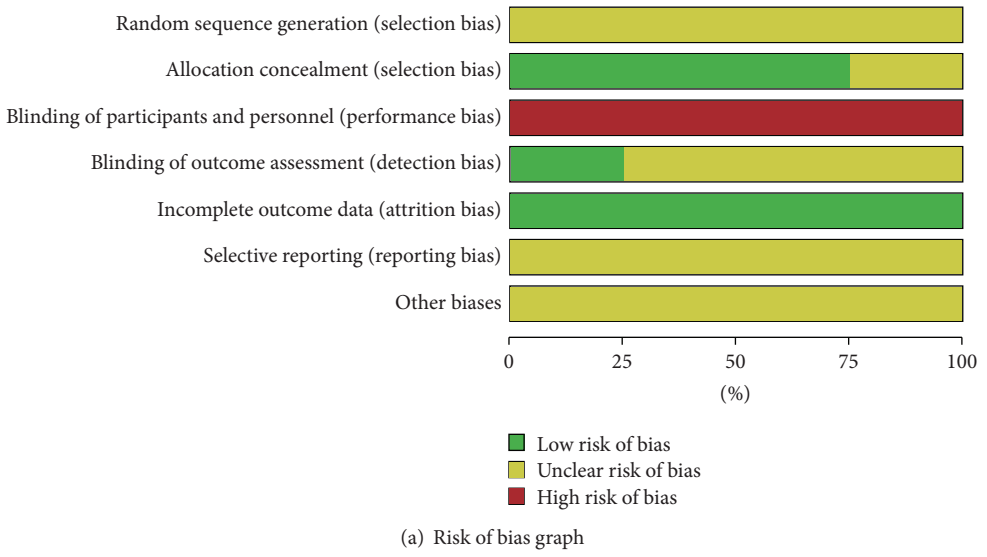
(e)

Figure 2: (a) Comparison of 1-month survival rates between patients taking entecavir and those taking lamivudine. (b) Comparison of 2-month survival rate between patients taking entecavir and those taking lamivudine. (c) Comparison of 3-month survival rates between patients taking entecavir and those taking lamivudine. (d) Comparison of 6-month survival rates between patients taking entecavir and those taking lamivudine. (e) Comparison of 12-month survival rates between patients taking entecavir and those taking lamivudine.

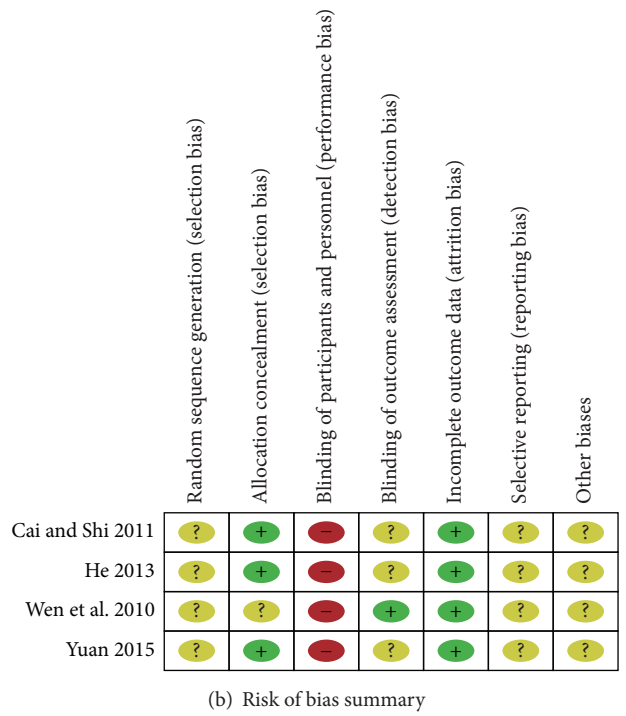
Table 2: Quality assessment of the eligible observational cohort studies.

Studies included	Selection				Comparability		Outcome			Scores
	1	2	3	4	5	6	7	8	9	
Hu et al., 2010 [25]	√	√	√	√	√	√		√		**7**
Gao et al., 2015 [26]	√	√	√	√	√	√		√	√	**8**
Cui et al., 2010 [22]	√	√	√	√	√			√		**6**
Chen et al., 2012 [27]	√	√	√		√			√		**5**
Lai et al., 2013 [28]	√	√	√		√	√		√		**6**
Zhang et al., 2014 [21]	√	√	√		√			√	√	**6**

For cohort studies, 1 indicates exposed cohort truly representative; 2 nonexposed cohort drawn from the same community; 3 ascertainment of exposure; 4 outcome of interest not present at start; 5 cohorts comparable based on the most important factors; 6 cohorts comparable on other factors; 7 quality of outcome assessment; 8 follow-up long enough for outcomes to occur; and 9 adequacy of follow-up of cohorts.



(a) Risk of bias graph



(b) Risk of bias summary

Figure 3: (a) Risk of bias graph: review of authors' judgments about each risk of bias item presented as percentages across all included RCTs. (b) Risk of bias graph: review of authors' judgments about each risk of bias item for each included study.

Table 3: Efficacy comparison of entecavir and lamivudine for dichotomous outcomes.

Outcome of interest	Number of studies	Entecavir		Lamivudine		Effect estimate		Heterogeneity	
		Sample size	Events	Sample size	Events	OR (95% CI)	p value	I^2 (%)	p value
Overall survival									
1 month	5	249	216	255	209	1.52 (0.92, 2.52)	0.1	0	0.97
2 months	4	186	135	184	120	1.48 (0.94, 2.32)	0.09	0	0.87
3 months	6	318	216	319	214	1.06 (0.75, 1.48)	0.75	0	0.87
6 months	3	193	143	214	158	0.98 (0.61, 1.57)	0.94	0	0.38
12 months	5	344	290	349	269	1.79 (1.17, 2.75)	0.008	0	0.76
HBV DNA negative									
1 months	6	382	251	353	155	2.85 (2.06, 3.94)	<0.00001	0	0.74
3 months	3	236	204	229	148	3.49 (2.20, 5.53)	<0.00001	0	0.83
12 months	3	215	208	201	156	8.61 (3.79, 19.59)	<0.00001	0	0.84
Recurrence of HBV	4	154	0	165	18	0.07 (0.01, 0.40)	0.003	0	0.93

OR: odds ratio; CI: confidence interval.

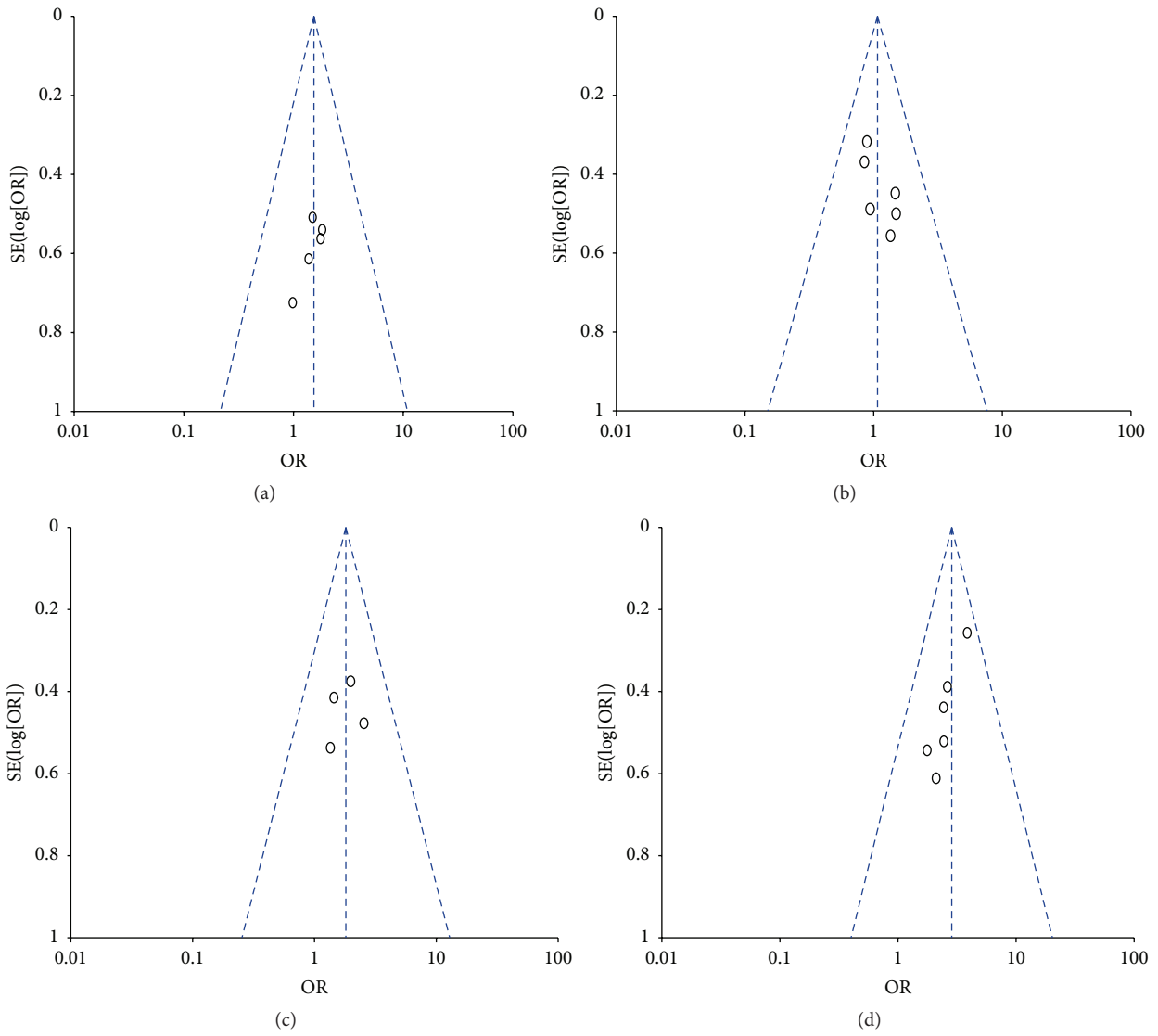


Figure 4: (a) Funnel plot of 1-month survival rate. (b) Funnel plot of 3-month survival rate. (c) Funnel plot of 12-month survival rate. (d) Funnel plot of the incidence of 1-month HBV negative status.

Table 4: Efficacy comparison of entecavir and lamivudine for continuous outcomes.

Outcome of interest	Number of studies	Sample size		Effect estimate		Heterogeneity	
		Entecavir	Lamivudine	MD (95% CI)	p value	I^2 (%)	p value
TBIL level							
1 month	3	134	126	−12.43 (−36.43, 11.58)	0.31	0	0.42
3 months	3	236	229	−1.69 (−6.66, 3.29)	0.51	0	0.82
12 months	3	215	201	−8.73 (−12.74, −4.72)	<0.0001	0	0.73
ALT level							
1 month	4	303	286	−4.96 (−10.07, 0.14)	0.06	0	0.64
12 months	3	215	201	−3.08 (−6.08, −0.07)	0.04	0	0.68
PTA level							
1 month	4	283	274	2.12 (0.42, 3.82)	0.01	21%	0.29
3 months	3	236	229	1.91 (−1.33, 5.15)	0.25	0	0.76
12 months	3	215	201	3.6 (−1.07, 8.26)	0.13	0	0.99

TBIL: total bilirubin; ALT: alanine aminotransferase; PTA: prothrombin activity; MD: mean difference; CI: confidence interval.

HBV DNA negative compared with lamivudine in hepatitis B-associated acute-on-chronic liver failure. This outcome seemed relatively stable irrespective of the duration of the antiviral therapy (Table 3).

3.3.3. Recurrence of HBV. Four studies comprising 154 patients taking entecavir and 165 patients using lamivudine reported the information of recurrence of HBV. No patients in entecavir group experienced a recurrence of HBV compared to 18 in the lamivudine cohort. Entecavir treatment significantly reduced the recurrence rate of HBV in patients with HBV-associated ACLF compared to patients taking lamivudine (0% versus 10.91%; OR: 0.07; 95% CI: 0.01, 0.40; p: 0.003). These data are suggestive of further benefits in long-term survival (Table 3).

3.3.4. TBIL Changes. Three studies reported the TBIL changes. No significant heterogeneity was found; the I^2 and p values of these studies at one-month, three-month, and twelve-month time points for TBIL changes were 0%, 0.42; 0%, 0.82; and 0%, 0.73, respectively. Therefore, the fixed effect model was applied. There were no significant differences between patients on entecavir and those using lamivudine in one-month and three-month TBIL changes (MD: −12.43, 95% CI: −36.43, 11.58, p: 0.31 for the former; MD: −1.69, 95% CI: −6.66, 3.29, p: 0.51 for the latter). However, TBIL reduction in the entecavir group was much more extensive than in those taking lamivudine at twelve months (MD: −8.73, 95% CI: −12.74, −4.72, $p < 0.0001$) (Table 4).

3.3.5. ALT Changes. Four trials reported the level of ALT in patients receiving antiviral therapy at one month. 303 patients were taking entecavir and 286 lamivudine. $I^2 = 0\%$ and $p = 0.64$ showed no significant heterogeneity and the fixed effect model was used. Our results showed a comparable effect between entecavir and lamivudine in reducing the level of ALT at one month (MD: −4.96, 95% CI: −10.07, 0.14, p: 0.06) (Table 4).

