Improvement of intestinal flora in model mice showing alcoholism by the administration of Stevia extract fermented with plant-derived lactic acid bacteria

(植物乳酸菌による生薬発酵技術を用いた腸内細菌叢の破綻と

その関連疾患に対する予防法の探索研究)

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Abstract

Bioactive substances with therapeutic potential can be found in medicinal plants used in Chinese medicine. Certain medicinal herbs have been utilized extensively, not just in Asian nations, but also in the USA and Europe, because Chinese medicine places an emphasis on treating the patient as an individual rather than just the ailment. The bioactive compounds found in plants are considerably reduced due to the lower bioavailability of glycosides as compared to aglycones, even though glycosides are typically used to enhance the stability and water solubility of bioactive compounds in plants. It has been shown that intestinal bacteria typically release aglycones from glycosyl residues of glycosides after intake. Microbes play a significant role in showing the effectiveness of medicinal plants containing the compounds, as they also transform the plant-derived substances using metabolite processes such as deglycosylation, dihydroxylation, reduction, C-ring breakage, and demethylation. Hence, utilizing the fermented herbal extract as a means of enhancing the efficacy of these medicinal herbs is an alluring option.

Correlations have recently been shown between the gut microbiome and obesity, liver diseases, and neuropsychiatric diseases. The dysbiosis of the gut microbiota may cause many kinds of illnesses. We have indicated that the administration of live lactic acid bacteria (LABs) cells potentially restores the symptoms of alcohol poisoning in an ethanol-fed mice model. It is interesting that there was a significant increase in the prevalence of a presumed category known as RF32, which has been proposed to have a positive association with the level of serum trimethylamine N-oxide (TMAO). Gut bacteria produce Trimethylamine (TMA) to some extent by utilizing dietary substances such as choline, carnitine, and betaine, which are subsequently converted into TMAO by enzymes in the liver. The link between TMAO and the likelihood of hepatic, cardiometabolic, and other long-term conditions is increasingly recognized; thus, limiting the production of microbial TMA through modifying the disrupted gut microbiota seems to be the most effective approach to preventing such diseases.

Although we have shown which aglycones are released from their glycosides and that therapeutic herbal extracts fermented with plant-derived LABs generate newly bioactive compounds, the substances for gut microbiota have not been investigated [1]. The present study determines whether the fermented

medicinal herbal extract can restore dysbiosis by using the level of TMA formation as an indicator.

Introduction

1.1. Stevia Leaf Effects

Stevia, a well-liked natural sweetener with low-calorie properties, belongs to the Asteraceae plant family. Because of their significant molecular size and pronounced polarity, it is improbable for steviol glycosides to be extensively absorbed or distributed through simple diffusion across biological membranes, which typically prefer small, lipophilic, and neutral molecules. Therefore, glycosides should be predicted to be less permeable. On the other hand, the glycoside diterpene steviol may be regarded as a therapeutic molecule, and it has adequate physicochemical characteristics for passive oral absorption. Recent *in vitro* studies of rebaudioside A, which is one steviol glycoside, and using the Caco-2 monolayer system, revealed that steviol glycosides had relatively low apparent permeability coefficients (P_{app}), but steviol is transported over monolayers significantly more quickly [2].

Steviol, the primary metabolite of corresponding glycosides, is easily absorbed in the lower intestine [3]. To put it differently, apart from a few rapidly degradable intermediate metabolites (such as steviolbioside), all steviol glycosides adhere to identical metabolic routes [4]. On the other hand, steviol does not seem to undergo conversion after contact with the human gut flora. Bacterial β-glucosidase facilitates the conversion of steviosides and rebaudiosides A, B, D, E, and M within the intestinal microbiota, primarily yielding steviol [5,6].

Based on the metabolomic analyses of intestinal contents, bile, and feces, the cecal flora breaks down steviol glycosides into steviol and sugars, which are then absorbed from the intestinal lumen and undergo enterohepatic circulation. The rapid absorption of steviol was evident as peak plasma levels were detected 15 minutes after the oral administration of a single dose of 45 mg/kg to rats. On the other hand, the levels of steviol in plasma rose gradually between 2 and 8 hours after the ingestion of a combination of stevioside, rebaudioside A, rebaudioside C, and dulcoside A. This indicates that the glycoside needs to undergo hydrolysis before it can be absorbed into steviol, and some glycosides are converted to aglycone at a faster rate than others [7].

According to certain studies, rebaudioside A is not effective in changing the variety of the gut flora but may partially affect the abundance of some genera in the flora. These findings may be caused by the bioconversion of rebaudioside A into steviol and glucose performed in the gastrointestinal system. Steviol is broken down, then absorbed, and transformed into steviol glucuronide, which helps the liver detoxify [8]. Numerous studies have demonstrated the beneficial effects of stevia leaf on hypertension, diabetes, metabolic syndrome, and its antibacterial and anticancer properties.

1.2. Effectiveness of Alcohol on Gut Microbiota

Alcohol misuse is a major global cause of disease and a significant risk for social and health issues. Alcohol, metabolized in the liver and lung, can lead to various health problems, including oxidative stress, toxicity, and the accumulation of fatty acid ethyl esters [9]. Acetaldehyde is believed to contribute significantly to alcohol-related disorders [10]. Recent findings suggest a potential connection between changes in the gut microbiota due to alcohol consumption and the development of alcohol-related illnesses. Interestingly, resuming alcohol intake can alleviate these diseases, but it also leads to alterations in the gut microbiota.

Due to a small amount of alcohol being absorbed in the mouth and esophagus, approximately 20 percent of it is gradually absorbed in the stomach, while the remaining 70 percent is absorbed from the small intestine[11]. The liver primarily metabolizes alcohol through the enzyme alcohol dehydrogenase (ADH), which converts alcohol into acetaldehyde and causes significant toxic harm to tissues and intestinal bacteria. Studies have shown that alcohol consumption can cause an imbalance in the gut microbiota [12,13] leading to a decrease in bacteria that produce short-chain fatty acids (SCFAs) and an increase in Gram-negative bacteria [14]. This imbalance can result in the disruption of the intestinal barrier due to the endotoxins produced by Gram-negative bacteria [15], ultimately leading to increased permeability of the gut mucosa [16].

Alcohol consumption can lead to alterations in the gut microbiota, which can impact the gastrointestinal tract and other organs. This is due to the increased permeability of the gut caused by alcohol, allowing bacteria and their byproducts to enter the circulatory system, both locally and throughout

the body. Alcohol intake may be particularly harmful to the liver, including cirrhosis, hepatitis, hepatic steatosis, liver fibrosis, and hepatocellular cancer. Mice given alcohol treatment for three weeks showed signs of intestinal bacterial overgrowth, liver impairment, and fat buildup. Moreover, changes in the composition of the gut microbiota were observed, with a decrease in the prevalence of Firmicutes and an increase in the presence of Bacteroidetes and Verrucomicrobia phyla [17]. These findings show that alcohol-induced liver damage is closely linked to the gut microbiota and that treating the alcohol-related liver illness may be important.

Alcohol changed gut microbiota and its behavior; thus, microbe-produced neurotransmitters such as dopamine, 5-hydroxytryptophan, and gamma-aminobutyric acid (GABA) are altered. The alterations also lead to changes in behavior in mammalian models, such as disruptions in emotional behavior, difficulties with memory, sleep issues, and feelings of depression [18]. This suggests that the gut microbiota plays a crucial role in regulating neuropsychiatric behaviors by the interaction of the brain–gut axis. These findings suggest that targeting the gut microbiota could potentially be a novel approach for treating brain damage caused by alcohol.

1.3. Trimethylamine

In the past, studies have focused on comparing the microbial composition of different disease stages to understand the connection between the microbiota and the development of cardiometabolic disorders. The relationship between dietary nutrients, the gut microbiota, and cardiometabolic disorders is interconnected, as evidenced by the recent finding that trimethylamine *N*-oxide (TMAO) could potentially play a role in the progression of atherosclerosis and cardiometabolic ailments [19]. The gut microbiota is essential to the TMAO production catalyzed by a specific microbial enzyme choline trimethylamine (TMA) lyase. The enzymes (TMA lyase) use different metabolic pathways to convert various dietary components that contain TMA molecules (such as choline, phosphatidylcholine, and L-carnitine) to TMA. After absorption by the host, hepatic flavin monooxygenase 3 (FMO3) transforms TMA into TMAO, which is then eliminated through the kidneys. Studies employing orally poorly absorbed antibiotics and the dietary consumption of isotopically tagged phosphatidylcholine have directly proven the necessary

function of gut microorganisms in the generation of TMAO in humans [20]. In human and animal studies, various bacterial families, including Desferriobacteriaceae, Anaerobacteriaceae, Prevotellaceaem [21] and Enterobacteriaceae [22,23], have been identified as being involved in the production of TMA/TMAO. Recent studies using bacteria found in the human gut found that strains have the ability to produce TMA in a laboratory setting. These strains include: *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *C. hathewayi*, *C. sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, and *Edwardsiella tarda*. These strains represent eight species, which belong to six different genera from two phyla (Bacillota and Pseudomonadota), and they exhibit significant consumption of choline[22]. Regarding choline, a recent work on sulfate-reducing bacteria *Desulfovibrio desulfuricans* that can degrade choline revealed the discovery of a cluster of genes (including *cutD*) involved in the free radical cleavage of the choline C-N bond to generate TMA [23]. Our diet is the main way we are exposed to the environment, and it greatly influences the makeup and functioning of the gut microbiome. The bacteria that reside in the gut and play a role in food digestion are known as the gut microbiota. In recent years, it has been demonstrated that the gut microbiota—the bacteria inhabiting the digestive system and contributing to the breakdown of food—are actively engaged in both inflammation and metabolism. A wide range of human diseases, such as diabetes, high blood pressure, cardiovascular disease, and renal disease, are intricately connected to these processes [20,24,25]. Based on our expanding understanding of how the microbiota affects nutritional intake, we currently think that the gut microbiota may be one of the major endocrine systems in the body, capable of creating metabolites that impact host health and illness. This result has significant implications for efforts to develop drugs that target the gut bacteria themselves instead of the human hosts in which they dwell, because the gut microbial TMAO pathway strongly correlates to human illness [26]. Additional research is necessary to explore potential pharmacological treatments that focus on the gut microbiota. This includes the discovery and creation of appropriate probiotics for human ingestion, along with effective dietary interventions and supplements.

