Milk fat globule membrane protects *Bifidobacterium longum* ssp. *infantis* ATCC 15697 against bile stress by modifying global transcriptional responses

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**ABSTRACT**

The milk fat globule membrane (MFGM) can protect probiotic bacteria from bile stress. However, its potential mechanism has not been reported. In this study, the viability, morphology and gene transcriptional response of *Bifidobacterium longum* ssp. *infantis* ATCC 15697 (BL_15697) stressed by bile salts with or without MFGM were investigated. It was shown that MFGM alleviated the reduction in BL_15697 population induced by 0.2% porcine bile stress and restored the population to the control levels. MFGM ameliorated the shrunken, fragmented appearance and irregular morphology of BL_15697 and maintained cell integrity disrupted by bile stress. RNA-sequencing results showed that MFGM increased transport of glucose and raffinose and decreased that of branched-chain amino acids (BCAA) in the presence of bile salts. MFGM stimulated the expression of genes involved in the synthesis of raffinose in galactose metabolism and the metabolism of BCAA, suggesting that MFGM stimulated the accumulation of raffinose and BCAA in the presence of bile. In addition, MFGM stimulated the expression of 2 bile efflux transporters under bile stress. Together, the multifactorial response helps BL_15697 excrete bile salts and maintain cellular integrity in response to bile stress. This study proposes a mechanism for the protection of BL_15697 against bile salt stress by MFGM, thereby providing a molecular basis for its application in incorporation of probiotics.

**Key words:** *Bifidobacterium longum* subsp. *infantis* ATCC 15697, transcriptomic analysis, bile salt stress, milk fat globule membrane

**INTRODUCTION**

The beneficial properties of *Bifidobacterium* depend on their ability to persist in the intestinal tract with high viability. Bile salt physiological concentration in human intestine gradient ranging from 2% to less than 0.05%, which is an important factor to shape intestine microbial community profile (Ruiz et al., 2013). Therefore, bile stress in the small intestine is a major challenge for sensitive and strictly anaerobic *Bifidobacterium*. Studies have been performed to explore the mechanism of bile resistance or tolerance in *Bifidobacterium* (An et al., 2014; Kelly et al., 2020). Transcriptional analysis of *Bifidobacterium* in response to bile stress revealed differential involvement of various biological processes, such as carbohydrate metabolism, ABC transporters, general stress response, and fatty acid metabolism (An et al., 2014; Bottacini et al., 2017; Wei et al., 2019). In addition, bile efflux systems are extremely important for bifidobacterial survival during bile salt exposure. Bifidobacterial bile efflux pumps, Bbr_0838 (*betA*) and BL1102 (*ctr*) have been shown to help *Bifidobacterium breve* and *B. longum* against bile stress (Gueimonde et al., 2009; Ruiz et al., 2012). The major pathways and genes in bifidobacteria regulated by bile stress are shown in Supplemental Table S1 ([https://doi.org/10.17632/zrb27bzsmz.1](https://doi.org/10.17632/zrb27bzsmz.1)).

Efforts have been made to improve bile resistance or tolerance to probiotics, including encapsulation (Martín et al., 2015; Rodrigues et al., 2020). Embedding probiotics in milk components or other matrices
is a potential way to protect them against bile stress (Zhang et al., 2021). The protective effects of embedding media hinge on their structure and composition. The milk fat globule membrane (MFGM), a 3-layered membrane wrapped around the milk fat globule, is composed of polar lipids, neutral triglycerides, and proteins, which are important in maintaining the stability of fat globules in milk (Li et al., 2021). The milk fat globule membrane plays an important role in human health, such as protecting the infant from pathogens or virus invasion, improving intelligence, and regulating of inflammation (Silva et al., 2021). Zhang et al. (2020) reported that MFGM protected Lactobacillus rhamnosus GG from bile stress and improved its viability in the intestine of mice. Usually, Bifidobacterium with multiple beneficial functions for human health is more sensitive to bile stress than Lactobacillus. Therefore, we wondered whether MFGM could help BI_15697 against bile stress, and it is important to understand the protective mechanism of MFGM in bile tolerance of probiotics. However, there is limited information regarding the transcriptional expression of bacterial genes responding to bile stress under the protection of MFGM.

The aim of this study is to evaluate whether MFGM affects the viability and morphology of BI_15697 under bile stress, and to identify genes that are restored by MFGM upon bile exposure. It was shown that the protective effect of MFGM on BI_15697 against bile tolerance is a multifactorial response that may be critical for survival and colonization of bifidobacteria in the human gut. The MFGM may be a potential matrix for the delivery of probiotics to the gut.

**MATERIALS AND METHODS**

**Supplemental Data**

Supplemental data associated with this article can be found online in the Mendeley Data Repository at https://doi.org/10.17632/zrb27bzsmz.1.

**Bacterial Strain and Growth Conditions**

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

Bifidobacterium longum ssp. infantis ATCC 15697 (BL_15697) was obtained from the China Center of Industrial Culture Collection (Beijing, China). Bifidobacterium infantis 35624 (BI_35624) was isolated from the Align Probiotic Supplement (Procter & Gamble Company). Bifidobacterium infantis Y46 (BI_Y46), Bifidobacterium bifidum Y10 (BB_Y10), B. bifidum Y22 (BB_Y22), and Bifidobacterium pseudocatenulatum YA (BP_YA) were isolated from feces of healthy, full-term, and breast-fed infants. Bifidobacteria were subcultured twice in de Man, Rogosa, and Sharpe broth (Qingdao Hope Bio-Technology Co. Ltd.) with 0.05% cysteine (MRSC) at 37°C for 18 h and were anaerobically (90% N₂, 5% CO₂, and 5% H₂) with 1% of the inoculation volume.

