Canadian Journal of Infectious Diseases and Medical Microbiology

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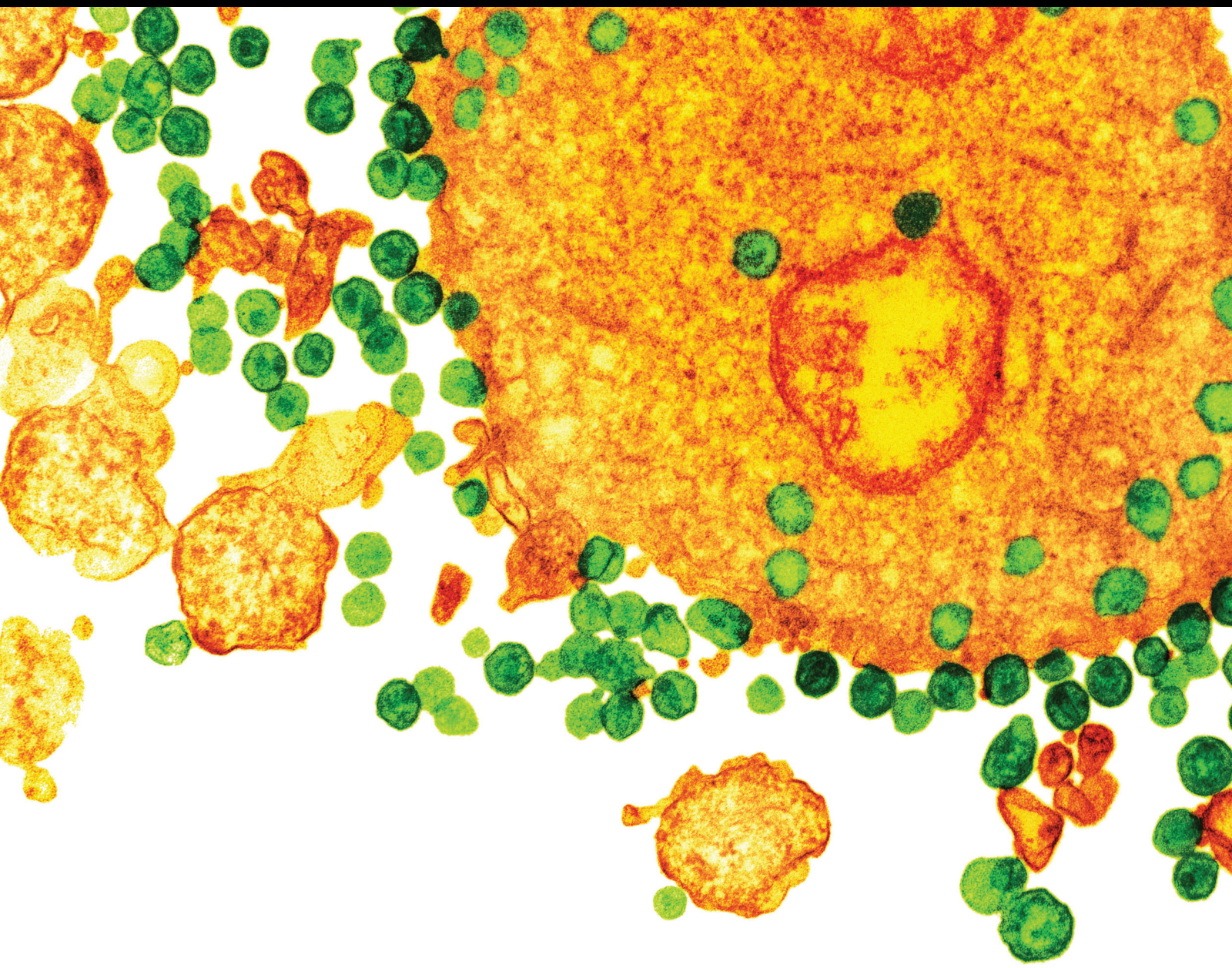

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Contents

Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2021, Article ID 9756586, 3 pages
https://doi.org/10.1155/2021/9756586

Corrigendum

Corrigendum to "Modulation of Short-Chain Fatty Acids as Potential Therapy Method for Type 2 Diabetes Mellitus"

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Received 23 May 2021; Accepted 23 May 2021; Published 8 June 2021

In the article titled "Modulation of Short-Chain Fatty Acids as Potential Therapy Method for Type 2 Diabetes Mellitus" [1], errors were identified in Figures 1 and 2 which were introduced during the preparation of the manuscript. The arrows for adipose tissue in Figure 1 should be pointing down for lipid buffering capacity and pointing up for inflammation. In Figure 2, the arrows for skeletal muscle should be pointing up. The authors confirm that this does not affect the results and conclusion of the article, and the revised figures are included below.

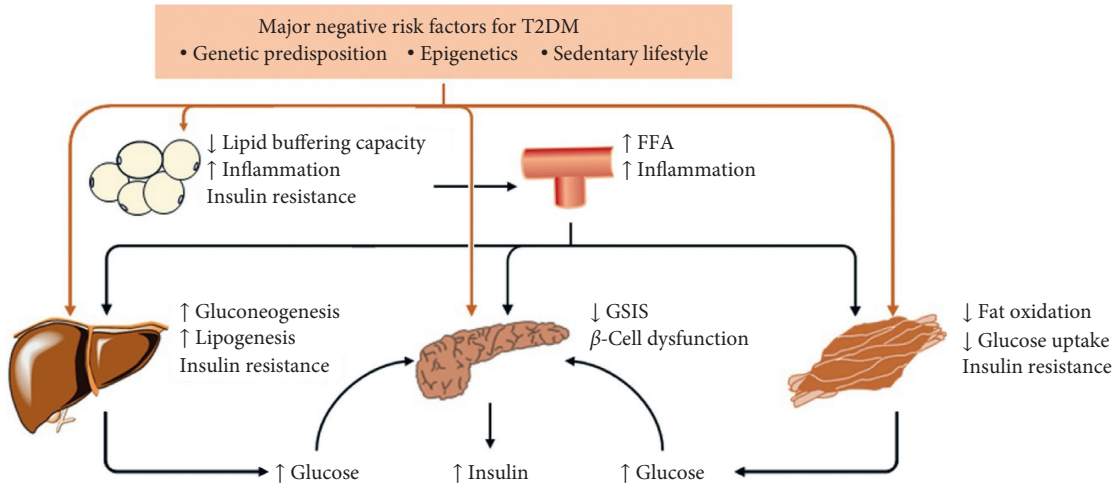

Figure 1: T2DM pathophysiology. A matrix of negative genetic, epigenetic, and lifestyle factors interact with one another and induce dysfunction of pancreatic β-cells and insulin resistance in the liver, skeletal muscle, or adipose tissue, thereby leading to the development of hyperinsulinemia and hyperglycemia. Moreover, once reduced lipid-buffering capacity in adipose tissue occurs, circulating lipid concentrations increase, leading to ectopic fat storage in the liver, skeletal muscle, and pancreas as well as the development of insulin resistance and dysfunction of pancreatic β-cells. In addition, inflamed adipose tissue results in a low-grade systemic inflammation, which contributes to the development of insulin resistance and T2DM. FFA, free fatty acid; GSIS, glucose-stimulated insulin secretion; T2DM, type 2 diabetes mellitus.

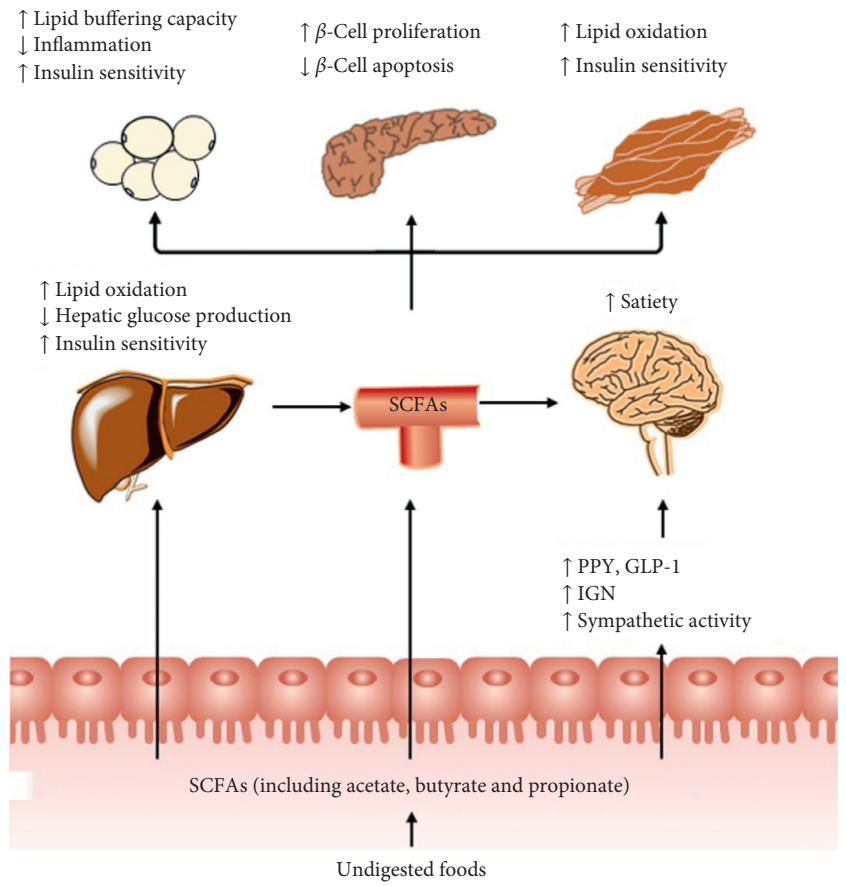

Figure 2: Impact of gut-derived SCFAs in T2DM. SCFAs (acetate, butyrate, and propionate) are produced from the fermentation of indigestible foods in the distal intestine by gut microbiota. In the distal gut, acetate, propionate, and butyrate stimulate the secretion of the "satiety" hormones GLP-1 and PYY in enteroendocrine-L cells, which leads to metabolic benefits upon satiety and glucose homeostasis. Furthermore, butyrate and propionate induce IGN and sympathetic activity, thereby beneficially leading to control of body weight and glucose homeostasis. Very little propionate and butyrate and a high concentration of acetate reach the circulation. They can also affect the metabolism and function of peripheral tissues directly (e.g., liver, adipose tissue, and muscle). Furthermore, circulating levels of acetate and propionate might cross the BBB and regulate satiety *via* CNS-related mechanisms. BBB, blood-brain barrier; CNS, central nervous system; GLP-1, glucagon-like peptide-1; GSIS, glucose-stimulated insulin secretion; IGN, intestinal gluconeogenesis; PYY, peptide YY; SCFAs, short-chain fatty acids; T2DM, type 2 diabetes mellitus.

References

[1] R. Tang and L. Li, "Modulation of Short-Chain Fatty Acids as Potential Therapy Method for Type 2 Diabetes Mellitus," *Canadian Journal of Infectious Diseases and Medical Microbiology*, vol. 2021, Article ID 6632266, 13 pages, 2021.

Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2021, Article ID 6666114, 10 pages
https://doi.org/10.1155/2021/6666114

Review Article

Faecalibacterium prausnitzii: A Next-Generation Probiotic in Gut Disease Improvement

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Received 20 November 2020; Revised 9 January 2021; Accepted 24 February 2021; Published 5 March 2021

Academic Editor: Tingtao Chen

The researchers are paying more attention to the role of gut commensal bacteria in health development beyond the classical pathogens. It has been widely demonstrated that dysbiosis, which means the alternations of the gut microbial structure, is closely associated with development of intestinal chronic inflammation-related diseases such as inflammatory bowel disease (IBD), and even infectious diseases including bacterial and viral infection. Thus, for reshaping ecological balance, a growing body of the literatures have proposed numerous strategies to modulate the structure of the gut microbiota, which provide more revelation for amelioration of these inflammation or infection-related diseases. While the ameliorative effects of traditional probiotics seem negligeable, emerging next generation probiotics (NGPs) start to receive great attention as new preventive and therapeutic tools. Encouragingly, within the last decade, the intestinal symbiotic bacterium *Faecalibacterium prausnitzii* has emerged as the "sentinel of the gut," with multifunction of anti-inflammation, gut barrier enhancement, and butyrate production. A lower abundance of *F. prausnitzii* has been shown in IBD, *Clostridium difficile* infection (CDI), and virus infection such as COVID-19. It is reported that intervention with higher richness of *F. prausnitzii* through dietary modulation, fecal microbiota transplantation, or culture strategy can protect the mice or the subjects from inflammatory diseases. Therefore, *F. prausnitzii* may have potential ability to reduce microbial translocation and inflammation, preventing occurrences of gastrointestinal comorbidities especially in COVID-19 patients.

1. Introduction

Probiotics are demonstrated to have potential anti-inflammatory and antiviral effects [1, 2]. The safety and clinical efficacy in reducing the severity and duration of upper respiratory tract infections of probiotics have been demonstrated [3]. An earlier review [4] confirmed the prophylactic and therapeutic effects of several lactic acid-producing bacteria strains on viral infection, which are reported to decrease titres of Ebola and cytomegalovirus and reduce respiratory and intestinal inflammation accordingly. Recently, Eguchi et al. evaluated the ability of *Lactobacillus gasseri* against respiratory syncytial virus (RSV) in a mice model [5]. The decreasing RSV titre and the diminishing expression of proinflammatory cytokines in the lung were significantly observed while interferons and interferon-stimulated genes were increased after the treatment. In addition, by summarizing 15 studies of *Lactobacillus rhamnosus GG* in the treatment of acute diarrhea, Szajewska and Kołodziej [6] concluded that *Lactobacillus rhamnosus GG* could reduce the severity of purging and the duration of diarrhea by about 1 day, and the most effective dose was more than 10^{10} CFU. According to a meta-analysis, a lower dose *Lactobacillus reuteri* was also reported to be effective in reducing the duration of diarrhea by approximately 1 day [7].

Although most traditional and widely used probiotics (e.g., *Bifidobacterium* spp. and *Lactobacillus* spp.) are safe, their effect on disease improvement is uncertain. Moreover, traditional probiotics are not disease-specific. Based on these situations, there is an urgent need for identification and

characterization of novel and disease-specific next generation probiotics (NGP). As one of the most common microbial species in the colon of healthy adults, *Faecalibacterium prausnitzii* (*F. prausnitzii*) constitutes over 5 percent of the overall total bacterial population [8]. The members of the genus *Faecalibacterium* are deemed symbiotic microorganisms, omnipresent in human and animal gastrointestinal tracts (GIT) [9]. Alterations in the abundance of *F. prausnitzii* have been commonly identified to be related to a number of human intestinal and metabolic diseases [10]. Therefore, due to the pervasiveness and immunomodulator, *F. prausnitzii* is not only an important predictor but also an influential contributor to intestinal health as well as the maintenance of gut homeostasis.

Studies have shown a certain correlation between the low abundance of *F. prausnitzii* and the increased incidence of inflammatory metabolic diseases such as inflammatory bowel disease [11], Crohn's disease [12], colitis [13] and some infectious diseases such as *Clostridium difficile* infection [14], human immunodeficiency virus (HIV) [15], and hepatitis B virus (HBV) [16]. On the other hand, supplementation with *F. prausnitzii* may contribute to the amelioration of specific metabolic disorders and inflammatory diseases [17–19].

Given the above, it is fair to speculate that these anti-inflammatory and antiviral effects can well lead to the prevention and/or relief of COVID-19-related symptoms, at least partially or in combination with other medicines. Thus, this raises a possibility that *F. prausnitzii* might be a new candidate probiotic which can be used in COVID-19 patients. Herein, we discuss recent advances in the understanding of the protective effects and mechanisms on infectious diseases of *F. prausnitzii* and its potential relevance in COVID-19 infection.

2. The Bionomics of *F. prausnitzii*

F. prausnitzii is one of the most abundant and widely distributed bacterial species inhabiting the human intestine, which has been consistently described as one of the most important butyrate producers found in the intestine [20]. Taxonomically, *F. prausnitzii* belongs to the *Firmicutes* phylum, the *Clostridia* class, and the *Ruminococcaceae* family, and the species is currently the only representative characterized within the genus [9]. Metabolically, as an anaerobe, *F. prausnitzii,* a non-spore-forming and non-motile rod that is Gram-positive, is exceedingly oxygen-sensitive [9]. It is difficult to survive even in an anaerobic environment, but adding riboflavin, cysteine or glutathione to the culture medium can improve its survival rate in a microaerobic environment [21]. *F. prausnitzii* can be divided into two lineages, line I and line II, and the differences in their physiological functions are still unclear [21].

A variety of monosaccharides can be used by bacteria as their energy sources, while the use of more complex carbohydrates varies from strain to strain. The nutrients can be obtained from the host or derived from other gut microbes cross-feeding. *F. prausnitzii* can use fructose, oligofructose, starch and inulin, but not arabinose, melibiose, raffinose, rhamnose, ribose, and xylose. Acetic acid can stimulate its growth and produce carbon dioxide, but not hydrogen [9]. The major fermentation products from glucoses and acetate by *F. prausnitzii* are formate, D-lactate, and butyrate [9].

The proportion of *F. prausnitzii* in gut microbiota is flexible affected by the colon physiological environment such as the pH, oxygen concentration, and cholate [21, 22]. Moreover, both improper diet and smoking will lead to a decreased count of *F. prausnitzii* [23]. In addition, the use of certain drugs can also modulate the abundance of *F. prausnitzii* in the intestine. For example, taking rifaximin can increase the level of *F. prausnitzii* [24], while the bacterium in the stool of Crohn's disease patients is reduced by taking infliximab and high doses of cortisol [25]. Studies have reported that butyrate produced by *F. prausnitzii* has a significant protective effect on enteritis [25]. As butyrate-producing bacteria, *F. prausnitzii* acts on the immune system and has anticancer effects [26, 27]. It can also improve the intestinal barrier, insulin sensitivity, and oxidative stress tolerance and reduce visceral sensitivity [28, 29].

Moreover, as one of the most abundant gut commensal bacteria, *F. prausnitzii* has the double effect of competitively inhibiting pathogenic bacteria and increasing the colonization of nonpathogenic bacteria [30], which could maintain a normal proportion of the gut microbiota. When *F. prausnitzii* is cocultured with *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*), which can also metabolize apple pectin, *F. prausnitzii* can produce more butyric acid than it alone [31]. This indicates that *F. prausnitzii* may rely on other gut microbiota including *B. thetaiotaomicron* for cross-feeding. Some studies found that the colonization of *F. prausnitzii* requires *B. thetaiotaomicron* and *Escherichia coli* (*E. coli*) to be preexisted in the intestine, which could prepare suitable conditions for *F. prausnitzii* by reducing redox potential and altering the composition of nutrients [32, 33]. In addition, *F. prausnitzii* and normal intestinal microecology can effectively prevent the proliferation of intestinal pathogenic bacteria such as *Escherichia coli*, *Clostridium*, and *Shigella*, which reduce the possibility of intestinal epithelium injury, thereby avoiding the activation of intestinal immune cells and the release of inflammatory factors [34].

Therefore, *F. prausnitzii* is a probiotic with an important protective effect on the human intestine and its reduction will lead to weakened intestinal anti-inflammatory and immune regulation capabilities.

Some characteristics of *F. prausnitzii* such as the absence of adhesion of epithelial cells [35], plasmids, antimicrobial [19, 36] and hemolytic activity, and the presence of DNAse activity [19] have been known to date. In addition, only the reference strain *F. prausnitzii* A2-165 [37] and the biofilm forming strain HTF-F [38] have been examined in vitro and in vivo for their beneficial anti-inflammatory effects. Some evidence points to this symbiotic intestinal bacterium, associated with intestinal barrier integrity and inflammation regulation, as an emerging "gatekeeper of the gut."

3. Multiskilled Commensal Bacterium *F. prausnitzii*

As a major member of the human microbiome, *F. prausnitzii* is a multiskilled commensal organism. It is distributed widely in the mammalian digestive tract. This bacterium has a variety of biological functions, such as regulating the immune response, suppressing inflammation, and promoting the integrity of the intestinal barrier.

3.1. Anti-inflammatory Effects. *F. prausnitzii* is a commensal bacterium with anti-inflammatory property, as demonstrated in a clinical trial [32]. Various studies have demonstrated decreasing abundance of *F. prausnitzii* in the gut could reduce protection against inflammatory interactions. This defensive mechanism possibly involves stimulating active molecules to secrete anti-inflammatory while inhibiting the secretion of proinflammatory cytokines. *F. prausnitzii* secretes anti-inflammatory molecules which can block the IL-1β-induced NF-$\kappa\beta$ signaling pathway, thereby reducing the production of interleukin IL-8 secreted by intestinal epithelial cells [39]. Additionally, *F. prausnitzii* can promote the secretion of IL-10 through peripheral blood monocytes, dendritic cells (DCs), and macrophages [37, 40] and consequently inhabit the synthesis of proinflammatory cytokines such as IFN-γ, TNF-α, IL-6, and IL-12 [41]. Through these mechanisms, the anti-inflammatory effect of *F. prausnitzii* in colitis may be realized, and through its anti-inflammatory properties, this bacterium may promote intestinal immune homeostasis.

3.2. Enhancement of Gut Barrier Function. Another key to intestinal development and maturity is the establishment of the integrity of the intestinal mucosa, which is not only essential for the absorption of nutrients, but also necessary for preventing bacteria and food antigens from entering the underlying tissues [42]. The metabolites released by *F. prausnitzii* have been shown to reduce the severity of inflammation by improving the function of the intestinal barrier and affecting paracellular permeability [43, 44]. Rossi et al. reported that the cell-free supernatant of *F. prausnitzii* can enhance the intestinal mucus barrier function by affecting the permeability of epithelial cells [40]. The improvement in permeability of *F. prausnitzii* appears to be related to the expression of certain tightly bound proteins [43]. It was investigated that *F. prausnitzii* could increase the levels of tight junction proteins occludin and E-cadherin and decrease colonic permeability, alleviating inflammation both in vitro and in vivo [45]. Moreover, *F. prausnitzii* may also help to maintain sufficient proportions of various cell types of secretory linage in the intestinal epithelium via the mucus pathway and O-glycan mucus formation [31].

3.3. Effects of Metabolites. Although we have confirmed the anti-inflammatory property of *F. prausnitzii* and its supernatant, the exact active substance and its mechanism have not been fully elucidated due to its complex composition. As an acetate consumer, *F. prausnitzii* has capacity to generate anti-inflammatory molecules such as butyrate and salicylic acids [32]. A growing body of the literature has reported that the main metabolites of *F. prausnitzii,* butyrate, play an important role in its anti-inflammatory activity. Butyrate is a short-chain fatty acid (SCFA) produced by intestinal microorganisms fermenting dietary fiber [46]. Moreover, *F. prausnitzii* has been consistently regarded as one of the main butyrate producers found in the intestine [47]. Butyrate provides energy (5–15% of the total calories) to the host and regulates the immune system, thereby protecting the host from pathogens [48].

Butyrate is secreted by intestinal microbiota and plays a significant role in intestinal physiology and body function. It is of great importance to prevent the invasion of pathogens, regulate the immune system, and reduce cancer progression [49]. As a representative of numerous pathways for electron disposal in the gut microbiota, *F. prausnitzii* can form butyrate, and its concomitant generation of NAD^+ and decreased ferredoxin is able to facilitate immune response modulation.

Salicylic acid is another metabolic product with anti-inflammatory effects delivered by *F. prausnitzii* [32]. As strong modulators of the inflammatory process, salicylic acid can also block the activation of NF-$\kappa\beta$ to inhibit the production of IL-8 as same as butyrate [50]. In the pharmaceutical industry, it is commonly recognized that salicylic acid can work as the forerunner of 5-aminosalicylic acid (5-ASA), which is a drug prescribed in the management procedure of IBD [24]. It has been documented in vitro that 10 mM of salicylic acid could reduce the level of IL-8 as well as the concentration found in the colon [32].

In addition, *F. prausnitzii* is capable of secreting anti-inflammatory substances including butyrate and salicylic acid, as previously described. Quévrain et al. reported another anti-inflammatory compound producing by *F. prausnitzii* called microbial anti-inflammatory molecule (MAM), which can inhibit the trigger of NF-κB in vitro and vivo as well [18, 34]. Since MAM operates actively at the center of signaling molecule IκB kinase α (IKKα), extra management of signal transduction molecules upstream or downstream of IKKα may significantly enhance its effects. In general, MAM could reach all over the body, and it can play a significant role in the regulation of inflammatory complications at anatomical locations outside of the intestine. The main mechanisms of *F. prausnitzii* are shown in Figure 1.

4. The Diseases Related to *F. prausnitzii*

As an important part of healthy human gut commensals, *F. prausnitzii* exerts significant actions on human health. Accumulating studies showed that the dysbiosis caused by the change of the count of *F. prausnitzii* in the intestine was closely related to the onset of some intestinal diseases such as IBD, irritable bowel syndrome (IBS), and colorectal cancer (CRC) [37, 51, 52].

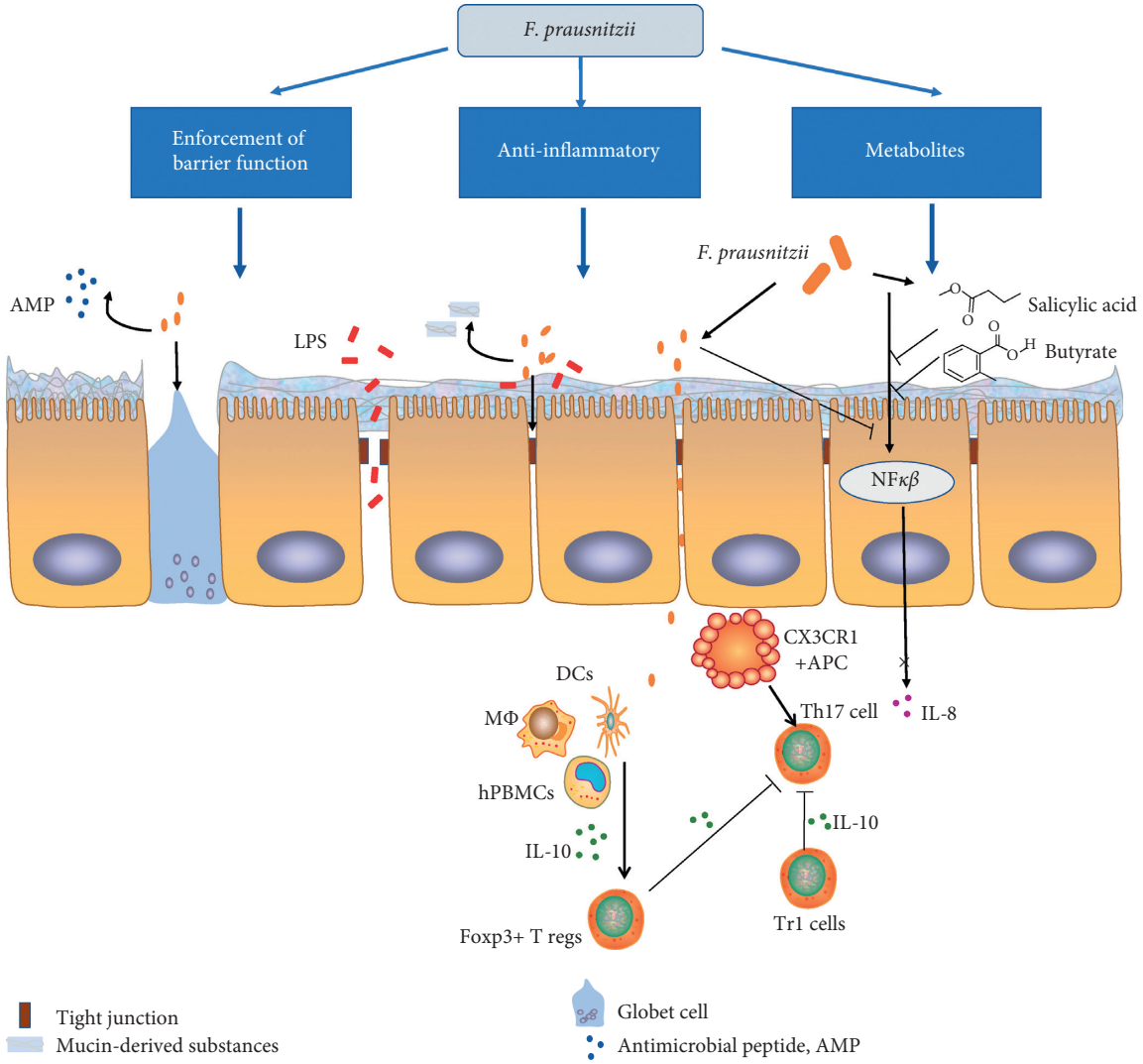

Figure 1: Mechanism underlying the anti-inflammation and enhancement of barrier function of *Faecalibacterium prausnitzii*. The effector molecules of *F. prausnitzii* encourage secretion of IL-10 from peripheral blood mononuclear cells (PBMCs), dendritic cells (DCs), and macrophages, and thus, proinflammatory cytokines are downregulated. The metabolites from *F. prausnitzii*, butyrate and salicylic acid, can block the secretion of IL-8 through inhibition of activation NF- *κβ* signaling and inflammation. The metabolites and effector molecules of *F. prausnitzii* can enhance the barrier function through providing growth factors from mucin degradation. *F. prausnitzii* could interact with the host by strengthening the intestinal barrier or modulating mucin turnover and immune responses.

4.1. IBD. Inflammatory bowel disease (IBD), whose two major forms are ulcerative colitis (UC) and Crohn's disease (CD) [53], is a chronic inflammation of the intestine induced by immune response under environmental conditions such as genetic susceptibility, diet, and antibiotic use. In the past few decades, plenty of clinical research data has shown that the composition and diversity of microbiota is modified in patients with IBD. Compared to that in healthy people, an increasing abundance of *Proteobacteria* was observed in the fecal microbiota of patients with active CD and UC, while lower fecal counts of *Firmicutes* were detected [37]. *F. prausnitzii*, as the most abundant bacteria in human intestine, possesses a small amount in CD and UC patients [54]. Machiels et al. found that there was a significant inverse association between the count of *F. prausnitzii* and disease activity in UC patients, even with the inactive disease [55]. Moreover, regarding the metabolites, decreasing short-chain fatty acids were observed in patients with UC [55]. Recently, Zhao et al. performed a systematic review and meta-analysis involving patients with UC and CD [56]. They found that both CD and UC patients had a lower abundance of *F. prausnitzii* than the healthy controls and a lower count of *F. prausnitzii* was detected in patients with active IBD in contrast with those in remission. In addition, it was reported

that patients receiving infliximab, a TNF-α blocker, showed an increase in *F. prausnitzii* population [57], which suggested a relationship in the pathomechanisms of IBD.

4.2. CDI. In addition to gut diseases, recent studies have shown the potential relevance between *F. prausnitzii* and infectious diseases such as CDI, HIV, and HBV. This will undoubtedly be a hotspot on *F. prausnitzii* worth studying in the future.

A study reported that patients with CDI had significantly fewer members of *F. prausnitzii* in their fecal microbiota than the healthy group [58]. Demirci et al. found that the amount of *F. prausnitzii* was reduced in patients with allergic diseases, which might suppress inflammation by decreasing proinflammatory cytokines such as IL-12 and increasing anti-inflammatory cytokine IL-10 [59]. Roychowdhury et al. revealed that supplementation with anti-inflammatory butyrate-producing commensal bacteria and prebiotic might help to promote innate immune responses and minimize bacterial burden and adverse effects during a course of antibiotic and *Clostridium difficile* exposure [60]. Moreover, a study observed increased *F. prausnitzii* in a cured recurrent CDI patient who had received fecal microbiota transplantation (FMT) 4.5 years ago.

Le Bastard et al. [61] discovered that ampicillin resulted in a sharp drop in bacterial species richness and diversity along with a fall in the percentage of *F. prausnitzii*. In mice receiving FMT, dysbiosis was immediately reversed with a significant increase in *F. prausnitzii*.

4.3. Virus-Infected Gut Dysfunction. Furthermore, Lu et al. found that gastrointestinal microbiota changes were linked to $CD4^+$ T-cell counts and immune activation in those with HIV [14]. In that study, *F. prausnitzii* is overrepresented in HIV-infected individuals who are immunological ART nonresponders or untreated compared to those immunological ART responders. Similarly, another study revealed that *F. prausnitzii* was depleted in HIV-positive persons on long-term ART compared to HIV-negative and the amount of *F. prausnitzii* has a negative correlation with gut dysfunction [15]. It has also been observed that the count of *F. prausnitzii* in asymptomatic carriers showed significant variation, and the variation range was considerably higher in patients with chronic hepatitis B and those with decompensated HBV cirrhosis in comparison with healthy controls [16]. These findings have revealed the potential connection between *F. prausnitzii* and viral infectious diseases, suggesting the possibility of *F. prausnitzii* as a targeted probiotic in the treatment of viral infectious diseases.

5. Strategies to Modulate the Abundance of *F. prausnitzii*

Due to the fact that all the *F. prausnitzii* strains from feces of healthy individuals showed positive anti-inflammation [62], it could be a promising target for therapeutic purpose. However, the production of medical supplements using obligate anaerobes, such as *F. prausnitzii*, is certainly a major challenge owing to the requirements for anaerobic conditions and mass production. Herein, we propose strategies to increase the abundance of *F. prausnitzii* from three perspectives.

5.1. Dietary Interventions Modulate F. prausnitzii. The structure of microbial communities of human beings depends, to a great extent, on the dietary factor, for bacterial composition in the gut is closely related to the available nutritional compounds [62]. The intake of the typical westernized diet which means a large amount of animal meat, sugar, animal fat, processed foods, and low fiber diet could reduce *F. prausnitzii*, whereas a high fiber diet with less meat can increase *F. prausnitzii* [63]. Hence, the abundance of *F. prausnitzii* can be regulated through the consumption of prebiotics and/or formulations. Treatment with inulin-type fructans and fructo-oligosaccharides has been demonstrated to increase the level of *F. prausnitzii* compared to placebo (maltodextrin) in patients [64]. Supplementation of prebiotic inulin-oligofructose also led to an increase of *F. prausnitzii* in healthy individuals [65]. An increase of *F. prausnitzii* was reported for red wine intake compared to baseline in male metabolic syndrome patients and healthy individuals [66]. Another study found that healthy men who consumed polydextrose or soluble corn fiber supplements had more *F. prausnitzii* than men who did not take fiber supplements, suggesting that this might be potential prebiotics [67]. In a research on the effects of a low-energy diet with prebiotic properties for patients with type 2 diabetes, *F. prausnitzii* increased by 34% compared to a placebo diet [68].

F. prausnitzii is reported to consume various diet including polysaccharides, such as the prebiotic inulin, arabinoxylans, resistant starch, fructan supplement, and ectins [63, 69]. As the most important modulators of gut microbiota, polysaccharides are generally consumed in the food because of their relative security, availability, and low price. A study demonstrated that increased consumption of polysaccharides had the potential to give advantage to individuals with a typical western-style diet, on condition that they take in enough dietary fiber [70]. As shown in several meta-analyses, the increased intake of dietary fiber greatly reduces the mortality risk [71].

5.2. Fecal Microbiota Transplantation. In recent years, there has been considerable interest in FMT, which is implemented by transferring the microbiota from healthy donors to people suffering from dysbiosis to restore eubiotic state. In an earlier study, van Nood et al. demonstrated that FMT could heal recurrent CDI, targeting the gut microbiota which can exert profound influence on the host metabolism [72]. Furthermore, researchers found that infusion of donor feces was significantly more effective for the treatment of recurrent CDI than the use of antibiotics. Moreover, in order to treat Crohn's disease and ulcerative colitis, Cui et al. proposed a step-up FMT strategy consisting of a FMT, then by further FMT steps or standard IBD prescriptions depending upon the patient's therapeutic response [73]. It is known that FMT can influence the growth of

Bacteroidetes and *Firmicutes*, especially *F. prausnitzii* [74]. A recent study investigates the safety and effectiveness of FMT in patients with mild or moderate UC by giving 47 patients treatments of fresh FMT. It shows that FMT resulted in clinical remission in patients with mild to moderate UC and that the remission may be associated with significantly increased levels of *F. prausnitzii* [75]. Sarrabayrouse et al. investigate changes in recipient intestinal mucosa upon contact with a fecal suspension (FS) obtained from a healthy donor by using a human explant tissue model and an in vivo mouse model. Interestingly, it shows that tissues with a low microbial load and a higher relative abundance of *Firmicutes* were more susceptible to FMT [76]. These studies suggest that *F. prausnitzii* can be a diagnostic and therapeutic candidate for the use of FMT in UC.

However, beneficial effects of FMT can be affected by dietary and host immune factors. The microbial structure of a healthy individual must be taken into account in time and in accordance with dietary, immune, and aging influences. In addition, the potential risk of the transmission of obesity and metabolic syndrome associated flora ought not to be ignored. FMT from the obese ones caused an increase in adiposity in mice, indicating the potential risk of transmission of some diseases associated flora [77]. Therefore, FMT needs further assessment.

5.3. Cultivate F. prausnitzii In Vitro. In addition to fecal microbiota transplantation and dietary regulation, how to cultivate *F. prausnitzii* in vitro is of vital importance. At present, the research on the isolation of *F. prausnitzii* and the exploration of the relationship between *F. prausnitzii* and diseases from the strain level are still in the preliminary stage at home and abroad. Therefore, the isolation and identification of *F. prausnitzii* and the screening of excellent strains that are correlated with human health and have strong biological activity have become an important prerequisite for further in-depth research on it. *F. prausnitzii* is extremely oxygen-sensitive, which may lose validity when exposed to the air for 2 minutes, and it is difficult to cultivate even in an anaerobic environment [9]. Adding riboflavin and cysteine or glutathione to the medium can make it grow in low oxygen environment [78]. In order to adapt to the micro-oxygen environment in the intestine, *F. prausnitzii* uses flavin and thiols as shuttle carriers inside and outside the cell to transfer electrons to oxygen and protect itself from oxidative stress [78]. Khan et al. found that the obligate anaerobic *F. prausnitzii* can be kept alive at ambient air for 24 h in a growth medium formulated with the antioxidants cysteine and riboflavin plus the cryoprotectant inulin [79]. It suggests that we can improve the growth of *F. prausnitzii* in vitro by changing the composition of the medium.

6. Potential Clinical Applications of *F. prausnitzii* in Diseases, Even in COVID-19 Infection

Since *F. prausnitzii* is extremely sensitive to changes in the intestinal environment, fecal- or mucosal-related *F. prausnitzii* can be regarded as a potential biomarker for diagnosis of intestinal diseases. However, a single bacterial species cannot be a universal biomarker for all types of diseases. Lopez-Siles proposed that the F-E index obtained by combining the abundance of *F. prausnitzii* and *E. coli* can be a better indicator than the single specie [80], which could discriminate between CD, IBS, and CRC [25, 52, 80], also distinguish CRC patients from other intestinal diseases. Since IBD is a chronic disease, many researchers paid their attention to the use of biomarkers to predict its prognosis. The lower CD activity index, C-reactive protein levels, and erythrocyte sedimentation rate have been demonstrated to be related to higher *F. prausnitzii* counts in feces [12]. Furthermore, disease remission could recover the abundance of *F. prausnitzii* in feces [13, 25]. However, the current research studies on the characteristics and functions of this bacteria are not enough intensive and extensive. There is a need for more in-depth study on the functional activity of *F. prausnitzii* and its potential as a biomarker.

In addition, as a treatment strategy, transplantation of *F. prausnitzii* has been widely used in dysbiosis of the intestinal flora that is associated with the inflammation, autoimmune disease, and infectious diseases. Butyrate-producing bacteria have been demonstrated to prevent translocation of endotoxic, which is a compound produced by the gut microbiota and has been reported to drive insulin resistance [81]. Sokol et al. [13] designed an in vitro experiment to prove that human immune cells with *F. prausnitzii* can show a potential anti-inflammatory response in the gut. At the same time, they revealed that the transplantation of *F. prausnitzii* in mice could shield the gut epithelium from destruction and inhibit gut inflammation induced by experimental reagents. Additionally, several research studies have demonstrated that oral *F. prausnitzii* has an anti-inflammatory effect in IBD mice models [82, 83]. Overall, transplantation of gut microbiota particularly *F. prausnitzii* from a healthy individual to subjects with metabolic syndrome or intestinal inflammation could modulate dysbiosis and inhibit downstream proinflammatory response.

Furthermore, respiratory viral infections have been reported to be associated with altered gut microbial structure, which predispose patients to secondary bacterial infections [84]. Probiotics may be an effective adjuvant strategy for the treatment and prophylaxis of viral infections including COVID-19. Numerous experts and scholars have proposed the use of probiotics to participate in the treatment of COVID-19 [85, 86], so it is important to screen out new probiotics. Moreover, according to a recent science blog by the IASPP (International Scientific Association for Probiotics and Prebiotics), numerous researchers around the world are studying the susceptibility of the microbiome to COVID-19 and assessing the ability of various probiotic strains to reduce viral load through multiple mechanisms of action.

Researchers performed transcriptome sequencing on the bronchoalveolar lavage fluid of COVID-19 patients, and the results showed that the microbiota was dominated by pathogens or oral and upper respiratory commensal bacteria [87]. Furthermore, it has been demonstrated comorbidities generally associated with severe COVID-19 are closely related to alterations in bacteria taxa from the phyla *Bacteroidetes* and *Firmicutes* [88, 89], which were reported to regulate ACE2 expression in rodents.

To the best of our knowledge, ACE2 is known as the receptor for SARS-CoV-2 to enter the host, which is highly expressed in both the respiratory and gastrointestinal tract [90]. In addition, it plays a role in controlling intestinal inflammation and maintaining intestinal microbial ecology [91]. Interestingly, *Firmicutes* species seemed to have diverse roles in regulating ACE2 expression in the colon of mice models [92]. Moreover, Zuo et al. found that there was an inverse correlation between abundance of *F. prausnitzii* and COVID-19 severity [93]. In this study, *F. prausnitzii* was discovered to be one of the top bacterial species which show a negative correlation with COVID-19 severity.

Clinical data showed that older patients and those with underlying chronic diseases related to inflammation (such as hypertension, obesity, diabetes, and coronary artery disease) had higher SARS-CoV-2 mortality and morbidity [94, 95]. Interestingly, the abundance of *F. prausnitzii* was reported to be lower in these subjects compared with healthy individuals [96–98]. Hence, it seems reasonable that *F. prausnitzii* can be an add-on therapy for the management of COVID-19.

The possible role of *F. prausnitzii* abundance in COVID-19 infection in terms of gut integrity and inflammation needs to be further elucidated. Its potential prognostic and therapeutic value in SARS-CoV-2 infections is waiting to be explored.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (81771085) and Key Projects of Sichuan Provincial Department of Science and Technology (2020YFSY0008).

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Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2021, Article ID 6622092, 9 pages
https://doi.org/10.1155/2021/6622092

Research Article

Cytotoxin-Associated Gene A-Positive *Helicobacter pylori* Promotes Autophagy in Colon Cancer Cells by Inhibiting miR-125b-5p

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Received 18 November 2020; Revised 31 January 2021; Accepted 8 February 2021; Published 2 March 2021

Academic Editor: Tingtao Chen

Objectives. To investigate the effects of cytotoxin-associated gene A- (CagA-) positive *Helicobacter pylori* on proliferation, invasion, autophagy, and expression of miR-125b-5p in colon cancer cells. *Methods*. Colon cancer cells were cocultured with *H. pylori* (CagA+) to analyze the effects of *H. pylori* on miR-125b-5p and autophagy. Colon cancer cells infected with *H. pylori* (CagA+) were mimicked by transfection of CagA plasmid. The effects of CagA on the proliferation, invasion, and autophagy of colon cancer cells were analyzed. Cell counting kit-8 (CCK-8), clone formation, and Transwell assays were used to detect cell viability, proliferation, and invasion ability, respectively. Proteins and miRNAs were detected by western blotting and qPCR, respectively. *Results*. *H. pylori* (CagA+) inhibited expression of miR-125b-5p and promoted autophagy in colon cancer cells. MiR-125 b-5p was underexpressed in colon cancer cells after CagA overexpression. CagA promoted colon cancer cell proliferation, invasion, and autophagy. Overexpression of miR-125b-5p inhibited the proliferation, invasion, and autophagy of colon cancer cells and reversed the effects of CagA. *Conclusion*. *H. pylori* (CagA+) infection may promote the development and invasion of colon cancer by inhibiting miR-125b-5p.

1. Introduction

Colon cancer is a common digestive tract tumor that usually occurs in people aged 40–50 years. According to a report, colon cancer is one of the most common tumors in China, and survey statistics show that the incidence of colon cancer among young people is increasing [1–3]. Despite tremendous breakthroughs in the detection and treatment of colon cancer, the 5-year survival rate of colon cancer patients is still not satisfactory. Indeed, more than 50% of patients with colon cancer have distant metastasis at diagnosis, which is an important factor leading to poor prognosis [4, 5].

Helicobacter pylori (*H. pylori*) is the major virulence factor of chronic gastritis and peptic ulcers, which are closely related to the pathogenesis of gastric mucosa lymphoid tissue lymphoma and gastric cancer [6–8]. In recent years, it has been found that *H. pylori* may be associated with the pathogenesis of colon cancer and polyps. Zumkeller et al. first discovered through metastasis analysis that *H. pylori* infection is potentially linked to the pathogenesis of colon

cancer and adenomatous polyps [9]. Teimoorian et al. also found that *H. pylori* is associated with colon cancer and adenomatous polyps [10]. The genotype differences of *H. pylori* strains are important factors leading to different clinical outcomes after infection. There is also a higher risk of serious clinical consequences of infection with cytotoxin-associated gene A- (CagA-) positive *H. pylori* than with the negative strain [11–13].

MicroRNAs (miRNAs), small noncoding single-stranded RNAs, consist of approximately 22 nucleotides encoded by an endogenous gene. miRNAs can directly bind to target messenger RNA (mRNA) by recognizing and complementing the 3′-untranslated region (UTR). miRNAs lead to gene degradation or translation, thus downregulating the expression of target genes [14, 15]. Regulation of posttranscriptional gene expression of miRNAs plays important roles in tumorigenesis, metastasis, and drug resistance [16–18]. *H. pylori* may regulate the proliferation of gastric cancer cells by inhibiting miR-152 and miR-200b [19]. The level of miR-490-3p is also associated with the prognosis of patients with gastric cancer caused by *H. pylori* [20]. However, the mechanism of *H. pylori*-induced colon cancer is still unclear.

In this study, it was found that CagA-positive *H. pylori* might promote the proliferation, invasion, and autophagy of colon cancer cells by inhibiting miR-125b-5p, thereby inducing colon cancer.

2. Materials and Methods

2.1. Cell Culture and Plasmid Transfection. Colon cancer cell lines DLD-1 and SW620 (American Type Culture Collection, USA) were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Thermo Fisher, Waltham, USA), 50 U/mL penicillin, and 50 μg/mL streptomycin (15070063, Thermo Fisher, Waltham, USA).

Standard *H. pylori* (CagA+, VacA+) NCTCl1637 was provided by the Chinese Center for Disease Control and Prevention. An *H. pylori* suspension with a concentration of 1×10^5 CFU/mL was added at a ratio of 200 : 1 when colon cancer cells were grown to 80% confluence. Cell changes were observed at 24 h after coculture, and the expression of CagA protein was detected by western blotting. The autophagy-related proteins LC3B-II/LC3B-I, Beclin-1, and miR-125b-5p were detected by western blotting and qPCR, respectively.

H. pylori (CagA+) infection was simulated by transfection of the CagA plasmid (GenePharma). Transfection was carried out according to the kit instructions. The miR-125b-5p mimic overexpresses miR-125b-5p by plasmid transfection. Colon cancer cell lines DLD-1 and SW620 were divided into four groups: mimic-NC + OE-NC (miR-125b-5p negative control + CagA negative control), mimic-miR-125b-5p + OE-NC (miR-125b-5p overexpression + CagA negative control), mimic-NC + OE-CagA (miR-125b-5p negative control + CagA overexpression), and mimic-miR-125b-5p + OE-CagA (miR-125b-5p overexpression + CagA overexpression). For different experimental groups, 2 μL of Lipofectamine^{TM} 2000 (Invitrogen, Waltham, USA), 40 pmol of CagA plasmid, miR-125b-5p plasmid and negative control (NC) (GenePharma) were mixed in 50 μL of serum-free medium at room temperature for 15 min. The lipid compounds were diluted in 300 μL of serum-free medium and 600 μL of medium containing FBS to produce a 1 mL volume mixture and incubated with the cells at 37°C with 5% CO_2 for subsequent experiments.

2.2. QPCR. Total RNA was obtained using TRIzol (Invitrogen, Waltham, USA). The concentration and purity of the RNA were detected by a NanoDrop2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One microgram of RNA was reverse transcribed using a reverse transcription cDNA kit (Thermo Fisher Scientific, Waltham, USA) for the synthesis of cDNA. SYBR Green PCR Master Mix (Roche, Basel, Switzerland) was used to conduct the qPCR experiments using a PCR Detection System (ABI 7500, Life Technology, USA). The PCR cycle was as follows: pretreatment at 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, 60°C for 1 min, 60°C for 1 min, and 4°C for preservation. Comparative cycle threshold ($2^{-\Delta\Delta}$Ct) analysis was employed to determine the expression of the RNAs [21, 22]. The expression levels of GAPDH and U6 were used for normalization. Primer sequences of the genes used in this work are described in Table 1.

2.3. Cell Counting Kit-8 (CCK-8) Assay. The cells were adjusted to a density of 2×10^4 cells/mL and inoculated in 96-well plates (100 μL per well). Forty-eight hours after transfection, 10 μL of CCK-8 (Beyotime Institute of Biotechnology, Beijing, China) was added and cultured at 37°C for 2 h. The optical density (OD) at 450 nm was measured by a microplate reader (Tecan Infinite M200 Microplate Reader; LabX, Männedorf, Switzerland) to calculate the relative cell viability.

2.4. Clone Formation Experiment. A total of 1×10^3 cells were inoculated per well into 6-well plates. The cells were cultured in a 5% CO_2 incubator for 2 weeks at 37°C. After aspirating the medium, 500 μL of methanol solution was added to each well to fix the cells for 15 min, and then 1 mL of crystal violet dye solution was added for 20 min. An automatic image analyzer was used to scan and photograph the cells, and the clone formation numbers were tested.

2.5. Transwell Assay. A total of 3×10^4 cells were transferred into the upper chambers of a Transwell apparatus (8 μm, BD Biosciences, CA, USA). The bottom chamber was filled with a complete medium supplemented with 10% FBS. After incubation for 48 h, cells that did not invade through the membrane were swept away. Then, the cells were fixed with 20% methanol and stained with 0.2% crystal violet. Cells invading into the bottom chamber per field were counted under an inverted microscope.

2.6. Western Blotting. Protein was extracted by protein lysate (RIPA). A BCA kit was applied to analyze the protein

Table 1: Primer sequences.

Primer name	Sequence (5′-3′)
miR-125b-5p-forward	TCCCTGAGACCCTAACTTGTGA
miR-125b-5p-reverse	AGTCTCAGGGTCCGAGGTATTC
CagA-forward	ATAATGCTAAATAGACAACTTGAGCGA
CagA-reverse	TTAGAATAATCAACAAACATCACGCCAT
U6-forward	CTCGCTTCGGCAGCACA
U6-reverse	AACGCTTCACGAATTTGCGT
GAPDH-forward	GGGAGCCAAAAGGGTCAT
GAPDH-reverse	GAGTCCTTCCACGATACCAA

concentration. Protein was separated by SDS-PAGE at 110 V for 100 min and transferred to PVDF membranes. The PVDF membranes were blocked in 5% nonfat milk for 1 h at room temperature. The antibodies (CagA, ab224836, Abcam, San Francisco, USA; Bcl2, ab59348, 26 kD; cyclin D1, ab134175, 34 kD; E-cadherin, ab40772, 97 kD; N-cadherin, ab18203, 130 kD; LC3B-II/LC3B-I, ab48394, 19 kD/17 kD; GAPDH, ab8245,36 kD; Beclin-1, ab207612, 52 kD) were diluted at 1 : 1000 with 5% BSA and added to the cells overnight at 4°C. Then, the secondary antibody (sc-516102/sc-2357; Santa Cruz Biotechnology, Inc. Dallas, TX, USA) was diluted at 1 : 5000 and added to the cells at room temperature for 2 h. Protein blot bands were detected by Pierce™ ECL plus western blotting substrate (Thermo Fisher, Waltham, USA) in ChemiDoc MP (Bio-Rad, California, USA).

2.7. Statistical Analysis. All experimental data are presented as the mean ± SD, and $p < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 6.

3. Results

H. pylori (CagA+) inhibits miR-125b-5p and promotes LC3B-II/LC3B-I and Beclin-1 in colon cancer cells.

CagA protein expression was significantly increased after coculture of both the DLD-1 (Figure 1(a)) and SW620 (Figure 1(b)) colon cancer cell lines with *H. pylori* (CagA+). After coculture with *H. pylori* (CagA+), miR-125b-5p expression was significantly decreased in both DLD-1 (Figure 1(c)) and SW620 (Figure 1(d)) cells. The expression of the autophagy-related proteins LC3B-II/LC3B-I and Beclin-1 was significantly higher than that in the control group for both DLD-1 (Figure 1(e)) and SW620 (Figure 1(f)) cells. The results indicated that *H. pylori* (CagA+) inhibited the expression of miR-125b-5p and promoted the expression of LC3B-II/LC3B-I and Beclin-1 in colon cancer cells.

CagA overexpression inhibits miR-125b-5p in colon cancer cells.

An *H. pylori* (CagA+) infection model was constructed by transfecting CagA. The qPCR results showed that transfection of the CagA plasmid increased the expression of CagA and decreased miR-125b-5p, and transfection of miR-125b-5p increased the expression of miR-125b-5p, but it was still lower than that of the CagA negative control and did not affect the expression of CagA in either the DLD-1 or SW620 colon cancer cell lines (Figures 2(a) and 2(c)). The western blot results also showed that overexpression of miR-125b-5p did not affect the expression of the CagA protein in either the DLD-1 or SW620 colon cancer cell lines (Figures 2(b) and 2(d)). This indicated that the transfection experiment was successful. Moreover, overexpression of miR-125b-5p did not affect the infection efficiency of CagA but did reverse the inhibitory effect of CagA on miR-125b-5p.

CagA overexpression promotes the proliferation and invasion of colon cancer cells by inhibiting miR-125b-5p.

On the fifth day, overexpression of CagA significantly increased the viability of both DLD-1 (Figure 3(a)) and SW620 (Figure 3(d)) cells. miR-125b-5p overexpression significantly decreased the viability of both DLD-1 (Figure 3(a)) and SW620 (Figure 3(d)) cells and reversed the effect of CagA on their viability. Increased levels of CagA also significantly increased the proliferation of both DLD-1 (Figure 3(b)) and SW620 (Figure 3(e)) cells, whereas miR-125b-5p overexpression significantly decreased the proliferation of both DLD-1 (Figure 3(b)) and SW620 (Figure 3(e)) cells and reversed the effect of CagA on their proliferation. CagA overexpression significantly increased the invasion of DLD-1 (Figure 3(c)) and SW620 (Figure 3(f)) cells, and overexpressing miR-125b-5p significantly decreased the invasion of both DLD-1 (Figure 3(c)) and SW620 (Figure 3(f)) cells and reversed the effect of CagA on their invasion.

Higher levels of CagA increased the expression of the apoptosis-related protein Bcl2, the proliferation-related protein cyclin D1, and the invasion-related protein N-cadherin but decreased the expression of E-cadherin in both DLD-1 (Figure 4(a)) and SW620 (Figure 4(b)) cells. Moreover, overexpression of miR-125b-5p had the opposite effect and reversed the effects of CagA on Bcl2, cyclin D1, N-cadherin, and E-cadherin in both DLD-1 (Figure 4(a)) and SW620 (Figure 4(b)) cells. This indicated that CagA overexpression promoted the proliferation and invasion of colon cancer cells by inhibiting miR-125b-5p.

CagA overexpression promotes autophagy in colon cancer cells by inhibiting miR-125b-5p.

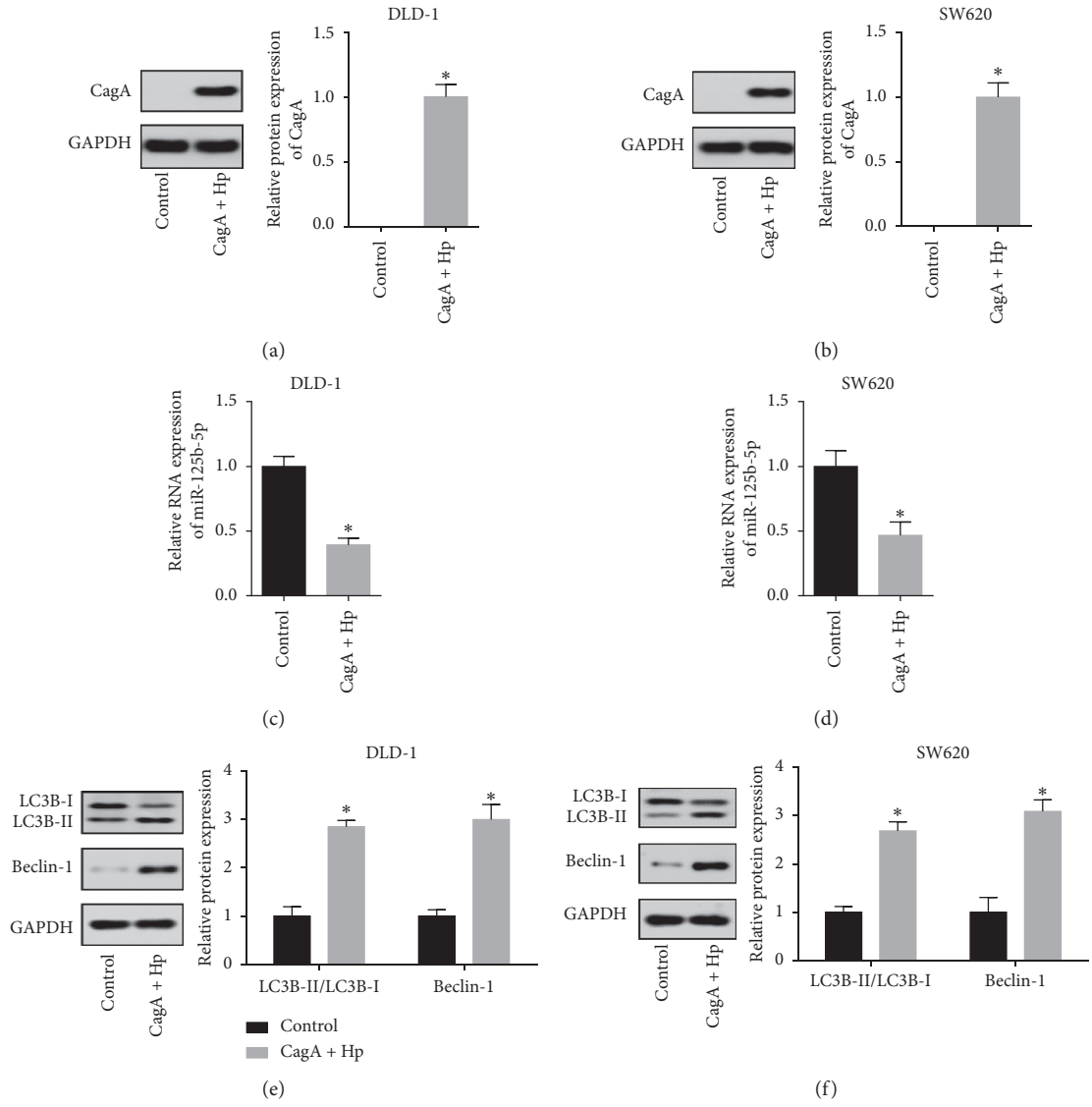

Figure 1: *H. pylori* (CagA+) inhibits miR-125b-5p and promotes LC3B-II/LC3B-I and Beclin-1 in colon cancer cells. (a) and (b) represent CagA protein expression was significantly increased after coculture of both the DLD-1 (a) and SW620 (b) colon cancer cell lines with *H. pylori* (CagA+). (c) and (d) represent that, after coculture with *H. pylori* (CagA+), miR-125b-5p expression was significantly decreased in both DLD-1 (c) and SW620 (d) cells. (e) and (f) represent the expression of the autophagy-related proteins LC3B-II/LC3B-I and Beclin-1 was significantly higher than that in the control group for both DLD-1 (e) and SW620 (f) cells. $^{*}p < 0.05$.

Overexpression of CagA promoted the expression of the autophagy-related proteins LC3B-II/LC3B-I in both DLD-1 (Figure 5(a)) and SW620 (Figure 5(b)) colon cancer cells. Moreover, the overexpression of miR-125b-5p inhibited the expression of LC3B-II/LC3B-I and reversed the effects of CagA on the expression of LC3B-II/LC3B-I in both DLD-1 (Figure 5(a)) and SW620 (Figure 5(b)) cells. This further indicated that CagA promoted autophagy by inhibiting the expression of miR-125b-5p, thus promoting the proliferation and invasion of colon cancer cells.

4. Discussion

H. pylori is considered a class I carcinogen, and its role in gastric cancer has been widely recognized. *H. pylori* also plays a role in other digestive tract tumors [13]. The genotype differences of *H. pylori* strains are important factors leading to different clinical outcomes after infection. The risk of serious clinical consequences with CagA-positive strains is significantly greater than that with CagA-negative strains [23]. Research from Europe and the United States has shown that the CagA gene is present in approximately 50–70% of *H. pylori* strains. The incidence and severity of gastrointestinal ulcers in patients infected with CagA + *H. pylori* are significantly higher than in those infected with CagA strains [24]. Researchers from China also showed that the detection rate of the CagA + strain is as high as 90% in patients with chronic gastritis [25]. After *H. pylori* infection, CagA is injected into the host cell through the CagPAI-type IV secretion system and phosphorylated, causing serious tissue

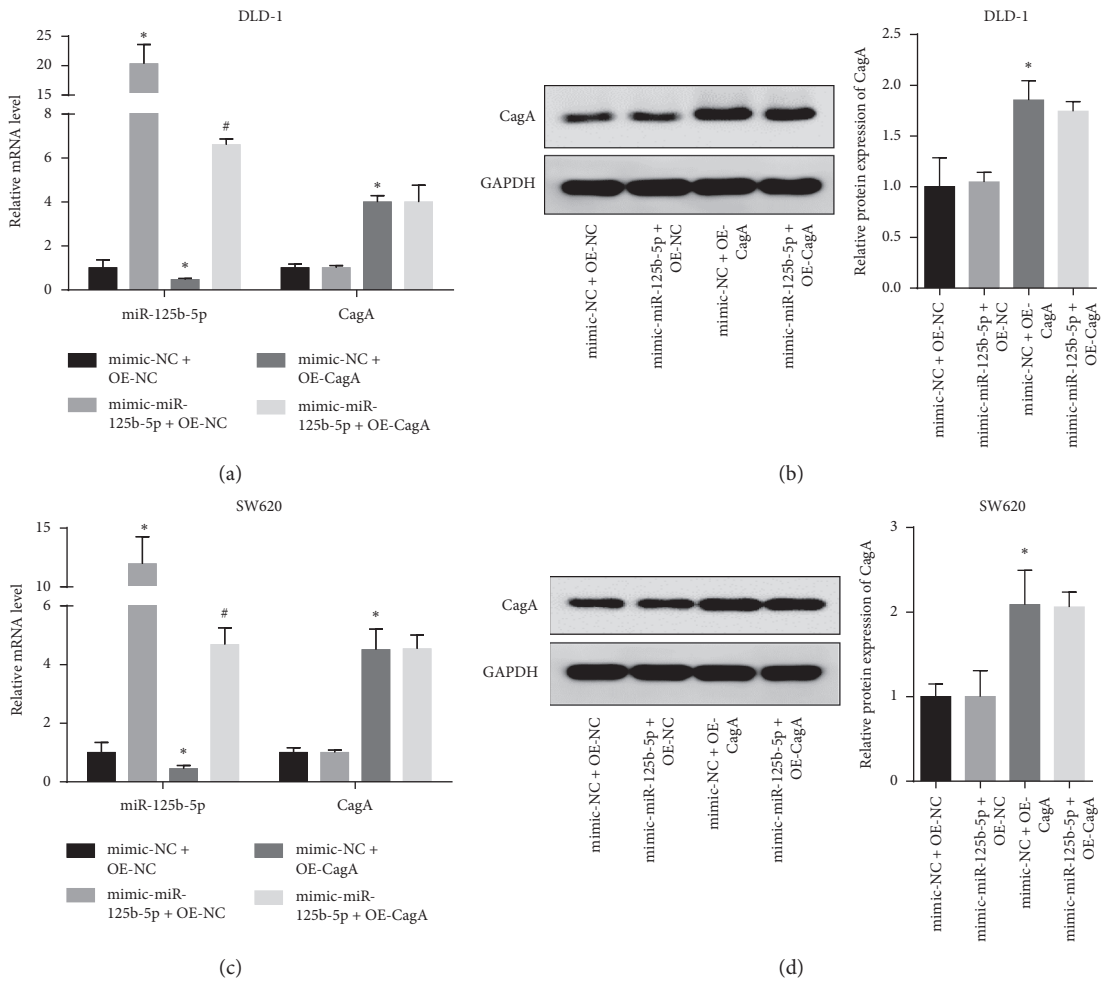

Figure 2: CagA overexpression inhibits miR-125b-5p, while miR-125b-5p does not affect CagA expression in DLD-1 and SW620 colon cancer cells. (a) and (c) represent the transfection of CagA plasmid decreased the miR-125b-5p level, while transfection of miR-125b-5p did not affect the CagA mRNA level in DLD-1 (a) and SW620 (c) cancer cell lines. (b) and (d) represent the transfection of miR-125b-5p did not affect the CagA protein level in DLD-1 (b) and SW620 (d) cancer cell lines. $^{*}p < 0.05$, compared with mimic-NC + OE-NC, and $^{\#}p < 0.05$, compared with mimic-NC + OE-CagA group.

inflammatory damage in the host and leading to abnormal cell function [26]. In addition, studies have confirmed in recent years that *H. pylori* can promote the epithelial–mesenchymal transition [27, 28].