1.4. Consequences of Lactic Acid Bacteria

Consuming alcohol enhances the quantity of gram-negative microorganisms in the digestive system

and affects the permeability of the intestines. Alcoholic liver damage (ALD) is caused by lipopolysaccharide (LPS), which is found in the outer membrane of Gram-negative bacteria. Partial fragments of the LPS escape from the intestinal lumen and then reach the liver through the portal vein. Proteins that bind to lipopolysaccharide (LPS) can attach to toll-like receptor 4 (TLR4), initiating a signal cascade that ultimately activates nuclear factor-kappa B (NF-κB) and subsequently activates Kupffer cells [27]. When the above-mentioned pathways are stimulated, activated Kupffer cells release reactive oxygen species (ROS), molecules of adhesion, chemokines, and pro-inflammatory cytokines, which can significantly harm liver function [28]. The gut microbiota takes an essential function in ALD; consequently, probiotic treatments that alter the microflora may be useful for preventing ALD.

By releasing cytosolic sand bile, the liver has the ability to impact intestinal function, while the mesenteric lymph nodes and portal vein enable the intestines to influence liver function [29]. Thus, the idea of the liver–gut axis was established. LABs may primarily safeguard against ALD by improving the integrity of the gut barrier, thereby reducing the concentration of LPS in the liver. Immune, mechanical, chemical, and biological barriers are examples of gut barriers. Maintaining a stable condition in the digestive system can help preserve the equilibrium of the intestinal microecological environment and ensure proper intestinal permeability.

The majority of bacteria that exist in the human body are located in the gastrointestinal tract, and the microorganisms in the gastrointestinal tract play a crucial role in the metabolic functions of the liver. A well-functioning gut microbiome can shield against the harmful effects of excessive alcohol intake, which can lead to liver damage, by reducing the presence of pro-inflammatory substances like LPS. Generally, lactobacilli have been reported to be able to balance the gut flora in several ways [30,31]. The mechanical barriers in the intestines, including the intestinal lining, the layer of mucus, and the tight connections, are essential for protecting the body from harmful substances like LPS and disease-causing bacteria. These barriers are vital for maintaining a healthy gut. Pro-oxidant enzymes also have a significant impact on generating ROS while processing alcohol. Through *in vitro* and *in vivo* experiments, LABs have been reported to have strong antioxidant activity and the ability to alleviate oxidative stress, enabling them to directly reverse the liver damage caused by alcohol. The phosphorylation of 5'-AMP-activated protein

kinase (AMPK), which is decreased by alcohol, is restored by LABs. This restoration leads to the reestablishment of acetyl-CoA carboxylase (ACC) activity and sterol regulatory element-binding protein (SREBP) 1, along with its mRNA expression. Consequently, the mRNA expression of peroxisome proliferator-activated receptor (PPAR)-α is restored, and alcohol-induced stearoyl–coenzyme A desaturase-1 (SCD1) is reduced. SCD1 is a crucial enzyme that is involved in the production of fatty acids [32]. Probiotics promote the development of mucosal cells in the small intestine, elevate the quantity of goblet and Paneth cells, augment the count of hydrochloric acid cells in the fundic glands, and enhance the stability of gastric acid pH [33]. The alterations enhance the thickness of the intestinal wall, the density of the intestinal villi, the depth of the intestinal mucosa, and the efficiency of the digestive enzymes [34]. Thus, by improving the capacity to absorb and digest nutrients in the liver, the intake of LABs may provide indirect restoration of ethanol-damaged liver tissue. Ethanol consumption leads to the direct stimulation of the gastric mucosa, which in turn affects the intestinal epithelial barrier and mucosal immune function. This can result in mucosal erosion, inflammatory cell infiltration, and significant damage to the mucosal surface [35]. Alcohol dehydrogenase (ADH) activity steadily declines with the onset of ALD. Consuming LABs can enhance the integrity of the stomach lining and the secretion capacity of epithelial cells. This is because these bacteria have the ability to attach to the cells lining the gastrointestinal tract and reduce the ethanol-induced stimulation of these cells. This mechanism decreases the amount of alcohol processed in the liver, indirectly protecting the organism from the alcohol.

1.5. Health and Flora Benefits of Fermented Foods

“Foods created by the intended microbial growth and enzymatic alteration of food components” is the definition of fermented foods. Numerous fermented foods have been demonstrated to have a range of positive health effects on the human body, such as immune system interaction, gut microbiota modification, and the creation of bioactive compounds. Many kinds of metabolites produced in fermented foods have positive effects.

Various types of compounds (peptides, oligosaccharides, free amino acids, modified polyphenols, organic acids, etc.) can be generated by microorganisms with various physiological actions during food

fermentation. Since these changes are done before food consumption, the fermentation process is a great way for enhancing not only nutrients but also health properties. After gaining entry, they have the ability to collaborate with other bacteria in order to produce microbial byproducts, such as vitamins, bacteriocins, and short-chain fatty acids, which serve various functions. It has been suggested that certain fermented food items or their components might offer potential advantages that are sometimes associated with changes in our microbiota [36]. This thesis will focus on the positive impact of fermented foods on the microbiota, discussing their health benefits and potential advantages.

Yogurt is an extensively researched fermented dairy item. It is obtained through the combined activity of two LAB strains (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) when added to milk. The European Food Safety Authority (EFSA) has acknowledged the positive impacts of yoghurt, stating that the presence of active cultures in yogurt can enhance the digestion of lactose in individuals with lactose dyspepsia. Yogurt consumption increases *S. thermophilus* and decreases *Bacteroides* abundances. Numerous animal model studies have demonstrated that prolonged consumption of yogurt has modified the proportion of Bacteroidetes and Firmicutes in the mouse microbiome and enhanced the prevalence of *Streptococcus* family members. This effect could potentially be linked to the presence of *S. thermophilus* in yogurt. Furthermore, certain human intervention studies have demonstrated that the intake of yogurt augments the quantity of certain microorganisms within the human microbiota. Notably, the prevalence of *Lactobacillus* is elevated, accompanied by a concurrent reduction in the diversity of *Bacteroides* species [37].

Cheese, also a fermented milk product, is similar in nature to yogurt in that both are made through a fermentation process, and both contain LABs that can be used for health benefits. However, cheese is more concentrated than yogurt, approximating a solid food, and is therefore more nutritious. The intake of cheese promotes the growth of bacteria that produce butyrate, which could potentially alleviate symptoms of atopic dermatitis. Extensive research has been conducted on the capacity of cheese and dairy products to induce alterations in the gut microbiota and produce beneficial effects on health. In a recent study that extensively examined the relationship between selecting nutritious foods and the composition of gut bacteria, a significant correlation was found between alpha diversity and the consumption of low-

fat cheese. Preclinical research has indicated higher amounts of bacteria that produce butyrate and lower levels of immune responses mediated by T-cell (T_{reg}) and IgE following the introduction of cream cheese to mice. These findings suggest a potential positive impact on the relief of atopic dermatitis. The fermenting bacteria in cheese can exist in the human gut for a certain period of time. Two intervention trials observed a notable rise in the population of fecal enterococci after the consumption of Camembert cheese. Additionally, the feces of individuals consuming the cheese contained microorganisms commonly found in Camembert cheese, including *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Geotrichum candidum*. Furthermore, *Leuconostoc mesenteroides* and *Geotrichum candidum* were detected in fecal samples after the intervention ended, specifically 15 days later [38].

Apart from fermented dairy products, there is limited data available regarding the control of the microbiota by alternative fermented foods derived from animals. Nonetheless, there is proof of the impact of fermented foods derived from plants on the microbiome of the digestive system and their possible correlation with health benefits. Regarding this matter, studies conducted on sauerkraut, kimchi, kombucha, and other fermented plant-based foods have revealed diverse impacts on the gut microbiota. However, further evidence is required to establish a definitive connection to their influence on human well-being.

Sourdough bread is a fermented food that has also been shown to have significant effects. Using a meta proteogenomic approach, researchers analyzed the impact of sourdough bread on the microbiome of rats and observed a decrease in bacterial taxa linked to a low-protein-eating regimen. In another study, the consumption of artisanal sourdough bread induced a different glycemic response than the consumption of industrial white bread [39]. These responses were individual specific and microbiologically relevant. The glycemic response of each individual can be anticipated by analyzing their microbiome profile before conducting the intervention study.