Three articles included data on ALT changes at twelve months, with 215 subjects prescribed entecavir and 201 receiving lamivudine. Patients on entecavir had more reduced levels of ALT at twelve months compared to those using lamivudine (MD: −3.08, 95% CI: −6.08, −0.07, p: 0.04). No apparent heterogeneity was found ($I^2 = 0\%$ and $p = 0.68$) (Table 4).

3.3.6. PTA Changes. Four studies, with a total of 283 subjects in their entecavir groups and 274 in lamivudine groups, reported the changes of PTA at one month. $I^2 = 21\%$ and $p = 0.29$ showed low heterogeneity and fixed effect model was applied. Patients taking entecavir presented higher rates of improvement of PTA than those with lamivudine after treatment for one month (MD: 2.12, 95% CI: 0.42, 3.82, p: 0.01) (Table 4).

Three studies reported the changes of PTA at three months and twelve months. No significant heterogeneity was measured between studies about PTA at three months and twelve months ($I^2 = 0\%$, $p = 0.76$ for the former; $I^2 = 0\%$ and $p = 0.99$ for the latter). Patients with entecavir showed no significant improvement of PTA compared to those with lamivudine for three months and twelve months (MD: 1.91, 95% CI: −1.33, 5.15, p: 0.25; MD: 3.6, 95% CI: −1.07, 8.26, p: 0.13, resp.) (Table 4).

3.4. Safety. No studies reported serious adverse events attributable to entecavir or lamivudine, nor did they report any drug-related viral mutation. All the patients tolerated the treatment without modification of dose or early discontinuation.

3.5. Sensitivity Analysis. The sensitivity analysis was performed to confirm the stability of the primary analysis by excluding studies one by one. We found out that the overall survival rate, HBV DNA negative, and recurrence of HBV did not change significantly with the exclusion of any single study.

4. Discussion

ACLF is a serious condition with a high mortality. Nearly two-thirds of patients may die without liver transplantation

[32, 33]. The mechanisms of HBV-associated ACLF are extremely intricate and complex and, as a result, not yet established. Nevertheless, one of the important mechanisms is the overactivity of immune response including the excess activity of HBcAg/HBeAg-specific T cells and involvement of B lymphocytes activity and peripheral glucocorticoid receptor expression [34].

Up to now, there has been no effective treatment for patients with HBV-associated ACLF. Therefore, it is critical to improve medical therapy for patients with HBV-associated ACLF as a key aim in extending periods of survival. Zhao et al.'s study suggested that HBV replication and mutation were the primary factor which may lead to chronic and acute liver failure [35]. As such, antiviral therapy by inhibition of HBV replication may be helpful in postponing the progression of liver failure and reducing the mortality of patients with HBV-associated ACLF. Though the efficacy of lamivudine and entecavir is controversial in the treatment of HBV-associated ACLF, recent studies had proven that both lamivudine and entecavir can decrease the mortality, improve the biochemical response, and effectively suppress the replication of HBV in patients with HBV-associated ACLF [14, 15]. Further studies have shown that a profound and rapid reduction of HBV DNA is effected by entecavir treatment but not by lamivudine. Accordingly, it appears that entecavir may be more efficacious than lamivudine in the treatment of HBV-associated ACLF.

In this meta-analysis, we made an efficacy comparison of entecavir and lamivudine in patients suffering from HBV-associated ACLF across ten eligible studies. The efficacy comparison outcomes were the overall survival rate, HBV DNA negativity, the recurrence of HBV, and the biochemical parameters (changes of TBIL, ALT, and PTA). The outcomes at different time points were different.

Though no statistically significant data was found, there was a discoverable tendency toward superiority of the entecavir therapy over the lamivudine in terms of survival rates at one, two, three, and six months. These outcomes are in accordance with Chen et al.'s study [27]. In the latter study, patients with entecavir and lamivudine had a similar accumulative survival rate during the first three months of treatment (66.7% versus 60%). However, patients with entecavir had significantly higher survival rate than those with lamivudine after treatment of twelve months. Those results suggest that entecavir outperforms lamivudine not in short-term survival but in that of the long term. Here, the short term was defined as not more than six months. The changes of TBIL and ALT were in line with the data on overall survival rates. Our results suggested a comparable efficacy in lowering the level of TBIL and ALT in subjects prescribed entecavir and those taking lamivudine, at least, in the short term. However, patients on entecavir acquired significantly lower TBIL and ALT levels after treatment for twelve months. PTA level at one month was significantly lower in the entecavir group than that in lamivudine subjects. Nevertheless, we found no significant difference in changes of PTA for patients with long-run use of entecavir and lamivudine. In general, both entecavir and lamivudine have the capacity to alleviate hepatic injury and improve liver function. But in the long run, entecavir may be superior to lamivudine in biochemical response. More patients on entecavir obtained HBV negative scores at one-, three-, and twelve-month time points than those on lamivudine, while, in addition, entecavir subjects had either very low or unmeasurable levels of HBV as compared to those on lamivudine. These results are attributed to entecavir's potency in suppressing HBV replication as well as the low level of mutations engendered by entecavir. These results are consistent with previous studies. As a whole, long-term use of entecavir could raise survival rates and improved patients' biochemical response, over and above its primary function to reduce virological replication rapidly for patients with HBV-associated ACLF.

However, there has been one perennial concern regarding entecavir administration in cases of chronic HBV-associated liver failure, which is acute lactic acidosis. A recent study reported that serious lactic acidosis occurred more often in patients with high MELD scores and multiorgan failure [36]. Patients in the eligible studies were mostly those with early- to mild-stage HBV-associated ACLF. Therefore, no serious adverse effects occurred as a result of the on occasion severe lactic acidosis in the meta-analysis.

The degree of hepatic necrosis, rather than the viral load, is the central determinant of short-term mortality in cases of HBV-associated ACLF [37, 38]. Therefore, the primary goal of antiviral therapy is as viral prevention in case of further liver transplantation and HBV reactivation. Moreover, cost may be a factor as lamivudine is cheaper than entecavir. As such, lamivudine may be a viable alternative in the first stages of treatment and restricted to short-term use. Routine switching to entecavir after liver function has improved or the adoption of the roadmap concept is reasonable treatment strategies for patients with HBV-associated ACLF [39].

Our study had a few limitations. Firstly, not all the eligible studies offered full datasets which satisfied requisite parameters. These gaps in data resulted in small sample sizes presenting different outcomes at different time points. The limited sample size itself might be deemed to weaken the validity of the conclusions. Secondly, there were only four randomized controlled trials and the quality of those RCTs was suboptimal. Therefore, higher-quality RCTs are a vital next step in validating our findings. Thirdly, all the eligible studies were conducted in China, rendering our conclusions potentially unsuitable for other populations. Fourthly, the conditions of eligible patients mainly are mild to moderate. Therefore, maybe our results are not conforming to patients with severe symptoms. Lastly, articles published in full text and published in English or Chinese were retrieved. There was a high probability of overlooking the eligible studies published in other languages or only in abstract.

5. Conclusion

In conclusion, short-term treatment with both entecavir and lamivudine are effective in increasing the survival rate and countering hepatic injury for patients with early-to-mild stage HBV-associated ACLF. But for long-term therapy,

entecavir has more advantages than lamivudine, whether measured by survival rates or clinical improvement. In addition, entecavir and lamivudine were equally well tolerated during the treatment.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Qi Liu was responsible for study conception and design. Jiao Yang and Hang Sun contributed with data acquisition and analysis. Jiao Yang wrote the paper. Qi Liu and Hang Sun critically revised the paper. All the authors read and approved the final paper.

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Research Article

Fish Oil Reduces Hepatic Injury by Maintaining Normal Intestinal Permeability and Microbiota in Chronic Ethanol-Fed Rats

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The aim of this study was to investigate the ameliorative effects of fish oil on hepatic injury in ethanol-fed rats based on the intestinal permeability and microbiota. Rats were assigned to 6 groups and fed either a control diet or an ethanol diet such as C (control), CF25 (control with 25% fish oil), CF57 (control with 57% fish oil), E (ethanol), EF25 (ethanol with 25% fish oil), and EF57 (ethanol with 57% fish oil) groups. Rats were sacrificed at the end of 8 weeks. Plasma aspartate aminotransferase (AST) and aminotransferase (ALT) activities, hepatic cytokines, and plasma endotoxin levels were significantly higher in the E group. In addition, hepatic histopathological analysis scores in the E group were significantly elevated. Rats in the E group also showed increased intestinal permeability and decreased numbers of fecal *Bifidobacterium*. However, plasma AST and ALT activities and hepatic cytokine levels were significantly lower in the EF25 and EF57 groups. Histological changes and intestinal permeability were also improved in the EF25 and EF57 groups. The fecal *Escherichia coli* numbers were significantly lower, but fecal *Bifidobacterium* numbers were significantly higher in the EF25 and EF57 groups.

1. Introduction

Chronic consumption of excessive ethanol leads to liver damage that may ultimately result in the development of alcoholic liver diseases (ALDs) including fatty liver, steatohepatitis, and cirrhosis [1]. Oxidative stress, lipid peroxidation, and inflammatory responses are all involved in the complex pathophysiological mechanisms of ALD [1]. There is an evolving concept that ethanol-induced dysbiosis disrupts the integrity of intestinal epithelium, resulting in intestinal inflammation and bacterial translocation [2]. Gut-derived endotoxin is a required cofactor, because an endotoxin-initiated hepatic necroinflammatory cascade leads to liver injury in ALD. Animal studies also showed that removal of the intestinal microflora with antibiotics prevents the occurrence of ALD [3–5]. Endotoxins are lipopolysaccharides (LPSs) derived from cell walls of gram-negative bacteria. Endotoxemia was found in ALD patients and gut leakage appears to be the cause of endotoxemia in ALD [6, 7]. Previous studies revealed that intestinal barrier hyperpermeability occurs only in alcoholics with ALD but not in those without liver disease [8]. Our previous study also indicated that synbiotics (combinations of probiotics and prebiotics) offers liver protection by improving the intestinal permeability and microbiota in rats with ethanol-induced liver injury [9]. Those findings mentioned above strongly suggest that intestinal barrier disruption induced by ethanol is the main mechanism of endotoxemia in ALD.

Dietary fish oil might be useful in preventing acute ethanol-induced fatty liver in animal models [10, 11]. Fish oil is rich in n-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which decrease blood triglyceride (TG) concentrations in hypertriglyceridemia patients and show protective effects against fatty liver [11].

It was also indicated that EPA and DHA were particularly effective in supporting the intestinal barrier integrity by improving natural resistance and reducing the permeability of allergic and inflammatory mediators such as interleukin- (IL-) 4 and interferon- (IFN-) γ [12]. In addition, recent studies suggested that fish oil may influence contents of the gut microbiota, especially increasing beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* [13, 14]. However, there is limited evidence from studies of the relationship between fish oil and ALD based on the viewpoint of the intestinal integrity and microflora.

Therefore, we hypothesized that fish oil may have protective effects against liver injury in ethanol-fed rats by improving the intestinal permeability and microbiota. This animal study of ethanol-induced liver injury was performed to investigate the proposed hypothesis.

2. Methods and Materials

2.1. Animals. Male Wistar rats which are 8 weeks old and weighing 280 g were used in this experiment (BioLasco Taiwan, Ilan, Taiwan). All rats were housed in individual cages in an animal room maintained at 22 ± 2°C and 50%~70% humidity with a 12 h light-dark cycle. Rats were allowed free access to a standard rodent diet (LabDiet ICN: AIN-76a Rodent Diet; PMI Nutrition International, St. Louis, MO, USA) and water during acclimation before the study. All procedures were approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

2.2. Study Protocol. Aspartate transaminase (AST) and alanine transaminase (ALT) activities in plasma were analyzed before the experiment. Rats were assigned to groups based on the AST and ALT activities to make sure that the liver function did not differ among groups at the beginning of the experiment. Experimental models of ALD are commonly generated by feeding animals the Lieber-DeCarli liquid diet, in which fats in the diet are rich in monounsaturated fatty acids (MUFAs) and low in polyunsaturated fatty acids (PUFAs) [15]. Therefore, in this study, fish oil was used to substitute for part of the olive oil in the Lieber-DeCarli liquid diet. That is, 36 male Wistar rats were divided into six groups and fed either a control diet or an ethanol-containing diet, in which the fat composition of both diets was adjusted with 25% or 57% fish oil substituted for olive oil. The groups included C (control), CF25 (control with 25% fish oil), CF57 (control with 57% fish oil), E (ethanol), EF25 (ethanol with 25% fish oil), and EF57 (ethanol with 57% fish oil). MUFA/PUFA ratios of the diets without fish oil and with 25% and 57% fish oil substitutions were 0.4, 0.7, and 1.5, respectively (Table 2). Rats in the E group were fed an ethanol-containing liquid diet, while rats in the C group were pair-fed an isoenergetic diet without ethanol. The ethanol liquid diet in this study contained 35% energy as ethanol which was modified from Lieber and DeCarli's ethanol liquid diet [15], while paired-fed control rats (C, CF25, and CF57 groups) received an equal amount of calories as their ethanol-fed counterparts (E, EF25, and EF57) by substituting ethanol-derived calories with maltodextrin. The compositions are shown in Table 1 and fish oil (VIVA Omega-3™) was provided by VIVA Life Science (Costa, Mesa, CA, USA). One gram fish oil contains 250 mg EPA and 178.6 mg DHA. A pair-feeding procedure was conducted in this study; that is, the amount of the liquid diet consumed by rats of the E group was measured and then equal energy of the diet was provided to rats of the other five groups the next day.

