Fermented milk containing *Lactobacillus casei* Zhang and *Bifidobacterium animalis* ssp. *lactis* V9 alleviated constipation symptoms through regulation of intestinal microbiota, inflammation, and metabolic pathways

Jicheng Wang,*¹ Xiaoye Bai,*¹ Chuantao Peng,*¹ Zhongjie Yu,¹ Bohai Li,¹ Wenyi Zhang,¹ Zhihong Sun,¹ and Heping Zhang†¹

Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Key Laboratory of Dairy Products Processing, Ministry of Agriculture, Inner Mongolia Agricultural University, Huhhot 010018, China

ABSTRACT

Studies suggest that probiotics and fermented milk can improve defecation in constipated patients. However, the mechanism of fermented milk containing probiotics on constipation remains poorly understood. Volunteers with chronic constipation symptoms were recruited and given 200 g/d of fermented milk containing *Lactobacillus casei* Zhang and *Bifidobacterium animalis* ssp. *lactis* V9 (PFM) for 4 wk. Clinical symptoms, cytokines, metagenomics, and metabolomics were evaluated in constipated participants before and after PFM intervention. After PFM intervention, we observed significant improvement of constipation symptoms. In the serum samples, the anti-inflammatory cytokine IL-10 increased and the proinflammatory cytokine C-reactive protein and lipopolysaccharides decreased. Metagenomics results showed that the increase of *B. animalis* was correlated with an increase in defecation frequency. Fatty acid biosynthesis and bile acid biosynthesis in stool samples as well as carnitine shuttle, vitamin E metabolism, and ascorbate and aldarate metabolism were identified as significantly altered metabolic pathways. Acylcarnitine, located on the carnitine shuttle pathway, had a significantly positive correlation with defecation frequency. It was speculated that PFM may contribute to alleviating constipation symptoms through 3 potential mechanisms: fine-tuning gastrointestinal microbiota, fighting inflammation, and regulating metabolic pathways.

Key words: probiotic fermented milk, constipation, gut microbiota, metagenomics, metabolomics, inflammation

INTRODUCTION

Constipation is a common and complex gastrointestinal disorder, affecting about 15% of the world's population (Ou et al., 2019). Functional constipation can be categorized into 3 groups (Andrews and Storr, 2011): normal-transit constipation, slow-transit constipation, and defecatory or rectal evacuation disorders. Due to the complexity of the causes of constipation, different approaches have been developed to treat constipation, including diet and lifestyle intervention, laxatives, and even surgery (Rao et al., 2016; Camilleri et al., 2017).

The beneficial effects of probiotic fermented milk have been widely reported, such as elimination of pathogens (Corr et al., 2009; Piewngam et al., 2018; Piatek et al., 2019), inhibition of tumor development (Hendler and Zhang, 2018), improvement of symptoms of patients with irritable bowel syndrome (Guyonnet et al., 2007; Agrawal et al., 2009), and rapid improvement of minor digestive symptoms (Marteau et al., 2019). There are also various bioactive metabolites in fermented milk, such as bioactive peptides (Mohanty et al., 2016), conjugated linoleic acid, and exopolysaccharides, which protect the host against dysbiosis and inflammation (Fernandez and Marette, 2018; Baars et al., 2019). Fermented milk containing probiotics has also been used for constipation relief. The consumption of kefir significantly reduced constipation of patients with mental and physical disabilities, and some individuals showed complete relief of constipation (Maki et al., 2018). Daily consumption of kefir also improved bowel satisfaction scores and accelerated colonic transit (Turan et al., 2014). Ozaki et al. (2018) found that the fermented milk containing *Lactococcus lactis* ssp. *cremoris* FC is beneficial for improving defecation and fecal properties (Ozaki et al., 2018). The numbers of various beneficial fecal bacteria increased in older individuals (>60 yr old) with regular use of fermented milk containing *Lactobacillus casei* strain Shirota (Aoyagi et al., 2019). In addition, daily administration of fer-

Received April 3, 2020.

Accepted July 20, 2020.

*These authors contributed equally to this work.

†Corresponding author: hepingdd@vip.sina.com

mented milk decreased the bacteria that can cause pro-inflammatory response in healthy volunteers (Toscano et al., 2017) and enhanced the activity of natural killer cells in older individuals (Lee et al., 2017). Fermented milk containing probiotics was also reported to be beneficial for healthy adults, possibly through increasing *Bifidobacterium* level (Redondo-Useros et al., 2019). However, fermented milk containing different probiotic strains leads to different microbiota characteristics. Redondo-Useros et al. (2019) reported that consumption of *Bifidobacterium*-containing probiotic fermented milk increased *Bifidobacterium animalis* levels, and consumption of *Lactobacillus*-containing probiotic fermented milk was associated with higher levels of *Bifidobacterium longum*.

Some studies have investigated the effects of fermented milk containing probiotics on improving gut motility and constipation symptoms (Massi, 2006; Zhang et al., 2010b; Leber et al., 2012; Dimidi et al., 2019); however, the underlying mechanisms remain largely unknown. One potential mechanism may be that the metabolites from probiotics and altered gut microbiota may modulate the generation of 5-hydroxytryptamine derivatives and stimulate the enteric nervous system, which results in increased gut transit time. In addition, inflammation is known to influence gut motility and thereby constipation. Studies suggest that chronic constipation was associated with the gut permeability (Leber et al., 2012), which links gut microbiota and immune response. *Lactobacillus casei* Zhang is a well-studied strain that can increase antioxidant enzyme activity (Zhang et al., 2010b); regulate cellular, humoral, and tumor immunity (Ya et al., 2008); improve blood lipid and liver lipid metabolism (Zhong et al., 2012); prevent type 2 diabetes (Zhang et al., 2014b); protect the liver; and prevent liver damage (Wang et al., 2013). Clinical studies also reported that *Lb. casei* Zhang helped maintain gut microbiota homeostasis, increasing the beneficial bacteria and reducing the potentially harmful bacteria in the intestinal tract (Kwok et al., 2014; Zhang et al., 2014a). It can also prevent upper respiratory tract infections by regulating intestinal flora and improve bowel movements (Hor et al., 2018). Although *B. animalis* ssp. *lactis* V9 has a high tolerance for gastric and bile acids (Liu, 2009), clinical experiments showed that *B. animalis* ssp. *lactis* V9 could improve diarrhea (Xu et al., 2019b) and enhance the host immunity (Xu et al., 2019a) through regulation of the gut microbiota.

The aim of this study was to investigate the effect of fermented milk containing *Lb. casei* Zhang and *B. animalis* ssp. *lactis* V9 (PFM) on clinical symptoms, immune factors, metagenomics, and metabolomics in

constipated participants. Due to the complexity of the mechanisms underlying constipation, the connections between constipation symptoms and factors in immunity, metagenome, stool, and serum metabolome were also evaluated.

MATERIALS AND METHODS

Ethics Approval

All participants gave their informed consent for inclusion before the study. The study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the Ethical Committee of Inner Mongolia Agricultural University (Hohhot, China).

Study Participant Recruitment

We recruited volunteers, who suffered from constipation symptoms in self-test and met the diagnostic criteria for functional constipation according to the Rome III criteria (Drossman and Dumitrascu, 2006), to participate in this study. People who were allergic to milk protein, used antibiotic within 15 d, or refused to provide informed consent were excluded. All volunteers were 20 to 40 yr old and without organic constipation, neuropathic constipation, and intractable constipation. Volunteers were provided with fermented milk (200 g/d) for 28 d without a particular dietary regimen during the study. They were required to report their current defecation status every 7 d. At the baseline, all volunteers performed a basic physical examination including height (cm), weight (kg), and systolic and diastolic blood pressure.