**Viability of Bifidobacteria**

As previously described by Zhang et al. (2020), MFGM (MFGM-10, Arla Food Ingredients, Aarhus, Denmark) and porcine bile (Shanghai Ruiyong Biotechnology Co. Ltd.) were dissolved in MRSC. Resusciated Bifidobacterium cells were washed with an equal volume of sterilized water and treated with MRSC, 5 g/L MFGM, 0.1% to 0.5% porcine bile, and 0.1% to 0.5% porcine bile and 5 g/L MFGM for 30 min, respectively.

**BI_15697 Morphology**

The BI_15697 cells grown in MRSC (M), MRSC with 5 g/L MFGM (F), MRSC with 0.2% bile (BE), and MRSC with 0.2% bile and 5 g/L MFGM (BF) were fixed, dehydrated, and sputter-coated with platinum-gold according to the previously reported method (Zhang et al., 2023). The samples were then observed by field emission-scanning electron microscopy (FE-SEM, Hitachi SU8010).

**RNA Extraction and Examination**

Total RNA from BI_15697 cells grown in M, BE, and BF for 30 min was extracted from 3 biological replicates using a TRIzol reagent (Invitrogen, Carlsbad) according to the manufacturer’s instructions. RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using a Nano Photometer spectrophotometer (Implen Inc.). The RNA concentration and integrity were evaluated using the Qubit RNA Assay Kit of the Qubit2.0 Flurometer and the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 System (Agilent Technologies).

**Library Preparation and RNA-Sequencing**

RNA samples were sequenced at Sangon Biotech Co. Ltd. (Shanghai, China), where mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Sequencing libraries were generated using the VAHTSTM mRNA-seq V2 Library Prep Kit for IL-
lumina according to the manufacturer’s recommendations, and index codes were added to assign sequences to each sample. Fragments of 150 to 200 bp in length were preferentially selected for amplification by PCR and purified using the AMPure XP System (Beckman Coulter). Library quality was assessed on the Agilent Bioanalyzer 2100 System, then quantified and pooled. The pooled cDNA libraries were sequenced on HiSeq XTen sequencers (Illumina, San Diego, CA).

Data Assessment and Quality Control

Sequenced data were evaluated using Fast QC (version 0.11.2). Raw reads were filtered using Trimmmomatic (version 0.36). The reads were aligned to the BI_15697 genome with GenBank accession number NC_011593 using HISAT2 (version 2.1.0) with default parameters. Each differential expression analysis between 2 different treatments was performed using the DESeq2 package (version 1.26.0, Bioconductor, Buffalo, NY). To obtain significant differential genes, screening conditions were set as follows: difference multiple $|\log_{2} FC| \geq 1$ and false discovery rate $q < 0.05$. Gene Ontology (GO) term enrichment analysis of differentially expressed genes (DEG) was performed using TopGO (version 2.24.0) with the associated GO terms. The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed using Cluster Profiler (version 3.0.5). Pathway enrichment analysis identified metabolic or signal transduction pathways that were more enriched in DEG compared with the whole genome background.

Real-Time Quantitative PCR Validation

The expression levels of selected genes were validated by real-time quantitative PCR (RT-qPCR). Primers for each gene were designed using the Primer 5 software and are listed in Table 1. Total RNA was prepared as described previously. Template cDNA was synthesized using Maxima Reverse Transcriptase (Thermo Scientific) from 200 ng of total RNA prepared as described previously. The RT-qPCR was performed using 2X SG Fast qPCR Master Mix (B639271, BBI, Roche, Switzerland) on a LightCycler480 II RT-qPCR system (Roche). Relative quantitation of RNA was calculated using the $2^{-\Delta \Delta \text{ct}}$ method (Schmittgen and Livak, 2008) with ileS as an endogenous control.

Statistical Analysis

All data are presented as the means ± standard deviation of 3 independent experiments. Data analysis was performed using SPSS 22.0 (IBM Corp.). The variance (one-way ANOVA) procedure and Duncan’s post hoc test were used to calculate statistical differences between groups. P-values <0.05 were considered significant.

RESULTS

Effect of MFGM on BI_15697 Population and Morphology Under Bile Stress

Bile salt tolerance is important for probiotics to exert their functional properties in the host intestine. The MFGM can improve the bile salt tolerance of *L. rhamnosus* GG (Zhang et al., 2020). Therefore, it was hypothesized that MFGM could improve viability of other probiotics under bile stress. The protective effects of MFGM on the BI_15697 cell numbers stressed by porcine bile (0.1%–0.3%) were evaluated (Figure 1A). The MFGM had no effect on BI_15697 cell numbers in the absence of porcine bile. Low concentrations of bile (0.1%) significantly reduced the numbers of BI_15697 cells, whereas the supplementation with MFGM (5 g/L) showed protective effects. The BI_15697 numbers decreased more, and the protective effect of MFGM became more pronounced as bile salt concentrations
increased. The higher concentrations of bile (0.2% and 0.3%) decreased BL_15697 cell numbers by 1.8 and 4.3 log cfu/mL after 30 min of treatment, whereas MFGM significantly increased cell numbers compared with the corresponding bile group. However, excessive bile salt (above 0.3%) caused most of the cell death, which was not suitable for RNA-sequencing (RNA-seq) analysis. Therefore, 0.2% porcine bile salt was selected for further analysis.