In this study, the effect of CagA + *H. pylori* on colon cancer cells was analyzed. First, it was discovered that *H. pylori* (CagA+) inhibited the expression of miR-125b-5p. Other studies have found that miR-125b-5p plays an important role in the inhibition of breast cancer, gallbladder cancer, esophageal squamous cell carcinoma, and other tumors [29–31]. Second, *H. pylori* (CagA+) infection was induced by transfection of the CagA plasmid, which showed that CagA promoted the expression of proliferation-related proteins and invasion-related proteins, thus promoting the proliferation and invasion of colon cancer cells. Finally, *H. pylori* (CagA+) infection promoted the expression of autophagy-related proteins. However, the overexpression of miR-125b-5p had the opposite effects and reversed the effects of CagA on proliferation, invasion, and autophagy. These results indicated that *H. pylori* (CagA+) might participate in the development and invasion of colon cancer by promoting autophagy, which can be inhibited by miR-125b-5p. Cao's study [32] showed that miR-125b-5p participates in the development of systemic lupus erythematosus and inhibits autophagy by targeting UVRAG. Xiao also reported that miR-125b-5p regulates autophagy [33].

Autophagy is the main pathway through which normal cells resist external stress and stimulation, but it has a dual effect on cancer cells. Autophagy promotes and inhibits the formation and development of tumors and plays different roles in different tumors and different stages of tumor development. In the early stage of tumor growth, the inhibition of autophagy activity can lead to the continuous growth of precancerous cells, indicating the role of autophagy in suppressing tumor growth; in the later stage of tumor growth, the tumor cells in the central ischemic area of the tumor experience poor nutrient status for a long duration. In the hypoxic state, autophagy provides energy support for the growth of tumor cells by degrading macromolecular substances, proteins, and organelles in the cell, which is

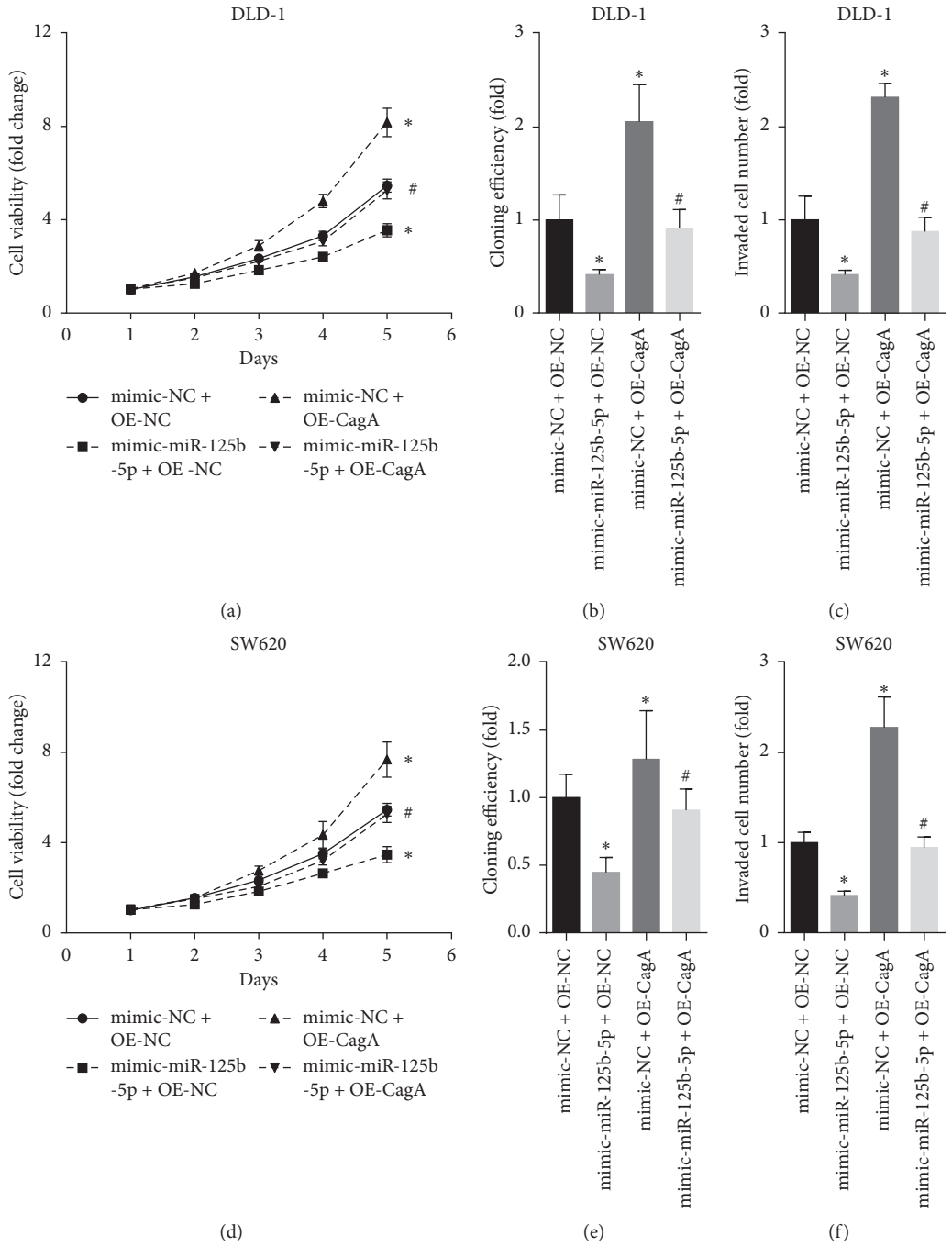

Figure 3: CagA overexpression promotes, while miR-125b-5p inhibits, the proliferation and invasion of colon cancer cells. (a) and (d) represent that on the fifth day, transfection of CagA plasmid significantly increased, while transfection of miR-125b-5p significantly decreased the viability of both DLD-1 (a) and SW620 (d) cells. (b) and (e) represent the transfection of CagA plasmid significantly increased, while transfection of miR-125b-5p significantly decreased the proliferation of both DLD-1 (b) and SW620 (e) cells. (c) and (f) represent the transfection of CagA plasmid significantly increased, while transfection f miR-125b-5p significantly decreased the invasion of DLD-1 (c) and SW620 (f) cells. $^{*}p < 0.05$, compared with mimic-NC + OE-NC, and $^{\#}p < 0.05$, compared with mimic-NC + OE-CagA group.

beneficial to the growth of tumor cells in a hypovascular environment [34]. Additionally, tumor cells can resist inflammatory reactions and acquire drug resistance through autophagy [35].

In conclusion, *H. pylori* (CagA+) inhibits the expression of miR-125b-5p in colon cancer cells and promotes autophagy. Overexpression of miR-125b-5p reverses the role of CagA in promoting the proliferation, invasion, and

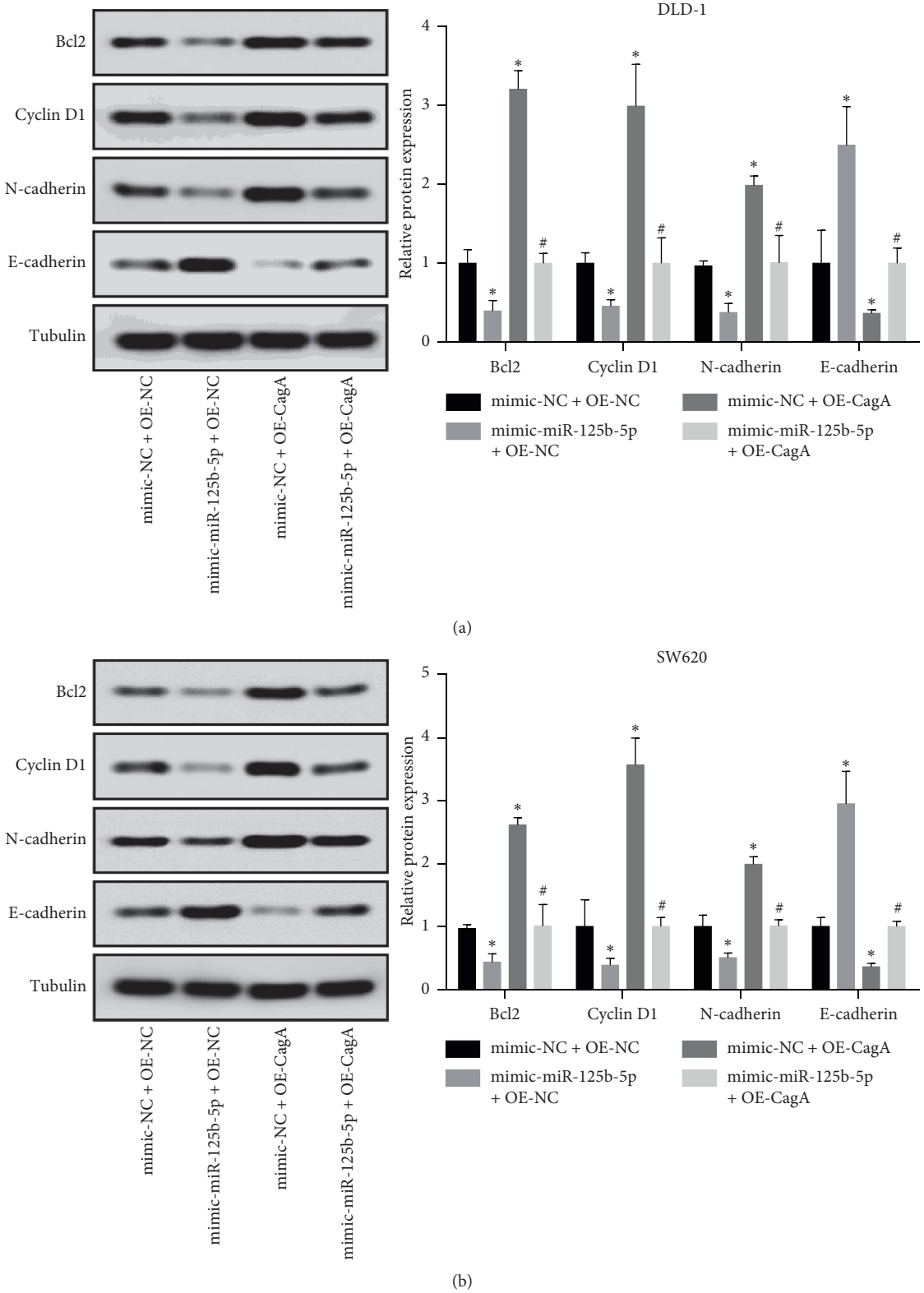

Figure 4: CagA overexpression promotes the proliferation and invasion of colon cancer cells by inhibiting miR-125b-5p. (a) and (b) represent the transfection of CagA plasmid increased the expression of the apoptosis-related protein Bcl2, the proliferation-related protein cyclin D1, and the invasion-related protein N-cadherin but decreased the expression of E-cadherin, while transfection of miR-125b-5p had the opposite effect and reversed the effects of CagA on Bcl2, cyclin D1, N-cadherin, and E-cadherin in both DLD-1 (a) and SW620 (b) cells. $^{*}p < 0.05$, compared with mimic-NC + OE-NC, and $^{\#}p < 0.05$, compared with mimic-NC + OE-CagA group.

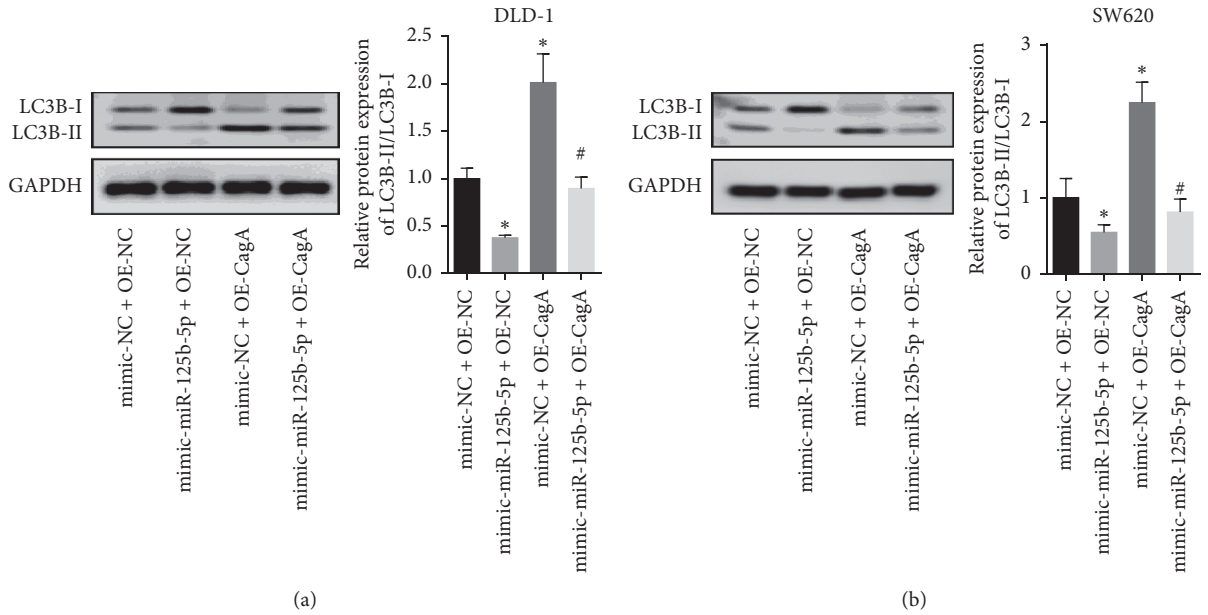

Figure 5: CagA overexpression promotes autophagy in colon cancer cells by inhibiting miR-125b-5p. (a) and (b) represent the transfection of CagA plasmid promoted, while transfection of miR-125b-5p inhibited the expression of LC3B-II/LC3B-I and reversed the effects of CagA on the expression of LC3B-II/LC3B-I in both DLD-1 (a) and SW620 (b) cells. $^{*}p < 0.05$, compared with mimic-NC + OE-NC, and $^{\#}p < 0.05$, compared with mimic-NC + OE-CagA group.

autophagy of colon cancer cells. This indicates that *H. pylori* (CagA+) infection may promote the development and invasion of colon cancer by inhibiting autophagy, but its specific mechanism needs further study.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported in part by the following funding sources: Sichuan Province Science and Technology Projects (2020YJ0190 and 2021YFH0148), Science and Technology Strategic Cooperation Programs of Luzhou Municipal People's Government and Southwest Medical University (2019LZXNYDJ27 and 2019LZXNYDJ30), Sichuan Science and Technology Grant for Overseas Students (19059), and Medical Research Project of Sichuan Province (Q16081).

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2021, Article ID 6627074, 7 pages
https://doi.org/10.1155/2021/6627074

Research Article

Serum Glycated Albumin Levels Are Affected by Alcohol in Men of the Jinuo Ethnic Group in China

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Received 20 November 2020; Revised 14 January 2021; Accepted 29 January 2021; Published 10 February 2021

Academic Editor: Yisong Qian

Aim. To investigate the effects of alcohol on serum glycated albumin (GA) levels in Chinese men. *Methods*. A total of 2314 male subjects from the Jinuo ethnic group in China were enrolled. Of these, 986 subjects drank alcohol frequently and 404 subjects did not. Lifestyle information was gathered by using a questionnaire, and measurements of blood pressure, body mass index, blood glucose level, liver function, and kidney function were collected. GA was measured by using an enzymatic method. Frequent drinking was defined as a history of drinking ethanol > 80 g/d within the past two weeks. Nondrinking was defined as no alcohol consumption in the past three months. Subjects with an alcohol intake between 0 and 80 g/d in the past two weeks were included in the drinking-occasionally group. Analysis of variance (ANOVA), correlation analysis, and linear regression were used to evaluate the effects of drinking on serum GA levels. Decision tree regression (DTR) algorithm was used to evaluate the effect of features (variables) on GA levels. *Results*. We found that male subjects who drank frequently had significantly lower serum GA levels than subjects who did not drink (13.0 ± 1.7 vs. 14.1 ± 3.7, $p < 0.05$). Spearman's correlation analysis calculated a coefficient of −0.152 between drinking and GA ($p < 0.005$). Linear regression established that drinking was an independent predictor for GA levels with a standardized regression coefficient of −0.144 ($p < 0.05$). Decision tree regression showed that the effect of drinking on GA levels (0.0283) is five times higher than that of smoking (0.0057). *Conclusions*. Frequent alcohol consumption could result in decreased GA levels in men of the Jinuo ethnic group in China.

1. Introduction

Serum glycated albumin (GA) is a nonenzymatic glycation product of albumin and glucose [1]. Since the half-life of albumin is 17–19 days, GA can reflect the average levels of blood glucose within the previous 2–4 weeks and can compensate for a deficiency of glycosylated hemoglobin (HbA1c), which reflects the average blood glucose level of the previous 8–12 weeks [2, 3]. GA has been widely used in clinical practice to assess blood glucose levels of patients before and after treatment, especially in patients who are at an early stage [4, 5]. GA has also been associated with chronic complications of diabetes mellitus (DM) and can be used as a predictor of DM complications [6, 7].

Previous work has demonstrated that GA levels are influenced by various factors [8, 9]. Concentrations of HbA1c are affected by erythrocyte replacement velocity. Similarly, GA levels are affected by the rate of serum albumin renewal. Albumin metabolism is affected by many factors. Alcohol consumption affects the metabolism of blood glucose and liver function [10]. Additionally, it has been reported that the concentration of glycated albumin in nondiabetic men in Japan can be reduced by drinking or smoking [11, 12]. However, the interaction between smoking and drinking has not been fully explored and the role of these two factors in patients with diabetes should be considered in clinical practice. It is known that drinking or smoking are common behaviors among Chinese men [13].

This study aimed to investigate the effects of drinking and smoking on GA levels among men in the Chinese community, including those with diabetes, impaired glucose regulation (IGR), and normal glucose tolerance (NGT).

2. Materials and Methods

2.1. Definitions. DM and IGR were diagnosed based on the diagnostic WHO 1999 criteria using the results of their 75 g oral glucose tolerance test (OGTT) without medical treatment. Drinking-frequently was defined as a history of drinking, the equivalent > 80 g/d of ethanol within the past two weeks. Nondrinking was defined as no alcohol consumption in the past three months. If the alcohol intake was between 0 and 80 g/d in the past two weeks, the subject was classified as drinking-occasionally. Smoking frequently was defined as smoking at least 1 cigarette per day for the past three months. If a subject had not smoked in the past three months, he was classified as no-smoking. Those who smoked some cigarettes but less than 1 cigarette daily in the past three months were classified as smoking-occasionally. Fibrosis index based on the 4-factor (FIB-4) value was used to evaluate liver fibrosis, FIB-4 = age × AST/PLT × $\sqrt{\text{ALT}}$ [14].

2.2. Study Subjects. From January to May 2012, a cross-sectional survey was conducted among men aged 18–75 years old from the Jinuo ethnic group in Yunnan Province, China. The study protocol was followed in accordance with the Helsinki Declaration and was approved by the Shanghai Jiao Tong University Affiliated Sixth People's Hospital's ethics committee. Informed consent was obtained from all participants prior to the survey. All subjects underwent an oral glucose tolerance test (OGTT), GA measurement, serum liver function, and renal function test.

2.3. GA Measurement. Liquid enzymatic determination of GA (Lucica®GA-L kit, Asahi Kasei Corporation, Tokyo, Japan) was performed using an Olympus AU2700™ Chemistry-Immune Analyzer. GA is represented as % with a detectable range of 3.2. – 68.1%, and the coefficient of variation (CV) was <3.0%.

2.4. Statistical Analysis. Analysis of variance (ANOVA) and linear trend tests were used to compare the differences between the groups and subgroups. Spearman's correlation analysis was used to observe the relationship between GA and other variables (drinking, smoking, etc.). Linear regression analysis was used to determine the effect these variables had on GA levels, with GA as the dependent variable and drinking status (nondrinking represented as 1, drinking-occasionally as 2, and drinking-frequently as 3), smoking status (no-smoking represented as 1, smoking-occasionally as 2, and smoking-frequently as 3), age, body fat percentage, fasting plasma glucose (FPG), 2h postprandial glucose (2hPG), albumin, glutamic-pyruvic transaminase (ALT), serum uric acid (sUA), FIB-4, and triglycerides (TG) as independent variables. All p values were double-tailed, and $p < 0.05$ was considered as statistically significant. SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

2.5. Feature Importance Analysis. Since linear regression cannot represent the nonlinear relationships between the GA and the independent variables, we used a decision tree regression (DTR) algorithm, a feature engineering method, to find the influence of these features/variables on GA levels. The following features such as drinking status, smoking status, age, body fat, 2hPG, albumin, ALT, sUA, and triglycerides were used. The FPG was not used because it has a significant correlation with 2hPG ($r = 0.51$, $p < 0.001$). The DTR model selects the tree's node (i.e., the feature) based on the decision criterion of mean squared error (between the true GAs and the GAs predicted by the model with the selected features). The importance of a feature refers to the (normalized) total reduction of the criterion brought about by that feature.

3. Results

A total of 2,314 male subjects were surveyed, of these 986 (42.6%) drank frequently and 404 (17.5%) did not drink; 1710 (73.9%) smoked frequently and 390 (16.9%) did not smoke; 169 (7.3%) neither drank nor smoked; and 825 (35.7%) both drank and smoked frequently.

The clinical data showed that systolic blood pressure (SBP), gamma-glutamyl transferase (γ-GT), total bilirubin test (TBIL), FIB-4, albumin, and TG levels were all significantly increased in the drinking-frequently group compared to the nondrinking group, while the 2-hour insulin (2hINS) levels were significantly lower ($p < 0.05$) (Table 1). There was a linear trend in SBP, albumin, TG, and 2hINS in these three groups. There was no significant difference in the levels of diastolic blood pressure (DBP), BMI, body fat, FPG, 2hPG, and fasting insulin among the three groups ($p > 0.05$).

The average serum GA levels for the whole study population were 13.2 ± 2.4%. The serum GA concentrations in the drinking-frequently group were significantly lower than those in the no-drinking group (13.0 ± 1.7 vs. 14.1 ± 3.7, $p < 0.05$). The subjects were further divided into subgroups based on their glucose tolerance status, age, smoking status, and body fat. GA levels of each subgroup of the drinking-frequently group, except the smoking-occasionally group, were significantly lower than those in the nondrinking group ($p < 0.05$). The results of the ANOVA analysis showed that there was a statistically significant difference in serum GA concentrations among the subgroups with different drinking statuses and that the serum GA concentrations in the no-drinking group, the drinking-occasional group, and the drinking-frequently group had a decreasing trend (Table 2). Subjects who smoked frequently had no significant difference in serum GA levels compared with those who were nonsmokers (13.2 ± 2.4 vs. 13.4 ± 2.5, $p = 0.453$, not listed in the table). Analysis of smoking status showed that there was also no linear trend in serum GA levels ($p = 0.180$, not listed in the table).

Table 1: Clinical characteristic by drinking status.

Parameter	Overall ($n = 2314$)	No-drinking ($n = 404$)	Drinking-occasionally ($n = 924$)	Drinking-frequently ($n = 986$)
DM	208 (9.0%)	27 (6.7%)	68 (7.4%)	113 (11.5%)*
IGR	494 (21.3%)	80 (19.8%)	187 (20.2%)	227 (23.0%)
Age (years)	40 ± 14	47 ± 15	37 ± 13	41 ± 14*
BMI (kg/m^2)	22.6 ± 3.4	22.4 ± 3.3	22.8 ± 3.5	22.5 ± 3.3
Body fat (%)	19.04 ± 5.74	18.87 ± 6.17	19.16 ± 5.71	18.99 ± 5.59
SBP (mmHg)	118 ± 17	118 ± 19	116 ± 16	119 ± 17
DBP (mmHg)	75 ± 12	74 ± 12	74 ± 12	77 ± 12*
FPG (mmol/L)	5.76 ± 1.25	5.90 ± 1.84	5.70 ± 1.16	5.75 ± 1.01
FINS (mU/L)	7.59 ± 9.95	7.24 ± 6.15	8.43 ± 13.27	6.95 ± 7.14
2hPG (mmol/L)	6.36 ± 3.03	6.28 ± 3.12	6.07 ± 2.64	6.67 ± 3.29*
2hINS (mU/L)	35.27 ± 31.62	40.08 ± 36.46	36.04 ± 32.93	32.56 ± 27.74*
HOMA-IR	2.00 ± 2.83	1.96 ± 2.29	2.19 ± 3.66	1.83 ± 2.03
TG (mmol/L)	1.87 ± 1.54	1.68 ± 1.22	1.83 ± 1.36	1.99 ± 1.79*
TC (mmol/L)	5.34 ± 1.13	5.20 ± 1.12	5.27 ± 1.05	5.46 ± 1.19*
HDL (mmol/L)	1.68 ± 0.59	1.56 ± 0.52	1.60 ± 0.51	1.81 ± 0.66*
LDL (mmol/L)	3.30 ± 0.90	3.21 ± 0.92	3.29 ± 0.87	3.31 ± 0.93
ALT (U/L)	36.8 ± 32.6	32.1 ± 14.0	34.6 ± 16.4	46.6 ± 45.2*
γ-GT (U/L)	76 ± 116	46 ± 63	58 ± 63	106 ± 156*
TBIL (mmol/L)	11.2 ± 5.8	10.6 ± 4.8	11.4 ± 6.3	11.3 ± 5.7*
Uric acid (umol/L)	396 ± 91	388 ± 96	397 ± 89	399 ± 91
FIB-4	1.46 ± 1.52	1.46 ± 0.91	1.16 ± 0.92	1.74 ± 2.04*
Albumin (g/L)	47.6 ± 3.3	46.8 ± 3.4	48.0 ± 3.2	47.6 ± 3.2*
GA (%)	13.2 ± 2.4	14.1 ± 3.7	13.2 ± 2.3	13.0 ± 1.7*

*Significantly different between the subjects who were not drinking and who were drinking frequently. 2hINS, 2h postprandial insulin; 2hPG, 2h postprandial plasma glucose; ALT, Alanine aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; FIB-4, fibrosis-4 score; FINS, fasting insulin; FPG, fasting plasma glucose; GA, glycosylated albumin; HDL, high density lipoprotein; HOMA-IR, homeostatic model assessment-insulin resistance; LDL, low density lipoprotein; SBP, systolic blood pressure; TBIL, total bilirubin; TG, triglycerides; γ-GT, γ-glutamyl transferase.

Table 2: Effects of drinking status on glycated albumin levels (%, mean ± SD).

Groups	Population	No-drinking	Drinking-occasionally	Drinking-frequently	p linear
Glucose regulation status	NGR ($n = 1612$)	13.4 ± 1.3	12.9 ± 1.2	12.7 ± 1.1*	<0.001
	IGR ($n = 494$)	13.9 ± 1.6	13.1 ± 1.2	12.9 ± 1.2*	<0.001
	DM ($n = 208$)	22.6 ± 10.1	16.5 ± 6.8	14.4 ± 3.7*	<0.001
FIB-4	<1.45 ($n = 1548$)	13.6 ± 3.1	12.9 ± 1.9	12.7 ± 1.6*	<0.001
	≥1.45 ($n = 766$)	14.8 ± 4.1	13.9 ± 2.9	13.3 ± 1.8*	<0.001
Age group	18–39 years ($n = 1178$)	13.0 ± 1.9	12.7 ± 1.2	12.5 ± 1.2*	<0.001
	40–59 years ($n = 872$)	14.1 ± 3.3	13.9 ± 3.5	13.1 ± 1.8*	<0.001
	≥60 years ($n = 264$)	15.7 ± 5.4	14.5 ± 2.2	14.2 ± 2.4*	0.001
Smoking status	No-smoking ($n = 390$)	13.7 ± 1.9	13.1 ± 3.1	13.0 ± 2.0*	<0.001
	Smoking-occasionally ($n = 214$)	15.2 ± 5.2	13.4 ± 1.6	13.2 ± 2.1	0.001
	Smoking-frequently ($n = 1710$)	14.3 ± 4.4	13.2 ± 2.2	12.9 ± 1.6*	<0.001
Body fat	<25% ($n = 1953$)	14.1 ± 3.6	13.2 ± 2.1	13.0 ± 1.4*	<0.001
	≥25% ($n = 361$)	14.2 ± 4.4	13.1 ± 3.2	12.7 ± 2.5*	0.003

*Significantly different between the subjects who were not drinking and who were drinking frequently. DM, diabetes mellitus; IGR, impaired glucose regulation; NGR, normal glucose regulation.

Spearman's correlation analysis revealed that GA levels were positively correlated with FPG, 2hPG, FIB-4, and age and negatively correlated with GA and BMI, body fat, γ-GT, albumin, smoking status, and drinking status, all of which were statistically significant. The correlation coefficients between GA and smoking status and drinking status were −0.040 ($p = 0.056$) and −0.152 ($p < 0.001$), respectively. After adjusting for age, FPG, 2hPG, BMI, body fat percentage, γ-GT, TBIL, albumin, and TG, the partial correlation coefficient between GA and drinking status was −0.149 ($p < 0.001$), and −0.049 between GA and smoking status ($p = 0.020$) (Table 3).

Linear regression analysis was performed using GA levels as the dependent variable and the age, body fat, FPG, 2hPG, albumin, ALT, TG, sUA, FIB-4, smoking status, and drinking status as independent variables. The method used in the regression model is "enter." The results demonstrated that drinking status was a predictor of GA in all three models with the increments of independent variables, whereas smoking status was not a predictor of GA. The standardized

Table 3: Correlation analysis of glycated albumin and other variables.

	Simple correlation		Partial correlation	
	r	p	r	p
BMI	−0.065	0.002	−0.054	0.010
Body fat	−0.057	0.007	−0.084	<0.001
FPG	0.667	<0.001	0.484	<0.001
FINS	−0.031	0.133	−0.005	0.799
2hPG	0.477	<0.001	0.413	<0.001
2hINS	−0.025	0.238	−0.054	0.011
TG	−0.013	0.532	−0.009	0.671
TC	−0.150	<0.001	−0.081	<0.001
HDL	−0.070	0.001	−0.105	<0.001
LDL	−0.145	<0.001	−0.045	0.031
ALT	−0.073	<0.001	−0.040	0.057
γ-GT	−0.042	0.045	−0.067	0.001
TBIL	−0.038	0.068	−0.020	0.329
UA	−0.098	<0.001	−0.074	<0.001
FIB-4	0.100	<0.001	−0.045	<0.032
Albumin	−0.177	<0.001	−0.060	0.004
Smoking status	−0.040	0.056	−0.049	0.020
Drinking status	−0.152	<0.001	−0.149	<0.001

Partial correlation: after adjusting for age. BMI: body mass index, FIB-4: fibrosis-4 score; FPG: fasting plasma glucose, FINS: fasting insulin, 2hPG: 2-hour postprandial glucose, 2hINS: 2-hour postprandial insulin, γ-GT: γ-glut amyl transferase, TBIL: total bilirubin, TG: triglycerides.

regression coefficients of drinking status were −0.134 in model 1 ($p < 0.001$), −0.167 in model 2 ($p < 0.001$), and −0.144 in model 3 ($p < 0.001$) with increasing independent variables. The linear regression models also showed that age, FPG, 2hPG, body fat, albumin, FIB-4, and TGs were all independent predictors of GA levels (Table 4).

Decision tree regression analysis was conducted to determine the effects of these features on GA levels. The results of this feature importance analysis using the decision tree algorithm are shown in Table 5. The effect of drinking on GA levels (0.0283) is five times higher than that of smoking on GA (0.0057). According to the importance of features analysis, the influence of drinking is much higher than that of smoking on GA levels.

4. Discussion

Like HbA1c, GA is a nonenzymatic glycation product; however, it can reflect the average blood glucose level of the previous 2–4 weeks because albumin has a shorter half-life. Therefore, it is a more suitable marker of the average blood sugar levels of anemia patients since GA is not affected by red blood cells or hemoglobin [15]. Moreover, GA is also more valuable in the assessment of neonatal diabetes glycemic control [16]. However, GA levels are not exclusively affected by the average blood glucose concentrations. A series of studies have found that GA levels are associated with factors such as age, body mass index, body fat, abdominal fat, and smoking status [11, 17, 18]. A previous study showed a link between GA concentration and smoking status but did not investigate the effects of drinking status [11]. Another study showed that increased alcohol consumption was associated with elevated blood glucose levels and decreased glycosylated hemoglobin and glycated hemoglobin concentrations in 300 nondiabetic men. Smoking and drinking are very common behaviors among Chinese men, and many Chinese smokers drink alcohol frequently. In order to avoid the effects of gender, our project has fully studied the effects of alcohol and smoking on GA levels in the male population of the general community, yielding some different results.

Jinuo ethnic residents in Yunnan, China, have been found to have a high prevalence of smoking/drinking [19]. Our study analyzed the association between GA levels and drinking as well as smoking in this regional population, where a high percentage of the population frequently drink and smoke. Our survey results showed that our study population had high frequency of smoking and drinking; 42.6% of the subjects drank frequently, 73.9% smoked frequently, and 35.7% both drank and smoked frequently. Pathophysiological data has indicated that alcohol can inhibit gluconeogenesis [20]. We have demonstrated that, compared with nondrinkers, those who drank frequently had similar FPG concentrations and significantly higher 2hPG and lower levels of GA. Meanwhile, the levels of GA showed a decreasing trend in individuals who were not drinking, drinking occasionally, and drinking frequently. Therefore, serum GA concentrations in those who drink frequently cannot be explained by blood glucose levels.

The subjects were further divided into subgroups based on glucose tolerance, age, smoking status, and body fat percentage. ANOVA analysis by subgroup showed that the GA levels were also significantly lower in subjects who were drinking frequently compared to those who were not drinking. These results further illustrate that the effect of alcohol consumption on serum GA levels is independent of blood glucose, age, body fat content, and smoking status. In contrast with the findings by Koga et al. [11], our results found that men who were not smoking showed no significant difference in GA levels compared with those who

Table 4: Linear regression to determine the variables associated with GA.

	Standardized coefficients	OR (95% CI)	p
Model 1			
Age	0.291	0.050 (0.043, 0.057)	<0.001
Body fat	−0.059	−0.025 (−0.041, −0.009)	0.003
Smoking status	0.002	0.007 (−0.122, 0.136)	0.911
Drinking status	−0.134	−0.443 (−0.577, −0.309)	<0.001
Model 2			
FPG	0.355	0.676 (0.603, 0.748)	<0.001
2hPG	0.288	0.180 (0.156, 0.204)	<0.001
Albumin	−0.157	−0.093 (−0.112, −0.074)	<0.001
Smoking status	0.006	0.015 (−0.069, 0.100)	0.724
Drinking status	−0.167	−0.434 (−0.522, −0.345)	<0.001
Model 3			
Age	0.191	0.026 (0.020, 0.031)	<0.001
Body fat	−0.121	−0.039 (−0.051, −0.028)	<0.001
FPG	0.381	0.722 (0.651, 0.793)	<0.001
2hPG	0.266	0.167 (0.143, 0.191)	<0.001
Albumin	−0.068	−0.040 (−0.061, −0.149)	<0.001
ALT	−0.074	−0.004 (−0.006, −0.002)	<0.001
TG	−0.048	−0.062 (−0.106, −0.018)	0.006
sUA	0.014	0.000 (−0.001, 0.000)	0.393
FIB-4	−0.053	−0.064 (−0.110, −0.018)	0.006
Smoking status	−0.010	−0.024 (−0.106, 0.059)	0.570
Drinking status	−0.139	−0.356 (−0.443, −0.270)	<0.001

2hPG, 2h postprandial glucose; ALT, Alanine aminotransferase; FPG, fasting plasma glucose; FIB-4: fibrosis-4 score; sUA, serum uric acid; TG, triglycerides.

Table 5: The importance of features on GA.

Features	The feature's importance
2h PG	0.416760
FIB-4	0.141971
Age	0.116355
sUA	0.091316
ALB	0.055419
BMI	0.055078
TG	0.054010
ALT	0.037208
Drinking status	0.026660
Smoking status	0.005224

2hPG, 2h postprandial glucose; ALT, Alanine aminotransferase; FPG, fasting plasma glucose; FIB-4: fibrosis-4 score; sUA, serum uric acid; TG, triglycerides.

smoked frequently. This may be because our study had a larger sample size.

In addition to smoking and sUA, our linear regression results showed that age, FPG, 2hPG, body fat, albumin, ALT, TG, FIB-4, and drinking status were all independent factors affecting serum GA levels. Age, FPG, and 2hPG are risk factors for elevated GA, while other factors are predictors of the decreased serum GA levels. Considering that there was no statistical difference between the smoking status and the correlation coefficient of GA, smoking status was not a predictor of serum GA level in the regression model. The result from decision tree regression is consistent with the results of the aforementioned statistical analysis, demonstrating that drinking status was more important than smoking status when applied as GA predictors.

Previous work has established that albumin is associated with inflammation and nutritional status [21], that GA is negatively correlated with C-reactive protein [16], and that thyroid function also impacts GA levels [22]. Our current analysis was unable to analyze these variables as our investigation lacked this information. Based on the results of the current study, we hypothesized that alcohol leads to a decrease in serum GA concentrations because albumin synthesis and metabolism may be affected by alcohol intake. The relationship between alcohol intake and GA needs further research. Considering the effect of alcohol on the liver, we observed a significant increase in FIB-4 in the frequent drinking group. FIB-4 was also found to be a predictor of GA decline. However, we lack a mechanism that would explain how alcohol consumption leads to an increase in FIB-4 and a further decrease in GA. TBIL and γ-GT are two indicators of liver damage and had higher concentrations in subjects who were drinking frequently compared to those who were not drinking. The detection of GA was not affected by TBIL or γ-GT but was affected by alcohol. In our study, blood samples were taken at least 8 h after fasting, so the effect of alcohol on GA test results can be ignored. A study by Inada and Koga [12] observed that drinking resulted in a decrease in GA and HbA1c. However, there is no evidence to support the hypothesis that the reduction in GA and HbA1c levels caused by drinking can slow the incidence of diabetic complications. Different from previous

study, in this study, more subjects were included, analyzing the relationship between GA and alcohol consumption in Chinese men at the first time.

In summary, we found that smoking status was not a significant factor contributing to a decrease in GA concentrations. GA levels were decreased in those who were drinking frequently in both the overall study population and the subgroups with DM, normal glucose tolerance, and impaired glucose regulation. GA levels of subjects who were drinking frequently had an absolute decrease of 0.628% (95% confidence interval: 0.456% to 0.800%) compared to subjects who were not drinking. This decrease was independent of blood glucose and body fat content and should be noted when making a clinical interpretation. Classifying patients by drinking statuses such as no-drinking, drinking-occasionally, and drinking-frequently is convenient to apply, and it could effectively account for the effect of drinking on GA levels in clinical practice.

It is well known that both a high-fat diet and alcohol can induce hepatic steatosis, inflammation, and fibrosis and can also change gut microbiota [23]. Furthermore, some complicated factors may have influence on this, including the changes in the gut microbiota or alcohol induced liver disease. Many studies have shown that abnormalities in the composition of the gut microbiota might contribute to the development of type 2 DM [24]. Additionally, the gut microbiota plays an important role in both nonalcoholic fatty liver disease and alcohol-related liver disease [25]. Alcohol may affect glucose metabolism in the liver by downregulating gluconeogenesis or changing hepatic lipids, inflammatory response, and oxidative stress by inducing steatosis [26, 27]. It may also affect gut microbiota diversity and interfere with the protective effect of beneficial bacteria [28]. Alcohol-related liver disease can also change the composition and function of the gut microbiota, and treatment of the gut microbiota can restore intestinal homeostasis and improve alcohol-related liver disease [29]. The ability of the gut microbiota to directly regulate GA levels is currently unclear and basic research should be done on this topic.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The Independent Ethics Community of Shanghai Sixth People's Hospital approved this study (REC no. 2015-28).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

WJ, YB, and FJ researched literature and conceived the study. QS, HL, and ML were involved in protocol development, gaining ethical approval, patient recruitment, and data analysis. CZ and XH wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript. C. Zhu and X. Hou contributed equally to this work.

Acknowledgments

The authors thank all the people that participated in the study. This research was funded by the Special Fund for Information Development of Shanghai Economic and Informatization Committee in 2017 (no. 201701014) and Shanghai Municipal Commission of Health and Family Planning (20154Y0059).

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2021, Article ID 6613903, 11 pages
https://doi.org/10.1155/2021/6613903

Research Article

Alleviation of Anxiety/Depressive-Like Behaviors and Improvement of Cognitive Functions by *Lactobacillus plantarum* WLPL04 in Chronically Stressed Mice

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Received 7 October 2020; Revised 18 November 2020; Accepted 17 January 2021; Published 31 January 2021

Academic Editor: Louis DeTolla

Background. Intestinal microorganisms play an important role in regulating the neurodevelopment and the brain functions of the host through the gut-brain axis. *Lactobacillus*, one of the most representative intestinal probiotics, produces important effects on human physiological functions. Our previous studies reveal that the *Lactobacillus plantarum* WLPL04 has a series of beneficial actions, such as antiadhesion of pathogens, protection from the harmful effect of sodium dodecyl sulfate, and anti-inflammatory stress on Caco2 cells. However, its effects on brain functions remain unknown. The present study aims to evaluate the potential effect of *L. plantarum* WLPL04 on anxiety/depressive-like behaviors in chronically restrained mice. *Methods*. Newly weaned mice were exposed to chronic restraint stress for four weeks and raised daily with or without *L. plantarum* WLPL04 water supplement. Animals were behaviorally assessed for anxiety/depression and cognitive functions. The 16S rRNA sequencing was performed to analyze the intestinal microbiota structure. The levels of the medial prefrontal cortical (mPFC) brain-derived neurotrophic factor (BDNF)/tropomyosin-related kinase B (TrkB) and serum 5-hydroxytryptamine (5-HT) were examined using Western blot and enzyme-linked immunosorbent assay. *Results*. The chronic stress-induced anxiety/depressive-like behaviors and cognitive deficits were significantly alleviated by the *L. plantarum* WLPL04 treatment. The 16S rRNA sequencing analysis showed that the chronic stress reduced the diversity and the richness of intestinal microbiota, which were rescued by the *L. plantarum* WLPL04 treatment. The levels of BDNF and TrkB in the mPFC and the concentration of 5-HT in the serum remained unchanged in chronically restrained mice treated with the *L. plantarum* WLPL04. *Conclusions*. The *L. plantarum* WLPL04 can rescue anxiety/depressive-like behaviors and cognitive dysfunctions, reverse the abnormal change in intestinal microbiota, and alleviate the reduced levels of 5-HT, BDNF, and TrkB induced by chronic stress in mice, providing an experimental basis for the therapeutic application of *L. plantarum* on anxiety/depression.

1. Introduction

The chronic stress in early life increases the susceptibility to a range of psychopathologies, including depression and anxiety [1]. Traditional psychotropic medications are controversial partly because the long-term effects to developing nervous systems have not been clearly established [2]. Recently, accumulating evidence indicates the existence of a microbiome-gut-brain axis and strong bidirectional communications among these structures [3, 4]. Stress in early life can alter the enteric microbiota [5], and intestinal bacterial infection can induce anxiety-like behaviors and cause memory deficits [6, 7]. Thus, the regulation of the intestinal microbiota is suggested to be an interesting strategy for the development of new therapy for mental diseases [8].

Probiotics are a group of active microorganisms and confer health benefits to the host via active interactions with

endogenous microbiota and gut cells when provided in appropriate amount [9]. Probiotics are also beneficial to patients suffering from psychiatric illness. In 2005, Logan and Katzman have used probiotics as adjunct therapy for depression for the first time [10]. Some strains of *Lactobacillus* and *Bifidobacterium* are shown to alleviate mood disorders and prevent stress-induced alterations in colonic microbiota [8, 11].

Lactic acid bacteria are regarded as safe and beneficial probiotics that may help prevent constipation, irritable bowel syndrome or Crohn's disease, and asthma or eczema in children [12, 13]. A recent study shows that the *Lactobacillus plantarum* NDC75017 alleviates the learning and the memory deficit in aging rats by reducing the mitochondrial dysfunction [14]. The *Lactobacillus plantarum* MTCC1325 strain, which produces acetylcholine, has potential antioxidant and anti-Alzheimer activities against the D-galactose-induced Alzheimer's disease [15, 16]. Most *L. plantarum* strains are isolated from fermented food, whereas the strains isolated from human breastmilk have some special features, such as regulating natural and acquired immune responses, treating infectious mastitis, having antimicrobial properties, and having beneficial effects for infants [17–19]. A previous study shows that the *L. plantarum* WLPL04 isolated from human breastmilk can increase the capabilities of human body against pathogens and may be a candidate probiotic for promoting host health [20]. The present study aims to assess the effects of *L. plantarum* WLPL04 on anxiety/depressive-like behaviors and cognitive functional deficits induced by chronic stress in young adult mice and analyze the underlying mechanism.

2. Materials and Methods

2.1. Animals. A total of 30 male C57BL/6 mice (approximately four weeks old at the start of the experiments) were used. All mice were randomly assigned to three groups (10 mice per group), including one control group and two chronic restraint-stressed groups. One of the stressed groups was provided with normal drinking water, and the other stressed group was provided with normal drinking water and *L. plantarum* WLPL04, which was kindly provided by State Key Laboratory of Food Science and Technology, Nanchang University, China. The final concentration of the *L. plantarum* WLPL04 in drinking water was 10^9 CFU/mL. The mice were housed in cages (3 or 4 mice per cage) under a constant temperature (23°C–25°C) and a 12 h light/dark cycle with ad libitum access to food (SHOOBREE, SPF-grade chow, Jiangsu Xietong Pharmaceutical Bio-Engineering Co., Nanjing, Jiangsu, China) and water (sterilized drinking water). Mice were placed in a plastic restrainer in their home cages for 3 h daily (from 11:00 to 14:00) for 28 consecutive days to establish chronic restraint stress [21]. All experiments were carried out in accordance with the principles of laboratory animal care and use approved by the Nanchang University Animal Care and Use Committee Guidelines.

2.2. Behavioral Assessments. Prior to behavioral tests, all mice were handled for five days and received 1 h accommodation to the experiment room before testing. The open field, elevated plus maze, and forced swimming tests were performed to analyze anxiety/depressive-like behaviors, and the novel object recognition and Barnes maze tests were used to evaluate the cognitive functions of the medial prefrontal cortex (mPFC).

2.2.1. Open Field Test. The open field test was performed in accordance with previously described procedures [22]. Each mouse was placed in an open field arena (45 cm × 45 cm × 40 cm, Med Associates, Vermont, USA) and allowed to freely explore the arena for 10 min to carry out the test. The locomotion of the mouse was recorded using a video capture software. The total distance and the routine traveled in the arena were measured. The open field arena was cleaned with 75% ethanol after each use.

2.2.2. Elevated Plus Maze Test. The elevated plus maze test was performed in a gray plastic cross-shaped maze (Med Associates, Vermont, USA) with 1.0 m elevation from the floor, two open arms (35 cm × 7 cm), and two closed arms (35 cm × 7 cm × 40 cm). The arms were connected by the center platform (7 cm × 7 cm). A mouse was placed in the intersection of the open and closed arms, facing an open arm, and allowed to freely explore the maze for 10 min. The behavioral exploration and the time spent in the open and the closed arms were video recorded for analysis. The maze arms were cleaned with 75% ethanol after each use.

2.2.3. Forced Swimming Test. The forced swimming test was performed in a Plexiglas cylinder (25 cm in height and 10 cm in diameter) containing water at height of 10 cm and temperature of 22°C–25°C. The water was changed between trials. Each mouse was allowed to swim for a maximum of 6 min, and the immobility time was recorded during the last 5 min of the trial. After the trial ended, the mouse was carefully dried with a cloth towel and kept under a heating fan for 1 h before placing back into the home cage.

2.2.4. Barnes Maze Test. The Barnes maze task is a spatial memory task. The maze apparatus (Techman, BMT-100, Chengdu, China) was a round platform (75 cm in diameter) with 18 evenly arranged holes (7 cm in diameter, Figure 1(a)). During the training, a target box was placed below one of the holes, which was labeled as the target hole. Training and testing were performed in a 500 lux light environment. The platform was cleaned with 75% ethanol after each use to avoid smelling interference with the next animal.

The day before the training, a mouse was placed in the target hole for 1 min to get familiar with the environment. For the spatial training, target holes were maintained in the same location relative to the extramaze cues on each trial. The trial was started by placing a mouse in the center of the platform. The mouse was covered with a cylinder. After 10 s, the mouse was allowed to freely explore the platform for

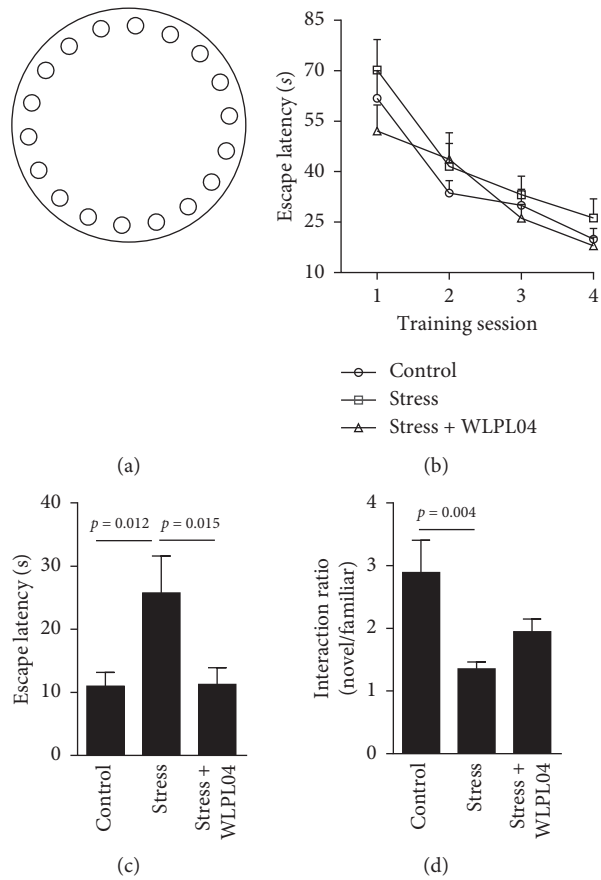

Figure 1: *L. plantarum* WLPL04 treatment alleviates the cognitive dysfunctions in chronically stressed mice. (a) Diagram of Barnes maze. (b), (c) Chronic stress impairs spatial memory but not spatial learning in the Barnes maze, and such memory deficit is rescued by the *L. plantarum* WLPL04 treatment. (d) Chronic stress impairs the novel/familiar object recognition, and such deficit is reversed by the *L. plantarum* WLPL04 treatment. $n = 7$ mice per group.

3 min. If the mouse found the target hole, the mouse was allowed to stay inside the target box for 30 s. If the mouse failed to find the target hole, the mouse was guided to the target box, and the environment light was turned off simultaneously. The mouse was permitted to stay in the box for 30 s. Each mouse was trained for three 3 trials each day for four consecutive days.

For the spatial memory testing, the target box was removed and each mouse underwent a probe trial on the fifth day. The mouse was placed on the center of the platform and allowed to freely explore the platform for 90 s. The latency to find the target box was recorded.

2.2.5. Novel Object Recognition Test. The novel object recognition task was performed in a square arena (30 cm × 30 cm × 45 cm). Each mouse was placed in the arena to explore for 1 min to get familiar with the environment. One day later, two identical objects were placed in two distinct corners of the arena, and the mouse was allowed to explore the arena for 10 min. On the next day, one of the two identical objects (familiar objects) was replaced by a novel object (nonfamiliar object). The novel object was different in shape and color with the familiar ones. The mouse was placed in the arena to freely explore for 10 min. The exploration behavior of the mouse was video recorded for analysis. The arena was cleaned with 75% ethanol after each use.

2.3. DNA Extraction and High-Throughput DNA Sequencing. After the chronic restraint stress, the mouse feces was collected from every cage and immediately placed in 1.5 mL screw-capped tubes for DNA extraction. Prior to the DNA extraction, each sample tube was added with sterilized phosphate-buffered saline (PBS) solution (6 ml, 0.05 M, pH 7.4), shaken for 5–10 min, and centrifuged for 5 min at 500 rpm. The deposits were collected in Eppendorf tubes, and such processes were repeated thrice. The deposits were suspended in 1.0 mL ddH_2O and centrifuged for 5 min at 14000 rpm. The resulting deposits were dissolved in 200 μl absolute ethanol (precooled at −20°C) and centrifuged for 2 min at 14,000 rpm. The supernatant was discarded, and the process was repeated thrice. The total DNA was extracted from the feces by using the TIANamp Bacteria DNA Kit (TIANGEN, DP302, Beijing, China) in accordance with the manufacturer's instructions. The extracted genomic DNA was sent to Personal, Inc. (Personal Bio Inc.,

Shanghai, China) for high-throughput sequencing and analysis.

The V3/4 region of the 16S rRNA gene was amplified using universal primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR product was extracted from 2% agarose gels, purified, and quantified. The sequencing was carried out using the Illumina MiSeq platform and 2 × 300 bp reagent kit for paired-end sequencing (GenBank accession number PRJNA673977). Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff, and chimeric sequences were identified and removed using the QIIME analysis tools (Quantitative Insights into Microbial Ecology, v1.8.0; http://qiime.org/).

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). The truck blood was collected and centrifuged at 3400 rpm for 20 min to measure the 5-HT level. The supernatant was collected and stored at −80°C for further analysis. The 5-HT level was measured using the commercial ELISA kit (ab133053, Abcam, Cambridge, UK). In brief, varying concentrations of standard and sample solutions were added into the ELISA plates. All ELISA measurements were performed in two replicates.

2.5. Protein Extraction and Western Blot. Brain tissues were quickly removed, washed with PBS, and homogenized with phenylmethanesulfonyl fluoride (Sigma-Aldrich, 78830, Wisconsin, USA). The total protein concentration was measured using the BCA protein assay kit (Thermo Scientific, 23235, New York, USA). Protein extracts were used for Western blot to quantify the levels of brain-derived neurotrophic factor (BDNF), tropomyosin-related kinase B (TrkB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After the measurement and the adjustment of protein concentration, samples were added with 4x loading buffer, heated at 100°C for 10 min, and loaded onto the SDS–PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Merck Millipore, ISEQ00010 and IPVH00010, Massachusetts, USA) for 2 h at 56 V in the transfer buffer. Membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBST) for 2 h at room temperature on an orbital shaker. The membranes were then cut into several parts in accordance with the goal protein molecular weight and incubated with primary antibodies (anti-BDNF antibody, 1 : 1000, Abcam, ab108319, Cambridge, UK; anti-TrkB antibody, 1 : 5000, Abcam, ab187041, Cambridge, UK; and anti-GAPDH antibody, 1 : 1000, BioRad, MCA4739, California, USA) overnight at 4°C. The membranes were washed thrice with TBST (5 min each time) and incubated with horseradish peroxidase- (HRP-) conjugated IgG secondary antibodies (goat anti-mouse IgG HRP, 1 : 3000, Cw0102s, Beijing, China; and goat anti-rabbit IgG HRP, 1 : 3000, Cw0103s, Beijing, China) for 2 h at room temperature. Signals were visualized using the Gel DocTM EZ System (BioRad, California, USA), and the relative levels of BDNF, TrkB, and GAPDH proteins were analyzed using the "Image J" Software (https://imagej.nih.gov/ij/).

2.6. Statistical Analysis. Statistical analysis was performed using the GraphPad Prism 6. Continuous variables were presented as the mean ± standard error of the mean. Results were analyzed using unpaired one-way and two-way ANOVA.

The Chao1 index was determined as follows:

$$S_{\text{Chao1}} = S_{\text{obs}} + \frac{n1\,(n1-1)}{2\,(n2+1)}, \tag{1}$$

where S_{Chao1} is the estimated number of OTUs (operational taxonomic units), S_{obs} is the observed number of OUTs, $n1$ is the number of OTU that have one sequence, and $n2$ is the number of OTU that have two sequences.

The Shannon index was commonly calculated as follows:

$$H_{\text{Shannon}} = -\sum_{i=1}^{R} p_i ln\, p_i, \tag{2}$$

where p_i is the proportion of characters belonging to the i^{th} type of letter in the string of interest, and R denotes the actual number of types.

3. Results and Discussion

3.1. L. plantarum WLPL04 Rescues Stress-Induced Anxiety/Depressive-Like Behaviors. The behavioral assessment with the open-field test showed that the chronically stressed mice spent significantly less time in the central area of the open field compared with the control mice, indicating that the chronically stressed mice exhibited the anxiety phenotype. This phenotype was not observed in the stress + WLPL04 group of mice (Figures 2(a) and 2(b), control: 22.22 ± 2.159 s; stress: 12.41 ± 1.115 s; stress + WLPL04 : 17.73 ± 1.798 s; and one-way ANOVA, F (2, 27) = 7.928, $p = 0.0020$).

The elevated plus maze test showed that the *L. plantarum* WLPL04 treatment could rescue the chronic stress-induced anxiety phenotype. The stressed mice spent significantly less time (control: 43.93 ± 7.197 s; stress: 23.61 ± 3.078 s; stress + WLPL04 : 34.13 ± 2.552 s; and one-way ANOVA, F (2, 27) = 4.571, $p = 0.0195$) and executed significantly less entries (control: 6.300 ± 1.146; stress: 3.300 ± 0.3000; stress + WLPL04 : 6.900 ± 1.286; and one-way ANOVA, F (2, 27) = 3.651, $p = 0.0395$) in the open arms of the maze compared with the control mice. This anxiety-like phenotype was not observed in the stress + WLPL04 group of mice (Figures 2(c) and 2(d)).

The forced swimming test was performed to examine the effect of the *L. plantarum* WLPL04 treatment on the depressive-like behavior. The stressed mice had significantly more immobility time (control: 37.17 ± 5.846 s; stress: 71.43 ± 8.680 s; stress + WLPL04 : 40.57 ± 5.467 s; and one-way ANOVA, F (2, 32) = 7.670, $p = 0.0019$) compared with the control mice, indicating that the chronically stressed mice exhibited the depressive-like phenotype. This phenotype was not observed in the stress + WLPL04 group of mice (Figure 2(e)).

Overall, these results suggested that the *L. plantarum* WLPL04 treatment could alleviate the anxiety and the

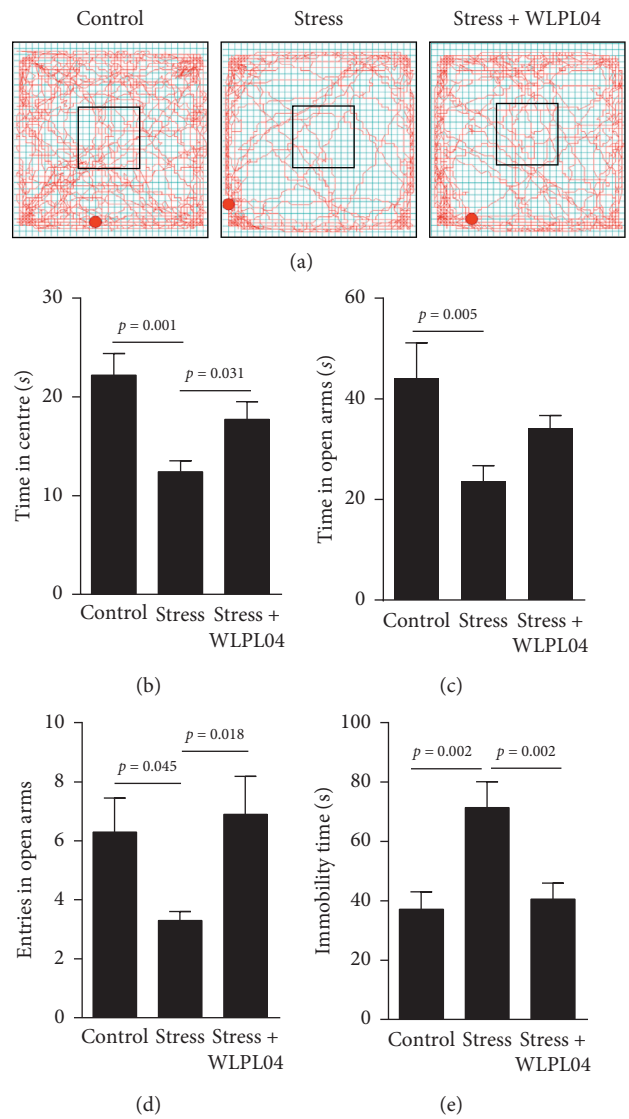

Figure 2: *L. plantarum* WLPL04 treatment reduces anxiety-like behaviors in chronically stressed mice. (a) The moving trajectory of the three groups of mice in the open-field test. (b) Chronically stressed mice exhibit significantly ($p < 0.01$) less time in the central area of the open field, and such behavioral phenotype is alleviated by the *L. plantarum* WLPL04 treatment. Chronically stressed mice have spent (c) significantly ($p < 0.01$) less time and (d) significantly ($p < 0.05$) fewer entries into the open arms in the elevated plus maze test, and such behavioral phenotypes are alleviated by the *L. plantarum* WLPL04 treatment. (e) Chronically stressed mice demonstrate significantly ($p < 0.01$) longer immobility time, and such behavioral phenotype is alleviated by the *L. plantarum* WLPL04 treatment. $n = 7 - 12$ mice per group.

depressive-like behavioral phenotypes induced by chronic stress.

3.2. L. plantarum WLPL04 Alleviates Stress-Induced Cognitive Functional Deficits. The Barnes maze and novel object recognition tests were performed to examine whether the *L. plantarum* WLPL04 treatment could alleviate the cognitive deficits induced by the chronic restraint stress. In the Barnes maze test, which measured the hippocampus and prefrontal cortex-dependent spatial learning and memory, the stressed and the control mice performed equally well during the training, indicating an intact ability of spatial learning (session 1: control: 61.85 ± 8.202 s; stress: 70.22 ± 9.081 s; stress + WLPL04 : 52.10 ± 7.771 s; session 2: control: 33.64 ± 3.709 s; stress: 41.59 ± 6.798 s; stress + WLPL04 : 43.70 ± 7.901 s; session 3: control: 30.01 ± 4.748 s; stress: 33.14 ± 5.496 s; stress + WLPL04 : 26.16 ± 4.148 s; session 4: control: 19.95 ± 3.223 s; stress: 26.20 ± 5.703 s; stress + WLPL04 : 17.94 ± 2.660 s; and two-way ANOVA, $F(6, 162) = 0.6035$, $p = 0.7272$). However, in the spatial memory in the probe trial, compared with the control mice, the stressed mice spent a significantly longer time (control: 10.96 ± 2.218 s; stress: 25.76 ± 5.814 s; stress + WLPL04 : 11.28 ± 2.641 s; and one-way ANOVA, $F(2, 39) = 4.693$, $p = 0.0149$) to find the target box, indicating impaired spatial

memory. Such impairment was not observed in the stress + WLPL04 group of mice, suggesting the protective effect of the *L. plantarum* WLPL04 on the spatial memory consolidation (Figures 1(b) and 1(c)).