Table 1: Composition of the experimental liquid diets in each group[1,2].

Ingredients	C	CF25	CF57	E	EF25	EF57
	Concentration (g/L (1000 kcal))					
Casein	41.4	41.4	41.4	41.4	41.4	41.4
L-cysteine	0.5	0.5	0.5	0.5	0.5	0.5
DL-methionine	0.3	0.3	0.3	0.3	0.3	0.3
Corn oil	8.5	8.5	8.5	8.5	8.5	8.5
Olive oil	28.4	21.3	12.2	28.4	21.3	12.2
Safflower oil	2.7	2.7	2.7	2.7	2.7	2.7
Fish oil	0	7.1	16.2	0	7.1	16.2
Choline bitartrate	0.53	0.53	0.53	0.53	0.53	0.53
Fiber	10	10	10	10	10	10
Xanthan gum	4	4	4	4	4	4
ICN: AIN-76 vitamins	2.5	2.5	2.5	2.5	2.5	2.5
ICN: AIN-76 minerals	2.6	2.6	2.6	2.6	2.6	2.6
Maltodextrin	115.2	115.2	115.2	25.6	25.6	25.6
Ethanol	0	0	0	50	50	50

[1]C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E: ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil.
[2]Casein, L-cysteine, DL-methionine, choline bitartrate, fiber, AIN-76 vitamins, AIN-76 minerals, and maltodextrin were purchased from ICN Biochemicals (Costa Mesa, CA, USA). Xanthan gum, ethanol, and glutamine were purchased from the Sigma-Aldrich (St. Louis, MO, USA). Corn oil and olive oil were purchased from the God Bene Enterprise (Yunlin, Taiwan). Safflower oil was purchased from the Taiwan Sugar Corporation (Taipei, Taiwan). Fish oil (VIVA Omega-3™) was provided by VIVA Life Science (Costa Mesa, CA, USA).

At the 7th week, an intestinal permeability test and microbial culture of feces were carried out for all rats. All rats were sacrificed at the 8th week of the experiment. Blood samples were collected in heparin-containing tubes and centrifuged (1200 ×g for 15 min at 4°C) to obtain plasma samples. All plasma samples were stored at −80°C until being assayed. Liver tissues were rapidly excised. Parts of the liver tissues were fixed in 10% formaldehyde and embedded in paraffin for a histopathological analysis. Other liver tissues were stored at −80°C for further analysis.

2.3. Plasma Biochemical Indicators of Liver Function. AST and ALT activities were analyzed as biochemical markers of liver function using the SYNCHRON CX System Hitachi 7170 (Hitachi High-Technologies, Tokyo, Japan).

2.4. Hepatic Histopathological Analysis. Liver tissues fixed with formalin were processed with hematoxylin-eosin (H&E) staining and Masson's trichrome staining. The H&E stain

Table 2: The fatty acid compositions of the experimental diets[1,2,3].

Fatty acid (mg)/39.6 g oil	C/E	CF25/EF25	CF57/EF57
Myristic acid (14:0)	8.2	7.9	7.4
Pentadecanoic acid (15:0)	0.0	0.0	0.0
Palmitic acid (16:0)	4668.0	3784.0	2651.0
Margaric acid (17:0)	14.0	12.2	9.9
Stearic acid (18:0)	1057.4	870.6	631.0
Nonadecanoic acid (19:0)	0.0	0.0	0.0
Arachidic acid (20:0)	165.9	135.4	96.3
Behenic acid (22:0)	69.4	59.0	45.6
Lignoceric acid (24:0)	35.1	30.2	23.9
Myristoleic acid (14:1)	0.0	0.0	0.0
Palmitoleic acid (16:1)	302.9	229.6	135.6
Oleic acid (18:1)	23323.7	18158.4	11538.2
Gadoleic acid (20:1)	131.0	110.9	85.2
Erucic acid (22:1)	2.0	2.0	2.0
Linoleic acid (18:2)	9358.2	8684.9	7821.9
Linolenic acid (18:3)	426.7	428.0	429.7
Octadecatetraenoic acid (18:4)	0.0	71.7	163.6
Arachidonic acid (20:4)	1.4	246.3	560.3
Eicosapentaenoic acid (20:5)	0.0	2185.4	4986.4
Heneicosapentaenoic acid (21:5)	0.0	93.0	212.2
Docosapentaenoic acid (22:5)	0.0	337.3	769.5
Docosahexaenoic acid (22:6)	0.0	1688.4	3852.4
Other fatty acids	9.2	9.2	9.2
Saturated fatty acids	6018.1	4899.3	3465.2
MUFAs	23759.6	18500.9	11760.9
PUFAs	9786.3	13734.9	18795.9
M/P	1/0.4	1/0.7	1/1.5

[1]C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E: ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil.
[2]The contents of fatty acid are shown as the weight (mg) in 39.6 g oil of the liquid diets (the sum of corn oil, olive oil, safflower oil, and fish oil).
[3]These data are based on Taiwan Food and Drug Administration, Ministry of Health and Welfare-food nutrient database established by Taiwan Food Industry Research and Development Institute and National Pingtung University of Science and Technology, Taiwan.

was used to evaluate liver damage including hepatocyte fatty change, inflammatory response, degeneration, and necrosis. Masson's trichrome stain was used to assess collagenous fibers. A semiquantitative histological evaluation was performed by a pathologist blinded to the treatment groups to evaluate the severity of hepatic injuries. The grading ranged from 0 to 4 where 0 = absent, 1 = trace, 2 = mild, 3 = moderate, and 4 = severe.

2.5. Inflammatory Response

2.5.1. Hepatic Cytokine Concentrations. Inflammatory cytokines including tumor necrosis factor- (TNF-) α, IL-1β, IL-6, and IL-10 levels were measured. Liver tissues (0.5 g) were homogenized in 1.5 mL ice-cold buffer (50 mM Tris (pH 7.2), 150 mM NaCl, and 1% Triton-X 100) plus 0.1% of a protease inhibitor. The homogenates were shaken on ice for 90 min and then the homogenates were centrifuged at 3000 ×g and 4°C for 15 min. The supernatant was analyzed with a DuoSet® rat TNF-α kit, a rat IL-1β/IL-1F2 kit, a rat IL-6 kit, and a rat IL-10 kit (R&D Systems, Minneapolis, MN, USA). Procedures followed the assay kit instructions. The optical density (OD) was read at 450 nm for all cytokines using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.6. Intestinal Permeability

2.6.1. Plasma Endotoxin Level. Plasma endotoxin levels were measured using a limulus amebocyte lysate assay kit (Pyrochrome® Cape Cod, East Falmouth, MA, USA) and procedures followed the manufacturer's instructions. The OD was read at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.6.2. Urinary Lactulose/Mannitol (L/M) Ratio. An oral sugar test was used to assess intestinal permeability [16]. Briefly, rats were intragastrically administered 2 mL of a sugar solution containing lactulose (100 mg/kg body weight (BW)), mannitol (6 mg/kg BW), and sucrose (200 mg/kg BW). Ten milliliter of lactated Ringer's solution was injected subcutaneously to rats before sugar administration to promote urine output. Then, rats were housed in metabolic cages individually and urine samples were collected for 5 h. Urinary sugar levels were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS, AB SCIEX QTRAP® 5500, Framingham, MA, USA). An increased urinary L/M ratio indicates that the intestinal permeability is elevated.

2.7. Microbiota Composition of Feces. In order to collect fresh feces, rats were anesthetized by ethyl ether inhalation and fecal samples were collected in an anaerobic dilution solution (4.5 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 0.5 g/L L-cysteine: HCl, 2 g/L gelatin, and 1 mL/L Tween-20). Fecal samples were followed by 10-fold serial dilutions (10^{-1} to 10^{-6}) to acquire different concentrations and 50 μL of the solution was inoculated onto agar by the spread plate method for plate counts. Certain microorganisms were isolated from fecal samples using different isolation media. CDC anaerobe blood agar plates (A01-12, Creative Media Products, Taiwan) were used to detect the total aerobic bacterial flora. Endo agar plates (Difco™ & BBL™, Becton, Dickinson and Company, Sparks, MD, USA) were used for detecting *E. coli*. *Lactobacillus* anaerobic MRS with bromocresol green (Difco™ & BBL™, Becton, Dickinson and Company, Sparks, MD, USA) was used to detect *Lactobacillus*. Modified *Bifidobacterium* iodoacetate medium-25 (Difco™ & BBL™, Becton, Dickinson and Company, Sparks, MD, USA) was used to detect *Bifidobacterium*. The number of colony forming units (CFU) of bacteria was quantified. Endo plates were incubated for 24 h at 37°C to count colonies of *E. coli*. CDC plates, modified MRS agar plates, and BIM-25 plates were incubated in anaerobic chambers for 48 h at 37°C to, respectively, count colonies of total aerobic bacterial flora, *Lactobacillus*, and *Bifidobacterium*.

Table 3: Effects of fish oil on the initial body weight (BW), final BW, liver weight, and relative liver weight in rats under chronic ethanol feeding[1,2,3].

Group	Initial BW (g)	Final BW (g)	Liver weight (g)	Relative liver weight (%)
C	339.5 ± 4.6^{a}	405.5 ± 5.5^{c}	9.0 ± 0.2^{a}	2.2 ± 0.1^{a}
CF25	335.5 ± 3.7^{a}	413.0 ± 5.9^{c}	10.1 ± 0.2^{bc}	2.4 ± 0.0^{a}
CF57	339.0 ± 5.6^{a}	419.8 ± 6.7^{c}	10.2 ± 0.2^{bc}	2.4 ± 0.1^{a}
E	328.8 ± 3.0^{a}	395.8 ± 7.1^{b}	10.9 ± 0.5^{c}	2.7 ± 0.1^{b}
EF25	326.8 ± 6.7^{a}	391.3 ± 8.8^{b}	9.7 ± 0.4^{ab}	2.8 ± 0.1^{b}
EF57	332.7 ± 6.4^{a}	394.2 ± 8.4^{b}	10.6 ± 0.4^{bc}	2.9 ± 0.1^{b}

[1]Values are expressed as the mean ± SEM. Means in the same column with different superscript letters significantly differ ($p < 0.05$).
[2]C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E, ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil.
[3]Relative liver weight: (liver weight/body weight) × 100%.

2.8. Statistical Analysis. Data are presented as the means ± standard error of the mean (SEM). A two-way analysis of variance (ANOVA) followed by Fisher's test was used to determine statistical differences among groups using SAS software version 8.0 (SAS Institute, Cary, NC, USA). Statistical significance was assigned at the $p < 0.05$ level.

3. Results

3.1. Food Intake, Growth Performance, and Liver Weight. Average liquid dietary intakes of the C, CF25, CF57, E, EF25, and EF57 groups were 63 ± 2, 62 ± 2, 62 ± 2, 65 ± 2, 63 ± 2, and 64 ± 2 g/rat/day, respectively. Average ethanol consumption of rats in the E, EF25, and EF57 groups was 3.2 ± 0.1, 3.1 ± 0.1, and 3.2 ± 0.1 g/day.

There were no significant differences in initial BWs among groups. However, the final BWs of the E, EF25, and EF57 groups were significantly lower than that of the C group ($p < 0.05$). Relative liver weights of the E, EF25, and EF57 groups were significantly higher than that of the C group (Table 3).

3.2. Plasma Biochemical Indicators of Liver Function. AST and ALT activities of the E group were significantly higher than those of the C group ($p < 0.05$), while these two parameters in the EF25 and EF57 groups were significantly lower than those of the E group ($p < 0.05$) (Table 4).

3.3. Hepatic Histopathological Analysis. Histopathological analysis scores were shown in Table 5. Hepatic fatty change, inflammation, and necrosis scores were significantly higher in the E group ($p < 0.05$), while scores of hepatic fatty change and inflammation were lower in the EF25 and EF57 groups than those in the E group ($p < 0.05$). On the other hand, hepatic degeneration and the necrosis score did not significantly differ between the EF25 and E groups, but the score of the EF57 group was significantly lower than that of the E group ($p < 0.05$). Moreover, the score of hepatic fibrosis was significantly higher in the E group compared to the C group. Conversely, only the fibrosis score of the EF57 group was significantly lower than that of the E group. The photomicrographs of the liver tissues showed that fatty change and inflammation were observed in the E group (Figure 1).

Table 4: Effects of fish oil on plasma aspartate transaminase (AST) and alanine transaminase (ALT) activities in rats under chronic ethanol feeding[1,2].

Group	AST (U/L)	ALT (U/L)
C	85.0 ± 1.6^{a}	53.2 ± 4.0^{ab}
CF25	84.7 ± 2.5^{a}	49.5 ± 3.7^{a}
CF57	91.3 ± 3.4^{ab}	50.0 ± 1.9^{ab}
E	197.8 ± 22.5^{d}	95.3 ± 15.7^{c}
EF25	122.0 ± 3.3^{bc}	76.2 ± 4.1^{b}
EF57	142.2 ± 14.7^{c}	71.2 ± 5.4^{b}

[1]Values are expressed as the mean ± SEM. Means in the same column with different superscript letters significantly differ ($p < 0.05$).
[2]C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E, ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil.

Masson's trichrome staining showed that collagenous fibers were shown in several biopsy specimens of the E group; however, few collagenous fibers were found in the other groups (Figure 2).