To test whether MFGM protects other bifidobacteria from bile stress, the viability of BL_Y46, BL_35624, BB_Y10, BB_Y22, and BP_YA incubated in bile salt with or without MFGM were assessed (Figure 1B and 1C). Similar to the changes in the viability of BL_15697, the addition of MFGM did not affect the viability of any of the bifidobacteria tested. Treatment with 0.3% of porcine bile salts significantly decreased the viability of BL_Y46, BL_35624, and BB_Y10, whereas MFGM increased their viability in the presence of bile salts. BB_Y22 and BP_YA were more tolerant to the bile salts, 0.3% bile salts slightly decreased their cell number, and supplementation with MFGM did not significantly affect viability. Therefore, BB_Y22 and BP_YA were exposed to 0.5% bile salts, and the protective effect of MFGM was demonstrated, showing that MFGM increased 0.58 log cfu/mL in BB_Y22 and 1.69 log cfu/mL in BP_YA as compared with those treated with 0.5% bile salts after 30 min of treatment. This result suggests that MFGM may protect Bifidobacterium from bile stress in general.

The morphologies of BL_15697 treated with MRSC, MFGM, 0.2% BE, and 0.2% BF for 30 min visualized by FE-SEM are shown in Figure 1B. In MRSC, BL_15697 showed a well-organized smooth-surfaced, intact structure with an adjacent slender rod shape. In the MFGM group, the morphology of BL_15697 was very similar to that grown in MRSC, with the MFGM surrounding the cell surface. In the case of BE, as shown in Figure 1D, the bacteria were shorter, thicker, and tended to clump together with a shrunken fragmented appearance and irregular morphology. When treated with BF, BL_15697 showed a slightly rough surface and intact rod-shaped morphology. This difference between MRSC and BF treatments was less pronounced than that between MRSC and BE treatments. Thus, the MFGM around BL_15697 maintained the bacterial integrity and protected the cells from the bile stress.

Transcriptomic Comparison of BL_15697 Under Different Conditions

To reveal the potential mechanism of MFGM alleviating the bile stress of BL_15697 at the molecular level, its gene transcription levels after MRSC, BE, and BF treatments, respectively, were analyzed by RNA-seq. Readings of 125,033,316, 108,402,870, and 168,013,710 were obtained when BL_15697 was treated with M, BE, and BF conditions, respectively. The M and BF treatments mapped 99% of the reads to the BL_15697 genome, while BE treatment mapped 98% of the reads. Further analysis showed an average 2,545, 2,556, and 2,495 out of the whole 2,637 genes in the genome were covered under the 3 conditions tested.

Real-time quantitative PCR analysis was performed to examine the transcriptional expression levels of 5 selected DEG, including BLON_RS10050 (encoding bile acid: sodium symporter family protein), BLON_RS09645 (ABC transporter ATP-binding protein), BLON_RS12315 (acyltransferase family protein), BLON_RS03240 (nucleoside hydrolase), and BLON_RS00920 (LacI family DNA-binding transcriptional regulator). The results showed that the BLON_RS10050 gene was significantly upregulated by 2.99-fold in BE treatment and 2.91-fold in BF treatment compared with M treatment. The BLON_RS09645 gene was downregulated by 2.61-fold in BE treatment and 2.82-fold in BF treatment compared with M treatment. The gene expression levels of BLON_RS12315 were downregulated 2.69- and 1.44-fold in BF treatment compared with BE treatment and M treatment, respectively. In addition, the changes of BLON_RS03240 and BLON_RS00920 were 2.75- and 2.79-fold in BF_vs_BE, and 2.58- and 1.74-fold in BF_vs_M, respectively. Taken together, the relative expression levels of these genes were consistent with the results of RNA-seq analysis (Table 2).

Transcriptomic Changes of BL_15697 Trigged by Bile Stress in MRSC

Comparing the RNA expression profiles of BL_15697 after BE and M treatments, 802 DEG were identified with 473 downregulated genes and 329 upregulated genes (|log2FC| ≥ 1 and q < 0.05; Figure 2A). Their putative functions were classified into different categories grouped by GO (Figure 3A). Gene Ontology analysis revealed that DEG were enriched in various biological processes, mainly in metabolic process, cellular process, localization, establishment of localization, biological regulation, regulation of biological process, and response to stimulus. Consistent with the biological process, the cellular components involved were membrane, cell, cell part, and membrane part. Enrichment analysis revealed catalytic activity, binding, and transporter activity in the molecular function category (Figure 3).

