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The Potential Function and Immunoregulatory Activity of Milk-Fat-Globule-Membrane Proteins in Zhongdian-Yak, Diqing Cattle, and Cattle-Yak Milk

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ABSTRACT: Milk-fat-globule-membrane (MFGM) proteins have important benefits to human health. This study aimed to elucidate the composition, potential function and immunoregulatory activity of MFGM proteins in Zhongdian-yak milk (ZM), Diqing cattle milk (DM), and cattle-yak milk (CM). 2254, 2788, and 2204 MFGM proteins were identified in ZM, DM, and CM, respectively. A total of 343, 554, and 487 differential proteins were identified in CM vs. ZM, DM vs. ZM, and DM vs. CM. These differential proteins were mainly annotated in immune regulation-related metabolic pathways. The MFGM proteins from the three kinds of milk promoted nitric oxide (NO) production in mouse macrophages and play an immunoregulatory role. Molecular docking and molecular dynamics analyses further revealed that highly abundant immune-related MFGM proteins interact with Toll-like receptors (TLR2 and TLR4) through hydrogen-bond-mediated stable binding, thereby exerting immunomodulatory effects. These findings provide new insights into the functional characteristics of MFGM proteins and offer a theoretical basis for the development of functional infant formula or dairy products.

Keywords: yak milk; proteomics; milk-fat-globule-membrane proteins; immunoregulatory; metabolic pathway

1. Introduction

Milk-fat-globule-membrane (MFGM) protein is a three-layer membrane protein that encapsulates natural milk fat globules (MFGs). It plays a vital role in maintaining the structural stability of MFGs. There are many kinds of MFGM proteins, exhibiting several physiological functions, such as improving immunity, promoting nerve development, anti-cancer and anti-inflammatory, and so on [1-4]. In particular, several MFGM proteins, such as lactadherin (MFG8), xanthine dehydrogenase (XDH), butyrophilin, and mucin-related proteins, have been reported to participate in immune regulation and host defense. Previous studies have shown that dietary supplementation with MFGM or its components can contribute to infant cognitive development, intestinal health, and immune function [5]. Therefore, MFGM proteins are increasingly regarded as promising functional milk-derived bioactive ingredients. Li et al. found that MFGM proteins are essential for the

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regulation of lipid breakdown during simulated gastrointestinal digestion in infants conducted in vitro [6]. Zhao et al. analyzed the MFGM proteins in human and Porcine milk. They identified a total of 3520 common and 956 significantly different MFGM proteins, some different MFGM proteins exhibiting immunomodulatory activities [7]. Lu et al. identified 312, 554, 175, and 143 MFGM proteins from human, bovine, goat, and yak milk, respectively. And they reported that the number of MFGM proteins in human milk was comparable to that in bovine milk but markedly different from those in goat and yak milk [8]. Hettinga et al. compared the differences between MFGM proteins in human and bovine milk and found that the human counterpart was composed of more immune active proteins, while the latter comprised more antibacterial proteins [9]. These studies showed that MFGM proteins exhibit several beneficial activities; however, the composition of MFGM proteins varied based on the growth environment, species, lactation behavior, and maternal diet.

Yak milk is an important source of nutrition for Tibetan people and known for its unique and rich nutritional composition. Cattle-yak is a hybrid of yak and cattle. It exhibits the characteristics of good milk quality and high milk yield. Yak, yellow cattle, and cattle-yak are raised in Tibetan areas. Therefore, yak milk, yellow cattle milk, and cattle-yak milk (CM) are all considered yak milk. Since the composition and potential physiological functions of yak milk have attracted a lot of attention, differences in the compositions and functions of yak milk, yellow cattle milk, and CM need to be assessed.