3.4. Inflammatory Response

3.4.1. Hepatic Cytokine Concentrations. Effects of fish oil on hepatic inflammatory cytokines in rats under chronic ethanol feeding are shown in Table 6. Chronic ethanol consumption (E group) led to a significant increase in hepatic TNF-α, IL-1β, IL-6, and IL-10 concentrations ($p < 0.05$). On the other hand, all of the hepatic cytokine levels measured in this study in the EF25 and EF75 groups were significantly lower compared to those of the E group ($p < 0.05$).

3.5. Intestinal Permeability

3.5.1. Plasma Endotoxin Level. Plasma endotoxin levels are presented in Table 7. Those of rats in the E group showed significant elevation compared to those of rats in the C group ($p < 0.05$). There was no significant difference in plasma endotoxin levels between the E and EF25 groups, but levels in the EF57 group showed a dramatic decrease ($p < 0.05$).

Table 5: Effects of fish oil on histopathological analysis of liver tissue in rats under chronic ethanol feeding[1,2].

Group	Fatty change		Inflammatory cell infiltration	Degeneration and necrosis	Fibrosis
	Macrovesicular	Microvesicular			
C	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
CF25	0.3 ± 0.2^{a}	0.2 ± 0.2^{a}	0.6 ± 0.2^{ab}	0.4 ± 0.2^{ab}	0.0 ± 0.0^{a}
CF57	0.5 ± 0.0^{a}	0.6 ± 0.2^{a}	0.6 ± 0.2^{ab}	0.6 ± 0.2^{ab}	0.4 ± 0.2^{ab}
E	2.0 ± 0.3^{d}	2.4 ± 0.2^{c}	2.6 ± 0.2^{e}	2.0 ± 0.0^{d}	2.2 ± 0.5^{d}
EF25	0.2 ± 0.2^{ab}	1.6 ± 0.2^{b}	1.4 ± 0.2^{cd}	1.4 ± 0.2^{cd}	1.6 ± 0.2^{cd}
EF57	0.4 ± 0.4^{abc}	1.4 ± 0.2^{b}	1.8 ± 0.4^{d}	1.0 ± 0.3^{bc}	1.0 ± 0.0^{bc}

[1]Values are expressed as the mean ± SEM. Means in the same column with different superscript letters significantly differ ($p < 0.05$).
[2]C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E, ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil.

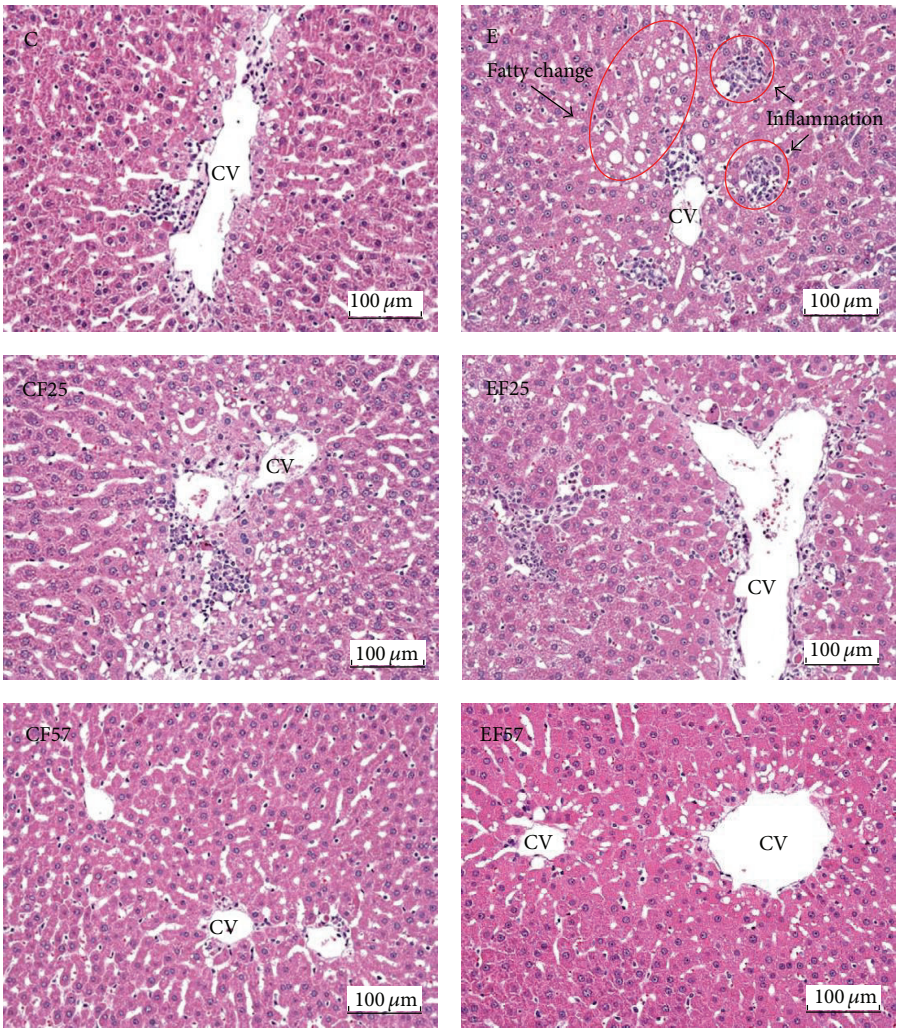

Figure 1: Representative photomicrographs of livers (H&E stain, magnification: ×200). C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E, ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil. CV, central vein. Fatty change and inflammation (arrows) occurred in E group while there were few histopathological changes in the other groups.

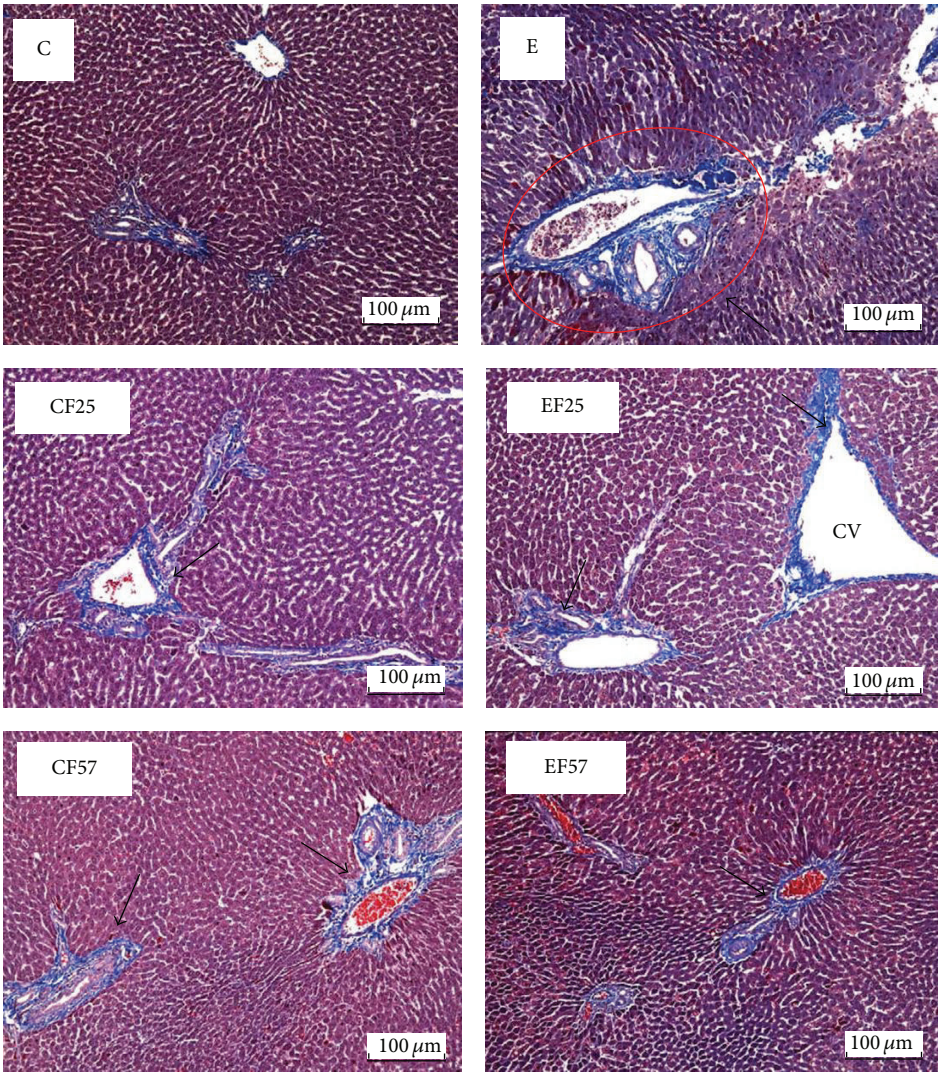

Figure 2: Representative photomicrographs of livers (Masson trichrome stain, magnification: ×200). C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E, ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil. CV, central vein. Collagenous fibers (arrows) were stained in several biopsy specimens of E group; however, few collagenous fibers were found in the other groups.

Table 6: Effects of fish oil on hepatic tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1β, IL-6, and IL-10 levels in rats under chronic ethanol feeding[1,2].

Group	TNF-α (pg/mg protein)	IL-1β (pg/mg protein)	IL-6 (pg/mg protein)	IL-10 (pg/mg protein)
C	60.4 ± 6.0^{a}	62.6 ± 3.0^{ab}	97.5 ± 5.7^{a}	91.3 ± 3.4^{a}
CF25	66.2 ± 5.1^{b}	67.1 ± 3.0^{b}	86.3 ± 7.6^{a}	87.6 ± 5.8^{a}
CF57	67.2 ± 3.2^{b}	59.3 ± 1.3^{ab}	91.5 ± 5.6^{a}	81.4 ± 4.1^{a}
E	89.0 ± 5.2^{c}	76.5 ± 2.5^{c}	118.1 ± 8.2^{b}	118.0 ± 5.4^{b}
EF25	59.5 ± 3.4^{a}	56.8 ± 2.6^{a}	94.0 ± 3.3^{a}	88.5 ± 4.6^{a}
EF57	75.2 ± 6.0^{b}	65.0 ± 3.8^{ab}	97.5 ± 5.9^{a}	92.9 ± 4.6^{a}

[1] Values are expressed as the mean ± SEM. Means in the same column with different superscript letters significantly differ ($p < 0.05$).
[2] C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E, ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil.

Table 7: Effects of fish oil on endotoxin concentrations in rats under chronic ethanol feeding[1,2].

Group	Endotoxin (EU/mL)
C	25.5 ± 2.6^{b}
CF25	26.9 ± 0.3^{b}
CF57	23.7 ± 1.7^{a}
E	30.4 ± 1.8^{c}
EF25	29.0 ± 3.6^{c}
EF57	21.2 ± 1.6^{a}

[1]Values are expressed as the mean ± SEM. Means in the same column with different superscript letters significantly differ ($p < 0.05$).
[2]C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E, ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil.

Table 8: Effects of fish oil on the lactulose/mannitol (L/M) ratio in rats under chronic ethanol feeding[1,2].

Group	L/M ratio
C	3.0 ± 0.0^{b}
CF25	3.2 ± 0.0^{b}
CF57	2.8 ± 0.1^{a}
E	3.4 ± 0.2^{c}
EF25	3.2 ± 0.2^{b}
EF57	2.9 ± 0.0^{ab}

[1]Values are expressed as the mean ± SEM. Means in the same column with different superscript letters significantly differ ($p < 0.05$).
[2]C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E, ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil.

3.5.2. Urinary L/M Ratio. The E group showed the highest urinary L/M ratio among all groups ($p < 0.05$). However, ratios were significantly lower in the EF25 and EF57 groups than that of the E group (Table 8).

3.6. Microbiota Composition of Feces. The number of fecal *E. coli* in the E group showed an increasing trend compared to the C group. On the other hand, numbers of *E. coli* in the EF25 and EF57 groups were significantly lower than that in the E group ($p < 0.05$). Additionally, there were no significant differences in the number of anaerobes or *Lactobacillus* among all groups. However, the number of *Bifidobacterium* in the E group was significantly lower than that in the C group ($p < 0.05$). Conversely, the EF25 and EF57 groups presented significantly high numbers of *Bifidobacterium* compared to the E group ($p < 0.05$) (Table 9).

4. Discussion

Average ethanol consumption in the E, EF25, and EF57 groups was 3.1~3.2 g/day. According to a conversion of animal doses to the human equivalent based on the body surface area, this amount of ethanol intake in rats is equivalent to 73.6 g/day/person [17, 18], which can be considered as a heavy drinker in human [19, 20].

Although the caloric intake was identical among the all groups, the final BWs were found lower in the ethanol intake groups (Table 3). This result indicated that nutrients absorption and the efficiency of calorie utilization were affected when ethanol was administered for a long period of time [21]. Relative liver weights in the ethanol intake groups also significantly increased, which is consistent with our previous report [22]. The current study further indicated that substituting fish oil for olive oil in the liquid diet had no effect on BWs or hepatomegaly in chronic ethanol feeding rats.