To investigate the molecular pathways affected by bile stress, pathway analysis was performed using
Figure 1. Viability and morphology of *Bifidobacterium longum* ssp. *infantis* ATCC 15697 (BL_15697). BL_15697 cell numbers in de Man, Rogosa, and Sharpe broth with cysteine (MRSC), MRSC with 5 g/L milk fat globule membrane (MFGM); MRSC with 0.1% to 0.3% porcine bile salt (0.1BE, 0.2BE, and 0.3BE); and MRSC with 0.1% to 0.3% bile salts and 5 g/L MFGM (0.1BF, 0.2BF, and 0.3BF) after 30 min of incubation (A). The cell numbers of bifidobacteria treated with 0.3% (B) and 0.5% (C) bile salts and MFGM after 30 min of incubation. Data were expressed as means ± SD from 3 independent experiments. Different letters above the bars indicate a significant difference at $P < 0.05$. Morphology of BL_15697 in MRSC (M), MRSC with 5 g/L MFGM (MFGM), MRSC with 0.2% bile (BE), and MRSC with 0.2% bile and 5 g/L MFGM (BF) observed by using field emission-scanning electron microscopy (D).
Cluster Profiler (Figure 4). The analysis revealed that the downregulated genes were significantly enriched in 8 pathways, including ABC transporters, galactose metabolism, amino sugar and nucleotide sugar metabolism, other glycan degradation, starch and sucrose metabolism, sphingolipid metabolism, phosphotransferase system (PTS), and lysosome. In contrast, upregulated genes were enriched in ABC transporters, AA biosynthesis, quorum sensing, 2-oxocarboxylic acid metabolism, nicotinate and nicotinamide metabolism, histidine metabolism, valine, leucine and isoleucine biosynthesis, fatty acid biosynthesis, and fatty acid metabolism.

Table 2. Transcriptional level of selected genes of BL_15697 measured by RNA sequencing (RNA-seq) and real-time quantitative PCR (RT-qPCR)\(^1\)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Description</th>
<th>BE vs. M (RNA-seq)</th>
<th>BE vs. M (RT-qPCR)</th>
<th>BF vs. BE (RNA-seq)</th>
<th>BF vs. BE (RT-qPCR)</th>
<th>BF vs. M (RNA-seq)</th>
<th>BF vs. M (RT-qPCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLON_RS10050</td>
<td>Bile acid: sodium symporter family protein</td>
<td>4.08</td>
<td>2.99 ± 0.42</td>
<td>—</td>
<td>0.89 ± 0.11</td>
<td>2.55</td>
<td>2.91 ± 0.12</td>
</tr>
<tr>
<td>BLON_RS09645</td>
<td>ABC transporter ATP-binding protein</td>
<td>−2.85</td>
<td>−2.61 ± 0.12</td>
<td>—</td>
<td>−0.026 ± 0.01</td>
<td>−3.07</td>
<td>−2.82 ± 0.07</td>
</tr>
<tr>
<td>BLON_RS12315</td>
<td>Aroyltransferase family protein</td>
<td>1.17</td>
<td>1.05 ± 0.02</td>
<td>−3.36</td>
<td>−2.69 ± 0.52</td>
<td>−2.20</td>
<td>−1.44 ± 0.24</td>
</tr>
<tr>
<td>BLON_RS03240</td>
<td>Nucleoside hydrolase</td>
<td>—</td>
<td>0.5 ± 0.02</td>
<td>3.26</td>
<td>2.75 ± 0.31</td>
<td>2.58</td>
<td>1.98 ± 0.02</td>
</tr>
<tr>
<td>BLON_RS00920</td>
<td>Lact family DNA-binding transcriptional regulator</td>
<td>−2.11</td>
<td>−1.68 ± 0.04</td>
<td>3.85</td>
<td>2.79 ± 0.12</td>
<td>1.74</td>
<td>1.94 ± 0.22</td>
</tr>
</tbody>
</table>

\(^1\)Transcriptional levels are indicated as the log2 fold change when BL_15697 was treated with de Man, Rogosa, and Sharpe broth with cysteine (MRSC; culture M), 0.2% porcine bile salts in MRSC (BE), or 5 g/L milk fat globule membrane and 0.2% porcine bile salts in MRSC (BF). A dash refers to no change analyzed by RNA-seq. The RT-qPCR values are means of 3 biological replicates with 3 technical replicates ± SD.

Figure 2. Summary of differentially expressed genes (DEG) of BL_15697 in de Man, Rogosa, and Sharpe broth with cysteine (MRSC; culture M), MRSC with 0.2% bile (BE), and MRSC with 0.2% bile and 5 g/L milk fat globule membrane (MFGM; BF). Volcano plot of DEG of (A) the BE group versus the M group (BE_vs_M); (B) the BF group versus the BE group (BF_vs_BE); (C) the BF group versus the M group (BF_vs_M). Venn diagram showing (D) overlap of DEG induced by bile salts in MRSC (blue), MFGM in bile salt broth (red), both bile and MFGM in MRSC (green), upregulated (E) and downregulated DEG (F) genes in 3 groups, DEG upregulated by bile stress but downregulated by MFGM (G), and DEG downregulated by bile stress but upregulated by MFGM (H).
MFGM Affected Gene Expressions of BI_15697 in the Presence of Bile Salts

Compared with the BE treatment, RNA expressions of BF-treated BI_15697 revealed 525 DEG, consisting of 381 downregulated genes and 144 upregulated genes (|\log_{2}\text{FC}| \geq 1 \text{ and } q < 0.05; \text{Figure 2B}). To elucidate the relationship between BE group and BF treatments, gene functional enrichment analysis was performed on the identified set of DEG. Gene Ontology analysis of DEG in BF_vs_BE was similar to that in BE_vs_M. Differentially expressed genes were enriched in different biological processes (Figure 3B). They were mainly enriched in metabolic process, cellular process, localization, establishment of localization, biological regulation, regulation of biological process, and response to stimulus. The major cellular components were membrane, cell, cell part, and membrane part. The molecular function category was highly represented by catalytic activity, binding, and transporter activity.