The Zhongdian yak represents a key yak breed in China, with its milk, known as Zhongdian-yak milk (ZM), being a distinctive local product. It has extremely high nutritional value [10]. Diqing cattle are a type of plateau livestock that exhibit excellent adaptability to the ecological conditions found in high-altitude regions. Cattle-yak is a dominant hybrid variety with yak as the female parent and cattle as the male parent. He et al. found that the contents of protein, fat, and total solids in ZM, Diqing yellow cattle milk (DM), and CM were significantly higher than those in Holstein cattle, Simmental cattle, and Yunling cattle milk [11]. Li et al. (2024) analyzed the whey proteins in ZM, DM, and CM using tandem mass tag (TMT) proteomics and found that cattle-yak whey proteins exhibited more functional activities [12]. However, there are few studies on the composition, function, and difference of MFGM proteins in ZM, DM, and CM. Therefore, the analysis of MFGM proteins in the milk of different species by proteomics can help elucidate the composition and function of their MFGM proteins and promote the utilization of MFGM proteins.

Proteomics is an important method for studying the composition and function of proteins in food products. Li et al. revealed the compositional complexity and functional diversity of MFGM proteins in Murrah, Nili-Ravi, and Mediterranean buffaloes through TMT proteomics analysis [13]. Jiang et al. demonstrated differences in proteomics and immunomodulatory activity of goat and cow MFGM proteins based on proteomics [14]. Liang et al. found that the MFGM protein composition of goat milk changed significantly across different lactation periods, and albumin played an important role during the entire lactation period [15]. Zhao et al. identified 3920 and 4001 MFGM proteins in human and pig milk using label-free quantitative proteomic approach [16]. They found that the differential MFGM proteins in human and pig milk were mainly annotated to peroxisome, complement, and coagulation cascades via the Kyoto

Encyclopedia of Genes and Genomes (KEGG) annotation. These studies demonstrate that proteomics is a reliable and powerful approach for comprehensively characterizing the composition, abundance, and potential biological functions of MFGM proteins. In particular, proteomics combined with bioinformatics analyses such as GO and KEGG annotation can systematically identify immune-related proteins and associated signaling pathways, thereby providing important insights into the functional characteristics and immunoregulatory potential of MFGM proteins. Therefore, an in-depth analysis of the composition and function of food by proteomics is also of certain significance for production and processing.

This study aimed to analyze the composition and function of MFGM proteins in ZM, DM, and CM using 4D label free proteomics. The study was aimed to (i) elucidate the MFGM protein profiles of ZM, DM, and CM; (ii) reveal the potential functions and related metabolic pathways of the MFGM proteins in ZM, DM, and CM; and (iii) analyze the *in vitro* immune activity and immunoregulatory mechanisms of the MFGM proteins in ZM, DM, and CM. The findings might help better understand the functions of and differences among the MFGM in ZM, DM, and CM, and might provide a theoretical basis for an optimal processing of Yunnan plateau characteristic milk.

2. Materials and methods

2.1. Sample collection

Milk samples from eighteen animals were collected concurrently from ZM, DM, and CM on a dairy farm located in Xianggelila City, Yunnan Province, China. Each group consisted of six healthy animals ($n=6$) at a similar lactation stage. These samples were categorized into three groups to serve as replicates, and milk was collected by milking device. Pooling was performed to minimize individual variation and enhance protein detection, given the limited sample size. Therefore, milk samples from every two animals within the same group were mixed in equal volumes, resulting in three pooled biological replicates for subsequent analysis. All of the animals were healthy and kept in a consistent feeding environment, receiving identical feed daily. After collection, the samples were immediately transported to the laboratory on ice and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

2.2. MFGM protein extraction

The proteins from the MFGM were isolated using a previously established method, with minor adjustments made to the procedure^[17]. Each milk sample was centrifuged at $8000\times g$ for 15 min. The layer of fat was gathered, rinsed three times with phosphate-buffered saline (PBS, Solarbio, Beijing, China), and subjected to centrifugation at $8000\times g$ for 15 minutes to eliminate any remaining casein and whey proteins. Subsequently, the fat was combined with an equivalent volume of STD buffer which contained 4% (w/v) sodium dodecyl sulfate (SDS, Sigma, St. Louis, MO, USA), 100 mM Tris-HCl (pH 7.6, Solarbio, Beijing, China), and 40 mM dithiothreitol (DTT, Sigma, St. Louis, MO, USA), then refrigerated overnight at $4\text{ }^{\circ}\text{C}$ for the milk fat to solidify. Following the melting process, the sample underwent incubation in a water bath set to

95 °C for 5 minutes before being centrifuged at 12000× g for 10 minutes. The layer of cream that formed on top was discarded, and the collected supernatant, which contained the MFGM proteins, was stored at −80 °C.