Regarding fermented soy products, numerous clinical studies have demonstrated their ability to modulate microbiota and produce diverse physiological effects. The effects of fermented soybean products on metabolic processes were studied in rodents, showing that fermented soybeans promote changes in fatty acid catabolism and major bacterial phyla in mice. The microbiota of mice was influenced by the

consumption of soymilk fermented with *Lactobacillus rhamnosus*, leading to an increase in certain types of bacteria like *Bacteroides*. These bacteria are known to produce isoflavone metabolites that are excreted in urine. Furthermore, the fermentation of soybeans with *Bacillus amyloliquefaciens* resulted in a decrease in hyperglycemia in a rat model of type 2 diabetes. This fermentation also led to an augmentation in Verrucomicrobiales populations and a decline in Enterobacteriales, along with various alterations in the microbiota [40]. Notably, positive effects on cognitive function and increases in *Lactobacillus* and *Bifidobacterium* populations were observed in mice after ingesting *Lactobacillus* plantarum-fermented soybeans. In a 12-week clinical trial, individuals with mild cognitive impairment demonstrated enhanced cognitive function after ingesting fermented items that contained L. *plantarum* C29. In conclusion, the consumption of fermented soy milk that includes *Lactobacillus casei* Shirota had a notable impact on the skin health of premenopausal women who were in good condition [41]. This resulted in a potential rise in the quantities of *Lactobacillus* and *Bifidobacterium*, while showing a potential decline in the quantities of Enterobacteriaceae and Porphyromonadaceae.

Some of the evidence mentioned above has demonstrated that fermented foods are important in promoting gut health. By consuming these foods and supplementing with these nutrients, it is possible to increase the number and variety of beneficial bacteria in the gut and, to some extent, suppress the abundance of pathogen-associated flora and maintain the balance of the gut flora, thereby promoting gut health. The gut microbiota is greatly influenced by diet, and gaining a better comprehension of how a nourishing diet interacts with a well-functioning gut microbiome will establish the basis for comprehending its significance in preventing and treating diseases. The benefits of any diet will depend heavily on the microbiome of an individual, and the way each person consumes a different diet based on their unique gut microbiome will lead us to a new era of eating patterns. In addition to gut health, there is a growing demand for products and foods that address multiple dimensions of health such as immunity, mood, weight management, skin management, etc., which are inextricably linked to the gut microbiome. Thus, groundbreaking research based on the importance of the gut microbiome may fundamentally change consumer preferences and the direction of the health food industry.

Objective

1. A comparison of unfermented herbal extracts in the inhibitory activity against the synthesis of trimethylamine (TMA).
2. The capacity to produce fermented herbal extracts with the potential to suppress TMA generation is a prerequisite for choosing the P-LAB in this laboratory.

3. To alleviate alcohol-poisoning symptoms and prevent associated diseases, as well as to investigate changes in gut flora.

Materials and Methods

2.1. Medicinal Herbal Extract Preparation

The dried and chopped medicinal plants were bought from Kojima Kampo in Osaka, Japan. Table 1 lists the twenty types of medicinal plants that were employed in this investigation. After being suspended in distilled water until the final concentration reached 5% (w/v), small pieces of the herb were heat treated for 30 minutes at 105 °C. The herbal debris was collected from the extracts through centrifugation, and the resulting supernatants were treated with a solution of sodium hydroxide (NaOH) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in order to lower their pH to 7.0. After being sterilized at a temperature of 121 °C for 20 minutes, the extracts were utilized as the culture media for LABs.

Table 1. Medicinal plants employed in this investigation.

ID	Lingua latīna	Japanese	Katakana
1	Glycyrrhizae Radix	甘草	カンゾウ
2	Leaves of Stevia rebaudiana		ステビア
3	Saussureae Radix	防風	ボウフウ
4	Glycyrrhiza uralensis Fisher		ウラルカンゾウ
5	Scutellariae Radix	黄芩	オウゴン
6	Armeniacae Semen	杏仁	アンニン
7	Sesami Semen	胡麻仁	ゴマニン
8	Lili Bulbus	百合	ビャクゴウ
9	Epimedii Herba	淫羊藿	インヨウカク
10	Cnidii Rhizoma	川芎	センキュウ
11	Polygoni Multiflori Radix	何首烏	カシュウ
12	Eriobotryae Folium	枇杷葉	ビワヨウ
13	Rhei Rhizoma	大黄	ダイオウ
14	Artemisiae Folium	艾葉	ガイヨウ
15	Atractylodis Lanceae Rhizoma	蒼朮	ソウジュツ
16	Moutan Cortex	牡丹皮	ボタンピ
17	Paeoniae Radix	芍薬	シャクヤク
18	Persicae Semen	桃仁	トウニン
19	Gardeniae Fructus	山梔子	サンシシ
20	Lycium Fructus	枸杞子	クコシ

2.2. Conditions for Culture and Bacterial Strains

Table 2 contains a list of the bacterial strains that were employed in this investigation. LAB strains were grown as stationary cultures in MRS broth (Merck KGaA, Darmstadt, Germany) at an appropriate temperature range of 28–45 °C. Next, using the same culture procedure and at the same temperature for 24 hours, each seed culture was injected at a rate of 1% (v/v) into the produced medicinal herbal extract. Following cultivation, every sample underwent sterilization for a duration of 20 minutes at a temperature of 121 °C. Subsequently, the centrifugation process was employed to eliminate the bacterial cell residue. The fermented medicinal plant extract was made from the obtained supernatant fluid. As an alternative, the unfermented extract was made using the supernatant fluid that was obtained from the therapeutic plant extract without any microbial inoculation.

Table 2. LAB strains utilized in this investigation.

Species	Strain	Cultivation temperature	Notes	References
Enterococcus avium	G-15	37°C	GABA	[42]
Enterococcus mundtii	15-1A	37°C	bacteriocin	[42]
Pediococcus pentosaceus	LP28	28°C	EPS, anti-obesity	[42]
	LY45	45°C	thermophilic, EPS	[43]
Lactobacillus amylovorus	PY45	45°C	thermophilic, EPS	[43]
Lactobacillus brevis	174A	28°C	bacteriocin	[44]
Lactobacillus paracasei	IJH-SONE68	28°C	EPS, anti-inflammation	[42][45]
Lactobacillus reuteri	BM53-1	28°C	anti-biofilm	[46]
Lactobacillus plantarum	SN13T	28°C	anti-constipation, improve liver function	[42,47]
Lactobacillus plantarum	SN35N	28°C	EPS, anti-virus infection	[42,48]

2.3. Creation of a Mouse Model for Alcohol Poisoning

The animal experiments performed in the current study were conducted in compliance with the experimental protocols authorized by Hiroshima University's Committee of Research Facilities for Laboratory Animal Science (Permit Number: A16-9-2). All experiments involving animals were conducted in accordance with Hiroshima University's Guidelines for the Treatment and Ethical Use of Laboratory Animals.

For this study, seven-week-old male C57BL/6JJmsSlc mice with age-specific pathogen-free (SPF) status were utilized. The mice were acquired from Shimizu Laboratory Supplies, Co., Ltd., located in Kyoto, Japan. Every mouse was kept in a plastic enclosure that maintained a temperature between 20 and 26 degrees Celsius, a humidity level ranging from 40 to 60 percent, and a light/dark cycle lasting for 12 hours. During a one-week period, the mice were given unlimited access to drinking water and were provided with regular rodent feed (MF diet, Oriental Yeast, Co., Ltd., Tokyo, Japan) to help them become familiar with their surroundings. Following the manufacturer’s instructions, the diet was subsequently substituted with an L10016 diet (Research Diet, New Brunswick, NJ, USA) created using Pre-Mix L10016A (Research Diet). To summarize, diets without ethanol, referred to as L10016 and L10015, were

created for the negative control group. These diets included the addition of 7.5 percent (v/v) ethanol from SAKURAO Brewery and Distillery Co., Ltd., Hiroshima, Japan, and 118.27 g/L maltodextrin 42 from Research Diet to Pre-Mix L10016A, respectively. Since the diets provided ample water content, the mice were kept without additional access to drinking water.

2.4. TMA Production Inhibitory Assay *in Vitro*

Following a two-week period of upbringing, the mice were euthanized by inhaling isoflurane, and the cecum was removed from both the non-ethanol-feeding and alcohol-poisoning model animals. After being incubated without oxygen at a temperature of 37 °C for a duration of 24 hours, a sample of the contents from the cecum was introduced into a modified Gifu anaerobic medium (GAM) broth provided by Nissui Pharmaceutical Co., Ltd. in Tokyo, Japan. After mixing the culture with the fermented herbal extract in a 1 to 4 ratio, it was incubated for an additional 24 hours under identical conditions. Following the elimination of the LAB cells from the cultured blend, the TMA content of the liquid was assessed using a modified Dyer method [49,50].

To summarize, the supernatant was combined with diluted neutral formalin (1:3) in a ratio of 1 mL to 0.2 mL. Then, dehydrated toluene (FUJIFILM Wako Pure Chemical Corporation) was added in a quantity of 2 mL, followed by the addition of a 25% (w/v) potassium hydroxide (KOH) solution (FUJIFILM Wako Pure Chemical Corporation) in a volume of 0.6 mL, all in a sequential order. The mixture was then vigorously vortexed for one minute. After standing at room temperature for five minutes, the toluene layer was dehydrated using sodium sulfate anhydride from FUJIFILM Wako Pure Chemical Corporation. After preparing a standard curve using TMA solutions ranging from 67.2 μM to 4.3 mM, the toluene extract was mixed with an equal volume of a 0.02% (w/v) solution of picric acid from FUJIFILM Wako Pure Chemical Corporation. The concentration of the resulting picrate was then determined spectrophotometrically at 450 nm.