Preparation of Probiotic Fermented Milk

The probiotic fermented milk was prepared with a standard fermentation process. Briefly, milk was exposed to 95°C for 5 min. Fermentation was started with 4 bacteria strains, including *Lactobacillus bulgaricus* ND02 (1×10^3 cfu/mL; Sun et al., 2011a), *Streptococcus thermophilus* ND03 (1×10^6 cfu/mL; Sun et al., 2011b), *Lb. casei* Zhang (1.0×10^5 cfu/mL; Zhang et al., 2010a), and *B. animalis* ssp. *lactis* V9 (4.0×10^7 cfu/mL; Sun et al., 2010), and incubated for 9 h at 37°C. The ND02 and ND03 strains were isolated from naturally fermented yak milk in Qinghai, China; Zhang is a novel probiotic strain identified and isolated from koumiss samples collected in Inner Mongolia, China; and V9 was isolated from the feces of a healthy Mon-

golian child in China. The initial pH was 6.5. The final compositions of PFM are listed in Table 1.

Questionnaire

Volunteers submitted a weekly stool diary in a self-test manner using an e-questionnaire from the baseline to the endpoint of the study to record the following defecation parameters: stool frequency, stool consistency (using the Bristol Stool Scale; **BSS**; Lewis and Heaton, 1997), incomplete sensation, unsuccessful attempts, straining, pain, and time to defecate during the 4-wk study. Except for stool frequency and stool consistency, defecation time was scored on a 5-point Likert scale (1 = <5 min, 2 = 5–10 min, 3 = 10–20 min, 4 = 20–30 min, 5 = 30 min); the other 4 indices (incomplete sensation, unsuccessful attempts, straining, and pain) were scored on a 5-point Likert scale (1 = never, 2 = moderate, 3 = severe, 4 = fairly severe, 5 = very severe).

Sample Collection

Stool and blood samples were collected before and after the study period. Immediately after the defecation of the volunteers, 10 to 15 g of feces was collected in a 50-mL enzyme-free sterile collection tube with an added appropriate amount of DNA protection solution (Takara Biomedical Technology, Beijing, China) using a sterile spoon for metagenomics analysis, and approximately 1 g of feces was collected in a 5-mL enzyme-free sterile collection tube with 3 mL of metabolite protection solution (Guangdong Longsee Biomedical Ltd., Guangzhou, China) for metabolomic analysis. The collected stool was transferred to the laboratory and stored in a refrigerator at -80°C until use.

Fasting venous blood (3–5 mL) was collected from each volunteer in the morning. Then, the blood was centrifuged at $1,238 \times g$ for 10 min at 4°C to obtain the serum, and the supernatant was carefully collected and divided into several units (100 μL). The serum samples

were frozen in liquid nitrogen, transported on dry ice, delivered to the laboratory, and stored at -80°C until use.

DNA Extraction

Bacterial DNA from 1 g of feces was extracted using the QIAamp Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality of the DNA was checked by spectrophotometric analysis (optical density ratio at 260/280 nm) using Nano-Drop One (Thermo Fisher Scientific, Waltham, MA) and agarose gel electrophoresis. All DNA samples were stored at 20°C before evaluation.

Metabolite Extraction

Each 200- μL serum sample was thawed at room temperature and placed in a 2-mL centrifuge tube; then, 800 μL of a mixture of methanol and acetonitrile (1:1 vol:vol) was added. It was vortexed for 1 min and placed at -20°C for 60 min. After centrifugation at $17,000 \times g$ at 4°C for 15 min, 500 μL of supernatant was dried with a vacuum at room temperature. Then, the dry sample was dissolved in 200 μL of 50% acetonitrile in H_2O and fully dissolved by vortex ultrasound. The dissolved samples were centrifuged again at 4°C at $17,000 \times g$ for 15 min, and 130 μL of supernatant was collected. Stool samples in a 5-mL enzyme-free sterile collection tube with 3 mL of metabolite protection solution were vortexed for 1 min. Then, 200 μL of the mixture was obtained and centrifuged at $17,000 \times g$ at 4°C for 15 min, and 130 μL of supernatant was collected. Ten microliters of clean supernatant from each serum sample or stool sample was mixed together as the quality control. Each extracted sample was passed through a 0.22- μm microporous membrane filter before analysis by ultrahigh-performance liquid chromatography electron spray ionization coupled with time-of-flight mass spectrometry and mainly used for analyzing the nonvolatile metabolites.

Cytokine Measurement

The serum samples were used for a sandwich ELISA analysis, and cytokines IL-1, IL-6, IL-8, IL-10, C-reactive protein (**CRP**), tumor necrosis factor- α , LPS-binding protein (**LBP**), LPS, cortisol, gastrin, motilin, 5-hydroxytryptamine, soluble CD14, and nuclear factor $\kappa\text{-B}$ were measured with corresponding commercially available ELISA kits (mlBio, Shanghai, China) according to the manufacturer's instructions. All assays were performed in duplicate, and final levels were the average of each pair of measurements.

Table 1. The composition of probiotic fermented milk

Ingredient	Content
<i>Lactobacillus casei</i> Zhang (cfu/mL)	$(4.13 \pm 0.03) \times 10^6$
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i> V9 (cfu/mL)	$(1.45 \pm 0.01) \times 10^8$
<i>Lactobacillus bulgaricus</i> ND02 (cfu/mL)	$(6.01 \pm 0.01) \times 10^7$
<i>Streptococcus thermophilus</i> ND03 (cfu/mL)	$(6.93 \pm 0.02) \times 10^8$
Protein (%)	3.0
Fat (%)	3.0
Carbohydrates (%)	11.5

Metagenomics

Paired-end metagenomic sequencing was performed on the Illumina HiSeq Ten platform with a read length of 150 bp. Metagenomics data were preprocessed to remove low-quality reads and contaminated reads. Cleaned reads were analyzed using Humann2 pipeline for taxonomical profiling and pathway analysis. Differential analysis was performed with 1-sided Wilcoxon rank sum test. Metagenomics data before the intake of fermented milk were treated as control and after the intake as treatment. The α -value cutoff for both the factorial Kruskal-Wallis test and the pairwise Wilcoxon test was 0.05. For Wilcoxon rank sum test, raw P -value of 0.05 was used as the cutoff. All statistical tests were performed using R (version 3.5.2; R Foundation for Statistical Computing, Vienna, Austria). Raw sequences reported in this article have been deposited in the National Center for Biotechnology Information Sequence Read Archive database under the accession number PRJNA612367.