In the novel object recognition test, which measures the prefrontal cortex-dependent cognitive function [23, 24], the control mice preferred to interact with the novel objects. By contrast, the stressed mice spent equal time interacting with novel and familiar objects, indicating a deficit in the novel object recognition. Such deficit was not observed in the stress + WLPL04 group of mice, suggesting that the *L. plantarum* WLPL04 could protect the cognitive ability for novel object recognition (Figure 1(d), control: 2.889 ± 0.5159; stress: 1.354 ± 0.1111; stress + WLPL04 : 1.946 ± 0.2031; and one-way ANOVA, $F\ (2,\ 18) = 5.620$, $p = 0.0127$).

Overall, these results suggested that the *L. plantarum* WLPL04 treatment could alleviate the cognitive deficits induced by chronic stress.

3.3. L. plantarum WLPL04 Reverses the Reduction in the Intestinal Microbiota Diversity Caused by Stress. The composition of intestinal microbiota was analyzed using the high-throughput DNA sequencing. The analysis of the relative abundance of the detected bacteria within each sample at the phylum level revealed a decrease in the abundance of Firmicutes and Actinobacteria and an increase in the abundance of Bacteroidetes in the stressed mice compared with those in the control mice (Figure 3(a)). Such changes were not observed in the stress + WLPL04 group of mice (Figure 3(a)). The Chao1 and the Shannon analyses showed that the species richness (Chao1) and the microbiota diversity (Shannon) in the feces were reduced by the chronic restraint stress, and such reductions were somehow reversed upon probiotic treatment with the *L. plantarum* WLPL04 (Figures 3(b) and 3(c), Chao1: control: 930.0 ± 49.00; stress: 662.0 ± 11.00; stress + WLPL04 : 772.3 ± 21.36; one-way ANOVA, $F\ (2,\ 4) = 18.70$, $p = 0.0093$; Shannon: control: 6.700 ± 0.2900; stress: 5.325 ± 0.08500; stress + WLPL04 : 5.990 ± 0.3262; and one-way ANOVA, $F\ (2,\ 4) = 4.606$, $p = 0.0917$). Thus, the chronic stress reduced the intestinal microbiota diversity, and such reduction could be reversed by the *L. plantarum* WLPL04 treatment.

3.4. L. plantarum WLPL04 Reverses the Decrease in the Serum 5-HT Level Caused by Stress. The 5-HT, a key element in the gut-brain axis, acts as a neurotransmitter in the central and the enteric nervous systems. The 5-HT plays an important role in learning, memory, and emotion regulation in the central nervous systems. The analysis of the serum 5-HT level revealed a significant decrease in the 5-HT level in stressed mice compared with that in the control mice. This decrease was not observed in the stress + WLPL04 group of mice (Figure 4, control: 130.6 ± 9.672 ng/mL; stress: 90.78 ± 8.945 ng/mL; stress + WLPL04 : 122.7 ± 12.65 ng/mL; and one-way ANOVA, $F\ (2,\ 6) = 4.004$, $p = 0.0786$), suggesting that the *L. plantarum* WLPL04 could produce a protective effect on the chronically stressed mice by maintaining the serum 5-HT at a normal level.

3.5. L. plantarum WLPL04 Prevents the Stress-Induced Decrease in BDNF and its Receptor TrkB. The BDNF, a member of neurotrophic factors, plays an important role in the nervous system development and learning/memory function [25]. Previous reports show that early-life events regulate the expression of neurotrophic factors [26]. Here, the expression levels of BDNF and its receptor TrkB in the mPFC of mice were measured using the Western blot. As shown in Figure 5, the chronic stress significantly reduced the levels of BDNF and the TrKB protein, and these reductions were not detected in the stress + WLPL04 group of mice (BDNF : control: $100.0 \pm 2.699\%$; stress: $65.57 \pm 7.401\%$; stress + WLPL04 : $99.68 \pm 9.028\%$; one-way ANOVA, $F\ (2,\ 6) = 8.182$, $p = 0.0193$; TrkB : control: $100.0 \pm 7.444\%$; stress: $50.64 \pm 2.565\%$; sStress + WLPL04 : $82.82 \pm 7.694\%$; and one-way ANOVA, $F\ (2,\ 6) = 15.54$, $p = 0.0042$). These results suggested that the *L. plantarum* WLPL04 could prevent the stress-induced decrease in BDNF and its receptor TrkB.

4. Discussion

The present study shows that the chronic restraint stress induces anxiety/depressive-like behaviors and results in cognitive deficits, causes an abnormal change in the intestinal microbiota, and reduces the levels of 5-HT, BDNF, and TrkB. These changes can be alleviated with the *L. plantarum* WLPL04 treatment, providing an experimental basis for the therapeutic application of *L. plantarum* on anxiety/depression and cognitive dysfunctions.

A growing body of studies suggests that the probiotic treatment can reverse the stress-induced intestinal dysbiosis and behavioral abnormality. The beneficial effects of probiotics include promoting host digestion, supporting the immune system, and managing the intestinal microbiota [27, 28]. *Lactobacillus* and *Bifidobacteria*, as the representative bacteria of Firmicutes and Actinobacteria, respectively, are reported to reduce anxiety symptoms in patients with chronic fatigue syndrome [29]. A probiotic formulation combining *L. acidophilus* Rosell-52 and *Bifidobacterium longum* Rosell-175 exerts a beneficial effect on the gastrointestinal symptoms in individuals affected by chronic stress [30]. The oral administration of *B. longum* 1714 or *Bifidobacterium breve* 1205 for six weeks reduces anxiety-like behavioral phenotypes in anxious BALB/*c* mice [31]. Several factors increase the risk of depression, including stress and environment and gut microbiota [32, 33]. Our study shows that four-week chronic restraint stress could cause depression. Previous studies show that gut microbiota can modulate depressive-like behavior. Mice treated with a combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 present improvements in depression-like behavior [34]. CCFM1025 treatment significantly reduced anxiety and depression-like behaviors induced by chronic unpredictable mild stress [35]. Mice received 1×10^9 cfu·*L. rhamnosus* daily for four weeks could

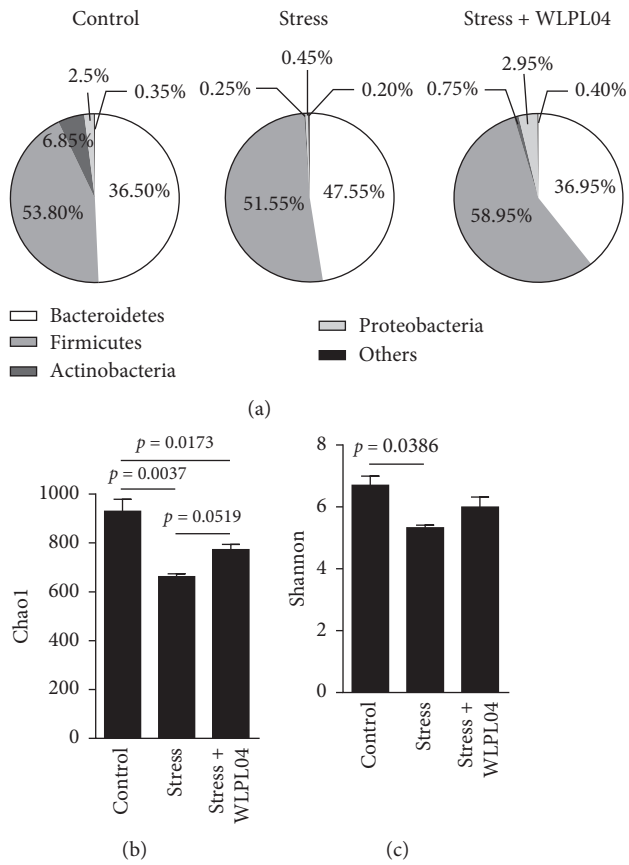

Figure 3: *L. plantarum* WLPL04 treatment rescues the negative change in the intestinal microbiota. (a) Aggregate microbiota composition at the phylum level in the fecal samples of experimental mice. Chronic stress significantly reduces the number of Firmicutes, and such negative effect is reversed by the *L. plantarum* WLPL04 treatment. Bacterial diversity and species richness as indicated by the (b) Chao1 and the (c) Shannon indices. The chronic stress tends to destroy the diversity and destroy the species richness, and such negative effects are rescued by the *L. plantarum* WLPL04 treatment. $n = 12$ mice per group.

alleviate anxiety and depression-related behaviors [36]. Data from the open-field test and elevated plus maze test in our study also show that *L. plantarum* WLPL04 alleviated anxiety and depressive-like behaviors induced by chronic restraint stress, suggesting its antianxiety and antidepression effect of *L. plantarum* WLPL04.

The link between microbiome composition and neurodevelopment has been proposed for a long time [4]. Microbiota has been reported to influence the neurodevelopment. The alterations of the gut microbiota may affect the neurodevelopment and could be mediated by microbiota via microbiota-gut-brain axis [37, 38]. Neurodevelopment-related molecular, such as BDNF, has been found to be related with the microbiota-gut-brain axis, and they are susceptible to modulations [4, 39]. The microbiota-gut-brain axis is reported to regulate neuropsychiatric diseases [40]. Alterations of gut microbiota could influence strongly on the neurodevelopment. The *L. helveticus* NS8 treatment improves cognitive deficit and anxiety-like behaviors in hyperammonemia rats. The two-month administration of *L. plantarum* MTCC1325 ameliorates the cognitive deficits in Alzheimer's disease [16]. The long-term treatment of *Lactobacillus paracasei* K71 may alleviate the age-dependent cognitive decline in mice [41]. The dysbiosis and behavioral deficits caused by prenatal stress can be prevented by treating the dam and the offspring mice with *Lactobacillus*-containing probiotics and indigenous *Lactobacillus reuteri*, respectively [42, 43]. In the present study, the *L. plantarum* WLPL04 treatment rescues cognitive deficits in chronically stressed mice, providing evidence that this probiotic treatment can benefit the host by alleviating stress-induced cognitive disorders.

The stress exposure is known to significantly change the gastric acid secretion, gastrointestinal motility, and mucous levels, which can influence the ability of microbes to colonize within the gastrointestinal tract. The stress can alter the composition of intestinal microbiota. For instance, Bailey et al. have reported that a social stressor reduces the relative abundance of Bacteroidetes in mice [5]. The change in the microbiota diversity can be detected as early as 2 h after stress exposure [44]. A study in nonhuman primates indicates that stress during pregnancy affects the infant gut microbiota by reducing *Bifidobacteria* and *Lactobacilli* [45]. The present study shows a consistent result, indicating that the chronic restraint stress induces changes in bacterial species and diversity.

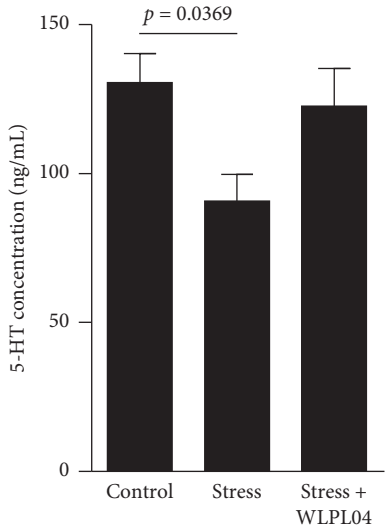

Figure 4: Chronic stress causes a decrease in the serum 5-HT concentration, and such effect is reversed by the *L. plantarum* WLPL04 treatment. Three independent experiments were conducted in each sample group.

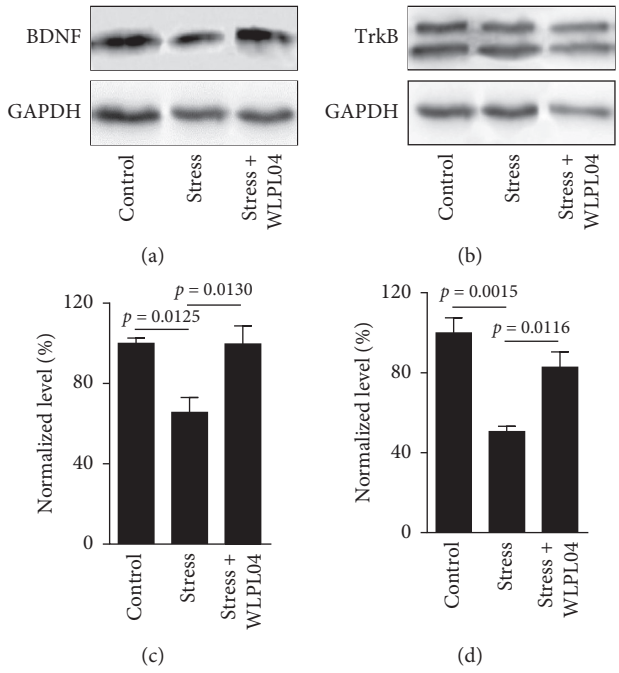

Figure 5: Protein levels of brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase *B* (TrkB) in the mPFC of mice. Chronic stress reduces the expressions of (a), (c) BDNF and (b), (d) TrkB, and such effects are rescued by the *L. plantarum* WLPL04 treatment. Three independent experiments were conducted in each sample group.

Although research shows the validity of probiotics, many scholars remain cautious. Suez et al. have found that the potential postantibiotic probiotic benefits may be offset by a compromised gut mucosal recovery [46]. Zmora has confirmed that the empiric probiotic supplementation may be limited and persistently affect the gut mucosa, meriting the development of new personalized probiotic approaches [47]. The effects of probiotic may be dependent on the strain. A probiotic formulation exhibits beneficial effects for stressed animals but has no benefit or may cause harm to normal ones [48]. Lactic acid bacteria are often considered to promote health but are reported to be associated with bad outcomes, including susceptible to inescapable electric stress [49], increased severity of psychotic dysfunctions [50], and high levels of proinflammatory [51]. Lactic acid bacteria are also reported to be relatively abundant in persons with schizophrenia and bipolar disorders [52, 53]. The discrepancy may be explained by differences in study design and

methodologies. In the present study, the *L. plantarum* WLPL04 is supplied as drinking supplement for chronically stressed mice, and results provide further evidence that this probiotic treatment can rescue the intestinal microbiota composition and support the hypothesis that the *L. plantarum* WLPL04 can benefit the host by alleviating stress-induced mood disorders. The 5-HT is produced in the brain and the gastrointestinal tract and can be detected in the blood. Gershon and Tack have reported that the gastrointestinal tract contains most of the body's 5-HT [54]. The synthesis and the metabolism of 5-HT in the brain and periphery are believed to be independent. To our knowledge, 90% of the 5-HT is produced by enterochromaffin cell and stored in granule cells [55]. When stimulus factors are involved, the 5-HT stored in granule cells is released into the blood [56]. The 5-HT produced in the brain cannot pass the blood-brain barrier and cannot enter blood.

The microbiota is involved in regulating the host's 5-HT level. Germ-free mice have significantly low serum serotonin [57]. Approximately 50% of the gut-derived 5-HT is regulated by the gut microbiota. Spore-forming bacteria, which are dominated by the Clostridiaceae and the Turicibacteraceae [57], produce short-chained fatty acids and tryptamine, which influence TPH-1 expression, 5-HT synthesis, and/or 5-HT release [58, 59]. The long-term diet supplementation with *L. paracasei* K71 elevates the serum serotonin level [41]. Consistent with those of the previous studies, our results show that the *L. plantarum* WLPL04 can reverse the serum 5-HT level of chronically restrained mice. The *L. plantarum* WLPL04 may affect the 5-HT synthesis in the gut and rescue the body's 5-HT concentration to a physiological level in the stressed mice.

The serotoninergic system plays an important role in the mood regulation [60]. The 5-HT has long been recognized as a key contributor to the regulation of mood and anxiety and is strongly associated with the etiology of major depression [61]. Early studies demonstrate that disabling the serotonergic system completely either by preventing the 5-HT production or by lesioning the 5-HT neurons leads to widespread behavioral consequences ranging from cognitive deficits to avoidance behaviors [52–65]. Increasing the bioavailability of the 5-HT in serotoninergic synapses effectively alleviates depressive symptoms [66]. In the present study, the serum 5-HT concentration is significantly decreased in the chronically stressed mice, and such decrease is alleviated by the *L. plantarum* WLPL04 treatment.

As the most abundant neurotrophic factor, the BDNF affects several aspects of brain functions. The expression of BDNF is dependent on the activity and regulated by internal and environmental factors. Increasing studies have shown that the stress downregulates the expression of BDNF [67–69]. The intestinal microbiota regulates the level of BDNF in the central nervous system [70]. For example, the administration of antimicrobials transiently alters the composition of microbiota and reduces the expression of BDNF in the hippocampus of mice [69]. Moreover, the hippocampal BDNF expression is upregulated after the mice are orally administered with the probiotic *B. longum* 1714 [31]. Consistently, the present study has found that the chronic restraint stress reduces the expression of BDNF and its receptor TrkB in the mPFC and impairs mPFC-dependent cognitive functions, and such effects are alleviated by the supplementation of the *L. plantarum* WLPL04.

5. Conclusions

In summary, the *L. plantarum* WLPL04 treatment can alleviate anxiety/depressive-like behaviors, the abnormal change in intestinal microbiota, and the reduced levels of 5-HT, BDNF, and TrkB induced by chronic stress, providing an experimental basis for its therapeutic application on anxiety/depressive mood disorders.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by the Natural Science Foundation of Jiangxi Province (Grant nos. 2016BAB215206, 20171ACB20002, and 20171BAB204019).

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2021, Article ID 6655414, 9 pages
https://doi.org/10.1155/2021/6655414

Research Article

A Comparative Transcriptomic Analysis of Human Placental Trophoblasts in Response to Pathogenic and Probiotic *Enterococcus faecalis* Interaction

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Received 5 October 2020; Revised 17 December 2020; Accepted 12 January 2021; Published 28 January 2021

Academic Editor: Maria De Francesco

With the ability to cross placental barriers in their hosts, strains of Gram-positive *Enterococcus faecalis* can exhibit either beneficial or harmful properties. However, the mechanisms underlying these effects have yet to be determined. A comparative transcriptomic analysis of human placental trophoblasts in response to pathogenic or probiotic *E. faecalis* was performed in order to investigate the molecular basis of different traits. Results indicated that both *E. faecalis* Symbioflor 1 and V583 could pass through the placental barrier *in vitro* with similar levels of invasion ability. In total, 2353 (1369 upregulated and 984 downregulated) and 2351 (1233 upregulated and 1118 downregulated) DEGs were identified in Symbioflor 1 and V583, respectively. Furthermore, 1074 (671 upregulated and 403 downregulated) and 1072 (535 upregulated and 537 downregulated) DEGs were only identified in Symbioflor 1 and V583 treatment groups, respectively. KEGG analysis showed that 6 and 9 signaling pathways were associated with interactions between Symbioflor 1 and V583. GO analysis revealed that these DEGs were mainly related to cellular and metabolic processes and biological regulation. However, 28 and 44 DEGs were classified into terms associated with placental and embryonic development in Symbioflor 1 and V583 treatment groups, respectively. Notably, 9 and 25 unique DEGs were identified only in Symbioflor 1 and V583 treatment groups, respectively. A large proportion of transcriptional responses differed when compared between pathogenic and probiotic *E. faecalis* interaction, and several unique DEGs and signal pathways were identified in the two different groups. These data enhance our understanding of how different traits can be affected by pathogenic and probiotic *E. faecalis* and the mechanisms underlying these effects.

1. Introduction

Enterococcus faecalis is a Gram-positive bacterium that is commonly found in a variety of different matrices including the alimentary tract and foods. This bacterium has received substantial attention due to the fact that it can exert both beneficial and pathogenic effects [1]. Certain *E. faecalis* strains are regarded as commensal bacteria or even probiotics for creating a healthy gut environment; however, other strains are considered to be dangerous as they can lead to a variety of nosocomial infections and diseases [2, 3]. Previous researches have shown that *E. faecalis* is able to pass through biological barriers and can subsequently mediate a variety of effects in the host [4, 5]. A review by Goldenberg et al. confirmed that various enterococci could transmit to fetus and cause stillbirth [6]. We have previously demonstrated that *E. faecalis* OG1RF can pass through the placental barrier of pregnant mice, translocate into the fetus, and then affect both fetal and placental growth and development [7]. We demonstrated that *E. faecalis* OG1RF induces placental and embryonic development retardation, stress and stimulus response activation, apoptosis, immune response disorder, and cell adhesion disintegration of placental trophoblasts through various signaling pathways using real-time PCR and DNA microarray [8]. *E. faecalis* has also been identified in meconium obtained from healthy neonates. The presence of this type of bacteria in meconium could initiate gut colonization as an adaptation to the fetal gut to prepare the fetus

for life outside the mother [9]. Albesharat et al. found that *E. faecalis* was present in the feces of mothers and their babies and hypothesized that the initial bacterial colonization of the infant occurs via vertical transmission from mothers to infants [10]. The close relationship between two typical *E. faecalis* strains (Symbioflor 1 [11] and V583 [12]) has been studied in significant detail with regards to human pregnancy. *E. faecalis* Symbioflor 1 (SymbioPharm, Herborn, Germany) is recognized as a probiotic strain that can alleviate the symptoms of irritable bowel syndrome by improving the microbial balance in the intestine. *E. faecalis* V583 is a vancomycin-resistant prototype clinical isolate that causes opportunistic nosocomial infection worldwide. Meanwhile, a human placental choriocarcinoma BeWo cell lines, a widely recognized *in vitro* placental model [13], was adopted. The BeWo cell line is regularly used to study a range of placental functions, including transplacental transport and infection caused by viruses and bacteria. DEGs of BeWo cells associated with *E. faecalis* invasion were identified by using DNA microarray, and transcriptomic profiling was compared by using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [14].

In this study, we first constructed an interaction model between two different *E. faecalis* strains and BeWo cells. Then, we compared the invasion abilities (adhesion ability and internalization ability) between the two *E. faecalis* strains. Subsequently, we performed comparative transcriptomic profiling by using a DNA microarray (Illumina Human HT-12 v4 Expression Bead Chip), GO analysis, and KEGG pathway analysis. The main objective of this study was to gain insights into the differences in transcriptional regulation between strains of *E. faecalis* that can exhibit dualistic behavior towards host health. The study was designed to identify the different traits that might be affected by putative pathogenic or probiotic *E. faecalis*. The identification of such traits is crucial as this information may ultimately contribute to the future development of strategies for the prevention and treatment of invasion and infection caused by different *E. faecalis* strains.

2. Materials and Methods

2.1. Bacterial Strains and Cell Lines. The bacterial strains used in this study are listed in Table 1. In brief, strains of *E. faecalis* strains, clinical pathogen V583 (ATCC 700802), and probiotic Symbioflor 1 (DSM 16431) were cultured in Trypticase soy broth (TSB) at 37°C for 24 h with shaking at 180 rpm. The bacteria were then harvested by centrifugation at 6000 rpm for 10 min, washed twice with sterile PBS (0.01M, pH 7.4), and reconstituted in cell culture medium DMEM/F-12 (Solarbio, Beijing, China) to yield a concentration of 10^9 CFU/mL before use.

The human choriocarcinoma cell line BeWo was purchased from Action-award Biotech Co., Ltd. (Guangzhou, China) and cultured as described previously [8]. In brief, BeWo cells were cultured in DMEM/F-12 medium supplemented with 10% (v/v) FBS (Gibco, Grand Island, NY) at 37°C under a 5% CO_2 atmosphere, until approximately 80%–90% cell confluence.

2.2. Invasion Assays. Invasion assays were performed in accordance with our previous study [8]. BeWo cells were transferred into 24-well plates (Corning, NY) and cultured for 24–48 h until a confluent monolayer was obtained. A 5 μL aliquot of resuspended bacteria was then added to each well containing 495 μL of cell culture medium and incubated for 60 minutes.

The total number of invading bacteria was determined by twice dip-washing with sterile PBS to remove free bacteria and lysing the BeWo cells with 500 μL of 0.5% Triton X-100/PBS to release the internalized bacteria. Serial dilutions were spread onto Trypticase soy agar (TSA) plates and incubated at 37°C overnight. The numbers of internalized bacteria were then determined by adding 100 μg/mL of gentamicin and 50 μg/mL of penicillin and incubating for 60 min to kill any viable extracellular bacteria that were still present. The cells were then washed twice, lysed, and the bacterial count determined as described above. P values were calculated using Student's t-test.

2.3. DNA Microarray and Data Analysis. Incubation experiments were performed as described in our previous study [8]. In brief, BeWo cells were grown in 24-well plates and cultured for 24–48 h until 80%–90% confluency. *E. faecalis* V583 and Symbioflor 1 were then harvested and resuspended in DMEM/F-12 cell culture medium to 10^9 CFU/mL. A 5 μL aliquot of resuspended *E. faecalis* V583 and Symbioflor 1 was then added to each well containing 495 μL of cell culture medium and incubated for 4 h. The same amount of cell culture medium, but without any *E. faecalis* strains, was used as a negative control. Total RNA was extracted using TRNzol Total RNA Reagent (TIANGEN, Beijing, China). DNA microarrays were performed by Beijing EMTD Technology Development Co., Ltd. using an Illumina Human HT-12 v4 Expression BeadChip system (Illumina, Inc., San Diego, CA). In brief, RNA was adjusted to a concentration of 200 ng/μL, followed by first and then second strand cDNA synthesis. Double-stranded cDNA was then purified with a filter cartridge, and cRNA was synthesized by T7 RNA polymerase transcription *in vitro*. Following purification and quantification, the cRNA was hybridized with the BeadChip, washed, scanned, and analyzed. Illumina expression data were deposited in the NCBI Gene Expression Omnibus (GEO) database under the accession number GSE75626. DEGs were analyzed using GO and KEGG pathway databases.

2.4. Statistical Analysis. Unless specified, all experiments were performed in triplicate. All data were analyzed using statistics programs contained in SigmaPlot 11.0 (Systat Software, San Jose, CA).

Table 1: *Enterococcus faecalis* strains used in this study.

Strain	Number	Country	Source	Isolation	Characteristics	References
Symbioflor 1	DSM 16431	Germany	Nonhospitalized person	Feces	Probiotic	[11]
V583	ATCC 700802	USA	Hospitalized patient	Blood	Pathogen, Ery^R, Gen^R, and Van^R	[12]

*Ery, erythromycin; Gen, gentamicin; Van, vancomycin; R, resistance.

3. Results

3.1. Invasion Ability of E. faecalis. The number of *E. faecalis* strains present in BeWo cells was determined using invasion assays. As shown in Figure 1(a), 8.42 ± 0.13 Log_{10} CFU/mL of Symbioflor 1 and 8.66 ± 0.18 Log_{10} CFU/mL of V583 were identified in BeWo cells after incubation under the same conditions. These results showed that incubation conditions did not cause any deviations in the detection of invasion ability.

As demonstrated in Figure 1(b), the invaded cell counts of Symbioflor 1 and V583 strains were 6.76 ± 0.20 and 6.86 ± 0.20 Log_{10} CFU/mL, respectively, while those of internalized Symbioflor 1 and V583 were 2.48 ± 0.35 and 2.06 ± 0.50 Log_{10} CFU/mL, respectively (Figure 1(c)). These results showed that there was no significant difference in the *in vitro* invasion ability when compared between the two strains ($P > 0.05$). These data indicated that both the pathogenic and probiotic *E. faecalis* strains could pass through the placental barrier. Similar observations were noted with regards to the *in vitro* invasion ability when compared between the two strains.

3.2. Differential Gene Expression Profile Analysis. DNA microarray techniques were used to compare the gene expression patterns of untreated BeWo cells with those infected by *E. faecalis*. After applying cutoffs for induction (ratio > 2.0-fold) and suppression (ratio < 0.5-fold), out of a total of 47,231 genes on the BeadChip, a total of 2353 DEGs, including 1369 upregulated genes and 984 downregulated genes were identified in the Symbioflor 1 treatment group. In the V583 treatment group, a total of 2351 DEGs, including 1233 upregulated genes and 1118 downregulated genes, were identified. Furthermore, 1279 of the total number of DEGs were found to be common to both treatment groups, thus accounting for 54.36% and 54.40% of the DEGs in Symbioflor 1 and V583 treatment groups, respectively (Figure 2(a), Tables S1 and S2).

In addition, 698 of the total number of upregulated DEGs were found to be common to both strains, thus accounting for 50.99% and 56.61% of the DEGs in the Symbioflor 1 and V583 treatment groups, respectively (Figure 2(b)). Furthermore, 581 of the downregulated DEGs were found to be common to both strains, thus accounting for 59.04% and 51.97% of the DEGs in the Symbioflor 1 and V583 treatment groups, respectively (Figure 2(c)).

In general, both pathogenic and probiotic *E. faecalis* treatment groups showed a similar number of total DEGs. However, the number of upregulated and downregulated genes varied significantly in both groups. Hence, DEGs were then mapped using the KEGG database for signal pathway analysis to gain further understanding of their biological function.

3.3. Signal Pathway Analysis of DEGs. According to signal pathway analysis of DEGs, six main terms associated with BeWo cells in response to invasion by Symbioflor 1, including the MAPK signaling pathway, Jak-STAT signaling pathway, adherens junction, T cell receptor signaling pathway, p53 signaling pathway, and pathogenic *Escherichia coli* infection. For the V583 treatment group, nine main terms were associated with the response of BeWo cells to invasion, including the MAPK signaling pathway, leukocyte transendothelial migration, p53 signaling pathway, T cell receptor signaling pathway, apoptosis, ErbB signaling pathway, adherens junction, B cell receptor signaling pathway, and pathogenic *Escherichia coli* infection. According to the results obtained as shown in Table 2, five common terms were identified in both treatment groups. However, the Jak-STAT signaling pathway in particular was only observed in the Symbioflor 1 treatment group, whereas the ErbB signaling pathway, apoptosis, B cell receptor signaling pathway, and leukocyte transendothelial migration were all identified in the V583 treatment group.

3.4. Bioinformatic Analysis of DEGs. DEGs were characterized functionally by comparison against GO database and classified into three different categories, namely, the biological process, cellular component, and molecular function. For the Symbioflor 1 treatment group, DEGs were categorized into 293 terms in the biological process, 58 terms in cellular component, and 70 terms in molecular function (Table S3). For the V583 treatment group, the DEGs were categorized into 267 terms in the biological process, 59 terms in cellular component, and 62 terms in molecular function (Table S4). According to the comparative analysis of relevant data from both treatment groups, the top three genes were classified under the GO biological process categories related to the same terms used for the cellular process, metabolic process, and biological regulation (Tables S3 and S4).

In particular, for the Symbioflor 1 treatment group, a total of 28 genes were classified into terms associated with placental and embryonic development: placental development, embryonic placental development, in utero embryonic development, and the embryonic process involved in female pregnancy (Table 3). However, for the V583 treatment group, a total of 44 genes were classified into terms associated with placental and embryonic development, namely, placental development, chordate embryonic development, in utero embryonic development, embryonic development ending in birth or egg hatching, embryonic cranial skeleton morphogenesis, and the embryonic process

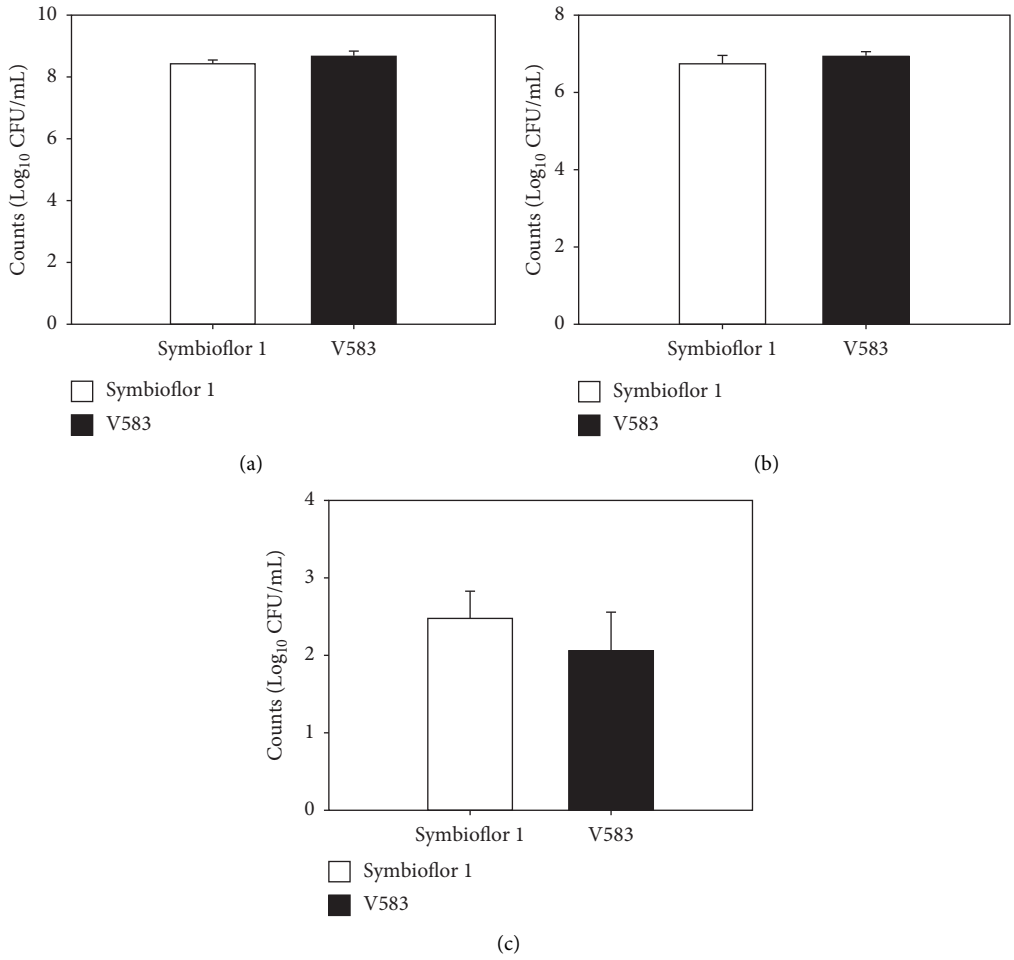


Figure 1: Invasion ability of *E. faecalis* strains associated with BeWo cells. (a) Colony forming units (CFUs) of *E. faecalis* strains after incubation; (b) CFUs of invaded *E. faecalis*; (c) CFUs of internalized *E. faecalis*. Values represent mean ± SD. *P* values were calculated using Student's *t*-test.

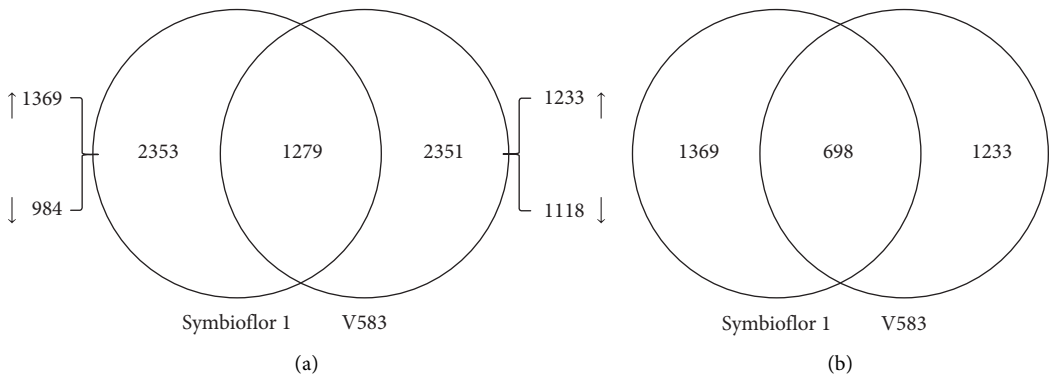
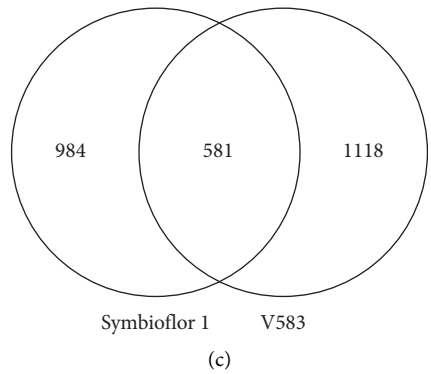


Figure 2: Continued.



(c)

Figure 2: Distribution of DEGs of BeWo cells in response to different *E. faecalis* strains. Venn diagram showing the number of unique and common DEGs in BeWo cells in response to Symbioflor 1 and V583 infection. (a) Total DEGs; (b) upregulated genes; (c) downregulated genes.

Table 2: DEGs containing relevant terms of the signal pathway generated by KEGG pathway analysis.

Group	Term	Genes
Symbioflor 1 group	hsa04010: MAPK signaling pathway	FGFR4, PDGFB, GNA12, PPM1A, MKNK2, PPM1B, MAP3K7, MAX, FOS, MAP3K5, MAP3K3, HSPA7, MAP2K7, MYC, HSPA8, RASA2, EGFR, TAOK1, TGFBR2, DUSP5, ATF4, RPS6KA4, MAPK13, MAPK14, JUN, RAP1A, PLA2G6, MAPK7, GADD45B, GADD45A, PLA2G2D (31)
	hsa04630: Jak-STAT signaling pathway	PTPN6, CRLF2, LEPR, CBL, SOCS4, BCL2L1, SOCS5, IL7R, IL10, STAT3, LEP, GH2, ZFP91, STAT4, SPRY1, EP300, IL10RB, IL5RA, SPRED1, MPL, MYC, PIK3R1 (22)
	hsa04520: adherens junction	EGFR, LOC646821, PTPN6, BAIAP2, TGFBR2, CTNND1, ACP1, WAS, CTNNB1, VCL, MAP3K7, PVRL4, EP300, PVRL1, SORBS1, PVRL2 (16)
	hsa04660: T cell receptor signaling pathway	PTPN6, NFKBIB, CBL, MALT1, IL10, MAP3K7, FOS, NCK2, MAPK13, MAPK14, PAK4, JUN, ZAP70, MAP2K7, PIK3R1 (15)
	hsa04115: p53 signaling pathway	ZMAT3, RPRM, SESN2, CCNG2, CDK2, CCNB1, PPM1D, CDKN1A, CDKN2A, BAX, RRM2, GADD45B, GADD45A (13)
	hsa05130: pathogenic *Escherichia coli* infection	LOC646821, NCK2, YWHAZ, KRT18, LOC399942, TUBA3E, WAS, ITGB1, CTNNB1, TTLL3 (10)
V583 group	hsa04010: MAPK signaling pathway	FGFR1, FGF18, PDGFB, MRAS, PPP3R1, PPM1A, MKNK2, CACNB3, GNG12, PPM1B, NFKB2, FOS, MAP3K5, CASP3, NFATC4, MYC, HSPA8, RASA2, EGFR, NTF4, TAOK1, TGFBR2, DUSP5, ATF4, DUSP2, JUN, GADD45G, RAP1A, MAPK9, GADD45B, PLA2G3, PLA2G2D, GADD45A (33)
	hsa04670: leukocyte transendothelial migration	ACTB, F11R, LOC646821, NCF4, SIPA1, CTNND1, ITGB1, ITGAM, CTNNB1, VCL, PTK2, CYBB, PTK2B, RAP1A, PIK3R3, PIK3R1, LOC284620 (17)
	hsa04115: p53 signaling pathway	ZMAT3, SESN2, CCNG2, CDK2, CCNE2, PPM1D, CDKN1A, CASP3, CDKN2A, RRM2, GADD45G, BAI1, APAF1, MDM4, GADD45B, GADD45A (16)
	hsa04660: T cell receptor signaling pathway	BCL10, PTPN6, NFKBIE, NFKBIB, CBL, PPP3R1, IL10, FOS, NCK2, JUN, PAK4, MAPK9, NFATC4, PIK3R3, PIK3R1, NFATC1 (16)
	hsa04210: apoptosis	IAP, AIFM1, PPP3R1, BAD, BCL2L1, CAPN2, CASP6, TNFSF10, CASP3, RIPK1, PRKAR1B, IL1RAP, APAF1, PIK3R3, PIK3R1 (15)
	hsa04012: ErbB signaling pathway	EGFR, CBL, BAD, NCK2, PTK2, CDKN1A, CDKN1B, PAK4, JUN, GAB1, MAPK9, PIK3R3, MYC, PIK3R1 (14)
	hsa04520: adherens junction	ACTB, EGFR, LOC646821, FGFR1, PTPN6, TGFBR2, CTNND1, ACP1, VCL, CTNNB1, PVRL4, EP300, PVRL1, PVRL2 (14)
	hsa04662: B cell receptor signaling pathway	BCL10, PTPN6, IFITM1, NFKBIE, NFKBIB, PPP3R1, FOS, JUN, NFATC4, PIK3R3, PIK3R1, BLNK, NFATC1 (13)
	hsa05130: pathogenic *Escherichia coli* infection	ACTB, LOC646821, NCK2, KRT18, ARPC3, LOC399942, TUBA3E, ITGB1, CTNNB1, TTLL3 (10)

involved in female pregnancy. According to the results obtained (Table 3), 19 DEGs were found to be common to both treatment groups.

In addition, the fold-changes for these DEGs are shown in Figure 3. For the Symbioflor 1 treatment group, eight out of nine unique DEGs were upregulated, while one gene (*MSX1*) was downregulated. For the V583 treatment group, 10 out of 25 unique DEGs were upregulated, while 15 genes were downregulated. Furthermore, the most common DEGs showed a similar tendency to vary in each of the treatment

Table 3: DEGs classified into placenta and embryonic development of the GO biological process category.

Group	Term	Genes
Symbioflor 1 group	GO: 0001701~in utero embryonic development	MAFG, MAFF, XRCC2, GABPA, GNA12, SPINT1, EGLN1, BCL2L1, MBNL1, ITGB1, TPM1, CITED2, HES1, NDEL1, MSX1, PSMC4, HAND1, HSF1, TEAD4, PKD1, MKL2, LOC652826 (22)
	GO: 0001890~placenta development	VWF, PPARD, HSF1, HAND1, CCNF, PLCD3, SPINT1, HS6ST1, EGLN1, PRDX3, CITED2 (11)
	GO: 0001892~embryonic placenta development	HSF1, HAND1, SPINT1, EGLN1, CITED2 (5)
	GO: 0060136~embryonic process involved in female pregnancy	HSF1, CITED2 (2)
V583 group	GO: 0043009~chordate embryonic development	GNA13, SYVN1, XRCC2, NDST1, EGLN1, BCL2L1, ITGB1, TPM1, CITED2, HSF1, PKD1, MKL2, FLVCR1, BCL10, MAFF, ADAM10, ZNF830, TGFBR2, SPINT1, MBNL1, LIG4, GAS1, CAPN2, BRCA1, HES1, DLX2, TULP3, EP300, TSC1, PSMC4, SP3, HOXB6, MNX1, TGIF1, RIPPLY1, FOXC1, APAF1, LOC652826, POFUT1 (39)
	GO: 0009792~embryonic development ending in birth or egg hatching	Same as GO: 0043009~chordate embryonic development (39)
	GO: 0001701~in utero embryonic development	GNA13, XRCC2, SYVN1, EGLN1, BCL2L1, TPM1, ITGB1, CITED2, HSF1, PKD1, MKL2, FLVCR1, MAFF, ADAM10, ZNF830, SPINT1, LIG4, MBNL1, CAPN2, HES1, TULP3, PSMC4, SP3, FOXC1, LOC652826 (25)
	GO: 0001890~placenta development	VWF, PPARD, CYP27B1, HSF1, SP3, CCNF, PLCD3, SPINT1, EGLN1, CITED2 (10)
	GO: 0048701~embryonic cranial skeleton morphogenesis	DLX2, TULP3, NDST1, TGFBR2, GAS1 (5)
	GO: 0060136~embryonic process involved in female pregnancy	HSF1, SP3, CITED2 (3)

groups. However, the *PPARD* gene in particular differed from all other genes as it was upregulated in Symbioflor 1 and downregulated in V583.

4. Discussion

In our previous studies, we demonstrated the ability of *E. faecalis* OG1RF to translocate both intestinal and placental barriers and demonstrated the molecular mechanisms responsible for these actions by DNA microarray analysis [7, 8]. However, the influence of *E. faecalis* in human pregnancy has not been fully elucidated, as different *E. faecalis* strains are expected to exhibit multiple roles. The objectives of the present study were to investigate and compare the different influences of putative pathogenic or probiotic *E. faecalis* on human placental trophoblast cells. Hence, two typical strains, representing the different lifestyles of this species, were evaluated for comparison: Symbioflor 1 and the pathogen V583. To the best of our knowledge, researchers have yet to investigate the influence of putative pathogenic or probiotic *E. faecalis* on human placental trophoblast cells by comparative transcriptomic analysis.

Our invasion assays demonstrated that both the pathogenic V583 strain and the probiotic Symbioflor 1 strain could adhere and internalize into human placental trophoblast cells. Furthermore, regardless of their diverse origins and lifestyles, these strains were found to show similar *in vitro* invasion ability. This observation was also consistent with our recent study of *E. faecalis* OG1RF in which 6.32 ± 0.10 Log_{10} CFU/mL of bacteria were seen to invade and 2.23 ± 0.29 Log_{10} CFU/mL were seen to internalize [8]. Similarly, Peng et al. found that the same three *E. faecalis* strains showed a similar adhesion rate but exhibited a different translocation rate in Ptk6 epithelial cell monolayers [15]. Bierne et al. reported that *E. faecalis* has the ability to internalize into intestinal LoVo cells [16]. These results demonstrated that different *E. faecalis* strains are able to pass through both intestinal and placental barriers, and their differences in invasion ability may be related to the cell model selected.

The influence of *E. faecalis* on human placental trophoblast cells was further investigated using DNA microarray analysis. We previously illustrated the biological effects and associated molecular mechanisms of *E. faecalis* OG1RF on placental function using a BeadChip microarray [8]. DNA microarrays have a distinct advantage over other techniques as they can provide both qualitative and quantitative data for a vast numbers of DEGs with high levels of sensitivity [17]. Li et al. used a DNA microarray to compare gene expression patterns between untreated and Aa-LPS-treated BeWo cells [18]. The changes in gene expression in human trophoblasts that pose a direct impact to placental and fetal health are now recognized as biomarkers [19]. According to the comparative results of DNA microarray analysis, the total number of DEGs appeared to be similar for both Symbioflor 1 and V583 treatment groups. Furthermore, the number of genes identified was slightly higher than that previously found in *E. faecalis* OGIRF [8]. However, almost half of the DEGs (both upregulated and downregulated) were unique to each group suggesting that different strains exhibit different effects on the placenta. According to our KEGG results, the V583 strain exhibited a greater number of signaling pathways than the Symbioflor 1 strain, thus indicating that pathogenic

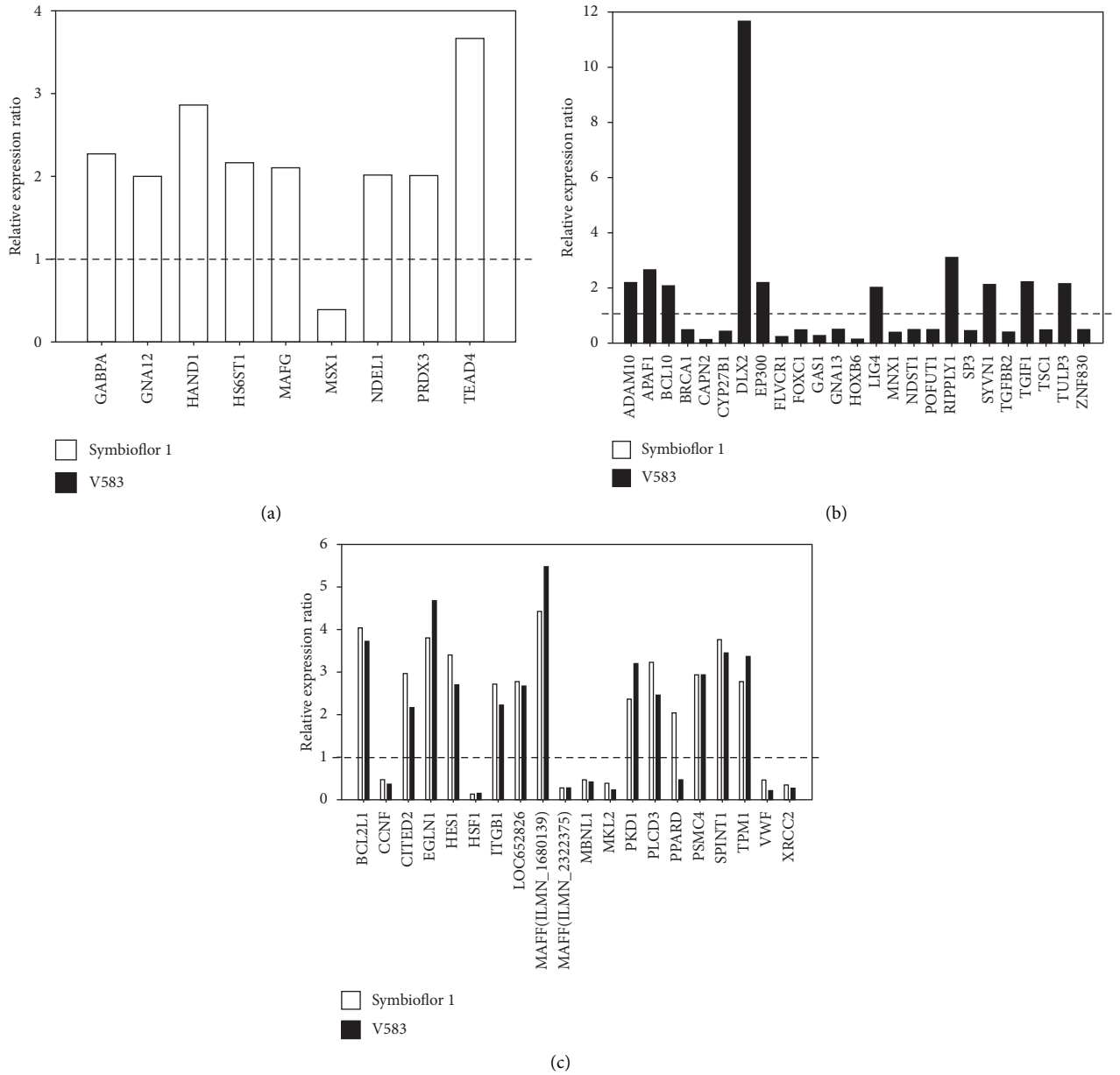


Figure 3: Fold change of DEGs classified into placenta and embryonic development. (a) Unique DEGs in the Symbioflor 1 treatment group; (b) unique DEGs in the V583 treatment group; (c) common DEGs in the Symbioflor 1 and V583 treatment groups. The relative expression ratio for each gene is presented in the histogram. A ratio greater than zero (>1) indicates upregulation of gene expression, and a ratio below zero (<1) indicates downregulation.

E. faecalis may in particular cause a stronger response in BeWo cells. Moreover, apoptosis, ErbB, B cell receptor, and leukocyte transendothelial migration signaling pathways were only activated in the V583 group. Similarly, pathogen-induced apoptosis in human placental trophoblasts can also lead to septicemia during pregnancy [18]. A previous study showed that the B cell receptor signaling pathway and leukocyte transendothelial migration were also found in the host cell infected by *Streptococcus pneumoniae* [20]. In particular, PPAR (peroxisome proliferator-activated receptor delta) is critically essential for placental development and function. This typical nuclear receptor has also been suggested to increase the placental fatty acid uptake [21]. Furthermore, studies have shown that PPAR-δ-deficiency mice offspring can lead to growth retardation and impairment of neural development [22]. Our present results showed that PPARD was upregulated in the Symbioflor 1 treatment group but downregulated in the V583 treatment group. These results suggest that the presence of pathogenic *E. faecalis* may stunt fetal and placental growth and development. This result is in high agreement with our previous studies showing that pregnant mice given oral doses of *E. faecalis* OG1RF exhibit changes in terms of their fetal and placental growth and development [7, 8]. Moreover, both ADAM10 and APAF1

were exclusively upregulated in the V583 treatment group but not in the Symbioflor 1 treatment group. Previous studies have demonstrated that ADAM10 can mediate E-cadherin shedding and regulate epithelial cell-cell adhesion, thus exerting a direct impact on early embryonic development *in vivo* [23]. APAF1 is considered as a key player in apoptosis during embryonic development [24]. We also found that CAPN2, POFUT1, and GAS1 were all downregulated in the V583 treatment; these proteins have been shown to play an important role in embryonic development [25–27]. In particular, mouse embryos lacking Pofut1 have been shown to exhibit defects in their cardiovascular system [26]. Martinelli et al. found that Gas1 mutant pups were only 3/4 the size of their control littermates [27] and showed similar abnormalities as fetal mice infected with *E. faecalis* OG1RF [7].

5. Conclusions

Our present analysis identified that a large proportion of transcriptional responses in BeWo cells differed when compared between infection caused by pathogenic and probiotic *E. faecalis*. Several unique DEGs and signal pathways were identified in the two strains. These data constitute a strong basis for understanding the mechanisms underlying the differential effects caused by pathogenic and probiotic strains of *E. faecalis*.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This research project was supported by the National Natural Science Foundation of China (31570075 and 31770133), Health Young and Middle-aged Backbone Personnel Training Project of Fujian Provincial Health Commision (2019-ZQNB-21), Foundation for Program of Department of Education from Fujian Province (JAT190849), and Research Program of Xiamen Medical College (K2015-09). The authors thank EditSprings for linguistic assistance during the preparation of this manuscript.

Supplementary Materials

Table S1. Differential gene expression profile of BeWo cell in response to *Enterococcus faecalis* Symbioflor 1 invasion. Table S2. Differential gene expression profile of BeWo cell in response to *Enterococcus faecalis* V583 invasion. Table S3. Differentially expressed genes affected by *Enterococcus faecalis* Symbioflor 1 classified with GO database. DEGs were characterized functionally by comparison against GO database and classified into three different categories, namely, the biological process, cellular component, and molecular function. Table S4. Differentially expressed genes affected by *Enterococcus faecalis* V583 classified with GO database. DEGs were characterized functionally by comparison against GO database and classified into three different categories, namely, the biological process, cellular component, and molecular function. (*Supplementary Materials*)

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2021, Article ID 6658674, 7 pages
https://doi.org/10.1155/2021/6658674

Review Article

Gut Microbiota-Derived Metabolites in the Development of Diseases

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Received 20 October 2020; Revised 12 December 2020; Accepted 30 December 2020; Published 12 January 2021

Academic Editor: Yisong Qian

Gut microbiota is increasingly recognized as a metabolic organ essential for human health. Compelling evidences show a variety set of links between diets and gut microbial homeostasis. Changes in gut microbial flora would probably contribute to the development of certain diseases such as diabetes, heart disease, allergy, and psychiatric diseases. In addition to the composition of gut microbiota, the metabolites derived from gut microbiota have emerged as a pivotal regulator in diseases development. Since high-fat and high-protein diets substantially affect the gut microbial ecology and human health, the current review summarizes the gut microbiota-derived metabolites such as short-chain fatty acids (SCFAs), amino acids, and their derivatives and highlights the mechanisms underlying the host responses to these bioactive substances.

1. Introduction

Intestine is a complex ecosystem harboring a diversity of microbial community known as the gut microbiota. Gut microbiota has recently emerged as a virtual endocrine organ producing multiple compounds, which maintains the homeostasis and influences the function of the human body. The gut microbiota community is predominantly composed of two phyla: Firmicutes and Bacteroidetes [1–4]. Emerging data show that an aberrant gut microbiota composition is associated with several diseases, such as metabolic disorders and inflammatory bowel disorder (IBD) [5]. Prebiotic feeding (e.g., with inulin-type fructans and some polyphenols) strongly increases the presence of *A. muciniphila* and improves metabolic disorders [6]. Conversely, some studies in mice have reported an increased abundance of *A. muciniphila* on the ingestion of a high-fat high-sucrose diet [7]. The host diets are believed to regulate the composition of gut microbiota and microbiota-derived metabolites, which causes a crosstalk between the host and its microbiome. A growing body of research has focused on the microbially produced metabolites such as short-chain fatty acids (SCFAs), amino acids, and their derivatives cometabolized by the host [8]. Implications from the diet–microbiota–host interactions highlight the therapeutic potential for preventing and treating certain diseases. Koh et al. identifies imidazole propionate as a microbially produced histidine-derived metabolite that is present at higher concentrations in subjects with type 2 diabetes [9]. In this review, we will describe the microbial origin of several key metabolites produced from diets and their remarkable effects on host physiology.

2. Synthesis of Short-Chain Fatty Acids

Dietary fibers but also proteins and peptides, which escape digestion from host enzymes in the upper gut, are metabolized by the microbiota in the cecum and colon [10]. Short-chain fatty acids are the metabolites of dietary fibers metabolized by intestinal microorganisms [11]. Protein fermentation can also contribute to the SCFAs pool but mostly gives rise to branched-chain fatty acids such as isobutyrate, 2-methylbutyrate, and isovalerate exclusively originating from branched-chain amino acids valine, isoleucine, and leucine

[12]. However, branched-chain amino acids (BCAAs) have been proposed as potentially harmful microbially modulated metabolites [13–15]. The acetate (C2), propionate (C3), and butyrate (C4) are the most abundant (≥95%) SCFAs, which are saturated aliphatic organic acids that consist of one to six carbons. Acetate, propionate, and butyrate are present in an approximate molar ratio of 3 : 1 : 1 in the colon and stool [15, 16]. In the cecum and large intestine, 95% of the produced SCFAs are rapidly absorbed by the colonocytes, while the remaining 5% is secreted in the feces. The SCFAs are not distributed evenly, which means they are decreased from proximal colon to distal colon [17]. Changing the distribution of intestinal flora and thus the distribution of metabolites may be of a great effect in the treatment of diseases because there is a concentration threshold for acetate's different impacts on the host.

Conceptually, the simplest way to synthesize an organic molecule is to construct one carbon at a time. The biochemical events that underlie the condensation of two one-carbon units to form the two-carbon compound, acetate, have intrigued chemists, biochemists, and microbiologists for many decades [18]. Gut microbiota produce acetate from (1) the pyruvate pathway which can produce acetyl-CoA as the precursor for acetate (2) and the Wood–Ljungdahl pathway which is composed of two branches: (1) the C1-body branch (also known as eastern branch) via reduction of CO_2 to formate and (2) the carbon monoxide branch (the western branch) via reduction of CO_2 to CO [19]. End product of this pathway is acetyl-CoA which is formed by formate, CO, and the extra methyl group. Another major SCFA butyrate has a particularly important role as the preferred energy source for the colonic epithelium and a proposed role in providing protection against colon cancer and colitis [20]. The two molecular of acetyl-CoA are converted to butyryl-CoA; then, the butyryl-CoA is turned into butyrate by some gut microbe with phosphotransbutyrylase and butyrate kinase. Interestingly, some microbes possess an enzyme called butyryl-CoA: acetate-CoA transferase, which transforms acetate and butyryl-CoA into acetyl-CoA and butyrate. There is a connection between acetate and butyrate distinctly, which suggests the complexity of the relationship between metabolites and leads us to think the significance of this metabolite transformation for the survival of bacteria. The significance may even play an important role in disease development. Propionate is produced in the human large intestine by microbial fermentation and may help maintain human health that includes antilipogenic, serum cholesterol levels lowering, anti-inflammatory, and anticarcinogenic functions [21–23]. There are three major microbially produced ways [24]: (1) acrylate pathway, (2) propanediol pathway, and (3) succinate pathway, which involves three genes: *lcdA* (encoding lactoyl-CoA dehydratase), *pduP* (encoding propionaldehyde dehydrogenase), and *mmdA* (encoding methylmalonyl-CoA decarboxylase), respectively Figure 1. Of note, diet fibers are not the only source for SCFAs. Analysis of metagenome data also suggested that butyrate can be synthesized from proteins via the lysine pathway [25]. Consequently, there are bacteria with different functions in the intestinal tract, and they perform their own duties. Some of them provide specialized supports for other functional bacteria or intestinal cells such as producing nutrients such as SCFAs. These kinds of bacteria are just like producers in the ecosystem. And these bacteria are promising to be migrated objects for fecal microbiota transplantation (FMT).

2.1. Regulation of Glucose/Energy Metabolism by SCFAs. The regulation of glucose metabolism by SCFAs is determined by multiple mechanisms. A recent study suggested that acetate, in the form of neutralized AcOH, activated AMPK (5′-AMP-activated protein kinase) in rat hepatocytes [26]. Activation of the hepatic AMPK pathway decreased gene expression of the gluconeogenic enzymes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK). AMPK mediates glucose uptake and free fatty acid oxidation in skeletal muscle and inhibits gluconeogenesis, glycolysis, lipogenesis, and cholesterol formation in the liver. Propionate, itself a substrate of IGN (intestinal gluconeogenesis), activates IGN gene expression via a gut-brain neural circuit involving the fatty acid receptor FFAR3 [27]. Though there is a plausible contradiction that SCFAs play opposite roles in gluconeogenesis. SCFAs have beneficial effects on glucose and energy homeostasis beyond question. The FFAR3 reporter is strongly expressed in the main, large population of enteroendocrine cells throughout the GI tract (gastrointestinal tract) but surprisingly also in neurons of both submucosal and myenteric ganglia. In contrast, the FFAR2 is expressed only in a subpopulation of the enteroendocrine cells but very strongly in a large population of leukocytes in the lamina propria throughout the small intestine [28]. SCFAs can activate FFAR2/3 (GPCR43/41) in colon cells to secrete PYY (peptide YY) or GLP-1 (glucagon-like peptide-1) into plasma. It is proved that GLP-1 can promote the secretion of insulin and inhibit the secretion of glucagon. The PYY can improve glucose uptake and utilization of periphery tissues [29]. What is more, another G protein-coupled receptor TGR5 responsive to bile acids can fine-tune energy homeostasis as a part of the BA–TGR5–cAMP–D2 signaling pathway [30] that can be targeted to improve metabolic control. The PYY or GLP-1 secreted by the intestinal cells mediate the nucleus tractus solitarius (NTS) in the brain via the vagus nerve and the circulatory system. Then, the signal is transmitted to ARC (arcuate nucleus) in the hypothalamus to enhance the satiety [31]. What is more, SCFAs existing in human cerebrospinal fluid work as an important energy source for glial cells and initiate peripheral effects such as enhanced leptin production by adipose tissue or diminished ghrelin production in the stomach [32]. Further investigations are needed to elucidate the complicated gut-microbiota-brain axis and the potential of gut-microbiota-targeted strategies, such as dietary interventions and faecal microbiota transplantation (FMT) that help patients to live a healthy weight throughout life. Some researchers have improved pseudomembranous colitis by faecal microbiota transplantation [33].

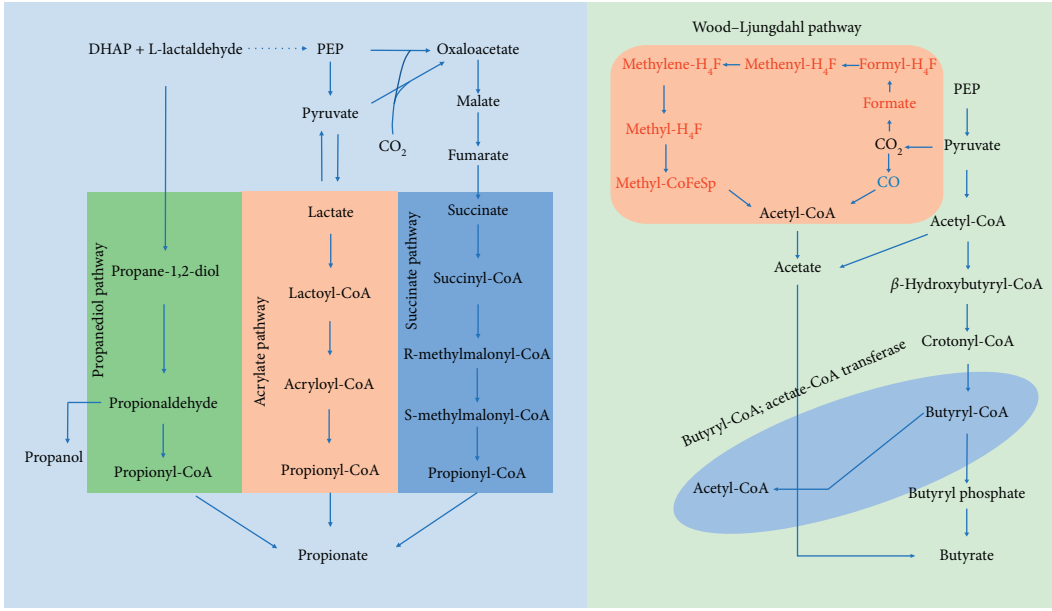

Figure 1: Pathways for biosynthesis of propionic acid, acetic acid, and butyric acid in microorganism.