In this study, plasma AST and ALT activities were measured to evaluate liver damage caused by chronic ethanol feeding. The enzyme activities in the E group were significantly higher than those in the C group after 8 weeks of ethanol feeding (Table 4). In addition, based on the histopathological analysis and photomicrographs of the livers (Table 5, Figure 1), fat accumulation was observed in the E group. This can be explained by enhancing free fatty acid mobilization from adipose tissues and increased biosynthesis of lipids, as previously suggested [22]. Furthermore, an inflammatory response also occurred in the E group (Table 5, Figure 1). These findings confirmed that ethanol consumption for 8 weeks led to liver injury in the present study. On the other hand, AST and ALT activities were significantly lower in rats fed the fish oil and ethanol-containing diet at the end of the study (Table 4). Similarly, according to results of the liver pathology (Table 5, Figure 1), we found that ethanol-induced fat accumulation and inflammation in the liver were alleviated by the dietary fish oil replacement. It was indicated that fish oil accelerates catabolism of very-low-density lipoprotein in the liver [23]. Therefore, we speculated that partial substitution of fish oil may have mitigated the liver damage by eliminating fat accumulation and then successively decreasing inflammation because of reduced fatty changes in the liver. On the other hand, we also found that only higher fish oil replacement (EF57 group) significantly reduced ethanol-induced fibrosis (Table 5, Figure 2). It is important to confirm the effects of fish oil on hepatic fibrosis-related factors, such as transforming growth factor- (TGF-) β1 in future studies.

Activation of the innate immune response by chronic ethanol exposure plays a major role in initiating and promoting alcoholic liver injury. Several steps, including intestinal bacterial LPS, increased gut permeability, endotoxemia, and Kupffer cell activation, trigger the inflammatory reaction [24]. Stimulation of Kupffer cells induces oxidative stress and produces proinflammatory cytokines, such as IL-1, IL-6, and TNF-α, that cause hepatocellular damage [25]. These cytokines lead to apoptosis and necrosis of hepatocytes and consequently result in liver injury [26, 27]. Anti-inflammatory cytokines are usually secreted with or after the production of proinflammatory cytokines, which in turn maintain homeostasis of immune response and protect the liver against injury [28]. IL-10 is an anti-inflammatory cytokine secreted by Kupffer cells and peripheral blood monocytes. In this study, we found that concomitant with the upregulated hepatic proinflammatory cytokines, IL-10 level was also elevated in the E group (Table 6). We speculated that IL-10 as an anti-inflammatory cytokine was secreted in response to the release of proinflammatory cytokines, because Kupffer cells

Table 9: Effects of fish oil on fecal microbiota composition in rats under long-term ethanol feeding[1,2,3].

Group	Anaerobe (CFU/g)	*Escherichia coli* (CFU/g)	*Lactobacillus* (CFU/g)	*Bifidobacterium* (CFU/g)
C	7.4 ± 0.2^{a}	6.1 ± 0.3^{abc}	6.8 ± 0.2^{a}	6.7 ± 0.1^{c}
CF25	7.3 ± 0.1^{a}	6.0 ± 0.2^{ab}	6.9 ± 0.2^{a}	6.4 ± 0.1^{bc}
CF57	7.1 ± 0.1^{a}	6.4 ± 0.1^{bc}	6.7 ± 0.5^{a}	6.5 ± 0.1^{c}
E	7.4 ± 0.3^{a}	6.7 ± 0.2^{c}	6.2 ± 0.2^{a}	6.0 ± 0.2^{ab}
EF25	7.1 ± 0.2^{a}	5.8 ± 0.3^{a}	6.3 ± 0.4^{a}	6.5 ± 0.2^{c}
EF57	6.9 ± 0.2^{a}	5.8 ± 0.1^{ab}	6.5 ± 0.2^{a}	6.6 ± 0.1^{c}

[1]Values are expressed as the mean ± SEM. Means in the same column with different superscript letters significantly differ ($p < 0.05$).
[2]C, control; CF25, control with 25% fish oil substituted for olive oil; CF57, control with 57% fish oil substituted for olive oil; E, ethanol; EF25, ethanol with 25% fish oil substituted for olive oil; EF57, ethanol with 57% fish oil substituted for olive oil.
[3]CFU, colony forming units.

were activated by chronic ethanol consumption. It might be concluded that there is an imbalance between pro- and anti-inflammatory mediators regulation during chronic ethanol exposure [29]. On the other hand, hepatic TNF-α, IL-1β, IL-6, and IL-10 concentrations in the EF25 and EF57 groups were significantly lower than those in group E (Table 6). Results showed that fish oil normalize hepatic pro- and anti-inflammatory cytokine secretions in rats under chronic ethanol abuse. This cytokine-lowering effect of fish oil might be one of the reasons explaining the minor inflammatory cell infiltration based on the hepatic histopathological analysis in the EF25 and EF57 groups (Table 5). A previous study indicated that fish oil could reduce ethanol-induced fatty liver and hepatic production of the inflammatory cytokines, IL-6 and TNF-α, consistent with the reduction of IL-6 having a protective effect against ethanol-induced hepatic steatosis [10]. This was confirmed by hundreds of references that the active ingredients in fish oil are EPA and DHA, which can competitively inhibit the secretion of proinflammatory interleukins, because EPA is converted into anti-inflammatory prostaglandins (PGs) of the PGE3 series [10].

In order to investigate relationships between intestinal integrity and ethanol-induced liver damage, plasma endotoxin levels and the intestinal permeability were measured. In this study, both the plasma endotoxin level and urinary L/M ratio were significantly higher in the E group (Tables 7 and 8). The mechanisms responsible for the influences of ethanol feeding on increasing intestinal permeability were proposed previously [30]. Firstly, chronic ethanol exposure promotes intestinal gram-negative bacteria growth which may result in accumulation of endotoxin. Secondly, intestinal bacteria and epithelial cells metabolize ethanol that may result in acetaldehyde accumulation, which in turn increased tyrosine phosphorylation of tight junction and adherens junction proteins and thus enhance the intestinal permeability to endotoxin [12]. Thirdly, ethanol induces the production of nitric oxide and superoxide that may decrease stable polymerized tubulin and increase disassembled tubulin levels and subsequently disrupt the intestinal barrier function. The increased intestinal permeability to peptidoglycan can initiate an inflammatory response in the liver [30].

The findings of this study revealed that chronic ethanol intake increased intestinal permeability, enhanced endotoxin translocation from intestines to the liver and systemic circulation, and triggered inflammatory response in the liver. However, ethanol exposure with 25% or 57% fish oil substituted for olive oil showed significantly lower plasma endotoxin levels (only in the EF57 group) and urinary L/M ratio (in both the EF25 and EF57 groups) (Tables 7 and 8). Previous study found that n-3 fatty acids changed the lipid environment in tight junction membrane microdomains, prevented the redistribution of tight junction proteins, and reduced the inflammation induced by TNF-α [31]. Therefore, we surmised that fish oil substitution for olive oil in rats fed the ethanol-containing liquid diet normalized the intestinal permeability and has lower plasma endotoxin level especially when higher fish oil was administered.

Normal intestine permeability and gut microbes are both important in maintaining intestinal health. It was indicated that species of *Bacteroides*, *Porphyromonas*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, and *E. coli* are the most frequent ones in the intestinal tract [28]. In this study, fecal microbes were analyzed to reflect gut microbes. We found that slightly higher numbers of *E. coli* and significantly lower numbers of *Bifidobacterium* in the E group were found (Table 9). This result was consistent with our previous report [9]. A clinical study also showed that alcoholic subjects had a significant reduction in fecal *Bifidobacterium* numbers with a trend towards increased *E. coli* [32]. In this study, we showed for the first time that the partial replacement of olive oil with fish oil significantly decreased numbers of *E. coli* and increased numbers of *Bifidobacterium* in stools and may tend to restore the bowel flora in rats under chronic ethanol feeding. However, the fecal microbiota is very complicated. It is important to improve the accuracy of the fecal microbiota analysis not only in quantitative technology but also in flora species. Comparison of the bacterial 16S ribosomal RNA gene sequence will be needed in our future studies.

At last, fish oil substitution has not been shown the dose-response relationship in ameliorating alcohol-induced liver damage according to the direct evidence of liver damage, that is, the histopathological analysis (Table 5). Only the score of fibrosis was significantly different between the EF25 and EF57 groups.

In this study, the olive oil was partially substituted by fish oil in the diet according to MUFA/PUFA ratios.

The MUFA/PUFA ratio of 57% fish oil substitutions is 1.5 which is recommended in the human diet. Thus, we suggested that heavy drinkers should take care of the dietary fat sources, especially n-3 PUFA, for preventing the hepatic injury. If fish oil is considered to be a supplement, the dosage should be calculated according to the individual dietary patterns.

5. Conclusions

In conclusion, chronic ethanol feeding increased intestinal permeability, resulted in unbalanced fecal microbiota composition that may elevate plasma endotoxin levels, and consequently contributed to liver damage. However, substituting fish oil, especially provided in high dose, for olive oil under ethanol exposure normalizes the intestinal permeability and fecal microbiota composition, thus providing a low plasma endotoxin level and inflammatory responses, which exert ameliorative effects on ethanol-induced liver injuries in rats.

Disclosure

Jiun-Rong Chen is first author.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Suh-Ching Yang and Jiun-Rong Chen designed the study. Ya-Ling Chen, Hsiang-Chi Peng, Yu-An Lu, Hsiao-Yun Chang, Hsiao-Yun Wang, and Yu-Ju Su carried out the experiments. Hsiao-Li Chuang measured the plasma endotoxin levels. Suh-Ching Yang and Yu-An Lu wrote the original paper.

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Research Article

Expressions of Matrix Metalloproteinases (MMP-2, MMP-7, and MMP-9) and Their Inhibitors (TIMP-1, TIMP-2) in Inflammatory Bowel Diseases

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Crohn's disease (CD) and ulcerative colitis (UC) belong to a group of inflammatory bowel diseases (IBD). The aim of our study was to evaluate the expression of MMP-2, MMP-7, MMP-9, TIMP-1, and TIMP-2 in ulcerative colitis and Crohn's disease. The study group comprised 34 patients with UC and 10 patients with CD. Evaluation of MMP-2, MMP-7, MMP-9, TIMP-1, and TIMP-2 expression in tissue samples was performed using immunohistochemistry. The overexpression of MMP-9 and TIMP-1 was dominant in both the glandular epithelium and inflammatory infiltration in UC patients. In contrast, in CD subjects the positive expression of MMP-2 and TIMP-1 was in glandular tubes while mainly MMP-7 and TIMP-2 expression was in inflammatory infiltration. Metalloproteinases' expression was associated with the presence of erosions, architectural tissue changes, and inflammatory infiltration in the lamina propria of UC patients. The expression of metalloproteinase inhibitors correlated with the presence of eosinophils and neutrophils in UC and granulomas in CD patients. Our studies indicate that the overexpression of metalloproteinases and weaker expression of their inhibitors may determine the development of IBD. It appears that MMP-2, MMP-7, and MMP-9 may be a potential therapeutic target and the use of their inhibitors may significantly reduce UC progression.

1. Introduction

Crohn's disease (CD) and ulcerative colitis (UC) belong to a group of inflammatory bowel diseases (IBD). These are chronic diseases of, as yet, unknown etiology, in which various inflammatory mediators, such as proteolytic enzymes including metalloproteinase, cytokines, and growth factors, and a number of cells including leukocytes and stromal cells are involved [1]. In UC the chronic inflammation, mainly limited to the mucosa of the colon and rectum, may cause crypts and mucosal ulceration [2]. CD may affect the entire gastrointestinal tract, in particular its ileocecal region [3]. Inflammation involves the entire intestinal wall leading to fibrosis and fistulae [2]. In both diseases there exists a significant risk of developing cancer [2].

Metalloproteinases (MMPs) belong to a large group of proteolytic zinc-dependent enzymes which are involved in the remodeling and degradation of extracellular matrix (ECM) by cleavage of one or more of its components. They are synthesized and secreted by cells in an inactive form. The structures of the enzymes are very similar: they consist of a predomain comprising a signal peptide, a catalytic domain containing the zinc binding motif, and a hemopexin-like domain [4]. This family of proteinases presently includes 24 enzymes which have been divided into 6 subgroups based on domain organization and substrate preference: collagenases

(MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11, and MMP-18), matrilysins (MMP-7, MMP-26), membranous MMP (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), and other MMPs not yet assigned to any group (MMP-11, MMP-12, MMP-19, MMP-20, MMP-23, and MMP-28) [5]. The enzymes are involved both in physiological states such as embryogenesis or wound healing and in pathological conditions, for example, rheumatoid arthritis, atherosclerosis, and tumor metastasis [6–8].

The activity of the metalloproteinases is controlled by macroglobulins and, predominantly, Tissue Inhibitors of Metalloproteinases (TIMPs). There are currently four known inhibitors referred to as TIMP-1, TIMP-2, TIMP-3, and TIMP-4 [9]. They consist of two structurally and functionally distinct domains. The N-terminal domain is an effective inhibitor of all MMPs through binding to the enzymes' catalytic portion containing zinc. The C-terminal domain has at least two separate enzyme binding sites, one for gelatinases and the other one for stromelysins [9]. Tissue inhibitors affect all MMPs with the exception of TIMP-1 which does not inhibit MT1-MMP (Membrane-Type 1-Matrix Metalloproteinase). Properties of TIMP-3, however, are distinguished from the remaining tissue inhibitors as studies have proven it to be a better inhibitor of ADAM-17 (TACE), ADAM-10, and ADAM-12 than of MMP [10]. The inhibitors bind to MMPs in a stoichiometric ratio of 1 : 1. The expression of TIMPs as well as MMPs must be controlled to maintain physiological functions in tissue remodeling. Disturbances of this balance may lead to diseases of uncontrolled ECM component formation such as neurological and cardiovascular diseases, ulcers, and tissue fibrosis [9]. Still there is little known about expression of MMP-2, MMP-7, MMP-9, TIMP-1, and TIMP-2 in IBD patients, which may help to better understand the disease and to be used as therapeutic targets.

