Intake of *Bifidobacterium lactis* Probio-M8 fermented milk protects against alcoholic liver disease

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ABSTRACT

Alcoholic liver disease (ALD) is a liver disease caused by long-term heavy drinking, which is characterized by increased inflammation and oxidative stress in the liver and gut dysbiosis. The purpose of this study was to investigate the protective effect of administering ordinary and probiotic- (containing the *Bifidobacterium animalis* ssp. *lactis* Probio-M8 strain; M8) fermented milk to rats. Several biochemical parameters and the fecal metagenomes were monitored before (d 0) and after (d 42) the intervention. Our results confirmed that alcohol could cause significant changes in the liver levels of the proinflammatory cytokine IL-1 β , antioxidation indicators, and liver function-related indicators; meanwhile, the gut bacterial and viral microbiota were disrupted with significant reduction in microbial diversity and richness. Feeding the rats with Probio-M8-fermented milk effectively maintained the gut microbiota stability, reduced liver inflammation and oxidative stress, and mitigated liver damages in ALD. Moreover, the Probio-M8-fermented milk reversed alcohol-induced dysbiosis by restoring the gut microbiota diversity, richness, and composition. Four predicted fecal metabolites (inositol, tryptophan, cortisol, and vitamin K2) increased after the intervention, which might help regulate liver metabolism and alleviate ALD-related symptoms. In short, our data supported that consuming Probio-M8-fermented milk effectively mitigated ALD. The protective effect against ALD could be related to changes in the gut microbiome after probiotic-fermented milk consumption. However, such observation and the causal relationship among probiotic milk consumption, changes in gut microbiome, and disease alleviation would still need to be further confirmed. Nevertheless,

this study has shown in a rat model that consuming probiotic-fermented milk could protect against ALD.

Key words: alcoholic liver disease, probiotics, metagenomics, bacteriophage, gut microbiota

INTRODUCTION

Alcoholic liver disease (ALD) is considered to be one of the leading causes of liver cirrhosis and liver-related death all over the world (Louvet and Mathurin, 2015). The disease has gradually become a common cause that seriously threatens human health (Stärkel et al., 2018). At present, there is no known treatment that can inhibit the progression of liver disease to liver cirrhosis in heavy drinkers, and abstinence from alcohol in heavy drinkers does not totally resolve previously done liver damage and related diseases (Gao and Bataller, 2011). A previous clinical study showed that multivitamins and thiamine were effective for preventing chronic alcohol abuse, but they had no effect on liver cirrhosis (Bruha et al., 2012). It was reported that ALD could be treated by inhibiting tumor necrosis factor (TNF)- α , antioxidant therapy, and stimulating liver regeneration, but the overall effect was not satisfactory, and the treatment was costly (Liu et al., 2021).

Alcohol intake was found to disrupt colonic homeostasis and cause gut dysbiosis (Dubinkina et al., 2017; Wang et al., 2020). It can also damage the shielding function and the integrity of the gastrointestinal tract (Li et al., 2021). At the same time, the intake of alcohol can lead to a decrease in the number of bile acid-producing bacteria in the intestine. Interactions between the host liver and gut microorganisms are known to play determining roles in the outcome of ALD treatment (Bajaj, 2019). As an important immune organ of the body, the liver has the ability to activate immune cells in response to gut metabolites and pathogen-derived signals, thereby regulating the secretion of bile acids and the inflammatory responses of the liver (Ma et al., 2018). As one of the main causes of liver injury,

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alcohol not only damages the liver directly, but also promotes the entry of inflammatory cytokines and endotoxins into the liver, which injures the liver indirectly (Xiao et al., 2019).

An in-depth study of the gut microbiota has suggested that the gut-liver axis is the main pathway for the onset and development of ALD, and that the gut-liver axis regulates gut immune responses, intestinal barrier function, and liver and systemic inflammation (Szabo, 2015). Thus, the gut microbiota may be a promising target for the treatment of ALD. Li et al. (2020) used berberine to regulate gut microbes to induce the expansion of immune cells to combat ALD. Ming et al. (2020) found that camel milk could alleviate the gut dysbiosis caused by acute alcohol, thereby alleviating liver inflammation. Ferrere et al. (2017) found that gut microbes raised the sensitivity of alcohol-fed mice to ALD, and fecal microbiota transplantation or intake of prebiotics could prevent alcohol-induced liver damage in mice with ALD via regulating the gut microbiota structure.