2.5. Tests on Animals to Determine How Fermented Stevia Extracts Affect Alcohol-Poisoning Model Mice

After the period of familiarization, the male SPF C57BL/6JJmsSlc mice were split into four experimental groups, each comprised of five mice. One group was given only the L10015 diet (which does not contain ethanol; referred to as the negative control group, NC). Another group was given only the L10016 diet (which contains ethanol; referred to as the positive control group, PC). One group was given the L10016 diet along with the simultaneous administration of unfermented stevia extract (referred to as the unfermented group, Unf). Lastly, a group was given the L10016 diet along with the simultaneous administration of fermented stevia extract (referred to as the fermented group, Fer). Prior to commencing the experiment, every mouse was weighed and distinguished by the diverse hues of markings on their tails using Animal Marker felt pens (manufactured by Fuchigami Kikai Co., Ltd., Kyoto, Japan). During the trial, the Unf and Fer groups were administered a 100 μL portion of the stevia extract samples daily using an oral feeding needle. Both the PC and NC groups were given an equal amount of sterile water instead of the extract.

After the experiment, the mice were euthanized using isoflurane (FUJIFILM Wako Pure Chemical Corporation), and samples of cecum and blood were obtained from every mouse. At Oriental Yeast Co., Ltd. in Tokyo, Japan, the levels of cholinesterase (ChE), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total cholesterol (T-Cho) in the serum were determined. As previously mentioned, capacity of each cecum to produce TMA was assessed and contrasted.

2.6. Evaluation of the Cecal Microbiome

The Bioengineering Lab conducted analyses on the Illumina MiSeq sequence platform and used MiSeq Reagent Kit v3. A study was conducted by a Kanagawa-based company (Co., Ltd., Japan) to analyze the microbiota of cecal contents using a paired-end technique with a read length of 300 bp (Illumina Inc., San Diego, CA, USA) based on 16S rRNA. In short, the DNA from the cecal contents of

each group was extracted and purified using an MPure-12 Automated Nucleic Acid Purification System and an MPure Bacterial DNA Extraction Kit obtained from MP Biomedicals in Santa Ana, CA, USA. The V3–V4 region of the 16S rRNA genes was amplified by using the primer sets 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-Nn-CCTACGGGNGGCWGCAG-3′ and 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-Nn-GACTACHVGGGTATCTAATCC-3′. Amplification was performed with Ex Taq HS (Takara Bio Inc., Shiga, Japan) according to the following conditions: 2 minutes at 94 °C, followed by 20–25 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 30 seconds at 72 °C. Finally, there will be a 5-minute extension period at a temperature of 72 °C. After adhering to the guidelines provided by the manufacturer, the amplified fragments underwent purification with the assistance of an Agencourt AMPure XP kit (Beckman Coulter Inc., Brea, CA, USA). To perform a second PCR reaction with the primer sets, a 2 μL portion of each resulting solution containing purified fragments was used as a template. The sequences 5′-AATGATACGGCGACCACCGAGATCTACAC-X_8-ACACTCTTTCCCTACACGACGC-3′ and 5′-CAAGCAGAAGACGGCATACGAGAT-X_8-GTGACTGGAGTTCAGACGTGTG-3′ were subjected to the following conditions: initial denaturation at 94 °C for 2 minutes, followed by 20–25 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 30 seconds at 72 °C. Finally, there was a 5-minute extension period at 72 °C. Following the previously mentioned purification process, the amplified samples were sent to the analytical device. The taxonomy assignment and sequencing analysis were performed using the QIIME 2.0 pipeline [51].

Results

3.1. Effect on the TMA Productivity of the Medicinal Herbal Extract

Earlier, the examination of microbiota was conducted on samples from the cecum of mice that were given ethanol to cause dysbiosis. This examination showed that the proportion of the presumed RF32 group, which has been associated with the level of TMAO in the blood [52], increased due to the consumption of ethanol[47]. Initially, our objective was to assess the TMA composition of the cecum samples from mice in the PC group, which were fed ethanol, and compare them to mice in the NC group, which were not given ethanol. Nevertheless, spectrophotometric testing was unable to quantify the amounts of TMA [49,50]; therefore, it may have resulted from measuring limitations. As a result, we assessed and contrasted the TMA generation in the cecum sample cultured broth (Figure 1). The results indicated that ethanol consumption altered the contents of the cecum, resulting in a significant increase in latent TMA production (approximately 15 times higher). Consequently, we utilized the TMA manufacturing level as an indicator for cecal dysbiosis.

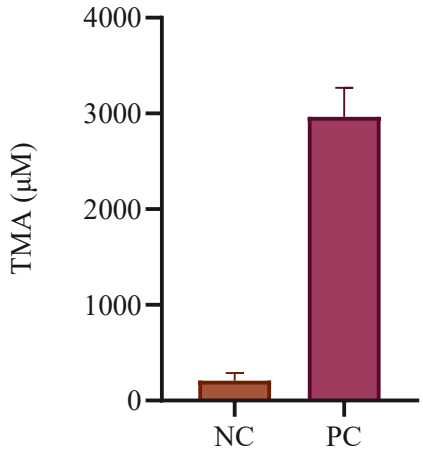

Figure 1. Variations in the amounts of trimethylamine (TMA) in the cecum sample cultured broth. Cecum samples were collected from mice in the positive control group (PC) and the negative control group (NC) that were given ethanol. The data is shown as the average ± standard error (S.E.).

Next, employing 20 different types of extract, it was determined whether the medicinal herbal extract affected the latent TMA productivity (Figure 2). The synthesis of TMA was reduced by specific herbal extracts, especially in Gardeniae Fructus (ID 19) and Lycium Fructus (ID 20), when compared to the sample without extracts. On the other hand, extracts from Glycyrrhizae Radix (ID 1) and stevia (ID 2) exhibited distinct TMA inducibility.

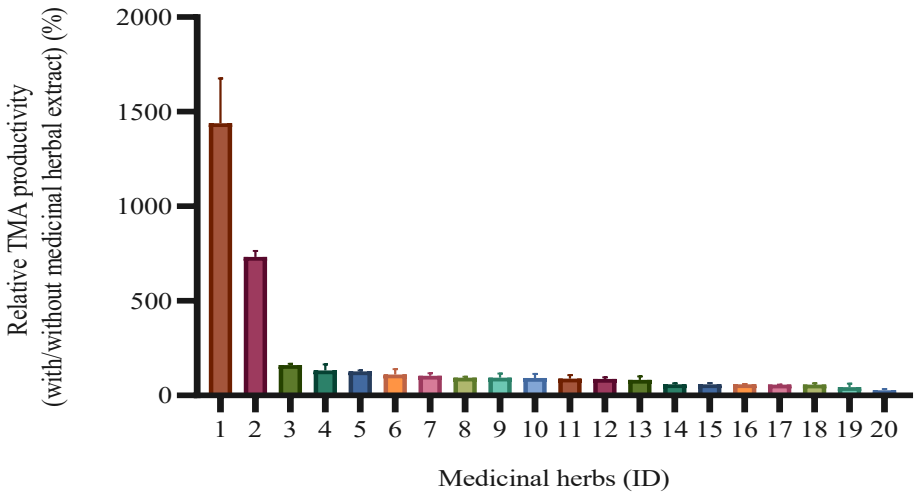

Figure 2. A study was conducted to compare the relative TMA production rates in cecum samples taken from mice that were administered ethanol along with non-fermented therapeutic herbal extracts, focusing on their latent productivity. In Section 2, the IDs of the therapeutic plants are compiled. The information is displayed as the average ± S.E. (n = 2).

3.2. Modification of Inhibitory Activity in the Medicinal Herbal Extract

The anti-TMA generation efficacy before and after LAB fermentation was compared using extracts from Gardeniae and Lycium fructus, based on the findings in the previous chapter. During this study, we fermented the herbal extracts using 10 distinct LAB strains that were isolated by our research team and identified as advantageous for promoting human health (Table 1). LP28, LY45, and SN35N strains exhibited greater reduction capabilities against TMA production as compared to the unfermented Gardeniae Fructus extract (Figure 3A). Conversely, the anti-TMA activity of the fermented extracts seemed to decrease in Lycium Fructus (Figure 3B). The discovery of these results prompted the use of these three strains for further examination.

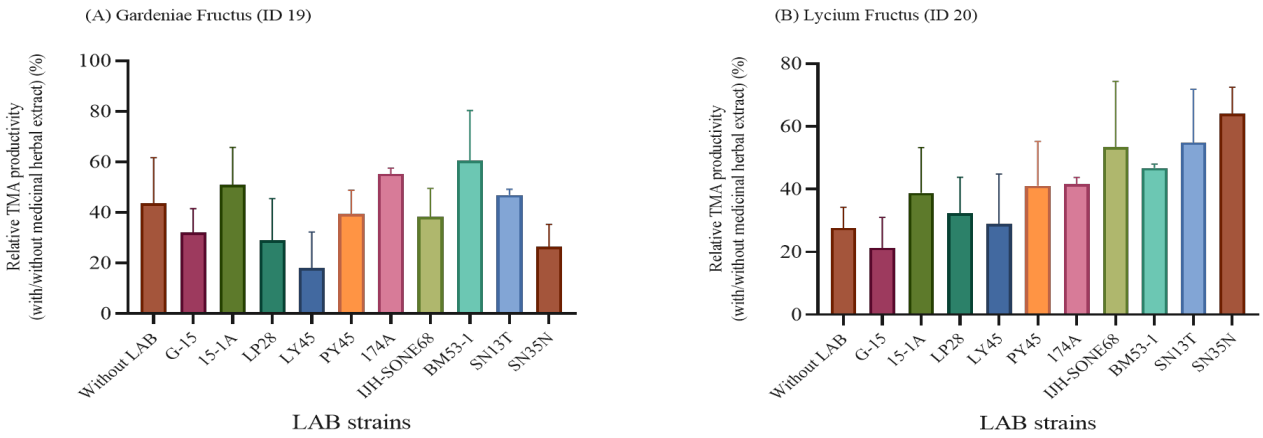

Figure 3. The comparative latent TMA yields of Gardeniae Fructus (A) and Lycium Fructus (B) extracts, which were fermented by lactic acid bacteria (LAB), in the cecum samples collected from mice that were given ethanol. The information is displayed as the average ± S.E. (n = 2).