The aim of our study was to evaluate the immunohistochemical expression of MMP-2, MMP-7, MMP-9, TIMP-1, and TIMP-2 in patients with UC and CD in correlation with histo- and clinicopathological parameters.

2. Materials and Methods

2.1. Materials. The study was performed in conformity with the Declaration of Helsinki for Human Experimentation and received approval by the Local Bioethics Committee of the Medical University of Bialystok.

The study groups consisted of 34 patients (25 male, 9 female) with UC and 10 patients (7 male, 3 female) with CD. The study materials were obtained from core needle biopsy, embedded in paraffin blocks acquired in the Second Department of General Surgery and Gastroenterology at the Medical University of Bialystok in the years 2003–2005. Study included 17 patients under the age of 18 diagnosed with UC and none with CD. We found 17 patients with UC and 10 with CD to be over 18 years of age.

2.2. Histopathological Examination. Sections were stained with hematoxylin and eosin (H&E) and subjected to routine histopathological assessment. The presence of epithelial dysplasia was noted and classified as negative, indefinite, low, and high grade. Indefinite dysplasia was observed in 15 cases of UC and in 5 cases of CD, whereas low-grade dysplasia was observed in 10 cases and high-grade dysplasia in 3 cases of UC. Low-grade dysplasia was present in only 1 case of CD. Disease activity was assessed according to the Geboes criteria [11]. Inactive disease was observed in 9 cases of UC and in 2 cases of CD. Active inflammation was present in 5 cases of UC and 4 cases of CD, whereas chronic disease was noted in 20 cases of UC and 4 cases of CD (Table 1).

2.3. Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue slides were cut on a sliding microtome into 4 μm thick sections. Sections were deparaffinized in serial xylene and rehydrated in alcohol. Only the MMP-9 antigen was not diluted in any buffer. To visualize the antigens of MMP-2 and TIMP-1, sections were heated in a microwave oven for 20 min in EDTA buffer at pH = 9. TIMP-2 was treated with citrate buffer (pH 6). Endogenous peroxidase was blocked for 5 minutes. Next, they were incubated with anti-human antibodies: mouse monoclonal antibody of Matrix Metalloproteinase 2 (clone 17B11, Novocastra, UK; dilution 1 : 60); mouse monoclonal antibody of Matrix Metalloproteinase 7 (clone 111433, R&D Systems, USA; dilution 1 : 75); mouse monoclonal antibody of Matrix Metalloproteinase 9 (clone 15W2, Novocastra, UK; dilution 1 : 80); mouse monoclonal antibody of Tissue Inhibitor of Matrix Metalloproteinase 1 (clone 6F6a, Novocastra, UK; dilution 1 : 150); mouse monoclonal antibody of Tissue Inhibitor of Matrix Metalloproteinase 2 (clone 46E5, Novocastra, UK; dilution 1 : 20). In each case, incubation continued for 1 hour at room temperature. Antibodies for metalloproteinase and their inhibitors were specific for human pro and active forms. Following the reaction in the streptavidin-biotin system (Biotinylated Secondary Antibody, Streptavidin-HRP, Novocastra, UK) the antigen-antibody complex was visualized with the use of chromogen 3,3-diaminobenzidine (DAB, Novocastra, UK). All sections were counterstained with hematoxylin.

2.4. Statistical Analysis. The statistical analysis was conducted using the STATISTICA 10.0 program (StatSoft, Cracow, Poland). Student's t-test was used to compare the two groups. Correlations between the parameters were calculated with Spearman's rank correlation coefficient tests. The p value < 0.05 was considered statistically significant.

3. Results

3.1. Expression of Matrix Metalloproteinases (MMP-2, MMP-7, and MMP-9) in Glandular Epithelium and Inflammatory Cells of UC and CD. A weak expression of MMP-2 was dominated in both the inflammatory infiltration and glandular epithelium of 73.3% of patients with UC. Moderate and high reactions of MMP-2 protein were present in a greater proportion in glandular tubes (16.7% and 6.7%, resp.) than in the inflammatory cells (9.1% and 0%, resp.). In Crohn's disease, the expression of MMP-2 in the glandular epithelium was strong in 60% of cases while its reaction in inflammatory cells was weak in 81.8% of cases. The expression

Table 1: Demographic and histological characteristics of the study group.

Disease	N	Age		Gender		Localization			Grade of dysplasia				Disease activity		
		<18	>18	F	M	1	2	3	N	IN	L	H	IA	A	CH
Ulcerative colitis (UC)	34	17	17	9	25	14	7	13	8	15	10	3	9	5	20
Crohn's disease (CD)	10	0	10	3	7	6	3	1	4	5	1	0	2	4	4

F: female, M: male; 1: proctitis in UC, colon in CD, 2: left-sided colitis in UC, colon + rectum in CD, and 3: pancolitis in UC, rectum in CD; grade of dysplasia: N: negative, IN: indefinite, L: low, and H: high; disease activity: IA: inactive, A: active, and CH: u

Table 2: Immunohistochemical expression of MMP-2, MMP-7, and MMP-9 in glandular tubes and inflammatory cells in tissues of ulcerative colitis and Crohn's disease.

Protein expression	Ulcerative colitis (UC)								Crohn's disease (CD)							
	Glandular cells (% *of cases*)				*Inflammatory cells* (% *of cases*)				*Glandular cells* (% *of cases*)				*Inflammatory cells* (% *of cases*)			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
MMP-2	3.3	73.3	16.7	6.7[1]	18.2	72.2	9.1	0	20	10	10	60[2]	9.1	81.8	9.1	0
MMP-7	54.9	29	16.1	0	6.4	35.5	32.3	25.8	0	60	30	10	0	20	20	60
MMP-9	6.7	33.3	23.3	36.7[3]	3.2	32.3	25.8	38.7[5]	16.7	50	25	8.3[4]	33.3	41.7	16.7	8.3[6]

1 versus 2 = 0.009; 3 versus 4 = 0.042; 5 versus 6 = 0.003.
0: absent, 1: weak, 2: moderate, and 3: strong.

of MMP-2 in glandular epithelium was significantly higher in CD compared to UC ($p = 0.009$). The expression of MMP-7 in the glandular epithelium was absent in 54.9% as opposed to the expression observed in inflammatory cells (weak: 35.5%, moderate: 32.3%, and strong: 25.8%). We found a weak expression in the glandular epithelium (60%) and strong reaction in inflammatory infiltration in patients with CD (60%). The expression of MMP-9 in patients with UC was weak in 33.3% of glandular epithelium and in 32.3% of inflammatory infiltration. Strong expression of MMP-9 was observed in 38.7% with inflammatory infiltration and 36.7% in the glandular epithelium. We found weak expression of MMP-9 in glandular epithelium (50% of cases) and in 41.7% in inflammatory infiltration of CD patients (Figure 1). Expression of MMP-9 was statistically higher in both glandular epithelium and inflammatory infiltration of UC compared to CD patients ($p = 0.042$, $p = 0.003$). (Results are shown in Table 2.)

3.2. Expression of Matrix Metalloproteinase Inhibitors (TIMP-1, TIMP-2) in Glandular Epithelium and Inflammatory Cells of UC and CD. We found strong expression of TIMP-1 in 62.5% of cases of glandular epithelium and in 37.5% of cases in cells of inflammatory infiltration in patients with UC. Expression of TIMP-1 was strong in 66.7% of the glandular epithelium in contrast with weak reaction in 55.6% of patients with CD.

A positive reaction of TIMP-2 was higher in the glandular epithelium than in infiltration of inflammatory cells in patients with UC. We found weak expression of TIMP-2 in the glandular epithelium (62.5%) and the absence or favorable reaction of TIMP-2 in infiltration of inflammatory cells (66.7% of cases) (Table 3).

3.3. The Correlation between Expression of Matrix Metalloproteinases (MMP-2, MMP-7, and MMP-9) and Histopathological Parameters. The statistical analysis showed a positive correlation between MMP-2 expression in the glandular epithelium of UC patients and the presence of erosions or ulcers ($p = 0.048$, $R = 0.377$). There was also a trend of increased expression of MMP-2 in the glandular epithelium concurrent with progression of changes in tissue architecture and the presence of neutrophils in the lamina propria ($p = 0.073$, $R = 0.344$; $p = 0.074$, $R = 0.349$, resp.). There was a correlation between the predominant weak expression of MMP-2 in the inflammatory infiltration and the presence of neutrophils in the lamina propria ($p = 0.041$, $R = 0.388$). No correlation was found between MMP-2 expression in the glandular epithelium and inflammatory infiltration in CD patients and the histopathological features.

The expression of MMP-7 in the glandular epithelium of UC patients positively correlated with the occurrence of erosions ($p = 0.027$, $R = 0.449$). In CD patients, a strong positive correlation was found between MMP-7 expression in the glandular epithelium and the location of the lesion ($p = 0.000$, $R = 0.898$).

No statistically significant relationship was established between MMP-9 in the glandular epithelium and inflammatory infiltration in patients with UC and CD. We observed a trend towards decrease in expression of MMP-9 protein and marked architectural tissue changes in patients with UC ($p = 0.064$, $R = -0.361$).

3.4. The Correlation between Expression of Matrix Metalloproteinase Inhibitors (TIMP-1, TIMP-2) and Histopathological Parameters. In UC patients, MMP-2 expression in the lamina propria of inflammatory infiltration correlated with presence of neutrophils, whereas TIMP-1 expression depended on the presence of eosinophils in the lamina propria ($p = 0.021$, $R = 0.588$) and neutrophils in the glandular epithelium ($p = 0.029$, $R = 0.0563$). In contrast, in CD patients TIMP-1 expression in the glandular epithelium was inversely related to the patients' age ($p = 0.049$, $R = -0.669$) while its response in the inflammatory infiltrate was associated with the presence of granulomas ($p = 0.016$, $R = 0.848$).

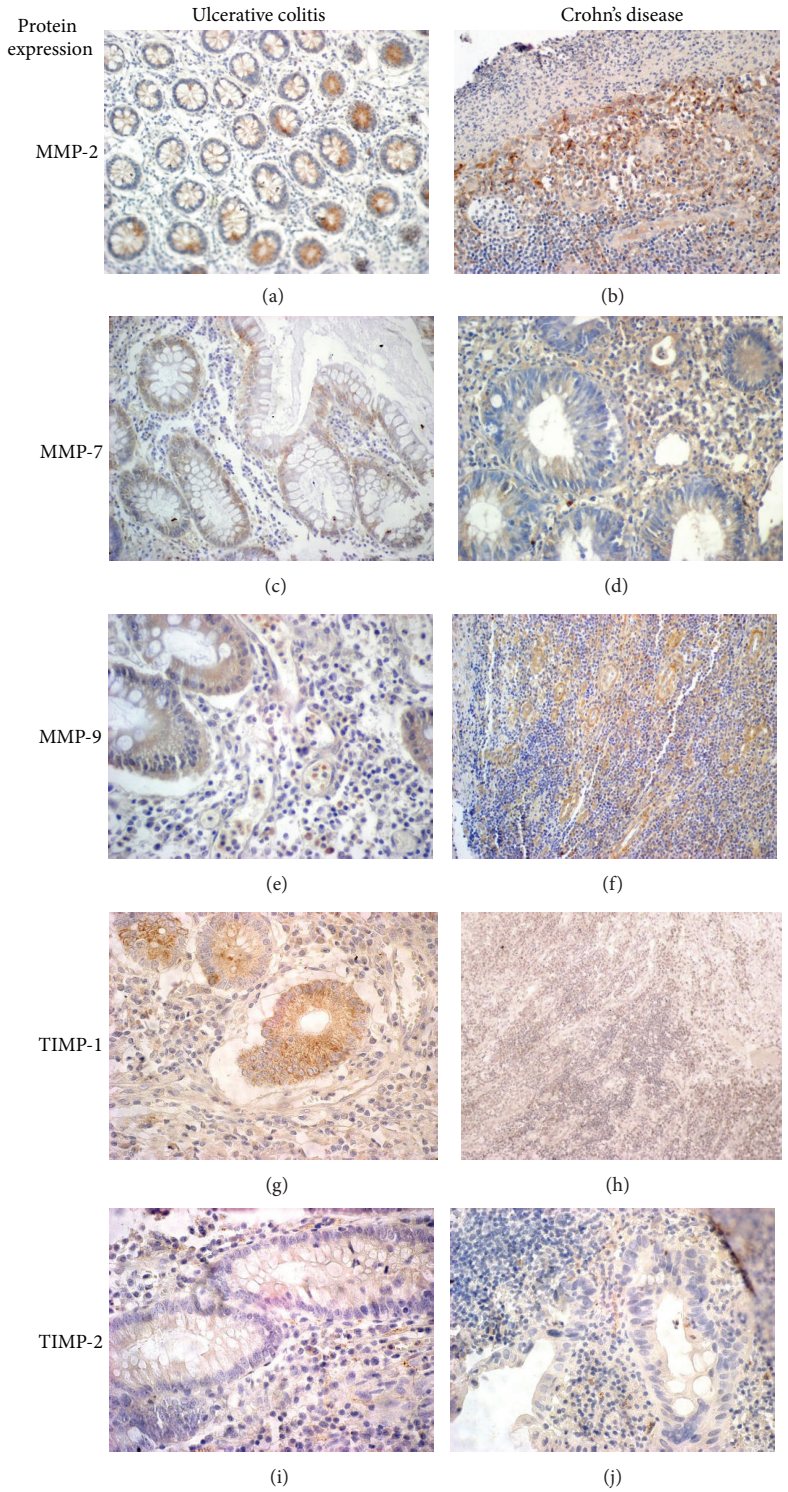

Figure 1: Immunohistochemical expression of MMP-2, MMP-7, MMP-9, TIMP-1, and TIMP-2 in glandular tubes and inflammatory cells in tissues of ulcerative colitis ($N = 34$) and Crohn's disease ($N = 10$). MMP-2 expression was weak in glandular tubes in patients with UC and positive reaction of this protein in inflammatory cells of CD (a, b). The positive expression of MMP-7 in glandular cells in both diseases, but there is more frequent expression observed in stroma of CD (c, d). Moreover, MMP-9 reaction was strong positive in glandular epithelium of UC and moderate in the stromal cells of CD patients (e, f). The strong expression of TIMP-1 in glandular cells and inflammatory infiltrate in both diseases compared to lack of or weak reaction of TIMP-2 protein (g, h, i, and j).