Bifidobacterium animalis ssp. *lactis* Probio-M8 (hereinafter referred to as Probio-M8) is a probiotic strain isolated from the breast milk of a healthy mother (Liu et al., 2020). Cao et al. (2021) found that Probio-M8 could alleviate Alzheimer's disease symptoms in APP/PS1 mice via gut microbiota regulation. Xia et al. (2021) found that mixed probiotics (containing Probio-M8) could improve muscle inflammation and antioxidation induced by overtraining through LPS-TLR4/NF- κ B signal pathway. Yogurt is a popular drink worldwide, and habitual consumption of yogurt has been found to inversely associate with the prevalence of newly diagnosed nonalcoholic fatty liver disease (Zhang et al., 2020). It was reported that daily yogurt consumption reduced liver steatosis in patients with nonalcoholic fatty liver disease (Bakhshimoghaddam et al., 2018). Therefore, it might play a role in liver protection. Moreover, yogurt is a suitable food matrix for delivery of probiotic strains including Probio-M8 (Wang et al., 2021). Thus, it is worth investigating if probiotic yogurt could also alleviate ALD.

This study hypothesized that the intake of probiotic-fermented milk (versus ordinary fermented milk without exogenous supplementation of probiotic bacteria) could confer added beneficial effects in protecting against ALD via influencing the host-gut microbiota. Therefore, this study constructed an ALD model in Wistar rats by feeding the alcohol-containing Lieber-DeCarli diet. The role of probiotics in liver function protection was evaluated by a multitude of physiological, inflammatory, and biochemical parameters. Meanwhile, fecal metagenomic sequencing was performed to track changes in the fecal bacterial and viral microbiota composition, as well as

metagenomic potential before and after the trial. This study confirmed the beneficial effects of Probio-M8 in protecting the liver function and maintaining the stability of gut bacteriophage diversity in rats fed a diet with alcohol.

MATERIALS AND METHODS

Ethics Statement

All experimental procedures involving animal subjects were approved by the Ethics Committee of the Inner Mongolia Agricultural University [no. (2020)040].

Production of Fermented Milk

To ensure the stability of the fermented milks during the entire period of trial, they were all produced and supplied by Mengniu Dairy Co. Ltd. using industrial methods developed by Mengniu Dairy Co. Ltd. with regular starter strains, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, with or without applying the probiotic strain, Probio-M8 (a final concentration of 10^8 cfu/mL in the Probio-M8-fermented milk). The fermented milks were kept at 4°C after production until rat feeding.

Rats and Diet

A total of 36 male Wistar rats (8 wk old) were purchased from Beijing Vital River Laboratory Animal Technologies Co., Ltd. The rats were allowed to acclimatize in the animal facility (constant temperature between 22 and 24°C; constant humidity of 50–60%) before the trial started. Water and standard chow diet were provided ad libitum during the acclimatization period. During the trial period, the control rats were given Lieber-DeCarli diet (TP4030) without addition of ethanol, whereas rats of the noncontrol groups were fed with the complete recipe of Lieber-DeCarli diet (Guo et al., 2018). The complete Lieber-DeCarli feed (purchased from Trophic Animal Feed High-Tech Co., Ltd.) was a high-fat alcohol-containing liquid diet that contained ethanol. During the adaptation period, liquid feed with 1% ethanol was fed to the rats initially, and then the ethanol concentration was increased by 1% every day until it reached 5%. Afterward, the highest ethanol concentration was given throughout the feeding period.

Trial Design

After the acclimatization, the rats were randomized into 4 groups, which were fed (1) normal diet

(**C** group); (2) Lieber-DeCarli diet (**M** group); (3) Lieber-DeCarli diet with Probio-M8-fermented milk (**P** group); (4) Lieber-DeCarli diet with regular fermented milk (**Y** group), respectively. Each group contained 9 rats; however, one rat of the Y group died accidentally during the experimental period. For the fermented milk-receiving groups, 4 mL of fermented milk were given daily by oral gavage. The treatment lasted 6 wk. The rats were weighed weekly.

Collection and Processing of Blood and Liver Samples

Fecal samples were collected at d 0 and 42. At d 42, the rats were killed. Fecal samples were collected at 0 d and 42 d (Figure 1A). For blood collection, the rats were fasted for 16 h beforehand. After they were anesthetized, blood (about 250 μ L) was collected by retro-orbital bleeding. Collected blood samples were stored in sterile centrifuge tubes. Sera were obtained from the collected whole blood samples after centrifugation at $3,000 \times g$ for 15 min at 4°C. Serum samples were stored at -80°C until further analysis.

One day after completing the serum and stool sampling, the rats were briefly anesthetized in a glass chamber in a mixture of 5% isoflurane and ambient air before cervical dislocation. The rat liver was collected and stored at -80°C before further analysis. Liver homogenates were prepared for ELISA. Briefly, around 100 μ g of liver tissue were transferred to a new chilled tube. Pre-chilled PBS (1 mL) with 10 μ L of protease inhibitors was added. Liver tissues were homogenized at low speed for ~ 20 s on wet ice. Samples were transferred into microcentrifuge tubes and centrifuge at $14,000 \times g$ at 4°C for 15 min. The supernatants were collected for ELISA.