2.2. Relationship between Cancer and SCFAs. Each nucleosome contains a nucleosome core, composed of an octameric complex of the core histone proteins, which forms a spool to wrap 145–147 bp of DNA [34]. The nucleosome core with about 165 bp of DNA together with the linker histone is called the chromatosome. The level of histone acetylation can influence the DNA replication; thereby, it determines cell proliferation in some way. There are two enzymes called histone acetyltransferase (HAT) and histone deacetylase (HDAC), which promote gene transcription and inhibit gene transcription, respectively. When losing the steady state of some gene expressions regulated by these two enzymes, the cells get high-risk differentiating into cancer cells. The SCFAs are one of the well-known HDAC inhibitors (HDACi) which have been used for cancer therapy. Among these SCFAs, the butyrate is the most pop and promising modulator of cancer and immune homeostasis. The butyrate is the primary energy source for colonocytes by transporting into colonocytes, entering the mitochondria, and undergoing β-oxidation to acetyl-CoA. Consequently, the acetyl-CoA enters the TCA cycle resulting in the reduction of NAD + to NADH, which enters the electron transport chain culminating in ATP production with CO_2 as a byproduct. Thus, butyrate has been shown to stimulate cell proliferation in a low concentration as a HAT activator. However, the nutritional function of butyrate is also important that it is proven to have a regulation on autophagy when the colonocytes are in an energy-deprived state via AMPK and p27 [35]. Naturally, butyrate also exerts antiproliferative and anticancer effects when tumor cell lines are exposed to it in vitro, primarily through HDAC inhibition. The Warburg effect (aerobic glycolysis) indicates that the cancerous colonocytes prefer glucose rather than butyrate as the energy substrate. For this reason, butyrate could be accumulated to a high concentration where it can protect against colorectal cancer as HDACi. It is interesting that butyrate has chance to play a HDACi role just because of the "strange food preferences" of cancerous colonocytes. The strange food preferences mean that cancerous colonocytes prefer glucose as the energy source rather than butyrate. In conclusion, the butyrate has totally different functions in different situations. A recent study showed that gut microbial production of butyrate stimulated polyp formation in a genetic mouse model of colorectal cancer ($Apc^{Min/+}Msh2^{-/-}$) [36]. The keypoint is that the polyp formation is considered as the marker of colorectal cancer. Thereby, the accurate relationship between butyrate and host disease development is not totally rigorously studied yet. One day, using butyrate as clinical application should take more individual differences and situations into account.

2.3. Relationship between Short-Chain Fatty Acids and Inflammation. SCFAs can modulate the progression of inflammatory diseases either by inhibiting histone acetylase (HDAC) activity, and thereby affecting gene transcription, or through the activation of metabolite-sensing G-protein coupled receptors (GPCRs) such as GPR43. Numerous works have proven that the SCFAs are related to decreasing of cytokines such as IL-6 and IL-8 in human macrophages [37] and TNFα in peripheral blood mononuclear cells (PMBCs) [38]. These inflammation-related phenomena are bound up with the HDAC inhibition role of SCFAs. There are general two steps for maturation of cytokines: proinflammatory and inflammatory. Thus, the anti-inflammatory effect of SCFAs could mediate inflammation by inhibiting gene which encodes cytokines or the mediator involved in production of mature inflammatory cytokines. Butyrate and propionate are found inhibiting the NF-kB pathway which is activated to release inflammatory cytokines. Thus, the inhibition role of SCFAs to HDAC may work through modulating NF-kB activity via controlling DNA

transcription [38]. Macrophage is one kind of white blood cells, derived from monocytes. Once stimulated, macrophages rapidly produce a large number of TNF-α, IL-1β, IL-6, NO, and arachidonic acid derivatives [39]. Numerous studies have established a role for butyrate that it inhibits macrophage migration mediated by LPS via reducing the transcriptional activity of Src (a nonreceptor tyrosine kinase) [40]. Regulatory T cells (Tregs) are considered sensitive to HDAC inhibition, which may be resulted by increased Foxp3 (forkhead box P3) induction through acetylation at *FoxP3* locus [41, 42]. Foxp3 is a transcription factor necessary for Treg development and function. Thus, SCFAs could mediate proliferative and functional capabilities of Tregs via *Foxp3*. The butyrate could slightly diminish the proliferation of Tregs but enhance the inhibitory ability on T cell proliferation mediated by CTLA-4 [43]. SCFAs are also important for regulating effector T cells such as $CD4^+$ and $CD8^+$ T cells particularly in regards to increased function and differentiation.

The integrity of epithelial is important for intestinal homeostasis because a leaky intestinal mucosal barrier allowed more intestinal microbial metabolites appear at where they should not be, which may initiate lots of unnecessary inflammation. SCFAs showed increasing anti-inflammatory IL-18 secretion by intestinal epithelial cells (IECs) [44], and IL-18 is a cytokine promoting gut epithelial integrity [45]. Butyrate-stimulated signaling of GPR109A could induce differentiation of Tregs and IL-10-producing T cells [46]. While GPR43 is activated by all three SCFAs, GPR109A is activated only by butyrate [47, 48]. Acetate was shown to promote the release of ROS (reactive oxygen species) when added on mouse neutrophils by activating GPR43 [49]. ROS is thought to upregulate or inhibit inflammation in a concentration-dependent manner. Therefore, the specific functions of SCFAs on controlling inflammation are supposed to be discussed in multiple views. The GPR43 also activates the NLRP3 inflammasome, which is critical for intestinal homeostasis. There are two stages of NLRP3 inflammasome including priming phase and signal activation [50]. The GPR43 activated by acetate initiate the hyperpolarization due to K+ efflux or successive to Ca^{2+} mobilization happening, which activates the NLRP3 inflammasome [44]. This is consistent with the downstream increasing IL-18. This beneficial role on epithelial integrity was confirmed in a model of dextran sulphate sodium-(DSS-) induced colitis in vivo in which the protective role of dietary fiber was mediated through NLRP3 activation in the epithelial compartment following GPCR activation [44]. In general, the SCFAs are multifunctional gut microbial metabolites that are of benefit to the host. The applications of it on different diseases should be more cautious because of its multifunction, which could initiate other chain reactions that we do not hope.

2.4. Aromatic Amino Acid Metabolites. The human digestive system will hydrolyze the proteins from all kinds of food into amino acids with the help of various proteases. A growing body of knowledge [51, 52] is accumulating by metabolomics that points the gut microbiota is also a mediator of the host health status via amino acids metabolism. The aromatic amino acid is called essential amino acid including tyrosine, tryptophan, and phenylalanine, which cannot be synthesized in vivo. Histidine is also an aromatic amino acid because of its imidazole ring. Microbially produced imidazole propionate from histidine is proven to impair insulin signaling through mTORC1 [9]. Thus, interactions among the gut microbiota, diet, and the host potentially contribute to the development of metabolic diseases and deserve more research.

2.5. Tryptophan Metabolites. Since the tryptophan (Trp) is not produced by animal cells, human rely on exogenous, mostly dietary intake. Tryptophan and its derivatives, bioactive small molecules, originate from nutrition- and environmental-related sources or are endogenously produced and modulated by the host and its microbiota. The three currently most studied pathways of tryptophan metabolism involved in host-microbiota interactions are as follows [51]: (1) the direct transformation of tryptophan into several molecules, including ligands of the aryl hydrocarbon receptor (AhR), by the gut microbiota; (2) the kynurenine pathway (KP) in both immune and epithelial cells via indoleamine 2,3-dioxygenase (IDO); and (3) the serotonin (5-hydroxytryptamine (5-HT)) production pathway in enterochromaffin cells via Trp hydroxylase 1. We focus on the first pathway because this is an article about gut microbes. The dominant products are indole and its derivatives. Indole, as an interspecies and interkingdom signaling molecule, plays important roles in bacterial pathogenesis and eukaryotic immunity, and indole concentrations of up to 1.1 mM are produced by indole-producing bacteria in the mouse, rat, and human gut [53, 54]. Most indole derivatives are considered as ligands for AhR (aryl hydrocarbon receptor) such as indole-3-acetaldehyde (IAAld) and indole-3-aldehyde (IAld). The AhR recognizes xenobiotics as well as natural compounds such as dietary components and microbiota-derived factors. AhR affects T cell differentiation and Th17 development and upregulates the IL-22 level to maintain the immune homeostasis in the intestinal tract [51, 55]. This is consistent with that there are lots of ligands existing in human gut such as indole as well as its derivatives. Some scientists find that highly adaptive *lactobacilli* are expanded and produce an AhR ligand (indole-3-aldehyde) that contributes to AhR-dependent IL-22 producing. The resulting phenomenon also provides antifungal resistance to fungus *Candida albicans* [56]. The uncovered mechanism provides us a new sight into interactions among host, indigenous bacteria, and harmful foreign pathogens. Other Trp-derived indole derivatives such as I3S (indoxyl-3-sulfate) reduced *Ccl2* and *Nos2* expressions in astrocytes in an AhR-dependent manner [57]. The article also reports that the AhR expression could be enhanced by the IFN-I signaling in astrocytes with upregulation of genes associated with IFN-I signaling. These findings suggest that it is promising to limit

the central nervous system (CNS) inflammation by combing drugs and diets therapy because of the important roles of astrocytes during CNS injury and disease. Another derivative, indole 3-propionic acid (IPA), is proved as an agonist for the pregnane X receptor (PXR) and downregulated the enterocyte inflammatory cytokine tumor necrosis factor-α (TNF-α) via toll-like receptor 4 (TLR4) [58]. In general, tryptophan derivatives are almost harmless to humans and may have an organ-specific or species-specific interaction with the host.

2.6. Phenylalanine Metabolites. The phenylalanine absorbed by the host is either utilized by the host or intestinal microbiota. Mostly, diet phenylalanine is digested into tyrosine with the help of phenylalanine hydroxylase (PAH) and then involved in melanin metabolism. The left is converted to phenylpyruvic and phenylacetic with the help of phenylalanine ammonia lyase (PAL). The patients with phenylketonuria caused by the accumulation of toxic metabolites of phenylalanine have interferences in these two enzymes. Some researchers [59] characterize a pathway from the gut symbiont *Clostridium sporogenes* that generates phenylalanine acid metabolites. This species either metabolizes phenylalanine to corresponding propionic acid derivatives phenylpropionic acid (PPA) with the enzymes encoded by *fldH, fldBC*, and *acdA* or phenylacetic acid (PAA) with the enzyme encoded by *porA*. A recent study [60] shows that PAA serves as the precursor of the gut microbiota-generated metabolite phenylacetylglutamine (PAGln), and phenylacetylglycine (PAGly) would promote cardiovascular disease- (CVD-) relevant phenotypes via host G protein-coupled receptors (GPCRs), including a2A, a2B, and b2-adrenergic receptors (ADRs). It is also worth mentioning that the production of PAGln and PAGly is species-specific, which means the PAA could be the precursor of different phenylalanine derivatives in different biological intestines with different microflora. All these results indicate that what matters are the enzymes that the microbes have, not the microbes themselves. Thus, it will be interesting to create an engineering bacterium which can turn toxic metabolites of phenylalanine into beneficial metabolites with its special enzyme ratio. In this way, people could prevent some diseases induced by losing certain enzyme such as phenylketonuria and enjoy food without any menace from the "rear."

3. Conclusion

The gut microbiome has attracted increasing attention over the last 15 years. However, the abundance of metagenomic data generated on comparing diseased and healthy subjects can lead to the erroneous claim that a bacterium is causally linked with the protection or the onset of a disease. In fact, during the development of diseases, people are constantly changing their eating habits. Thus, the gut microbiome is changing too. We still need more work to go beyond the simple associations, and we need to provide as much as possible more complex analyses (e.g., multiomics and time series measurements) if we want to finally approach the final causality. For example, *P. copri* is found having the opposite effect in diabetes [13, 61]. This is consistent with that there are lots of research studies indicating that gut microbiota brings damage or benefits to host. There are lots of confounding factors that affect the specific role of gut microbiota. Thus, the targeted screening of gut microbiota could be realized by the host through diet control or fecal microbiota transplantation.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that they have no conflicts of interests.

Authors' Contributions

Nan Qi is a lead contact.

Acknowledgments

This work was supported by grants from the National Key Research and Development Program of China (2018YFA0900404), National Natural Science Foundation of China (31870864), and Fundamental Research Funds for the Provincial Universities of Zhejiang (RF-B2020003).

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2021, Article ID 6632266, 13 pages
https://doi.org/10.1155/2021/6632266

Review Article

Modulation of Short-Chain Fatty Acids as Potential Therapy Method for Type 2 Diabetes Mellitus

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Received 9 November 2020; Revised 7 December 2020; Accepted 18 December 2020; Published 4 January 2021

Academic Editor: Meng-Hao Huang

In recent years, the relationship between intestinal microbiota (IM) and the pathogenesis of type 2 diabetes mellitus (T2DM) has attracted much attention. The beneficial effects of IM on the metabolic phenotype of the host are often considered to be mediated by short-chain fatty acids (SCFAs), mainly acetate, butyrate, and propionate, the small-molecule metabolites derived from microbial fermentation of indigestible carbohydrates. SCFAs not only have an essential role in intestinal health but might also enter the systemic circulation as signaling molecules affecting the host's metabolism. In this review, we summarize the effects of SCFAs on glucose homeostasis and energy homeostasis and the mechanism through which SCFAs regulate the function of metabolically active organs (brain, liver, adipose tissue, skeletal muscle, and pancreas) and discuss the potential role of modulation of SCFAs as a therapeutic method for T2DM.

1. Introduction

The alarmingly high worldwide incidence of type 2 diabetes mellitus (T2DM) and its complications has made it one of the major causes of death. T2DM is a major health issue worldwide. The International Diabetes Federation has estimated that 463 million adults worldwide are living with diabetes currently; 90% of whom have T2DM. This estimate is projected to be 700 million by 2045 [1].

Insulin resistance in insulin-sensitive tissues such as the liver, muscle, and adipose tissue and dysfunction of pancreatic β-cells can contribute to the development of hyperglycemia, hyperinsulinemia, insulin resistance, and T2DM [2]. Over the past two decades, information on abnormal signaling by adipocytes and subclinical inflammation that contributes to the prediabetic state has expanded understanding of the complexity of T2DM pathophysiology beyond the classic triumvirate of pancreatic β-cells, skeletal muscle, and the liver (Figure 1) [3].

Intestinal microbiota (IM) has a vital role in the modulation of glucose homeostasis and the pathogenesis of metabolic diseases, including T2DM [3, 4]. IM composition is shifted away from species that produce butyrate in people with prediabetes or T2DM compared with that in controls [5, 6]. Insulin sensitivity is improved in obese individuals after receiving transplantation of fecal microbiota from lean donors, which is associated with an increase in the abundance of acetate- or butyrate-producing bacteria [7, 8]. Animal studies support a causal role for IM in the development of obesity, insulin resistance, and T2DM [9, 10]. In addition, alterations in IM have been associated with the development of diabetes-related chronic low-grade inflammation [11, 12].

Clinical trials have indicated that an increase in the intake of nondigestible carbohydrates (dietary fiber) is a possible nutritional strategy to modulate IM, thereby preventing and alleviating the disease phenotypes of T2DM [13–16]. Such dietary fiber supports the growth of symbiotic

bacteria. In return, fermentation of these indigestible carbohydrates by these bacteria produces short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate. The beneficial effects of dietary fiber are often considered to be mediated by SCFAs through the provision of energy sources and reduction of inflammation, as well as regulation of glucose homeostasis and energy homeostasis [4]. The beneficial effect of SCFAs on glucose control, lipolysis, resting-energy expenditure, body weight, and insulin sensitivity has been shown in animals [17–19] and humans [13, 20–22]. SCFAs have been shown to increase insulin sensitivity and promote glucose homeostasis, so modulation of SCFAs could provide a unique approach to T2DM treatment.

In this review, we discuss recent studies that provide evidence for the role of microbial SCFAs (acetate, propionate, and butyrate) in T2DM pathogenesis. We provide an overview of the biological properties of SCFAs and their impact on metabolic homeostasis. The effects of SCFAs and nondigestible carbohydrates on the metabolism and function of the gut-brain axis, liver, adipose tissue, skeletal muscle, and pancreas in relation to energy homeostasis, insulin sensitivity, and insulin secretion are also discussed (Figure 2) [23]. Finally, we discuss the potential of SCFAs as novel therapeutics for T2DM.

2. Overview of SCFAs

2.1. Metabolism and Systemic Concentrations of SCFAs. SCFAs are saturated fatty acids with chain lengths of 1–6 carbon atoms. Due to a lack of the enzymes essential for digestion of dietary fiber in the human gut, SCFAs are the primary metabolites from the fermentation of incompletely hydrolyzed dietary foods by specific gut microbiota in the colon through various pathways (Table 1) [4]. The total concentration of SCFAs in the gut is 0.5–0.6 mol per day [24] depending on the diet, bacterial composition of the gut, and intestinal transit time [25]. Acetate (C2), propionate (C3), and butyrate (C4) are the most abundant SCFAs found in the gut (≥95%) with a molar ratio of roughly 3 : 1 : 1, respectively [3]. The SCFAs produced in the gastrointestinal tract are absorbed rapidly by colonocytes, with only <10% excreted in feces [26]. SCFAs are absorbed by colonocytes mainly through four transport mechanisms: passive diffusion; exchange with bicarbonate; transport by monocarboxylate transporters (MCTs); through sodium-coupled MCT1 [27].

After being absorbed by colonocytes, SCFAs are used as substrates in mitochondrial β-oxidation and the citric acid cycle to generate energy [28]. Among SCFAs, butyrate is the primary energy source for colonocytes [4], and propionate is a gluconeogenic substrate [17]. SCFAs that are not metabolized in colonocytes are transported to the liver through the portal circulation, where SCFAs are used as energy substrates for hepatocytes by acetyl-CoA synthetases (ACS) [28]. In addition, in the liver, acetate and butyrate are substrates for the synthesis of cholesterol and long-chain fatty acids [29], and propionate is converted into glucose through the tricarboxylic acid (TCA) cycle [27]. Uptake of propionate and butyrate in the liver is significant, whereas acetate uptake in the liver is negligible [3]. This situation arises because of the low substrate affinity of hepatic mitochondrial ACS1 (which can activate acetate) and the absence of cytosolic ACS2 in the liver, which is present in other organs (e.g., heart and skeletal muscles), where it can be utilized as fuel [28]. SCFAs absorbed in the sigmoid colon and rectum can also reach the systemic circulation directly through the inferior vena cava [23]. Consequently, only 40%, 10%, and 5% of microbial acetate, propionate, and butyrate, respectively, reach the systemic circulation. The plasma concentration (in μM) of acetate, propionate, and butyrate has been estimated to be 19–160, 1–13, and 1–12, respectively [23].

In addition, SCFAs can cross the blood-brain barrier (BBB) *via* MCTs to inform the brain of the intestinal metabolic state [30]. In the brain, acetate is used as an important energy source for astrocytes [25]. The concentration of acetate and propionate in the cerebrospinal fluid of healthy individuals is ~31 μM and ~62 μM, respectively [31, 32]. It has been shown that intravenous or colonic infusions of acetate lead to ~3% and ~2% acetate taken up by the brain, respectively [33]. However, butyrate uptake in the brain is very low (only 0.006% of the injected dose in primates) [34]. Moreover, no measurable brain uptake of acetate has been detected up to 76 min after intravenous injection in humans [25].

In summary, SCFAs are small-molecule metabolites produced from microbial fermentation of indigestible carbohydrates. Butyrate and propionate are metabolized mainly in the colon and liver, whereas acetate is the main SCFA to enter the circulation. In addition, circulating levels of acetate and propionate can cross the BBB, but uptake of SCFAs in the brain is very low.

2.2. Cellular Signaling Pathways of SCFAs. SCFAs are used not only as essential energy sources but also function as signaling molecules because they activate orphan G protein-coupled receptors (GPRs) and inhibit histone deacetylases (HDACs). In this way, they exert several effects to improve metabolic homeostasis and energy homeostasis. The interactions of SCFAs with specific cellular signaling pathways have a potentially key role in SCFAs-mediated regulation of T2DM pathogenesis and are described below.

2.2.1. GPR Activation. GPR41 and GPR43 are the best-studied SCFA receptors, which have been identified as free fatty acid (FFA) receptor 3 (FFAR3) and FFAR2, respectively [35]. GPRs are seven transmembrane-spanning proteins that detect extracellular molecules and induce intracellular signaling cascades and cellular responses involving different G protein heterotrimers or arrestins [36]. If GPRs are activated by ligands, the Gα subunit of the heterotrimers (which bears most responsibility for coupling with receptors) disassociates from the G$\beta\gamma$ subunits and further affects intracellular signaling proteins depending on the type of Gα subunit (e.g., $G\alpha_{i/o}$ and $G\alpha_{q/11}$) [37]. GPR41 couples with pertussis toxin-sensitive $G\alpha_{i/o}$ proteins. GPR43 couples not only with $G\alpha_{i/o}$ but also with the pertussis toxin-insensitive $G\alpha_{q/11}$ proteins [38]. Activation of GPR41 and GPR43 by SCFAs *via* $G\alpha_{i/o}$

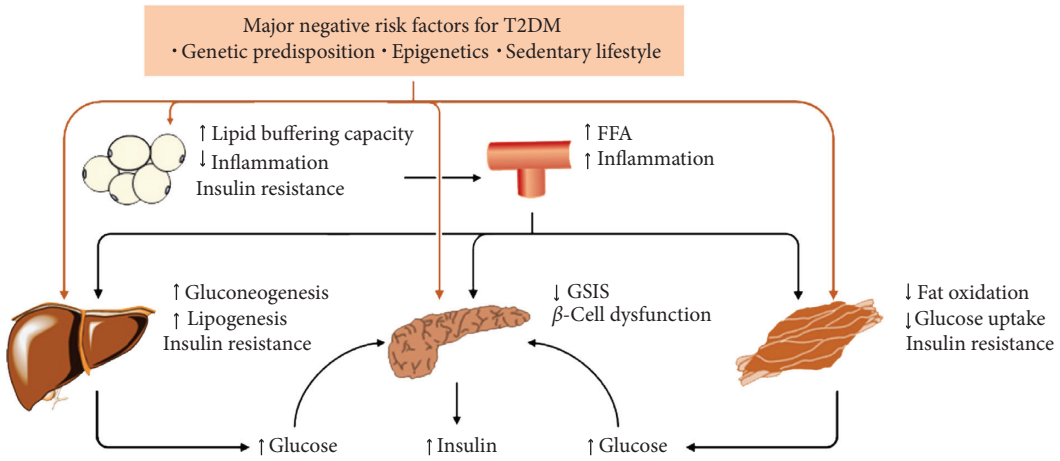

Figure 1: T2DM pathophysiology. A matrix of negative genetic, epigenetic, and lifestyle factors interact with one another and induce dysfunction of pancreatic β-cells and insulin resistance in the liver, skeletal muscle, or adipose tissue, thereby leading to the development of hyperinsulinemia and hyperglycemia. Moreover, once reduced lipid-buffering capacity in adipose tissue occurs, circulating lipid concentrations increase, leading to ectopic fat storage in the liver, skeletal muscle, and pancreas as well as the development of insulin resistance and dysfunction of pancreatic β-cells. In addition, inflamed adipose tissue results in a low-grade systemic inflammation, which contributes to the development of insulin resistance and T2DM. FFA, free fatty acid; GSIS, glucose-stimulated insulin secretion; T2DM, type 2 diabetes mellitus.

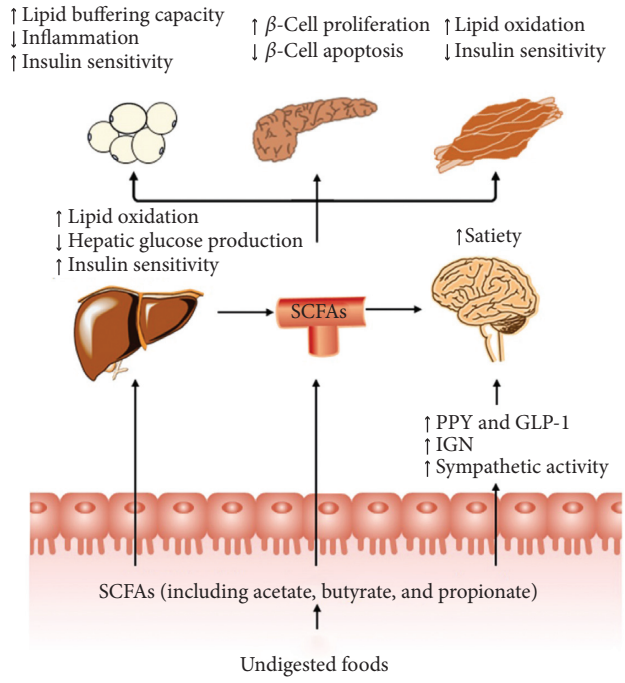

Figure 2: Impact of gut-derived SCFAs in T2DM. SCFAs (acetate, butyrate, and propionate) are produced from the fermentation of indigestible foods in the distal intestine by gut microbiota. In the distal gut, acetate, propionate, and butyrate stimulate the secretion of the "satiety" hormones GLP-1 and PYY in enteroendocrine-L cells, which leads to metabolic benefits upon satiety and glucose homeostasis. Furthermore, butyrate and propionate induce IGN and sympathetic activity, thereby beneficially leading to control of body weight and glucose homeostasis. Very little propionate and butyrate and a high concentration of acetate reach the circulation. They can also affect the metabolism and function of peripheral tissues directly (e.g., liver, adipose tissue, and muscle). Furthermore, circulating levels of acetate and propionate might cross the BBB and regulate satiety *via* CNS-related mechanisms. BBB, blood-brain barrier; CNS, central nervous system; GLP-1, glucagon-like peptide-1; GSIS, glucose-stimulated insulin secretion; IGN, intestinal gluconeogenesis; PYY, peptide YY; SCFAs, short-chain fatty acids; T2DM, type 2 diabetes mellitus.

inhibits the activity of adenylate cyclase (AC), which leads to a reduction of cyclic adenosine monophosphate (cAMP) generation. GPR43 activation by SCFAs *via* $G\alpha_{q/11}$ activates phospholipase C (PLC), promotes activation of inositol trisphosphate (IP3) receptors located on the endoplasmic reticulum, and leads to Ca^{2+} release from the endoplasmic

Table 1: Precursors, biosynthetic pathways, and producers of SCFAs.

SCFAs	Precursors	Pathways	Producers
Acetate	Pyruvate	Acetyl-CoA pathway	Most intestinal bacteria, such as *Bacteroides* spp., *Prevotella* spp., *Ruminococcus* spp., *Bifidobacterium* spp., and *Akkermansia muciniphila*
		Wood–Ljungdahl pathway	*Clostridium* spp., *Streptococcus* spp., and *Blautia hydrogenotrophica*
Propionate	Phosphoenol-pyruvate	Succinic pathway	*Bacteroides* spp., *Dialister* spp., *Phascolarctobacterium succinatutens*, and *Veillonella* spp.
		Acrylic pathway	*Coprococcus catus* and *Megasphaera elsdenii*
	Deoxyhexose	Propanediol pathway	*Ruminococcus obeum*, *Roseburia inulinivorans*, and *Salmonella* spp.
Butyrate	Acetyl-CoA	Acetate CoA-transferase pathway	*Faecalibacterium prausnitzii*, *Eubacterium hallii*, and *Roseburia* spp.
		Butyrate kinase pathway	*Coprococcus catus* and *Coprococcus comes*
	Proteins	Lysine pathway	*Odoribacter splanchnicus* and *Alistipes putredinis*

reticulum (Figure 3) [35]. G$\beta\gamma$ subunits also activate various molecules, such as the isoform of AC and phospho-inositide-3-kinase. Moreover, β-arrestin-2 (a negative regulator of GPR signaling) is recruited by GPR43 activation, which desensitizes GPR signaling *via* G proteins and induces the endocytosis of GPRs [39]. In addition, β-arrestin functions as a "scaffold protein" to link GPRs to intracellular signaling pathways and consequently activates the mitogen-activated protein kinase (MAPK) cascade [40].

The potencies of individual SCFAs in activating GPR43 in humans are ordered as $C2 = C3 > C4$, and those for GPR41 are ordered as $C3 > C4 > C2$ [35]. GPR43 is expressed in adipose tissue, intestines, pancreatic β-cells, and immune tissues [35, 41]. GPR41 is expressed in adipose tissues, intestines, the peripheral nervous system, and immune cells [35, 41]. Thus, GPR43 and GPR41 have important roles in the SCFAs-induced beneficial effects of various physiological functions and systemic glucose homeostasis.

2.2.2. HDAC Inhibition. HDACs are a group of proteases that deacetylate histones and nonhistone proteins, ensuring that they can negatively charge DNA, "curl" chromatin, and inhibit gene transcription. The opposing enzymes, histone acetyltransferases (HATs), transfer the acetyl group of acetyl-CoA to histones, dissociate DNA from histone octamers, relax the nucleosome structure, make transcription factors bind to DNA-binding sites, and activate gene transcription. HATs and HDACs maintain acetylation of histone and nonhistone proteins in dynamic equilibrium to regulate physiological functions, such as inflammation, pancreas development, glucose metabolism, and insulin signaling [24, 42]. However, overexpression and aberrant recruitment of HDACs are associated with T2DM pathogenesis [43].

SCFAs are natural inhibitors of HDACs. SCFAs can act directly on HDACs by entering cells through transporters or act indirectly on HDACs through GPR activation [44]. Evidence highlighting the beneficial effects of SCFA-mediated HDAC inhibition in T2DM has arisen mostly from studies using butyrate. Butyrate has been shown to inhibit HDAC3, suppress peroxisome proliferator-activated receptor (PPAR)-α expression, and induce hepatic fibroblast growth factor 21 (*Fgf21*) transcription, which promotes lipid oxidation, triglyceride (TG) clearance, and ketogenesis in the liver [45]. Furthermore, the butyrate-mediated inhibition of HDAC increases nuclear factor erythroid 2-related factor 2 (*Nrf2*) expression *via* the coactivator P300 at the *Nrf2* promoter [46], which has been shown to lead to an increase of its downstream targets to protect against diabetes-induced oxidative stress and inflammation in diabetic mice [47]. In addition, the deacetylase inhibition induced by butyrate also enhances mitochondrial activity [48].

Propionate and acetate can also improve T2DM by inhibiting HDACs [49]. In 3T3-L1 adipocytes, propionate (20 mM) was shown to increase the rate of lipolysis *in vitro* through HDAC inhibitory activity to a similar extent as that by butyrate (5 mM). However, acetate (5 mM) did not affect lipolysis [49]. This may be because of the high mitochondrial and lipogenic demand for two-carbon acetyl units from exogenous acetate in adipocytes, leaving it to contribute to histone acetylation only sparingly [50]. However, acetate can inhibit HDACs in the liver, leading to amelioration of hepatic lipid dysregulation and enhancement of insulin sensitivity in diabetic rats [51] Moreover, acetate released from histone deacetylation can be "recaptured" to supply the acetyl units for HATs [50], indicating a complex role of acetate in histone acetylation.

Altogether, the interaction with GPRs and/or inhibition of HDACs have critical roles in the beneficial effects of SCFAs in T2DM pathogenesis. However, understanding of how SCFAs inhibit HDACs and regulate posttranslational modifications is in its preliminary stages. Future studies should make use of epigenetics and transcriptomics to obtain comprehensive understanding of the part played by SCFAs in T2DM pathogenesis.

3. Beneficial Effects of SCFAs on Energy and Glucose Homeostasis

A deficiency of SCFAs has a central role in T2DM development [52]. A metagenome-wide association study of IM in Chinese patients with T2DM showed a moderate degree of intestinal dysbiosis with a lower abundance of butyrate-producing bacteria [5]. Consistently, fecal-metagenome analyses of European women with T2DM have revealed significant depletion of butyrate-producing microbiota,

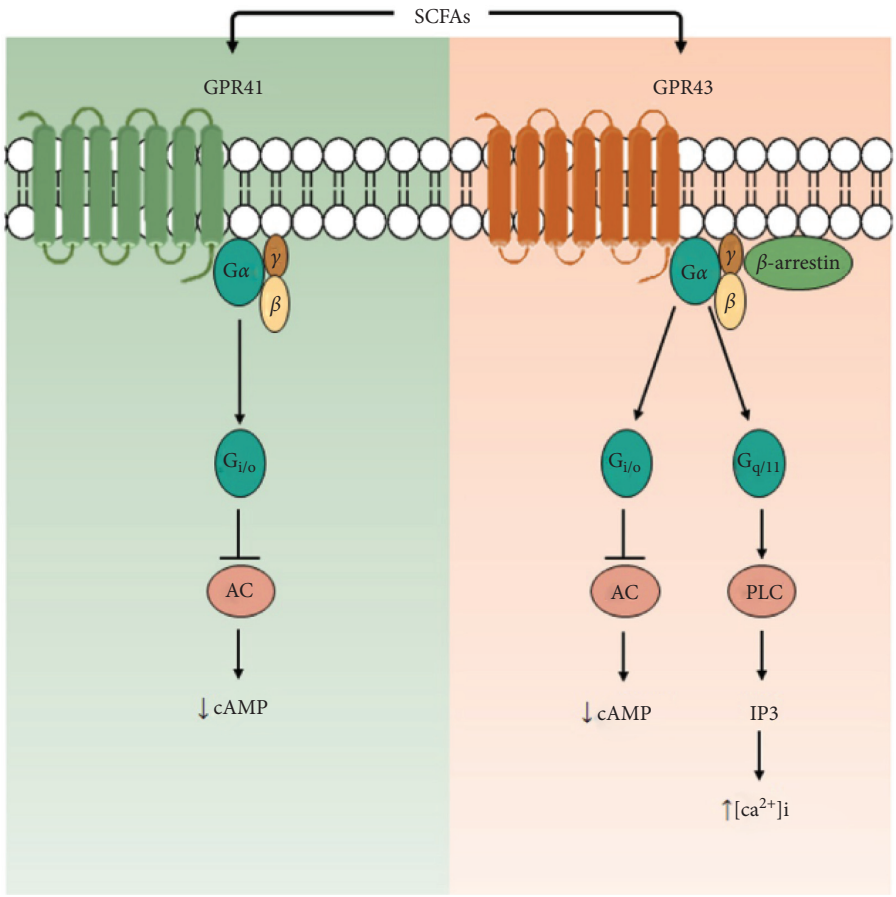

Figure 3: Signaling pathways of GPR41 and GPR43 activated by SCFAs. The signaling pathway downstream of each receptor is illustrated. AC, adenylate cyclase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; GPR, G protein-coupled receptor; IP3, inositol trisphosphate; PLC, phospholipase C; SCFAs, short-chain fatty acids.

which exhibited a negative correlation with serum levels of insulin, C-peptide, and TG [6].

The production of SCFAs induced by dietary fiber and resistant starch can improve insulin sensitivity and glucose homeostasis in humans. Supplementation with high amylose-resistant starch has been shown to reduce body fat, increase levels of acetate, early-phase insulin, and glucagon-like peptide- (GLP-) 1, and increase the number of gut microbes that produced acetate in volunteers with normal body weight [53]. Rye-based bread supplemented with resistant starch type 2 increased insulin sensitivity, fasting levels of peptide YY (PYY), GLP-2, acetate, butyrate, and total SCFAs in healthy middle-aged individuals [54]. Furthermore, administration of dietary fiber in T2DM patients enhanced a group of SCFA producers and improved glycated hemoglobin levels, partly *via* increased GLP-1 production, which resulted in T2DM alleviation [13].

Direct administration of SCFAs can influence the homeostasis of glucose metabolism and optimize insulin sensitivity. Acute oral administration of sodium propionate increased resting-energy expenditure and was accompanied by increased whole-body lipid oxidation, in fasted healthy volunteers [21]. Rectal administration of SCFAs mixtures for four days increased fasting fat oxidation, energy expenditure, and plasma levels of PYY and decreased fasting free-glycerol concentrations in normoglycemic overweight men [20].

Overall, the clinical data stated above suggest that modulation of SCFAs could prevent or alleviate T2DM. Nonetheless, those are preliminary results from small-sample studies on the effects of SCFAs on the host's metabolism. More prospective studies involving much larger and more diverse sample sets are needed to investigate further the effects of long-term administration (through different modes of administration) of SCFAs on T2DM.

3.1. SCFAs Regulate the Brain's Control of Energy Homeostasis. SCFAs derived from the gut can positively influence the effect of the brain in controlling energy homeostasis and glucose homeostasis. These include reduced energy intake, body weight, hepatic glucose production, and improved insulin sensitivity, all of which reduce T2DM. SCFAs affect the gut-brain axis by regulation of secretion of metabolic hormones, induction of intestinal gluconeogenesis (IGN),

stimulation of vagal afferent neurons, and regulation of the central nervous system (CNS) [25].

3.1.1. Regulation of Secretion of Metabolic Hormones. Modulation of the hormones associated with satiety is one of the best-studied mechanisms by which SCFAs regulate appetite and energy intake. Studies [13, 55, 56] have shown that plasma levels of GLP-1 and PYY in overweight adults are increased after acute rectal infusion of sodium acetate [57, 58] or SCFAs mixtures [20] or an oral insulin-propionate ester [59]. SCFAs can trigger the secretion of GLP-1 and PYY from enteroendocrine-L cells [60–64] through GPR41 and GPR43 and/or GPRs-independent signaling by being metabolized to adenosine triphosphate (ATP) as a colonocyte energy source [65]. Production of these gut hormones leads to activation of appetite- and food intake-related brain activity *via* humoral and neural pathways [25]. GLP-1 is an anorexigenic incretin hormone that enhances glucose-dependent insulin secretion [66]. The interaction between circulating levels of GLP-1 and food reward-related central nervous activity in the dorsolateral prefrontal cortex can achieve body weight loss in obese individuals [67]. PYY is costored and cosecreted with GLP-1 by enteroendocrine-L cells [35]. PYY is another anorexic neuropeptide that has been shown to inhibit gastrointestinal movement, suppress appetite, and improve the survival and function of pancreatic β-cells, with obvious benefits for T2DM [68].

Moreover, SCFAs can affect the secretion of other metabolic hormones, including leptin and ghrelin. The BBB and vagus nerve are implicated in the effect of these hormones on the brain [69, 70]. Leptin is an anorexic hormone secreted from adipose cells and activates hypothalamic proopiomelanocortin neurons to inhibit food intake [71]. However, the effect of SCFAs on the regulation of leptin production seems controversial [69]. *In vitro* studies have demonstrated consistently that SCFAs stimulate leptin secretion in adipocytes through GPR41 activation. *In vivo* studies have shown that body fat (rather than SCFAs) is the main driver for leptin synthesis [69]. Ghrelin is the main "hunger" hormone. It is produced by ghrelin cells in the stomach and duodenum and activates hypothalamic somatostatin neurons to promote feeding [70]. Acute increases in levels of colonic-derived SCFAs by ingestion of inulin reduce ghrelin levels in lean and obese individuals [72]. However, chronic intragastric infusion of acetate has been found to activate the vagal nervous system and, in turn, stimulate ghrelin secretion in rats, which may promote hyperphagia and metabolic syndrome [73].

Hence, SCFAs may exert beneficial effects on appetite suppression and lower energy intake mainly by regulation of metabolic hormones such as GLP-1, PYY, leptin, and ghrelin. Further investigations on the direct impact and underlying mechanism of action of SCFAs on these hormones are needed to clarify the mechanism through which SCFAs affect energy homeostasis.

3.1.2. IGN Induction. IGN is a brain signal derived from the intestine that plays an important part in glucose homeostasis and energy homeostasis [74]. IGN is induced during the postabsorptive period [17, 74]. It can induce beneficial effects on metabolism, such as a decrease in food intake, acquisition of a food preference, rapid-phase secretion of insulin, and reduction of hepatic glucose production, *via* gut-brain glucose signaling [75]. Because of intense glycolysis in the intestine, physiological portal hypoglycemia occurs during the postabsorptive period. This signals to the brain *via* sodium-coupled glucose cotransporter 3 in the hepatic-portal area (a key link in the portal glucose-sensing process) and promotes the reonset of hunger [74, 75]. IGN activation counterbalances the lowering of portal-area glucose, resulting in hunger inhibition. Interestingly, the hunger-curbing effect of the portal glucose signal induced by IGN involves activation of afferents from the spinal cord and specific neurons in the parabrachial nucleus, rather than the afferents from the vagal nerves [74, 76].

The SCFAs butyrate and propionate activate IGN *via* complementary metabolic processes [17, 77]. Butyrate-induced activation of IGN is mediated by an increase in ATP, which increases intracellular cAMP, but not *via* $G\alpha_{i/o}$- or $G\alpha_{q/11}$-mediated mechanisms [17]. Propionate (itself a gluconeogenic substrate) activates GPR41 in periportal nerves. It stimulates a gut-brain neural circuit that induces IGN by promoting the local release of vasoactive intestinal peptides [77] and upregulation of methylmalonyl-CoA mutase (the key enzyme in propionate metabolization) [17]. The position of SCFAs upstream of IGN-mediated gut-brain glucose signaling indicates that this function can activate the portal nervous system and its related benefits.

3.1.3. Stimulation of Vagal Afferent Neurons. SCFAs can suppress energy intake by stimulating vagal afferent neurons directly. Oral (but not intravenous) administration of butyrate reduces food intake by activating the gut-brain neural circuit, resulting in inhibition of orexigenic neuropeptide Y neurons in the hypothalamus and neurons within the tractus solitarius and dorsal vagal nuclei [19]. Furthermore, intraperitoneal administration of three SCFAs was shown to reduce food intake by activating vagal afferents in fasted mice in the order $C4 > C3 > C2$ [78]. This effect was attenuated by systemic capsaicin treatment and hepatic-branch vagotomy that desensitized vagal afferents. Moreover, butyrate-induced sympathetic activity increased phosphorylation of extracellular signal-regulated kinase-1/2 and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) signaling in nodose ganglion neurons [78]; this could have been mediated by GPR41 activation in nodose ganglion neurons [79]. Moreover, the gut-brain neural circuit induced by SCFAs may also promote fat oxidation by activating brown adipose tissue [19]. Surprisingly, chronic intragastric infusion of acetate activated vagal nervous and stimulated ghrelin secretion in rats, which increased caloric intake and weight gain [73]. A recent study revealed that activation of the right (but not the left) upper-gut vagal sensory ganglion stimulated the parabrachio-nigral pathway (which regulates food

consumption) in mice [80]. Thus, investigating whether these asymmetric gut-brain pathways of vagal origin might be a mechanism that mediates the differential effects of SCFAs on vagal activity would be worthwhile.

3.1.4. CNS Regulation. Only a few rodent studies have demonstrated that SCFAs derived from the colon can cross the BBB directly and affect the CNS, which are related to appetite and energy intake. Intracerebroventricular injections of acetate were shown to reduce food intake significantly at 1-2 h after injection [33]. In the hypothalamus, acetate is oxidized in the TCA cycle, leading to inactivation of AMP-activated protein kinase (AMPK) and simultaneous inhibition of acetyl-CoA carboxylase (ACC). This action stimulates proopiomelanocortin neurons and suppresses agouti-related peptide neurons, thereby inducing appetite inhibition [33]. Importantly, due to the invasive nature of the studies on the effects of SCFAs on brain function, those studies were limited to *in vitro* and animal studies. Whether colonic-derived SCFAs have a similar role in the human CNS merits further investigation.

In summary, the studies mentioned above provide evidence for the therapeutic benefit of SCFAs on energy homeostasis *via* regulation of appetite-regulating hormones and sympathetic activity. In addition, butyrate and propionate might promote metabolic benefits on glucose homeostasis and body weight *via* induction of IGN, and acetate might directly induce appetite inhibition *via* a central mechanism in the CNS.

3.2. SCFAs Induce Preservation of Hepatic Metabolic Function. The gut-liver axis is involved in the beneficial effect of SCFAs on T2DM chiefly by preserving the metabolic function of the liver, including decreasing hepatic glucose production [81] and lipid accumulation [82], modulating hepatic mitochondrial function, and increasing glucose uptake and glycogen synthesis in hepatocytes [83].

SCFAs sustain hepatic metabolic function and insulin sensitivity mainly *via* an AMPK-dependent pathway [84]. AMPK is a necessary regulator for maintaining the homeostasis of the metabolism of energy, glucose, and lipids in the liver. AMPK and its downstream fatty acid oxidation genes increased by acetate administration alleviate hepatic lipid accumulation in mice suffering from nonalcoholic steatohepatitis [85]. Butyrate-induced AMPK increases levels of PPAR coactivator (PGC)-1α or ACC in insulin-resistant hepatocytes and in mice [18, 86], which modulate the mitochondrial functions and increased use of substrates (especially fatty acids), leading to reduction of intracellular lipid accumulation. Moreover, propionate activates AMPK in human HepG2 hepatocytes, resulting in downregulation of expression of the gluconeogenesis-related genes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase [81]. Importantly, knockdown of GPR43 expression prevents propionate-induced phosphorylation of AMPK [81]. Activation of GPR43 by SCFAs induces expression of $G\alpha_{i/o}$ and $G\alpha_{q/11}$, as well as recruitment of β-arrestin-2. Although $G\alpha_{i/o}$ reduces the production of cAMP from ATP, which can inhibit AMPK activation, the $G\alpha_{q/11}$-induced increase in $[Ca^{2+}]_i$ activates Ca^{2+}/calmodulin-dependent protein kinase β-dependent phosphorylation of AMPK [81]. Moreover, a recent study showed that β-arrestin-2 also contributes to the GPR43-induced activation of AMPK [86]. In summary, SCFAs-induced activation of AMPK can be attributed to GPR43-induced $G\alpha_{q/11}$ activation and β-arrestin-2 recruitment.

In addition to AMPK activation, SCFAs-induced activation of GPR43 can promote glucose uptake and glycogen metabolism in the liver. In db/db mice and HepG2 cells, butyrate administration was shown to upregulate expression of two glucose transporters and inhibit protein kinase B (Akt) expression which, in turn, activated glycogen synthase kinase 3. This process increased glycogen storage significantly in mice and HepG2 cells [83].

Furthermore, as a broad-spectrum HDAC inhibitor, butyrate also exhibits beneficial effects in the liver by an epigenetic mechanism involving HDAC inhibition. The inhibition of HDAC2 induced by butyrate upregulates expression of hepatic GLP-1R and subsequently promotes GLP-1-dependent activation of insulin pathways. Subsequently, this action stimulates lipid oxidation and improves hepatic steatosis and insulin sensitivity [87]. Butyrate inhibits HDAC3 and HDAC4 significantly, accompanied by an increase in the number of genes participating in β-oxidation of fatty acids, which promotes the biogenesis and function of mitochondria in insulin-resistant hepatocytes [86]. In addition, butyrate induces PPARα activation with enhanced histone H3 acetyl K9 (H3K9Ac) modification on its promoter by HDAC1 inhibition, which leads to upregulation of *Fgf21* expression, and enhanced fatty acid oxidation [45, 88].

Taken together, the results stated above suggest that colonic-derived SCFAs might indirectly affect liver function and metabolism by interacting with GPR43 and inhibiting HDAC. These actions might affect hepatic glucose and glycogen metabolism, fatty acid oxidation, and mitochondrial function, with activation of AMPK, Akt, and PPARα being mediators of these effects. Results from *in vitro* and animal studies seem promising, but there is a dearth of clinical research and very little integration of human and animal studies. Thus, it is necessary to investigate the mechanism through which SCFAs affect metabolic function in human livers.

3.3. SCFAs Improve Dysfunction in Adipose Tissue. Adipose tissue is the most abundant energy store (in the form of TG) in the human body. Adipose tissue is a lipid-buffering mass that increases plasma TG clearance and inhibits the release of fatty acids into the circulation [23]. If energy expenditure is lower than energy intake, the TG stored in adipose tissue and the rate of lipolysis increase. These actions lead to an overflow of lipids, accumulation of lipids in other peripheral tissues, and adipose tissue inflammation, which contribute to T2DM development. SCFAs can regulate lipid metabolism and reduce inflammation [89] in adipose tissue.

SCFAs can alter lipid metabolism in adipose tissue by promoting lipolysis and inhibiting lipogenesis, with activation of AMPK and β3-adrenergic receptors (ARβ3) in mice [90, 91], pigs [92], and rabbits [93, 94]. Clinical data have suggested the metabolic effects of SCFAs on the dysfunction of adipose tissue, as indicated by decreased free-glycerol concentrations [20] or eliminated FFA in plasma [95]. Acetate induces upregulation of lipolysis-related factors [93, 94], which may due to activation of the GPR43-AMPK pathway [81, 86]. Butyrate administration also activates ARβ3-mediated lipolysis in adipose tissue by enhancing acetylation of lysine 9 on histone H3 of the ARβ3 promoter [91].

Furthermore, SCFAs can promote adipogenesis in rabbit adipocytes [93, 94] and 3T3-L1 adipocytes *in vitro* [96]. Treatment with acetate, propionate, or butyrate accelerated the differentiation of 3T3-L1 adipocytes by upregulating expression of the enzymes of fatty acid metabolism, including lipoprotein lipase, adipocyte fatty acid-binding protein 4, fatty acid transporter protein 4, and fatty acid synthase (FAS) [96]. GPR41/43-mediated MAPK signaling may be involved in SCFA-induced adipocyte differentiation in rabbits by upregulating downstream adipocyte-specific transcription factors, including PPARγ and differentiation-dependent factor 1 [93, 94]. Nevertheless, clinical studies suggest that SCFAs are not correlated with adipocyte differentiation. Acetate and propionate do not affect the differentiation of human preadipocytes [97]. More evidence is needed to clarify the effects of SCFAs on human adipogenesis. However, propionate inhibits adipogenic differentiation of human chorion-derived mesenchymal stem cells (cMSCs), which is elicited by silencing of GPR43 expression [98]. Since almost all of the tissues contain varying proportions of MSCs, inhibiting the adipogenic differentiation of MSCs with SCFAs may be a way to inhibit the undesirable formation of adipocytes throughout the body.

Few studies have evaluated the direct effect of SCFAs on adipose tissue inflammation. Adipose tissue inflammation plays a part in the development of insulin resistance and T2DM [3, 23]. Macrophages are the most studied of the adipose-derived immune populations. They are believed to be major sources of inflammatory cytokines in response to a high-fat diet (HFD) and obesity [99]. Butyrate reduces macrophage infiltration in adipose tissue in mice, which results in the improvement of insulin sensitivity [100]. Moreover, *in vitro* studies have revealed that GPR41/43-induced activation of $G\alpha_{i/o}$ protein is involved in the way propionate reduces tumor necrosis factor-α release in macrophages [89]. Those data suggest that SCFAs might counteract adipose tissue inflammation directly.

In summary, SCFAs can increase the lipid-buffering capacity of adipose tissue by promoting lipolysis, inhibiting lipogenesis, and promoting adipogenesis of adipose precursor cells, but inhibiting adipogenic differentiation of cMSCs (which have been identified in numerous tissues). SCFAs may prevent chronic low-grade inflammation by reducing macrophage infiltration in adipose tissue. Most evidence has been obtained from *in vitro* studies of adipocytes derived from animals, which cannot directly reflect the status of SCFAs in human adipocytes. Hence, future research should focus on the metabolic function of SCFAs in humans and human-cell models.

3.4. SCFAs Enhance Insulin Sensitivity in Skeletal Muscle. In addition to inhibition of ectopic fat storage by reducing circulating lipid concentrations, SCFAs might also contribute to improvement in skeletal muscle insulin sensitivity by decreasing fatty acid synthesis and increasing lipolysis in skeletal muscle. SCFAs can increase the lipid-oxidation capacity of skeletal muscle by improving mitochondrial function [101, 102]. Mitochondria are essential for maintaining energy homeostasis in skeletal muscle by adaptive reprogramming to meet the demands imposed by an increased lipid supply [103]. Supplementation with butyrate enhances mitochondrial biogenesis in skeletal muscles as indicated by upregulation of expression of most mitochondrial DNA-encoded genes. This action may be involved in GPR41/43 and PGC-1α pathways [101]. The butyrate-mediated activity of HDAC inhibitors may also induce nucleosome positioning, which is associated with improving β-oxidation and insulin sensitivity [102]. Moreover, SCFAs can decrease fatty acid synthesis by downregulating mRNA expression of FAS, ACC, and PPARσ in longissimus dorsi [92]. However, studies on the effects of SCFAs on the metabolic function of human skeletal muscle are lacking. Therefore, future research should pay attention to SCFAs uptake in skeletal muscle as well as their effect and mechanism of action on oxidative metabolism in human muscle.

3.5. SCFAs Regulate Pancreatic Function. Besides the indirect effect of SCFAs on insulin secretion *via* the parasympathetic nervous system and regulation of circulating lipid concentrations, studies have suggested the direct effect of SCFAs on pancreatic β-cells. *In vitro* [104] and animal [105, 106] studies revealed that propionate and butyrate inhibited the apoptosis of pancreatic β-cells and promoted their proliferation, which led to an increase in pancreatic β-cell mass and improved glucose homeostasis. This effect may be related to the SCFAs-mediated HDAC inhibitory activity inducing activation of the MAPK pathway [106], and inhibition of the endoplasmic reticulum stress-related protein kinase R-like ER kinase (PERK)-CCAAT/enhancer-binding protein homologous protein (CHOP) pathway [105]. The MAPK pathway has pivotal roles in the proliferation and differentiation of pancreatic β-cells [106] and PERK-CHOP pathway has an important role in the apoptosis of pancreatic β-cells [105].

Moreover, SCFAs might influence pancreatic function by regulating insulin secretion. Dietary supplementation with propionate has been shown to increase glucose-stimulated insulin secretion (GSIS) in humans, an effect that is dependent upon $G\alpha_{q/11}$-mediated signaling consequent to GPR43 activation [104]. However, propionate inhibited glucose-dependent insulin secretion, which occurred through a $G\alpha_{i/o}$ pathway [107]. Interestingly, butyrate supplementation reduced insulin secretion at a basal condition (2.8 mM glucose) but increased GSIS (16.7 mM

glucose) released by pancreatic β-cells isolated from HFD mice [82]. Acetate has been shown to strongly reduce plasma insulin levels and GSIS from isolated perfused pancreas tissue from rats [108]. The discrepancies mentioned above may be related to the ability of SCFAs to activate GPR41 and GPR43. Studies on GPR41-or GPR43-knockout mice have found that GPR41 and GPR43 are involved in the insulin secretion activity of pancreatic β-cells [109, 110]. The SCFAs induced GPR41 to activate $G\alpha_{i/o}$ signaling pathways, which reduced cAMP levels in pancreatic β-cells and led to reduced insulin secretion from pancreatic β-cells [111]. Inconsistently, the SCFAs-induced activation of GSIS was attributed to GPR43-and $G\alpha_{q/11}$-dependent actions, which increased $[Ca^{2+}]_i$ and induced insulin secretion [109, 112, 113]. Thus, SCFAs modify the balance between GPR41 and GPR43 signaling in pancreatic β-cells and, therefore, may "fine-tune" insulin secretion to maintain metabolic homeostasis.

The data mentioned above illustrate the ability of SCFAs to regulate pancreatic function and glucose homeostasis. The signaling induced by SCFA-mediated HDACs inhibition contributes to the protection of pancreatic β-cells by inhibiting their apoptosis and promoting their proliferation. In addition, SCFAs might regulate insulin secretion through GPRs pathways. However, the physiological importance of the GPR-based dual-coupled signaling mechanism in insulin secretion is not fully understood. Therefore, more studies are needed for further investigation of the mechanism of the effects of the SCFA-GPR axis on the control of insulin secretion and functioning of pancreatic β-cells.

4. Conclusions and Perspectives

A wide range of preclinical evidence strongly suggests that an increase in SCFAs could be a potential therapeutic method to prevent and/or alleviate T2DM. Evidence in humans is circumstantial, but clinical data indicate the possibility of SCFAs as novel therapeutic agents for T2DM. Observational and intervention studies provide evidence that SCFAs might induce appetite inhibition and affect energy homeostasis by regulating the secretion of appetite-regulating hormones, inducing IGN, stimulating sympathetic activity, and regulating CNS. SCFAs might regulate glucose homeostasis by decreasing glucose production, increasing glucose uptake and glycogen synthesis in the liver, increasing pancreatic β-cell mass, and regulating insulin secretion. Furthermore, SCFAs might improve lipid metabolism by increasing the lipid-buffering capacity of adipose tissue and reduce inflammation in adipose tissue, as well as enhancing fatty acid oxidation and mitochondrial function in the liver and skeletal muscle.

Clinical studies have indicated a causal role for SCFAs in metabolic health. However, the metabolic consequences of direct administration of SCFAs in humans are incompletely understood. Clinical trials are needed to verify these effects on humans. Due to the instability of the SCFAs dose delivered to the target, a novel targeting method for colonic delivery of SCFAs should be developed to achieve more consistent and reliable dosing. The gut-host signal axis may be more resistant to such intervention by microbial SCFAs (especially in the insulin-resistant phenotype), so this method should be tested for ≥3 months. In addition, due to interindividual variability in microbiota and metabolism, factors that may directly affect host substrate and energy metabolism, such as diet and physical activity, should be standardized or at least assessed. Moreover, advanced metabolomics, epigenetics, metatranscriptomics, and metagenomics approaches may provide insight into the impact of SCFAs on maintaining insulin sensitivity and metabolic homeostasis in humans. These emerging technologies may offer great potential for the eventual therapeutic translation of SCFAs in T2DM.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors express sincere thanks to their department and all colleagues for their support. This work was supported by National Natural Science Foundation of China (no. 81790631).

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2020, Article ID 6675206, 8 pages
https://doi.org/10.1155/2020/6675206

Review Article

The Applications of Nanopore Sequencing Technology in Pathogenic Microorganism Detection

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Received 9 October 2020; Revised 4 December 2020; Accepted 18 December 2020; Published 31 December 2020

Academic Editor: Tingtao Chen

Infectious diseases are major threats to human health and lead to a serious public health burden. The emergence of new pathogens and the mutation of known pathogens challenge our ability to diagnose and control infectious diseases. Nanopore sequencing technology exhibited versatile applications in pathogenic microorganism detection due to its flexible data throughput. This review article introduced the applications of nanopore sequencing in clinical microbiology and infectious diseases management, including the monitoring of emerging infectious diseases outbreak, identification of pathogen drug resistance, and disease-related microbial communities characterization.

Infectious disease leads to significant health care burden in the world. Although we have made great progress in the prevention and control of infectious diseases [1], the emerging of new pathogen and reemerging of classical pathogens epidemics still pose serious threats to human health. The number of cases of respiratory infections and *tuberculosis* in the world in 2017 was as high as 17.9 billion [2]. At the same time, new pathogens are still emerging, including the outbreak of SARS-CoV in 2002–2003 [3, 4], Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 [5], Ebola virus in West Africa [6], and the recent global epidemic of SARS-COV-2 [7], with 30-day hospital mortality as high as 20% [8]. Diagnosis and control of infectious diseases is still a protracted war.

Characterization of infectious-causing microorganisms is essential for surveillance, treatment, and control of infectious diseases. Difficulty to isolate and culture microorganisms, complex of community composition, and high level of genomic plasticity impede the understanding of infectious diseases [9]. Microbial detection based on traditional culture method is still regarded as the gold standard in clinical. However, it is time-consuming and is limited to cultivable pathogens [10]. The method of RT-qPCR is rapid, specific, and economic; however, it is limited to known pathogens, resulting in missed diagnosis of unknown pathogens [11]. Furthermore, it is unable to detect the mutation of the pathogen (Table 1). The plasticity of pathogen genome endows them with the ability of mutation adaptation under environmental pressure (antibiotic exposure, etc.) [22], and these mutations are often disadvantageous to the host, which may lead to the increase in antibiotic resistance or virulence [23], thus becoming a threat to antibacterial chemotherapy.

The rise of DNA sequencing technology provides an effective way to better understand infectious diseases. Over the past decades, next-generation sequencing (NGS) has become popular in clinical microbiology research and clinic settings. The method of metagenomic NGS has the ability to accurately detect all microorganisms in the sample without culturing within 24 hours. With the development of NGS technology, the detection cost is gradually decreased [24]. However, the detection result could not be analyzed immediately because the sequencing principle of NGS is based on the assemble of short reads. The bioinformatics analysis

Table 1: Advantages and disadvantages of common pathogen detection methods.

Testing methods	Advantages	Disadvantages	Turnaround time	Reference
Culture	Easy to obtain, low cost, simple operation	Sensitivity is affected by antibiotics, limited use in fastidious organisms, time-consuming, unable to detect the gene mutation	Usually takes 5–7 days	[12, 13]
Polymerase chain reaction (PCR) (e.g., direct PCR and multiplex PCR)	Rapid, simple operation, accurate quantification, low cost	Limited to detection of known pathogens, depends on primer design, but the primer is not always effective, unable to detect the gene mutation	One to several hours	[14]
Targeted NGS	High throughput, potential for quantitation highly specific amplification of selected organism types, be able to detect gene mutation	PCR amplification is needed, complicated to operate and long time to result, limited to not covering the entire gene region, depends on hypothesis requiring primers that may not always work, accurate taxonomic identification depends on the quality and completeness of the reference databases	One to several days	[15–17]
Metagenomic NGS	High throughput, no amplification, no bias testing, direct application to clinical samples, potential for discovery of unknown pathogens, be able to detect gene mutation	High cost, long time to result, complicated to operate, vulnerable to human background pollution, difficult to analyze complex genome structure	One to several days	[16, 18]
Nanopore sequencing	Ultralong reads and real-time data, accurate species resolution, direct sequencing of DNA and RNA, high throughput and inexpensive, rapid, portable, and easy to operate, be able to parse complex genome structure, be able to detect gene mutation	Relatively high error rate, quality of sequencing is affected by library quality and sequencing inhibitors	Several hours	[19–21]

could be done only after the complete of the sequencing. Due to the short reads, it is difficult to parse the complex genome structure of microorganisms which usually contain many duplicates [25].

In recent years, innovations in third-generation sequencing technology, represented by Oxford Nanopore Technologies (ONT) and SMRT Pacific Biosciences (PacBio), have been able to produce longer reads in real time [26, 27]. Its extralong reads allow it to assemble the microbial genome in its entirety. The ONT is a portable equipment which allows the identification of pathogenic microorganisms on-site with lower cost per run and is more convenient to clinical laboratories [20]. Compared to NGS, the turnaround time of nanopore is shorter (Table 1). From sample collection to data acquisition, it takes only a few hours [28, 29]. At present, nanopore sequencing platforms have appeared in many clinical microbiology laboratories. Here, we highlight the applications of nanopore sequencing technique in infectious diseases, including monitoring of emerging infectious diseases outbreak, identification of pathogen drug resistance, and disease-related microbial communities characterization.

1. Rapid Identification of Pathogenic Microorganisms

Rapid and accurate identification of pathogenic microorganisms is a key link in the diagnosis and treatment of clinical infectious diseases. Currently, culture is still the main method for pathogen detection in clinic. However, it takes a long turnaround time, often 5–7 days [12]. Although NGS platforms such as Illumina have been used to track infectious diseases in hospitals, the inability to detect on-site is one of the drawbacks. The nanopore sequencing technology based on ONT platform allows the identification of pathogenic microorganisms on-site and is suitable for a wide range of clinical samples, including difficult-to-cultivate pathogens and opportunistic pathogens [30–33] (Table 2).

Due to the diversity of pathogens that can cause infection, the etiological diagnosis of infectious diseases is challenging. Several studies have shown that, in the detection of culture-positive samples, nanopore sequencing results show a high degree of consistency with microbiological culture results [30, 31, 36]. At the same time, information on possible bacterial pathogens was also obtained in culture-

Table 2: Nanopore sequencing can quickly identify pathogens in various clinical samples.

Sample type	Nucleic acid type	Diagnosis	Sequencing methods	Turnaround time	Pathogen
Blackbird brain tissues	DNA	—	—	—	*Usutu virus* [21]
Whole blood	RNA	—	—	<24 h	*Ebola virus* [20]
Plasma	RNA	—	Metagenomic sequencing	<6 h	*Chikungunya virus* [29]
Whole blood	RNA	—	Metagenomic sequencing	<6 h	*Ebola virus* [29]
Serum	RNA	—	Metagenomic sequencing	<6 h	*Hepatitis C virus* [29]
Cerebrospinal fluid	DNA	Bacterial meningitis	16S sequencing	3 h	*L. monocytogenes*: *S. pneumoniae*, *P. aeruginosa*: *K. pneumoniae, etc.* [34]
Sputum	DNA	Community-acquired pneumonia	16S sequencing	5 h	*Haemophilus influenzae pneumonia* [30]
Faeces	DNA	Necrotizing enterocolitis	Shotgun metagenomic sequencing	<5 h	*Klebsiella pneumoniae and Enterobacter cloacae* [28]
Sonication fluid	DNA	Prosthetic joint infections	Metagenomic sequencing	—	*Staphylococcus aureus, Cutibacterium acnes, Staphylococcus epidermidis, etc.* [35]

—, not given.

negative samples [36]. In addition, the composition of fungal communities can also be fully characterized by nanopore-based metagenomics [32]. Because of its speed and sensitivity, this method may be more effective than conventional diagnostic tests in the diagnosis of infectious diseases, and this may provide valuable information for further tracking of pathogens missed or undetectable by conventional microbial culture. Importantly, for critically ill patients, nanopore sequencing can provide the required results within feasible time range of clinical diagnosis [36, 37], which can better serve the clinic.