2.3. Protein digestion

A mixture consisting of 100 µL of protein and 400 µL of pre-chilled acetone (Sinopharm Chemical Reagent Co., Shanghai, China) was allowed to precipitate at 20 °C overnight. Following this, the sample was centrifuged for 10 minutes at 12,000 rpm and 4 °C, and the supernatant was carefully moved to a clean Eppendorf (EP) tube. Next, 200 µL of 80% acetone that had been pre-cooled was added to the EP tube. The resulting mixture underwent two washing steps before being centrifuged again for 10 minutes at 12,000 rpm at a temperature of 4 °C. The supernatant was subsequently transferred to a fresh EP tube, while the protein precipitate was solubilized using an ultrasound water bath for a duration of 10 minutes. To achieve a final concentration of 5 mM, 1 µL of DTT was added to reduce the disulfide bonds. The solution was subsequently incubated at 55 °C for a duration of 10 minutes. After the sample was cooled to room temperature, the disulfide bonds were reduced and subsequently alkylated by adding 2 µL of iodoacetamide (Solarbio, Beijing, China) to reach a final concentration of 10 mM. This mixture was then kept in the dark for 15 minutes. Following the alkylation step, trypsin (Solarbio, Beijing, China) was prepared in a resuspension buffer at a concentration of 0.5 µg/L and allowed to incubate at room temperature for 5 minutes. After ultracentrifugation at 1,000 rpm and 37 °C, the proteins were incubated overnight. After drying, each fraction was completely dissolved in 0.1% formic acid (FA) and subsequently analyzed using liquid chromatography-mass spectrometry (LC-MS).

2.4 Identification and Quantification via Nano LC-MS/MS

Briefly, 200 ng of peptides from each sample were isolated and assessed using a nano-UPLC (nanoElute2) paired with a timsTOF Pro2 instrument (Bruker) equipped with a nano-electrospray ion source. The separation utilized a reversed-phase column (PePsep C18, 1.9 µm, 75 × 15 cm, Bruker, Germany). The mobile phases consisted of H₂O with 0.1% formic acid (phase A) and acetonitrile (ACN) containing 0.1% formic acid (phase B). A 45-minute gradient was implemented for sample separation at a flow rate of 300 nL/min. The mass spectrometer utilized the data-dependent acquisition (DDA) mode known as parallel accumulation serial fragmentation (PaSEF) for the purpose of DDA data collection, with the scanning range set from m/z 100 to m/z 1700 for MS1. In the course of PASEF MS/MS scanning, the energy of impact rose consistently in relation to ion mobility, starting at 20 eV ($1/K0 = 0.6 \text{ Vs/cm}^2$) and reaching 59 eV ($1/K0 = 1.6 \text{ Vs/cm}^2$).

2.5. Multivariate and bioinformatic analyses

The raw MS files provided by the vendor were analyzed utilizing the SpectroMine software (version 4.2.230428.52329) along with its integrated Pulsar search engine. MS spectra lists were matched against the species-specific UniProt FASTA databases (uniprot-Mus musculus-reviewed-10090-2022-11.fasta), applying carbamidomethyl [C] as a constant modification, while oxidation (M) and acetyl (Protein N-term) were set as

variable modifications. A maximum of two missed cleavages was permitted. The false discovery rate (FDR) for both PSM and peptide levels was established at 0.01. Peptide identification was conducted with an initial precursor mass deviation of up to 20 ppm, along with a fragment mass deviation of 20 ppm. All remaining parameters were configured to their default settings.