To further confirm the impact of LAB fermentation on the herbal extracts, we analyzed the suppressive effect on TMA formation of all the investigated botanical extracts both before and after undergoing LAB fermentation (Figure 4). Among the three strains, the LY45-fermented extracts appeared to have better activity. It is noteworthy that LAB fermentation significantly reduced the TMA production found in the unfermented extracts of stevia and Glycyrrhizae Radix, particularly when the LY45 strain was used for fermentation. Due to the high concentration of glycyrrhizin in Glycyrrhizae Radix, which has been shown to counteract antihypertensive drugs through its pseudo-aldosterone effect [53,54], the next *in vivo* verification experiment utilized the LY45-fermented stevia extract.

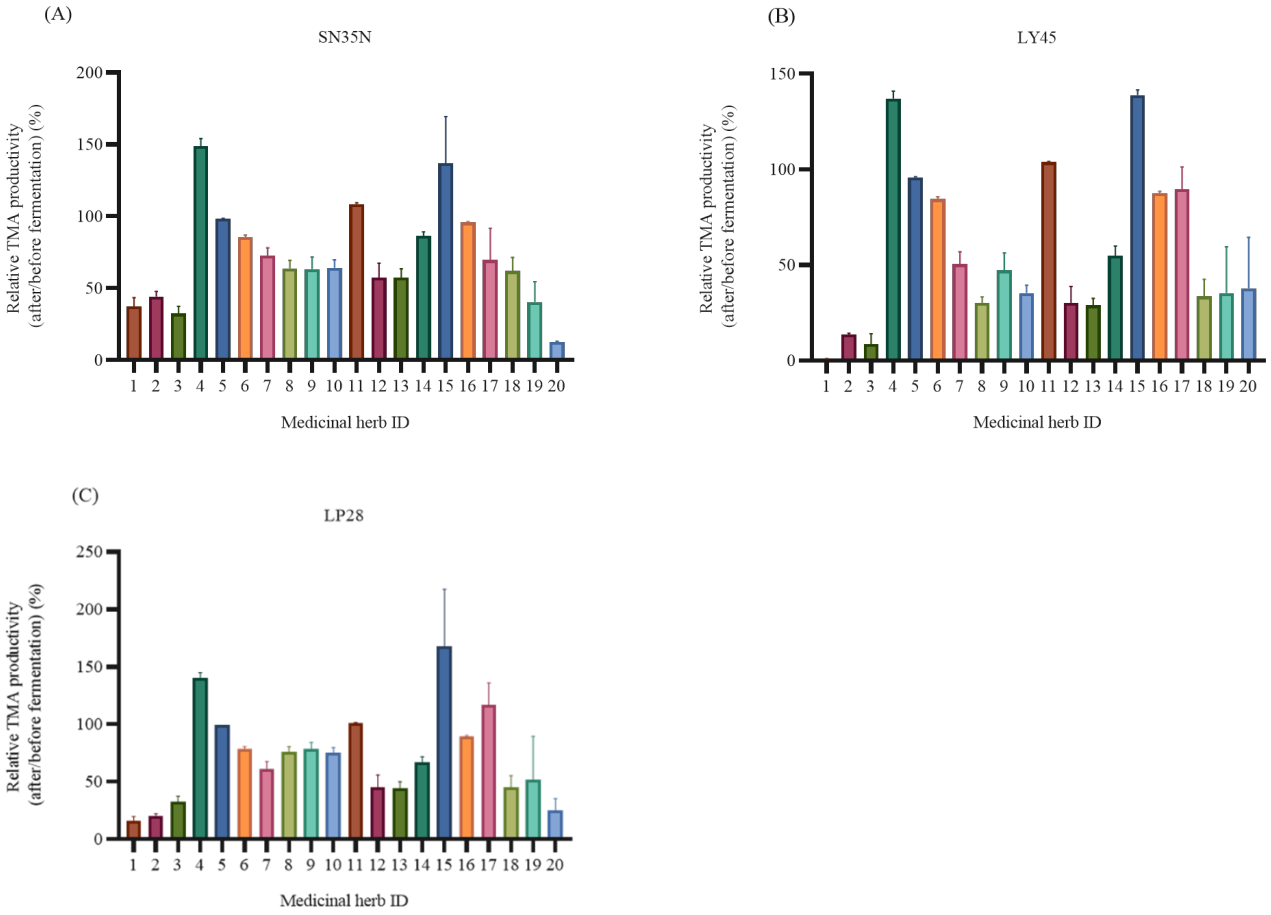

Figure 4. Mice fed ethanol and administered therapeutic herbal extracts were used to evaluate the TMA productivities of cecum samples fermented with *L. plantarum* SN35N (A), *P. pentosaceus* LY45 (B), and *P. pentosaceus* LP28 (C). In Section 2, the IDs of the therapeutic plants are compiled. The data is presented as the mean ± standard error (S.E.) (n = 2).

3.3. Improving the Effectiveness of Fermented Stevia Extracts on Model Mice Intoxicated with Alcohol

The effects of LY45-fermented or unfermented stevia extracts on recovery before and after fermentation were analyzed in mice that were orally given an alcohol-poisoning model. This analysis is shown in Figure 5. A significant increase ($p < 0.05$) was observed in hepatic parameters AST and ALT when comparing the ethanol-fed group (positive control, PC) with the negative control group (NC, without ethanol feeding). The enhancement was not noteworthy in the unfermented category (Unf), but it was remarkable in the fermented category (Fer) (Figure 5A, C). A similar result was found in LDH, but no

noteworthy distinctions were found across the therapy groups (Figure 5E). A statistically significant ($p < 0.05$) reduction in the increased TMA production observed in the PC group was observed in the Fer group when comparing the latent TMA productivities of the cecal contents collected from each mouse. There were no obvious treatment-related impacts in the other items.

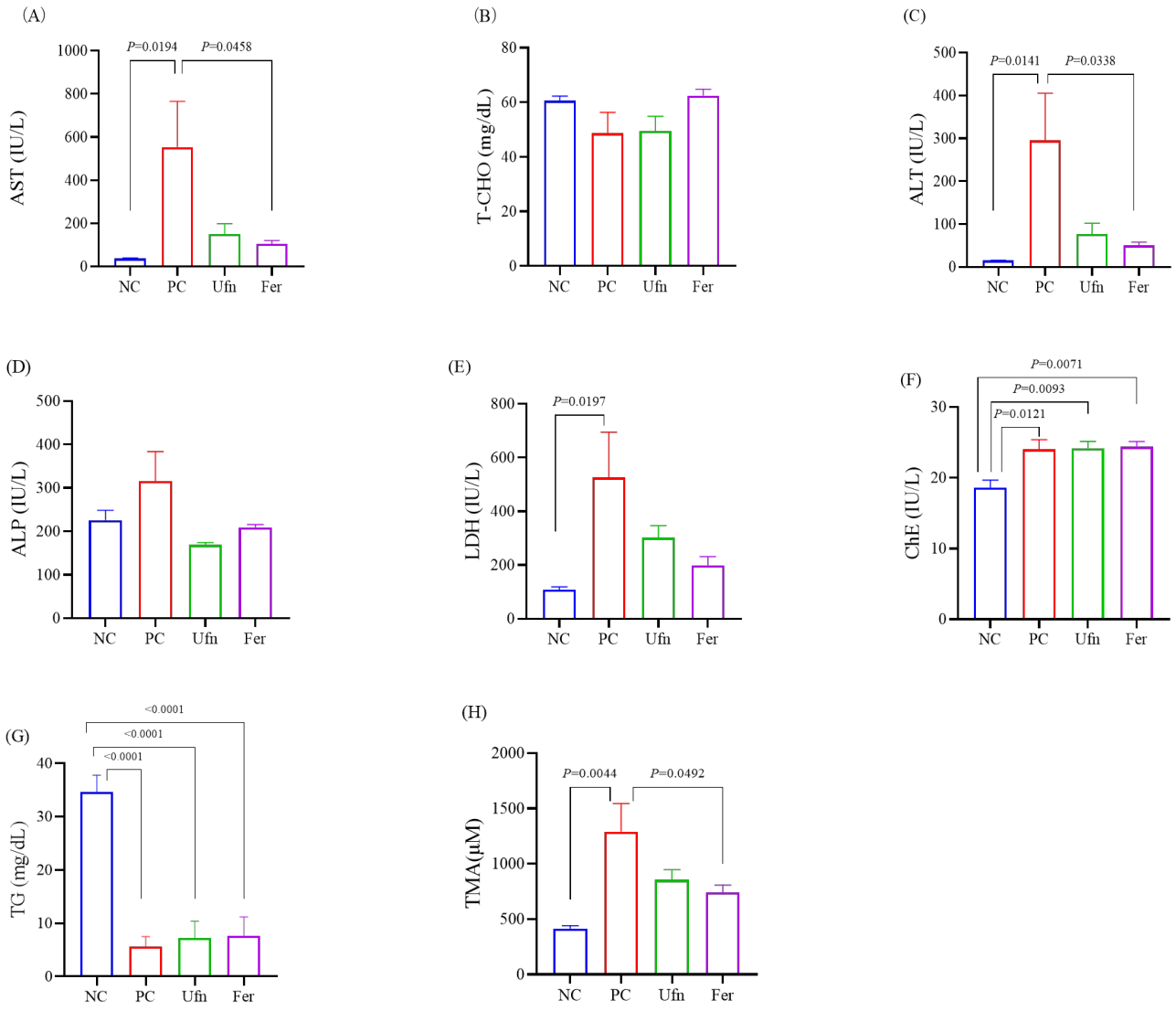

Figure 5. The serum levels of cholinesterase (AST) (A), total cholesterol (T-Cho) (B), alanine aminotransferase (ALT) (C), alkaline phosphatase (ALP) (D), lactate dehydrogenase (LDH) (E), cholinesterase (ChE) (F), and triglyceride (TG) (G) in each experimental group varied, along with the latent TMA productivities of the cecum samples (H). Samples were collected from mice that consumed ethanol, including those given unfermented or fermented stevia extract (labeled Unf and Fer respectively), mice that received ethanol and no ethanol (NC), and mice fed ethanol and PC. The information is displayed as the average ± S.E. (n = 5). The Tukey–Kramer multiple comparison test was used for statistical analysis.