The microbial pathways significantly regulated by the BF_vs_BE treatments are shown in Figure 4. These pathways include peptidoglycan biosynthesis, arginine biosynthesis, aminoacyl-tRNA biosynthesis, histidine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, homologous recombination, folate biosynthesis, and carbon fixation pathways in prokaryotes were significantly downregulated by MFGM in the presence of bile salts (Figure 4C). Compared with the BE treatment, the BF treatment upregulated the pathways of ABC transporters, starch and sucrose metabolism, galactose metabolism, bacterial secretion system, and protein export (Figure 4D).

DISCUSSION

MFGM Attenuated the Reduction in Cell Number and Morphology of BI_15697 Induced by Bile Salt

*B bifidum* is the dominant strain in the intestinal tract of newborns and its abundance in the human gut changes with age, diet, and genetics (Ley et al., 2006). The administration of *B. bifidum* for immunomodulatory activities has been studied (Lim and Shin, 2020). It is documented that supplementation with BI_15697 regulates the gut microbiota and reduces lipopolysaccharide-induced intestinal inflammatory cytokines and alleviates colonic and serum endotoxins (Rodes et al., 2014; Wickramasinghe et al., 2015; Chichlowski et al., 2020). Tolerance of gastric and bile salts stress in the intestine is a prerequisite for BI_15697 to enter the intestine and exert its probiotic function.

Because BI_15697 is sensitive to bile salts, cell counts decreased with increasing bile salt concentration. Specifically, the cell numbers decreased from 9.27 log cfu/mL after M treatment to 3.74 log cfu/mL after 0.3% bile salt treatment. Under MFGM protection, this value increased to 8.06 log cfu/mL, indicating that the M treatment significantly improved the bile salt tolerance of BI_15697.
salt tolerance of BI_15697. In addition, BF treatment ameliorated the shrunken-fragmented appearance and irregular morphology of BI_15697 and improved cell integrity under bile stress.

MFGM Regulated Transcriptional Expression of BI_15697 Response to Bile Stress

Studies have revealed gene expression profiles of Bifidobacterium under different stress and nutritional conditions (An et al., 2014; Zabel et al., 2020; Liu et al., 2021). The DEG of B. breve UCC 2003 (B. longum BBMN68) in response to the bile stress are involved in various biological processes such as carbohydrate, AA, and nucleotide metabolism and bile efflux systems, stress response, and transport systems (Ruiz et al., 2012; An et al., 2014; Kelly et al., 2020). Similar to previous studies, RNA-seq analysis of BI_15697 treated with MRS and bile salts showed that bile salt stress regulated the genes involved in membrane transport, metabolism of AA, carbohydrates, cofactors and vitamins, nucleotide, and glycans biosynthesis, and metabolism. However, there is no report on the mechanism by which MFGM protects probiotics against bile stress. Therefore, it is speculated that MFGM restore some gene expression of BI_15697 induced by bile salt stress. Thus, this study focused on the overlap of the DEG between BE_vs_M and BF_vs_BE treatments (Figure 2).

The Venn diagram showed 193 DEG in the overlap between the BE_vs_M and BF_vs_BE treatments (Figure 2D). Among them, 11, 28, 79, and 75 genes were in the overlap between BE_vs_M up and BF_vs_BE up (Figure 2E), BE_vs_M down and BF_vs_BE down (Figure 2F), BE_vs_M down and BF_vs_BE up (Figure 2G), and BE_vs_M up and BF_vs_BE down (Figure 2H), respectively. In these groups, 5, 6, 17, and 33 DEG participated in KEGG metabolic pathway, respectively. The relative expression levels of these genes are shown in Supplemental Table S2 (https://doi.org/10.17632/zrb27bzsmz.1).

Figure 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the differentially expressed genes (DEG) identified in groups of BE_vs_M (A and B) and BF_vs_BE (C and D). Enrichment factor indicates the number of DEG associated with the KEGG analysis divided by the total number of DEG. The size of the dots represents the number of DEG associated with the KEGG analysis, and the color represents the P-value. M = cells cultured in de Man, Rogosa, and Sharpe broth with 0.05% cysteine (MRSC); BE = cells cultured in MRSC with 0.2% bile; BF = cells cultured in 0.2% bile with 5 g/L milk fat globule membrane.
**MFGM Stimulated Overexpressed Genes of BI_15697 Response to Bile Stress**

Five genes were upregulated by both treatments of BE_vs_M and BF_vs_BE, namely *ftsY*, *clpB*, *dnaK*, *BLON_RS12580*, and *BLON_RS12585* (Supplemental Table S3, https://doi.org/10.17632/zrb27bzsmz.1). The gene *ftsY* (encoding signal recognition particle-docking protein molecular chaperone FtsY) is involved in the targeting and insertion of nascent membrane proteins into the cytoplasmic membrane and acts as a receptor for the complex formed by the signal recognition particle and the ribosome-nascent chain. Both *clpB* (encoding ATP-dependent chaperone) and *dnaK* (encoding molecular chaperone DnaK) are involved in the longevity regulation pathway. The expression of *clpB* and *dnaK* in this present study proved previous reports that expression of molecular chaperones (*dnaK* and *clpB*) was induced by the adverse condition including bile stress (De Dea Lindner et al., 2007; Sánchez et al., 2008). *BLON_RS12580* and *BLON_RS12585* (encoding ABC transporter ATP-binding protein) are multidrug efflux pumps acting as ABC transporters. These results suggest that the supplementation of MFGM induced further upregulation of these genes, and partially attenuated bile salt damage, as demonstrated by cell viability and morphology results.