Analysis of Biochemical Indicators and Inflammatory Cytokines

The levels of inflammatory cytokines (TNF- α , IL-6, and IL-1 β) and antioxidation factors [glutathione peroxidase (**GSH-PX**), glutathione (**GSH**), superoxide dismutase (**SOD**), and malondialdehyde (**MDA**)] were determined by ELISA. The ELISA kits were purchased from Cloud-Clone Corp. (CCC), and the manufacturer's protocols were used. The ELISA plates were read at the wavelength of 450 nm by an ELISA plate reader (CCC). The levels of liver function [total protein, albumin, globulin, total bile acid (**TBA**), aspartate aminotransferase, alanine transaminase (**ALT**), alkaline phosphatase (**ALP**), total bilirubin (**TBIL**), direct bilirubin (**DBIL**), and indirect bilirubin (**IBIL**)] were evaluated using commercially available diagnostic

kits (Rong Sheng) using a Chemray 240 automated chemistry analyzer (Rayto).

DNA Extraction and Metagenomic Shotgun Sequencing

After thawing the fecal samples, 1 g of fecal sample of each sample was weighed and used for metagenomic DNA extraction. The metagenomic DNA was extracted with the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. The purity and quality of the extracted DNA were ensured by a NanoDrop spectrophotometer (Thermo Scientific) and agarose gel electrophoresis. The DNA libraries were prepared by using the NEBnext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). The fecal microbial metagenomic DNA libraries were used for 2×150 bp paired-end whole-metagenome sequencing using the Illumina HiSeq X Ten platform.

Bioinformatic Analysis

KneadData (ver.0.10.0) software (<https://pypi.org/project/kneaddata/#files>; McIver et al., 2018) was used for quality control to remove rat sequences (O'Connor et al., 2020).

MetaPhlan2 (ver. 2.0) was used to profile the species-level microbial taxonomy of rat fecal microbiota using default settings via the search engine Bowtie2 ver. 2.2.9 (Langmead and Salzberg, 2012; Truong et al., 2015). The high-quality reads obtained were assembled into contigs using MegaHit ver. 1.0 (Li et al., 2016). QUAST ver. 5.0.0 was used to evaluate the results of metagenomic assembling (Gurevich et al., 2013). MetaBAT2 ver. 2.12.1 (Kang et al., 2019), VAMB ver. 1.0 (Nissen et al., 2021), and Maxbin2 ver. 2.0 (Wu et al., 2016) were used to bin the assemblies using a minimum scaffold length threshold of 2,000 bp. Das tool v 1.1.2 (Sieber et al., 2018) was used to join contigs assembled by MetaBAT2, VAMB, and Maxbin2. Finally, metagenome-assembly genome (**MAG**) meeting the quality requirements (completeness $>80\%$ and contamination $<5\%$) were selected. The depth of coverage required for the binning was determined by mapping the raw reads to the genome assemblies with Bowtie2, followed by calculating the corresponding read depth of each individual scaffold with samtools ver. 1.9 (Li et al., 2009) together with the jgi_summarize_bam_contig_depths function in MetaBAT2. The completeness and contamination of each MAG were estimated with CheckM ver. 1.0.18 (Parks et al., 2015) using the lineage_wf workflow. Subsequently, high-quality MAG were selected for downstream analysis.

B

Body weight of Wistar rats

Figure 1. Animal experiment. (A) Experimental design. (B) Changes in BW of rats. Data are expressed as mean $\pm$ SD. C = control receiving Lieber-DeCarli diet without alcohol; M = model with Lieber-DeCarli alcohol diet; P = Lieber-DeCarli alcohol diet with probiotic *Bifidobacterium animalis* Probio-M8; Y = Lieber-DeCarli alcohol diet with non-Probio-M8-containing yogurt.

Figure 2. Biochemical indicators. (A) Liver function indexes in different groups (C = control receiving Lieber-DeCarli diet without alcohol; M = model with Lieber-DeCarli alcohol diet; P = Lieber-DeCarli alcohol diet with probiotic *Bifidobacterium animalis* Probio-M8; Y = Lieber-DeCarli alcohol diet with non-Probio-M8-containing yogurt). (B) Liver levels of inflammatory cytokines. (C) Liver and (D) plasma levels of antioxidant factors. TBA = total bile acid; ALT = alkaline transaminase; ALP = alkaline phosphatase; TBIL = total bilirubin; DBIL = direct bilirubin; IBIL = indirect bilirubin; GSH = superoxide dismutase; GSH-PX = glutathione peroxidase; MDA = malondialdehyde; GSH = glutathione. The upper and lower edges represent the interquartile range of the entire dataset. Midlines represent the median of the entire data set. Whiskers represent the lower 25% and upper 25% of the range of data values, excluding outliers. Dots illustrate the data values.