3.4. Alterations of Cecal Microbiota

In this experiment, alcohol intake led to a decrease in Deltaproteobacteria but an increase in Gammaproteobacteria, and overall, the abundance of the phylum Proteobacteria was increased (Figure 6). Additionally, the cecal microbiota of the alcohol-poisoning model mice that were given the samples also were subjected to comparison summaries (as shown in Figure 7 and Table 3). Following the administration of ethanol, our focus was on the groups that exhibited an amplified (became discernible or multiplied by at least four times) or diminished (became undetectable or decreased by at least four times) response. The use of ethanol greatly decreased the prevalence of various genera (*Allobaculum*, *Sutterella*, *Lactobacillus*, *Adlercreutzia*, *Anaerofustis*, and *Prevotella*); however, among them, only *Adlercreutzia* was observed to be recovered by consuming either fermented (Fer) or unfermented (Unf) stevia extracts. Conversely, whereas ethanol feeding significantly elevated the ratios of the genera *Escherichia*, *Bacteroides*, *Enterococcus*, *SMB53* (putative), and *Dorea*, these effects vanished when fermented stevia extract was fed. The extracts failed to recover the heightened amounts of *Lactococcus*, *Clostridium*, and *Staphylococcus* that were also observed in the ethanol-fed group (PC). It was not restored in the Fer group, unlike the Unf group, except in the *Streptococcus* genus, which also thrived in the PC group. Additionally, the alteration in *Bacteroides* was also improved by the unfermented stevia extract.

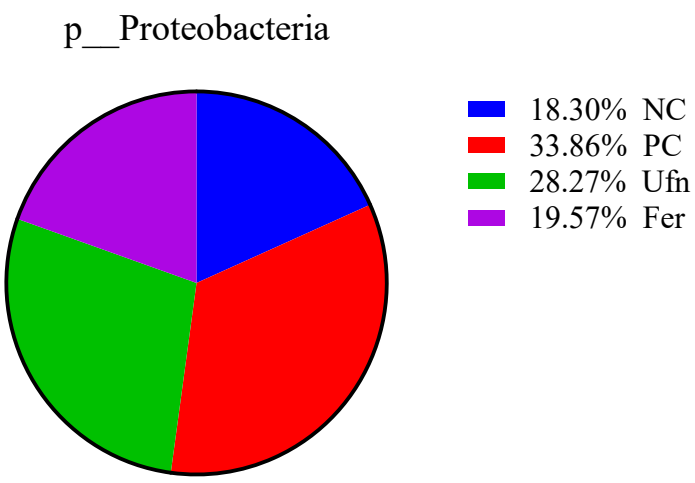

Figure 6. The percentage of the total bacterial population for which the phylum Proteobacteria accounts.

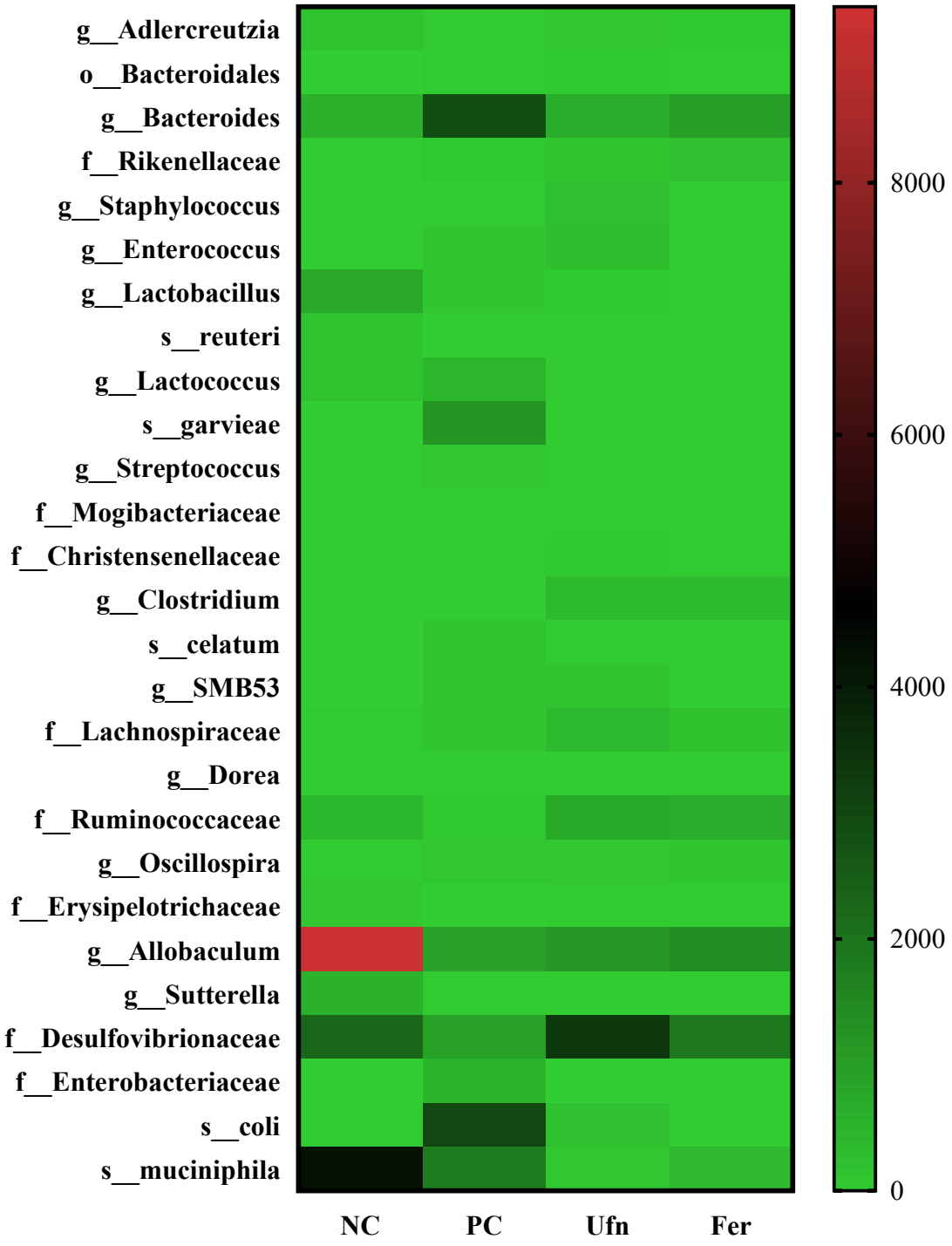

Figure 7. To identify the cecal microbiota in each group, the normalized relative abundance ratio was determined using the V3–V4 region of 16S rDNA. Areas of deeper red and green, respectively, denote greater and lower levels. The experimental design for each group is identical to that shown in Figure 5.

Table 3. A synopsis of compositional changes in the cecal microbiota.

	Phylum	Restore	Class	Restore	Oder	Restore	Family	Restore	Genus	Restore	Species	Restore
		Fer Unf		Fer Unf		Fer Unf		Fer Unf		Fer Unf		Fer Unf
Increased			Gammaproteobacteria	†	Enterobacteriales	†	Enterobacteriaceae	†	*Escherichia*	†	*Escherichia coli*	†
			Bacilli		Bacillales		Enterococcaceae	†	*Bacteroides*	† †	*Mucispirillum schaedleri*	
							Streptococcaceae		*Enterococcus*	†	*Lactococcus garvieae*	†
							Clostridiaceae		*Lactococcus*			
							Staphylococcaceae		*Clostridium*			
							Rikenellaceae		*Staphylococcus*			
							Bacteroidaceae	† †	*SMB53*	†		
							Mogibacteriaceae	†	*Streptococcus*	†		
									Dorea	†		
Decreased	Actinobacteria	† †	Erysipelotrichi		Erysipelotrichales		Erysipelotrichaceae		*Allobaculum*		*Akkermansia muciniphila*	
			Verrucomicrobiae		Burkholderiales		Alcaligenaceae		*Sutterella*		*Lactobacillus reuteri*	
			Betaproteobacteria		Coriobacteriales	† †	Lactobacillaceae		*Lactobacillus*			
			Coriobacteriia				Desulfovibrionaceae	† †	*Adlercreutzia*	† †		
			Deltaproteobacteria	† †			Coriobacteriaceae	† †	*Anaerofustis*			
							Christensenellaceae	† †	*Prevotella*			
							Eubacteriaceae					
							Prevotellaceae					

Comparing the ethanol-fed bacteria group to the non-ethanol-diet control group revealed changes in the proportions of the bacteria belonging to the groups as shown in the table.