Metabolomics

Mass spectrometry analysis with stool and serum samples was performed with Agilent 6545A QTOF (Agilent Technologies, Santa Clara, CA) in positive and negative modes separately. The raw data were preprocessed with XCMS Online (<https://www.sogou.com/link?url=hedJjaC291MICZF9uGXo0PnuRScp6ODmBVZ1xkmcoSU>). Minimum and maximum peak widths were 10 and 60 s, respectively. Signal-to-noise ratio threshold was 6. m/z diff (the m/z tolerance allowed for spectral features) was set to 0.01. Mass traces were retained if they contained at least 3 peaks with intensity ≥ 500 . The width of overlapping m/z slices was 0.015 to create peak density chromatograms and group peaks cross-samples. The results from XCMS Online were uploaded to MetaboAnalyst, and MS Peaks to Pathways module was used for pathway analysis (<https://www.metaboanalyst.ca/MetaboAnalyst/upload/PathUploadView.xhtml>). Mass accuracy was set to 10.0 ppm and P -value cutoff was set to 0.05 in mummichog implementation. The statistical analysis module was applied to do data filtering and preprocessing. Metabolite abundances were filtered by mean intensity and preprocessed by sum normalization and range scaling. Data from positive mode and negative mode were mixed and processed together.

Statistical Analysis

All statistical tests were performed using R (version 3.5.2); $0.01 < P < 0.05$ and $P < 0.01$ were considered

to be statistically significant and extremely significant, respectively. For all analysis visualized with heatmaps, R package ComplexHeatmap (2.3.1) and ggpubr (0.2) were executed. For metagenomics analysis, R package phyloseq (1.26.1) and in-house package xviz (1.0.5) were used. When investigating associations between features, Spearman correlation was performed and results were visualized with R package psych (1.8.12). Paired Wilcoxon rank sum test was performed to do differential analysis in metagenomics, metabolomics, and inflammatory factors data.

RESULTS

Twenty-six participants provided questionnaires at wk 0, including 5 men and 21 women aged 28.50 ± 5.52 yr (mean $\pm$ SD) and with a body mass index (kg/m^2) of 23.66 ± 3.72 (Supplemental Table S1, <https://doi.org/10.3168/jds.2020-18639>). Of the participants, 26 provided questionnaires at wk 3, and 16 completed the questionnaires at wk 4. Due to the dropout of participants at wk 4, we used questionnaires at wk 3 as a surrogate for constipation symptom records of wk 4 for omics data analysis. Twenty-four of the 26 participants provided stool samples at wk 0 and wk 4, and 18 of the 24 provided blood samples. Figure 1 depicts the experimental approach and design.

Alleviation of Constipation Symptoms

The participants showed diverse constipation symptoms at wk 0 (Supplemental Table S2, <https://doi.org/10.3168/jds.2020-18639>). Among the 24 participants with stool samples collected, 16.67% ($n = 4$) had a defecation frequency of <3 times/wk. About 71% of participants ($n = 17$) had no fewer than 2 unsuccessful defecatory attempts, 25% ($n = 6$) had a BSS stool consistency score (Lewis and Heaton, 1997) of 1 or 2, 79% ($n = 19$) had an incomplete sensation of defecation score above 2 (scaling from 1–5), 66.7% ($n = 16$) had a straining score above 2 (range: 1–5), 50% ($n = 12$) had a defecation pain score of more than 2 (range: 1–5), and 8% ($n = 2$) had a defecation time score of more than 3.

After the 4-wk intervention of PFM, we observed significant improvement of constipation symptoms, including defecation frequency ($P = 0.03$), incomplete defecation ($P = 1.9 \times 10^{-5}$), defecation pain ($P = 0.0012$), defecation time ($P = 0.0014$), straining during defecation ($P = 5 \times 10^{-7}$), and unsuccessful defecation attempts ($P = 0.063$; Figure 2). The proportion of BSS 4 increased from 16.00% ($n = 4$) to 69.23% ($n = 18$) after a 1-wk intervention of PFM, and afterward the proportion of BSS 5 increased (Supplemental Figure S1, <https://doi.org/10.3168/jds.2020-18639>).

Figure 1. Overview of study design: 26 participants were recruited and given probiotic fermented milk (200 g/d, 28 d). Their defecation conditions were recorded weekly. Stool samples from 24 individuals and blood samples from 18 individuals were collected at wk 0 and 4.

Alleviation of Inflammation After 4-wk Intervention of Probiotic Fermented Milk

The intake of probiotic fermented milk changed the relative abundance of a few inflammatory markers (Figure 3). We observed 2 significantly decreased inflammatory markers (CRP, $P = 0.031$; LPS, $P = 0.0023$) and 2 significantly increased cytokine markers (LBP, $P = 0.028$; IL-10, $P = 0.015$). For the remaining cytokines, the significant changes were not observed; the data are shown in Supplemental Table S3 (<https://doi.org/10.3168/jds.2020-18639>).

Modulation of Intestinal Microbiota After 4-wk Intervention of Probiotic Fermented Milk

The α -diversity between wk 0 (class A) and wk 4 (class B) groups was compared using the InvSimpson index (Figure 4A), and there was no significant difference (Wilcoxon $P = 0.695$). Also, β -diversity between wk 0 (class A) and wk 4 (class B) groups was compared using a principal component analysis (PCA) plot (Figure 4B), and there was no clear separation. Furthermore, hierarchical clustering of the microbiome profiles also showed that samples from the same individuals were clustered together using Bray Curtis distance, with the exception of participants 23 and 40 (Supplemental Figure S2, <https://doi.org/10.3168/jds.2020-18639>).

In addition, pathway analysis using humann2 identified no significant results between the pre- and postintervention groups. Nonetheless, the variation of taxa may depend on the baseline of the individuals. To confirm this, we performed a 2-sided Wilcoxon rank sum test and identified 11 significantly different species together (raw $P < 0.05$; Figure 4C). Hierarchical clustering of the identified species showed that after a 4-wk intervention of probiotic fermented milk, 6 species decreased and 5 species increased, including *Strep. thermophilus*, *B. animalis*, and *Lactobacillus acidophilus*, among which the patterns of *B. animalis* were individual specific and correlated with the improvement of defecation frequency (Pearson correlation $r = 0.45$, $P = 0.03$; Figure 4C-E; Supplemental Table S4, <https://doi.org/10.3168/jds.2020-18639>). We failed to observe significant correlations between significantly different species and the other symptoms (Figure 4D; Supplemental Table S4).

Alterations in the Stool and Blood Metabolic Pathways After 4-wk Intervention of Probiotic Fermented Milk

To identify the metabolic pathways that were significantly altered after a 4-wk intervention of probiotic fermented milk, we performed a metabolic pathway analysis using MetaboAnalyst, a web-based tool for

Figure 2. Changes in constipation symptoms, including frequency, incomplete defecation sensation, pain, straining, time, and unsuccessful attempts, were reported from wk 0 to 4; ns indicates $P > 0.05$; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$. Each dot represents 1 individual; each box numbered 0, 1, 2, 3, and 4 represents wk 0, 1, 2, 3, and 4, respectively. In each box, the middle line denotes the median, the upper line denotes the third quartile, and the lower line denotes the first quartile.

Figure 3. Differential analysis of inflammatory cytokines from pre- and postintervention groups. Comparisons show 4 selected cytokines between wk 0 (class A) and wk 4 (class B). In each box, the middle line denotes the median, the upper line denotes the third quartile, and the lower line denotes the first quartile. Dots denote outliers. LBP = LPS-binding protein; CRP = C-reactive protein.

metabolomic data interpretation (Xia et al., 2012). In contrast to the metagenomics profile, intake of PFM dramatically changed the stool metabolomics profile, and the PCA of the stool metabolic profile provided a clear difference between the pre- and postintervention groups (Figure 5A). Then, we performed pathway analysis using the mummichog algorithm (Li et al., 2013) included in MetaboAnalyst (Chong et al., 2018), which takes into consideration both the number of detected metabolic features in individual pathways and their alterations between cases and controls. We identified de novo fatty acid biosynthesis and vitamin E metabolism as 2 metabolic pathways of interest, as both exhibited lower *P*-values and greater pathway effect (Figure 5B).