The taxonomy of each MAG was annotated against the National Center for Biotechnology Information (NCBI) nonredundant Nucleotide Sequence Database using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The phylogeny was built using the 400 universal PhyloPhlAn markers listed in a previous publication (Pasolli et al., 2019). Then, the MAG were clustered to define species-level genome bin (SGB) using dRep ver. 2.2.4 (Olm et al., 2017). Secondary metabolite clusters were annotated using antiSMASH 5.0 (Blin et al., 2019). Kyoto Encyclopedia of Genes and Genomes (KEGG) was annotated using eggNOG-mapper (Huerta-Cepas et al., 2017).

Vibrant v1.2.1 (Kieft et al., 2020) and Deep-virfinder v 1.0 (Ren et al., 2020) were used to identify prophages in the contigs. CD-HIT v 4.8.1 (Fu et al., 2012) was used to construct a nonredundant gene catalog. The completeness and contamination of each MAG were estimated with CheckV ver. 1.0.18 (Nayfach et al., 2021).

Nucleotide Sequence Availability

The sequence data set was deposited in the NCBI Sequence Read Archive database (project number PRJNA769784, <http://www.ncbi.nlm.nih.gov/bioproject/769784>).

Statistical Analysis

The R package vegan was used for α and β diversity analysis. The Wilcoxon rank sum test was used to measure statistical differences in α diversity, β diversity, and species between groups. R software was used to perform an unpaired Wilcoxon rank sum test to evaluate differences in plasma factors and inflammatory cytokines between groups. R package Psych was used to perform Spearman's test. Canonical correlation analysis was performed with Matlab 2016b software. Other multivariate analyses, including principal component analysis and principal coordinate analysis (PCoA) were performed with R software (v 4.0.3; <https://www.r-project.org/>). Data were visualized with R software.

RESULTS

Body Weight

The BW of rats in the C group receiving the regular diet gradually increased (Figure 1B), reaching a maximum value at d 42 (mean $\pm$ SD; 567.43 ± 31.41 g). In contrast, the BW of rats receiving the Lieber-DeCarli diet or the Lieber-DeCarli diet plus fermented milk with or without probiotic supplementation did not show an

obvious increasing trend throughout the trial period (BW ranged from 432.07 ± 31.55 to 463.18 ± 37.94 g), and no significant difference was observed in the BW among the 3 Lieber-DeCarli diet-receiving groups.

Liver or Plasma Levels of Physiological and Biochemical Indexes

No significant difference was observed in the liver levels of total protein, albumin, globulin, aspartate aminotransferase and albumin/globulin ratio across all 4 groups ($P > 0.05$; Supplemental Figure S1A; <http://dx.doi.org/10.6084/m9.figshare.17978003>). The liver levels of TBA, ALT, and TBIL were significantly lower in the C group than the M group and fermented milk-receivers ($P < 0.05$). Significantly lower levels of liver ALP, DBIL, and IBIL were detected in P group than M group ($P < 0.05$), but no significant difference was seen in these 3 parameters between the P and C groups ($P > 0.05$; Figure 2A; Supplemental Figure S1A).

The liver level of IL-1 β was significantly higher in the M group compared with the C group ($P < 0.001$) and the fermented milk-receivers ($P = 0.002, 0.008$); no significant difference was observed in the liver IL-1 β level between the nonalcohol-diet receivers and the fermented milk-receivers ($P > 0.05$). In contrast, no significant difference was observed in hepatic levels of TNF- α and IL-6 across the 4 groups (Figure 2B). These results suggested that long-term alcohol intake could stimulate individual proinflammatory factors (e.g., IL-1 β) in the rat liver, whereas consuming fermented milks with or without probiotics could alleviate alcohol-induced overshooting of IL-1 β in the liver. No significant difference was observed in the plasma levels of IL-1 β , TNF- α , and IL-6 across the 4 groups ($P > 0.05$; Supplemental Figure S1B).