The term *increased* pertains to the categories that became detectable or experienced an increase fourfold or greater only after ethanol consumption.

The term *decreased* pertains to the categories that, following the consumption of ethanol, either became undetectable or decreased by a factor of four or greater.

The proportions denoted by the term *restore*, indicated by a dagger (†), are achieved by the utilization of fermented (Fer) or unfermented (Unf) stevia extract.

Discussion

Our previous study demonstrated that in mice showing fatal symptoms of alcohol poisoning, the presence of live LAB cells (specifically the SN13T strain) plays a crucial role in rebalancing the gut microbiota and safeguarding the host against dysbiosis [47]. Model mice with disrupted cecal microbiota may lead to the production of TMA, which is produced from choline in the gut flora and is crucial for maintaining brain and nervous system activities in addition to the structural integrity of the body [55]. TMAO is enzymatically generated from TMA in the liver and is a well-known risk indicator for several chronic illnesses [19,56–58]. Unbalanced gut flora may accelerate TMA bioconversion from choline, resulting in choline insufficiency and the following significant risk of hepatic inflammation. Additionally, the acceleration of serum TMAO also induces liver damage and hepatic steatosis [55,59–61]. Our findings suggest that such latent dangers are caused by cecal microbiota suffering from ethanol-induced dysbiosis.

The present study has shown that LY45-fermented stevia extract can effectively inhibit TMA production in cecal microbiota cultivation, as observed in both laboratory and living organism experiments, with statistical significance ($p < 0.05$). Interestingly, while unfermented stevia extract demonstrated some success in restoring AST, ALT, and TMA levels in animal experiments, an *in vitro* test indicated that the identical unfermented extract notably amplified latent TMA production (Figure 2). The decrease in TMA production may be caused by the digestion, breakdown, and metabolic reactions of the constituents in the unfermented stevia extract during gastrointestinal processing. We also observed an improvement in hepatic indices (AST and ALT) in the unfermented stevia extract through *in vivo* experiments. This observation may be due to the fact that stevia-derived glycosides are degraded to steviol during the digestion process, and the absorbed steviol is partially bio-converted into steviol glucuronide, which has a positive detoxifying impact on the liver[8]. The improvement effects observed were not due to substances produced by the LY45 strain itself, but rather to the metabolites obtained from the components present in the stevia extract produced by microorganisms during the process of fermentation/metabolism. On the other hand, through *in vitro* analysis, the fermented stevia extract was also found to be effective in preventing TMA formation. This implies that the LY-45 strain can convert stevia glycosides to active

components in addition to steviol. This demonstrates the effectiveness of the LY-45 strain throughout the fermentation process; however, our results do not show an intrinsic probiotic effect on the LY-45 strain, since the direct effect on the intake of the LY-45 strain was not tested using the same model mice.

Recent studies have shown that the concept of a brain–gut axis is a promising avenue for treating Alzheimer's disease (AD). Restoring the proper balance of gut flora has the potential to ameliorate central nervous system dementia. However, the neuroprotective processes resulting from gut flora have not been completely understood due to the intricacy of brain–gut axis mechanisms. TMAO, which is a microbiota-dependent metabolite, has been reported to be a causative factor of a number of disorders, including type II diabetes, atherosclerosis, and thrombosis. Because AD is associated with all of these disorders, we have presumed that TMAO and AD are correlated with each other. The process of producing TMAO has been linked to certain groups of microorganisms, including Firmicutes, Ruminococcaceae, *Escherichia*, *Bifidobacterium*, and *Akkermansia*. The formation of oxidized TMA (TMAO) also correlates with high choline consumption and insulin resistance. Elevated plasma TMAO levels can lead to the penetration of this compound into the central nervous system, consequently causing immune responses and subsequent neuroinflammation, which disrupts the integrity of the blood–brain barrier. TMAO has been demonstrated to regulate the signaling pathway of TXNIP-NLPR3, PERK/Akt/mTOR, Sirtuin1/p53/p21/Rb, NLRP3/ASC/caspase1, PERK/eIF2α/ER-stress, and SIRT3-SOD2-mtROS. TMAO can also enhance the production of Aβ and the hyperphosphorylation of tau proteins and triggers apoptosis, inflammation, endoplasmic reticulum stress, and ROS [62]. Therefore, for the development of novel medications for the above-mentioned disorders, inhibiting the action of TMA, which is a crucial marker for TMAO formation, is very promising.

The conversion of choline to TMA is greatly influenced by the presence of gut microbiota. One way of modifying the gut flora ratio is decreasing the quantity of gut bacteria that can produce TMA, and the purpose seems to be achieved by the fermented stevia extract. The liver converts TMA into TMAO. The elevated liver-associated levels of AST and ALT seen in mice given alcohol indicate some liver damage, despite not quantifying the amount of TMAO in the bloodstream directly. Our results indicate that the liver receives benefits from steviol and its metabolites, such as glucuronide; thus, we hypothesize that the

blood TMAO concentration was likewise decreased, followed by stopping the development of all linked illnesses. Figure 4 showed the differences in TMA generation between the pre-fermentation and post-fermentation stages of Glycyrrhizae, Saussureae, and stevia extracts. Among them, (1) the presence of glycyrrhizic acid in Glycyrrhizae Radix may complicate therapeutic uses in the future; (2) the primary active ingredients of Saussureae Radix are volatile oil, coumarin, and chromone; and (3) investigations into the pharmacological action of stevia have mostly examined its antipyretic, analgesic, and anti-inflammatory characteristics. Therefore, stevia was chosen as a candidate because we hypothesized that Saussureae Radix has minimal bearing on enterohepatic metabolic disorders.

The bioactive compounds in herbal remedies may be converted by the gut microbiota, and the therapeutic effects of plant extracts can be influenced by the makeup of the microbiota. Recent findings have indicated that the makeup of gut microbiota plays a crucial role in determining the efficacy of immune checkpoint inhibitors that specifically target programmed cell death protein 1 (PD-1), such as the monoclonal antibody Nivolumab, in combating cancer. In this case, the effectiveness is anticipated to be dependent on the increase in the proportion of Th1 cells as compared to Treg cells and tumor-specific CD8+ T cells, which is influenced by various bacterial species [63].

Since the animal experiment in this study was conducted on inbred SPF mice, intestinal conditions of all mice were quite alike. The mice that were given ethanol performed positively when exposed to both the unfermented stevia extract and the fermented one. Nevertheless, if the resident gut bacteria, which have the ability to convert the precursor into an active form, are not present, symptoms may worsen instead of having no impact whatsoever. The use of safe microorganisms, such as probiotics including LABs, in fermenting natural products or herbal medicinal precursors is expected to be a promising method for preventing problems caused by individual variations in response to medicinal compounds that necessitate the bioconversion process.

Lately, there has been a great deal of attention given to Firmicutes and Bacteroidetes, the predominant bacterial phyla in the gastrointestinal tract. SCFA synthesis of Gram-positive bacteria from the phylum Firmicutes is crucial for the nourishment and metabolic processes of their hosts. Firmicutes bacteria can control appetite and fullness by indirect interaction with other tissues and organs through their metabolites.

On the other hand, Bacteroidetes bacteria are associated with immunological control. By stimulating the production of cytokines, Bacteroidetes LPSs and flagellin interact with cellular receptors to improve immune responses. An increase or decrease in the Firmicutes/Bacteriophages (F/B) ratio is considered an ecological disorder, with the former being commonly linked to obesity and the latter to inflammatory bowel disease (IBD). The ratio is widely acknowledged to have a substantial influence on the maintenance of a healthy balance in the gut. Nutritional and immunosuppressive qualities of probiotics, which are defined as living microorganisms, can help host health if taken in sufficient quantities. This fact is well supported by research, showing how probiotics relate to F/B ratios, obesity, and IBD. Probiotics can help restore dysbiosis and protect against obesity and IBD. However, it is crucial to choose the right strain or combination of probiotics, as they vary in their impact on the F/B ratio[64]. Although there is no statistical significance, we also found some changes in the F/B ratio in our results. The concept of the F/B ratio is widely debated and has some association with physical well-being, although there is no apparent link between the ratio and particular disorders. Furthermore, the importance of a particular group of genus and species for health needs to be better defined. An ecologically dysfunctional or unstable gut microbial community structure is indicated by the elevated abundance of Proteobacteria in the gut. Besides external enteropathogenic Proteobacteria, the natural flora of a healthy mammalian gut also consists of various commensal bacteria from this phylum. These bacteria appear to be benign when in small proportions, whereas in certain intestinal environments they are capable of triggering inflammatory responses or even metabolic disorders. Nevertheless, the long-term enrichment of Proteobacteria in the gastrointestinal tract could indicate an imbalanced and precarious structure of the microbial community or a diseased condition of the host. Hence, employing time-based monitoring instead of conducting cross-sectional studies could potentially provide a more accurate assessment of disease risk by considering the proportion of Proteobacteria in the gastrointestinal tract.