Similar to what had been observed in the stool metabolome, PCA using serum metabolomics data also discerned postintervention samples from preintervention ones (Figure 6A). Five pathways with high significance and high pathway effect were identified and included bile acid biosynthesis, xenobiotics metabolism, ascorbate (vitamin C) and aldarate metabolism, carnitine shuttle, and tryptophan metabolism (Figure 6B). More detailed results of pathway analysis are listed in Supplemental Table S5 (<https://doi.org/10.3168/jds.2020-18639>), and significantly different metabolites from the corresponding metabolic pathway were identified.

Furthermore, the correlation between constipation symptoms and significantly different metabolites was analyzed. The result showed that acylcarnitine, located on the carnitine shuttle pathway, had a significantly positive correlation with defecation frequency as shown in Figure 7; significant correlations between other significantly different metabolites and constipation symptoms were not observed.

DISCUSSION

Improvement of Defecation Frequency via Fine-Tuning the Intestinal Microbiota Composition After Intake of PFM

Studies reported the difference of gut microbiome composition between healthy controls and constipated patients (Ohkusa et al., 2019). The gut microbiota is thought to be an important factor associated with gut motility (Pozuelo et al., 2015), and regulation of the gut microbiota by probiotics can improve bowel movement frequency in up to 70% of constipated patients, which

Figure 4. Diversity analysis of metagenomics samples from pre- and postintervention groups. (A) Comparison of α -diversity between wk 0 (class A) and wk 4 (class B) groups; the α -diversity index presented is inverse Simpson. (B) Comparison of β -diversity between wk 0 (class A) and wk 4 (class B) groups. PC = principal component. (C) Species detected as significantly differently abundant between wk 0 and wk 4 by Wilcoxon rank sum test ($P < 0.05$). (D) Pearson correlations between change of 2 specific species (wk 0–4) and improvements in constipation phenotypes (wk 0–4). The cross symbol indicates that the P -value of that Pearson correlation was not significant ($P > 0.05$). (E) Scatter plot of relative abundance of *Bifidobacterium animalis* and defecation frequency from wk 0 (class A) to wk 4 (class B). In each box, the middle line denotes the median, the upper line denotes the third quartile, and the lower line denotes the first quartile. Each dot represents 1 individual.

consequently improves constipation symptoms (Zhu et al., 2014; Pozuelo et al., 2015). In our study, there were positive correlations between the abundances of *Strep. thermophilus* and *B. animalis* and the improvement of defecation frequency; that is, the increase of relative abundance of *B. animalis* and *Strep. thermophilus* accompanied an increase in defecation frequency. Some has research reported that *B. animalis* can improve gut motility, shorten the colonic transit time, and improve defecation frequency (Kim et al., 2015; Pozuelo et al., 2015). We speculated that *B. animalis* may contribute to the improvement of defecation frequency through improving gut motility and speeding up transit constipation. Some research has reported that *Strep. thermophilus* may have a beneficial role in preventing diarrhea (Uriot et al., 2017); however, the role of *Strep. thermophilus* in improving constipation is not yet established. This is the first report of the positive correlation between the abundance of *Strep. thermophilus* and the improvement of defecation frequency. Coincidentally, *B.*

animalis and *Strep. thermophilus* were the major bacterial species contained in the PFM. However, whether the increased *B. animalis* and *Strep. thermophilus* stem from *B. animalis* V9 and *Strep. thermophilus* ND03 in PFM or accelerated by the PFM is still unknown. In addition, it has been reported that *Lactobacillus* strains can increase the abundance of *Bifidobacterium* strains (Redondo-Useros et al., 2019). Therefore, *Lb. casei* Zhang from PFM may contribute to the increase of *B. animalis* and indirectly participate in the improvement of constipation.

Anti-Inflammatory Properties of PFM

A few studies suggest that the immune system is involved in the regulation of gut motility and that gut mucosal inflammation modulates the sensory and motor functions of the gut (Collins, 1996). Our data suggested that cytokine markers CRP, LPS, LBP, and IL-10 were significantly changed after a 4-wk intake

Figure 5. Stool metabolomics analysis using samples from pre- and postintervention groups. (A) Principal component (PC) analysis plot using results from positive (pos) mode (up) and negative (neg) mode (down). Data were scaled to unit variance and centered. Distance to model (dmodx) test is used to show the chance of the sample being an outlier. (B) De novo fatty acid biosynthesis and vitamin E metabolism were among the top pathways enriched in stool metabolomics data.

of PFM. The acute-phase protein CRP is produced by hepatocytes and is widely used as inflammation biomarker in clinical practice for differentiating inflammatory bowel disorders from constipation-predominant functional bowel disorder (Poullis et al., 2002). Some constipated participants in our study had a high level of CRP, suggesting an inflammation-related bowel disorder. The decrease of CRP after intake of PFM may reflect the amelioration of inflammation-related bowel disorder. Supplementation with *Lactobacillus casei* was reported to reduce serum CRP level and improve health conditions in patients with rheumatoid arthritis (Alipour et al., 2014). We speculate that the decrease of CRP after intake of PFM may contribute to amelioration of constipation symptoms.

Another acute-phase protein, LBP, has been suggested as a marker of infection (Schumann and Zweigner, 1999). In addition, this protein plays a role in the innate immune response. It can bind to LPS and thereafter bring LPS to the CD¹⁴ receptors on the monocyte-macrophage cell lineage; then, CD¹⁴ receptors interact with toll-like receptor-4, initiating cytokine production (Heumann and Roger, 2002; Palsson-McDermott and O'Neill, 2004). In our study, LBP level was increased after intake of PFM. Mokhtari et al. (2019) found that the increase of LBP was associated with probiotic intake,

and LBP is involved in the inactivation of proinflammatory cytokines to express a physiologic defense against infection (Lamping et al., 1998). Therefore, it was speculated that the probiotics in PFM increased LBP, which then initiated cytokines that inactivated the pro-inflammatory cytokine. Interleukin-10 is known as an anti-inflammatory cytokine that prevents inflammation damage to the host (Saraiva and O'Garra, 2010). Smits et al. (2005) reported that *Lactobacillus reuteri* and *Lb. casei* were able to induce IL-10-producing regulatory T cells by priming human dendritic cells. Taken together, it is likely that after intake of PFM, the increase of LBP and IL-10 levels and decrease of CRP level may contribute to the amelioration of inflammation, which further contributes to the improvement of gut motility.

Some Key Metabolic Pathways Regulated by Intake of PFM Seem to Improve Gut Motility

We observed that the fatty acid biosynthesis was the most significantly altered metabolic pathway in the stool metabolic profile, and furthermore, most significantly different metabolites were related to arachidonic acid metabolism and biosynthesis of unsaturated fatty acids. Some research has reported that unsaturated fatty acids can accelerate the recovery of gut motility.