The rate of false findings was set at 0.01 for both PSM and peptide concentrations. The identification of peptides was performed with an initial precursor mass deviation allowed up to 10 ppm along with a particle mass deviation of 0.02 Da. Differentially expressed MFGM proteins were identified using a t-test, followed by multiple testing correction with the Benjamini-Hochberg method to control the false discovery rate (FDR). Proteins with an adjusted *P*-value < 0.05 and a fold-change threshold of >1.2 (upregulated) or <0.83 (downregulated) were considered significantly differentially expressed. Functional classifications were performed using gene ontology (GO; <http://geneontology.org/>) and KEGG (<https://www.genome.jp/kegg/>) pathways, analyzed via the online tool for annotation, visualization, and integrated discovery (DAVID; <http://david.abcc.ncifcrf.gov/home.jsp>). The variance of the differentially expressed proteins was assessed with SPSS software (Version 25.0, SPSS Inc., Chicago, IL), treating *P* values < 0.05 as statistically significant.

2.6. RAW264.7 cell viability assay

The viability of RAW264.7 cells was assessed through colorimetric analysis utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Solarbio, Beijing, China) [7]. In summary, a density of 1.5×10^4 RAW264.7 cells per well was introduced into 96-well plates and allowed to stabilize for 12 hours. After discarding the medium, 100 μ L of the sample, prepared at different concentrations in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA), was added to each well. The incubation of the plate lasted for 20 hours. Following this, each well received 20 μ L of a 5 mg/mL MTT solution, which was prepared in PBS, and the plate was then incubated for an additional 4 hours under the same conditions. Once the medium was discarded, 150 μ L of dimethyl sulfoxide (DMSO, Solarbio, Beijing, China) was introduced to each well, after which the plate was shaken at 25 °C for 10 minutes until all solid components were completely dissolved. Absorbance readings of the samples were taken at 490 nm using a microplate reader (BioTek Gen5, USA). The calculation for cell viability was performed using the equation:

$$\text{Cell viability (\%)} = (A_s/A_c) \times 100$$

where A_s is the optical density of the sample and A_c is the optical density of the control.

2.7. Nitric oxide (NO) assay

Cells of the RAW264.7 line were plated in 96-well plates at a density of 5×10^4 cells per well. The control group consisted of cells that were not subjected to any treatment. For negative control, the cells were dissolved in the Lipopolysaccharides (LPS, Solarbio, Beijing, China) solution. After 12 hours of incubation, the cells were exposed to different concentrations (0, 12.5, 25, 50, and 100 μ g/mL) of MFGM proteins for an additional 12 hours. This was succeeded by incubation either with or without LPS (1 μ g/mL) for 10 hours. The supernatants were subsequently gathered, and the levels of nitric oxide (NO) were evaluated utilizing the

Griess reagent (Beyotime Biotechnology, Shanghai, China). Absorbance readings were taken at 540 nm with a microplate reader, and concentrations were determined using a standard curve for sodium nitrite.

2.8. Molecular docking

The crystal structures of MFG EGF and factor V/VIII domain containing (A0AAA9RUL8, MFGE8, AlphaFold ID: AF-A0AAA9RUL8-F1), xanthine dehydrogenase/oxidase (P80457, AlphaFold ID: AF-P80457-F1), TLR2 (Q9QUN7, XDH, AlphaFold ID: AF-Q9QUN7-F1), and TLR4 (Q9QUK6, AlphaFold ID: AF-Q9QUK6-F1) were found in the Protein Structure Database (<https://www.alphafold.ebi.ac.uk/>). Finally, the HDOCK server (<http://hdock.phys.hust.edu.cn/>) was used for molecular docking analysis^[18].

2.9. Molecular dynamics (MD) simulations

Molecular dynamics (MD) simulations were conducted using the AMBER 22 software package to evaluate the structural stability of four protein–receptor complexes: MFGE8–TLR2, MFGE8–TLR4, XDH–TLR2, and XDH–TLR4. Prior to the production runs, energy minimization was performed in two stages: 2,500 steps of the steepest descent method, followed by 2,500 steps of the conjugate gradient algorithm to eliminate unfavorable contacts.

Subsequently, the systems were gradually heated from 0 K to 298.15 K over 200 ps under the NVT ensemble at a constant volume. During this stage, temperature was increased at a uniform rate to ensure system stability. Equilibration was carried out in two phases: first, a 500 ps NVT simulation was performed to allow for uniform distribution of solvent molecules; second, a 500 ps NPT simulation was conducted to equilibrate the entire system under isothermal–isobaric conditions.