In a healthy gut, the immune system tightly regulates its response to maintain a symbiotic relationship with commensal bacteria. The presence of a positive feedback loop is indicated by this potential. Environmental or host factors (e.g., selective disruptions of homeostasis caused by low-fiber diets and acute or chronic inflammation) result in ecological dysregulation of the abundant population of

Proteobacteria in the gastrointestinal tract. Host inability to regulate the growth of Proteobacteria can lead to uncontrolled proliferation, which, in turn, can make it easier for inflammatory or external pathogens to invade. This is especially true when the microbial community is a small fraction and has reduced resistance to colonization[65]. Hence, approaches to disrupting feedback loops might involve enhancing the collaboration between the gut microbiota and the host. Given that most studies have described the microbial community status in the context of its relevance to host physiology for future inflammatory and metabolic interventions, there is a need to first attend to the abundance of Proteobacteria to develop effective therapeutic approaches.

Unlike an earlier study, which suggested that the probiotic effect of live SN13T cells may ameliorate dysbiosis[47], the stevia extract fermented with LY45 possessed efficacy without live cells. This implies that gut microbiota enhancement may be influenced by various substances apart from viable probiotic cells. Indeed, the results of this investigation demonstrated that giving fermented stevia extract to the alcohol-fed model mice decreased the number of *Escherichia* and *Enterococcus* genera. The most common species in the genus *Escherichia*, non-pathogenic *E. coli*, is known to be a component of the resident gut microbiota that helps healthy adult humans produce vitamin K and defend against external infections [66]. *E. coli*, the most widely studied indole-synthesizing organism, can be used in a model system to understand a variety of regulatory mechanisms (repression, transcriptional attenuation, and feedback repression). Indoles play a positive role in promoting human well-being by regulating the gut barrier, supporting intestinal balance through immune cell activation that triggers the release of anti-inflammatory substances like IL-22 [67]. Additionally, they hinder the growth of harmful bacteria and boost the production of mucins, thereby strengthening the protective function of the mucus barrier. In addition, indoles also play a key role in regulating the intestinal microecology. By impeding detrimental types of bacteria and modifying the severity of intestinal pathogenic bacteria, they impact gene expression, ultimately mitigating conditions like hemorrhagic colitis. In the meantime, indole compounds and their variations play a vital role in the activation of aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR)-mediated pathways that have anti-inflammatory effects [68]. Inflammation is regulated by indole-3-propionic acid through the downregulation of enterocyte tumor necrosis factor-α (TNF-α) using PXR,

while also upregulating mRNA that encodes growth inhibitors to control intestinal permeability and intestinal barrier function. However, indoles are also two-sided coins, and some derivatives have specific disadvantages. For example, indole sulfate produced by the hepatic metabolism of indoles is nephrotoxic and cardiovascular toxic at high concentrations and can lead to multi-system dysfunction by promoting pathological changes such as oxidative stress and inflammation [69]. Different concentrations of indoles exert different physiological functions. Indoles have a vital function in maintaining gut balance and promoting human well-being. Numerous gut bacteria have been recognized for their ability to convert tryptophan into indoles through metabolism. However, it is possible that some bacteria still have not been identified. Therefore, in the future, the utilization of metabolomics and macroeconomics will be necessary to gain a deeper understanding of indole-producing bacteria that are currently unknown, as well as their related metabolic pathways. Although symbiotic *E. coli* is generally non-pathogenic, excessive increases may lead to an increase in indoles, which are beneficial in certain amounts; however, in excess, some indoles can also be toxic to the gastrointestinal, nervous, and cardiovascular systems.

Furthermore, it is acknowledged that the genus *Enterococcus* is a common element of the inherent microbiome, and certain strains of *Enterococci* have been utilized as probiotic treatments for diverse ailments [66]. Due to the various autotrophies of LAB strains, it is challenging to anticipate whether sugars, vitamins, and minerals, which can stimulate the proliferation of countless bacteria, would persist post-fermentation. Ongoing research aims to elucidate the substances that enhance the observed circumstances [70]. Thus, some compounds generated during fermentation could aid in the regeneration of the gut microbiota.

Fermented stevia extract also corrected the elevated changes in *Dorea* and genus putative *SMB53* (family Clostridiaceae) that were detected from the microbiota analysis. Recent findings have unveiled the connection between the *SMB53* category and levels of TMA and TMAO, as well as their correlation with obesity, type II diabetes, and hepatic steatosis [71–74]. Moreover, this group has been identified as a pro-inflammatory entity within the gastrointestinal system. Furthermore, the *SMB53* genus is more prevalent in *Trim28*$^{hep-/-}$ mice, which is an epigenetic instability model that bears hepatocellular cancer [75]. Although animal experiments have not been conducted on those disorders, it is expected that

fermented stevia extract, which enhances TMA generation, will be beneficial for inflammatory disorders related to the *SMB53* genus. Reduced gut *SMB53* levels in patients with Alzheimer's disease have been reported. In our mouse experiment, the abundance of *SMB53* was elevated, accompanied by an increase in alcohol consumption, but the fermented stevia extract restored it to normal. We hypothesize that fermented stevia extract can prevent or treat Alzheimer's disease by controlling the quantity of *SMB53* and maintaining normal levels.

Cirrhosis and fatty liver disease [76,77] may be linked to two species, namely *Dorea longicatena* and *Dorea formicigenerans* [78,79]; furthermore, different stages of obese non-alcoholic fatty liver disease (NAFLD) have demonstrated a correlation with diverse *Dorea* species [80,81]. Therefore, more investigation into the identification of those *Dorea* species will provide fresh perspectives on the management of intestinal dysbiosis.

A significant pathogen in aquaculture, *Lactococcus garvieae*, often causes outbreaks and negatively impacts productivity. This species is uncommon and has little ability to infect humans. There is conflicting evidence about the connection between human diseases and aquaculture outbreaks [82]. However, due to the physical similarities of *Lactococcus garvieae* to other genera, it is readily ignored. The current results also showed an increase in *Lactococcus garvieae*, which was reduced by the fermented stevia extract. Mogibacteriaceae have been reported to increase in the intestines of mice on a high-fat diet [83] and may be associated with metabolic disease, which was only ameliorated by fermented stevia extract. The genus *Adlercreutzia*, which decreased in the group fed with alcohol, had its abundance restored by both fermented and unfermented stevia extract. Studies have shown a decrease in the abundance of *Adlercreutzia equolifaciens* in patients with liver disease and ulcerative colitis [84]. Therefore, it can be inferred that *Adlercreutzia* plays a significant role in maintaining a healthy state and is associated with anti-inflammatory effects. Regardless of fermentation, stevia extract also restored the altered abundance of Coriobacteriaceae and Christensenellaceae caused by ethanol feeding. Important metabolic processes, such as the conversion of bile acid, steroids, and phytoestrogens, are known to be carried out by the Coriobacteriaceae family, which has been studied in relation to metabolic disorders [85]. The family Coriobacteriaceae is thought to have a role in a number of biological host activities, including lipid and

bile acid metabolism, glucose homeostasis, and bile acid homeostasis. This suggests that there may be advantages to the increasing presence of the family Coriobacteriaceae. Christensenellaceae is negatively correlated with BMI and metabolic syndrome and is reduced in patients with IBD [86]. However, it is increased in patients with Parkinson's syndrome and multiple sclerosis. It is closely related to host health and is used therapeutically to improve health by regulating flora.

Conclusion

This study has shown that stevia leaf extract can be fermented by *Pediococcus pentosaceus* LY45, which can improve blood ALT and AST levels as well as the latent TMA production of cecal content. Mice given ethanol showed substantial improvement in these parameters. Our findings imply that stevia extract fermented with LY45 might be valuable for creating substrates that rebuild the gut flora. Regretfully, the types of substrates that were created during the fermentation of the extract remain unknown. Our findings also suggest that fermenting a medicinal plant with LABs has a great deal of potential to produce beneficial chemicals that can prevent and treat gut dysbiosis.

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Abbreviations

TMAO	trimethylamine *N*-oxide
TMA	trimethylamine
LAB	lactic acid bacteria
ADH	alcohol dehydrogenase
SCFA	short-chain fatty acid
GABA	gamma-aminobutyric acid
FMO3	flavin monooxygenase 3
LPS	lipopolysaccharide
ALD	alcoholic liver disease
LBP	lipopolysaccharide-binding proteins
TLR	Toll-like receptors
NF-κB	nuclear factor-kappa B
ROS	reactive oxygen species
AMPK	activated protein kinase
ACC	Acetyl-CoA carboxylase
SREBP1	Sterol regulatory element-binding protein
PPARα	Peroxisome proliferator-activated receptor alpha
SCD1	stearoyl coenzyme A desaturase 1
EFSA	European Food Safety Authority
T_{reg}	T-cell
SPF	specific-pathogen-free
ChE	cholinesterase
LDH	lactate dehydrogenase
AST	aspartate aminotransferase
ALT	alanine aminotransferase
ALP	alkaline phosphatase
T-Cho	total cholesterol
AD	Alzheimer's disease
F/B	Firmicutes/Bacteriophages ratio
IBD	inflammatory bowel disease
TXNIP	thioredoxin-interacting protein
NLRP3	NLR-family pyrin domain-containing protein 3
ASC	Apoptosis-associated speck-like protein containing a caspase-recruitment domain
PERK	protein kinase RNA-like endoplasmic reticulum kinase
eIF2α	Eukaryotic Initiation Factor 2 alpha
ER	Endoplasmic Reticulum

Rb	retinoblastoma protein
mtROS	Mitochondrial ROS
SOD2	superoxide dismutase 2
PERK	Protein kinase R-like endoplasmic reticulum kinase
mTOR	mammalian target of rapamycin
Akt	Protein kinase B (PKB)

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