The emergence of new pathogens and mutations in existing pathogens leading to unpredictable outbreaks will continue to pose challenges to public health [38]. Metagenome sequencing based on ONT is a powerful tool for tracking disease outbreaks and transmission dynamics. Nanopore technology has shown its stability in the effective identification and monitoring of zoonotic infections such as Usutu virus [21], Ebola virus [20], Zika virus [39], and yellow fever virus [40]. At the same time, without relying on expensive facilities and instruments, the nanopore sequencing platform allows it to operate in a variety of extreme environments [41, 42]. In particular, the pocket sequencer MinION launched by ONT is more portable and cheaper for on-site sequencing and genetic analysis of outbreaks. More recently, the outbreak of COVID-19 has captured global attention. In January of this year, the *SARS-COV-2* (WH-Human_1) genome sequence was published for the first time in China and shared globally [43]. Later, Wang et al. innovatively developed Nanopore Targeted Sequencing (NTS) detection method [44], which obviously promoted the confirmation of infected patients. At the same time, the device detects outbreaks in real time, allowing researchers to track how the disease spreads and the rapid evolution of infectious agents [20]. This has brought great benefits to the prevention and control of the epidemic. Among them, the Chinese Center for Disease Control uses 3 sequencing technologies, including nanopore sequencing. Six patients' (COVID-19) samples were used for genome-wide phylogenetic analysis which describes the origin of the virus and, at the same time, through the homology model infer that the virus may have human angiotensin-converting enzyme 2 (ACE2) receptor binding properties [45]. The combination of nanopore sequencing data with epidemiological findings can help identify infected populations and guide outbreak control decisions. The discovery of this important information made a great contribution in tracing the source of the outbreak and in the management of patients.

Another advantage of nanopore sequencing is that the direct RNA sequencing could be realized. RNA of many viruses, including all influenza viruses and polio, was used as a repository for genetic information [29, 46]. In 2015, the RNA virus was sequenced for the first time using nanopore sequencing, and in just four hours, a reliable sketch of the influenza genome was produced [47]. What is more surprising, the influenza virus genome has been reported to be detected in the form of natural RNA [48]. Previous RNA sequencing of almost all depended on RNA reverse transcription or amplification [49]. Nanopore technology provides a more convenient method for sequencing RNA molecules in natural situation. Furthermore, direct sequencing of RNA molecules can help to reveal mysterious epigenetic RNA modifications, and some modifications may be the source of pathogen resistance [50]. It is not hard to imagine the potential for such portable sequencing tools, without dedicated devices and high-end computing resources, to detect RNA viruses directly from clinical samples during a viral pandemic or outbreak. This allows researchers to conduct field investigations and obtain clinically useful information in a very short period of time [51].

In addition, another typical application is the analysis of outbreak-related isolates using nanopores to reveal evolutionary history and indicate mutation information. Currently, the sensitivity of previous diagnostic methods using the *M* gene has been reduced due to the new gene mutation in the H1N1 and H3N2 virus [52]. The whole genome of clinical samples of influenza A virus was sequenced by nanopores, the relevant isolates were analyzed, and the mutation information was indicated [53]. Later, two genes PB2 and NS were identified and selected as new diagnostic targets for influenza A virus detection. At the same time, due to the interaction between pathogen and host, it shows the ability to adapt to mutation frequently [22]. It is also necessary to continuously update genomic information and screen for possible outbreak-related event strains [54]. Moreover, by analyzing the genome sequence of pathogens, candidate genes of vaccine antigens can be screened out, which can contribute to the development and design of subsequent vaccines. Overall, nanopore sequencing can monitor outbreaks and provide increasingly accurate and timely guidance for outbreak management, prevention, and control and for the evolution research.

2. Detection of Antibiotic Resistance

Identifying key characteristics of pathogens such as antimicrobial resistance (AMR) and pathogenicity is critical for therapeutic implications [55]. Repeated sequences of genomes and mobile genetic elements such as plasmids often contain important drug resistance and pathogenicity elements, yet such complex genomes are difficult to assemble in their integrity [56]. Nowadays, with the prevalence of NGS, there is still a gap in the understanding of the virulence and AMR of bacteria. In the early study, the potential of MinION to resolve bacterial antibiotic resistance islands was described although the accuracy rate was only 72% [57]. This may be due to the low coverage of the genome in the early operation of MinION. With the continuous upgrading and improvement of ONT technology and the upgrading of chips, the detection sensitivity of DNA single bases has been greatly improved [58]. In 2017, MinION alone was used to detect antibiotic resistance genes in three clinical isolates of *Klebsiella pneumoniae*, with an assembly accuracy of 99% [59]. At the same time, with the improvement of sequencing depth, the accuracy of nanopore assembly will be improved further. ONT released a new R10 chip in July 2019 that claims to have a Q50 level of common sequence accuracy for its nanopores—equivalent to one error per 100,000 bases, or 99.999 percent accuracy. In general, nanopore sequencing has great advantages in rapid identification of pathogens and analysis of antimicrobial resistance.

It is important to note that mobile genetic elements (MGEs) also often carry a large amount of resistance information, and the capture and analysis of these MGEs can explain and refine antibiotic resistance phenotypes in clinical isolates [60]. However, the widely used NGS assembly of MGEs is often highly fragmented and can result in omissions [61]. This impedes the proper identification of plasmids, phages, and virulence factors. However, the long reading length of nanopore sequencing shows great advantage in this aspect. In one study, three plasmids from *Klebsiella pneumoniae* were isolated, and AMR genes were obtained by nanopore sequencing alone, with an assembly accuracy of about 99% [59]. More significantly, the study was sufficient to describe antibiotic resistance information on plasmid DNA at a low reading depth in as little as 20 minutes, balancing the relationship between turnaround time and accuracy. Shiga toxin-producing *E. coli* (STEC) is a highly pathogenic hemorrhagic pathogen with high incidence rate and high lethality. The genome is rich in plasmids, phages, and virulence genes [62, 63]. Recently, 3 STEC genomes were sequenced by Illumina and MinION platforms. The MinION data provided genomic location information for 20 phages, while in Illumina sequencing data, individual phages could not be reconstructed [64]. Meanwhile, MinION sequencing found that one strain carried multiple AMR genes, all in a tiny plasmid [64]. Equally significant, the hybrid metagenomic assembly of OPERA-MS has been developed for use in the study of intestinal metagenomes of patients treated with antibiotics. The mobile elements of the metagenome in the stool sample were successfully assembled with long reading data provided by nanopore sequencing [65]. Among them, new antibiotic resistance has been found with no homology with known sequences, which is of great value in the clinical environment. In summary, nanopore sequencing can identify AMR gene carried by pathogenic bacteria more effectively in a shorter time. This could help clinicians make decisions about customizing antibiotics rather than broad-spectrum ones.

3. Description of Disease-Related Microbial Community

Under normal circumstances, each person's body is a rich ecological environment in which the human body and microbial community are finely balanced. However, changes in internal physiological and pathological conditions and external interference can easily upset this balance [66, 67]. In many cases, clinical infections are complex [68], particularly in the lower respiratory and intestinal tracts, where microorganisms are abundant, and the coinfections are not surprising [69]. In the study by Charalampous and collaborators, mixed infections in lower respiratory tract samples were successfully determined by nanopore metagenomics [31].

A common understanding is that microbial diversity is directly related to disease [70, 71]. Metagenomic sequencing is a powerful tool for characterization of microbial community [72]. In the previous studies of metagenomics, Illumina platform has contributed to high accuracy, but its reading of highly fragmented short sequences easily leads to the wrong assembly of genome repeat regions [73]. Nanopore-based sequencers have the ability to overcome this limitation by producing long reads and allowing highly complete genome sequences [74]. In one study, nanopore metagenomic sequencing and Illumina metagenomic sequencing were simultaneously applied to the microbial

simulated community, and the results showed that the two platforms submitted similar answers for identification at the level of microbial species classification and abundance [28]. However, for species that are highly similar at the genome level, ONT Platform shows a more accurate species abundance [35].

Targeted sequencing based on 16S RNA gene has also been widely used in microbial classification and diversity research. NGS-based 16S rRNA sequencing strategy only analyzes hypervariable regions (V1-V2 or v3-v4) [75]. Microbial diversity may be largely underestimated. ONT platform provides more accurate identification of bacteria by analyzing the full-length sequence of 16S rRNA gene; at the same time, more accurate taxonomy and clearer developmental science are available [76, 77].

4. Conclusions and Future Perspectives

At present, nanopore sequencing has been successfully applied to the monitoring and management of the outbreak of new infectious diseases, identification of drug-resistant pathogenic microorganisms, and identification of disease-related microbial community characteristics, which provides a feasible solution for solving the current epidemic problem. However, it must be acknowledged that this may not be the best option for common pathogens but can improve or fill the gaps in conventional diagnostic tools. And it is important that the personnel to operate should be appropriate for good collection of samples, nucleic acid separation, library preparation, sequencing, and data processing. At the same time, external quality control needs to be established. Moreover, the clinical samples are very complex, there is a high proportion of nucleic acid contamination in the host, and the vulnerable microbial population may be covered [31, 78]. Although strategies to enrich microbial nucleic acids are being developed, the methods of sample preparation still need to be optimized [79, 80]. The accuracy of genome assembly remains a focus of concern. It is therefore urgent to improve the resolution of single base especially for the small genome with high mutation. Of course, the combination of nanopore sequencing and other short reads sequencing is also a potential solution, which can help obtain higher quality genomic information [81–83], but it is believed that, with the further development of the technology, nanopore sequencing alone can provide enough accurate results.

Disclosure

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

XZ, SW, and SY conceived the idea. XZ and SY drafted the manuscript. SW, SY, and FY revised the manuscript. All authors read and approved the final manuscript. Xiaojian Zhu and Shanshan Yan contributed equally to this work.

Acknowledgments

This work was supported by the Open Project of Key Laboratory of Prevention and Treatment of Cardiovascular and Cerebrovascular Diseases, Ministry of Education (grant no. XN201817), Science and Technology Innovation Outstanding Young Talents Training Program of Jiangxi Province (grant no. 20192BCBL23017), and Youth Jinggang Scholars Program in Jiangxi Province.

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2020, Article ID 6682020, 7 pages
https://doi.org/10.1155/2020/6682020

Research Article

Analysis of the Salivary Microbiome in Obstructive Sleep Apnea Syndrome Patients

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Received 16 October 2020; Revised 2 December 2020; Accepted 10 December 2020; Published 23 December 2020

Academic Editor: Tingtao Chen

Background. Oral microbiota plays an important role in oral and systemic diseases, while few reports referred to obstructive sleep apnea syndrome (OSAS). Thus, this study aimed to explore the different salivary microbiome in patients with OSAS and controls. *Materials and Methods*. Saliva was collected from 15 OSAS patients and nine healthy controls, and bacterial genomic DNA was extracted for 16S rRNA amplicon sequencing based on the Illumina platform. *Results*. The alpha and beta diversities were not significantly different between patients with OSAS and controls. The main phyla in the two groups were Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, and Fusobacteria, which accounted for 95% of the abundance. The main genera were *Streptococcus*, *Rothia*, *Actinomyces*, *Prevotella*, and *Neisseria*. Based on the genus and operational taxonomic units, *Peptostreptococcus*, *Alloprevotella*, and *Granulicatella* were enriched in controls, while only *Scardovia* species were significantly more abundant in patients with OSAS. *Conclusions*. There was no significant difference in the relative abundance of bacteria between OSAS and controls. So, further studies will need to focus on the metagenome of bacteria in OSAS patients.

1. Introduction

Obstructive sleep apnea syndrome is a sleep-breath disorder caused by upper airway obstruction and characterized by recurrent hypoxemia and daytime sleepiness [1, 2]. It can occur at any age and lead to poor quality of life [3]. Moreover, the prevalence and burden of OSAS are expected to increase in the future. The underlying pathology of OSAS may be intermittent hypoxemia and hypercapnia of the upper airway, leading to local and systemic inflammatory responses [4, 5].

The etiology of OSAS is not understood clearly. Some studies have shown that there is a close relationship between microorganisms and OSAS. Intermittent hypoxia regulates hypoxia/reoxygenation cycles in the upper airway and gut, which increases the bacterial diversity in OSAS patients. Based on this, microbial changes can affect the inflammatory process in different mucosal tissues, including nasal fluid [6], tonsil [7], and gut [8]. The relative abundances of *Streptococcus*, *Prevotella*, and *Veillonella* were higher in the nasal fluid of severe OSAS patients than those in healthy subjects. *Streptococcus salivarius*, *Prevotella* spp., and *Terrahaemophilus aromaticivorans* were more common on adenoids from patients with OSAS [7]. Additionally, the altered microbiome in severe OSAS patients was associated with inflammatory biomarkers (e.g., inflammatory cells, interleukin IL-8, and IL-6) [6]. Also, proinflammatory cytokines were elevated in the saliva and serum of OSAS patients [9]. All this proved that microbial-host interaction might play an important role in immune response in OSAS patients.

Bacteria, which have colonized every location of the human body, are linked to human health. The oral cavity is an important exchange medium between exogenous substances and the internal environment [10]. Many studies

have focused on the link between oral microbiome and systemic disease [11], for example, chronic kidney disease [12], diabetes [13], and obesity [14], because OSAS can also cause some complications such as hypertension and other cardiovascular diseases [15]. Thus, specific bacterial microenvironments may be present in the oral cavity of OSAS patients. However, there are only a few studies concerning this issue [16, 17]. The two studies demonstrated that OSAS is closely linked with periodontitis and the buccal mucosal microbiome was significantly altered in pediatric OSAS patients, respectively.

Saliva, as a representative sample of the oral ecosystem has become an important sample matrix in bioanalytics and reflects systemic conditions [18]. Salivary microbiota is associated with oral and systemic diseases and mediated inflammatory responses. Therefore, we aimed to explore the bacterial composition and community structure of saliva in patients with severe OSAS and controls with 16S rRNA high-throughput sequencing to determine the relation between OSAS and oral microbiome.

2. Materials and Methods

2.1. Subjects. The study included unrelated subjects suspected of having OSAS who were admitted to the Sleep Center of the Affiliated Sixth People's Hospital, Shanghai Jiao Tong University meanwhile. Ten subjects who did not snore were also recruited to complete the overnight polysomnography test. The Institutional Ethics Committee of the Hospital of Shanghai Jiao Tong University approved the study (protocol reference number 2018-KY-013 (K)). Informed consent was obtained from all participants. All participants were asked to complete a uniform questionnaire containing questions regarding current and previous illnesses and medical treatments. Subjects with the following indexes were excluded: systemic diseases (e.g., hypertension and diabetes), oral disease (e.g., periodontitis and dental caries), smoking, antibiotics applied for less than three months, and any treatment with adenoids.

The diagnostic criterion of OSAS patients was the apnea-hypopnea index (AHI; mean number of apneas or hypopneas per hour), with apnea defined as the cessation of flow for > 10 s and hypopnea as $a > 50\%$ reduction inflow. An AHI of 5–15 is considered mild, 16–30 is moderate, and >30 is considered severe. Finally, only severe OSAS patients and healthy subjects were included. The diagnostic evaluator has a rich clinical experience for eight years and has been employed as a Technical Section Member of the Chinese Medical Doctors Association Sleep Medicine Specialized Committee.

2.2. Saliva Collection. After the overnight polysomnography test, all subjects were required not to drink and eat for two hours and clean their mouth of obvious food residue on the oral mucosa and teeth before sampling. Unstimulated saliva (2 ml) was collected in a 50 ml sterile tube, placed on ice, delivered to the laboratory. Each sample was centrifuged at 8000 rpm for 10 min at 4°C, and the precipitate was collected and stored at −80°C for DNA extraction.

2.3. DNA Extraction and Sequencing. The 24 saliva precipitates were digested by lysozyme (20 mg/ml) and digested at 37°C for 30 min, and then bacterial genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions. The DNA quality was determined by the $OD_{260/280}$ ratio (1.8–2.0), using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, USA). DNA integrity was verified by 1% agarose gel electrophoresis. High-quality DNA was stored at −20°C for further sequencing.

An equal DNA concentration (10 ng/μl) from 24 samples was used for 16S rRNA gene amplification of V3–V4 regions (primers: F341 : ACTCCTACGGGRSGCAGCAG, R806 : GGACTACVVGGGTATCTAATC) [19] with an 8 bp unique index inserted at the 5′ end to distinguish the different samples. PCR was performed using a KAPA HiFi HotStart ReadyMix PCR Kit; the products were purified using an AxyPrep DNA Gel Extraction Kit (Axygen, USA). The extracted products were quantified with Qubit 3.0 and real-time PCR to conduct libraries following the instructions according to the manufacturer's instructions. The libraries were sequenced using the Illumina HiSeq PE250 platform by Shanghai Realbio Technology Co., Ltd. (Shanghai, China). The raw sequence data have been submitted to the NCBI with the accession number PRJNA544600.

2.4. Data Processing. The raw sequence data were assigned to each sample using the unique barcode sequence. The paired-end reads were assembled using Pandaseq software [20] based on the overlap region. High-quality reads were selected using Usearch software (version 7.0190) based on the following criteria: an average value was >20, a base number containing $N < 3$, and an average length was 220–500. After singletons were filtered, the clean reads were clustered into operational taxonomic units (OTUs) by 97% similarity using UPARSE [21], and chimeras were removed using Usearch. The sequence with the highest abundance was selected from each OTU and used as the representative sequence of the OTU. The representative sequence was classified against the Ribosomal Database Project (RDP) database and Human Oral Microbiome Database to assign microbial taxa (phylum, class, order, family, and genus) for each sample.

All subsequent analyses were conducted using QIIME (version 1.9.1) [22]. The OTUs of 24 samples were used for further analysis. To identify the alpha diversity and beta diversity, the same number of clean sequences was chosen from each sample to reduce the sequence depth factor. Principal coordinates analysis (PCoA) was performed to analyze the structure of microbial communities in controls and patients with OSAS based on the OTU level.

2.5. Statistical Analysis. Clinical data (age, body mass index (BMI), and apnea-hypopnea index (AHI)) were compared by independent-samples t-test. The index of sex was compared by the chi-square test, and the other indexes of mean

SaO_2, minimum SaO_2, and oxygen desaturation index (ODI) were compared by the Wilcoxon rank-sum test, respectively, using SPSS software (version 19.0). The alpha diversity (Chao1, Observed OTUs, PD whole tree, Shannon) and bacterial composition were tested using the Wilcoxon rank-sum test. LEfse analysis was based on Wilcoxon rank-sum test and with the threshold of logarithmic linear discriminant analysis (LDA) score set to 2.0. The PCoA of beta diversity was performed by the ANOSIM test. P value < 0.05 was considered statistically significant.

3. Results

3.1. Basic Information. Nine controls and 15 patients with severe OSAS patients were chosen for this study. There were no significant differences in sex, age, and body mass index (BMI) between the two groups (Table 1). The AHI and ODI indexes in OSAS patients were significantly higher than those in the controls. Because OSAS patients have apnea at night, which will lead to hypoxia, so the mean SaO_2 and minimum SaO_2 indexes were significantly lower in OSAS patients than those in the controls.

Sequencing yielded 862,398 clean reads after quality assessment and filtering, with an average of 35,933 reads per sample. The number of OTUs per sample was 152–251.

3.2. Biodiversity of the Salivary Microbiome. We conducted alpha and beta diversity analyses to explore the microbial community composition and structure in OSAS patients and controls. The observed OTUs and the Chao index, which represent the microbial richness, and the Shannon index were no significant differences between OSAS patients and controls (Figure 1).

Beta diversity is used to describe phylogenetic differences in microbial communities between diseased and controls. This method can present the bacterial difference between two groups based on the distance. As shown in Figure 2, the OSAS and healthy samples overlapped, and there was no apparent difference in distribution between the two groups, with the principal components of 26.52% and 22.14%. ANOSIM analysis also showed that there was no significant difference in bacterial composition and structure between the controls and patients with OSAS ($P > 0.05$) (Figure 2). PCoA analysis was conducted using weighted UniFrac distances based on the OTU level, which showed that there was a similar bacterial environment between controls and patients with OSAS.

3.3. Bacterial Composition of Saliva in OSAS Patients and Controls. We analyzed the relative abundance of microbial taxa at the phylum, class, order, family, and genus levels. The main phyla in the two groups were Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, and Fusobacteria, which accounted for 95% of the abundance. At the genus level, *Streptococcus*, *Rothia*, *Actinomyces*, *Prevotella,* and *Neisseria* were the most abundant in the two groups, and there were no significant differences in these genera between the two groups (Figure 1). The proportions of *Peptococcus*, *Peptostreptococcus*, *Alloprevotella*, and *Granulicatella* were less abundant in patients with OSAS compared with controls; only the genus *Scardovia* had a significantly higher abundance in patients with OSAS (Figure 3).

Table 1: The basic clinical information of OSAS patients and control subjects.

Characteristics	OSAS	Controls	P value
No. of subjects	15	9	—
Age (years)	47.0 ± 9.5[a]	40.2 ± 9.4[a]	0.103
Sex (male/female)	13/2	6/3	0.326
BMI[d] (kg/m^2)	27.0 ± 3.8[a]	28.5 ± 6.4[a]	0.453
AHI[c] (events/h)	54.4 ± 19.0[a]	2.7 ± 1.2[a]	<0.001
Mean SaO_2 (%)	93 (91–94)[b]	96 (95–97)[b]	0.007
Minimum SaO_2 (%)	74 (67–81)[b]	93 (91–94)[b]	<0.001
ODI[e] (events/h)	58.0 (47.0–69.1)[b]	3.4 (1.7–5.2)[b]	<0.001

[a]Values are presented as means ± SD. [b]Values are presented as mean (with 95% confidence intervals) as appropriate. [c]AHI = apnea-hypopnea index. [d]BMI = body mass index. [e]ODI = oxygen desaturation index. The age and BHI and AHI indexes were compared by independent-samples t-test, and the sex index was compared by the chi-square test; the other indexes of mean SaO_2, minimum SaO_2, and ODI indexes were compared by the Wilcoxon rank-sum test, respectively, using SPSS software.

The linear discriminant analysis effect size (LEfse) method was used to analyze the influence of bacteria on health and disease, with LDA > 2 labeled based on the OTU level, which considered the statistical significance and biological correlation. This method revealed the influence of significantly different bacteria on the two groups. Genera such as *Peptostreptococcus*, *Alloprevotella*, and *Granulicatella* were enriched in healthy controls, while *Scardovia* was significantly more abundant in patients with OSAS (Figure 4).

4. Discussion

Our study revealed the bacterial composition and diversity of saliva microbiome in adult OSAS, demonstrating that the bacterial microenvironment of saliva was relatively stable compared with controls. No significant difference in the alpha diversity of the salivary microbiome was indicated between patients with severe OSAS and controls in our results. Moreover, genera such as *Peptostreptococcus*, *Alloprevotella*, and *Granulicatella* were enriched in healthy controls while *Scardovia* was enriched in patients with OSAS. We know that the oral cavity does not belong to the upper airway system, so the saliva environments of patients with OSAS and controls may be similar.

OSAS is a significant risk factor for hypertension, cardiovascular disease, and metabolic disorders such as obesity [14] and diabetes [13]. Bacteria play an important role in these processes. It is possible that OSAS leads to gut hypoxia and hypercapnia and increased sympathetic activity, which results in gut dysbiosis [23, 24]. A study has showed higher relative abundances of *Porphyromonas* and *Aggregatibacter* and elevated proinflammatory cytokines in patients with OSAHS compared with controls without OSAHS [25]. Recent studies have also shown that due to the reflux of oropharyngeal oral secretions during sleep, oral bacteria were detected in the nasal lavage of patients with OSAS [6]. The oral environment represents an interface between the

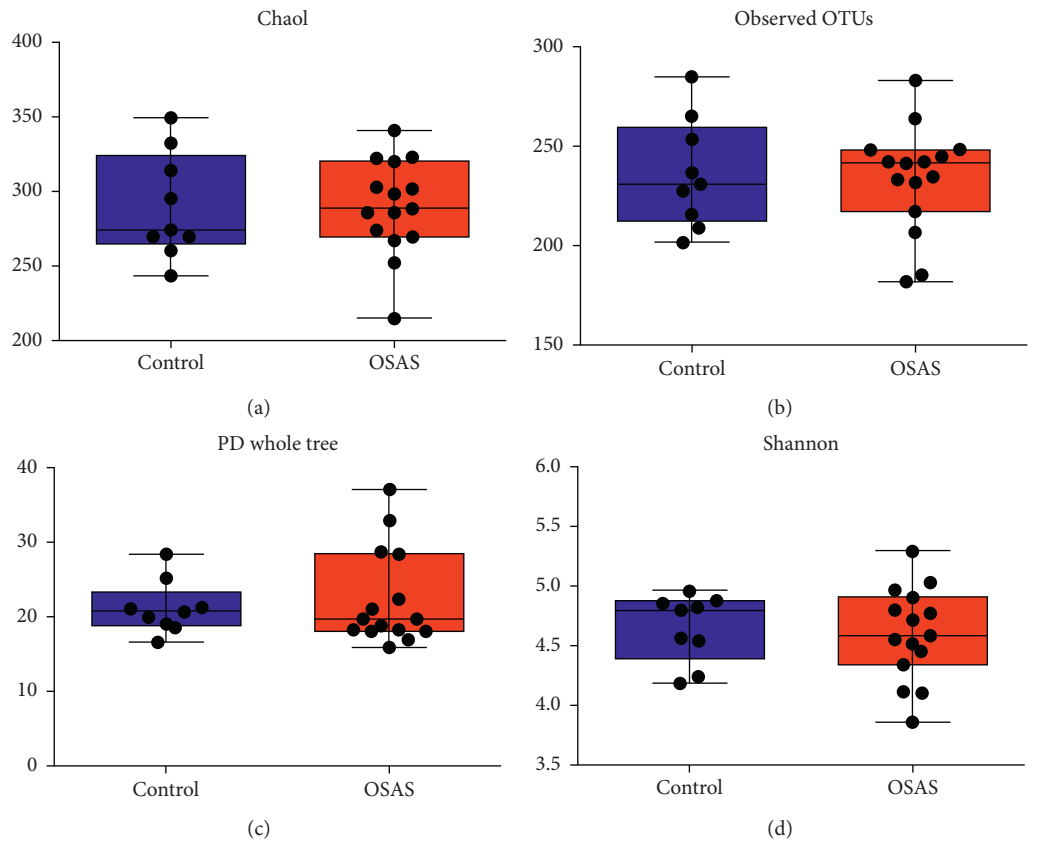

Figure 1: Comparison of salivary microbiome alpha diversity in patients with severe obstructive sleep apnea syndrome (OSAS) and controls. (a, b) Community richness (chao1 and observed operational taxonomic units), (c) comparison of the bacterial evolutionary distance, and (d) the Shannon index. All of the indexes were tested using the Wilcoxon rank-sum test. The significant difference was set by $P < 0.05$. Box and whisker plots were indicated medium, minimum, and maximum values. All the samples were shown in the plot.

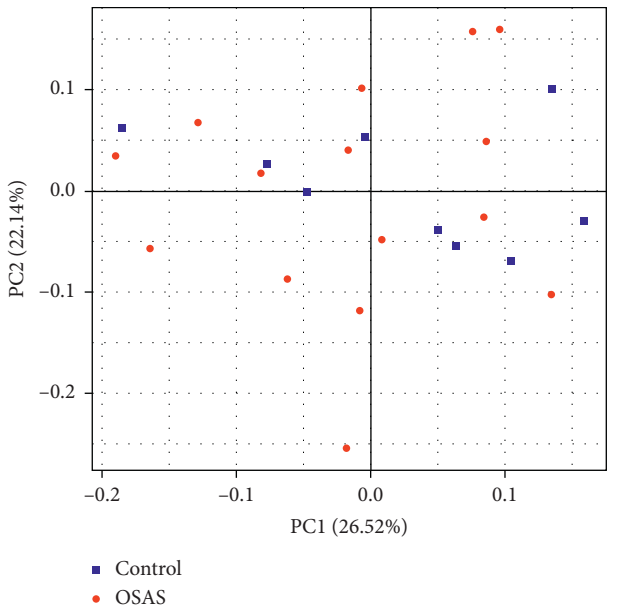

Figure 2: PCoA analysis of the salivary microbiome of patients with severe OSAS and controls using principal coordinate analysis. The analysis was based on UniFrac distance. Significant differences were assessed using Anosim analysis. Significance was indicated by $P < 0.05$.

internal and external environments, and it is easily influenced by the internal environment. The metabolic products of oral bacteria can be recycled into the blood circulation and are involved in local and systemic immune responses, which may accelerate the progression of systemic diseases [26, 27]. It has also been shown that patients with OSAS experience local (upper airway) and peripheral (systemic) inflammation. Thus, maintaining a relatively good oral environment is important for patients with OSAS.

Studies found that the composition and the metabolomics profile of the oral microbiome were significantly altered in pediatric OSAS [17]. Although there were no significant differences in the overall phylogenetic structure of the salivary microbiome, several bacteria were also altered because of the oxygen environment. *Peptostreptococcus*, *Alloprevotella*, and *Granulicatella* were enriched in controls, while *Scardovia* was enriched in OSAS patients. *Peptostreptococcus* is the most common Gram-positive anaerobe found in the oral cavity of healthy and patients. Many diseases are caused by this bacterium, including endocarditis [28] and root canal infections [29]. *Alloprevotella* is anaerobic Gram-negative rods isolated from the normal oral and intestinal bacterial population. Although not considered pathogenic, the strains were saccharolytic ability and produced acetic and succinic acids in the oral cavity [30]. All of the above identified genera were common in the samples

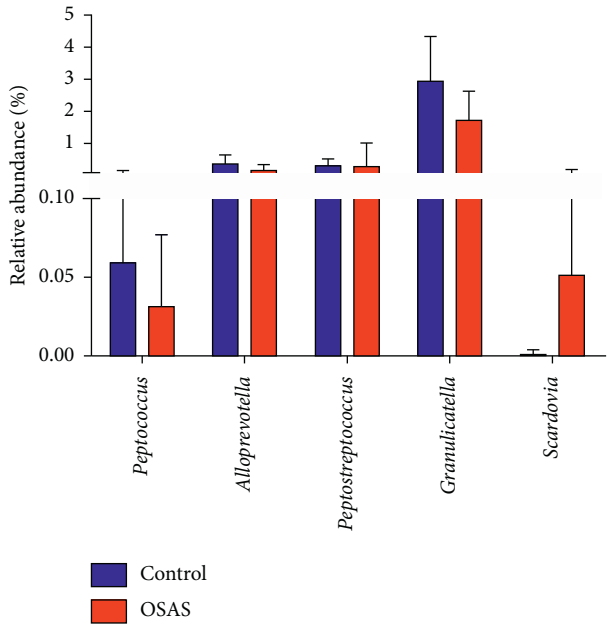

Figure 3: Significantly different genera in patients with severe OSAS and controls. The significantly different genera (prevalence > 50% in all samples in each group) between the two groups were accessed using the Wilcoxon test based on the relative abundance values. The significant difference was set by $P < 0.05$. The error bars were presented as mean.

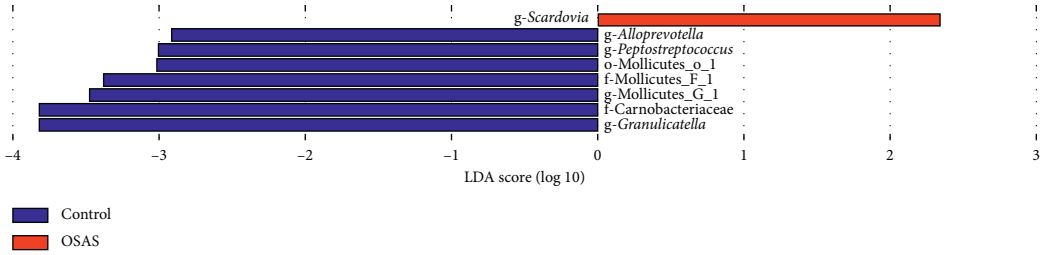

Figure 4: Taxonomic differences in salivary samples from patients with severe OSAS and controls. The enriched bacteria between the two groups based on linear discriminant analysis effect size analysis (LEFse). LDA > 2 is labeled.

obtained from controls. Some species in the genus *Scardovia* were found to be related to dental caries [31], which indicated that OSAS patients might tend to have oral disease. The oral condition change was caused by a combination of bacteria rather than by a single bacterium. Although there was no difference in the salivary microbiome of the controls compared with OSAS patients, OSAS patients should also pay attention to oral health, because they can be easily infected.

There are some limitations to the study. First, the sample size was relatively small to reach our conclusion. Thus, a larger validation study and metagenomic analysis would be useful to support our results. Second, a dietary diary should be considered to reduce the influence of food on the oral salivary microbiome. Finally, different oral microbiota was present at different oral sites; therefore, saliva collection alone cannot represent the entire oral microbiome.

5. Conclusion

This study applied 16S rRNA gene sequencing technology to analyze the salivary microbiome between OSAS patients and controls. The results showed that there were no significant differences in the bacterial diversity and phylogenetic structure of the salivary microbiota. However, the relative abundances of *Peptococcus*, *Peptostreptococcus*, *Alloprevotella*, and *Granulicatella* were lower and only the genus *Scardovia* was enriched in the saliva samples from OSAS patients compared with controls.

Data Availability

The data used to support the findings of the study are available from the corresponding author upon request, and the raw data had been uploaded to the NCBI with the accession number PRJNA544600.

Conflicts of Interest

The authors have declared no conflicts of interest.

Authors' Contributions

All the authors participated in the design and implementation and analysis and interpretation of the results and

wrote the manuscript. All the authors gave final approval before submission. Peizeng Jia and Jianyin Zou contributed equally to this work.

Acknowledgments

The authors would like to thank Xuting Che (Realbio Technology, Co., Ltd., Shanghai) for their technical assistance and Yunyan Xia for her assistance. This study was supported by grants-in-aid from the National Key Project of Research and Development Program of China (grant no.2017YFC0112500) and the National Science Foundation China (grant no. 81600792).

Supplementary Materials

SFigure 1: bacterial composition at the phylum and genus level. (A) Phylum level and (B) genus level. (*Supplementary Materials*)

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2020, Article ID 6678872, 13 pages
https://doi.org/10.1155/2020/6678872

Research Article

Improved High-Throughput Sequencing of the Human Oral Microbiome: From Illumina to PacBio

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Received 21 October 2020; Revised 6 November 2020; Accepted 18 November 2020; Published 11 December 2020

Academic Editor: Tingtao Chen

Background. A comprehensive understanding of the commensal microflora and its relation to health is essential for preventing and combating diseases. The aim of this study was to examine the structure of the oral microbiome by using different sequencing technologies. *Material and Methods*. Five preschool children with no symptoms of oral and systemic diseases were recruited. Samples of saliva were collected. A 468 bp insert size library was constructed on the MiSeq platform and then subjected to 300 bp paired-end sequencing. Libraries with longer insert sizes, including a full-length 16S rDNA gene, were sequenced on the PacBio RS II platform. *Results*. A total of 122.6 Mb of raw data, including 244,967 high-quality sequences, were generated by the MiSeq platform, while 134.6 Mb of raw data, including 70,030 high-quality reads, were generated by the PacBio RS II platform. Clustering of the unique sequences into OTUs at 3% dissimilarity resulted in an average of 225 OTUs on the MiSeq platform; however, the number of OTUs generated on the PacBio RS II platform was 449, far greater than the number of OTUs generated on the MiSeq platform. A total of 437 species belonging to 10 phyla and 60 genera were detected by the PacBio RS II platform, while 163 species belonging to 12 phyla and 72 genera were detected by the MiSeq platform. *Conclusions*. The oral microflora of healthy Chinese children were analyzed. Compared with traditional 16S rRNA sequencing technology, the PacBio system, despite providing a lower amount of clean data, surpassed the resolution of the MiSeq platform by improving the read length and annotating the nucleotide sequences at the species or strain level. This trial is registered with NCT02341352.

1. Introduction

The human oral microbiome comprises over 700 prevalent taxa at the species level, including a large number of opportunistic pathogens involved in periodontal, respiratory, cardiovascular, and systemic diseases [1–5]. Identification of oral microorganisms at the species level is the basis and prerequisite for analyzing microbial communities of the oral cavity. The 16S rRNA gene is considered the gold standard for phylogenetic studies of microbial communities and high-throughput sequencing of the 16S rRNA gene could provide snapshots of microbial communities, revealing phylogeny and the abundances of microbial populations across diverse ecosystems [6, 7]. For this reason, the sequencing techniques had become an important tool for understanding the biology and functional characterization of oral microorganisms.

The emergence of the next-generation sequencers (NGS) and their sequencing by synthesis have drastically transformed the way scientists delve into the relationship between microbiome and related diseases [8]. Since then, many studies have used the NGS technologies, such as Roche/454 [9], ABI/Solid, Illumina [10], and its upgrade platforms including Illumina/HiSeq and MiSeq for microbial ecosystem analysis [9–15]. When it comes to the resolution and accuracy of the sequencing results, lengths and quantity of reads are very important factors [16–18]. Unfortunately, the NGS came with this drawback. Compared with the previous methods (e.g., Sanger sequencing), the reads generated are short. This became a major challenge for the assembly,

especially in the case of large repetitive genomes [19]. Thus, in spite of the low cost and extremely high-throughput, the NGS platform is sometimes less accurate as a result of short read lengths and long repeats present in multiple copies [17]. Besides, although the explosion of sequence data brought about by high-throughput sequencing technologies is highlighting a richness of microbes not previously anticipated, not all of the novel organisms discovered by the NGS can be named by taxonomists because the existing tools are not sufficient to provide species names or phylogenetic information for the millions of short reads [20]. Operational taxonomic units (OTUs) at the 97% similarity is recognized as providing differentiation of bacterial organisms below the genus level [12]; however, it was still inaccurate for the reason that this level of clustering defines either microbial species or strains.

Third-generation sequencing (TGS), PacBio single molecule, real-time (SMRT) sequencing technology circumvented this problem by greatly increasing read lengths that have the ability to sequence the full length of the 16S rRNA gene [16, 18]. It involves a DNA fragment sequenced by a single DNA polymerase molecule connected to the bottom of a zero-mode waveguide [18]. During DNA synthesis, each of the nucleotides is illuminated upon incorporation, which can enable for identification. The PacBio RS II can yield average sequence reads of greater than 2500 bp; however, some research data show that circular consensus sequencing (CCS) of shorter fragments (<1500 bp) can decrease the sequencing errors [21]. Some studies have shown that the longer reads generated from sequencing the entire 16S rRNA gene provide a higher resolution of organisms and higher estimates of richness [17]. A previous study has shown that PacBio outperformed the other sequencers such as Roche 454 and MiSeq in terms of the length of contigs and reconstructed the greatest portion of the genome when sequencing the genome of *Vibrio parahaemolyticus* [22]. However, there have been few studies that aim at comparing the next-generation sequencing technology with PacBio RS II in oral microbiome. In this study, we explore the microbiota of oral cavity using sequences amplified V3-V4 and the V1-V9 small subunit ribosomal RNA (16S) hypervariable regions by two different platforms. The aim of this study was to evaluate the performance of TGS technology PacBio RS II in comparison with NGS technology Illumina/MiSeq for the structure of oral microbiome in 5 healthy preschool children in China.

2. Materials and Methods

2.1. Patient Information. Five preschool children aged 63–74 months, lacking evidence of oral and systematic diseases were recruited based on a list of exclusion criteria on Nov 26, 2014. The subjects with a history of chronic antibiotic used within 8 weeks before enrollment were excluded from the study. All subjects' legally authorized representatives provided written informed consent upon enrollment. The study was approved by the Institutional Review Board of the Affiliated Stomatology Hospital of Zhejiang University in accordance with the Declaration of Helsinki principles.

2.2. Saliva Sampling and Isolation of Bacterial DNAs. The subjects were instructed neither to eat and drink nor to perform any oral hygiene procedure two hours before sampling. Saliva samples were collected from all subjects in the morning between 9 : 00 am and 11 : 00 am.

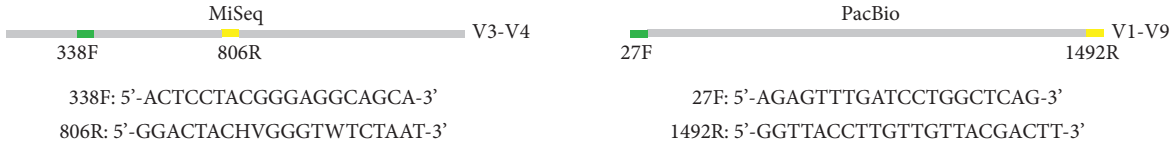
Unstimulated saliva samples were collected according to a protocol, modified from a previous study. The children were initially asked to rinse their mouth thoroughly with deionized water prior to sampling, followed by collection of at least 5 mL unstimulated saliva in a plastic cup. Finally, the samples were transferred into sterile cryogenic vials. Then, the samples were placed into liquid nitrogen and stored at −80°C until use.

Bacterial DNAs were extracted using the E.Z.N.A.™ Soil DNA Kit (Qiagen, Omega, USA), according to the instructions of the manufacturer. The enriched microbial DNAs were purified by ethanol precipitation. DNA concentration was measured using NanoDrop, and its molecular size was estimated by agarose gel electrophoresis. DNAs were stored at −20°C until use.

2.3. PCR Amplification of the 16S rRNA Gene. PCR amplification of the 16S rRNA gene hypervariable V3-V4 regions was performed with universal bacterial primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The V1-V9 hypervariable region was performed with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The products were extracted with the AxyPrep DNA Gel Extraction kit (Qiagen, USA) and were then examined by agarose gel electrophoresis. According to the electrophoretic results, the PCR products were quantified by Quantifluo™-ST (Promega, USA). Then, the products from different samples were then mixed at equal ratios for pyrosequencing on the two different platforms.

2.4. DNA Library Construction and Sequencing. Construction of DNA library was carried out by following the manufacturer's instructions (Illumina and PacBio). A 468 bp insert size library was constructed on the MiSeq platform and then applied to 300 bp paired-end sequencing. Libraries with longer insert size (1540 bp) were performed on the PacBio RS II platform, including full length of 16S rDNA gene. Barcoded 16S rRNA amplicons (V3-V4 and V1-V9 hypervariable regions) of the five Chinese children were sequenced on MiSeq and PacBio RS II platforms, respectively. Raw data were generated, and low-quality reads were then removed by quality control (Figure 1).

2.5. Bioinformatic Analysis. We used QIIME software to cluster filtered reads into operational taxonomic units (OTUs) from PacBio and MiSeq platforms [23] by applying a 97% identity threshold relative to a centroid sequence. The generated OTUs were used for alpha-diversity (Shannon and Simpson), richness (Chao, ACE), coverage, and rarefaction curves using Mothur software (version v.1.30.1) [24]. We



338F: 5'-ACTCCTACGGGAGGCAGCA-3'
806R: 5'-GGACTACHVGGGTWTCTAAT-3'

27F: 5'-AGAGTTTGATCCTGGCTCAG-3'
1492R: 5'-GGTTACCTTGTTGTTACGACTT-3'

MiSeq				
Insert size (bp)			468	
Sequencing length (bp)			PE300	
Sample ID	Reads	Sequences	Bases	Average length
1	49212	56649	25312520	446.83
2	50054	56238	25086400	446.08
3	41843	48103	21409856	445.08
4	48838	54939	24489025	445.75
5	55020	58892	26279440	446.23

PacBio				
Insert size (bp)			1540	
Sequencing length (bp)			Full length of 16SrRNA gene	
Sample ID	Reads	Sequences	Bases	Average length
1	7894	10656	15570128	1461.2
2	8790	10772	15866046	1472.9
3	16198	21044	31096186	1477.7
4	12350	15542	22794132	1466.6
5	24798	33310	49260212	1478.8

Figure 1: Sequencing results from 5 oral samples. Partial 16S amplicons (V3-V4) were sequenced on the Illumina/MiSeq; and full-length 16S amplicons (V1-V9) were sequenced on PacBio.

then assigned the resulting OTUs using a BLAST-based method implemented in QIIME, employing the SILVA (version 119) database as the reference for taxonomic analysis [25, 26]. The species-level operational taxonomic units (OTUs) and relative richness of phylum, class, order, family, genus, and species for each sample between the two platforms were compared. Statistical analysis was performed using SPSS for Windows (version 19.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Increased Diversity of Oral Microbiota Sequenced by TGS. By high-throughput pyrosequencing of 5 samples synchronously on two different platforms, a total of 122.6 Mb raw data including 244,967 high-quality sequences were generated by the MiSeq platform, while 134.6 Mb raw data including 70,030 high-quality reads were generated by the PacBio RS II platform. For the MiSeq platform, 99.99% of the clean reads distribution ranged from 401 to 500 bp, and for the PacBio RS II platform, 94.24% of the clean reads were distributed from 1401 to 1600 bp.

The average lengths of quality reads were 446 bp and 1471 bp on MiSeq and PacBio RS II platforms, respectively. With accurate read lengths of 1471 base pairs, the PacBio system opens up the possibility of identifying microorganisms to the species level in oral cavity (Figure 1).

A slightly higher coverage was observed in the PacBio RS II platform, and the level of coverage indicated that the 16S rRNA gene sequences identified by the two sequencing platforms represented the majority of bacterial sequences present in the oral saliva samples. The rarefaction curves and richness indices (Chao and ACE) that estimated the richness of the total oral microbiota also show that enough sequencing data were generated by the two platforms (Figures 2 and 3).

Clustering the unique sequences into OTUs at 3% dissimilarity resulted in an average of 225 OTUs on the MiSeq platform; however, the number of OTUs generated on the PacBio RS II platform was 449, almost twice as that of the MiSeq platform (Figure 3). Other indices (Chao estimate and Ace index) revealed that the PacBio RS II platform detected more species. The comparisons of alpha-diversity indices (Shannon and Simpson) of the oral microbiota were significantly different between the two platforms. The Shannon index of the MiSeq group was lower than that of the PacBio RS II group, and the Simpson index of the MiSeq group was higher than that of the PacBio RS II group. It was demonstrated that the PacBio RS II platform exhibited a significant higher level of α-diversity when compared with the MiSeq platform (Figure 4). In spite of less clean reads, the PacBio RS II system discovered more species than the MiSeq sequencing platform (Figures 2 and 3).

3.2. Taxonomic Analysis of Different Platforms. 437 species derived from 10 phyla, 17 classes, 24 orders, 31 families, and 60 genera were detected by the PacBio RS II platform, while 163 species derived from 12 phyla, 21 classes, 29 orders, 42 families, and 72 genera were detected by the MiSeq platform.

At the phylum level, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and TM7 shared 95.7% of oral microbiome and 1.17% of oral bacteria cannot be classified by the MiSeq platform. However, on the PacBioRS II platform, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, *Actinobacteria*, and TM7 comprised 99.96% of the community and all of the bacteria were annotation to phylum (Figure 5(a)).

The overall structure of oral microbiota for each platform at the phylum level is shown in Figure 6(a). Ten phyla were shared by the two platforms, and Candidate_division_SR1 were found only on the MiSeq platform.

At the class level, the majority of the sequences of MiSeq belonged to *Betaproteobacteria*, *Bacteroidia*, *Negativicutes*, *Actinobacteria*, and *Bacilli*, which contributed 93.3% of the whole community. The unknown and unclassified class proportion accounted for 0.79%. For the PacBio platform, *Betaproteobacteria*, *Bacilli*, *Negativicutes*, *Gammaproteobacteria*, and *Epsilonproteobacteria* shared 92.9% of oral

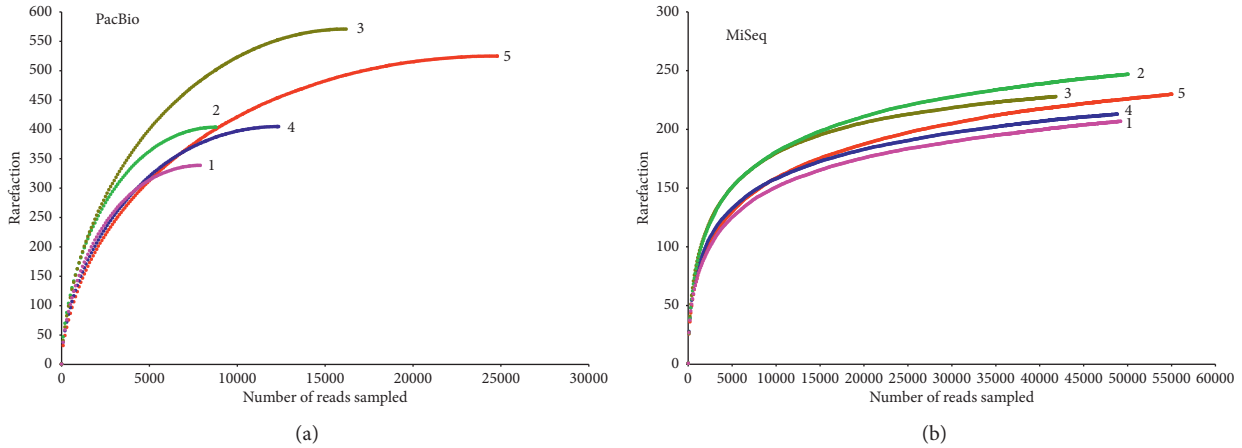


Figure 2: Rarefaction curves for (a) PacBio and (b) MiSeq platforms. The average number of OTUs in each sample was calculated. Samples from the two platforms displayed similar phylogenetic diversity at a 97% identity level.

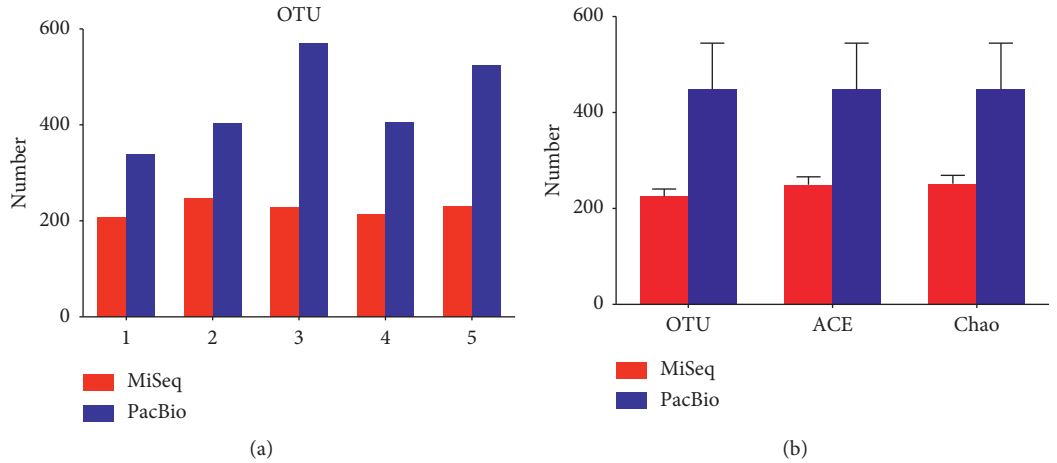


Figure 3: Richness of oral saliva. (a) OTU distribution of the 5 samples sequenced by MiSeq and PacBio platforms. (b) Comparison of OTU number and richness indices (Chao and ACE) between PacBio and MiSeq platforms. Different colors indicate different platforms.

microbiome and a minuscule proportion (0.25%) of unknown classes was generated (Figure 5(b)). The overall structure of oral microbiota for each platform at the class level was shown in Figure 6(b). *Betaproteobacteria* accounted for the largest proportion of the total community in both of the two groups, while the abundance of the abundance of Bacilli and Bacteroidia were different between the two platforms.

At the order level, *Neisseriales*, *Bacteroidales*, *Selenomonadales*, *Lactobacillales*, *Fusobacteriales*, *Pasteurellales*, and *Clostridiales* dominated the community in both groups (Figure 5(c)). The overall structure and portion of oral microbiota for each platform were shown in Figure 6(c). The unknown and unclassified order proportion sequencing by MiSeq was 0.79%; however, only 0.25% order was unclassified by the PacBio platform (Figure 6(c)).

At the family level, *Neisseriaceae*, *Prevotellaceaes*, *Veillonellaceae*, *Streptococcaceae*, *Pasteurellaceae*, and *Fusobacteriaceae* shared 82.4% and 88.0% of oral microbiome by the MiSeq and PacBio platforms, respectively (Figure 5(d)). 0.39% and 0.25% of oral bacteria were unknown or cannot be classified by the MiSeq and PacBio platform, respectively (Figure 6(d)).

At the genus level, the majority of the sequences of the two platforms belonged to *Neisseria*, *Prevotella*, *Veillonella*, *Streptococcus*, *Haemophilus*, and *Fusobacterium*, which contributed 79.4% and 86.8% of the MiSeq and PacBio community. The unknown and unclassified genera of the MiSeq platform accounted for 0.68% (Figure 5(e)). The overall structure and portion of oral microbiota for each platform were shown in Figure 6(e).

At the species level, 68 species were shared by the two platforms and 368 species were detected only by the PacBio RS II platform. Forty-two genera cannot be classified into special strains on the MiSeq platform, which accounted for nearly half of the whole community (Figure 6(f)); however, only 0.03% of microorganisms were unidentified when using the PacBio RS II platform.

Figure 5(f) shows the top 15 species generated by the two platforms. As is shown in the figure, unlike the other levels, there was a distinction between the most abundant bacteria sequenced by the two platforms. Speculation was

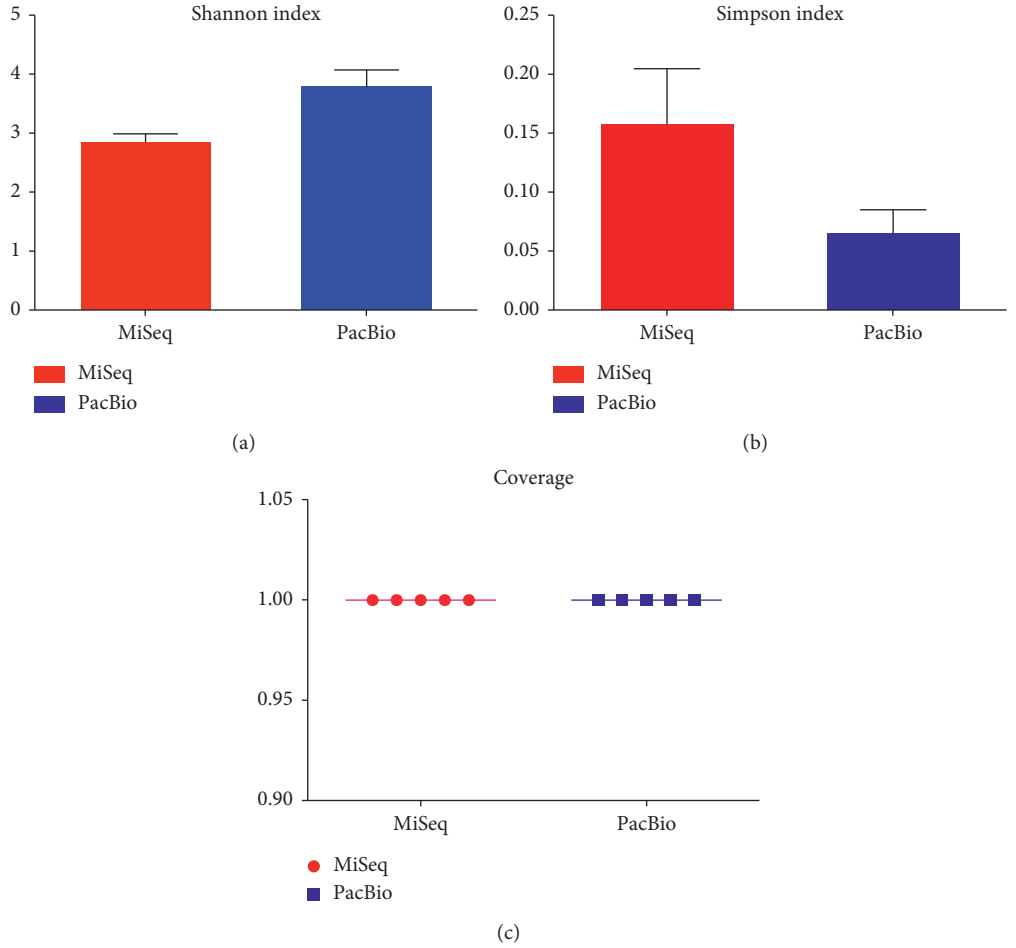


FIGURE 4: Comparison of α-diversity and coverage between MiSeq and PacBio platforms. (a) Shannon index, which can reflect how many OTUs there are in saliva and simultaneously take into account how evenly the OTUs are distributed among the oral microbiome. (b) Simpson index, which is used to measure the degree of concentration when oral microbiota are classified into OTUs. (c) Coverage, which is calculated from the length of the original genome (G), the number of reads (N), and the average read length (L) as $N*L/G$.

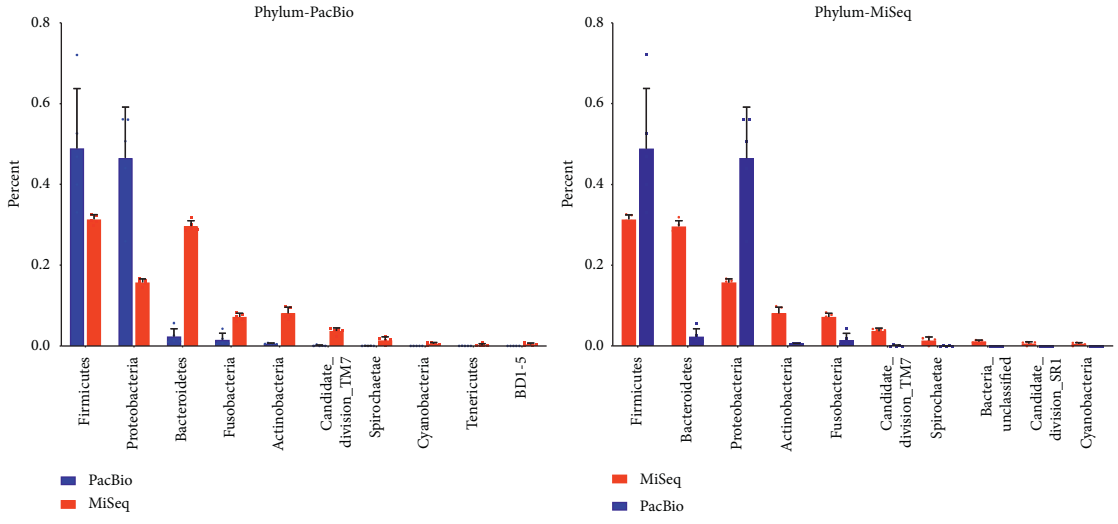
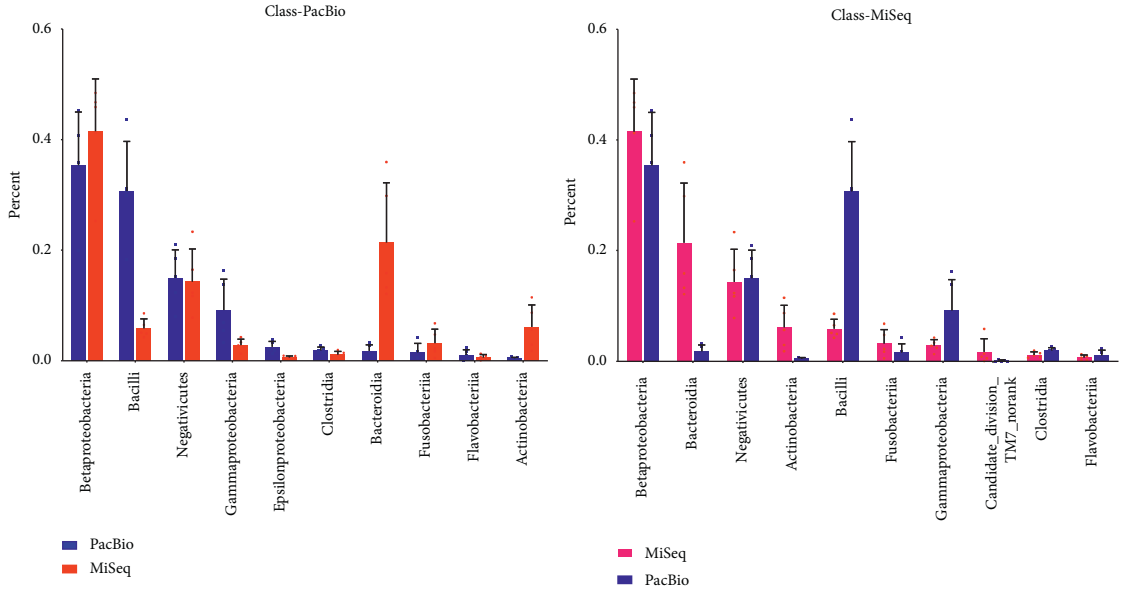
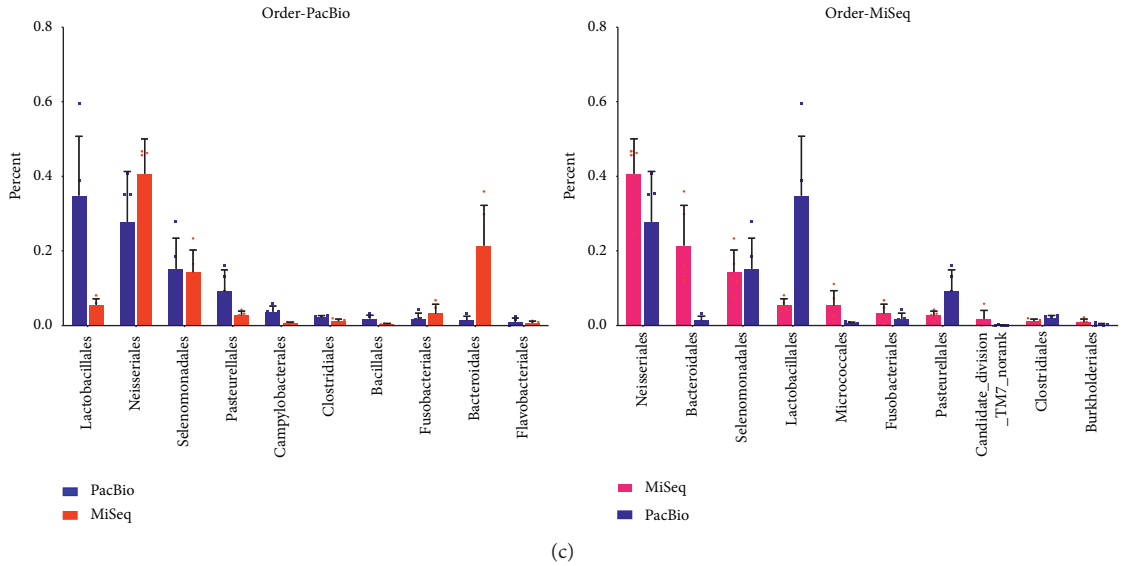


FIGURE 5: Continued.



(b)



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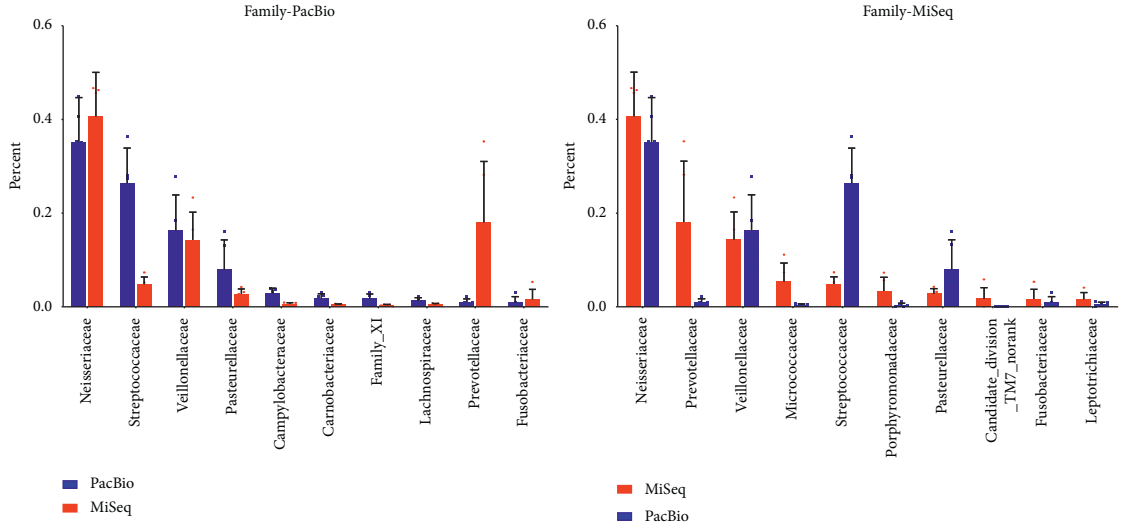

(d)

Figure 5: Continued.