Figure 6. Serum metabolomics analysis using samples from pre- and postintervention groups. (A) Principal component (PC) analysis plot using results from positive (pos) mode (up) and negative (neg) mode (down). Data were scaled to unit variance and centered. Distance to model (dmodx) test is used to show the chance of the sample being an outlier. (B) Bile acid biosynthesis was among the top pathways enriched in serum metabolomics ($P < 0.05$). Carnitine shuttle, which was enriched in stool metabolomics, was also found in serum metabolomics.

ity (Zhang et al., 2011). Bile acid biosynthesis is the top altered metabolic pathway enriched in serum metabolomics analysis, and a few studies have reported

that bile acid metabolism in constipated patients was changed (Abrahamsson et al., 2008; Maneerattanaporn and Chey, 2011; Rao, 2018). Bile acids serve as physi-

Figure 7. The correlation between defecation frequency and abundance of acylcarnitine after consuming fermented milk containing *Bifidobacterium animalis* V9 and *Lactobacillus casei* Zhang. A = wk 0 group; B = wk 4 group. The correlation analysis between constipation symptoms and significantly different metabolites was analyzed with Spearman's rank correlation coefficient.

ological laxatives and can activate TGR5 expressed by enterochromaffin cells, which further release 5-hydroxytryptamine and calcitonin gene-related protein (Snape et al., 1980; Keely et al., 2007), and bile acids were suggested to improve gut motility via the enteric nervous system (Karlström et al., 1986). These results indicate that probiotic yogurt may regulate bile acid biosynthesis to improve gut motility and therefore alleviate constipation symptoms. Carnitine shuttle is another interesting pathway we identified in serum metabolomics, and carnitine plays a central role in producing energy. Weaver et al. (1992) reported the gut dysmotility issue in infants with carnitine deficiency, and carnitine supplementation resolved the symptoms. Murata et al. (2017) also reported the effects of carnitine on reducing the severity of constipation. Therefore, carnitine may improve gut motility through replenishing energy. Vitamin E metabolism and ascorbate and aldarate metabolism also were identified as significantly altered metabolic pathways. Vitamin E is a potent antioxidant and anti-inflammatory element, which may regulate constipation symptoms through manipulation of inflammation (Singh et al., 2005). In the ascorbate and aldarate metabolism, D-glucuronic acid can be converted into ascorbate through a sequence of enzyme-driven steps, which can inhibit inflammatory reaction (Yao et al., 2015). In summary, we deduced that intake of PFM may improve gut motility through regulating fatty acid biosynthesis, bile acid biosynthesis, and carnitine

shuttle and therefore alleviate constipation symptoms. In addition, vitamin E metabolism and ascorbate and aldarate metabolism have anti-inflammatory effects and improve gut motility.

We evaluated the effect of PFM on clinical symptoms, immune factors, metagenomics, and metabolomics in constipated participants and observed significant improvement of constipation symptoms. We propose that PFM may help alleviate constipation symptoms through 3 potential mechanisms: fine-tuning gastrointestinal microbiota, fighting inflammation, and regulating metabolic pathways (Figure 8). Specifically, intake of PFM fine-tuned gastrointestinal microbiota; the increase of relative abundance of *B. animalis* and *Strep. thermophilus* accompanied defecation frequency increase, and *Strep. thermophilus* and *B. animalis* may improve defecation frequency. The anti-inflammatory cytokines (LBP and IL-10) increased and the proinflammatory cytokine CRP decreased, which indicates that PFM may have anti-inflammatory properties and therefore improve the immune system. Fatty acid biosynthesis, bile acid biosynthesis, carnitine shuttle, vitamin E metabolism, and ascorbate and aldarate metabolism were identified as significantly altered metabolic pathways. Among these metabolic pathways, acylcarnitine, located on the carnitine shuttle pathway, had a significantly positive correlation with defecation frequency, which can alleviate constipation symptoms. In conclusion, PFM may help alleviate constipation

Figure 8. Potential mechanism of probiotic fermented milk in alleviating constipation. Fermented milk containing *Bifidobacterium animalis* V9 and *Lactobacillus casei* Zhang may alleviate constipation symptoms by improving gut motility through regulation of intestinal microbiota, inflammation, and metabolic pathways. CRP = C-reactive protein; LBP = LPS-binding protein.

symptoms through 3 potential mechanisms: fine-tuning gastrointestinal microbiota, fighting inflammation, and regulating metabolic pathways.

ACKNOWLEDGMENTS

The authors thank all the participants for their assistance in this project. All authors participated in the design, analysis, writing, or revision of the manuscript. Jicheng Wang contributed to data collection. Chuantao Peng performed data analysis. Chuantao Peng and Xiaoye Bai prepared the final draft. All authors read and approved the final version of the paper. This study was supported by the National Natural Science Foundation of China (31720103911), Inner Mongolia Science and Technology Major Projects (zdxz2018018) and China Agriculture Research System, China (CARS-36). The authors declare no competing financial or conflict of interest.