Finally, a 100 ns production run was performed under NPT conditions with periodic boundary conditions. The non-bonded cutoff distance was set to 10 Å, and long-range electrostatic interactions were treated using the Particle Mesh Ewald (PME) method. The SHAKE algorithm was applied to constrain all bonds involving hydrogen atoms, allowing a 2 fs integration time step. Temperature coupling was achieved using the Langevin thermostat with a collision frequency (γ) of 2 ps^{−1}, and the system pressure was maintained at 1 atm. Simulation trajectories were recorded every 10 ps for subsequent analysis.

2.10. Statistical Analysis

The analysis of the data employed both one-way and two-way analysis of variance (ANOVA). A significance level of $P \leq 0.05$ was deemed statistically significant. The statistical evaluations were conducted utilizing GraphPad Prism version 9.4.1 and Origin 2021 software. Results are presented as means $\pm$ standard deviation (SD).

3. Results and discussion

3.1. Identification of MFGM proteins in ZM, DM, and CM

The MFGM proteins in ZM, DM, and CM were identified via 4D label-free proteomics. 3085 MFGM proteins were identified, with specific counts of 2254, 2788, and 2204 MFGM proteins found in ZM, DM, and

CM, respectively. As shown in Figure 1A, 1806 proteins were common to all three MFGM proteins, while 90, 571, and 69 unique MFGM proteins were identified in ZM, DM, and CM, respectively.