The levels of liver antioxidant indexes, including SOD ($P = 0.010$ – 0.025), GSH ($P < 0.001$ in all cases), and GSH-PX ($P < 0.001$ in all cases), were significantly higher in the C group compared with the M group and the 2 fermented milk groups, which was likely a direct effect of the intake of Lieber-DeCarli diet (Figure 2C). The intake of fermented milk with or without Probio-M8 did not prevent from alcohol-induced suppression of liver antioxidant enzymes. In contrast, the levels of GSH-PX were significantly lower in the fermented milk-receivers compared with those just receiving the Lieber-DeCarli diet ($P = 0.002, 0.031$). The level of liver MDA was significantly higher in the M group compared with the nonalcohol-diet receivers and the fermented milk-receivers ($P \leq 0.001$; Figure 2C); meanwhile, the liver MDA levels of the fermented milk groups were significantly lower than that of the M group and the

nonalcohol-diet group ($P < 0.001$). Such results suggested the intake of fermented milk could significantly reduce alcohol-induced liver lipid peroxidation, as MDA was one of the end products of lipid peroxidation of biological membranes. The level of liver MDA could reflect the degree of oxidative damage of liver cells indirectly.

The plasma level of GSH of the P group was significantly higher than the C group ($P = 0.015$) and the M group ($P = 0.019$), and the mean GSH level of the Y group was numerically higher than the C group and the M group (Figure 2D). The plasma GSH-PX levels showed similar trends, except that significantly more GSH-PX were detected in the plasma of both fermented milk groups compared with the nonprobiotic-containing fermented group ($P \leq 0.001$). Moreover, the Probio-M8-containing fermented milk group had significantly more plasma GSH-PX compared with the Y group ($P < 0.001$).

Changes in Fecal Microbiota After Fermented Milk Intervention

Alcohol intake not only directly affected the liver metabolism, but also exerted a great effect on the overall fecal microbiota structure (Figure 3). The fecal microbiota diversity and richness (represented by Shannon diversity index and J index) in rats of C group and the 2 fermented milk groups increased significantly ($P < 0.05$) at d 42, but such changes were not observed in M group ($P > 0.05$; Figure 3A and B), suggesting that alcohol intake prevented the increase in the gut microbiota diversity and richness, whereas ingesting fermented milk with or without probiotics countered such effect. A PCoA (Bray-Curtis distance; Supplemental Figure S2A, <http://dx.doi.org/10.6084/m9.figshare.17978003>; Figure 3C) was performed to visualize changes in fecal microbiota structure of each group before and after treatment (d 0 and 42, respectively), and significant changes were observed in all groups ($P < 0.05$). It is worth noting that the magnitude of change in the β diversity at d 42 compared with d 0 varied between groups, with greater variations in the fecal microbiota of C group ($P < 0.001$) and P group ($P < 0.05$) compared with the M group (Supplemental Figure S2B). The fold-change in Shannon diversity index before and after the treatment was not significantly different between groups (Wilcoxon rank sum test, $P > 0.05$; Supplemental Figure S2C), but it correlated positively with the Bray-Curtis dissimilarities (Supplemental Figure S2D), indicating that a more remarkable change in the community structure tended to have a more obvious improvement in the α diversity of the microbial community. The results of canonical correlation

analysis further confirmed that there was significant change in the fecal microbiota structure in each group at d 42 compared with d 0 (Figure 3D). Moreover, the fecal microbiota of P group was more distant from M group and Y group, which clustered together, suggesting the probiotic-containing yogurt affected the rat fecal microbiota more significantly than nonprobiotic yogurt (Figure 3D).

The most prevalent species in most rat microbiome of samples collected throughout the study period were *Bacteroides massiliensis* (10.61%), *Lactobacillus reuteri* (8.23%), *Lactobacillus johnsonii* (7.62%), and *Lactobacillus murinus* (7.25%; Supplemental Figure S3A; <http://dx.doi.org/10.6084/m9.figshare.17978003>). It is worth noting that the relative abundance of *Akkermansia muciniphila* in the M group and Y group ($P < 0.05$ in both cases) but not C group and P group ($P > 0.05$ in both cases) increased significantly after the treatment (Supplemental Figure S3B). Significantly fewer *Lactobacillus reuteri* and *Bacteroides uniformis* were detected in C group than M group and Y group ($P < 0.05$ in both cases), but such differences were not seen between C group and P group ($P > 0.05$).

Changes in Fecal Virome After Fermented Milk Intervention

Then, the influence of Probio-M8-containing fermented milk on the fecal bacteriophage microbiota was evaluated (Figure 4). Alpha diversity analysis showed that continuous alcohol intake significantly decreased the Shannon index and the J index of rat fecal phages ($P < 0.05$; Figure 4A and B), whereas ingesting Probio-M8-fermented milk or nonprobiotic-containing fermented milk effectively prevented the drop in virome diversity and richness ($P < 0.05$). Indeed, except for M group, the diversity and richness of gut bacteriophages in other groups showed a nonsignificant upward trend ($P > 0.05$). However, on the PCoA score plot, no distinct grouping was seen, suggesting minor or no difference in the fecal virome structure between groups (Figure 4C).