REFERENCES

- Abrahamsson, H., A.-M. Östlund-Lindqvist, R. Nilsson, M. Simrén, and P.-G. Gillberg. 2008. Altered bile acid metabolism in patients with constipation-predominant irritable bowel syndrome and functional constipation. *Scand. J. Gastroenterol.* 43:1483–1488. <https://doi.org/10.1080/00365520802321212>.
- Agrawal, A., L. A. Houghton, J. Morris, B. Reilly, D. Guyonnet, N. Goupil Feuillerat, A. Schlumberger, S. Jakob, and P. J. Whorwell. 2009. Clinical trial: The effects of a fermented milk product containing *Bifidobacterium lactis* DN-173 010 on abdominal distension and gastrointestinal transit in irritable bowel syndrome with constipation. *Aliment. Pharmacol. Ther.* 29:104–114. <https://doi.org/10.1111/j.1365-2036.2008.03853.x>.
- Alipour, B., A. Homayouni-Rad, E. Vaghef-Mehrabany, S. K. Sharif, L. Vaghef-Mehrabany, M. Asghari-Jafarabadi, M. R. Nakhjavani, and J. Mohtadi-Nia. 2014. Effects of *Lactobacillus casei* supplementation on disease activity and inflammatory cytokines in rheumatoid arthritis patients: A randomized double-blind clinical trial. *Int. J. Rheum. Dis.* 17:519–527. <https://doi.org/10.1111/1756-185X.12333>.
- Andrews, C. N., and M. Storr. 2011. The pathophysiology of chronic constipation. *Can. J. Gastroenterol.* 25(Suppl. B):16B–21B.
- Aoyagi, Y., R. Amamoto, S. Park, Y. Honda, K. Shimamoto, A. Kushi, H. Tsuji, H. Matsumoto, K. Shimizu, K. Miyazaki, S. Matsubara, and R. J. Shephard. 2019. Independent and interactive effects of habitually ingesting fermented milk products containing *Lactobacillus casei* strain Shirota and of engaging in moderate habitual daily physical activity on the intestinal health of older people. *Front. Microbiol.* 10:1477. <https://doi.org/10.3389/fmicb.2019.01477>.
- Baars, T., C. Berge, J. Garssen, and J. Verster. 2019. The impact of raw fermented milk products on perceived health and mood among Dutch adults. *Nutr. Food Sci.* 49:1195–1206. <https://doi.org/10.1108/NFS-12-2018-0347>.
- Camilleri, M., A. C. Ford, G. M. Mawe, P. G. Dinning, S. S. Rao, W. D. Chey, M. Simrén, A. Lembo, T. M. Young-Fadok, and L. Chang. 2017. Chronic constipation. *Nat. Rev. Dis. Primers* 3:17095. <https://doi.org/10.1038/nrdp.2017.95>.
- Chong, J., O. Soufan, C. Li, I. Caraus, S. Li, G. Bourque, D. S. Wishart, and J. Xia. 2018. MetaboAnalyst 4.0: Towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res.* 46:W486–W494. <https://doi.org/10.1093/nar/gky310>.
- Collins, S. M. 1996. The immunomodulation of enteric neuromuscular function: Implications for motility and inflammatory disorders. *Gastroenterology* 111:1683–1699. [https://doi.org/10.1016/S0016-5085\(96\)70034-3](https://doi.org/10.1016/S0016-5085(96)70034-3).
- Corr, S. C., C. Hill, and C. G. M. Gahan. 2009. Understanding the mechanisms by which probiotics inhibit gastrointestinal pathogens. *Adv. Food Nutr. Res.* 56:1–15. [https://doi.org/10.1016/S1043-4526\(08\)00601-3](https://doi.org/10.1016/S1043-4526(08)00601-3).
- Dimidi, E., A. Zdanaviciene, S. Christodoulides, S. Taheri, P. Louis, P. I. Duncan, N. Emami, R. Crabbé, C. A. De Castro, P. McLean, G. E. Bergonzelli, K. Whelan, and S. M. Scott. 2019. Randomised clinical trial: *Bifidobacterium lactis* NCC2818 probiotic vs placebo, and impact on gut transit time, symptoms, and gut microbiology in chronic constipation. *Aliment. Pharmacol. Ther.* 49:251–264. <https://doi.org/10.1111/apt.15073>.
- Drossman, D. A., and D. L. Dumitrascu. 2006. Rome III: New standard for functional gastrointestinal disorders. *J. Gastrointest. Liver Dis.* 15:237–241.
- Fernandez, M. A., and A. Marette. 2018. Novel perspectives on fermented milks and cardiometabolic health with a focus on type 2 diabetes. *Nutr. Rev.* 76(Suppl 1):16–28. <https://doi.org/10.1093/nutrit/nuy060>.
- Guyonnet, D., O. Chassany, P. Ducrotte, C. Picard, M. Mouret, C.-H. Mercier, and C. Matuchansky. 2007. Effect of a fermented milk containing *Bifidobacterium animalis* DN-173 010 on the health-related quality of life and symptoms in irritable bowel syndrome in adults in primary care: A multicentre, randomized, double-blind, controlled trial. *Aliment. Pharmacol. Ther.* 26:475–486. <https://doi.org/10.1111/j.1365-2036.2007.03362.x>.
- Hendler, R., and Y. Zhang. 2018. Probiotics in the treatment of colorectal cancer. *Medicines (Basel)* 5:101. <https://doi.org/10.3390/medicines5030101>.
- Heumann, D., and T. Roger. 2002. Initial responses to endotoxins and Gram-negative bacteria. *Clin. Chim. Acta* 323:59–72. [https://doi.org/10.1016/S0009-8981\(02\)00180-8](https://doi.org/10.1016/S0009-8981(02)00180-8).
- Hor, Y.-Y., L.-C. Lew, A. S.-Y. Lau, J.-S. Ong, L.-O. Chuah, Y.-Y. Lee, S.-B. Choi, F. Rashid, N. Wahid, Z. Sun, L.-Y. Kwok, H. Zhang, and M.-T. Liong. 2018. Probiotic *Lactobacillus casei* Zhang (LCZ) alleviates respiratory, gastrointestinal & RBC abnormality via immuno-modulatory, anti-inflammatory & anti-oxidative actions. *J. Funct. Foods* 44:235–245. <https://doi.org/10.1016/j.jff.2018.03.017>.
- Karlström, L., J. Cassuto, M. Jodal, and O. Lundgren. 1986. Involvement of the enteric nervous system in the intestinal secretion induced by sodium deoxycholate and sodium ricinoleate. *Scand. J. Gastroenterol.* 21:331–340. <https://doi.org/10.3109/00365528609003083>.
- Keely, S. J., M. M. Scharl, L. S. Bertelsen, L. R. Hagey, K. E. Barrett, and A. F. Hofmann. 2007. Bile acid-induced secretion in polarized monolayers of T84 colonic epithelial cells: Structure-activity relationships. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292:G290–G297. <https://doi.org/10.1152/ajpgi.00076.2006>.
- Kim, S.-E., S. C. Choi, K. S. Park, M. I. Park, J. E. Shin, T. H. Lee, K. W. Jung, H. S. Koo, and S.-J. Myung. 2015. Change of fecal flora and effectiveness of the short-term VSL#3 probiotic treatment in patients with functional constipation. *J. Neurogastroenterol. Motil.* 21:111–120. <https://doi.org/10.5056/jnm14048>.
- Kwok, L. Y., L. Wang, J. Zhang, Z. Guo, and H. Zhang. 2014. A pilot study on the effect of *Lactobacillus casei* Zhang on intestinal microbiota parameters in Chinese subjects of different age. *Benef. Microbes* 5:295–304. <https://doi.org/10.3920/BM2013.0047>.
- Lamping, N., R. Dettmer, N. W. Schröder, D. Pfeil, W. Hallatschek, R. Burger, and R. R. Schumann. 1998. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria. *J. Clin. Invest.* 101:2065–2071. <https://doi.org/10.1172/JCI2338>.
- Leber, B., N. J. Tripolt, D. Blattl, M. Eder, T. C. Wascher, T. R. Pieber, R. Stauber, H. Sourij, K. Oettl, and V. Stadlbauer. 2012. The influence of probiotic supplementation on gut permeability in patients with metabolic syndrome: An open label, randomized pilot study. *Eur. J. Clin. Nutr.* 66:1110–1115. <https://doi.org/10.1038/ejcn.2012.103>.