Table 3: Immunohistochemical expression of TIMP-1 and TIMP-2 in glandular tubes and inflammatory cells in tissues of ulcerative colitis and Crohn's disease.

Protein expression	Ulcerative colitis (UC)								Crohn's disease (CD)							
	Glandular cells (% *of cases*)				*Inflammatory cells* (% *of cases*)				*Glandular cells* (% *of cases*)				*Inflammatory cells* (% *of cases*)			
	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
TIMP-1	18.8	12.5	6.3	62.5	25	31.2	6.3	37.5	22.2	11.1	0	66.7	22.2	55.6	22.2	0
TIMP-2	10	45.5	31.8	18.2	40.9	31.8	9.1	18.2	12.5	62.5	12.5	12.5	66.7	11.1	0	22.2

0: absent, 1: weak, 2: moderate, and 3: strong.

The statistical analysis of TIMP-2 expression in the glandular epithelium of UC patients showed a significant correlation with the age of patients ($p = 0.029$, $R = 0.466$) who were divided into two groups: 1: <18 and 2: ≥18 years of age. In group 2, expression was moderate whereas in group 1 weak expression was dominant. We did not observe a relationship between TIMP-2 in the glandular epithelium and inflammatory cells and histopathological features.

4. Discussion

A series of destructive as well as regenerative processes occur in both UC and CD. Different levels of metalloproteinase and their inhibitor expression are observed depending on their stage of advancement. Our studies have shown a tendency towards weak expression of MMP-7 in glandular epithelium and a predominantly moderate or strong response in inflammatory infiltration in approximately 60% of UC patients. In contrast, Rath et al. [12] and Newell et al. [13] demonstrated an increase in MMP-7 mRNA levels which correlated with disease severity and degree of dysplasia in UC patients. Our study has shown positive expression of MMP-7, both in the glandular tubes and in inflammatory infiltrate in all CD patients which increased with the incidence of erosions. Inflammatory cells including neutrophils which are responsible for maintaining a local inflammatory response and stromal degradation may be the source of MMP-7.

Our research has demonstrated mainly weak expression of MMP-2 in the inflammatory infiltration and a much stronger response in the glandular epithelium of patients with UC and CD. von Lampe et al. [14] and Pirilä et al. [15] also confirmed the overexpression of MMP-2 in colonic mucosa of UC patients. Furthermore, Sim et al. [16] observed the upregulation of MMP-2 mRNA in patients with CD. It has been proven that the overexpression of MMP-2 in cultured intestinal epithelial cells determines the integrity of the protective barrier whereas its downregulation affects the sensitivity of the mucosa and may determine the occurrence of colitis [17]. In our study, we have observed that the expression of MMP-2 in glandular epithelium correlated with the presence of erosions. By contrast, MMP-2 immunoreactivity in the inflammatory infiltrate in UC patients positively correlated with the presence of neutrophils in the lamina propria. MMP-2 expression in both glandular epithelium and inflammatory infiltration, dependent mainly on mesenchymal cells, neutrophils, and eosinophils, determines the disorganization of protective structures, the decomposition of collagen types IV and V, and the degradation of the stromal tissue of IBD patients [18, 19].

Continuous inflammatory response in the intestinal mucosa in the course of IBD appears to be an important therapeutic target. The majority of animal colitis models have been based on chemically induced models with the dextran sulfate sodium-induced colitis model being the most widely used due to its similarities with human ulcerative colitis. In experimental studies, the dextran sulfate sodium- (DSS-) induced colitis has shown a lack of MMP-9 expression in healthy intestinal mucosa which is upregulated in inflamed mucosa of IBD [20]. DSS-induced colitis studies have proved that MMP-9 activity increases in homogenates of colonic mucosa in UC and is dependent on TNF-alpha [21, 22]. Studies in animal models have confirmed that the lack of MMP-9 −/− expression in the DSS-induced colitis determines a reduction in inflammation and damage to the intestinal mucosa [23, 24]. In our study, we have observed mainly positive expression of MMP-9 in both glandular epithelium and inflammatory infiltration in UC. Mao et al. [21] also demonstrated significantly higher expression of MMP-9 in the colonic mucosa of UC patients compared to the control group. In addition, activity of the protein has increased in homogenates of the inflamed mucosa of both UC and CD [25]. Furthermore, the statistical analysis of our research data has confirmed a tendency towards increased MMP-9 expression in UC patients who displayed changes in the architecture of the colonic tissue. It has been proved that MMP-9 is produced by a variety of inflammatory cells, in particular polymorphonuclear leukocytes (PMNL), and secreted in response to local inflammation [26]. We therefore believe that the lasting activation of the MMP-9 protein expression and the chronic inflammation of the lining of the colon in UC patients may lead to a loss of structural tissue integrity and may determine tissue damage. In contrast to UC patients, we have demonstrated low expression of MMP-9, in particular in the inflammatory infiltration, and its slightly higher immunoreactivity in the glandular tubes in CD patients. Our observations are contrary to Bailey et al. [20] who observed positive reaction of MMP-9 in PMNL present in the lamina propria more and less in the submucosa and muscularis propria. Our findings and the available literature reports suggest that MMP-9 plays an important role in the pathogenesis of colitis and may be a potential target for anti-inflammatory therapy [27, 28].

TIMP-1 and TIMP-2 are responsible for controlling the activity of Matrix Metalloproteinases, thus maintaining the

correct balance in the remodeling and degradation of ECM. Wang and Yan [29] reported positive expression of TIMP-1 in 80–89% of cases of inflamed ulcerative changes and intact colon mucosa in UC patients and in 75% of cases of normal colon mucosa. Rath et al. [12] reported significantly higher presence of TIMP-1 in the inflamed mucosa of adult IBD patients. In contrast, Mäkitalo et al. [30, 31] confirmed positive expression of TIMP-2 in epithelial cells and stroma in adult patients while lack of expression of TIMP-1 was found in all the studied cases of pediatric UC patients. Despite their enhanced activity in both diseases, it appears that tissue inhibitors expressions are too weak or their activation occurs too late to prevent the development of either condition.

The immunohistochemical analysis of our research data indicates that the overexpression of metalloproteinases and much weaker activation of the inhibitors in tissue samples may determine the development of IBD. A significant correlation has been established in UC patients, in particular between the increased expression of metalloproteinases and the examined histopathological markers which determine disease progression. It seems that MMP-7, MMP-2, and MMP-9 may be potential therapeutic targets and the use of their inhibitors may significantly reduce disease progression in UC patients. Nevertheless, the present findings should be confirmed in a larger study group in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Prealbumin/CRP Based Prognostic Score, a New Tool for Predicting Metastasis in Patients with Inoperable Gastric Cancer

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Background. There is a considerable dissimilarity in the survival duration of the patients with gastric cancer. We aimed to assess the systemic inflammatory response (SIR) and nutritional status of these patients before the commencement of chemotherapy to find the appropriate prognostic factors and define a new score for predicting metastasis. *Methods.* SIR was assessed using Glasgow Prognostic Score (GPS). Then a score was defined as prealbumin/CRP based prognostic score (PCPS) to be compared with GPS for predicting metastasis and nutritional status. *Results.* 71 patients with gastric cancer were recruited in the study. 87% of patients had malnutrition. There was a statistical difference between those with metastatic ($n = 43$) and those with nonmetastatic ($n = 28$) gastric cancer according to levels of prealbumin and CRP; however they were not different regarding patient generated subjective global assessment (PG-SGA) and GPS. The best cut-off value for prealbumin was determined at 0.20 mg/dL and PCPS could predict metastasis with 76.5% sensitivity, 63.6% specificity, and 71.4% accuracy. Metastatic and nonmetastatic gastric cancer patients were different in terms of PCPS ($P = 0.005$). *Conclusion.* PCPS has been suggested for predicting metastasis in patients with gastric cancer. Future studies with larger sample size have been warranted.

1. Introduction

The majority of patients with gastric cancer have a poor overall survival. Therefore, finding the appropriate prognostic factors will help to improve clinical approach on patients that will lead to accurate decision making and planning for supportive care. This will possibly increase the rate of survival [1].

Weight loss and performance status are usually used to predict survival and treatment outcomes in patients with inoperable gastric adenocarcinoma [2], but the degree to which they are associated with poor prognosis is not well defined and performance status does not provide an objective measurement [3, 4]. Studies have shown that the presence of malnutrition and a systematic inflammatory response cause a short survival, reduced response rate, and higher risk for treatment-induced complications in patients with malignancy [5, 6].

Recently, the host nutritional and immune status have been evaluated by the Glasgow Prognostic Score (GPS), which is a combination of serum C-reactive protein (CRP) as an index for systemic inflammatory response and an important factor for the development and progression of neoplasms [7] along with serum albumin which has been proposed as a prognostic factor in a variety of cancers. GPS has prognostic importance independent of tumor stage in number of malignancies including gastrointestinal cancer [4, 8–10].

On the other hand, prealbumin is a remarkable prognostic factor for treatment outcomes and/or nutritional

Table 1: General characteristics of the patients and disease.

		Male	Female
Age (mean ± SD)	62.13 ± 14.39	63.43 ± 13.75	57.00 ± 16.24
Gender (*n*, %)			
Male	56 (79%)		
Female	15 (21%)		
Anatomic area (%)			
GEJ*/proximal stomach	38 (54%)	29 (51%)	10 (64%)
Distal stomach	33 (46%)	27 (49%)	5 (36%)
Stage (%)			
3	28 (39%)	21 (37%)	6 (40%)
4	43 (61%)	35 (63%)	9 (60%)
BMI# (mean ± SD)	21.08 ± 3.99		
Metastasis			
Metastatic	43 (61%)	21 (37%)	6 (40%)
Nonmetastatic$	28 (39%)	35 (63%)	9 (60%)
SGA A	13%	15%	9%
SGA B	49%	46%	58%
SGA C	38%	39%	33%
PG-SGA (mean ± SD)	16.07 ± 5.02	15.76 ± 5.17	16.92 ± 4.70

*Gastroesophageal junction.
**Type of gastric adenocarcinoma.
#Body mass index.
$Unresectable gastric cancer.

status of colon [11], esophagus [12], ovarian [13], and lung cancers [14, 15]. Recently, we studied the nutritional status of patients with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) during induction chemotherapy and its effects on chemotherapy-related complications in which prealbumin was found as the common biomarker for better treatment outcomes in both groups of patients with acute leukemia [16]. Here, systemic inflammatory response of the patients with inoperable gastric adenocarcinoma (IGA) was investigated by GPS, while their nutritional status was assessed using patient generated subjective global assessment (PG-SGA) as well as the serum levels of albumin, prealbumin, transferrin, CRP, and total lymphocyte count (TLC). Then a new prognostic score, prealbumin/CRP based prognostic score (PCPS), was introduced for predicting metastasis in this group of patients based on serum prealbumin and CRP and compared with PG-SGA and GPS.

2. Materials and Methods

2.1. Study Population. A convenient sample of 71 patients with inoperable gastric adenocarcinoma was recruited in this prospective study before the onset of chemotherapy between February 2013 and March 2014 (Table 1). The Human Ethics Committee of Tabriz University of Medical Sciences approved the study and written informed consent was obtained from all the patients before the commencement of the study. Patients with history of other malignancies, autoimmune disease, chronic renal or hepatic disease, diabetes, and thyroid disorders and those who were taking anti-inflammatory drugs were excluded from the study. The Tumor-Node-Metastasis (TNM) classification of malignant tumors was used for staging the tumors. All patients received the same chemotherapy regimen as described below.

The chemotherapy regimen was as follows: docetaxel, 75 mg/m^2 i.v. (one-hour infusion) on day 1; cisplatin, 75 mg/m^2 i.v. (one-hour infusion) on day 1; and 5-fluorouracil (5-FU) i.v. (continuous infusion) on days 1–5, to be repeated every 3 weeks for 6 cycles.

2.2. Biochemical Analyses. Venus blood samples were taken after an overnight fasting and the serum was separated and stored at −70°C for future analysis. Hitachi 917 automated equipment was used for measuring albumin concentration and serum CRP, prealbumin, and transferrin were analyzed using the Minineph Human kits (Birmingham, UK).