**MFGM Further Reduced the Downregulated Genes Expression of BI_15697 in Response to Bile Stress**

Six genes were downregulated in both BE_vs_M and BF_vs_BE treatments, namely *recR* (encoding recombination protein RecR), *BLON_RS09125* (encoding metal ABC transporter permease), *BLON_RS13785* (encoding 7-cyano-7-deazaguanine synthase), *BLON_RS12530* (encoding DUF442 domain-containing protein), *BLON_RS02445* (encoding ABC transporter ATP-binding protein), and *BLON_RS00420* (encoding serine hydrolase; Supplemental Table S3). These genes are involved in 6 different pathways, including homologous recombination, ABC transporters, folate biosynthesis, lipopolysaccharide biosynthesis, β-lactam resistance, and peptidoglycan biosynthesis. This could be due to the presence of MFGM, which has a certain protective effect on the bacteria. Therefore, BI_15697 does not need these genes to survive the bile stress in the presence of MFGM.

**Downregulated Genes Response to Bile Salts was Stimulated by MFGM.** The transcriptional changes of some DEG induced by BE_vs_M and BF_vs_BE treatments were diametrically opposite. Genes upregulated elicited by BE_vs_M treatments were downregulated by BF_vs_BE treatments and vice versa. It is speculated that these genes with opposite expression may be repaired by MFGM.

Seventeen downregulated genes induced by BE_vs_M treatments but upregulated by BF_vs_BE treatments were mainly involved in the pathways of ABC transporters (*BLON_RS12305*, *BLON_RS12735*, *BLON_RS12740*, *BLON_RS12745*, and *ugpC*), galactose metabolism (*BLON_RS12755*, *BLON_RS00720*, and *BLON_RS12720*), PTS (*ptsP*), and quorum sensing (*BLON_RS03285* and *BLON_RS03290*) pathways (Supplemental Table S4, https://doi.org/10.17632/zrb27bzsmz.1).

The sugar ABC transporter substrate-binding protein encoded by *BLON_RS12305* is a putative substrate-binding protein of the chitobiase transport system. The sn-glycerol-3-phosphate ABC transporter ATP-binding protein UgpC (*BLON_RS12835*) is involved in the transport of glucose/galactose oligomer/maltoligosaccharide/raffinose/stachyose/melibiose. The ABC transporters encoded by *BLON_RS12735*, *BLON_RS12740*, and *BLON_RS12745* are carbohydrate ABC transporter permease, sugar ABC transporter permease, and extracellular solute-binding protein, respectively. They play a key role in the raffinose/stachyose/melibiose transport system. *BLON_RS00720* encodes glycoside hydrolase family 13 protein, and *BLON_RS12720* encodes α, α-phosphotrehalase, which catalyzes the conversion of sucrose to D-fructose or D-glucose. *BLON_RS12755* encodes α-galactosidase, which catalyzes raffinose to synthesize D-galactose and D-glucose. Thus, it is speculated that MFGM has restored raffinose metabolism and transport, which was impaired by bile stress.

As a prebiotic, raffinose selectively promotes the growth of bifidobacteria. It can also promote the growth of other genera through direct catabolism or cross-feeding of prebiotics. *Bifidobacterium* can preferentially consume raffinose, which promotes its growth. However, the role of raffinose in increasing resistance of *Bifidobacterium* to adverse conditions has not been reported. Studies have shown that raffinose exerts various functions under stress conditions, and the deposition of raffinose can increase the tolerance of plant to salinity, drought, or cold stress (Yan et al., 2022). Thus, we postulate that in the presence of MFGM, the increased expression of related enzymes of raffinose metabolism and transport allows *Bifidobacterium* to accumulate more raffinose, thus improving its tolerance to bile salts. Therefore, further studies are needed to confirm the mechanism by which prebiotic raffinose improves the tolerance of probiotic bacteria to adverse environment.

The gene of *ptsP* encoding phosphoenolpyruvate-protein phosphotransferase (*ptsI*) in the PTS was decreased 2.72-fold after exposure to bile salts but
increased 8.64-fold in the presence of MFGM (Supplemental Table S4). The enzyme ptsI, a prokaryotic protein as the first enzyme (EI) serves as an energy coupling protein for the porters of the PTS responsible for the internalization and simultaneous phosphorylation of monosaccharides. ptsI transfers a phosphate group from phosphoenolpyruvate (PEP) via HPr to a histidine in the next enzyme (EII) in this pathway. PEP is an energy source and phosphoryl donor (Brackenbury and Isom, 2011). Therefore, the overexpression of ptsP induced by MFGM in the presence of bile salts may provide more energy for the growth of BI_15697. Furthermore, it has been reported that ptsI is present on the surface of the pneumococcus and functions as an adhesin (Mizrachi Nebenzahl et al., 2016). In contrast, MFGM prolonged the retention time of L. rhamnosus GG in the intestine of Balb/c mice in a previous study (Zhang et al., 2020). Thus, it is concluded that MFGM upregulated the expression of ptsP, which probably played the role of adhesin in the presence of bile salts in the intestine.

**Upregulated Genes Response to Bile Salts was Decreased by MFGM.** The expression of 33 genes was increased by BE_vs_M treatments but decreased by BF_vs_BE treatments. KEGG pathway analysis showed that these DEG were mainly in the pathways of AA biosynthesis, galactose metabolism, and ABC transporters (Supplemental Table S5, https://doi.org/10.17632/zrb27bzsmz.1).