The composition of fecal virome in rats was then analyzed. Taxonomic profiling showed that the fecal phages belonged to 10 families, which were Siphoviridae, Retroviridae, Podoviridae, Picornaviridae, Phycodnaviridae, Myoviridae, Mimiviridae, Microviridae, Inoviridae and Herelleviridae (Figure 4D). The 2 most abundant phages in our data set belonged to Siphoviridae and Myoviridae. At d 42, the average relative abundance of Retroviridae and Mimiviridae in the M group increased significantly compared with C group and the P group ($P < 0.05$), whereas the average relative abundance

Figure 3. Gut microbiota diversity in rats. (A) Shannon index. (B) J index. The upper and lower edges represent the interquartile range of the entire dataset. Midlines represent the median of the entire data set. Whiskers represent the lower 25% and upper 25% of the range of data values, excluding outliers. Dots illustrate the data values. (C) Principal component (PC) analysis based on Bray-Curtis distance. (D) Canonical correlation analysis of gut microbiota structure (Distance = Euclidean distance). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. AC or BC = control receiving Lieber-DeCarli diet without alcohol at d 0 or 42; AM and BM = model with Lieber-DeCarli alcohol diet at d 0 or 42, respectively; AP and BP = Lieber-DeCarli alcohol diet with probiotic *Bifidobacterium animalis* Probio-M8 at d 0 or 42, respectively; AY and BY = Lieber-DeCarli alcohol diet with non-Probio-M8-containing yogurt at d 0 or 42, respectively.

of Siphoviridae decreased significantly ($P < 0.05$). A screening for clustered regularly interspaced short palindromic repeats spacer sequence in the metagenome revealed matching genome of one or more phages. The number of matches between M group and C group decreased rapidly, whereas the numbers of matches of P group and Y group number were similar to that of the nonalcoholic group (Figure 4E). Procrustes analysis showed that changes in the fecal bacterial and viral microbiota were cohesive ($P = 0.001$, Figure 4F).

Correlation Between SGB With Physiological and Biochemical Indicators

The gut microbiota plays an important role in regulating inflammation and antioxidation. Thus, correlations between SGB with antioxidation factors, inflammatory cytokines, and liver function index were analyzed (Figure 5). Procrustes analysis showed that changes in SGB were cohesive to the physiological and biochemical indexes ($P = 0.001$; Figure 5A), implicat-

ing potential association between the host's gut microbiota with inflammation and antioxidation. Significant correlations were found between some dominant SGB (average relative abundance >1%) with antioxidation factors, inflammatory cytokines, and liver function index (Spearman's test, $r > 0.6$, $P < 0.05$; Figure 5B). Moreover, SGB correlated significantly and positively with most of the liver function index, plasma antioxidant factors, and plasma inflammatory cytokines (Spearman's test, $r > 0.6$, $P < 0.05$), but SGB correlated significantly and negatively associated with most of the liver antioxidant factors and liver inflammatory cytokines (Spearman's test, $r < -0.6$, $P < 0.05$). For example, *Clostridium celatum* correlated significantly and positively with most liver function indexes (e.g., ALT, TBA, DBIL; $r > 0.6$, $P < 0.05$), but correlated negatively with antioxidant factors (GSH-PH and IL-6;

$r < -0.6$, $P < 0.05$). It is worth noting that significantly fewer *Clostridium celatum* sequences were found in the P group than the M and Y groups, and its relative abundance was significantly lower in the Y group than in the M group.

To further explore how the gut microbiota affected liver function, potential fecal metabolites were predicted from the SGB genome (Figure 6). A total of 15 gut metabolites relating to the pathways of tryptophan synthesis, quinolinic acid degradation, acetate synthesis I, glycolysis (preparatory phase), and others (Figure 6A) were found. The inositol, tryptophan, cortisol, and vitamin K2-related pathways are known to regulate liver metabolism through the gut-liver axis. At the same time, significant differences were found in the relative abundance of 6 of the 22 identified SGB in this study between groups, which were Bin0004, Bin0926,

Figure 4. Diversity and composition of gut virome in rats. (A) Shannon index. (B) J index. (C) Principal component (PC) analysis based on Bray-Curtis distance. (D) Composition of major phage families in rat fecal viromes. (E) Potential phage infection of fecal bacteria. (F) Procrustes analysis of rat fecal virome and microbiota. * $P < 0.05$. C = control receiving Lieber-DeCarli diet without alcohol; M = model with Lieber-DeCarli alcohol diet; P = Lieber-DeCarli alcohol diet with probiotic *Bifidobacterium animalis* Probio-M8; Y = Lieber-DeCarli alcohol diet with non-Probio-M8-containing yogurt. BC = control receiving Lieber-DeCarli diet without alcohol at d 42; BM = model with Lieber-DeCarli alcohol diet at d 42; BP = Lieber-DeCarli alcohol diet with probiotic *Bifidobacterium animalis* Probio-M8 at d 42; BY = Lieber-DeCarli alcohol diet with non-Probio-M8-containing yogurt at d 42. Error bars represent SD.