- Lee, A., Y. J. Lee, H. J. Yoo, M. Kim, Y. Chang, D. S. Lee, and J. H. Lee. 2017. Consumption of dairy yogurt containing *Lactobacillus paracasei* ssp. *paracasei*, *Bifidobacterium animalis* ssp. *lactis* and heat-treated *Lactobacillus plantarum* improves immune function including natural killer cell activity. *Nutrients* 9:558. <https://doi.org/10.3390/nu9060558>.
- Lewis, S. J., and K. W. Heaton. 1997. Stool form scale as a useful guide to intestinal transit time. *Scand. J. Gastroenterol.* 32:920–924. <https://doi.org/10.3109/00365529709011203>.
- Li, S., Y. Park, S. Duraisingham, F. H. Strobel, N. Khan, Q. A. Soltow, D. P. Jones, and B. Pulendran. 2013. Predicting network activity from high throughput metabolomics. *PLOS Comput. Biol.* 9:e1003123. <https://doi.org/10.1371/journal.pcbi.1003123>.
- Liu, G. 2009. A novel limiting strain energy strength theory. *Trans. Nonferrous Met. Soc. China* 19:1651–1662. [https://doi.org/10.1016/S1003-6326\(09\)60084-4](https://doi.org/10.1016/S1003-6326(09)60084-4).
- Maki, R., M. Matsukawa, A. Matsuduka, M. Hashinaga, H. Anai, Y. Yamaoka, K. Hanada, and C. Fujii. 2018. Therapeutic effect of lyophilized, kefir-fermented milk on constipation among persons with mental and physical disabilities. *Jpn. J. Nurs. Sci.* 15:218–225. <https://doi.org/10.1111/jjns.12189>.
- Maneerattanaporn, M., and W. D. Chey. 2011. Targeting bile acids in the treatment of constipation. *Expert Rev. Gastroenterol. Hepatol.* 5:657–659. <https://doi.org/10.1586/egh.11.63>.
- Marteau, P., B. Le Névé, L. Quinquis, C. Pichon, P. J. Whorwell, and D. Guyonnet. 2019. Consumption of a fermented milk product containing *Bifidobacterium lactis* CNCM I-2494 in women complaining of minor digestive symptoms: Rapid response which is independent of dietary fibre intake or physical activity. *Nutrients* 11:92. <https://doi.org/10.3390/nu11010092>.
- Massi, M. 2006. Effects of probiotic bacteria on gastrointestinal motility in guinea-pig isolated tissue. *World J. Gastroenterol.* 12:5987–5994. <https://doi.org/10.3748/wjg.v12.i37.5987>.
- Mohanty, D. P., S. Mohapatra, S. Misra, and P. S. Sahu. 2016. Milk derived bioactive peptides and their impact on human health—A review. *Saudi J. Biol. Sci.* 23:577–583. <https://doi.org/10.1016/j.sjbs.2015.06.005>.
- Mokhtari, Z., Z. Karbaschian, A. Pazouki, A. Kabir, M. Hedayati, P. Mirmiran, and A. Hekmatdoost. 2019. The effects of probiotic supplements on blood markers of endotoxin and lipid peroxidation in patients undergoing gastric bypass surgery: A randomized, double-blind, placebo-controlled, clinical trial with 13 months follow-up. *Obes. Surg.* 29:1248–1258. <https://doi.org/10.1007/s11695-018-03667-6>.
- Murata, S., K. Inoue, T. Aomatsu, A. Yoden, and H. Tamai. 2017. Supplementation with carnitine reduces the severity of constipation: A retrospective study of patients with severe motor and intellectual disabilities. *J. Clin. Biochem. Nutr.* 60:121–124. <https://doi.org/10.3164/jcbln.16-52>.
- Ohkusa, T., S. Koido, Y. Nishikawa, and N. Sato. 2019. Gut microbiota and chronic constipation: A review and update. *Front. Med. (Lausanne)* 6:19. <https://doi.org/10.3389/fmed.2019.00019>.
- Ou, Y., S. Chen, F. Ren, M. Zhang, S. Ge, H. Guo, H. Zhang, and L. Zhao. 2019. *Lactobacillus casei* strain Shirota alleviates constipation in adults by increasing the pipercolinic acid level in the gut. *Front. Microbiol.* 10:324. <https://doi.org/10.3389/fmicb.2019.00324>.
- Ozaki, K., T. Maruo, H. Kosaka, M. Mori, H. Mori, Y. Yamori, and T. Toda. 2018. The effects of fermented milk containing *Lactococcus lactis* ssp. *cremoris* FC on defaecation in healthy young Japanese women: A double-blind, placebo-controlled study. *Int. J. Food Sci. Nutr.* 69:762–769. <https://doi.org/10.1080/09637486.2017.1417977>.
- Pålsson-McDermott, E. M., and L. A. J. O'Neill. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113:153–162. <https://doi.org/10.1111/j.1365-2567.2004.01976.x>.
- Piatek, J., H. Sommermeyer, A. Ciechelska-Rybarczyk, and M. Bernatek. 2019. In-vitro pathogen inhibition: Comparing the inhibitory effects of a complex multistrain synbiotic with simple probiotics containing the yeast *Saccharomyces boulardii* or *Lactobacillus rhamnosus* bacteria. *bioRxiv*. <https://www.biorxiv.org/content/10.1101/543819v1>.
- Piewngam, P., Y. Zheng, T. H. Nguyen, S. W. Dickey, H.-S. Joo, A. E. Villaruz, K. A. Glose, E. L. Fisher, R. L. Hunt, B. Li, J. Chiou, S. Pharkjaksu, S. Khongthong, G. Y. C. Cheung, P. Kiratisin, and M. Otto. 2018. Pathogen elimination by probiotic *Bacillus* via signalling interference. *Nature* 562:532–537. <https://doi.org/10.1038/s41586-018-0616-y>.
- Poullis, A. P., S. Zar, K. K. Sundaram, S. J. Moodie, P. Risley, A. Theodossi, and M. A. Mendall. 2002. A new, highly sensitive assay for C-reactive protein can aid the differentiation of inflammatory bowel disorders from constipation- and diarrhoea-predominant functional bowel disorders. *Eur. J. Gastroenterol. Hepatol.* 14:409–412. <https://doi.org/10.1097/00042737-200204000-00013>.
- Pozuelo, M., S. Panda, A. Santiago, S. Mendez, A. Accarino, J. Santos, F. Guarner, F. Azpiroz, and C. Manichanh. 2015. Reduction of butyrate- and methane-producing microorganisms in patients with irritable bowel syndrome. *Sci. Rep.* 5:12693. <https://doi.org/10.1038/srep12693>.
- Rao, S. S. C. 2018. Treating constipation with bile: A new target. *Lancet Gastroenterol. Hepatol.* 3:520–521. [https://doi.org/10.1016/S2468-1253\(18\)30166-3](https://doi.org/10.1016/S2468-1253(18)30166-3).
- Rao, S. S. C., K. Rattanakovit, and T. Patcharatrakul. 2016. Diagnosis and management of chronic constipation in adults. *Nat. Rev. Gastroenterol. Hepatol.* 13:295–305. <https://doi.org/10.1038/nrgastro.2016.53>.
- Redondo-Useros, N., A. Gheorghe, L. E. Díaz-Prieto, B. Villavisencio, A. Marcos, and E. Nova. 2019. Associations of probiotic fermented milk (PFM) and yogurt consumption with *Bifidobacterium* and *Lactobacillus* components of the gut microbiota in healthy adults. *Nutrients* 11:651. <https://doi.org/10.3390/nu11030651>.
- Saraiva, M., and A. O'Garra. 2010. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10:170–181. <https://doi.org/10.1038/nri2711>.
- Schumann, R. R., and J. Zweigner. 1999. A novel acute-phase marker: Lipopolysaccharide binding protein (LBP). *Clin. Chem. Lab. Med.* 37:271–274. <https://doi.org/10.1515/CCLM.1999.047>.
- Singh, U., S. Devaraj, and I. Jialal. 2005. Vitamin E, oxidative stress, and inflammation. *Annu. Rev. Nutr.* 25:151–174. <https://doi.org/10.1146/annurev.nutr.24.012003.132446>.
- Smits, H. H., A. Engering, D. van der Kleij, E. C. de Jong, K. Schipper, T. M. M. van Capel, B. A. J. Zaat, M. Yazdanbakhsh, E. A. Wierenga, Y. van Kooyk, and M. L. Kapsenberg. 2005. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *J. Allergy Clin. Immunol.* 115:1260–1267. <https://doi.org/10.1016/j.jaci.2005.03.036>.
- Snape, W. J. Jr., S. Shiff, and S. Cohen. 1980. Effect of deoxycholic acid on colonic motility in the rabbit. *Am. J. Physiol.* 238:G321–G325. <https://doi.org/10.1152/ajpgi.1980.238.4.G321>.
- Sun, Z., X. Chen, J. Wang, P. Gao, Z. Zhou, Y. Ren, T. Sun, L. Wang, H. Meng, W. Chen, and H. Zhang. 2010. Complete genome sequence of probiotic *Bifidobacterium animalis* ssp. *lactis* strain V9. *J. Bacteriol.* 192:4080–4081. <https://doi.org/10.1128/JB.00369-10>.
- Sun, Z., X. Chen, J. Wang, W. Zhao, Y. Shao, Z. Guo, X. Zhang, Z. Zhou, T. Sun, L. Wang, H. Meng, H. Zhang, and W. Chen. 2011a. Complete genome sequence of *Lactobacillus delbrueckii* ssp. *bulgaricus* strain ND02. *J. Bacteriol.* 193:3426–3427. <https://doi.org/10.1128/JB.00369-10>.
- Sun, Z., X. Chen, J. Wang, W. Zhao, Y. Shao, L. Wu, Z. Zhou, T. Sun, L. Wang, H. Meng, H. Zhang, and W. Chen. 2011b. Complete genome sequence of *Streptococcus thermophilus* strain ND03. 193:793–794. <https://doi.org/http://doi.org/10.1128/JB.01374-10>.
- Toscano, M., R. De Grandi, V. L. Miniello, R. Mattina, and L. Drago. 2017. Ability of *Lactobacillus kefir* LKF01 (DSM32079) to colonize the intestinal environment and modify the gut microbiota composition of healthy individuals. *Dig. Liver Dis.* 49:261–267. <https://doi.org/10.1016/j.dld.2016.11.011>.