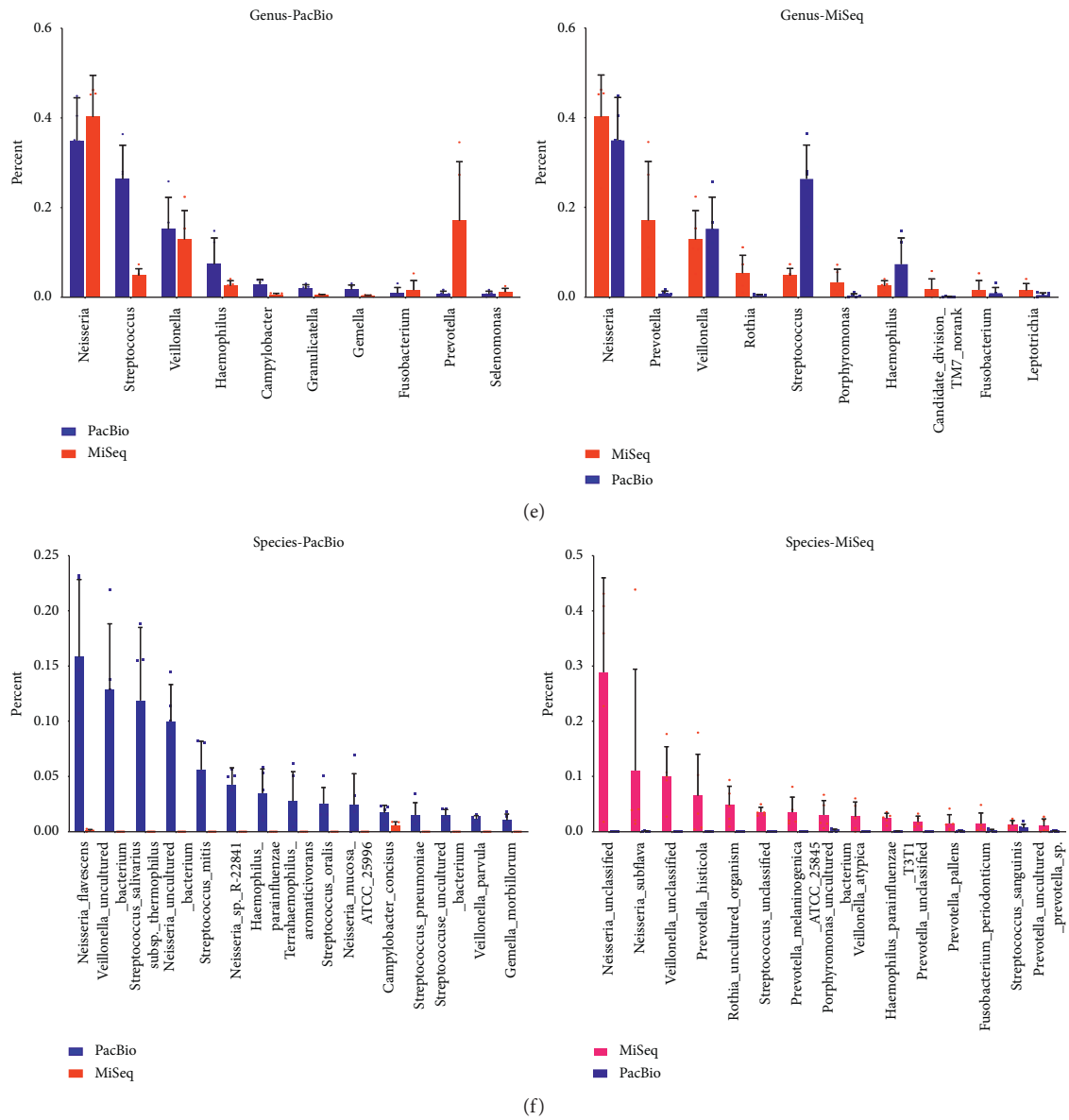

Figure 5: The relative abundance of top 10 phyla, classes, orders, families, and genera and top 15 species. (a) Top 10 phyla. (b) Top 10 classes. (c) Top 10 orders. (d) Top 10 families. (e) Top 10 genera. (f) Top 15 species.

that a large proportion of the total bacteria was unclassified by the MiSeq platform. The structure and composition of saliva microbiota shown in Figure 7 lists comparison of some species sequenced by the two platforms. As is shown in Figure 7, unclassified species accounts for a considerable proportion on the MiSeq platform. The PacBio RS II platform, by contrast, had higher resolution and could provide more information at the species level.

For species of *Actinomyces*, 16.2% of the bacteria was unclassified by the MiSeq platform. *Actinomyces odontolyticus* and *Actinomyces uncultured bacterium* were shared by the two platforms, and 7 unique species were generated only by the PacBio RS II platform (Figure 7(a)).

As to the species of *Campylobacter*, 2.2% of the bacteria was unclassified by the MiSeq platform. *Campylobacter concisus* and *Campylobacter showae* were shared by the two platforms, and 8 species were unique to the PacBio RS II platform (Figure 7(b)).

For species of *Rothia*, 10.1% of the bacteria was unclassified by the MiSeq platform. *Rothia uncultured bacterium* was the only species shared by the two platforms, and 8 unique species were generated only by the PacBio RS II platform (Figure 7(c)).

When it comes to *Haemophilus*, 5.7% of the bacteria was unclassified by the MiSeq platform. *Haemophilus parahaemolyticus*, *Haemophilus parainfluenzae T3T1*, and *Haemophilus uncultured bacterium* were shared by the two

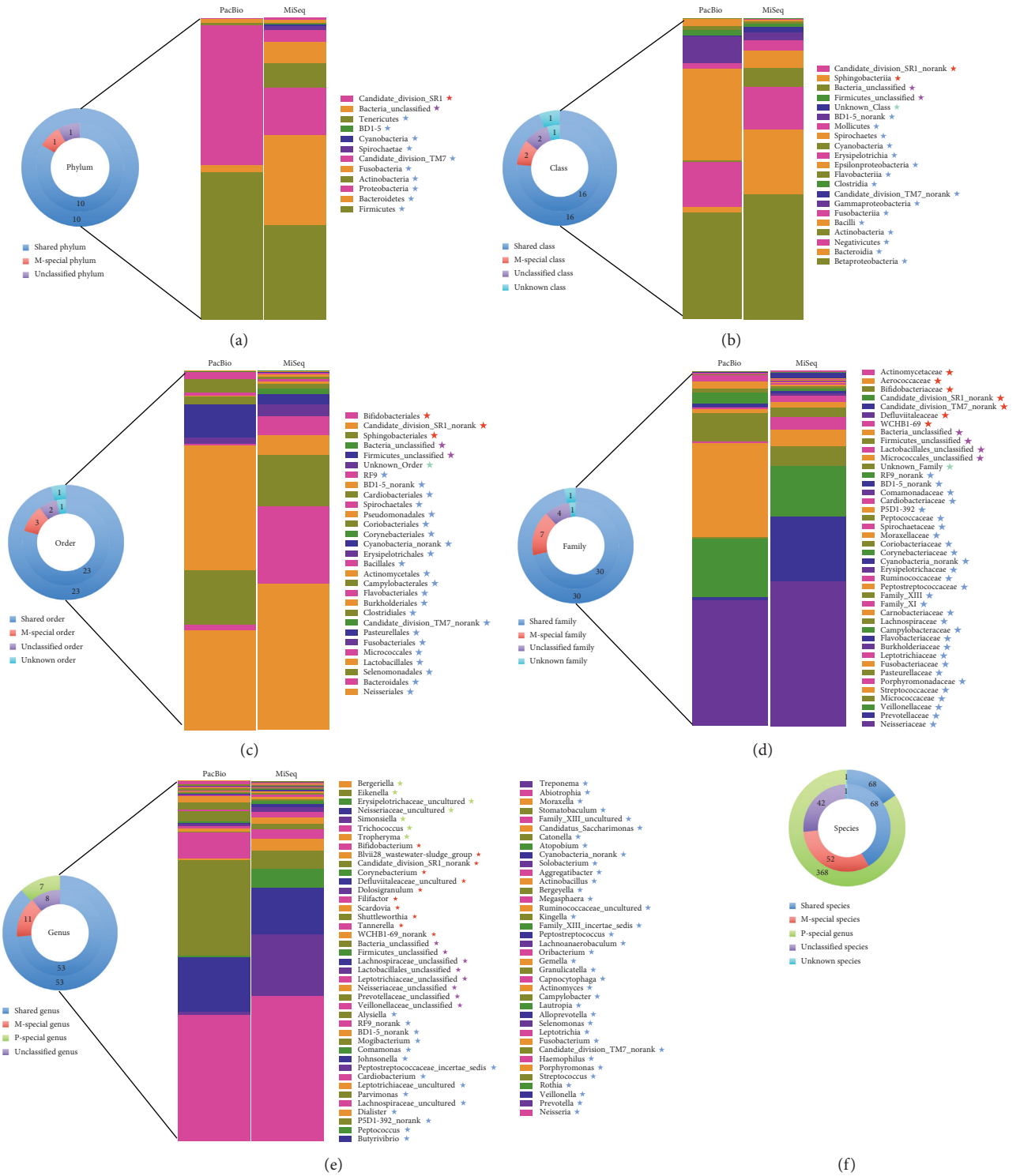

Figure 6: Community structures sequenced by PacBio and MiSeq platforms. ■ represents the number of organisms shared by the two platforms and the detail taxonomy information was shown on the right bar chart. The star of the same color represents the names of the shared organism. ■ represents the number of organisms generated only by MiSeq platforms and the detail taxonomy information was shown on the right bar chart. The star of the same color represents the names of the organism only generated by MiSeq. ■ represents the number of unclassified organism and the detail taxonomy information was shown on the right bar chart. The star of the same color represents the names of the unclassified organism. ■ represents the number of unknown organism and the detail taxonomy information was shown on the right bar chart. The star of the same color represents the names of the unknown organism. ■ represents the number of organisms generated only by PacBio platforms and the detail taxonomy information was shown on the right bar chart. The star of the same color represents the names of the organism only generated by PacBio. (a–f) represents phylum, class, order, family, genus and species level, respectively.

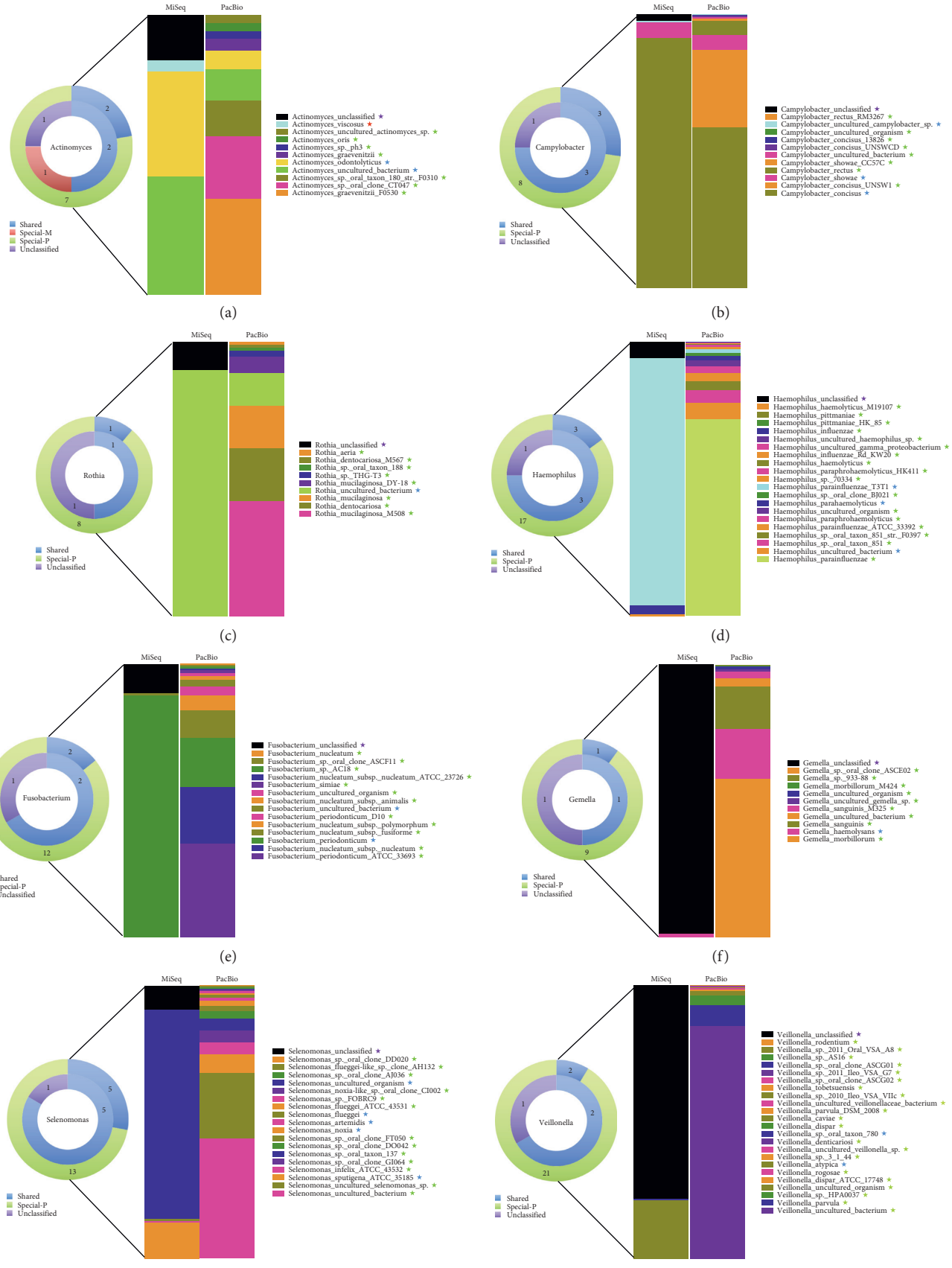

Figure 7: Continued.

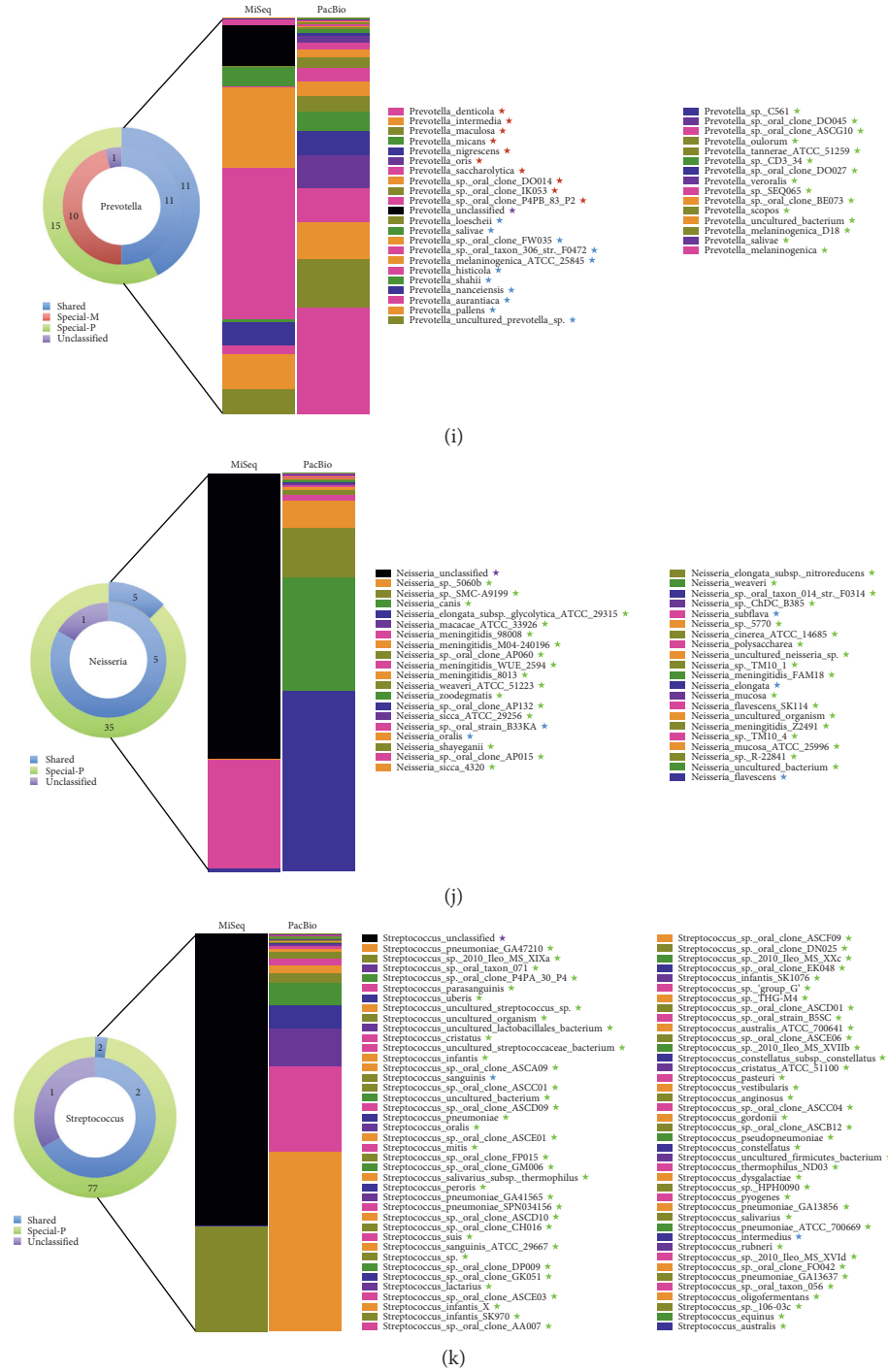

Figure 7: Structure and composition of some particular species sequenced by PacBio and MiSeq platforms. The outer ring of the chart represents the number of species sequenced by the PacBio platform. The inner ring, on the opposite, represents the number of species sequenced by the MiSeq platform. ■ represents the number of species shared by the two platforms, and the detail information was shown on the right bar chart. The star of the same color represents the name of the shared species. ■ represents the number of species generated only by the MiSeq platform, and the detail information was shown on the right bar chart. The star of the same color represents the name of the species only generated by MiSeq. ■ represents the number of unclassified species, and the detail information was marked on the right bar chart. The star of the same color represents the name of the unclassified species. ■ represents the number of species generated only by the PacBio platform, and the detail information was shown on the right bar chart. The star of the same color represents the name of the species only generated by PacBio. (a–k) represents the species of *Actinomyces*, *Campylobacter*, *Rothia*, *Haemophilus*, *Fusobacterium*, *Gemella*, *Selenomonas*, *Veillonella*, *Prevotella*, *Neisseria*, and *Streptococcus*, respectively.

platforms, and 17 unique species were generated only by the PacBio RS II platform (Figure 7(d)).

For species of *Fusobacterium*, which are among the most abundant bacteria in healthy oral cavity, 10.8% of the bacteria was unclassified by the MiSeq platform. *Fusobacterium periodonticum* and *Fusobacterium uncultured bacterium* were shared by the two platforms, and 12 unique species were generated only by the PacBio RS II platform (Figure 7(e)).

Figure 7(f) shows the composition of *Gemella* sequenced by different platforms. The comparison of sequencing results between MiSeq and PacBio RS II indicates that *Gemella haemolysans* was the only species shared by both the platforms and up to 98.8% species were unclassified by the MiSeq platform. Nine unique species were generated only by the PacBio RS II platform.

For species of *Selenomonas*, 8.6% of the bacteria was unclassified by the MiSeq platform. Five species including *Selenomonas uncultured organism*, *Selenomonas flueggei*, *Selenomonas artemidis*, *Selenomonas noxia*, and *Selenomonas sputigena ATCC 35185* were shared by the two platforms, and 7 unique species were generated only by the PacBio RS II platform (Figure 7(g)).

As to species of *Veillonella*, 78.1% of the bacteria was unclassified by the MiSeq platform. *Veillonella atypica* and *Veillonella* sp. *oral taxon 780* were shared by the two platforms, and 21 unique species were generated only by the PacBio RS II platform (Figure 7(h)).

For species of *Prevotella*, 10.5% of the bacteria was unclassified by the MiSeq platform. 11 species including *Prevotella loescheii*, *Prevotella salivae*, *Prevotella* sp. *oral clone FW035*, *Prevotella* sp. *oral taxon 306 str. F0472*, *Prevotella melaninogenica ATCC 25845*, *Prevotella histicola*, *Prevotella shahii*, *Prevotella nanceiensis*, *Prevotella aurantiaca*, *Prevotella pallens*, and *Prevotella uncultured prevotella* sp. were shared by the two platforms. The number of unique species generated by the MiSeq and PacBio RS II platform were 15 and 11, respectively (Figure 7(i)).

For species of *Neisseria*, which are the most abundant species of the community in this study, 71.7% of the bacteria was unclassified by the MiSeq platform. Five species including *Neisseria* sp. *oral strain B33KA*, *Neisseria oralis*, *Neisseria subflava*, *Neisseria elongata*, and *Neisseria flavescens* were shared by the two platforms. The number of unique species generated by PacBio RS II platform was up to 35 (Figure 7(j)).

Streptococcus is a gram-positive bacterium belonging to the phylum *Firmicutes*, which is found to be associated with many kinds of oral diseases, such as caries [18, 27], pneumonia, bacteremia, and meningitis [28, 29]. In this study, 73.4% of *Streptococcus* was unclassified by the MiSeq platform. Only the two species *Streptococcus intermedius* and *Streptococcus sanguinis* were shared by the two platforms. The number of unique species generated by the PacBio RS II platform was up to 77 (Figure 7(k)).

4. Discussion

A number of research studies have presented evidence for using childhood oral microbiome to predict future oral and systemic diseases [30]. Therefore, it is very important for us to find a suitable sequencing method to study oral microbiome. In this study, the oral saliva microbiome of five healthy Chinese children was evaluated using the NGS and TGS. The oral microbiome composition sequenced by the two platforms was basically identical from phylum to genus level. The structure of oral microbiome at the species level, however, showed a significant difference between the two platforms. The possible reason we speculate is that a large amount of short reads generated by the MiSeq platform cannot be resolved in spite of the development of the assemblers, such as the Celera Assembler, SOAPdenovo, and Allpath-LG. As a result, a very large proportion of bacteria was unclassified by the MiSeq sequencing technology. The longer reads sequenced on the PacBio platform gave more phylogenetic resolution than 400–500 bp fragments that contain fewer hypervariable regions.

Compared with our previous study on the structure of oral microbiome in healthy children, the top 10 phyla, genera, and species are consistent [18]. However, when compared with other studies, there are some differences with our results [31]. In this respect, we speculated that oral microbiome is linked to age, race, and region at the species level. Some studies have also demonstrated that the oral microbiota are better defined based on age, gender, oral niches, and even the body size [32, 33]. Recent findings indicate that the oral ecosystem of healthy children is highly heterogeneous and dynamic with substantial changes in microbial composition over time and only few taxa persisting across the age [34]. PacBio RS II sequencing, one platform of TGS, has the ability to provide longer sequences and reads generated from sequencing the entire 16S rRNA gene. Compared with the previous NGS, this platform can establish a higher estimate of richness and provide the ability to identify organisms at a higher taxonomic and phylogenetic resolution [17, 18, 35]. At the same time, some studies have shown that the PacBio sequencing error rate is in the same range of the previously widely used Roche 454 sequencing platform and the current MiSeq platform [36, 37]. More importantly, a recent study presented a high-throughput amplicon sequencing methodology based on PacBio CCS that measures the full-length 16S rRNA gene with a near-zero error rate [38].

Compared with the traditional 16S rDNA sequencing of the MiSeq platform, the PacBio RS II technology improved its read length and annotated the nucleotide sequence of oral bacteria to the species level. PacBio RS II may be optimal for oral microbiome sequencing due to its long reads and high performance, while platforms such as Illumina MiSeq will provide cost-efficient methods for sequencing projects.

Previous research studies had compared the TGS PacBio platform with the NGS Roche 454 pyrosequencing platform. Amplicons of the 16S rRNA gene from the environmental

samples from streambed habitats, rocks, sediments, and a riparian zone soil were analyzed [16, 17]. In this study, we focus on the oral microbiome of healthy Chinese children and compare the amplicons of the 16S rRNA gene between PacBio and MiSeq platforms. As the exact composition of the microbiome from the five Chinese children were unknown, it is still difficult to assess the accuracy of the PacBio RS II platform at the species level. Next, we would enroll a known isolate as a positive control in high-throughput sequencing, which can provide the quality assurance of quantifying error rates when analyzing environmental communities.

5. Conclusions

In our study, oral microbiome of healthy Chinese children was explored. For oral microbiome studies, if the goal is identifying all species in a sample, PacBio appears to have superior performance to MiSeq. However, if the goal is to simply quantify relative differences in diversity, either platform would be appropriate. In this article, we have compared the difference between the two platforms, however, with the limited sample size, the study does not provide a statistic conclusion, and more in-depth studies with larger group sizes are needed to validate these results.

Data Availability

The data sets used and/or analyzed during the current study available from the corresponding author on reasonable request. The authors have deposited the raw data and clean data (after QC and human reads removal) to the NCBI under accession number PRJNA445629.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the Science and Technology Project of the Zhejiang Provincial Department of Education (Grant no. Y201737984) and the Natural Science Foundation of Zhejiang Province, China (Grant no. LQ19H140002). This article is dedicated to Hui Chen, who guided us to accomplish this project.

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2020, Article ID 8867548, 9 pages
https://doi.org/10.1155/2020/8867548

Research Article

A Clinic Trial Evaluating the Effects of Aloe Vera Fermentation Gel on Recurrent Aphthous Stomatitis

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Received 27 August 2020; Accepted 12 November 2020; Published 7 December 2020

Academic Editor: Christian Bautista

Recurrent aphthous stomatitis (RAS) is the most common disorder in the oral mucosa that affects the daily quality of life of patients, and there is currently no specific treatment. In the present study, we developed aloe vera fermentation gel under the action of probiotics on aloe vera. In total, 35 patients with the history of aphthous stomatitis were enrolled to explore the potential benefits of aloe vera fermentation gel to treat RAS, and the healing-promotion effects were recorded and compared; microbial compositions in different groups were tested by high-throughput sequencing. Our results indicated that the duration of healing time of the aloe group showed potentially better effects because of the higher proportion of 4–6 day healing time (35% vs. 20%) and lower proportion of 7–10 day healing time (65% vs. 80%) compared with that of the chitosan group. Also, the use of aloe vera fermentation gel could return oral bacteria to normal levels and reduce the abundance of harmful oral bacteria including *Actinomyces*, *Granulicatella*, and *Peptostreptococcus*. These results suggest that aloe vera fermentation gel has the ability to treat patients with RAS and has positive prospects in clinical applications.

1. Introduction

Recurrent aphthous stomatitis (RAS) is a common disorder characterized by single or multiple ulcers with clear boundaries usually at the lingual margin, cheek, and lip; RAS affects 10–20% of the population and can heal automatically in about 10 days. Despite the high prevalence, the aetiological mechanism is not completely clear at present, but several risk factors, e.g., genetic susceptibilities, immune disorders, infections, vitamin and trace element deficiencies, systemic diseases, hormonal imbalances, mechanical damage, and stress, have been shown to be associated with the occurrence and progression of this disease [1, 2]. RAS has seriously affected the daily quality of life of patients (speaking, eating, and swallowing), but there is still no specific curative management for RAS, although topical medications, consisting of preservatives and anti-inflammatory/analgesics, aim to reduce pain degree and inflammation, while promoting disease healing [3]. Therefore, it is necessary to develop an effective agent for the treatment of RAS.

The oral cavity harbours many distinct microbial communities dominated by Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, and Bacteroidetes; disturbances of the oral microbiota contribute to the prognosis of a series of oral diseases, including mucositis and periodontitis and may be related to the aetiopathogenesis of RAS [4–6]. Given this, the intervention of saliva microbiota composition could play a potential role in the treatment of RAS [7–9]. In recent years, the concept of probiotics has gradually entered people's vision, has been used in the treatment of various oral diseases such as periodontitis and mucositis, and can be used as an adjunctive in treating RAS [10–13]. Aloe vera is a cactus-like plant that has been broadly used as

a cosmetic moisturiser, toothpaste, food flavouring, and preservative in the pharmaceutical and food fields; it can also be used in medicine for its effects such as wound healing, anti-inflammation, antioxidant, antibacteria, antifungal, antiviral, and antitumour properties [1, 14]. Moreover, previous studies indicated that aloe vera showed the ability to treat RAS evidenced by reduced pain and healing time [1].

Considering the therapeutic efficacies of aloe vera and probiotics, the probiotic fermentation products of aloe vera show promise in RAS treatment, as fermentation by probiotics can result in new compounds which have the potential for health-modulation and produce lots of metabolites including lactic acid, an antioxidant that can strengthen the beneficial effects of probiotics and aloe vera [15]. Moreover, our previous studies demonstrated that aloe vera fermentation showed strong antibacterial, antioxidant, and anti-inflammatory activities and possessed a strong burn injury healing effect in vivo [14, 16].

In the present study, the aloe vera fermentation gel was produced and used for RAS patients to evaluate its effects on shortening the healing time and restoring microbial diversity in the oral cavity.

2. Materials and Methods

2.1. Aloe Vera Fermentation Gel Preparation. *Lactobacillus plantarum* MH-301 (provided by Harbin Meihua Biotechnology Co, Ltd, Harbin, Heilongjiang, PR China) was cultured in 5 ml Man–Rogosa–Sharpe (MRS) medium 10–16 h for static cultivation at a temperature of 37°C. Then, *L. plantarum* MH-301 was cultured in fresh MRS medium for amplification cultivation overnight at 37°C. The aloe vera leaves were cut off and mashed after being washed thoroughly. Sterile water (1 : 1) and edible glucose (5% of aloe vera mass) were added to the mashed aloe vera. The overnight cultured probiotic was centrifuged at 8000 g for 5 min and washed with sterilised phosphate-buffered saline (PBS) 2–3 times; the probiotics were resuspended by adding them to sterilised PBS at a level of 5% of aloe vera. The probiotics were inoculated into the inactivated aloe vera solution and incubated for 36–72 h at 37°C for fermentation until the pH reached 3–4. The aloe vera fermentation gel was prepared by adding gelatine powders to the aloe vera solution and conserved at 4°C.

2.2. Ethical Statement. This trial was conducted at the Affiliated Stomatological Hospital of Nanchang University, China, in 2019. The trial was approved by the Ethical Committee of Affiliated Stomatological Hospital of Nanchang University (No. 2019–008) and had been registered at the Clinical Trail Registry (identifier: ChiCTR1900023903). All participants signed a written informed consent form, and all methods were performed in accordance with the approved guidelines.

2.3. Participants and Selection Criteria. Forty-six patients were enrolled, and 35 patients (12 male and 23 female) aged 18–60 years were selected from the Affiliated Stomatological Hospital of Nanchang University patients, and an additional 10 healthy people were selected as the negative control. All patients were enrolled 2–5 days after the occurrence of first oral ulcer. Inclusion criteria consisted of the following: (a) clinical examination and confirmed history of recurrent oral ulcer, (b) recurrence of the oral ulceration, and (c) patients in good condition with no serious systemic disease. Eligible persons were excluded if they were administered antibiotics or glucocorticoids or accepted periodontal or dental treatments that interfered with the results of our experimental drugs.

2.4. Trial Design. The baseline information of patients, including individual information (age and gender) and characteristics of ulcers (duration of healing time), were reported for all patients. Oral inspections were conducted by one inspector of the Affiliated Stomatological Hospital of Nanchang University. All patients were divided into two groups. For the first group, patients were applied with aloe vera fermentation gel, reported as the AA group. The remaining patients were using chitosan gel (AC group). All patients were informed to apply a layer of gel on the surface of the ulcer every day after each meal (three times each day) until the ulcer disappeared. The duration of recovery for all patients in the two groups was recorded. The saliva of patients was collected before the use of gels as a positive control (PC group) and collected again after the use of drugs once the ulcers disappeared. Additionally, 10 healthy individuals were enrolled into this experiment as the negative control group (NC group) and their saliva was collected and conserved for further high-throughput sequencing.

2.5. DNA Extraction and High-Throughput Sequencing. Saliva samples were taken and stored at −80°C. The combination of genomic DNA kits and a bead method was used, and the concentration and quality of DNA were determined by ultraviolet photometer. The V4 regions of the 16S rDNA genes of each sample were amplified by PCR amplification using designed primers of 515F/806R (515F, 5′-GTGCCAGCMGCCGCGGTAA-3′; 806R, 5′-GGAC TACVSGGGTATCTAAT -3′) according to the conserved regions in the sequence. Amplified DNA products were sequenced by an Illumina MiSeq platform, and the raw data were conserved in the form of FASTQ (Gene Bank accession number PRJNA656084).

2.6. Data Analysis. Paired-end reads from the original DNA fragments were processed by FLASH software (v1.2.7, http://ccb.jhu.edu/software/FLASH/) and QIIME software (v1.8.0, http://qiime.org/). Sequences with ≥97% similarity were regarded as the same operational taxonomic units (OTUs). The compositions and relative abundances of each sample at the phylum level and the genus level were analysed using the QIIME software. The QIIME software (v1.8.0) was also used to analyse the *a*-diversity (within samples, indexes of observed OTUs) and *ß*-diversity (between samples, PCoA). According to the obtained OTU abundance matrix, the total

number of OTUs in each sample (group) was calculated and visualised through the Venn diagram using *R* software.

All data were reported as mean ± SD, and results were analysed using SPSS20.0 (Chicago, IL) and GraphPad Prism (v6.0) via one-way ANOVA, chi-squared test, and unpaired *t* test. All tests were two-tailed, and the p value of 0.05 was considered to be statistically significant.

3. Results

3.1. Participants. A total of 46 volunteers (16 male and 30 female) were enrolled in the study. During the clinical test analysis, 10 in the AA group and 1 in the AC group were excluded due to missing data. Subsequently, 7 in the AA group and 4 in the AC group were excluded as the DNA extraction failed. Additionally, 10 healthy individuals were enrolled for high-throughput sequencing. The flow diagram is shown in Figure 1.

3.2. Aloe Vera Fermentation Gel Accelerated the Healing of RAS. The AA and AC groups were fixed by age and gender ($p = 0.186$, $p = 0.411$, respectively). The mean healing time for aloe vera fermentation gel and chitosan gel was 7.40 ± 1.85 days and 7.93 ± 1.84 days, respectively. Additionally, all patients were healed 4–10 days after the occurrence of ulcers; the healing time was divided into 4–6 days and 7–10 days to analyse the healing effects in the AA and AC groups based on clinical experience. The proportion of patients with a 4–6 day healing time in the AA group was higher than in the AC group (35% vs. 20%, respectively; $p = 0.728$), whereas the proportion of patients with a 7–10 day healing time in the AA group was lower than in the AC group (65% vs. 80%m respectively; $p = 0.931$) (Table 1). Thus, aloe vera fermentation gel had a potentially better wound healing effect than chitosan gel.

3.3. The α- and ß-Diversities of the Oral Microbial Community. From the 58 communities in the NC, PC AA, and AC groups, 6,345,619 filtered clean tags (average of 109,407.22 filtered clean tags per sample) and 18,739 OTUs (average of 323.09 OTUs per sample) were obtained from all samples (data not shown). The common OTU Venn diagram suggested that there are 270 common core OTUs found in all groups; the numbers of unique OTUs in the NC, PC, AA, and AC groups was 1,360, 2,360, 1,103, and 742, respectively (Figure 2(a)). As shown in Figure 2(b), observed species were analysed to estimate the α-diversity of the bacterial communities; no significance was observed in the NC (336.86 ± 294.46), PC (301.89 ± 195.29), AA (304.68 ± 130.14), and AC (283 ± 132.50) groups. The principal coordinates analysis (PCoA) was used to evaluate the OTU relationship between different groups (Figure 2(c)), and the microbial diversities in the PC group were significantly different from those in the NC group; treatment with the aloe vera fermentation gel could alter the microbial diversities toward to the NC group.

3.4. Comparison of the Oral Microbiota Composition. As shown in Figure 3, the relative abundance of bacteria was compared between the NC, PC, AA, and AC groups. The results suggested that the bacteria from the genera Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes were dominant and comprised >93% of oral bacteria in all groups at the phylum level. Additionally, we analysed the relative abundance of Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes, respectively. The results showed that the abundance of Firmicutes (37.98% vs. 69.87%) and Actinobacteria (7.33% vs. 13.44%) were increased in the PC group compared with that in the NC group, whereas the abundance of Proteobacteria (29.34% vs. 12.84%) and Bacteroidetes (18.81% vs. 0.64%) was decreased in the PC group. After treatment, the relative abundance of Proteobacteria (22.14%) and Actinobacteria (9.32%) was changed toward to the normal level in the AA group. At the genus level, the ten most abundant genera were Streptococcus, Haemophilus, Actinomyces, Neisseria, Gemella, Granulicatella, Peptostreptococcus, Prevotella, Rothia, and Alloprevotella, accounting for >74% of all bacteria (Figure 4). In the PC group, the relative abundance of Streptococcus (22.85% vs. 48.97%), Actinomyces (5.40% vs. 10.10%), Gemella (2.30% vs.4.63%), Granulicatella (1.70% vs. 4.70%), and Peptostreptococcus (1.78% vs. 5.59%) was increased compared with that of the normal NC group, while the abundance of Haemophilus (14.75% vs. 2.83%) and Neisseria (9.48% vs. 2.57%) was decreased in the PC group compared with that of the NC group. The use of aloe vera fermentation gel (Haemophilus (8.96%), Actinomyces (6.43%), Neisseria (5.39%), Granulicatella (3.98%), and Peptostreptococcus (1.63%)) returned to normal levels in the AA group.

4. Discussion

RAS causes pain and difficulties with eating, speaking, and swallowing, thereby affecting the patients' quality of life [17]. In consideration of the beneficial effects and safety of herb medicines, it is meaningful to develop a new herb medicine in the treatment of RAS.

In the present study, we developed the aloe vera fermentation gel, and its effects on RAS were evaluated and compared with the approved chitosan gel on the market in 35 patients from the Affiliated Stomatological Hospital of Nanchang University. The results suggested that the oral ulceration in these patients could disappear within 10 days, with 35% of patients recovered within 4–6 days and 65% of patients recovered by 7–10 days. However, only 20% of patients using chitosan gel were recovered within 4–6 days, and 80% of patients were recovered by 7–10 days (Table 1), indicating that the aloe vera fermentation gel had potentially better wound healing benefits than chitosan gel.

For RAS, wound healing and anti-inflammation are important for patients. Aloe vera is a cactus-like plant that has been widely used in medicine to treat burn injuries, cutaneous wounds, and oral ulceration, which makes it a good candidate for the treatment of RAS [18, 19]. The reasons for the shortened healing time in the AA group may

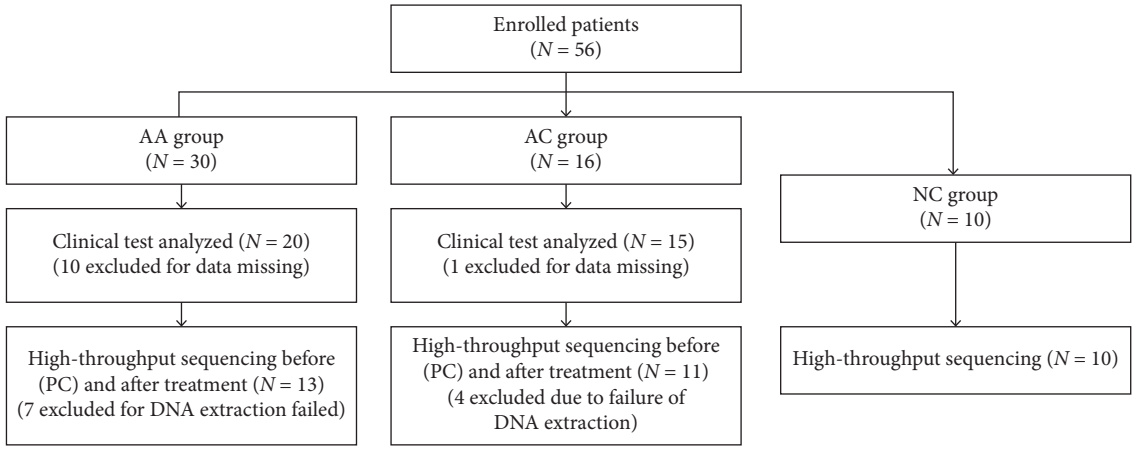

Figure 1: Flow diagram of the trial; forty-six patients with RAS were enrolled into our trial, and then 11 patients (10 in AA and 1 in AC) were excluded due to data missing from the clinical test analysis. Moreover, 11 patients (7 in AA and 4 in AC) were excluded from high-throughput sequencing analysis as the DNA extraction failed. Additionally, 10 healthy individuals were selected for high-throughput sequencing as a negative control group.

Table 1: Patients' baseline information and characteristics.

Variable	AA group	AC group	p value
Percentage of total enrollment, No (%)	20 (57.14)	15 (42.86)	—
Male; female, n: n (%: %)	8 : 12 (40.00 : 60.00)	4 : 11 (26.67 : 73.33)	0.411
Age, y	30.5 y26.6	26.0756.44	0.186
Duration of healing time, no. (%)			
4–6 days	7 (35.00)	3 (20.00)	0.728
7–10 days	13 (65.00)	12 (80.00)	0.931

AA, patients treated with aloe vera fermentation gel. AC, patients treated with chitosan gel.

be due to the effectiveness of aloe vera for promoting wound healing and anti-inflammation and the strong anti-inflammatory, immunomodulatory, antioxidative, and antibacterial effects endowed by probiotics during aloe vera fermentation [20, 21]. RAS is a chronic inflammatory disease, and the benefits of aloe vera have been shown in the treatment of this disease [22]. Inflammation is a dynamic process with proinflammatory cytokines, and aloe vera shows anti-inflammatory benefits by inhibiting inflammatory processes and proinflammatory cytokines [23]. For example, Aloe vera can inhibit the leukocyte infiltration, eicosanoid formation, and generation of inflammatory mediators including histamine and bradykinin [1]. Aloe vera contains a series of components such as acemannan which have wound healing potential by enhancing the repair process and epithelial cell proliferation via the induction of factors contributing to wound repair including fibroblasts and collagen [1, 24]. Furthermore, the immunomodulatory effects of aloe vera also suggest its potential benefit for RAS treatment [1, 19]. The antioxidant components in aloe vera also enhance the anti-inflammatory effects by inhibiting the production of reactive oxygen metabolites, therefore preventing oxidative stress [25]. Probiotics are live microorganisms that can confer a healthy benefit on the host and can be used as an adjunctive in various diseases due to wound healing effects and antimicrobial effects against various pathogens [13, 26]. The topical use of probiotics can be used as an antagonist against wound pathogens and enhance wound healing effects, by decreasing the pathogen load, and can be used in the treatment of burn infections and chronic ulcers [26]. Immune system dysfunction favours the occurrence of inflammatory reactions and the appearance of RAS [27]. Evidence indicates that probiotics can modulate the immune response along with anti-inflammation to influence the progression of RAS [27]. The anti-pathogenic properties, together with tissue repair, and immunomodulatory, and anti-inflammatory properties of probiotics, make them an attractive option in RAS [28]. In particular, the combination of aloe vera and probiotics during aloe vera fermentation confers a better effect.

The human oral cavity belongs to the second-most abundant source of microbiota after the gastrointestinal tract, and previous studies have shown that the oral microbiota in healthy individuals are different from the microbiota observed in patients with various oral diseases and oral dysbiosis [6, 29–31]. Our results suggest that the aloe vera fermentation gel can not only shorten the healing time but also alter the speed at which bacterial compositions return to normal levels (Figures 3 and 4). The relative abundances at the phylum level were analysed; the first six phyla composed of Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Fusobacteria, and Spirochaetes were consistent with those in the previous studies of oral microbiota [6]. We found that Firmicutes was significantly

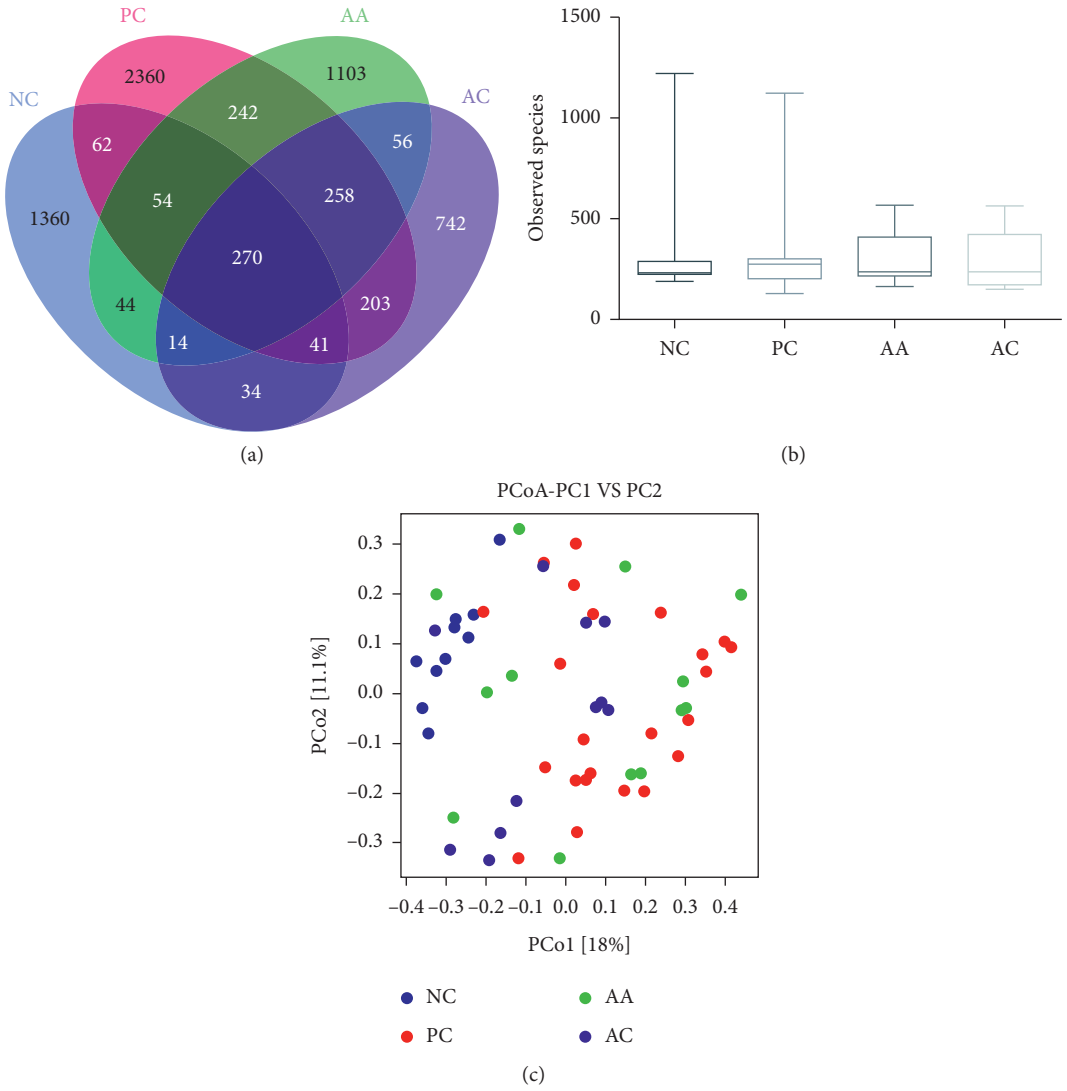

Figure 2: Evaluation of aloe vera fermentation gel on α-diversity (within samples), β-diversity (between samples), and Venn diagram representation. (a) Common OTUs' Venn diagram. (b) Observed species. (c) Principal coordinates analysis (PCoA).

increased, whereas Proteobacteria and Bacteroidetes were significantly reduced in RAS patients. The relative abundances of Actinobacteria were increased in the PC group and decreased after treatment with aloe vera fermentation gel, which indicates that this drug has potential benefit with regard to returning relative abundances to normal levels. The relative abundances at the genus level were also compared between groups. The decreased *Haemophilus* and *Neisseria* and increased *Actinomyces*, *Granulicatella*, and *Peptostreptococcus* in the PC group and normalised abundances in the AA group demonstrated that aloe vera fermentation gel had benefits in the maintenance of microbial compositions [32]. Additionally, the decreased *Haemophilus* and *Nesseria* correlated the results of a previous study showing that decreased Proteobacteria, containing *Haemophilus* and *Nesseria*, is related to oral diseases such as gingivitis and cancer [33–36]. *Actinomyces* has long been recognised as a causative agent of actinomycosis as it increases the pathogenicity by attacking broken or necrotic tissues and is related to the incidence of RAS, with high abundance indicating poor prognosis for RAS [37, 38]. Also, the previous studies have shown that *Granulicatella* is raised in patients with oral inflammatory diseases [39, 40]. From the community alternations, we concluded that ulceration could change the bacterial compositions and lasts for the entire ulcerative process. Nonetheless, the intervention of aloe vera fermentation gel could normalise some of the bacteria at the phylum and genus levels and decrease the abundance of harmful oral bacteria which indicate good prognosis and suggest that our new drug has the potential to be used in the clinical setting.

In conclusion, this clinical trial showed that Aloe vera fermentation gel effectively facilitated the healing process and normalised microbiota disorders of RAS. It can reduce the abundance of harmful oral bacteria including *Actinomyces*, *Granulicatella* and *Peptostreptococcus*, which implies a better prognosis. Therefore, it could improve the quality of life for patients with RAS. This offers a direction for future

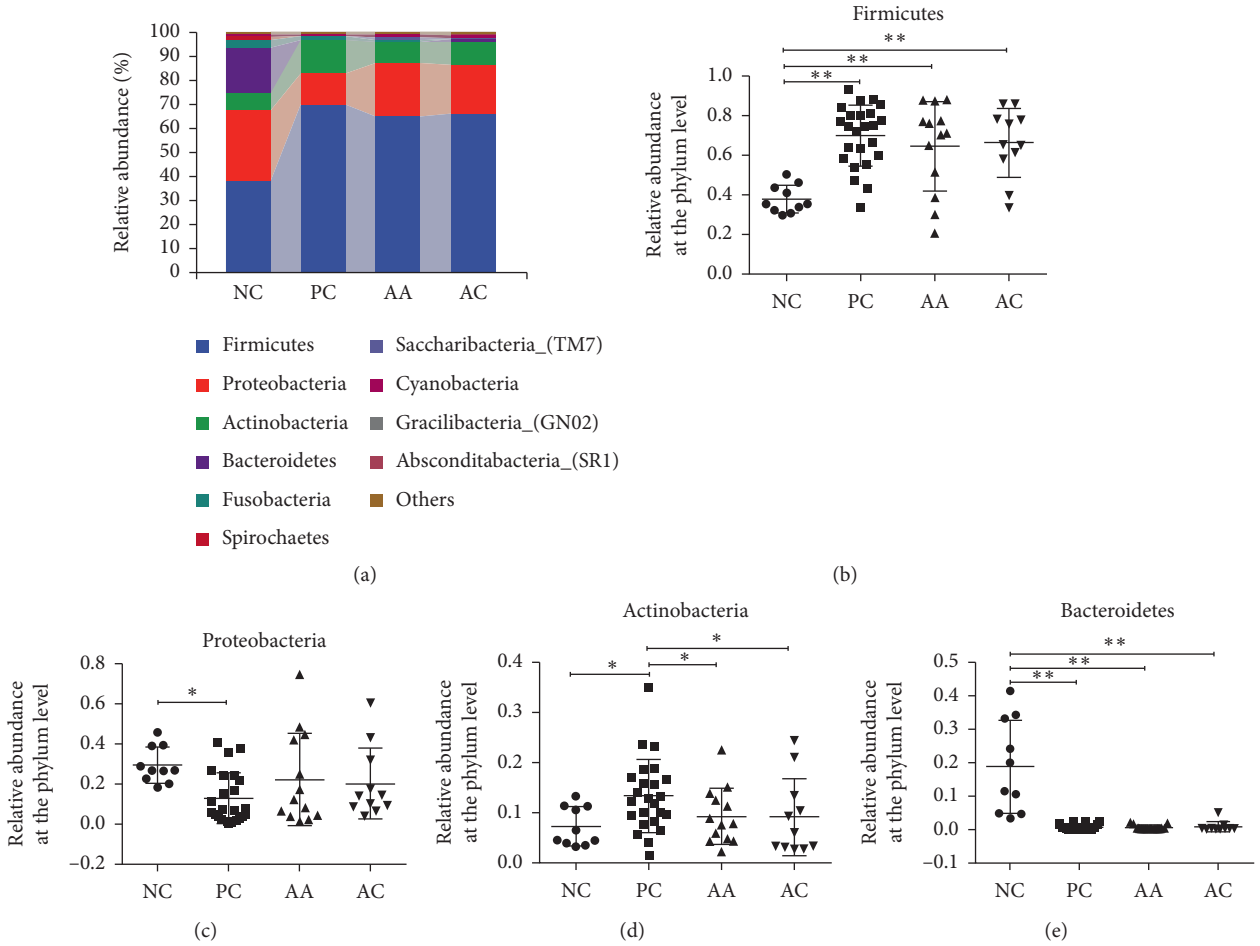

Figure 3: Evaluation of the aloe vera fermentation gel on the bacterial compositions at the phylum level. (a) The relative abundances of the top 10 bacteria at the phylum level. The relative abundances of (b) Firmicutes, (c) Proteobacteria, (d) Actinobacteria, and (e) Bacteroidetes. All data are shown as mean ± SD. Significant differences are denoted by $^{*}p < 0.05$ and $^{**}p < 0.01$.

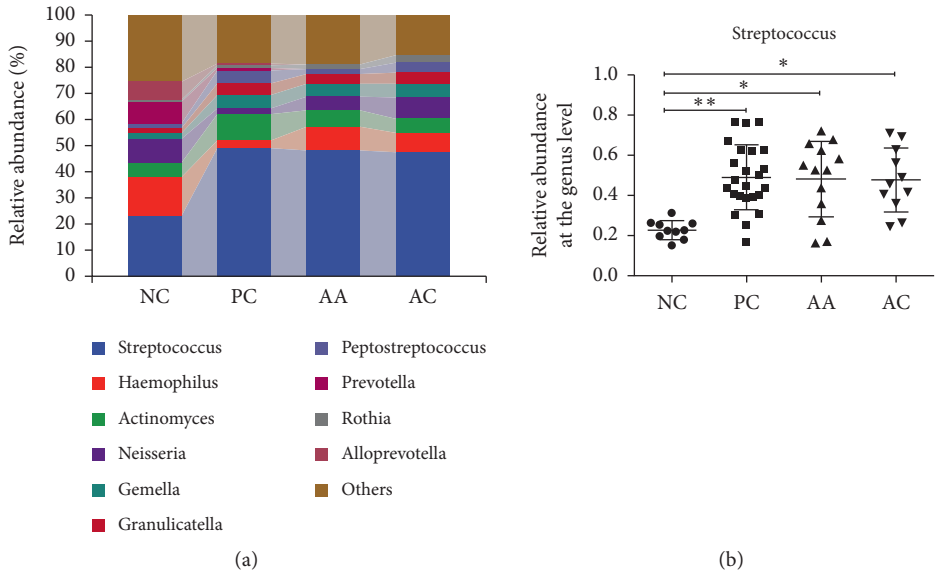

Figure 4: Continued.

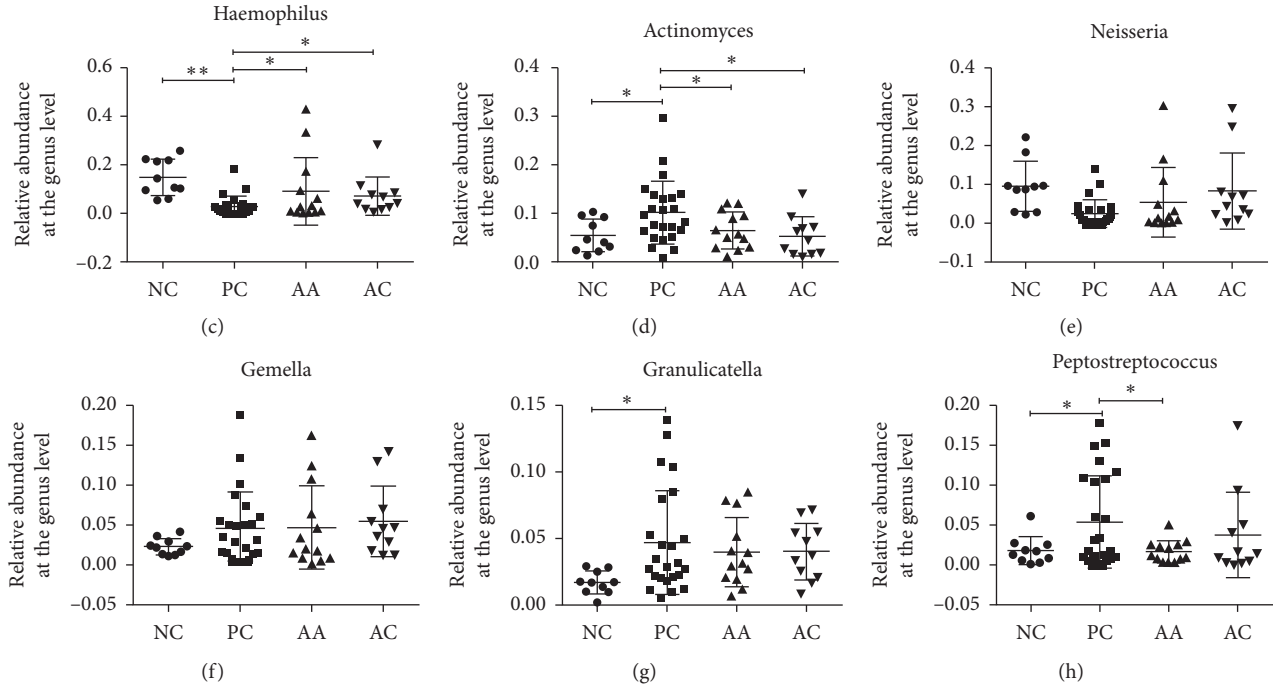

Figure 4: Evaluation of the aloe vera fermentation gel on the bacterial compositions at the genus level. (a) Taxonomic profiles at the phyla level in 58 saliva samples of the top 10 genera at the genus level. The relative abundances of (b) *Streptococcus*, (c) *Haemophilus*, (d) *Actimomyces*, (e) *Neisseria*, (f) *Gemella*, (g) *Granulicatella*, and (h) *Peptostreptococcus*. All data are shown as mean ± SD. Significant differences are denoted by $^{*}p < 0.05$ and $^{**}p < 0.01$.

research, and provides a potential drug for clinical use. However, the effects of Aloe vera fermentation gel were evaluated in just two aspects, so further underlying mechanism tests are needed to accelerate the clinical application of this drug.

Data Availability

The deidentified raw data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The trial was approved by the Ethical Committee of Affiliated Stomatological Hospital of Nanchang University (No. 2019–008) and had been registered at the Clinical Trail Registry (identifier: ChiCTR1900023903).

Consent

All participants signed a written informed consent form, and all methods were performed in accordance with the approved guidelines.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by the grants from the National Natural Science Foundation of China (no. 82060638), the Major Academic Discipline and Technical Leader of Jiangxi Province (no. 20194BCJ22032), and "Double Thousand Plan of Jiangxi Province" (Superior Science and Technology Innovation Team of Jiangxi Province).

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2020, Article ID 8837156, 13 pages
https://doi.org/10.1155/2020/8837156

Research Article

The Impact of Age and Pathogens Type on the Gut Microbiota in Infants with Diarrhea in Dalian, China

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Received 14 September 2020; Revised 25 October 2020; Accepted 11 November 2020; Published 30 November 2020

Academic Editor: Tingtao Chen

Objective. Diarrhea in infants is a serious gastrointestinal dysfunction characterized by vomiting and watery bowel movements. Without proper treatment, infants will develop a dangerous electrolyte imbalance. Diarrhea is accompanied by intestinal dysbiosis. This study compared the gut microbiota between healthy infants and diarrheic infants. It also investigated the effects of age and pathogen type on the gut microbiota of infants with diarrhea, providing data for the proper treatment for diarrhea in infants. *Materials and Methods*. DNA was collected from the fecal samples of 42 Chinese infants with diarrhea and 37 healthy infants. The healthy infants and infants with diarrhea were divided into four age groups: 0–120, 120–180, 180–270, and 270–365 days. Using PCR and 16S rRNA high-throughput sequencing, the diarrhea-causing pathogens in these infants were identified and then categorized into four groups: *Salmonella* infection, *Staphylococcus aureus* infection, combined *Salmonella* and *Staphylococcus aureus* infection, and others (neither *Salmonella* nor *Staphylococcus aureus*). *Results*. The species diversity of gut microbiota in diarrheic infants was significantly reduced compared with that in healthy infants. Infants with diarrhea had a lower abundance of *Lactobacillus* spp. and *Bacillus* spp. ($P < 0.001$) and a significant richness of *Klebsiella* spp. and *Enterobacter* spp. ($P < 0.001$). Similar gut microbiota patterns were found in diarrheic infants in all four age groups. However, different pathogenic infections have significant effects on the gut microbiota of diarrheic infants. For instance, the relative abundance of *Klebsiella* spp. and *Streptococcus* spp. was significantly increased ($P < 0.001$) in infants infected with *Staphylococcus aureus*; meanwhile, the richness of bacteria such as *Enterobacter* spp. was significantly increased in the *Salmonella* infection group ($P < 0.001$). *Conclusion*. The microbiota in infants with diarrhea has changed significantly, characterized by decreased species diversity and abundance of beneficial bacteria and significant increase in the proportion of conditional pathogens. Meanwhile, the gut microbiota of infants with diarrhea at different ages was similar, but different pathogenic infections affect the gut microbiota characteristics. Therefore, early identification of changes in gut microbiota in infants with diarrhea and the adoption of appropriate pathogen type-specific interventions may effectively alleviate the disease and reduce adverse reactions.

1. Introduction

As a serious gastrointestinal dysfunction, infant diarrhea has become a global public health problem. Without proper treatment, the child will have an electrolyte imbalance that can be life-threatening [1]. Diarrhea was the second cause of childhood mortality, according to the last WHO bulletin, published in 2018 (https://www.who.int/gho/publications/world_health_statistics/2018/en/). *Rotavirus*, *Shigella* spp., and *Salmonella* spp. were the three leading causes of diarrheal deaths in infants [2]. Also, *Staphylococcus aureus* was one of the common pathogens of infectious diarrhea [3]; the peptidoglycan and toxins of *S. aureus* can induce infantile diarrhea [4].

The gut microbiota community is symbiotic with the host and changes dynamically with the host's age and physiological status as well as environmental factors [5, 6]. The homeostasis of the intestinal microbiota plays a vital role

in human health, specifically by promoting the digestion and absorption of food, maintaining the host's immune balance, metabolism, and homeostasis of the intestinal barrier [7, 8]. In addition, the emergence of probiotics, prebiotics, and other products provides new means of preventing and treating clinical diseases [9, 10].

However, once the host's intestinal microecological balance was broken, various intestinal diseases will follow [11]. For instance, infantile diarrhea was one of the most common metabolic diseases related to the infant's gut microecological balance. More research has found that gut dysbiosis has an impact on the occurrence and development of diarrhea. For example, The et al. have reported a consistent elevation of *Fusobacterium mortiferum*, *Escherichia*, and reduced *Bifidobacterium pseudocatenulatum* in infants with diarrhea [12]. Another research found *Bifidobacterium* and *Lactobacillus* species to be decreased in Colombian children with diarrhea [13]. However, few reports compared the gut microbiota characteristics in infants of different ages or with different pathogens.

This study aimed to (a) identify the differences in the gut microbiota composition between diarrheic (under one year) and healthy infants, (b) characterize the gut microbiota in diarrheic infants of different ages, and (c) examine the effect of different pathogenic bacteria on the intestinal microbiota of infants with diarrhea. This study can clarify gut microbiota changes in infants with diarrhea and provide a specific reference for the additional diagnosis and treatment.