2.3. Immunological Analyses. The systemic inflammatory response was measured using a combination of serum C-reactive protein and albumin as follows: patients with C-reactive protein ≤10 mg/L and albumin ≥3.5 mg/dL were allocated a score of 0; patients with one of these parameters abnormalities were allocated a score of 1; and those with both abnormalities, C-reactive protein >10 mg/L and albumin <3.5 g/dL, were allocated a score of two (Table 2). Then a new score was defined and called the prealbumin/CRP based prognostic score (PCPS) and it was compared with conventional GPS for evaluating the inflammatory status of patients and predicting metastasis. The PCPS was constructed using prealbumin and C-reactive protein with the same cut-off value for CRP and 0.20 mg/dL for prealbumin.

Table 2: Classification of prealbumin/CRP based prognostic score (PCPS).

Prealbumin (mg/dL)	CRP (mg/L)	PCPS
0.20<	<10	0
0.20<	10≤	1
<0.20	<10	1
<0.20	10≤	2

Similar categorization was used for allocating scores of 0, 1, and 2 to the patients.

2.4. Nutritional Assessment. BMI was computed as weight (kg)/height (m^2). The scored PG-SGA was completed by all the patients with the help of a trained oncology nurse. PG-SGA consists of the history of weight changes, food intakes, and the contributing factors, activities, physical examination, and the metabolic stress which affects the nutritional requirements. Based on scored PG-SGA, patients were scored at 0-1 (with no need of nutritional intervention) and there was a progressive need for nutritional support, so that those who scored ≥9 required immediate symptom management and/or nutritional support. PG-SGA also provided a categorical assessment as PG-SGA A (well-nourished), PG-SGA B (moderate malnutrition), and PG-SGA C (severe malnutrition) [5].

2.5. Statistical Analyses. Quantitative variables were presented as mean (standard deviation [SD]) or median (percentile 25–percentile 75) based on the normality of the distribution, and qualitative variables were reported as frequency (%). The best cut-off point value for prealbumin was determined using receiver operating characteristic (ROC) analysis, considering the optimal sensitivity and specificity to calculate the Youden Index ((specificity + sensitivity) − 1). The likelihood ratios (LRs) and area under curve (AUC) were shown as a measure of metastasis prediction adequacy using prealbumin concentration. Then regression tree analysis was used to measure sensitivity, specificity, and accuracy of the new score, PCPS, for prediction of metastasis in IGA patients.

The association between categorized variables was examined using Chi-square test or Fisher exact test. Independent-samples Kruskal-Wallis test was used to determine the association between scored PG-SGA and categorized variables.

The significance level was considered 0.05 by doing a two-tailed analysis. SPSS software (SPSS Inc., Chicago, IL) was used for performing statistical analyses.

3. Results

General characteristics of patients are shown in Table 1. Seventy-nine percent ($n = 56$) of the patients were male and 21% ($n = 15$) were female with an average of 62.13 ± 14.39 and 21.08 ± 3.99 for age and BMI, respectively. Twenty-eight patients (39%) had locally advanced unresectable gastric cancer (stage 3) (Figure 1) and 43 patients (61%) had distance metastasis (stage 4) (Figure 2). According to categorized PG-SGA, 13% of patients were well-nourished (PG-SGA A), while 49% of them were moderately (PG-SGA B) and 38% severely (PG-SGA C) malnourished. Considering PG-SGA A as an index for well-nourished category and PG-SGA B and PG-SGA C for some degrees of malnutrition, 87% of patients suffered from malnutrition before the beginning of chemotherapy. The mean score for PG-SGA was 16.07 ± 5.02, an indicative of need for immediate nutritional support.

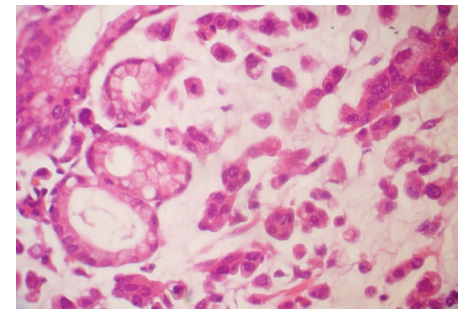

Figure 1: Microscopic scheme of metastatic diffuse type gastric cancer.

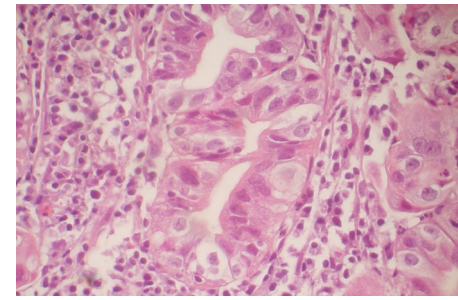

Figure 2: Microscopic scheme of nonmetastatic intestinal type gastric cancer.

Then metastatic and nonmetastatic patients were compared in terms of BMI, scored PG-SGA, visceral proteins, CRP, and TLC. There was a statistical difference between them according to prealbumin and CRP; $P = 0.012$ and $P = 0.004$, respectively (Table 3).

The best cut-off value for prealbumin was determined at 0.20 mg/dL for differentiating metastatic from nonmetastatic status using ROC analysis (Table 4). Using regression tree analysis, PCPS could predict metastasis with 76.5% sensitivity, 63.6% specificity, and 71.4% accuracy (considering scores 0 and 1 in one category and score 2 in the second category).

There was no statistical association between GPS, PCPS, and categorized PG-SGA, although 72% of the patients with severe malnutrition had a score of 2 for PCPS (Table 5). Moreover, the distribution of scored PG-SGA was the same across the categories of GPS and PCPS ($P = 0.527$ and $P = 0.334$).

There was a statistical difference in PCPS score between metastatic and nonmetastatic gastric cancer patients ($P = 0.009$), while they were not different in terms of GPS (Table 6). Moreover, there was no statistical association between anatomic location and site of gastric adenocarcinoma and PCPS ($P = 0.701$ and $P = 0.956$, resp.).

Table 3: Comparison between patients with metastatic and nonmetastatic inoperable gastric adenocarcinoma based on indicators of nutritional and inflammatory status.

	Metastatic	Nonmetastatic[$]	Mean difference (95% CI)	*P* value
BMI[*]	21.94 ± 3.94	22.13 ± 4.11	0.18 (−2.43 to 2.81)	0.885
Albumin[*]	3.57 ± 0.74	3.94 ± 0.68	0.36 (−0.30 to 0.76)	0.070
Prealbumin[*]	0.14 ± 0.06	0.20 ± 0.10	0.06 (0.01 to 0.09)	**0.012**
Transferrin[*]	218.48 ± 119.68	279.07 ± 135.75	60.59 (−5.75 to 126.94)	0.073
CRP[**]	37.60 (15.59–85.16)	15.61 (5.52–30.01)		**0.004**
TLC[*]	1.18 ± 0.49	1.27 ± 0.54	0.09 (−0.19 to 0.37)	0.516
PG-SGA[**]	17 (13–20.50)	17 (11–19)	0.564	0.564

[*] Mean ± SD, *P* value based on independent-samples *t*-test.
[**] Median (percentiles 25–75), *P* value based on Mann-Whitney test (only *P* value was reported).
[$] Unresectable gastric cancer.

Table 4: Receiver operating characteristic (ROC) analysis and optimum cut-off point of prealbumin for predicting metastasis in patients with inoperable gastric adenocarcinoma.

	PA	AUC	SEN	SPE	PPV	NPV	LR^+	LR^-
mets	0.20	0.68 (0.54–0.82)[*]	77.1% (61.0–87.9)	52.2% (33.0–70.8)	71.1% (55.2–83.0)	60.0% (38.7–78.1)	1.61 (1.01–2.56)	0.44 (0.21–0.90)

Mets: metastasis; [*] 95% confidence interval (CI); PA: prealbumin (mg/dL); AUC: area under the curve; SEN: sensitivity; SPE: specificity; LR^+: positive likelihood ratio; LR^-: negative likelihood ratio; NPV: negative predictive value; PPV: positive predictive value.

Table 5: The association between GPS, PCPS, and categorized PG-SGA.

	PG-SGA[*] A (%)	PG-SGA B (%)	PG-SGA C (%)	*P* value[**]
GPS[#]				
0	40	19	33	
1	20	52	25	
2	40	29	42	0.527
PCPS[$]				
0	40	14	14	
1	40	19	14	
2	20	67	72	0.334

[*] Patient generated subjective global assessment.
[**] *P* value was calculated based on independent-samples Kruskal-Wallis test.
[#] Glasgow Prognostic Score.
[$] Prealbumin/CRP based prognostic score.

Table 6: The differences between metastatic and nonmetastatic patients with inoperable gastric adenocarcinoma in terms of PG-SGA, GPS, and PCPS.

	Metastatic	Nonmetastatic	*P* value[**]
PG-SGA[*] (%)			
A	12	19	
B	48	44	
C	40	37	0.820
GPS[#] (%)			
0	16	36	
1	40	41	
2	44	23	0.153
PCPS[$] (%)			
0	9	27	
1	15	37	
2	76	36	**0.009**

[*] Patient generated subjective global assessment.
[**] *P* value was calculated based on exact Chi-square test.
[#] Glasgow Prognostic Score.
[$] Prealbumin/CRP based prognostic score.

4. Discussion

Malnutrition and systemic inflammatory response are common in patients with cancer and they both have significant impact on patients' quality of life, treatment outcomes, prognosis, and survival [4, 5, 15, 17]. In this study, the nutritional and inflammatory status of patients with inoperable gastric adenocarcinoma were assessed using known scores of PG-SGA, a valid tool for nutritional assessment of patients with cancer and GPS, a score for measuring systemic inflammation, and an independent prognostic score in different kinds of malignancies including gastrointestinal cancer [4, 8–10]. Eighty-seven percent of patients had some degrees of malnutrition and 65% of them had GPS scores of 1 or 2, but there was no significant difference between patients with metastatic and nonmetastatic gastric cancer in terms of PG-SGA or GPS.

Comparing patients with metastatic and nonmetastatic gastric cancer, it was found that they were different statistically according to prealbumin and CRP and not albumin. The results of this study confirm the findings of previous studies that showed that baseline levels of prealbumin had a significant correlation to overall survival in patients with advanced colorectal cancer [11] and esophageal cancer [12]. In a study

on patients with non-small cell lung cancer [15] and epithelial ovarian carcinoma [18] prechemotherapy concentrations of prealbumin were associated with response to treatment and outcomes. Moreover, Ho et al. found that low level of prealbumin was an independent prognostic factor for overall survival in cancer patients and its assessment has been suggested to be considered as a part of palliative care setting [18].

Notably, Inoue et al. investigated the association between the serum levels of rapid turnover proteins (RTPs) and the prognosis in patients with advanced cancer receiving total parenteral nutrition. They found that there was a significant association between RTPs' concentration and survival in cancer patients and among RTPs and prealbumin had the most correct prognosis rate with 91.9% compared to transferrin and retinol binding protein [19]. The short prealbumin half-life of ≈2 days in the blood circulation makes it a more sensitive biomarker for the assessment of nutritional state compared to albumin with much longer half-life [20, 21]. It should be mentioned that we studied the nutritional status of patients with ALL and AML during induction chemotherapy and its impact on chemotherapy-related complications in which prealbumin was the common biomarker for better treatment outcomes in both groups of patients with acute leukemia [16].

On the other hand, CRP is an indicator of systemic inflammatory response and studies have shown the independent prognostic value of elevated serum levels of CRP in solid tumors including gastroesophageal cancer [6]. Given the evidences, this study designed a new prognostic score based on prechemotherapy concentrations of prealbumin and CRP, after determining the best cut-off point value of prealbumin by ROC analysis at 0.20 mg/dL for differentiating metastatic from nonmetastatic status named PCPS. This score could predict metastasis with 76.5% sensitivity, 63.6% specificity, and 71.4% accuracy. Noteworthy, the patients with metastatic and nonmetastatic gastric cancer were significantly different according to PCPS unlike the conventional score of GPS. However, there was no significant association between GPS, PCPS, and categorized PG-SGA which may be due to the relatively small sample size and the fact that 87% of the patients (metastatic and nonmetastatic) already had malnutrition (before the onset of chemotherapy). So PCPS could not be considered as a tool for assessment of nutritional status in patients with inoperable gastric adenocarcinoma.

The small sample size was one of the possible limitations, as it was a single center study and a large number of eligible patients declined inclusion as a result of their critical conditions. Moreover, there was no official registration system, so the patients could not be followed to determine the survival rate. However, this study presented a composite score of prealbumin and CRP suggesting that it may be a strong prognostic score in patients with inoperable gastric adenocarcinoma. Although it is early to propose the replacement of GPS with PCPS, assessment of the host inflammatory response and nutritional status with PCPS and evaluation of its association with response to treatment, prognosis, complications, and survival in patients with different kinds of cancer is warranted.

Abbreviations

GPS: Glasgow Prognostic Score
CRP: C-reactive protein
ALL: Acute lymphoblastic leukemia
AML: Acute myeloid leukemia
PG-SGA: Patient generated subjective global assessment
TLC: Total lymphocyte count
PCPS: Prealbumin/CRP based prognostic score
RTPs: Rapid turnover proteins.

Conflict of Interests

The authors declare that there is no conflict of interests.

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