- Turan, İ., Ö. Dedeli, S. Bor, and T. İlter. 2014. Effects of a kefir supplement on symptoms, colonic transit, and bowel satisfaction score in patients with chronic constipation: A pilot study. *Turk. J. Gastroenterol.* 25:650–656. <https://doi.org/10.5152/tjg.2014.6990>.
- Uriot, O., S. Denis, M. Junjua, Y. Roussel, A. Dary-Mouro, and S. Blanquet-Diot. 2017. *Streptococcus thermophilus*: From yogurt starter to a new promising probiotic candidate? *J. Funct. Foods* 37:74–89. <https://doi.org/10.1016/j.jff.2017.07.038>.
- Wang, Y., Y. Li, J. Xie, Y. Zhang, J. Wang, X. Sun, and H. Zhang. 2013. Protective effects of probiotic *Lactobacillus casei* Zhang against endotoxin- and D-galactosamine-induced liver injury in rats via anti-oxidative and anti-inflammatory capacities. *Int. Immunopharmacol.* 15:30–37. <https://doi.org/10.1016/j.intimp.2012.10.026>.
- Weaver, L. T., S. R. Rosenthal, W. Gladstone, and H. S. Winter. 1992. Carnitine deficiency: A possible cause of gastrointestinal dysmotility. *Acta Paediatr.* 81:79–81. <https://doi.org/10.1111/j.1651-2227.1992.tb12085.x>.
- Xia, J., R. Mandal, I. V. Sinelnikov, D. Broadhurst, and D. S. Wishart. 2012. MetaboAnalyst 2.0—A comprehensive server for metabolomic data analysis. *Nucleic Acids Res.* 40:W127–W133. <https://doi.org/10.1093/nar/gks374>.
- Xu, H., W. Huang, Q. Hou, L.-Y. Kwok, W. Laga, Y. Wang, H. Ma, Z. Sun, and H. Zhang. 2019a. Oral administration of compound probiotics improved canine feed intake, weight gain, immunity and intestinal microbiota. *Front. Immunol.* 10:666. <https://doi.org/10.3389/fimmu.2019.00666>.
- Xu, H., F. Zhao, Q. Hou, W. Huang, Y. Liu, H. Zhang, and Z. Sun. 2019b. Metagenomic analysis revealed beneficial effects of probiotics in improving the composition and function of the gut microbiota in dogs with diarrhea. *Food Funct.* 10:2618–2629.
- Ya, T., Q. Zhang, F. Chu, J. Merritt, M. Bilige, T. Sun, R. Du, and H. Zhang. 2008. Immunological evaluation of *Lactobacillus casei* Zhang: A newly isolated strain from koumiss in Inner Mongolia, China. *BMC Immunol.* 9:68. <https://doi.org/10.1186/1471-2172-9-68>.
- Yao, W., L. Zhang, Y. Hua, P. Ji, P. Li, J. Li, L. Zhong, H. Zhao, and Y. Wei. 2015. The investigation of anti-inflammatory activity of volatile oil of *Angelica sinensis* by plasma metabolomics approach. *Int. Immunopharmacol.* 29:269–277. <https://doi.org/10.1016/j.intimp.2015.11.006>.
- Zhang, J., L. Wang, Z. Guo, Z. Sun, Q. Gesudu, L. Kwok, Menghe-bilige, and H. Zhang. 2014a. 454 pyrosequencing reveals changes in the faecal microbiota of adults consuming *Lactobacillus casei* Zhang. *FEMS Microbiol. Ecol.* 88:612–622. <https://doi.org/10.1111/1574-6941.12328>.
- Zhang, Q., J.-C. Yu, W.-M. Kang, and G.-J. Zhu. 2011. Effect of ω -3 fatty acid on gastrointestinal motility after abdominal operation in rats. *Mediators Inflamm.* 2011:152137. <https://doi.org/10.1155/2011/152137>.
- Zhang, W., D. Yu, Z. Sun, R. Wu, X. Chen, W. Chen, H. Meng, S. Hu, and H. Zhang. 2010a. Complete genome sequence of *Lactobacillus casei* Zhang, a new probiotic strain isolated from traditional homemade koumiss in Inner Mongolia, China. *J. Bacteriol.* 192:5268–5269. <https://doi.org/10.1128/JB.00802-10>.
- Zhang, Y., R. Du, L. Wang, and H. Zhang. 2010b. The antioxidative effects of probiotic *Lactobacillus casei* Zhang on the hyperlipidemic rats. *Eur. Food Res. Technol.* 231:151–158. <https://doi.org/10.1007/s00217-010-1255-1>.
- Zhang, Y., X. Guo, J. Guo, Q. He, H. Li, Y. Song, and H. Zhang. 2014b. *Lactobacillus casei* reduces susceptibility to type 2 diabetes via microbiota-mediated body chloride ion influx. *Sci. Rep.* 4:5654. <https://doi.org/10.1038/srep05654>.
- Zhong, Z., W. Zhang, R. Du, H. Meng, and H. Zhang. 2012. *Lactobacillus casei* Zhang stimulates lipid metabolism in hypercholesterolemic rats by affecting gene expression in the liver. *Eur. J. Lipid Sci. Technol.* 114:244–252. <https://doi.org/10.1002/ejlt.201100118>.
- Zhu, L., W. Liu, R. Alkhouri, R. D. Baker, J. E. Bard, E. M. Quigley, and S. S. Baker. 2014. Structural changes in the gut microbiome of constipated patients. *Physiol. Genomics* 46:679–686. <https://doi.org/10.1152/physiolgenomics.00082.2014>.

ORCIDS

- Jicheng Wang <https://orcid.org/0000-0003-0118-5628>
 Xiaoye Bai <https://orcid.org/0000-0003-0476-5492>
 Wenyi Zhang <https://orcid.org/0000-0001-5530-4210>
 Heping Zhang <https://orcid.org/0000-0003-4329-8006>