2. Materials and Methods

2.1. Subjects and Sample Collection. A total of 42 diarrheic infants and 37 healthy infants under the age of one were recruited from the Center for Disease Control of Xigang District, Dalian, China (Figure 1, Table 1). Infants with virus infection, such as rotavirus, were excluded from the study; all infantile diarrhea cases were caused by prokaryotic infection. Meanwhile, infants with diarrhea were in the early stages of diarrhea and had not been treated with antibiotics. The fecal samples were collected from each infant and immediately stored at −20°C until transfer to the laboratory on dry ice and then stored at −80°C before use.

The study was approved by the ethical committees of Dalian Medical University, Dalian, China. Patients have filled out the informed consent form before sample collection.

2.2. Fecal DNA Extraction, PCR Amplification, 16S rRNA Sequencing, and Analysis. The microbial genomic DNA from the fecal samples was extracted using the E.Z.N.A.® Stool DNA kit (Omega Bio-tek, Inc.). The DNA concentration was measured using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA). PCR was performed to amplify the V3 and V4 region of the 16S rRNA gene using the primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′); template DNA was absent in the negative control [14]. PCR products were monitored on a 2% agarose gel. The PCR fragments were sequenced on an Illumina HiSeq platform (Novogene, Beijing, China). The QIIME software 1.9 package was used to analyze sequences (Quantitative Insights Into Microbial Ecology, http://bio.cug.edu.cn/qiime/). Sequences having a 97% resemblance or higher were categorized as the same operational taxonomic units (OTUs). The alpha diversity of microbiota was evaluated by the Chao 1 index, observed species index, and abundance-based coverage estimator (ACE) index. The beta diversity was evaluated by nonmetric multidimensional scaling (NMDS) [15]. The ANOSIM similarity analysis was based on a nonparametric test to compare intragroup and intergroup differences [16]. Linear discriminant analysis Effect Size (LEfSe) was used to identify the bacterial taxa differentially represented between groups at different taxonomic levels. A linear discriminant analysis (LDA) was used to estimate the effect size of each deferentially abundant feature (LDA ≥ 4 was shown in figures) [17]. The datasets are publicly available (accession number: PRJNA611095).

2.3. Identification of Different Pathogens in Feces of Diarrheic Infants. The PCR amplification of the partial 16S rRNA gene of *Salmonella* and *Staphylococcus aureus* was performed. The forward (5′-GTG AAA TTA TCG CCA CGT TCG GGC AA-3′) and reverse primer (5′-TCA TCG CA CCG TCA AAG GAA CC-3′) were used to detect a 284-bp *Salmonella* gene fragment [18, 19]. Notwithstanding, *Staphylococcus aureus* infection was identified by the PCR with the forward (5′-AAC TCT GTT ATT AGG GAA GAACA-3′) and reverse (5′-CCA CCT TCC GGT TTG TCA CC-3′ [20]) primer. This way, we divided the samples into four categories according to pathogen type: *Salmonella*, *Staphylococcus aureus*, combined *Salmonella* and *Staphylococcus aureus*, and others (neither *Salmonella* nor *Staphylococcus aureus*) (Table 1).

2.4. Statistical Analysis. All the experiments were done in triplicate. The data were presented as arithmetic mean ± standard error of the mean (SEM). Community comparisons were evaluated using a Student's *t*-test with the GraphPad Prism Program (Version 8.1.0; GraphPad Software Inc., La Jolla, CA, USA) [21]. The QIIME was used to calculate the beta diversity distance matrix, and the *R* language vegan software package was applied to perform NMDS analysis and mapping [22]. A P value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Cohorts of Patients and Healthy Infants. The basic clinical information of 37 healthy infants and 42 diarrheic infants was collected, including gender, age, and number of samples in each category. 61.9% of infants with diarrhea have skin problems, such as pruritus and rash (Table 1). The healthy infants and infants with diarrhea were divided into four groups by age: 0–120, 120–180, 180–270, and 270–365 days. Using PCR and 16S rRNA sequencing, the diarrhea-causing pathogens in these infants were identified and divided into

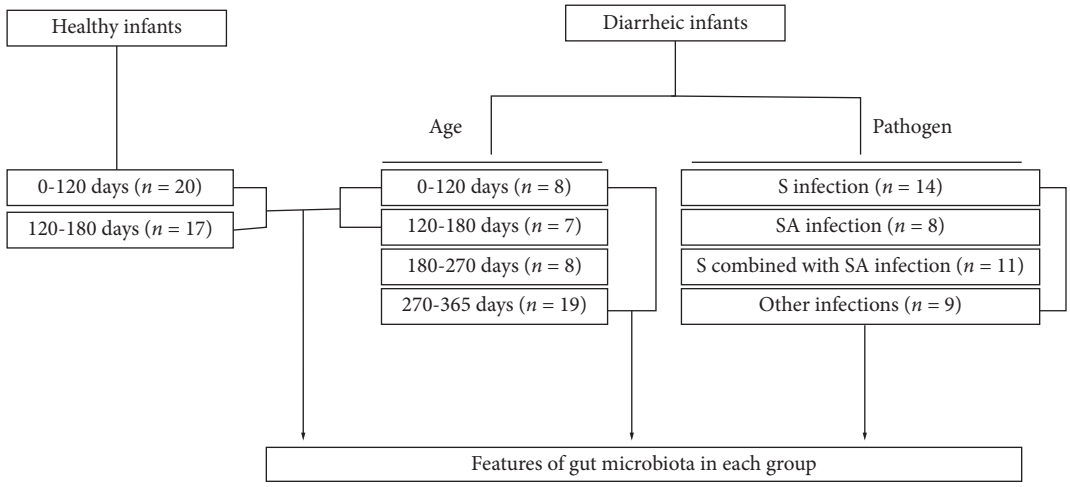

Figure 1: The flowchart of sample collection and grouping in this study. S: *Salmonella*; SA: *Staphylococcus aureus*.

Table 1: The basic clinical information on healthy infants and infants with diarrhea.

Characteristic		Healthy controls	Diarrheic infants
Number of samples	—	37	42
Gender	Male	24 (64.9%)	22 (52.4%)
	Female	13 (35.1%)	20 (47.6%)
Age	0–120 (days)	20 (54.1%)	8 (19.0%)
	120–180 (days)	17 (45.9%)	7 (16.7%)
	180–270 (days)	0	8 (19.0%)
	270–360 (days)	0	19 (45.2%)
Pathogen	S	0	14 (32.3%)
	SA	0	8 (19.0%)
	S.SA	0	11 (26.2%)
	Others	0	9 (21.4%)
Rash	—	0	26 (61.9%)

four categories, *Salmonella*, *Staphylococcus aureus*, combined *Salmonella* and *Staphylococcus aureus*, and others, which were neither *Salmonella* nor *Staphylococcus aureus* (Figure S1). To eliminate the effect of age on experimental results, we excluded 18 diarrheic infants to ensure no statistical difference in the arithmetic mean and SEM deviation of age between diarrheic and the healthy infants ($P = 0.897$, Figure 1).

3.2. The Alterations of Gut Microbiota Composition in Diarrheic Infants. The overlapping OTUs of the healthy infant's group and the diarrhea group were shown in a Venn diagram (Figure 2(a)). The 16S rRNA gene sequencing showed 977 and 744 unique OTUs in healthy and diarrheic infants, respectively, while 467 OTUs were common in both groups. According to OTU analysis, the bacterial communities in diarrheic and healthy infants tended to be heterogeneous (Figure 2(b)). The ACE, Chao 1, and observed species index showed that gut microbiota of diarrheic infants had significantly lower alpha diversity than those of healthy infants (all $P = 0.001$; Figures 2(c), 2(d) and 2(e)).

The NMDS calculation on ranking order was used for clustering the 79 samples into two distinct enterotypes (Figure 3(a)). The intergroup divergence was greater than intragroup divergence (Figure 3(b)), suggesting a significant difference in beta diversity between healthy infants and diarrheic infants. The LDA effect size (LefSe) algorithm was adopted to identify the bacterial groups that showed significant differences in abundance between the two groups. Comparisons between the two groups revealed that the *Firmicutes* phylum was significantly more abundant in healthy infants than diarrheic infants. At the genus level, the main abundant microbial genera shifted from *Lactobacillus* and *Bifidobacterium* in healthy infants to *Klebsiella* and *Streptococcus* in diarrheic infants (Figures 3(c) and 3(d)).

Distinct bacterial composition was observed between the healthy and diarrhea group. The microbiome contained 23 phyla, 168 families, and 370 genera in all fecal samples. Proteobacteria, Firmicutes, and Actinobacteria were the most abundant taxonomic groups. The relative abundance of Proteobacteria (44.67%) in diarrheic infants was substantially higher than healthy infants ($P < 0.001$), while the abundances of *Firmicutes* (24.27%) and *Actinobacteria* (22.14%) in diarrheic infants were lower ($P < 0.001$, $P = 0.031$, Figures 4(a), and 4(b)). At the family level (Figures 4(c), 4(d)), the relative abundance of Enterobacteriaceae (43.60%) in diarrheic infants was considerably higher than in healthy infants ($P < 0.001$). On the contrary, the relative abundance of Lactobacillaceae (3.03%) and Bifidobacteriaceae (2.05%) in diarrheic infants was notably lower than in healthy infants ($P < 0.001$, $P = 0.026$). At the genus level (Figures 4(e), 4(f)), the relative abundance of *Klebsiella* (16.57%) in diarrheic infants was higher than in healthy infants ($P = 0.001$). On the contrary, the relative abundance of *Lactobacillus* (3.03%) and *Bifidobacterium* (20.52%) in diarrheic infants was lower than in healthy infants ($P < 0.001$, $P = 0.025$).

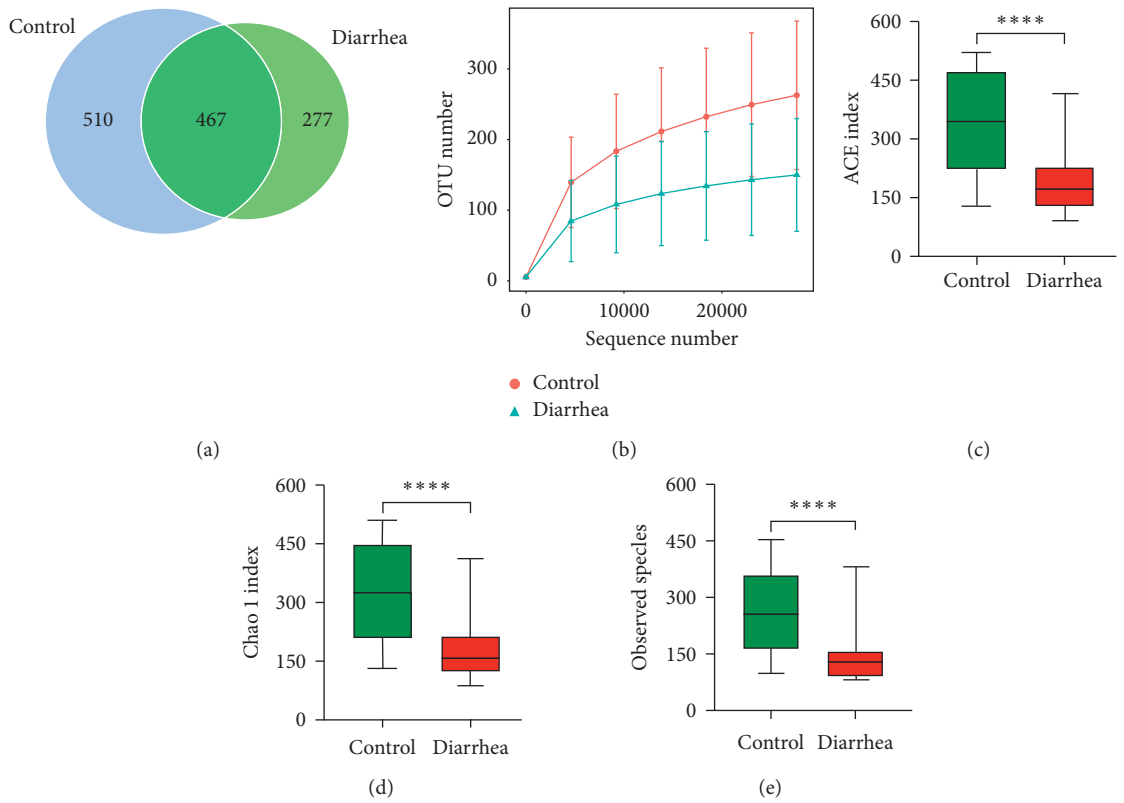

Figure 2: The alpha diversity of gut microbiota in healthy infants and diarrheic infants. (a) Venn diagram of OTUs in the two groups. (b) Observed species index in the two groups. (c–e) Comparing the alpha diversity indices (ACE, Chao 1, and observed species) based on the OTU profiles.

3.3. Characteristics of Gut Microbiota in Healthy and Diarrheic Infants at Different Ages. The healthy infants and infants with diarrhea were divided by age into four groups: 0–120, 120–180, 180–270, and 270–365 days. We investigated the characteristics of the gut microbiota of infants in the four age groups. The alpha diversity indexes showed that, in healthy infants, the diversity of gut microbiota of 120–180-day-old infants was significantly lower than that of 0–120-day-old infants ($P < 0.01$). Interestingly, different ages did not affect the diversity of the gut microbiota of diarrheic infants (Figure 5(a), $P > 0.05$). Based on the factor of age, NMDS clustering divided the samples of infants with diarrhea into four groups; however, the four groups did not separate clearly, suggested a similarity among the samples from different age groups (Figure 5(b)).

At the phylum level (Figure 5(c)), the relative abundance of Proteobacteria (54.67%) in diarrhea-1 (0–120 days) infants was individually higher than in diarrhea-4 (270–365 days) infants ($P = 0.027$). On the contrary, the relative abundance of Actinobacteria (7.29%) in diarrhea-1 (0–120 days) infants was lower than in diarrhea-3 (180–270 days) infants ($P = 0.031$) and diarrhea-4 (270–365 days) infants ($P = 0.013$). At the family level (Figure 5(d)), the relative abundance of Enterobacteriaceae (53.96%) in diarrhea-1 infants was significantly higher than in diarrhea-4 infants ($P = 0.025$). On the contrary, the relative abundance of Bifidobacteriaceae (5.07%) in diarrhea-1 infants was lower than in diarrhea-3 infants ($P = 0.024$) and diarrhea-4 infants ($P = 0.009$). At the genus level (Figure 5(e)), the relative abundance of *Bifidobacterium* (5.07%) in diarrhea-1 infants was lower than in diarrhea-3 infants ($P = 0.024$) and diarrhea-4 infants ($P = 0.009$). The relative abundance of *Klebsiella* (14.42%) in diarrhea-2 infants (120–180 days) was higher than in diarrhea-4 infants ($P = 0.031$). Other than the previously mentioned comparisons, the differences among the other groups of diarrheic infants were not statistically significant.

3.4. The Characteristics of Intestinal Microbiota in Diarrheic Infants Infected with Different Pathogens. According to Chinese health authorities, *Salmonella* and *Staphylococcus aureus* (SA) were the two common causes of infantile diarrhea in China (http://www.phsciencedata.cn/Share/zh-CN/index.jsp). Therefore, we identified the pathogens in the fecal samples of diarrheic infants and divided the samples by pathogen type: *Salmonella* (S), *Staphylococcus aureus* (SA), combined *Salmonella* and *Staphylococcus aureus* (S.SA), and others (no *Salmonella* or *Staphylococcus aureus*). Subsequently, we investigated the characteristics of the intestinal microbiota of diarrheic infants in the four groups. Regardless of the group, the Chao 1 index of diarrheic infants was reduced compared with healthy infants (Figure 6(a)). NMDS clustered the samples of infants with

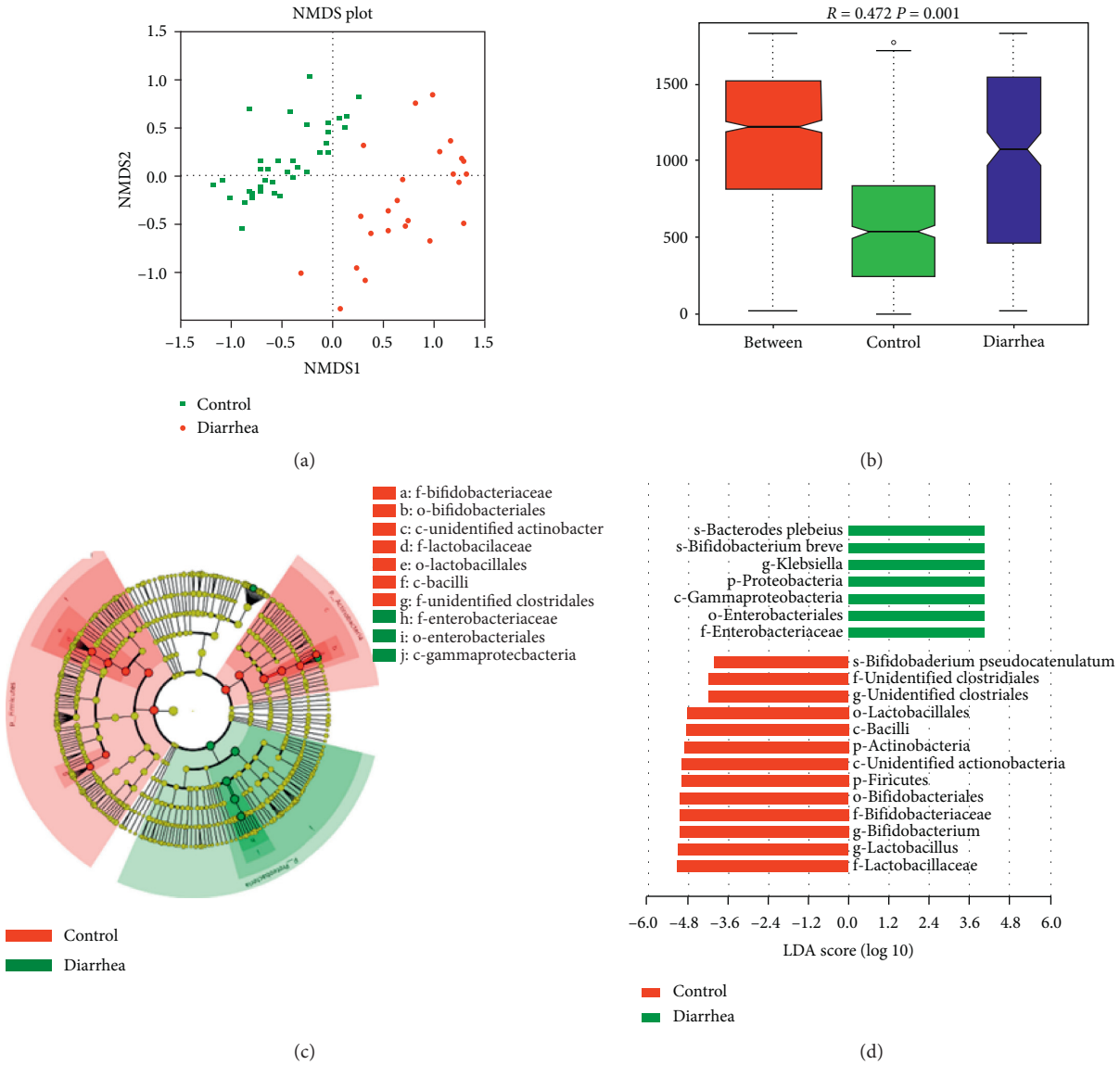

Figure 3: The beta diversity and predominant microbiota of healthy and diarrheic infants. (a) The nonmetric multidimensional scaling (NMDS) of beta diversity is calculated on ranking order. (b) The ANOSIM Similarity Analysis is based on a nonparametric test to compare intragroup and intergroup differences. (c) Cladogram indicating the phylogenetic distribution of microorganisms as related to group characteristics; the difference was shown in red for the healthy group and green for the diarrhea group. The diameter of each circle was proportional to the taxa's abundance. The strategy of multiclass analysis was not strict (at least one somewhat differential). The circle from inside to outside represented the phylogenetic level from domain to genus. (d) Indicator microbial groups within the two types of sediments with a linear discriminate analysis (LDA) value greater than 4.0. The color lump represented the microbes with a significant difference at different taxonomic levels. Red and green represented the healthy group and the diarrhea group, respectively. The *x*-axis represented the LDA score of the microbes. The *y*-axis represented the microbes, which were detected to be significantly different in the groups.

diarrhea into five groups; the *Salmonella* and *Staphylococcus aureus* groups were separated for healthy infants but did not separate from each other (Figure 6(b)).

At the genus level, the relative abundance of *Lactobacillus* and *Bifidobacterium* in the *Salmonella*, *Staphylococcus aureus*, combined *Salmonella*, and *Staphylococcus aureus* group was found significantly lower than in healthy infants ($P = 0.001$). In contrast, the *Bacteroides* and *Streptococcus* in diarrheic infants were more abundant ($P = 0.001$). Each group had different intestinal microbiota characteristics. For instance, *Bifidobacterium* and *Streptococcus* were predominant in the *Staphylococcus aureus* group, while the *Bifidobacterium* and *Bacteroides* account for a large proportion of bacteria in the *Staphylococcus aureus* group (Figure 6(c)). LEfSe analysis showed that, at the family level, the Ruminococcaceae and Enterobacteriaceae were differentially enriched in the *Salmonella* group (Figure 6(d)). In addition, at the family level, the most differentially abundant bacteria in healthy infants included Lactobacillaceae and Bifidobacteriaceae, while Streptococcaceae and Ruminococcaceae

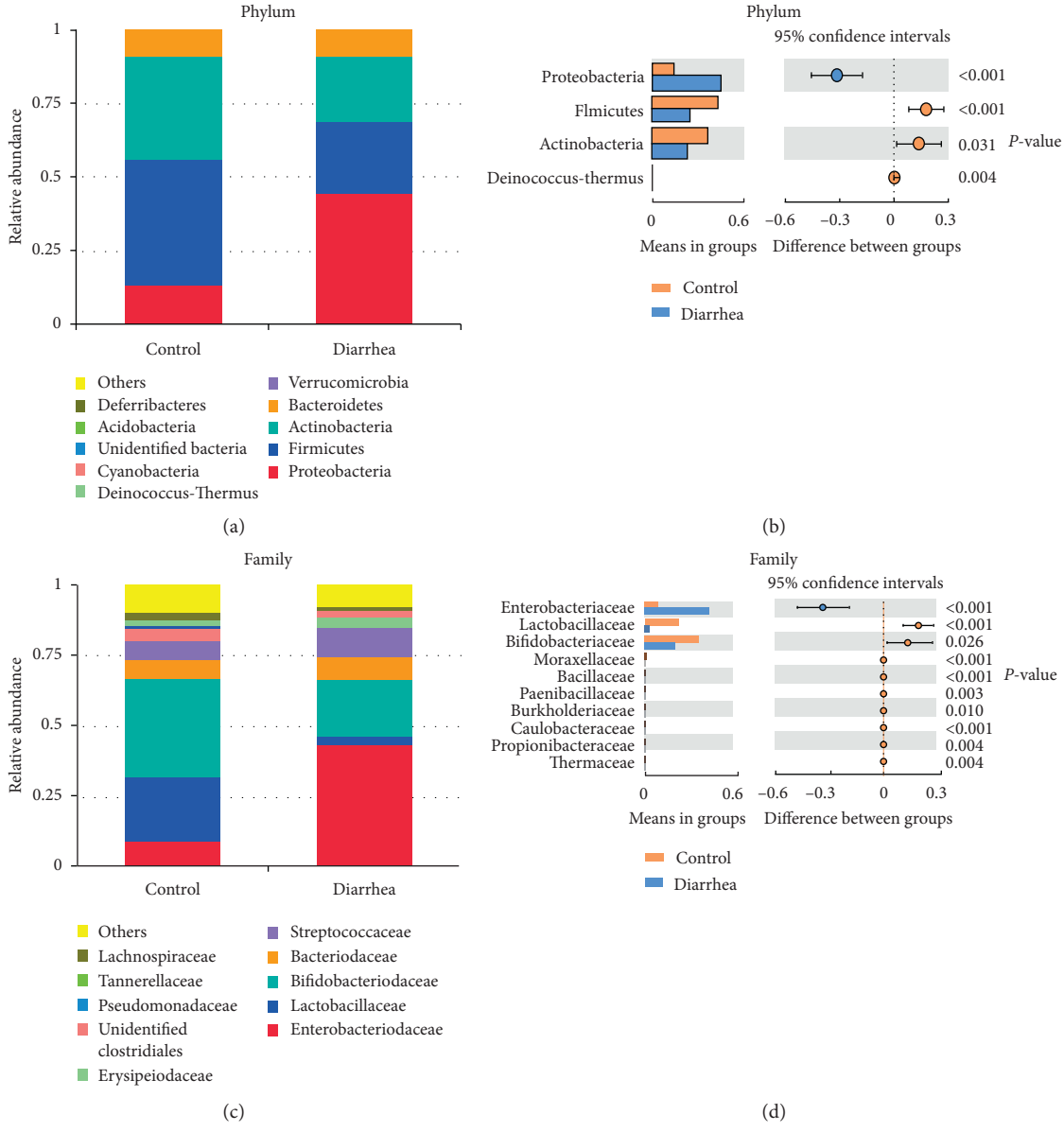

Figure 4: Continued.

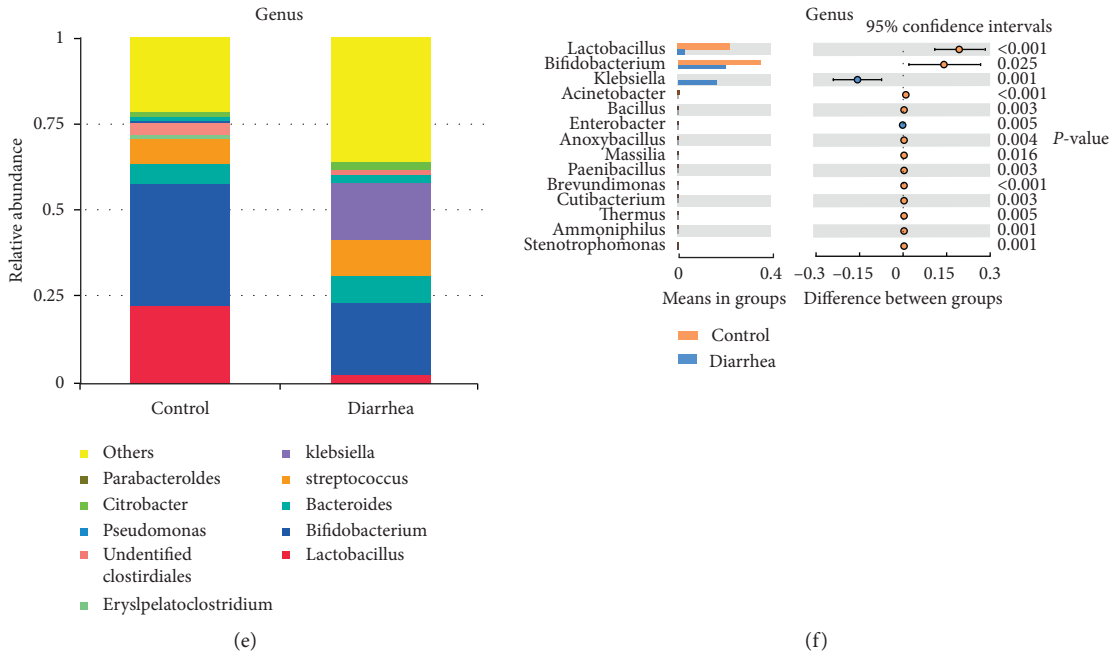

Figure 4: The shift in the gut microbiota of healthy infants and diarrheic infants. The relative abundance of the top 10 microbiota at the phylum (a), order (c), and genus level (e). The genera in the gut microbiota of healthy infants were strikingly different from those in diarrheic infants at the phylum (b), order (d), and genus level (f).

were overrepresented in the *Staphylococcus aureus* group (Figure 6(e)). At the genus level, *Klebsiella* and *Streptococcus* predominated in the *Staphylococcus aureus* group, while *Faecalibacterium* and *Subdoligranulum* were the predominant bacteria in the *Salmonella* group (Figure 6(f)).

There were changes in the gut microbiota of the infants infected with different pathogens compared with healthy infants. There were also differences in the gut microbiota between diarrheic infants infected by different pathogens. For instance, at the genus level (Figure 6(c)), the relative abundance of *Streptococcus* (14.93%) in the *Staphylococcus aureus* group was higher than in the *Salmonella* group ($P = 0.017$) and the other group ($P = 0.047$). Meanwhile, the relative abundance of *Enterococcus* (1.62%) in the *Staphylococcus aureus* group was higher than in the *Salmonella* group ($P = 0.023$). The relative abundance of *Klebsiella* (10.45%) in the combined *Salmonella* and *Staphylococcus aureus* group was higher than in the *Salmonella* group ($P = 0.039$).

4. Discussion

The homeostasis of the human gut microbiota has multiple positive effects on the host's health [23]. Microbes colonize the neonatal gut immediately following birth. The establishment and interactive development of the early gut microbiota play a vital role in infants' growth and health [24]. However, during the same period, owing to immune immaturity, the risk of illness will be high [25, 26]; for example, diarrhea often occurs.

Acute diarrhea was a diarrheal episode of presumed infectious etiology that begins quickly and lasts for fewer than 14 days [27]. Bacterial infections are a common cause of infantile diarrhea. Several different enteropathogenic agents can cause diarrhea in infants, such as enteroaggregative *Escherichia coli*, enteropathogenic *Escherichia coli*, *Salmonella*, *Shigella* spp., and *Staphylococcus aureus*, to name a few [28, 29].

Our study compared the characteristic of gut microbiota in healthy infants and diarrheic infants in multiple aspects. The results showed that the gut microbiota of infants with diarrhea changed significantly. First, compared with the healthy infants, the intestinal microbiota diversity of diarrheic infants was significantly decreased. Some research revealed that intestinal infection could affect the aerobic bacteria because they would spread through oxygen to obtain energy and metabolism [30–32]. Second, the Firmicutes accounted for a large proportion of bacteria in healthy infants and were beneficial to intestinal epithelial cells [33, 34]. However, in the diarrhea group, Proteobacteria was the predominant bacteria and the microbial signature of dysbiosis in gut microbiota. Third, our results showed that the relative abundance of *Lactobacillus* in the healthy infants was significantly higher than that in the diarrheic infants. On the other hand, Enterobacteriaceae had an opposite trend. Recent studies have found that intestinal dysbacteriosis was the leading cause of infantile diarrhea. Lactobacillaceae could alleviate the severity of diarrhea, whereas Enterobacteriaceae had an opposite effect [35]; our results are consistent with this finding.

Finally, when we compare the results of two groups at the genus level, we can find that *Lactobacillus* decreased and *Klebsiella* and *Enterobacter* increased with diarrhea. *Lactobacillus* are recognized as probiotics because of their health-

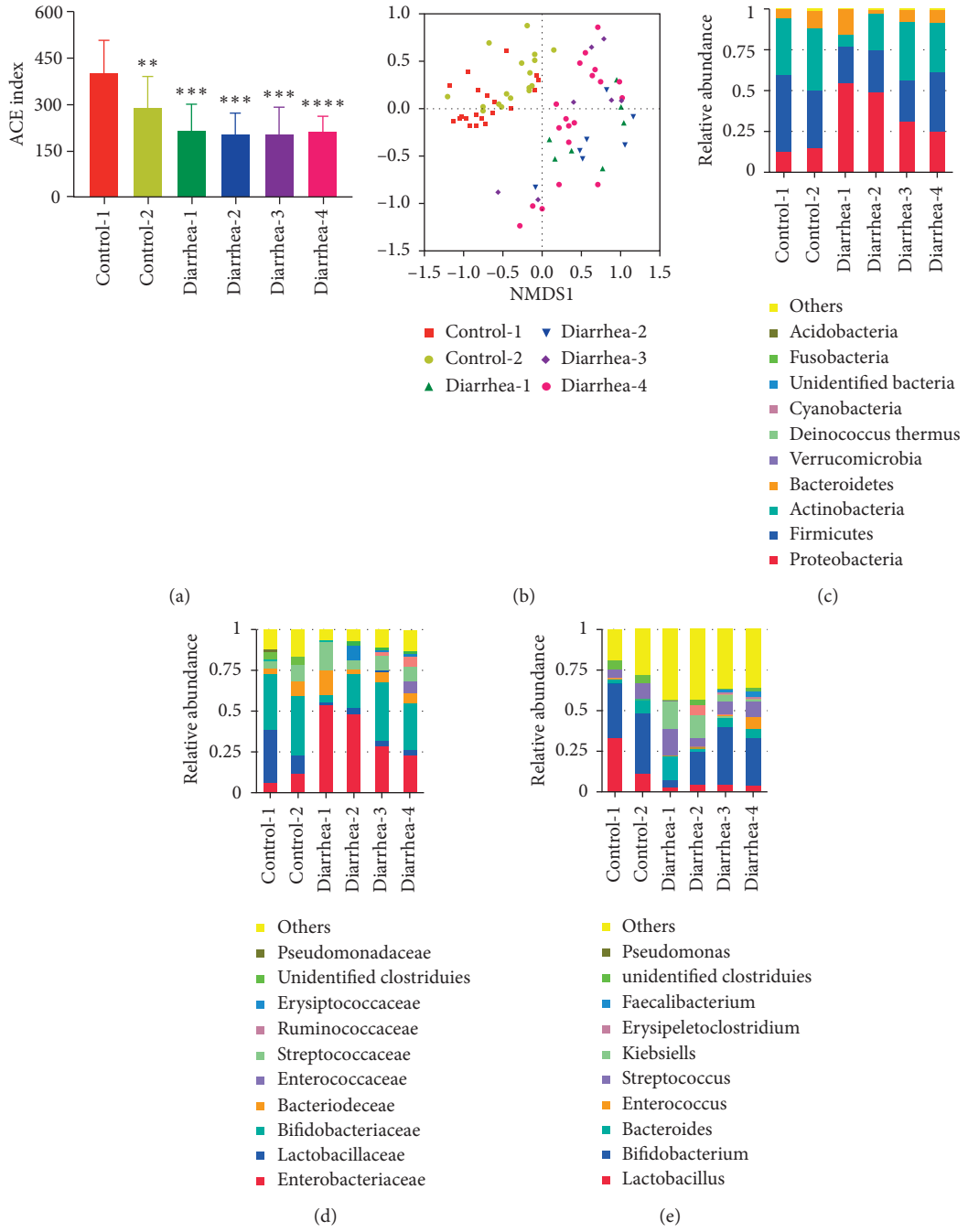

Figure 5: The gut microbiota of healthy infants and infants with diarrhea of different ages. (a). The comparison of the alpha diversity (ACE index) of various ages based on the OTUs profiles. (b). The NMDS of beta diversity is calculated on ranking order. The relative abundance of the top 10 microbiota at the phylum (c), order (d), and genus level (e). Control-1: 0–120-day-old healthy infants; control-2: 120–180-day-old healthy infants; diarrhea-1: 0–120-day-old diarrheic infants; diarrhea-2: 120–180-day-old diarrheic infants; diarrhea-3: 180–270-day-old diarrheic infants; diarrhea-4: 270–360-day-old diarrheic infants.

promoting effects [36]. *Lactobacillus* can, via competitive exclusion, enhance epithelial barrier function and produce antipathogenic compounds to protect the host [37]. Davoodabadi et al. studied different *Lactobacillus* strains to identify probiotic candidates for preventing intestinal infections caused by diarrheagenic *E. coli* [38]. Szajewska et al. found that probiotic *Lactobacillus* could significantly reduce the risk of antibiotic-associated diarrhea in children and adults [39, 40]. Numerous bacterial infectious agents have been implicated in AAD, including *Clostridium perfringens, Staphylococcus aureus,* and *Klebsiella oxytoca* [41]. *K. oxytoca* also causes infections of the respiratory and urinary tracts and soft-tissue and hepatobiliary infections [42].

We also explored the effects of different ages on the gut microbiota of infants with diarrhea. The results showed no

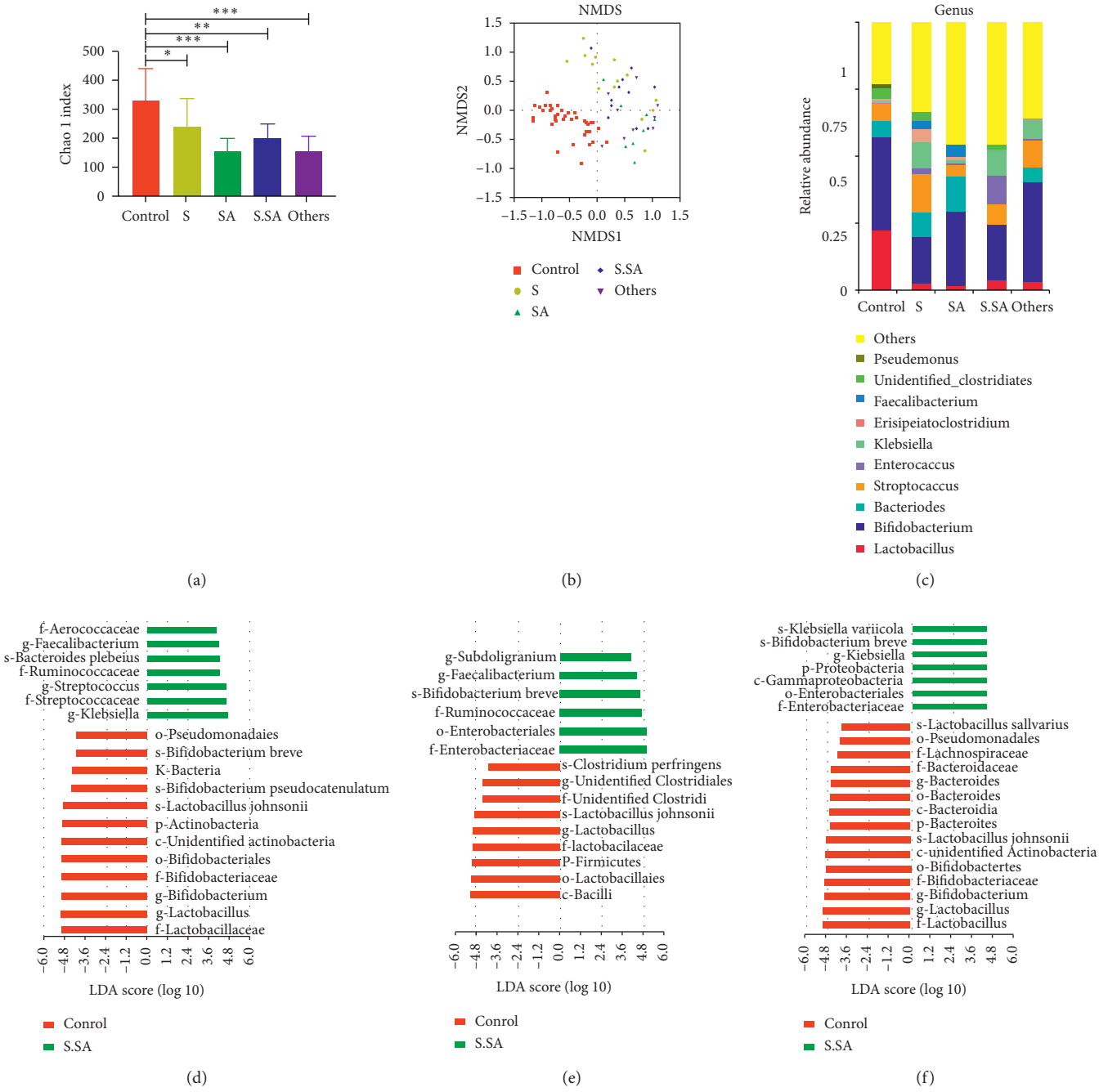

FIGURE 6: The gut microbiota of diarrheic infants infected by different pathogens. (a). The comparison of the alpha diversity (Chao 1 index) of different groups (control, *Salmonella* (S), *Staphylococcus aureus* (SA), combined *Salmonella* and *Staphylococcus aureus* (S.SA), and others) based on the OTU profiles. (b). The NMDS of beta diversity was calculated on ranking order. (c). The relative abundance of the top 10 microbiota at the genus level of different groups. (d–f). The LEFSe method analysis of the predominant bacteria between different groups.

significant differences in species diversity among diarrheic infants at different ages. Meanwhile, beta diversity results suggested similar intestinal microbiota in infants with diarrhea at different ages. Interestingly, when we compared the effect of age on the relative abundance of bacterial species, we found differences between the lower and upper age groups. *Bifidobacterium*'s relative abundance was low in the gut microbiota of diarrheic infants of 0–120 days; cesarean delivery may be a factor. Studies have reported that cesarean-section infants show reduced intestinal microbiota complexity and relatively low abundance of *Bifidobacterium* [43, 44].

Meanwhile, the relative abundance of *Bifidobacterium* increased significantly with age, possibly because the infants had been breastfed since birth. Some studies reported high levels of *Bifidobacterium* and *Lactobacillus* in the fecal samples of breastfed infants [45, 46]. Similarly, we found that, in healthy infants, the relative abundance of

Lactobacillus was low in the gut microbiota of diarrhea infants from 120 to 180 days, while *Bacteroides* increased. The reason may be that a newborn's intestine is aerobic, and only facultative anaerobic bacteria can grow. However, in just a few days, the intestinal cavity becomes anaerobic so that only *Bifidobacterium*, *Clostridium,* and *Bacteroides* can colonize [47]. In the first few weeks, the baby's intestinal microbiota resembles the mother's skin and vaginal microbiome, where *Enterococci*, Streptococcaceae, Lactobacillaceae, *Clostridium,* and *Bifidobacterium* predominate. In the first few months, the baby's diet is almost entirely milk, which is conducive to *Bifidobacterium,* the predominant microbiota at this stage [48, 49]. When solid food is introduced, the baby's gut microbiota will undergo a substantial change because the food contains various polysaccharides that are not easily digestible; as a result, the abundance of *Bacteroides* and *Clostridium* increases and *Bifidobacterium* and Enterobacteriaceae decrease [50, 51]. Therefore, it suggested that the changes in the gut microbiota of infants with diarrhea are related to the environment, feeding methods, and delivery methods and are not closely related to age from zero to one year.

Subsequently, based on PCR and sequencing results, we divided diarrheic infants into four groups: *Salmonella*, *Staphylococcus aureus*, *Salmonella,* and *Staphylococcus aureus* and others. The results of the microbiota analysis showed some differences between the groups. *Klebsiella* and *Staphylococcus* were the predominant bacteria in the *Staphylococcus aureus* group; meanwhile, Enterobacteriaceae was the most abundant bacteria in the *Salmonella* group. Therefore, diarrhea may be caused by mixed pathogenic bacterial infections. *Staphylococcus aureus* can secrete staphylococcal enterotoxin A (SEA) [52], which bind to MHC class II molecules and *T*-cell receptors to stimulate *T*-cell proliferation and activation by the variable region of β chain, leading to the uncontrolled increase of many proinflammatory cytokines. The superantigen SEA can cause fever, decrease immunity, and promote many other bacterial infections [53, 54]. Moreover, *Staphylococcus aureus*'s peptidoglycan can promote the occurrence of diarrhea by activating mast cells to release inflammatory substances [55]. Studies have reported reduced *Bacteroides* and increased *Klebsiella* in patients with *Staphylococcus aureus* infection [56, 57]; such finding is consistent with the results of this study. It is also reported that the use of antibiotics significantly increased the proportion of *Klebsiella* in intestine [58]. Hence, the cocolonization of *Staphylococcus aureus* and *Klebsiella* may be related to antibiotic exposure in the intestinal microenvironment. The relative abundance of Enterobacteriaceae increased, while the relative abundance of *Lactobacillus* decreased in the *Salmonella*-infected diarrheic infants; this trend is consistent with the previous finding on the characteristics of intestinal microbiota after *Salmonella* infection [59–61].

Similarly, patients receiving antibiotics also showed an increase in Enterobacteriaceae [62]. One might wonder why the *Salmonella* in diarrhea infants promotes Enterobacteriaceae. One possibility is that, after *Salmonella* infection, the pathogenic factors of *Salmonella* will be released to induce the host to develop a mucosal inflammation response [63]. As the host tries to eliminate the bacteria, it may cause "collateral damage" that destroys the human intestinal microecological balance, resulting in clinical symptoms like diarrhea.

In this study, we used high-throughput sequencing to investigate gut microbiota's characteristics in infants with diarrhea. We also compared the effects of different ages and different pathogens on the gut microbiota of diarrheic infants. The results showed that the gut microbiota of infants with diarrhea had changed significantly. Simultaneously, different pathogenic infections were found to affect the characteristics of gut microbiota in diarrheic infants; however, the intestinal microbiota of these infants at different ages was similar. This study was our first comprehensive analysis of the effects of different ages and different pathogen types on the gut microbiota in infants with diarrhea. It will provide some reference for the treatment of and nutritional adjustment for diarrheic infants. Indeed, the study's small sample size is a limiting factor; more samples are needed to verify our findings. Also, some infants were treated for skin problems, mostly eczema, followed by urticaria. Since rash in children is closely related to intestinal microbiota disorders and helper *T* cell imbalance [64, 65], the rash's underlying mechanism requires further study.

Data Availability

All the data that were used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

M. L. and B. H. conceived the study, designed the experiments, and revised the manuscript. Q. F. performed DNA extraction, analyzed the data, and drafted the manuscript. M.Y. coordinated in selecting field sampling sites and sample collection. H. L., Y. W., and X. L. performed DNA extraction. J. Y. discussed the data. All authors contributed to the critical revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors gratefully acknowledge Professor Li Wenzhe for his technical support. This research was funded by the Nutrition and Care of Maternal and Child Research Fund Project of Guangzhou Biostime Institute of Nutrition and Care (2019BINCMCF02); National Natural Science Foundation of China (no. 31900920); Liaoning Provincial Program for Top Discipline of Basic Medical Sciences, China; Research Project of Liaoning Provincial Department of Education (LZ2020066).

Supplementary Materials

Identification of *Salmonella* infection in infants with diarrhea by agarose gel electrophoresis. PCR product was 284 bp. (*Supplementary Materials*)

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Hindawi
Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2020, Article ID 8818989, 11 pages
https://doi.org/10.1155/2020/8818989

Research Article

Evaluation of the Antibacterial Activity and Probiotic Potential of *Lactobacillus plantarum* Isolated from Chinese Homemade Pickles

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Received 21 August 2020; Revised 12 October 2020; Accepted 31 October 2020; Published 19 November 2020

Academic Editor: Shengjie Li

This study investigated the antipathogenic activity and probiotic potential of indigenous lactic acid bacteria (LAB) isolated from Chinese homemade pickles. In total, 27 samples were collected from different sites in China. Fifty-nine yielded pure colonies were identified by 16S rRNA gene sequencing as LAB and were initially evaluated for the antibacterial activity in vitro. Initial screening yielded *Lactobacillus plantarum* GS083, GS086, and GS090, which showed a broad-spectrum antibacterial activity against food-borne pathogens, especially multidrug-resistant pathogens. Meanwhile, organic acids were mainly responsible for the antimicrobial activity of the LAB strains, and the most abundant of these was lactic acid (19.32 ± 0.95 to 24.79 ± 0.40 g/l). Additionally, three *L. plantarum* strains demonstrated several basic probiotic characteristics including cell surface hydrophobicity, autoaggregation, and survival under gastrointestinal (GI) tract conditions. The safety of these isolates was also evaluated based on their antibiotic susceptibility, hemolytic risk, bile salt hydrolase activity, and existence of virulence or antibiotic resistance genes. All strains were safe at both the genomic and phenotypic levels. Therefore, *L. plantarum* GS083, GS086, and GS090 are fairly promising probiotic candidates and may be favorable for use as preservatives in the food industry.

1. Introduction

Fermentation is a traditional method used to prolong the shelf life and improve the flavors of food [1]. Fermented foods such as pickles are widely utilized by most families in China and have been continually consumed for thousands of years. A variety of lactic acid bacteria (LAB) strains such as *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Pediococcus* are involved in the pickle fermentation phase and have crucial health-improving effects [2]. Currently, LAB are classified as "generally recognized as safe" microorganisms and are widely used in the food industry [3]. In addition to their probiotic functions, LAB can improve food flavor and nutritional value by generating aromatic compounds and converting isoflavone glucosides into aglycones [4, 5]. Therefore, screening probiotic LAB from fermented food has gained increasing attention in the recent years.

Microbiologic contamination is one of the most important reasons of food spoilage and/or reductions in its shelf life. Food contamination with food-borne pathogens such as *Escherichia* and *Salmonella* has led to severe infections, which can sometimes even be fatal [6, 7]. Moreover, antibiotics are commonly used to reduce the harm caused by microbial contamination. However, one problem associated

with the excessive use of antibiotics is the ongoing occurrence of multidrug-resistant pathogens [8]. These organisms cause persistent risks for the whole chain in the food industry, given that cannot easily be inactivated by chemical or physical methods. As a result, there is an urgent need to find favorable biological preservatives as promising alternatives to antibiotics.

LAB, not only as antagonists of pathogenic microorganisms but also producers of antimicrobial metabolites, have attracted much attention. Several studies have proven that LAB can inhibit the growth of pathogenic microorganisms via multiple mechanisms, including competitively inhibiting pathogen binding, enhancing the host immune system, and producing pathogen growth-inhibitory compounds such as organic acids, bacteriocins, and hydrogen peroxide [9]. Therefore, LAB could be candidate biological preservatives in the food industry. However, not all of these bacteria can be applied to the control of food-borne pathogens in the food industry as they might produce unfavorable flavors in food. However, LAB strains isolated from traditional fermented food are more likely to be accepted by customers.

Before LAB strains are potentially used as probiotics, their probiotic characteristics and safety should be considered [10]. Furthermore, a potent probiotic isolate should possess certain characteristics such as survival and colonization ability in different environments [11]. Furthermore, they should be able to withstand bile salts and the low pH of gastric juice and have adhesion ability, which could be helpful in colonizing the human host [12]. According to the FAO/WHO guidelines, probiotic microorganisms should be safe for humans, with the most important concerns being potential virulence and antibiotic resistance [10, 13]. Hence, the utility of LAB should be evaluated at both phenotypic and genomic levels.

Thus, this work investigated the antibacterial activity of indigenous LAB strains obtained from Chinese homemade pickles against food-borne and multidrug-resistant pathogens, combined with the characterization of the antibacterial metabolites produced by them, to reveal their probiotic potential.

2. Materials and Methods

2.1. Isolation and Characterization of LAB Strains. A total of 27 samples of traditional homemade pickles with different fermentation methods were collected around China. LAB were isolated according to the methods described by Yi et al. [14]. LAB species were confirmed by 16S rRNA sequencing, using the universal primers 27F : AGAGTTTGATCCTGGCTCAG and 1492R : ACGGCTACCTTGTTACGACTT [15], and evolution analysis was performed by the neighbor-joining method (MEGA X version 10.1.7) and visualized with iTOL (https://itol.embl.de/itol.cgi).

2.2. Antibacterial Activity of LAB Strains against Food-borne and Multidrug-Resistant Pathogens

2.2.1. Preparation of the Cell-Free Culture Supernatant. The cell-free culture supernatant (CFS) of LAB was prepared according to the method described by Muthusamy et al. [16]. LAB were statically cultured at 37°C for 24 h. Cell suspensions were centrifuged at 3,100 × g at 4°C for 15 min, and the supernatants were filtered through a sterilized 0.22 μm filter. The CFS samples were stored at 4°C before use.

2.2.2. Information on Food-Borne Pathogens and Culture Preparation. Eight common food-borne pathogens were selected as indicators in this study, including *Listeria monocytogenes* ATCC 19117, *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 8739, *Salmonella typhimurium* ATCC 14028, *Cronobacter sakazakii* ATCC 29544, and *Pseudomonas aeruginosa* ATCC 15442. Moreover, six multidrug-resistant bacteria were also selected as indicators including *L. monocytogenes* 1846–1, *B. cereus* 3311-2A, *S. aureus*117-2, *E. coli* 2624–2, *S. typhimurium* 54–9, and *C. sakazakii* cro 300A [6, 17–21]. All indicators were cultured overnight in LB broth at 37°C.

2.2.3. Antibacterial Spectrum of LAB Strains. The inhibitory activity of CFS produced by LAB was measured by the Oxford cup agar diffusion method [22]. Overnight cultures of indicator bacteria were diluted and spread onto nutrient agar plates. Then, 100 μl of CFS was added to sterile Oxford cups on the plates for coculture at 37°C for 24 h. Then, the diameter of the inhibition zone was measured by using a pair of Vernier calipers.

2.3. Antibacterial Metabolites Produced by L. plantarum Strains

2.3.1. Sensitivity of Antibacterial Metabolites to pH and Enzymes. To verify the pH sensitivity of the LAB strains, the pH of CFS was adjusted to 5.5 using 1.0 M NaOH. Similarly, CFS samples were inactivated by catalase, trypsin, pepsin, and proteinase K (2 mg/ml) at 37°C for 2 h. Residual antibacterial activity of the treated CFS was determined against *S. typhimurium* ATCC14028 (representative of Gram-negative bacteria [G^-]) and *L. monocytogenes* ATCC19117 (representative of Gram-positive bacteria [G^+]).

2.3.2. Quantification of Organic Acids by HPLC. Six types of organic acids in the CFS were measured by HPLC (Agilent, USA) according to Upreti et al. [23]. The data were processed using OpenLAB CDS ChemStation Edition TM software. The obtained peaks were compared with standards (purity ≥ 99%).

2.4. Probiotic Characteristics of L. plantarum Strains

2.4.1. Carbohydrate Fermentation Patterns. Fermentation patterns of LAB were tested with an API 50 CHL test based on 49 selected carbohydrate sources. Briefly, overnight cultures were suspended in 10 ml of the API 50 CHL medium, and each sample was applied onto cupels containing different carbohydrates on an API 50 CH test strip.

Fermentation patterns were determined after incubation for 24–48 h at 37°C.

2.4.2. Testing Tolerance to Gastrointestinal Tract Conditions. The gastric and pancreatic juices, used to simulate the digestive environment, were prepared according to the method described by Katarzyna and Alina [24]. Simulated gastric juice was prepared by dissolving 0.35% (w/v) pepsin in PBS, which was acidified to a pH of 2.0. Simulated pancreatic juice (pH 8.0) was composed of 1.1% (w/v) $NaHCO_3$ and 0.1% (w/v) trypsin. The simulated gastric and pancreatic juices were filtered through a sterilized 0.22 μm filter.

LAB cells were suspended in simulated gastric juice and incubated at 37°C for 3 h. Their viability was, then, determined by the flat colony counting method. The collected cells from the gastric phase were suspended in simulated pancreatic juice for 24 h, and then, the bacterial survival rate was estimated by the DeMan Rogosa Sharpe (MRS) agar plate enumeration method.

2.4.3. Cell Adhesion Activity. Autoaggregation was analyzed using a modified method reported by Ogunremi et al. [25]. The suspensions were mixed and incubated at room temperature for 4 h and, then, measured based on their absorbance at 600 nm. Calculations were based on equation (1) in the main text, where A = the absorbance at 0 h and A_t = the absorbance at 4 h.

$$x = \frac{A - A_t}{A} \times 100. \quad (1)$$

The cell surface hydrophobicity of LAB strains was evaluated by measuring the bacterial cell adhesion to the hydrocarbon xylene according to the method described by Rokana et al. [26]. The LAB cells were cultured overnight and collected by centrifugation at 12,400 × g at 4°C for 10 min. Cells were resuspended in PBS, and their absorbances were detected at 600 nm. Then, a 3 ml cell suspension sample was mixed with 1 ml of hydrocarbon xylene. After incubation at 37°C for 1 h, the absorbance of the obtained aqueous layer was determined at 600 nm. The percent hydrophobicity was measured based on the decrease in absorbance. Calculations were performed using equation (2) in the main text, where A = the absorbance at 0 h and A^* = the absorbance at 1 h.

$$x = \frac{A - A^*}{A} \times 100. \quad (2)$$

2.5. Safety Evaluation of L. plantarum Strains

2.5.1. Antibiotic Susceptibility Testing. The antibiotic susceptibility of LAB was determined by the broth microdilution method [27]. Nine types of antibiotics were tested, including chloramphenicol, erythromycin, rifampicin, tetracycline, gentamycin, clindamycin, imipenem, ampicillin, and vancomycin. They were dissolved in the respective diluents and prepared at different concentrations (from 0.5 to 1,024 μg/ml). Susceptible and resistant strains were defined according to the standards reported by EFSA [28].

2.5.2. Hemolytic and Bile Salt Hydrolase (BSH) Activity. The LAB were streaked on blood agar plates to evaluate hemolysis activity according to Lee [29]. The BSH activities of LAB were checked by culturing the bacteria on MRS agar containing 0.5% taurodeoxycholic acid under anaerobic conditions for 48 h. The area of bacterial colonies showing white precipitates was scored as bile salt hydrolase positive [30].

2.5.3. Whole-Genome Sequencing and Bioinformatic Analyses. Whole-genome sequencing was performed according to the method described by Pang [31]. High-quality reads were assembled using SPAdes v. 3.6.2 program, and putative open reading frames were predicted with Prokka 1.1.3. Functional annotation was performed by rescreening BLASTp against the Nonredundant Protein Database of the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The cyclic images and comparative genomic analyses were generated using the BLAST Ring Image Generator (BRIG), in which the sequence of *L. plantarum* WCFS1 was downloaded from the NCBI.

The presence of virulence factors in the genome of LAB was identified using the virulence factor database (VFDB, http://www.mgc.ac.cn/VFs/main.htm). The genetic determinants conferring antimicrobial resistance (AMR) in the genome were searched using two publicly available databases, namely, the Comprehensive Antibiotic Resistance Database (CARD, http://arpcard.mcmaster.ca) and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/).

2.6. Statistical Analysis. Statistical analysis was performed using GraphPad Prism version 8.01 software. All data are shown as the mean values ± standard deviations from triplicate samples. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Isolation and Identification of LAB Strains. In total, 59 LAB strains were isolated from 27 samples of pickles with different fermentation methods from around China (Figure 1). The results of 16S rRNA gene sequencing and homology searching using BLAST confirmed that these strains included *Lactobacillus* (42), *Lactococcus* (6), *Weissella* (5), *Enterococcus* (3), *Pediococcus* (2), and *Leuconostoc* (1).

3.2. Antibacterial Activity of L. plantarum Strains against Food-borne and Multidrug-Resistant Pathogens. As shown in Table S1, nine isolates showed antimicrobial activity against eight food-borne pathogens, including G^- and G^+ bacteria. These strains were screened, and their inhibitory activity against six multidrug-resistant bacteria was also evaluated.

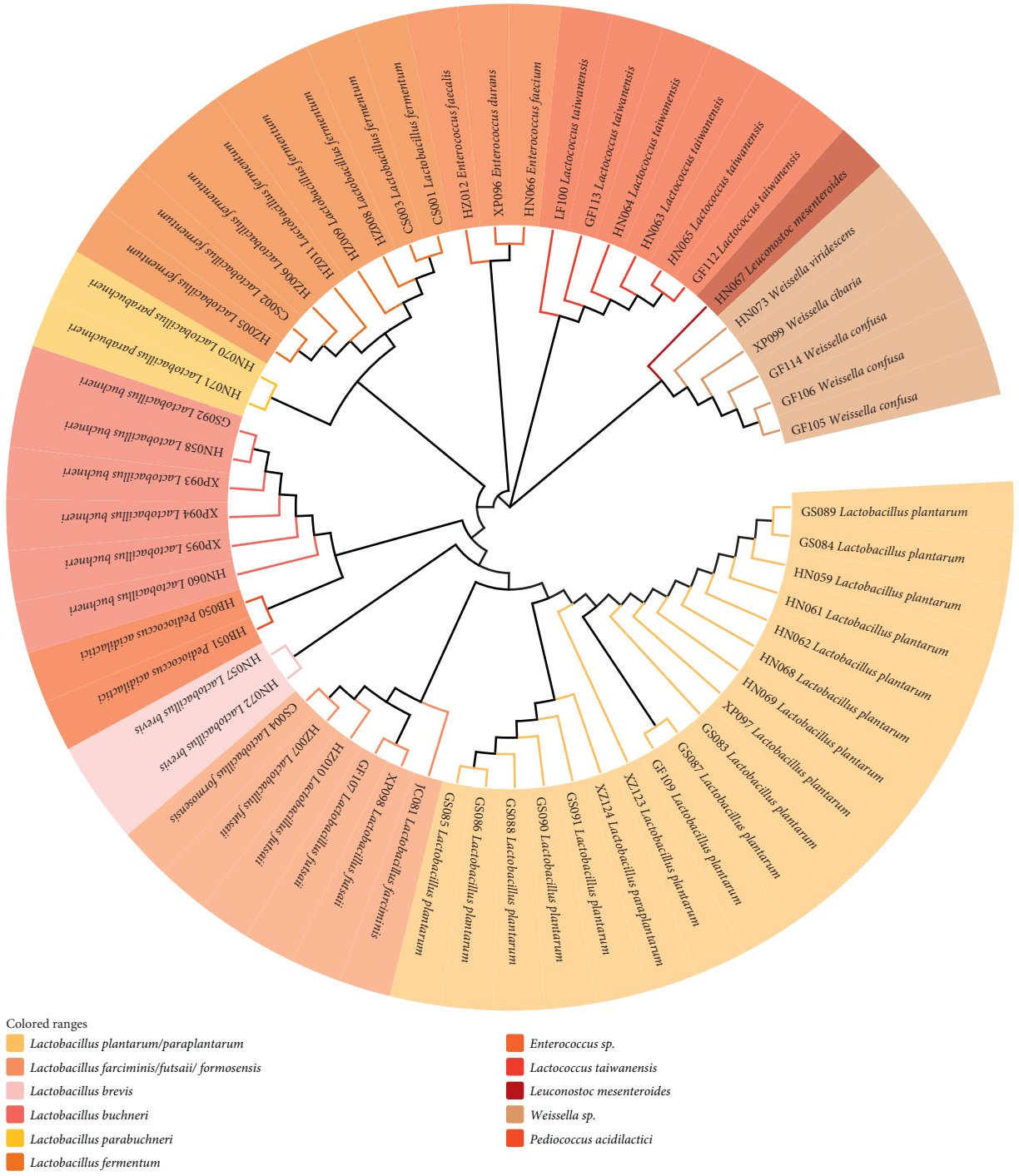

Figure 1: Circular phylogenetic tree based on the neighbor-joining method of 16S rRNA gene sequences of the isolated LAB. The scale bar represents 0.010-nucleotide substitutions per position.

Among them, three strains of LAB including *L. plantarum* GS083, GS086, and GS090 showed active resistance to all multidrug-resistant pathogens; these were subjected to further analyses (Figure 2).

3.3. Sensitivity to pH and Enzymes. The antibacterial activity of CFS samples of different *L. plantarum* strains only disappeared when they were neutralized at pH 5.5 (Table 1). In addition, the inhibitory effect of CFS after protease treatment was almost the same as that before treatment, suggesting that the antibacterial substances are not proteinaceous.

3.4. Organic Acids Produced by L. plantarum Strains. The organic acids in the CFS were detected, among which the most abundant was lactic acid, with concentrations ranging

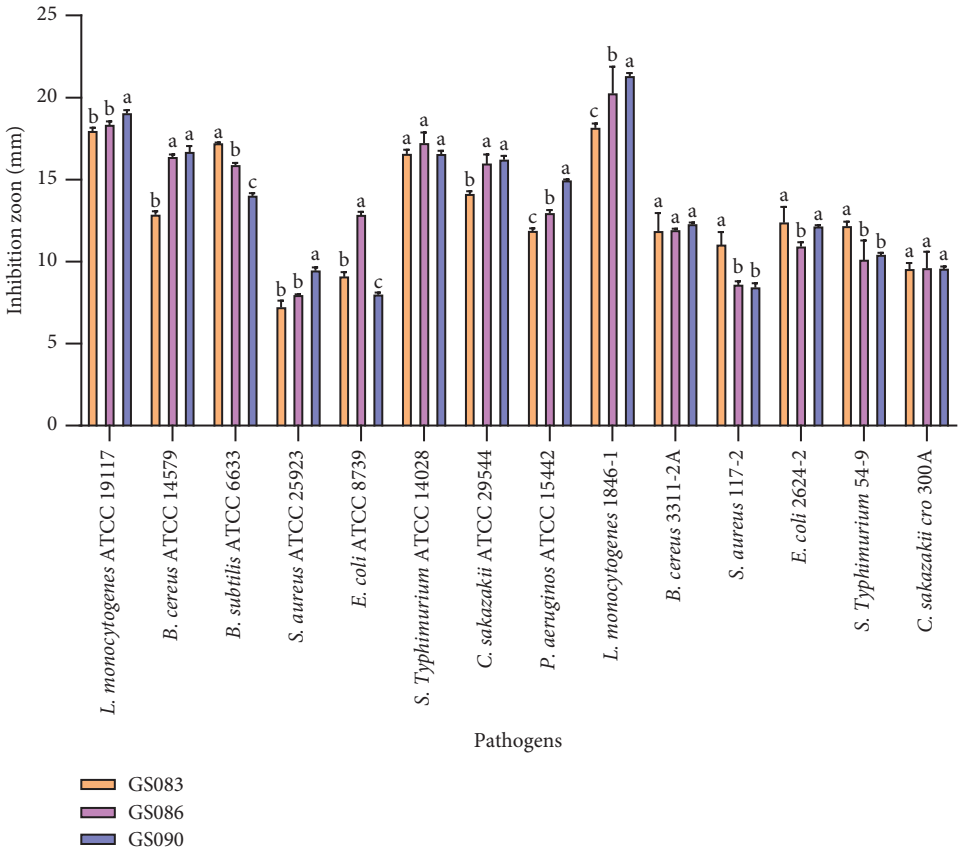

Figure 2: Antibacterial activity of *L. plantarum* GS083, GS086, and GS090 against 14 pathogen indicators. The zone of inhibition is expressed as the mean ± SD of three replicates.