Ten genes involved in AA biosynthesis were stimulated by bile stress but decreased by MFGM in the presence of bile salts. *hisH* (encoding imidazole glycerol phosphate synthase subunit HisH), *priA* (encoding bifunctional 1-(5-phosphoribosyl)-5-((5-phosphoribosylamino) methylideneamino) imidazole-4-carboxamide isomerase/phosphoribosylantranilate isomerase PriA), and *hisB* (encoding imidazolylglycerol-phosphate dehydratase HisB) were involved in histidine metabolism. *BLON_RS07135* (encoding bifunctional indole-3-glycerol phosphate synthase/tryptophan synthase subunit β) and *BLON_RS07130* (encoding tryptophan synthase subunit α) were involved in tryptophan biosynthesis. Additionally, both *argB* (encoding acetylglutamate kinase) and *argJ* (encoding bifunctional glutamate N-acetyltransferase/AA acetyltransferase argJ) were involved in arginine biosynthesis. Additionally, both *abr* (alanine racemase) and *gltB* (glutamate synthase large subunit) were involved in alanine metabolism. *BLON_RS12015* (encoding aminotransferase class I/II-fold pyridoxal phosphate-dependent enzyme) catalyzes transformation from L-cystathionine to L-homocysteine in cysteine and methionine metabolism. Those AA stimulated by bile stress could be potential nitrogen sources for BL_15697 under bile stress (An et al., 2014). The MFGM decreased some AA biosynthesis that was stimulated by bile salts, therefore. Furthermore, the decreased expression levels of genes related to the AA biosynthesis by MFGM were all significantly higher than those increased by bile salt stress. This may be due to the fact that MFGM protects BL_15697 from bile salts and acts as a nitrogen source to support BL_15697 growth in bile salts.

The expression of 7 DEG (*BLON_RS02945*, *BLON_RS02950*, *BLON_RS01680*, *BLON_RS01670*, *BLON_RS01665*, *BLON_RS02580*, and *BLON_RS07675*) involved in quorum sensing and 5 genes (*phnC*, *phnE* [*BLON_RS00120*, *phnE* [*BLON_RS00115*], *BLON_RS00130*, and *urtA*]) involved in ABC transporter were upregulated by bile stress but downregulated by MFGM in the presence of bile salts. Some genes were involved in different pathways; for example, *BLON_RS02945*, *BLON_RS02950*, and *BLON_RS07675* were involved in both quorum sensing and ABC transporters. *BLON_RS02945* and *BLON_RS02950* encode critical enzymes in the transport of branched-chain amino acids (BCAA). Data from the present study showed that the transcript level of *BLON_RS02945* and *BLON_RS02950* of BI_15697 was accelerated by bile stress but depressed by MFGM in the presence of bile. The expression of genes (*ilvC*, *leuD*, *leuC*, *RS10735*, and *RS05520*) involved in the biosynthesis of valine, leucine, and isoleucine was stimulated by bile but not affected by MFGM (Supplemental Table S6, https://doi.org/10.17632/zrb27bzsmz.1). These results suggest that the synthesis of BCAA might still increase, but its transport decreased, resulting in a high level of BCAA concentration in bacterial cells. It has been shown that the BCAA aminotransferase of *B. longum* NCIMB 8809 was stimulated by exposure to bile salts (Sánchez et al., 2005). However, the opposite phenomenon (i.e., under expression of a gene encoding ketol-acid reductoisomerase involved in BCAA synthesis) was observed in *B. longum* BBM168 in response to bile stress at the transcriptional and protein levels, respectively (An et al., 2014). It is speculated that this opposite result was due to the diverse regulatory mechanism of AA metabolism and transport in *Bifidobacterium* under different concentrations of bile salts. Branched-chain AA have been postulated as a mechanism to build hydrophobic AA of the cell membrane to protect cells against bile salt attack (Sánchez et al., 2005) and maintain the internal bacterial pH (Len et al., 2004). These results showed that more BCAA were accumulated in BI_15697 treated with bile salts in the presence of MFGM to help the bacteria resist bile salt attack.
Transcription of genes involved in the transport of oligopeptide (BLON_RS07675), phosphonate (phnC, phnE, and BLON_RS00130) and urea (urtA) was stimulated by bile salts but repressed by MFGM in the presence of bile salts. BLON_RS07675, encoding ABC transporter substrate-binding protein is one of the transport subunits from oligopeptide transport system OppA. It has been reported that peptide is accelerated by the presence of bile (Ruiz et al., 2009). The expression of genes including BLON_RS00125 (phnC, encoding phosphonate ABC transporter ATP-binding protein), BLON_RS00120 (phnE, encoding phosphonate ABC transporter, permease protein PhnE), BLON_RS00115 (phnE), and BLON_RS00130 (encoding phosphate/phosphite/phosphonate ABC transporter substrate-binding protein) in the phosphonate transport system were observed. The altered expression results suggest that the transportation of phosphonate was stimulated by bile salts but suppressed by MFGM. We postulate that BI_15697 required more nutrients including oligopeptide, phosphonate and urea to survive in the bile salts, while the presence of MFGM maintained the cell integrity and provides more nutrients for BI_15697 growth. BI_15697 was no longer required to transport these substances from the extracellular compartment resulting in a decrease in the expression of these ABC transporters. However, the comprehensive mechanism of phosphonate transport in Bifidobacterium response to bile salts has not yet been established.