Figure 5. Correlation between fecal microbiome and physiological and biochemical parameters. (A) Procrustes analysis of species-level genome bins (SGB) with physiological and biochemical index. (PC = principal component.) (B) Correlation between SGB with physiological and biochemical indexes (Spearman rank correlation test; $r > 0.6$, $P < 0.05$). Red solid and blue dotted lines represent positive and negative correlations, respectively. The darker the color, the stronger the correlation. Line thickness represents the magnitude of confidence level, and a thicker line represents a lower P -value. MAG = metagenome-assembly genome; TBA = total bile acid; ALT = alkaline transaminase; ALP = alkaline phosphatase; TBIL = total bilirubin; DBIL = direct bilirubin; IBIL = indirect bilirubin; SOD = superoxide dismutase; GSH-PX = glutathione peroxidase; MDA = malondialdehyde; GSH = glutathione.

Bin0933, Bin1249, Bin1870, and Bin2375 (Figure 6B). These differential abundant SGB and potential metabolites could possibly be the functional elements regulating responses in ALD.

DISCUSSION

Long-term heavy drinking can cause ALD, which is characterized by liver inflammation and imbalance of gut microbiota (Wrzosek et al., 2021). The process of alcohol metabolism and its derivatives could induce the body's inflammatory responses, oxidative stress, and nutritional imbalance (Zhu et al., 2019). In recent years, more and more studies have shown that the gut microbiota is an important participant in the occurrence and development of ALD by acting through the gut-liver axis and the gut-brain axis (Lim et al., 2017; Tripathi et al., 2018; Bajaj, 2019). Ingesting probiotics is a safe way to maintain colonic stability and restore a healthy gut microbiota (Cao et al., 2021). Therefore, this study analyzed the effect of consuming Probio-M8-fermented milk on rat gut microbiota in ALD, as well as its protective function in protecting against ALD through the gut-liver axis.

The BW of rats consuming an alcohol-containing diet was significantly lower than the control group, suggesting long-term alcohol intake would reduce nutrient absorption and protein accumulation. However, ingest-

ing fermented milk (both with and without probiotics) failed to improve the weight loss caused by alcohol consumption. Alcohol is addictive, and long-term alcohol intake might cause varying degree of health-related consequences ranging from acute poisoning to personality and behavior changes, as well as diet reduction and nutritional deficiencies (Higuera-de la Tijera et al., 2018). Alcohol intake also caused some degree of dysbiosis (characterizing by a significant decrease in the relative abundance of Bin0926 in fecal microbiota). The drastic reduction in BW gain could be a result of suppression of certain metabolic pathways (e.g., glycolysis) as the level of metabolites related to glycolysis decreased in the alcohol-diet group at d 42.

Our results also showed that alcohol exposure significantly increased the liver levels of the proinflammatory cytokine (IL-1 β), antioxidation factor (MDA), and liver function indicators (e.g., TBA, ALT, ALP, TBIL, DBIL, and IBIL), but significantly decreased the biochemical elements related to antioxidation (GSH, GSH-PX, and SOD). IL-1 β is a highly active proinflammatory factor that can activate inflammatory cells and accelerate inflammation; MDA and liver function index can reflect the degree of lipid peroxidation and liver injury, respectively. However, feeding Probio-M8-fermented milk effectively inhibited the increase of IL-1 β , ALP, DBIL, and IBIL in the liver, thereby alleviating liver inflammation and liver damage. Feeding Probio-M8-fermented

milk to the rats also significantly increased the plasma levels of GSH-PX and GSH, which may help reduce the oxidative stress response in the liver, thereby reducing alcohol damage to the liver. In summary, our results support that Probio-M8-fermented milk effectively reduced alcohol-induced liver inflammation, oxidative stress, and liver damage.