Table 1: Characterization of antibacterial compounds from three *L. plantarum* strains.

Treatment	Residual inhibitory activity*					
	S. typhimurium ATCC14028			*L. monocytogenes* ATCC19117		
	GS083	GS086	GS090	GS083	GS086	GS090
Control	+++	+++	+++	++++	++++	++++
5.5	—	—	—	—	—	—
Catalase	+++	+++	+++	++++	++++	++++
Trypsin	+++	+++	+++	++++	++++	++++
Pepsin	+++	+++	+++	++++	++++	++++
Proteinase K	+++	+++	+++	++++	++++	++++

*The diameter of the inhibition zone (mm): − < 6, 6 < + < 10, 10 < ++ < 14, 14 < +++ < 18, ++++ > 18.

Table 2: Quantitative detection of organic acids by HPLC (g/l).

Organic acid	*L. plantarum* GS083	*L. plantarum* GS086	*L. plantarum* GS090
Formic acid	0.53 ± 0.10^{Ac}	0.43 ± 0.24^{Ac}	ND^{*Bc}
Malic acid	0.36 ± 0.09^{Ac}	0.82 ± 0.11^{Ac}	0.49 ± 0.15^{Ac}
Lactic acid	19.32 ± 0.95^{Ba}	24.79 ± 0.40^{Aa}	19.85 ± 0.43^{Ba}
Acetic acid	ND^{Bc}	2.00 ± 0.71^{Ab}	2.15 ± 0.27^{Ab}
Citric acid	1.06 ± 0.38^{Ab}	0.55 ± 0.08^{Ac}	0.64 ± 0.20^{Ac}
Succinic acid	1.54 ± 0.15^{Ab}	1.29 ± 0.26^{Ab}	0.69 ± 0.01^{Bb}

*Not detected. A-C: different superscript small letters in the same row denote differences ($p < 0.05$). a-c: different superscript small letters in the same column denote differences ($p < 0.05$).

Table 3: Carbohydrate fermentation patterns of *L. plantarum* GS083, GS086, and GS090.

Carbohydrates	GS083	GS086	GS090
Glycerol	w	w	w
Erythritol	–	–	–
D-arabinose	–	–	–
L-arabinose	+	+	+
Ribose	+	+	+
D-xylose	–	–	–
L-xylose	–	–	–
Adonitol	–	–	–
Methyl-βD-xylopyranoside	–	–	–
Galactose	+	+	+
Glucose	+	+	+
Fructose	+	+	+
Mannose	+	+	+
Sorbose	–	–	–
Rhamnose	+	+	+
Dulcitol	–	–	–
Inositol	–	–	–
Mannitol	+	+	+
Sorbitol	+	+	+
Methyl-αD-mannopyranoside	+	+	+
Methyl-αD-glucopyranoside	–	–	–
N-acetylglucosamine	+	+	+
Amygdalin	+	+	+
Arbutin	+	+	+
Esculin	+	+	+
Salicin	+	+	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	+	+	+
Melibiose	+	+	+
Sucrose	+	+	+
Trehalose	+	+	+
Inulin	–	–	–
Melezitose	+	+	+
Raffinose	+	+	+
Amidon	w	–	w
Glycogen	–	–	–
Xylitol	–	–	–
Gentiobiose	+	+	+
Turanose	+	–	w
Lyxose	–	–	–
Tagatose	–	–	–
D-fucose	–	–	–
L-fucose	–	–	–
D-arabitol	–	–	w
L-arabitol	–	–	–
Gluconate	+	+	+
2-Keto-gluconate	–	–	–
5-Keto-gluconate	–	–	–

Fermentation results are indicated as follows: +, positive; w, weak positive; –, negative.

from 19.32 ± 0.95 to 24.79 ± 0.40 g/l (Table 2). Furthermore, formic acid was detected only in the CFS of *L. plantarum* GS083 and *L. plantarum* GS086. The CFS samples of all tested strains contained acetic acid except for that of *L. plantarum* GS086.

3.5. Carbohydrate Fermentation Patterns of L. plantarum Strains. Fermentation patterns of carbohydrate sources by each *L. plantarum* strain are summarized in Table 3. Differences in fermentation capability were observed as follows: GS083 weakly fermented amidon and did not ferment D-arabitol, while only fermenting turanose; GS090 weakly fermented amidon, turanose, and D-arabitol, resulting in a color transition from green to blue in the API indicator medium instead of yellow, whereas GS086 yielded completely negative results. Based on the patterns identified through the APIWEB database of BioMérieux, the identities (%) of GS083, GS086, and GS090 were 99.9% compared to *L. plantarum* group 1.

3.6. Tolerance of L. plantarum Strains to Gastrointestinal Tract Conditions. Each *L. plantarum* strain was tested for colonization of the GI tract by evaluating their survival in simulated gastric and pancreatic digestion environments (Table 4). All the isolates examined survived in both gastric and pancreatic digestion, which helps in colonizing the intestines. The population of *L. plantarum* strains was superior to 6.8 ± 0.20 lg cfu/ml at the end of these phases.

3.7. Cell Adhesion Activity of L. plantarum Strains. Different *L. plantarum* strains exhibited a high percentage of autoaggregation, ranging from $85.20 \pm 1.07\%$ to $88.01 \pm 1.40\%$ after 4 h incubation (Table 4). Meanwhile, these strains were tested for their cell surface hydrophobicity to estimate their adhesion ability. As shown in Table 4, the tested isolates showed different hydrophobicities.

3.8. Safety of L. plantarum Strains. All three *L. plantarum* strains met the requirements of MIC cutoff values suggested by the EFSA guideline on the antibiotic susceptibility of LAB (Figure 3). Three strains were susceptible to all analyzed antimicrobial agents (including chloramphenicol, erythromycin, rifampicin, tetracycline, gentamycin, clindamycin, imipenem, and ampicillin) with the exception of vancomycin, as an MIC of 512 mg/ml was observed for vancomycin.

The tested LAB strains did not exhibit any hemolytic effect on the blood agar (γ-hemolysis), supporting their safety in vivo (Table 4). Moreover, probiotics with BSH activity showed enhanced tolerance to the bile salts, accordingly lowering blood cholesterol and preventing hypercholesterolemia [32]. The qualitative assessment of bile salt hydrolase activity, indicated by the presence of white precipitation around the colonies in the three LAB strains studied, showed that all strains were positive for BSH activity (Table 4).

The genome assembly and annotation statistics are shown in Table 5. The genomic sequences of *L. plantarum* GS083, GS086, and GS090, respectively, had an identity of 99.18%, 99.16%, and 99.16% with the type genome of *L. plantarum* WCFS1 based on average nucleotide identity (ANI) [33]. CDS sequences of *L. plantarum* GS083, GS086, GS090, and WCFS1 were compared and mapped to the genome of *L. plantarum* WCFS1 (Figure 4). The result revealed that *L. plantarum* GS083 had more genes orthologous with those of *L. plantarum* WCFS1.

Table 4: Probiotic properties, hemolytic, and bile salt hydrolase activity of the selected strains.

Strain		GS083	GS086	GS090
Resistance to gastric and pancreatic juices*	0 h	9.9 ± 0.06	10.1 ± 0.21	9.9 ± 0.07
	3 h	7.5 ± 0.05	8.5 ± 0.12	8.2 ± 0.06
	24 h	6.8 ± 0.20	6.9 ± 0.04	7.3 ± 0.09
Cell surface hydrophobicity (%)		14.86 ± 1.05[b]	21.03 ± 2.01[a]	21.88 ± 1.44[a]
Autoaggregation (%)		85.20 ± 1.07[a]	87.49 ± 3.08[a]	88.01 ± 1.40[a]
Hemolytic activity		−	−	−
Bile salt hydrolase activity		+	+	+

*0 h- viability at the beginning of the assay, 3 h- gastric phase viability after simulation of gastric conditions, 24 h- pancreatic phase viability after simulation of pancreatic condition. a-b: different superscript small letters in the same row denote differences ($p < 0.05$). +: positive, −: negative.

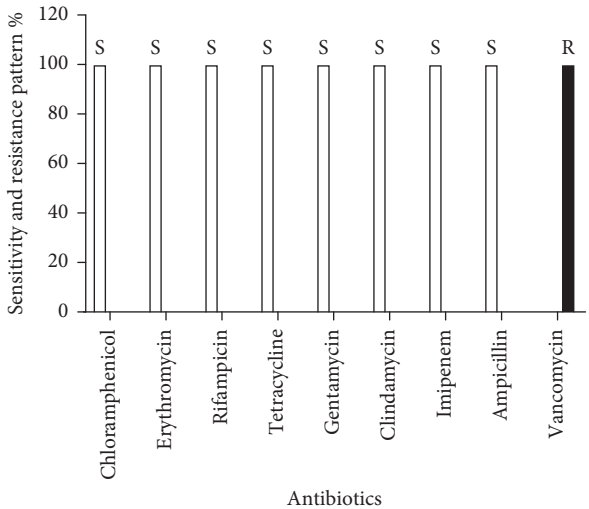

Figure 3: Antibiotic resistance of the selected *L. plantarum* strains. S represents susceptible; *R* represents resistance.

Table 5: Summary of the assembly and annotation statistics of three *L. plantarum* strains.

Strain	GS083	GS086	GS090
Genome size (bp)	3296019	3206156	3220543
GC content (%)	44.26	44.44	44.43
No. of coding sequences	3162	3097	3070
No. of rRNAs	8	8	8
No. of tRNAs	60	59	59

No virulence genes were found under the stringent criteria of >80% identity and >60% coverage. Using the default settings (perfect/strict option for CARD; 90% threshold and 60% minimum length for ResFinder) to search two AMR databases, CARD and ResFinder, no hits were obtained for AMR genes among the genomes of the three LAB strains, suggesting the safety of these isolates.

4. Discussion

Pickles have abundant microbiota and could be utilized as a source for obtaining novel probiotic strains [2, 34, 35]. Additionally, LAB isolated from pickles have many beneficial health effects, such as antibacterial [14] and immunomodulatory [35] activity. In this study, 59 LAB strains were isolated from Chinese pickles including those from the genera *Lactobacillus*, *Lactococcus*, *Weissella*, *Enterococcus*, *Pediococcus*, and *Leuconostoc*, and their antibacterial activity and probiotic potential were further investigated.

The antipathogen potency of CFS produced by LAB was tested. Here, 98.3% of the isolates had antibacterial activity against, at least, one food-borne pathogen, and 91.5% of LAB could inhibit the growth of both G^- and G^+ bacteria. Cervantes-Elizarrarás et al. found that 60% of isolates from aguamiel and pulque (10 strains) inhibited the growth of *E. coli* (G^-) and *S. aureus* (G^+) [36]. An interesting phenomenon was discovered, i.e., *L. monocytogenes* among all tested pathogens was the most sensitive to the CFS produced by LAB. This result was consistent with the findings of Ayala et al. [37]. The prevalence of multidrug-resistant strains of common bacterial pathogens is increasing worldwide [38]. Moreover, infections caused by resistant bacteria might lead to an increase in morbidity and mortality [39]. Some LAB can inhibit the growth of multidrug-resistant pathogens by producing antimicrobial compounds. For example, hydrogen peroxide and lactic acid produced by *L. fermentum* 3872 prevent infections caused by multidrug-resistant *Campylobacter* strains [40]. With this study here, *L. plantarum* GS083, GS086, and GS090, three newly identified LAB, showed prominent antibacterial activity against food-borne and multidrug-resistant pathogens.

LAB usually produce antimicrobial compounds comprising organic acids, hydrogen peroxide, and bacteriocin, among others [41]. Sensitivity tests suggested that the CFS samples from our isolates did not contain any compounds of a proteinaceous nature [42]. It is reasonable to infer that the antibacterial activity of the studied CFS samples might be attributed to organic acids which can also play a role during growth in GI tract conditions. This phenomenon was consistent with the results of previous studies. For example, Barbara et al. reported that organic acids produced by *L. plantarum* CRL 759 inhibit the growth of methicillin-resistant *S. aureus* and *P. aeruginosa* [43]. Furthermore, organic acids can penetrate the cell membrane, thereby affecting cell functions by acidifying the cytoplasm and inhibiting the activity of acid-sensitive enzymes [44]. To verify organic acid production by the three *L. plantarum* strains, the CFS samples were analyzed by HPLC; organic acids including lactic acid, formic acid, malic acid, acetic acid, citric acid, and succinic acid were found to be produced. It is worth mentioning that the highest amount of lactic acid was produced by the three *L. plantarum* strains,

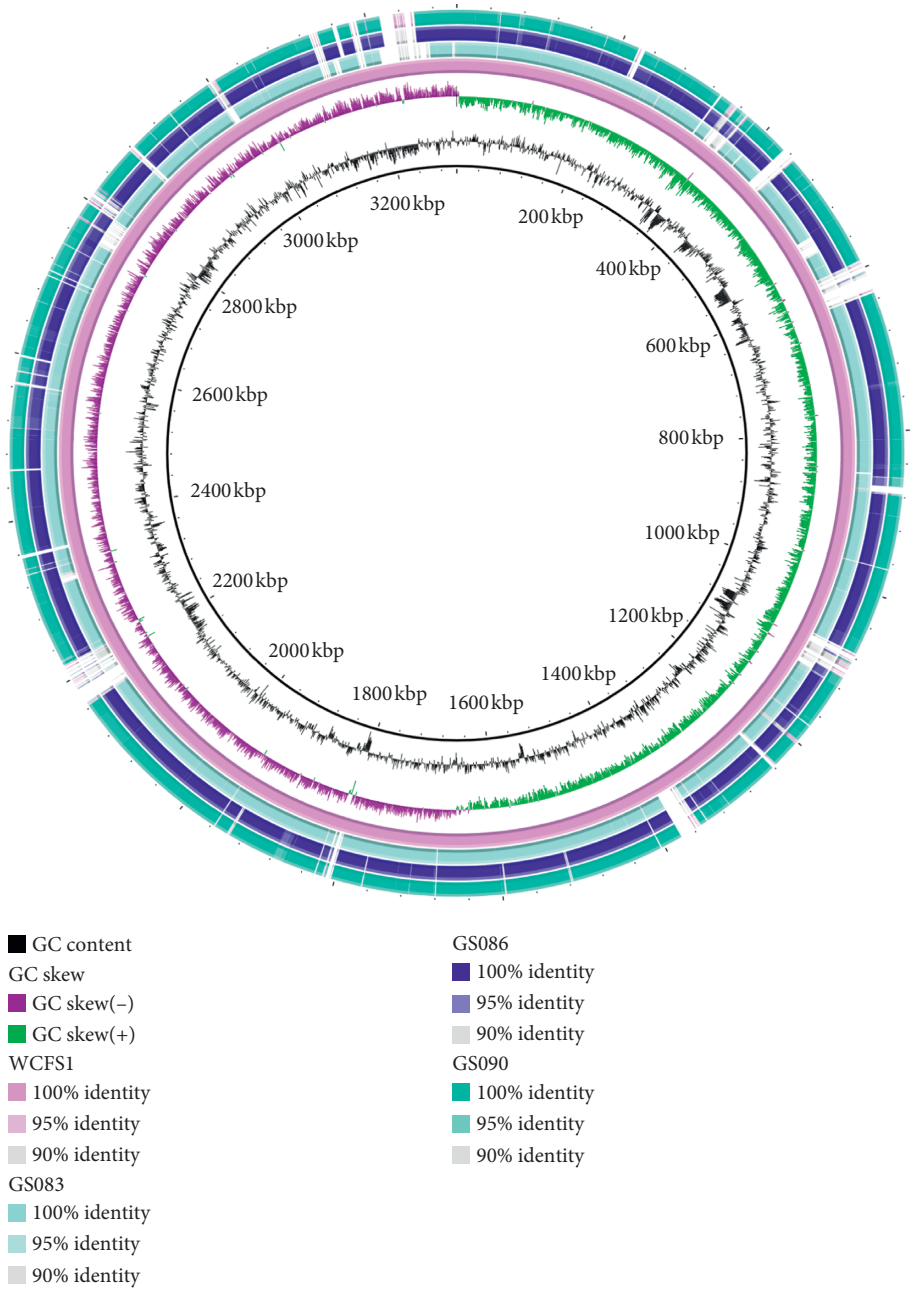

Figure 4: BLAST rings of CDS sequences of *L. plantarum* GS083, GS086, GS090, and WCFS1 mapped onto the genome of the *L. plantarum* WCFS1.

which is consistent with the finding of a study by Muthusamy et al. Lactic acid is one of the major metabolites produced by LAB and is usually produced at a higher level by LAB than other organic acids [16].

To better evaluate the probiotic potential of the three *L. plantarum* strains, several characteristics were tested. The secretion of gastric acid and transit through the stomach constitute a primary defense mechanism that all ingested microorganisms must overcome, including probiotics [45]. A simulated gastric and pancreatic digestion environment was generated to test the survival of three *L. plantarum* isolates under the harsh conditions present in the GI tract. The counts of viable LAB cells were within the range of regulations (6 lg to 10 lg per day) [46], and the three *L. plantarum* strains were deemed adequate to exert probiotic effects in vivo. The capacity of probiotic microorganisms to autoaggregate plays a pivotal role in the colonization of the host epithelia, a prerequisite that aids the host defense mechanisms against gut and skin infections [47]. Autoaggregation of higher than 40% is required for a strain to be a potential probiotic [48]. The percent autoaggregation for the three *L. plantarum* strains after 4 h was greater than 85%, indicating their good adhesion ability. In addition, the surface adhesion ability of bacteria also depends on their hydrophobicity [49]. The results showed that all isolates had hydrophobicity ranging from 14% to 25%, with GS090

having the highest level. Significant differences existed among the investigated strains, which might be attributed to differences in hydrophobic and hydrophilic extensions in the cell wall [31]. In addition, the probiotic properties of the three *L. plantarum* strains were illuminated based on their complete genome sequences.

The FAO/WHO recommended that as a safety measure, the antibiotic resistance profile of a proposed probiotic should also be evaluated [10]. *L. plantarum* GS083, *L. plantarum* GS086, and *L. plantarum* GS090 were only resistant to vancomycin. However, glycopeptide (vancomycin) resistance has been reported in LAB, which is not transferable to pathogens and is rather associated (in most cases) with their innate resistance resulting from the impermeability of their membrane, presumably through a resistance efflux mechanism [50]. Meanwhile, three *L. plantarum* strains showed nonhemolytic and BSH activities, which were recommended as safety characteristics for probiotic selection [51]. According to the whole-genome sequence analysis, the three *L. plantarum* strains lack virulence genes and antibiotic resistance genes. Taken together, these three strains are safe to be used as probiotics. Pickle is a traditional fermented vegetable food with long shelf life, and LAB play a key role in its fermentation.

5. Conclusions

In this study, LAB showed different antimicrobial activities against food-borne and multidrug-resistant pathogens isolated from Chinese homemade pickles, including *Lactobacillus*, *Lactococcus*, *Weissella*, *Enterococcus*, *Pediococcus*, and *Leuconostoc*. The three LAB isolates *L. plantarum* GS083, *L. plantarum* GS086, and *L. plantarum* GS090 were found to have a broad-spectrum antibacterial activity against all the tested pathogens. Furthermore, the three *L. plantarum* strains produced organic acids, including lactic acid, formic acid, malic acid, acetic acid, citric acid, and succinic acid, which are the major metabolites exerting negative effects on the growth of pathogens. Moreover, properties of gastrointestinal tolerance, cell adhesion, BSH, and the lack of multidrug resistance, hemolysis, virulence genes, and antibiotic resistance genes could also contribute to the probiotic potential of these three *L. plantarum* strains. The results ultimately indicate that *L. plantarum* GS083, GS086, and GS090 have potential for application as biological preservatives in the food industry.

Data Availability

All the data supporting the findings are incorporated within the article. Raw data can be presented by the principal investigator upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Y. Zeng and Y. Li contributed equally to the research.

Acknowledgments

This work was supported by the Department of Science and Technology of Guangdong Province (grant no. 2018B020205002) and Guangdong Academy of Sciences (grant nos. 2020GDASYL-20200301002).

Supplementary Materials

Supplementary Table 1: antibacterial spectrum of LAB strains against eight food-borne pathogens. Supplementary Table 2: antibacterial spectrum of LAB strains against six multidrug-resistant bacteria. The diameter of the inhibition zone (mm): - < 6, 6 < + < 10, 10 < ++ < 14, 14 < +++ < 18, ++++ > 18. (*Supplementary Materials*)

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Canadian Journal of Infectious Diseases and Medical Microbiology
Volume 2020, Article ID 8811477, 11 pages
https://doi.org/10.1155/2020/8811477

Research Article

MiR-155-Mediated Deregulation of GPER1 Plays an Important Role in the Gender Differences Related to Inflammatory Bowel Disease

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Received 9 August 2020; Revised 26 August 2020; Accepted 29 August 2020; Published 16 September 2020

Academic Editor: Meng-Hao Huang

Aim. The incidence and clinical manifestations of inflammatory bowel disease (IBD) are thought to have gender differences, which suggests that the estrogen signaling pathway and intestinal flora may play key roles in the pathogenesis of IBD. In IBD, microRNA-155 (miR-155) is upregulated and regulates *G* protein coupled estrogen receptor (GPER1), which affects the intestinal flora. The objective of this study was to investigate the role of the estrogen receptors and miR-155 in the pathogenesis of IBD. *Methods*. From July 2018 to July 2019, in the Department of Gastroenterology at Daping Hospital, Army Military Medical University, a total of 50 patients with IBD were included in this study, and 24 healthy examinees were randomly selected as the control group. Colonoscopies were performed, and clinical characteristics and blood samples were collected from all of the subjects. The serum cytokine levels in the patients with IBD and the health donors were detected by ELISA, and the estrogen receptor level measurements for all of the participants were assessed by immunohistochemistry (IHC) and quantitative real-time PCR (qPCR). The miR-155 levels were detected by qPCR in all of the participants, and miR-155$^{-/-}$ mice were used to investigate the mechanism of miR-155 in the pathogenesis of IBD. *Results*. The clinical characteristics and medications were different for the IBD patients when gender was considered. The male patients produced more proinflammatory cytokines, and while GPER1 expression was downregulated, miR-155 was upregulated in the patients with IBD. MiR-155 showed proinflammatory activity, while GPER1 showed an anti-inflammatory response during the pathogenesis of IBD. The miR-155$^{-/-}$ mice showed improvements in weight loss, survival, rectal bleeding, colon length, and histopathological changes compared with the wild-type mice. Furthermore, the male miR-155$^{-/-}$ mice showed increased inflammation compared to the female miR-155$^{-/-}$ mice in the above aspects. *Conclusion*. This study presents evidence indicating that miR-155 plays a key role in the pathogenesis of IBD for the different genders. MiR-155 was upregulated and showed proinflammatory activity, whereas GPER1 showed an anti-inflammatory response during the pathogenesis of IBD. The results demonstrated that more proinflammatory cytokines and reduced GPER1 levels were observed in the male IBD patients. Thus, miR-155 was involved in the regulation of GPER1 and induced gender differences in IBD patients. MiR-155 may be a potential marker for IBD-targeted therapy.

1. Introduction

Inflammatory bowel diseases (IBD), which include ulcerative colitis (UC) and Crohn's disease (CD), result from deregulated inflammation in a genetically susceptible host [1]. Recently, the incidence of IBD has dramatically increased worldwide, especially in Asian countries [2]. However, the pathogenesis of IBD is not fully clarified. Environmental influences, a disbalance of intestinal microbes, and genetic susceptibility are all involved in the pathophysiology of IBD. The gut microbiota changes the community structure and functional capacity throughout the development of IBD. The ratio of the incidence of IBD in men and women is 1 : 1.5 [3], but females are 60% less likely to develop inflammation-associated colon cancer compared to males [4]. Therefore, it was hypothesized that sex

hormones and their receptors may play a role in the pathogenesis of IBD and differences in the characteristics of IBD in males and females. Moreover, the formation of intestinal flora is influenced by the sex hormones that govern the gender differences [5].

Estrogen receptors, including the nuclear estrogen receptors (ERα and ERβ) and the membrane receptor (*G* protein coupled estrogen receptor, GPER1), are involved in the induction of inflammation [6]. GPER1 has a higher affinity for estrogen and could mediate rapid signal transduction and transcriptional events [7], and the stimulation of GPER1 activates the MAPK, PI3K and NF-κB-dependent signaling pathway as well as other cellular signaling pathways [8]. Although the activation of GPER1 reduces the production of inflammatory cytokines and increases the production of anti-inflammatory cytokines [9, 10], the precise role of GPER1 in the pathogenesis of IBD and its relationship with disease activity is not fully understood. Thus, the gut microbiome might be associated with the development of IBD. MicroRNA-155 is upregulated in a variety of inflammatory diseases and is thought to be a positive regulator of T-cell responses. MiR-155 is one of the most highly expressed miRNA in the serum samples of IBD patients, and probiotics improve intestinal inflammation by regulating miR-155 [11]. An miRNA database search revealed that GPER1 is a predicted target of MiR-155. Thus, our goal was to investigate the role of estrogen receptors in the pathogenesis of IBD and the effect of MiR-155 on the regulation of the estrogen receptor signaling pathway. This study investigated the potential mechanism of IBD, which contributes to the investigation of new therapeutic targets.

2. Materials and Methods

2.1. Participants and Colon Mucosa Sample Collection. A total of 74 patients were admitted to the Department of Gastroenterology at Daping Hospital from July 2018 to July 2019 and were included in this study. The age of participants ranged from 18 to 65 years old. The diagnosis of the patients with IBD was according to the criteria of Lennard-jones, a common diagnostic criterion, which includes clinical, endoscopic, histopathologic, and radiological diagnoses [12]. Patients were excluded from the study if any of the following conditions were met: minors, pregnancy, uncontrolled medical or other serious diseases, or psychiatric disease. Blood samples and colon specimens from an endoscopic biopsy were collected from 22 patients with Crohn's disease (CD) (15 men and 7 women), 28 patients with ulcerative colitis (UC) (16 men and 12 women), and 24 patients unrelated to the disease, which served as the control group (12 men and 12 women). This study was approved by the Ethics Committee of Army Medical Center, and all the participants provided written informed consent prior to the study initiation (chictr.org.cn, ChiCTR1800017211).

2.2. Classification of IBD and Disease Activity Scores. The classification of IBD was according to the Montreal classification. The Montreal classification for CD included age at diagnosis (A1 = <40y versus A2 = ≧40), disease behavior (B1 = nonstructuring and nonpenetrating versus B2 = structuring versus B3 penetrating), and disease location (L1 = ileal versus L2 = colonic versus L3 = ileocolonic versus L4 = upper). The Montreal classification for UC included extent (E1 = proctitis versus E2 = left-sided versus E3 = extensive) [13]. The disease severity of CD was measured according to Crohn's Disease Activity Index (CDAI) score [14]. CDAI scores of <150, 150–450 and > 450 were classified as the remission stage, mild, and moderate active stage and severe stage, respectively.

The Modified Mayo Disease Activity Index (MMDAI) was used for the clinical and research evaluation of UC [15]. Scores of ≤2, 3–10 and 11-12 were classified as clinical remission and mild, moderate, and severe activity, respectively.

2.3. Plasma Inflammatory Cytokine, Estradiol, and Testosterone Measurements. A total of 5 ml serum was collected without an anticoagulant from the patients with IBD and the health donors. The blood samples were centrifuged at 2750 rpm for 12 minutes at room temperature (RT). The serum was collected and stored at −80°C until use. All the hematological indexes were measured by an automatic biochemical analyzer. The levels of TNFα, IL-6, and IL-10 in the plasma were detected using a chemiluminescence immune detection system (Immulite1000, Siemens, Germany). The estradiol and testosterone levels were detected using an automatic chemiluminescence immunoanalyzer (DX1801, Beckman, USA). White blood cells (WBC), red blood cells (RBC), platelets (PLT), hemoglobin (Hgb), C reactive protein (CRP), and the erythrocyte sedimentation rate (ESR) were detected by an automatic biochemical analyzer (XE2100, Sysmex, Japan). Albumin levels were recorded by an automatic biochemical analyzer (DXC800, Beckman, USA).

2.4. Histopathology and Immunohistochemistry (IHC). The colon tissues obtained from the colonoscopies were fixed in formalin. The tissues were sectioned (5 μm) and mounted on glass slides. Hematoxylin and eosin staining was performed to evaluate colon inflammation. Paraffin-embedded sections were deparaffinized and dehydrated. Antigen retrieval was then performed in ethylenediaminetetraacetic acid buffer (EDTA; pH 9.0) for 20 minutes using a pressure cooker. The sections were incubated in an endogenous peroxidase blocker and a normal goat serum working fluid (ZSGB-Bio, Beijing, China) to block endogenous peroxidase and nonspecific responses. The sections were then incubated overnight at 4°C with primary antibodies against GPER1 (Abcam, Cambridge, UK), ERα (Proteintech, Wuhan, China), and ERβ (Proteintech, Wuhan, China). The next day, the secondary antibodies (ZSGB-Bio, Beijing, China) were applied. The antibody binding was visualized by DAB staining (Dako, Glostrup, Denmark), and the slides were counterstained with Mayer's hematoxylin before applying glass coverslips [16].

2.5. Reverse Transcription and Quantitative Real-Time PCR. Colon samples from the mice were homogenized with an appropriate amount of Trizol reagent (Omega, Georgia, USA) followed by the repeated addition to an adsorption column and were centrifuged. The total RNA was extracted from the tissue samples. Specific targeting primers (Sangon, Shanghai, China) were added for the cDNA synthesis by reverse transcriptase. The microRNA was reverse transcribed into cDNA using the Bulge-Loop MicroRNA qRT Primer (Ribobio, Guangzhou, China), and this was used as a template for real-time qPCR with the TB green premix Ex Taq II (Takara Bio, Japan). The gene specific primers were as follows: ERα, forward primer 5′-TACTGCATCAGATCCAAGGGAA-3′, reverse primer 5′-CCTCGGGGTAGTTGTACAC-3′; ERβ, forward primer 5′- GCTGAACGCCGTGACCGATG-3′, reverse primer 5′- ACGTGGGACAGGAGCATCAGG-3′; GPER1, forward primer 5′-TTCCGCGAGAAGATGACCATCC-3′, reverse primer 5′-TAGTACCGCTCGTGCAGGTTGA-3′; IL-6, forward primer 5′- AGA-CAGCCACTCACCTCTTCAG-3′, reverse primer 5′-TTCTGCCAGTGCCTCTTTGCTG-3′; IL-10, forward primer 5′-TCTCCGAGATGCCTTCAGCAGA-3′, reverse primer 5′- TCAGACAAGGCTTGGCAACCCA-3′; and TNFα, forward primer 5′- CGTGGAGCTGGCCGAG-GAG-3′, reverse primer
5′- GCAGGCAGAAGAGCGTGGTG.

2.6. Western Blotting. An immunoenzymatic (Western blot) method was used to detect the protein levels of GPER1, AKT1, and NF-κBp65 in the colon biopsy specimens. The entire colon samples from the mice were homogenized in lysis buffer with an appropriate amount of phenylmethylsulfonyl fluoride, and the lysates were centrifuged at 11,750×g for 12 min. Equal amounts of protein from each sample were subjected to SDS-PAGE. After electrophoresis, the proteins were blotted onto polyvinylidene difluoride membranes and were incubated with the primary antibodies against GPER1 (1 : 1000), AKT1 (1 : 1000), and NF-κBp65 (1 : 1000) (Abcam, Cambridge, UK) overnight at 4°C. The membranes were then incubated in the corresponding secondary antibodies (Abcam, Cambridge, UK). A freshly prepared ECL kit (Beyotime, Beijing, China) was used to visualize the proteins on the membrane in the darkroom [9]. The film was scanned, and the AlphaEaseFC software processing system was used to analyze for optical density values.

2.7. Mice and Reagents. The miR-155$^{-/-}$ mice were purchased from Jackson Laboratory and were housed under specific pathogen-free conditions. All the miR-155$^{-/-}$ mice used in our study were homozygous. SPF C57BL/6 mice obtained from the laboratory animal center at the Army Medical University were maintained and bred under specific pathogen-free conditions. All the mice were between 8 and 10 weeks old. Dextran sulfate sodium salt (DSS) colitis was induced by adding 3% DSS (Sigma-Aldrich) to the drinking water for 5 days, followed by regular drinking water for 5 days. Severity of colitis was assessed using the disease activity index (DAI), which is based on the loss of body weight, stool consistency, and hematochezia. The animal experiments conformed to guidelines of animal usage in research issued by the Army Medical University.

2.8. Statistical Analysis. GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA) software was used for the data and statistical analyses. Continuous variables are presented as the mean ± standard deviation. A T-test was used to compare the studied groups. Categorical variables are expressed as frequencies and percentages and were compared using $\chi 2$ tests. The statistical comparison using the four groups was performed using an analysis of variance (ANOVA) and a Mann–Whitney U test. A nominal p value <0.05 was considered statistically significant.

3. Results

The clinical characteristics of the male IBD patients were different compared to the female IBD patients.

Fifty patients (31 males and 19 females) who were diagnosed with IBD in the Department of Gastroenterology at Daping Hospital, Army Medical University, were enrolled in this study. In our study, the ratio of male to female was 1 : 0.47 for CD and was 1 : 0.74 for UC, respectively. The detailed demographic characteristics, laboratory characteristics, and treatment histories are shown in Supplementary Table 1. The female patients with UC were more likely than the male patients to develop extraintestinal manifestations (EIM) ($p = 0.05$), while the male patients with CD were more likely than the female patients to develop various complications ($p = 0.021$) (Supplementary Table 1). We further analyzed the complete medical history of the patients with IBD. In general, men suffering from CD were treated more frequently with biologics than females ($p = 0.047$) (Supplementary Table 1). The PLT count and ESR level in the male patients with CD were lower compared to the female patients ($p = 0.049$ and $p = 0.014$, respectively) (Supplementary Table 1).

3.1. Gender Differences and Disease Classification. The clinical characteristics of the male patients with IBD were different compared to the female patients, indicating that gender differences existed. Thus, in order to investigate whether there was a significant difference in the disease severity and classification, we analyzed the characteristics between the male and female patients with IBD. There was no significant gender-specific difference in the patients with CD and UC, in terms of disease characteristics, according to the Montreal classification. In addition, no significant differences in the activity of the disease were found between the male and female patients (Supplementary Table 2).

Since the clinical characteristics of the IBD patients showed a gender difference, but no significant difference was found for disease severity and classification between the male and female patients, we investigated whether there was a difference in the cytokine expression between the male and

female patients. As expected, the expression of the inflammatory cytokines IL-6 and TNFα was higher in both the serum and tissue of the patients with IBD compared to the control group ($p < 0.01$) (Figures 1(a), 1(c), 1(d), and 1(f)), and the expression of the anti-inflammatory cytokine IL-10 was significantly lower in both the serum and tissue compared to the control group ($p < 0.01$) (Figures 1(b) and 1(e)). In addition, the expression of IL-6 and TNFα in the male patients with IBD was significantly higher compared to the female patients with IBD ($p < 0.05$) (Figures 1(a), 1(c), 1(d), 1(f)).

3.2. GPER1 Is Associated with Gender Differences in IBD Patients. The clinical characteristics and the expression of TNFα and IL-6 showed significant differences between male and female IBD patients, indicating that the pathogenesis of IBD may involve sex hormones and estrogen receptors. Therefore, we evaluated the sex hormone levels in the IBD patients and the control group. The levels of both estradiol and testosterone in the IBD patients were lower than in the control group ($p < 0.05$) (Supplementary Figure 1A and 1B).

Given that the sex hormones were lower in the IBD patients, we next assessed the expression of the estrogen receptors, that is, ERα, ERβ, and GPER1, in the control and IBD groups. Compared to the control group, ERα expression in the IBD patients was not significantly different ($p > 0.05$), while ERβ expression was lower in the IBD patients compared with the control group ($p < 0.0001$) (Figures 2(a)–2(c)). However, there was no significant difference between the ERα and ERβ expression between the male and female IBD patients ($p > 0.05$) (Figures 2(a)–2(c)). In contrast, GPER1 expression was lower in the IBD patients compared to the control group ($p < 0.0001$). Moreover, its expression in the female patients was significantly higher compared to the male patients ($p < 0.0001$), indicating that GPER1 may be correlated with the gender differences observed in IBD (Figures 2(a) and 2(d)).

We further detected the signaling pathways downstream of GPER1. The expression of AKT1 and NF-κBp65 was significantly higher in the IBD patients than in the control group (Figure 2(e)). Furthermore, GPER1 was significantly decreased in male patients compared with female patients, while the expression of AKT1 and NF-κBp65 was higher in the male patients than in the female patients (Figure 2(e)).

3.3. MiR-155 Is Involved in the Mechanism Related to the Gender Differences between Male and Female IBD Patients. The expression of GPER1 was different between the male and female IBD patients, suggesting that GPER1 might be involved in the mechanism of the gender differences observed in IBD patients. MicroRNAs play very important roles in the regulation of protein function. Using a microRNA database search, we found that the 3′UTR of GPER1 contained a potential binding site for miR-155 (Figure 3(a)). Therefore, GPER1 was a predicted target of miR-155. Here, we also found that miR-155 expression was higher in patients with IBD compared to the control group ($p < 0.0001$), and miR-155 expression was also higher in the male IBD patients compared to the female patients ($p < 0.001$) (Figure 3(b)).

3.4. Knock-Out of MiR-155 Could Attenuate the Intestinal Inflammation in 3% DSS-Induced Colitis. MiR-155 was involved in the regulation of GPER1 and may induce the gender differences observed in patients with IBD. Thus, miR-155$^{-/-}$ mice were used to investigate the manifestations of colitis between the male and female mice. We observed that male and female miR-155$^{-/-}$ mice showed decreased inflammation compared to the WT mice in many aspects, including weight loss (Figure 4(a)), survival (Figure 4(b)), rectal bleeding (Figure 4(c)), colon length (Figure 4(d)), and histopathological changes (Figure 4(e)). However, the male miR-155$^{-/-}$ mice showed increased inflammation compared to the female miR-155$^{-/-}$ mice in the above aspects (Figures 4(a)–4(e)).

4. Discussion

A significant gender difference in the incidence of IBD has been reported [17]. Here, we demonstrated that male patients produce more TNFα, and, thus, we investigated the underlying mechanism of the gender differences observed in patients with IBD. GPER1 and miR-155 played important roles in the gender differences associated with IBD. MiR-155 played a proinflammatory role, while GPER1 was anti-inflammatory during the pathogenesis of IBD (Figure 5).

An imbalance of proinflammatory cytokines and anti-inflammatory cytokines leads to intestinal immune function and gut microbiome disorders, which is closely related to the pathogenesis of IBD. By analyzing both serum and tissue, we found that the levels of the proinflammatory cytokines IL-6 and TNFα were significantly higher in the patients with IBD compared to the control group, while the level of the anti-inflammatory cytokine IL-10 was lower than the control group. These findings were consistent with previous reports [18]. A disruption of the Th17/Treg balance by IL-6 is believed to be an important factor in the development of IBD [19]. Here, we found that the quantitative level of TNFα in the male patients was significantly higher than in the female patients. TNFα increases intestinal permeability in IBD by increasing the expression of epithelial myosin light chain kinase (MLCK) [20]. TNF also plays a role in the composition of the gut microbiome during development and affects the development of the immune system [21]. Anti-TNF-therapy regulates the gut microbiota and intestinal barrier function, which transforms the diversity of the gut microbiota in IBD patients toward the healthy population [22, 23]. Blocking TNFα is a successful targeted therapy for IBD. There were significant gender differences in TNFα expression, and it has been suggested that sex hormones may be related to the incidence of IBD.

More and more evidence reveals that E2 has a protective effect on inflammation-related diseases. Decreasing inflammation in mice is achieved by increasing the expression of E2 in mice, which stimulates the expression of antioxidant enzymes [24]. The concentration of estrogen metabolites

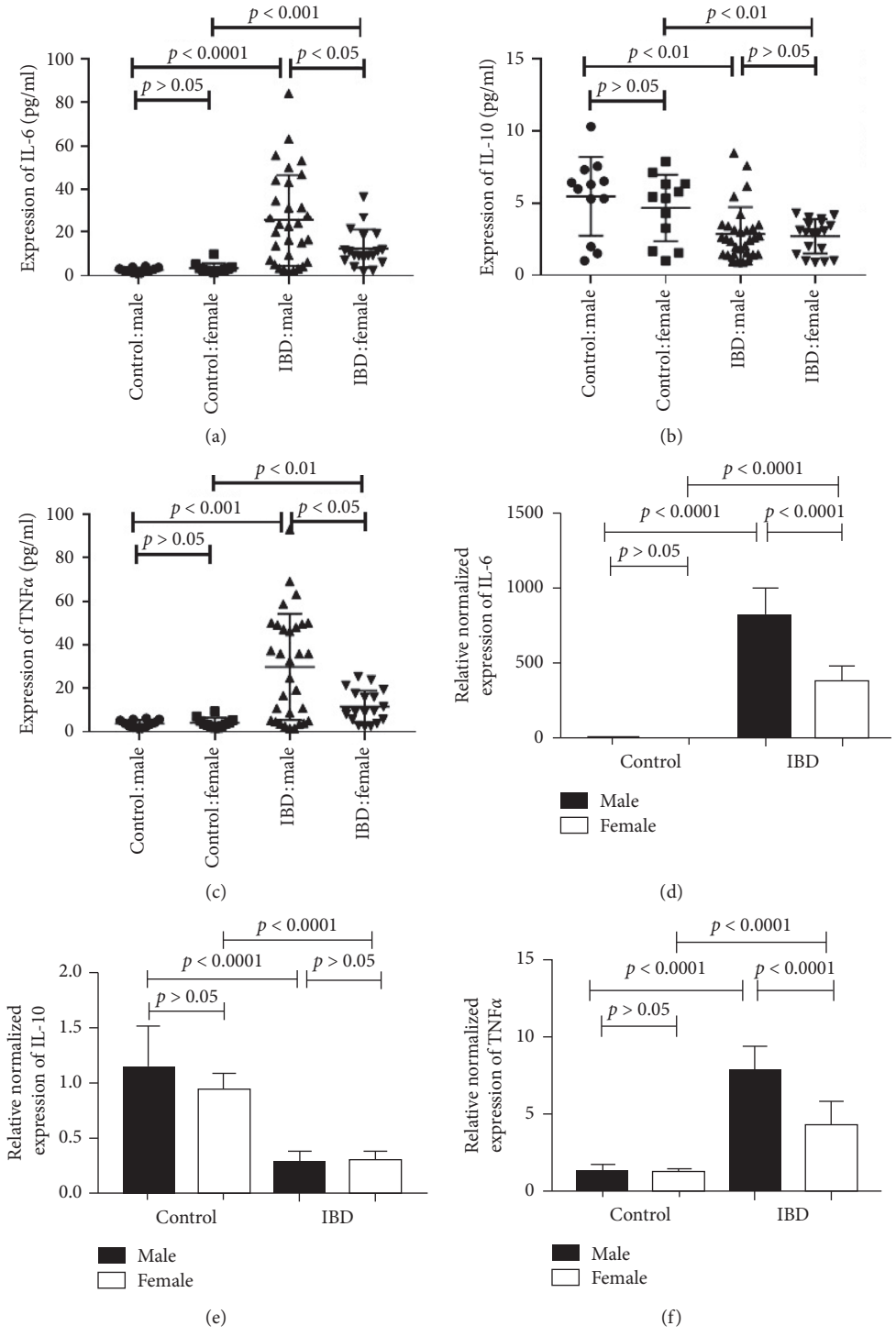
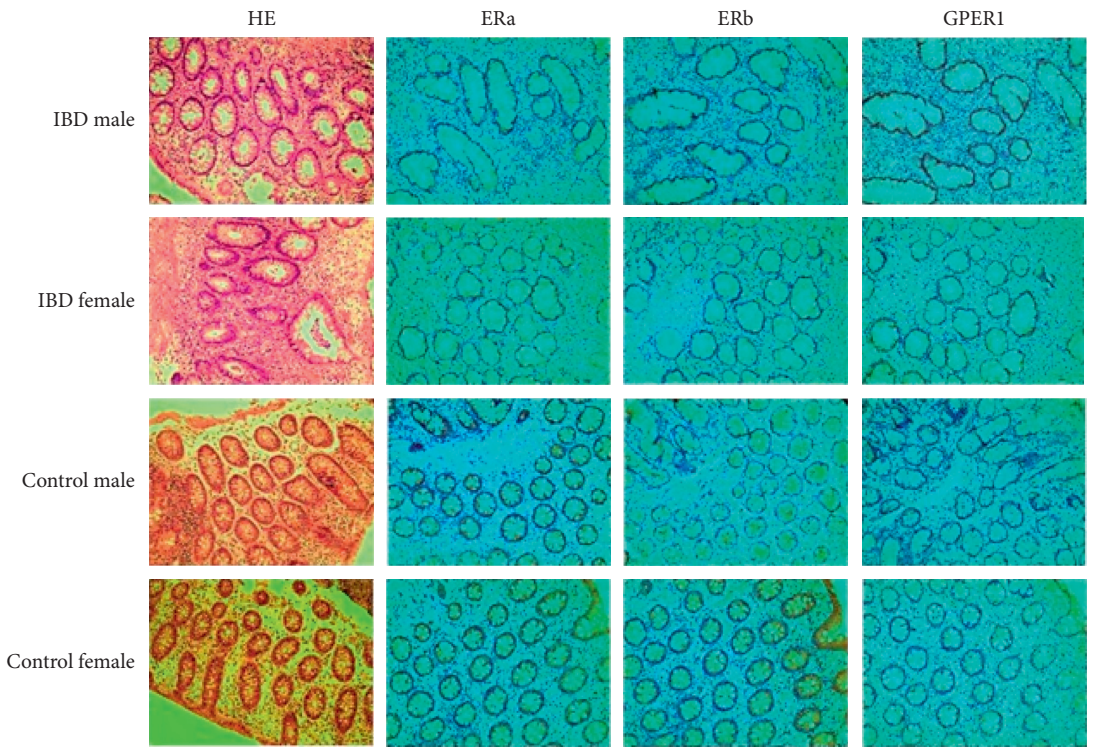
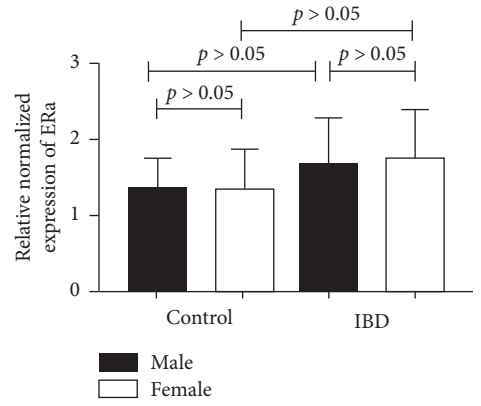


Figure 1: The expression of IL-6 and TNFα in male IBD patients is higher than in female patients. 1a and d expression of IL-6 in the serum (a) and tissue (d) of the IBD patients and control group. (b, e) Expression of IL-10 in the serum (b) and tissue (e) of the IBD patients and control group. (c, f) Expression of TNFα in the serum (c) and tissue (f) of the IBD patients and control. The values are presented as the mean ± SEM.

(EM) appears to be closely related to the diversity of the gut microbes. For example, 16-alpha-hydroxylation of estradiol is the metabolite most closely associated with gut microbes [25]. However, there is no relevant research related to this in IBD. In contrast, estrogen improves the barrier function in intestinal epithelial cells and mucosal healing by upregulating tight junction proteins and reduces the production of inflammatory cytokines in IBD [26]. Recently, the combination of estrogen and probiotics is suggested to improve gut leakage and repair intestinal barrier function [27]. One



(a)



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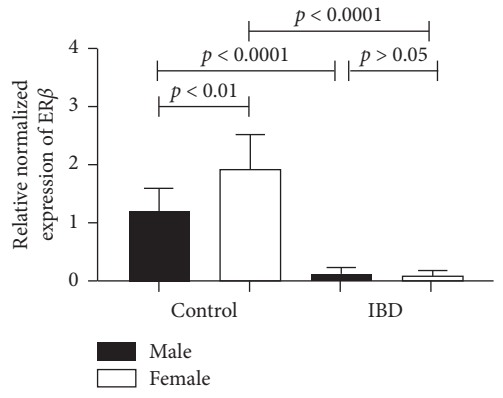

(c)

Figure 2: Continued.

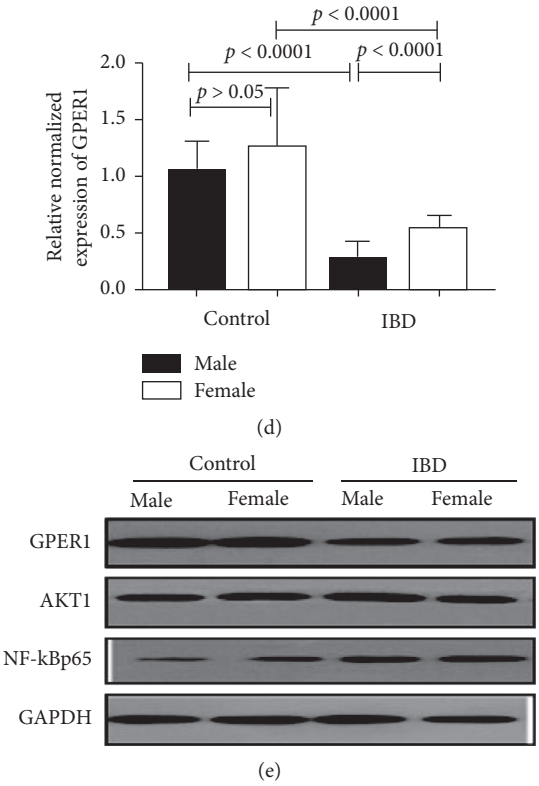

Figure 2: GPER1 is associated with gender differences in IBD patients. (a) Representative immunohistochemical (IHC) staining of the estrogen receptor protein in tissue (400×). (b) The mRNA expression of estrogen receptor ERα, ERβ (c), and GPER1 (d) in tissue. (e) The expression of GPER1, AKT1, and NF-κBp65 is assessed by a western blot. The values are presented as the mean ± SEM.

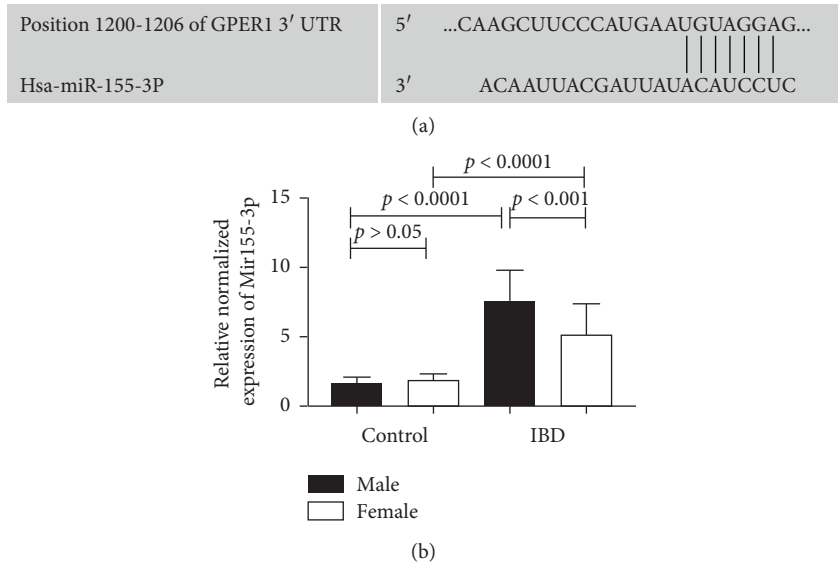

Figure 3: MiR-155 is involved in the regulation of GPER1. (a) GPER1 is a target of miR-155. (b) The expression of miR-155 in the colon tissues of IBD patients and the control group. The values are presented as the mean ± SEM.

mechanism of the protective effect of estrogen may be realized by the estrogen receptor, which is involved in the pathogenesis of IBD [4]. Our study demonstrated that, in male IBD patients, the level of E2 was significantly higher than in the control male participants. In order to further illustrate the role of the estrogen receptor, mediated by estrogen signal, in IBD, we tested the level of GPER1, ERα, and ERβ in the colon. There were no significant gender differences with respect to the expression of ERα and ERβ between the male and female IBD patients. However, GPER1

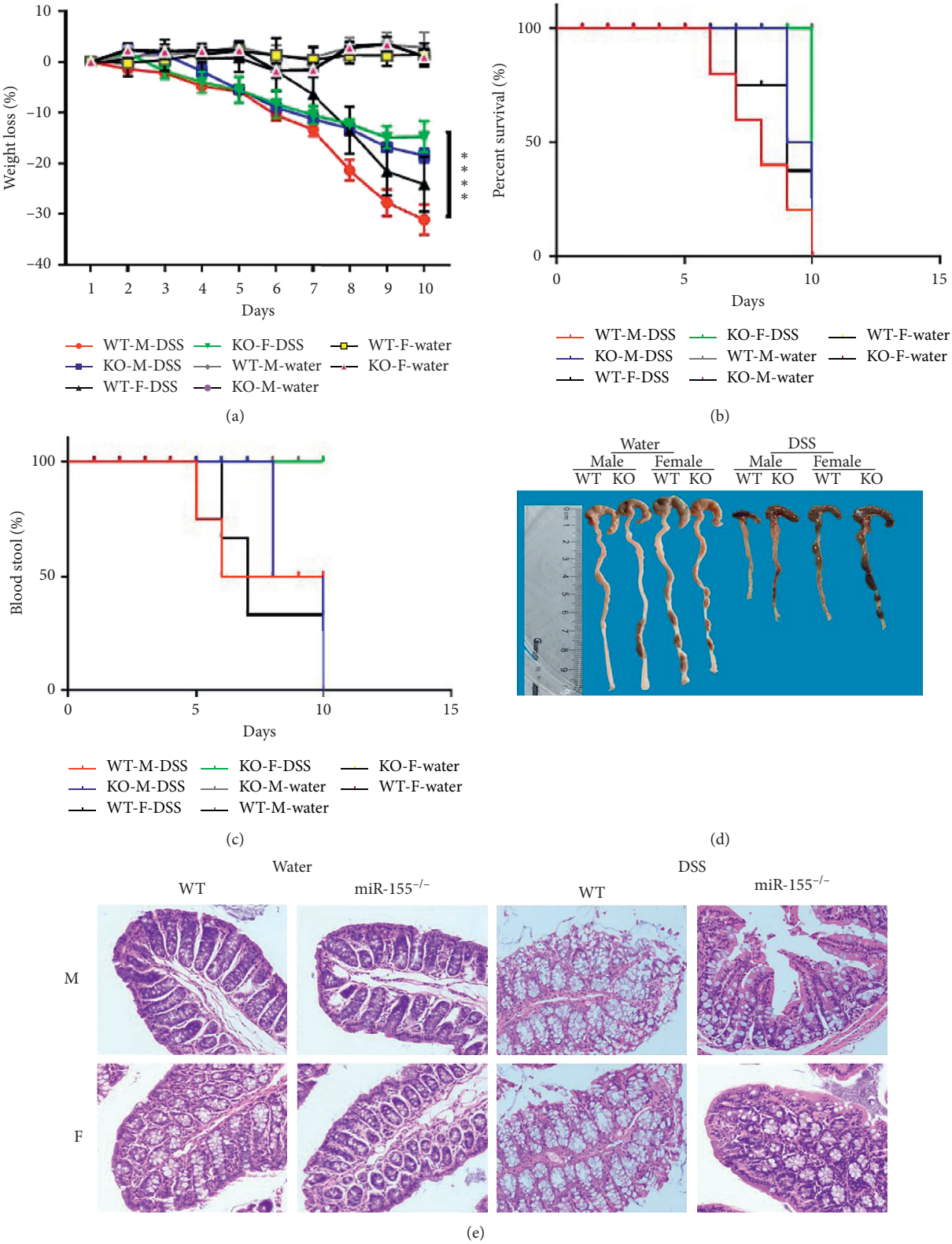

Figure 4: Knock-out of MiR-155 could attenuate the intestinal inflammation in 3% DSS-induced colitis. (a) Weight change, (b) Kaplan–Meier survival rate plot, (c) bloody stool, (d) representative gross colon appearance and colon length, and (e) representative H&E-stained colon cross sections (original magnification, 200×).

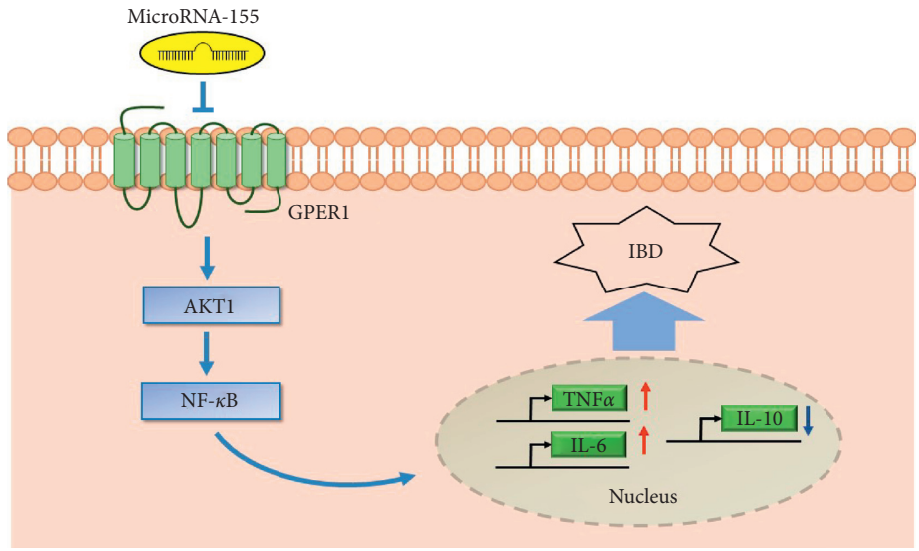

Figure 5: MiR-155 plays a proinflammatory role, and GPER1 is anti-inflammatory during the pathogenesis of IBD. In IBD patients, the proinflammatory cytokines IL-6 and TNFα were significantly higher, and the anti-inflammatory cytokine IL-10 was significantly lower.

expression was significantly lower in the IBD patients compared to the control group, especially in the male patients with IBD. GPER1 protein was expressed in human endothelial cells, monocytes, and macrophages [10, 28]. Our immunohistochemical analysis revealed that GPER1 was localized in the cytoplasm of the intestinal epithelial cells and goblet membranes, which suggested that the expression of GPER1 was closely related to the pathogenesis of IBD. Furthermore, our data demonstrated that AKT1 and NF-κBp65, which are downstream of the PI3K-Akt signaling pathway, were significantly upregulated in IBD patients. However, there was no significant difference between males and females. In IBD, the activation of GPER1 blocks the pathway dependent on proinflammatory cytokines, and the activation of GPER1 is thought to play a key role in intestinal inflammation [10]. However, recent evidence suggests that there are significant differences in the bacterial species richness between GPER1$^{-/-}$ and GPER1$^{+/+}$ rats. Compared with WT GPER1$^{+/+}$ rats, Gper1$^{-/-}$ rats exhibit significantly reduced levels of Clostridiales under the phylum Firmicutes. Thus, the deletion of GPER1 significantly alters gut microbes [29]. MicroRNAs also modulate gut-associated metabolism by regulating gut microbes. MiR155 is highly expressed in IBD, and it might share common molecular pathways with gut microbiota, such as *Ruminococcus* [30]. Thus far, no study has shown that miR-155 has gender differences affecting gut microbes, and this will be the next goal of our research group.

In order to further study the relationship between GPER1 and microRNAs, we predicted that GPER1 is a target of miR-155 based on a miRNA database. The 3′ UTR of GPER1 contains a potential binding site for miR-155. We further detected the level of miR-155 in tissue and demonstrated that the expression of miR-155 in IBD patients was significantly increased, especially in the male IBD patients, compared to the controls. In order to verify the existence of gender differences using animal experiments, we employed miR-155$^{-/-}$ mice and a DSS model of colitis. We found that the clinical and pathological scores of the miR-155$^{-/-}$ mice decreased, while those of male rats increased. These data suggested that the miR-155 deficiency reduced the intestinal inflammation of DSS mice. Furthermore, we demonstrated that the female miR155$^{-/-}$ mice were more effective at preventing inflammation.

MiR-155 is a multifunctional miRNA that is closely associated with inflammation. In particular, it is closely related to IBD and participates in the molecular changes linked to important targets and signaling pathways associated to this disease [31]. MiR-155 promotes macrophages to polarize into the M1 phenotype, which increases expression of M1 macrophages and decreases expression of M2 macrophages, thereby activating a proinflammatory pathway [32]. Probiotics improve inflammation in the colon by regulating the expression of miR-155, which is a marker involved in the colon immune response [33]. Some studies show that the clinical scores of miR-155$^{-/-}$ mice in the acute experimental colitis model group are lower than those in the wild-type control group, reversing the pathogenesis related to colitis and reducing the systemic and inflammatory cytokines [11]. Our data confirmed that the regulation of miR-155 in IBD patients of different genders was different, which might be due to the regulation of GPER1-induced inflammation. In some chronic inflammatory diseases, such as osteoporosis, atherosclerosis, and chronic inflammatory diseases, sex dimorphic characteristics are attributed to the role of the estrogen receptor. Previously, it was reported that the expression pattern of the estrogen receptor promotes sex dimorphism, which might protect women, but not men, from inflammation [33].

Gender differences in the gut microbiota have been reported in humans. The intestinal flora of men is dominated by *Bacteroidetes* and *Prevotella*, suggesting that the

difference in the gene expression of the sex chromosomes or gonadal hormone levels might affect the regulation of the gut microbiota [28]. The intestinal flora of women shows less proinflammatory functions, and, thus, females are less prone to inflammation [34]. Altogether, it is clear that the gender differences of the gut microbiota can influence the severity of IBD. Rodríguez-Nogales et al. showed that the administration of some probiotics, such as *Escherichia coli* Nissle 1917, *Lactobacillus fermentum,* and *Lactobacillus salivarius,* could attenuate the development of DSS-induced colitis by downregulating mir-155 expression [35, 36].

In conclusion, our study explored the prominent position of miR-155 in regulating GPER1 in patients with IBD of different genders. MiR-155 was a negative regulator of GPER1 in both genders. A deficiency of miR-155 led to a significant inhibition in inflammation levels for females compared with the males. Our research also had some limitations. Firstly, the underlying mechanism needs to be further clarified. Secondly, the population investigated in this study was relatively limited and, thus, needs to be further determined using a larger population. Thirdly, the gender differences related to miR-155-regulated intestinal flora need to be further confirmed. Finally, previous studies were mainly based on European and American populations, while this study mainly focused on Asian populations. In Asian populations, men are more likely to develop IBD than women [37]. More studies are needed in order to fully comprehend the mechanism of miR155 in IBD, but early results suggest that miR-155 might be a premarker for targeted therapy in patients with IBD, which warrants further studies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Xiaojuan Shao and Jintao Li contributed equally to this article.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Grant nos. 81700483, 31671251, and 81570497), Chongqing Research Program of Basic Research and Frontier Technology (Grant nos. cstc2017jcyjAX0302, and cstc2020jcyj-msxmX1100), and Army Medical University Frontier Technology Research Program (grant no. 2019XLC3051).

Supplementary Materials

Supplementary Table 1: demographic difference and laboratory finding between female and male IBD patient. Supplementary Table 2: classification of patients. Supplementary Figure 1: sex hormones of IBD patients decreased in IBD patients. Serum estradiol (A) and androgen (B) levels in serum. Values were shown as mean ± SEM. (*Supplementary Materials*)

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