The expression of BLON_RS11825 encoding ATP-grasp domain-containing protein was decreased by bile stress but stimulated by MFGM in the presence of bile salts. ATP-grasp domain-containing protein is responsible for catalyzing acetyl-CoA to generate malonyl-CoA, which is a key step for fatty acid metabolism. Transcriptional expression of ptsP and BLON_RS11825 indicated that bile stress decreased pyruvate accumulation but increased metabolic flux into fatty acid. This phenomenon was confirmed by the increased expression of enzymes (BLON_RS02210 and BLON_RS11815) related to fatty acid metabolism induced by the BE_vs_M treatments. Fatty acid metabolism plays an important role in the bile tolerance of Bifidobacterium. Some intermediates, such as cyclopropane fatty acid, have been shown to help bacteria survive the stress by decreasing membrane permeability (An et al., 2014). The overexpression of ptsP and low expression of BLON_RS11825 in the presence of MFGM response to bile salts could increase the efflux of pyruvate, which is involved in several metabolic pathways including BCAA biosynthesis. Therefore, an increased biosynthesis of pyruvate might result in the accumulation of BCAA, which protect bacteria from bile stress.

### MFGM Stimulated Overexpression of Bile Efflux Transporters Under Bile Stress

Studies have shown that bile efflux transporters, such as ctr and betA help protect bifidobacteria from bile stress (Price et al., 2006; Gueimonde et al., 2009). Two genes (BLON_RS10050 and BLON_RS08555) related to the bile efflux transporters were detected in this study (Supplemental Table S7, https://doi.org/10.17632/zrb27bzsmz.1).

The transcription of BLON_RS10050, encoding a protein of the bile acid: sodium symporter family, was 16.96-fold upregulated by bile stress, which was not affected by MFGM. BLON_RS10050 showed high homology to BBNM68_849 in B. longum BBMN68 and BL1102 in B. longum NCC2705. BBNM68_849 was identified as Na+/ bile acid symporter, which involved in the bile acid metabolic pathway and playing a role in a specific hydrolase or exclusion system (Hao et al., 2011). BL1102 (ctr), encoding a Na+-dependent nucleoside transporter, was a putative cholate transporter, shown to be resistant to bile salts, cholate and some antibiotics (Price et al., 2006). The mechanism of bacterial tolerance to bile salts is so complex that none of the known systems can fully explain it. The changes in BLON_RS10050 induced by the BE_vs_M and BF_vs_BE treatments indicated that BLON_RS10050 helped to expel bile salts from BL_15697 regardless of the presence of MFGMs.

The expression of BLON_RS08555, homology with BL0920 (betA) in B. longum NCC2705 and Bbr0838 in B. breve, was not affected by bile stress alone, but was increased 2.32-fold by the combination of MFGM and bile salts. This shows that in the presence of MFGM, 2 bile efflux pumps, Ctr and BetA, may be able to secrete more bile from cells. Therefore, BI_15697 may defend against bile salt attack and maintain cell integrity in the presence of MFGM. We hypothesize that overexpression of bile efflux pumps in Bifidobacterium may improve the bile salt tolerance of the bacterium, and we will confirm this in the future by constructing genetically engineered strains.

Based on the results of the present study, a model of MFGM protective mechanism is proposed for both BI_15697 and bifidobacteria response to bile stress (Figure 5). The MFGM restored the expression of genes related to ABC transporter, galactose metabolism and transport, BCAA transport, AA metabolism and stimulated the expression of 2 bile efflux transporters. The genes involved in the transport of glucose (ugpC) and raffinose were stimulated by MFGM, suggesting that the transport of glucose and raffinose was increased by MFGM in the presence of bile salts. In addition, the expression of genes related to the synthesis of raffinose...
in the galactose metabolism was stimulated by MFGM, which was inhibited by bile stress, which may lead to a greater accumulation of raffinose in BL_15697. The ABC transporters involved in oligopeptide, phosphonate, urea, and BCAA transport were stimulated by bile salts but inhibited by MFGM in the presence of bile salts. The expression of genes involved in BCAA metabolism were increased by bile salts but not affected by MFGM, suggesting that the accumulation of BCAA in the bile salts independent of MFGM helps BL_15697 survive in bile stress. Ten genes (priA, hisH, and hisB, BLON_RS07135, BLON_RS07130, argB, argJ, alr, gltB, and BLON_RS12015) that are involved in the biosynthesis of AA were stimulated by bile stress but decreased by MFGM in the presence of bile salts. In particular, 2 genes involved in bile efflux (BLON_RS08555 and BLON_RS10050) help BL_15697 export bile salts from BL_15697 in the presence of MFGM unlike BLON_RS10050 in the bile salt without MFGM. Overall, MFGM protects BL_15697 from bile stress by modifying global-transcriptional responses that help BL_15697 maintain cell integrity.

**CONCLUSIONS**

In brief, 0.2% porcine bile salts reduced the cell viability and damaged the cell integrity of B. longum ssp. infants ATCC 15697, which was restored by MFGM. The presence of MFGM restored the expression of genes related to ABC transporter, galactose metabolism and transport, BCAA transport, AA metabolism, and so on. This study comprehensively elucidated the mechanism involved in the bacterial protective ability of MFGM against bile stress, which provides a new theoretical basis for the development and application of MFGM that can resist the adverse environment in the host intestine.

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