The rat fecal microbiota before and after the intervention was analyzed by metagenomics technology, and obvious differences were observed in the gut microbiota composition and diversity between groups. Ingesting Probio-M8-fermented milk or non-probiotic-containing fermented milk maintained the fecal gut microbiota diversity and richness, which were drastically reduced by alcohol consumption. Such an effect was important in maintaining colonic homeostasis, particularly avoiding gut dysbiosis. A survey of patients with ALD found that the gut level of cytolysin-positive *Enterococcus faecalis* correlated with the severity of liver disease and the mortality rate of patients with alcoholic hepatitis, possibly by reducing hemolysin in the liver and alcohol-induced

ALD (Duan et al., 2019), suggesting the gut microbiota composition is associated with the prognosis in ALD. At the same time, our results showed that after alcohol intake, the intestinal abundances of *Lactobacillus reuteri* and *Bacteroides uniformis* increased significantly. The imbalance of *Lactobacillus reuteri* A9 could lead to gut bile acid metabolism dysregulation and gut homeostasis disruption (Jiang et al., 2019). Further analysis of the fecal microbiota structure revealed that feeding Probio-M8-fermented milk or nonprobiotic-containing fermented milk could counter alcohol-induced dysbiosis and help restore a healthy gut microbiota in the rats, and such effects were more obvious in the presence of Probio-M8. Consistent with the observations of the current work, alcohol-induced gut dysbiosis has been reported in rats, and the liver damage and gut dysbiosis could be protected by feeding aplysins (Xue et al., 2017). Chen et al. (2020) applied fish oil to mitigate alcohol-induced liver injury and gut dysbiosis. These observations together supported that the application of certain health-promoting products could help protect

Figure 6. Functional metagenomic analysis of rat fecal samples at d 42. (A) Distribution of predicted gut microbiota metabolites and their related pathways. The oblong symbol indicates the presence of the corresponding pathway in a specific differential species-level genome bin (SGB); MGB = gut-brain module; MF = gut metabolic modules. (B) Six significantly differential SGB were identified. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. C = control receiving Lieber-DeCarli diet without alcohol; M = model with Lieber-DeCarli alcohol diet; P = Lieber-DeCarli alcohol diet with probiotic *Bifidobacterium animalis* Probio-M8; Y = Lieber-DeCarli alcohol diet with non-Probio-M8-containing yogurt.

from deleterious health effects caused by long-term alcohol consumption.

Our results suggested that, similar to the beneficial effect on the gut bacterial microbiota, Probio-M8-fermented milk could maintain the diversity and richness of rat fecal virome, which were also drastically reduced by alcohol consumption. Changes in the fecal virome microbiota were consistent with the variations in the fecal bacterial microbiota, as well as physiological and biochemical indicators of the host, suggesting that the gut virome and bacterial microbiota were likely rather stable under usual conditions, but they would fluctuate with external factors such as long-term alcohol intake, which could weaken the balance between the host-gut phages and bacteria interactions and decrease the stability of the gut microbiota. Duan et al. (2019) found that specific bacteriophage intervention could alleviate ALD via regulating the gut microbiota. The gut bacteriophage microbiota is believed to play a key role in shaping the gut microbial composition, driving bacterial diversity, and promoting horizontal gene transfer (Sutton and Hill, 2019). Thus, it is possible that modulating the gut virome was one of the mechanisms by which Probio-M8-fermented milk helped restore and maintain the stability of the rat gut microbiota and its metabolism.

We then analyzed changes in the predicted fecal metabolomic potentials that were related to the gut-liver axis. Our results revealed 4 potential metabolites that were closely related to liver metabolism and function, including inositol, tryptophan, cortisol, and vitamin K2. Inositol is a chemical messenger in the liver that regulates glycogen decomposition, gluconeogenesis, and pyruvate dehydrogenase activation, and it inhibits the growth of many kinds of cancers (Williamson et al., 1985). Inositol was also found to inhibit the occurrence and development of colorectal cancer and liver metastasis in BALB/c mice (Fu et al., 2016). On the other hand, vitamin K2 could block the beneficial effect of IFN- α -2b administered during early development of rat liver cancer (Vera et al., 2019). Moreover, the liver could reduce liver injury by degrading tryptophan, controlling heme synthesis and bilirubin metabolism, and producing immunomodulatory and active metabolites (Badawy, 2017). Cortisol could effectively inhibit the overshooting of IL-1 β in the liver and reduce liver inflammation (DeRijk et al., 1997). Some significant differential SGB (e.g., Bin2375, Bin1249, and B1870) possess one or a combination of the aforementioned pathways, which increased after fermented milk intervention. Our data supported that ingesting Probio-M8-fermented milk could combat ALD by regulating the gut microbiota and its metabolic pathways through the

gut-liver axis to reduce liver inflammation, oxidative stress, and liver injury.

CONCLUSIONS

This study analyzed the alleviating effect of administering Probio-M8-fermented milk in ALD rats. Our study confirmed that long-term alcohol intake could induce liver inflammation, oxidative stress, and liver damage, meanwhile destroying the gut homeostasis and causing gut dysbiosis (particularly reducing the diversity and richness of gut bacterial and viral microbiota). Administering Probio-M8-fermented milk could effectively mitigate ALD-associated symptoms and maintain gut homeostasis by restoring a healthy fecal microbiota and enhancing potential liver protective gut metabolites.

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