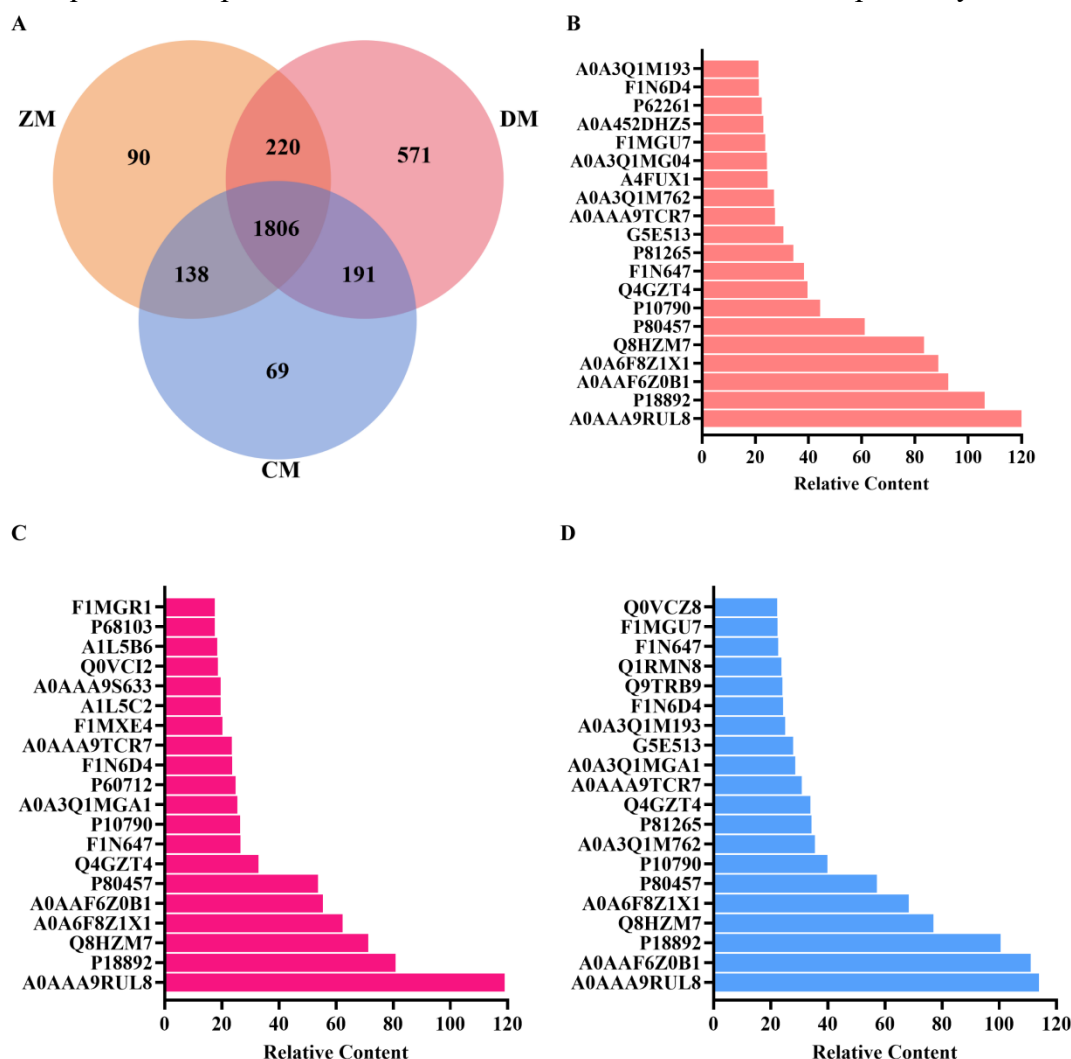


Figure 1. Venn diagram of the identified milk-fat-globule-membrane (MFGM) proteins in Zhongdian-yak milk (ZM), Diqing cattle milk (DM), and cattle-yak milk (CM); Top 20 high-abundance proteins in ZM (B), DM (C), and CM (D).

The top 20 high-abundance proteins identified in ZM, DM, and CM are shown in Figure 1B, C, and D, respectively. Eleven proteins, including MFG-E8 (A0AAA9RUL8), butyrophilin subfamily 1 member A1 (P18892), xanthine dehydrogenase/oxidase (P80457), and fatty acid synthase (F1N647), exhibited high abundance across ZM, DM, and CM. MFG EGF and factor V/VIII domain containing (MFG-E8) was the most abundant protein in all three samples. This protein exhibits a protective effect on the spontaneous fusion of MFGs in human milk and is essential for mammary gland development and phagocytosis of apoptotic cells [19, 20]. MFG-E8 also inhibits the progression of liver steatosis and inflammation, promotes the formation of muscle fibers, improves sarcopenia, and regulates the intestinal immune system of newborns [21, 22]. The subfamily 1 member A1 of butyrophilin (BTN1A1; P18892) enhances the secretion of milk fat and demonstrates specific immunomodulatory functions [16]. It plays an important role in milk fat secretion because of the continuous increase progression of the lactation cycle in Laoshan goat milk [15]. Xanthine dehydrogenase/oxidase (P80457) is closely related to oxidative stress, exhibits immune regulatory function,

and maintains metabolic homeostasis^[23]. Polymeric immunoglobulin receptor (P81265) has been identified in many kinds of milk and plays an important role in mucosal immunity as a transepithelial polyimmunoglobulin transporter^[14, 24]. These results indicated that the high-abundance proteins in ZM, DM, and CM exhibit immunomodulatory activity, indicating that the three kinds of milk can improve immunity.

3.2. Differentially expressed MFGM proteins in ZM, DM, and CM

The analysis of variations in MFGM proteins among the samples was conducted using principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) models. In the PCA model, the first and second components explained 35.56% and 18.16% of the total variance, respectively (Figure 2A). Conversely, in the OPLS-DA model, these components contributed 15.5% and 13.1% to the total variance (Figure 2B). The classification metrics R^2Y and Q^2 were found to be 0.998 and 0.704, respectively, suggesting that the OPLS-DA model demonstrated both reliability and satisfactory fit and predictability (Figure 2C). These results showed that the MFGM proteins in all three milk samples had good parallelism within the group, with marked differences between the groups. Elucidating the composition of MFGM proteins in different varieties of milk can help enhance our understanding of the functions of different milk varieties. Therefore, we screened differentially expressed MFGM proteins based on the following criteria: $P < 0.05$ and $FC \geq 1.2$ or ≤ 0.85 .

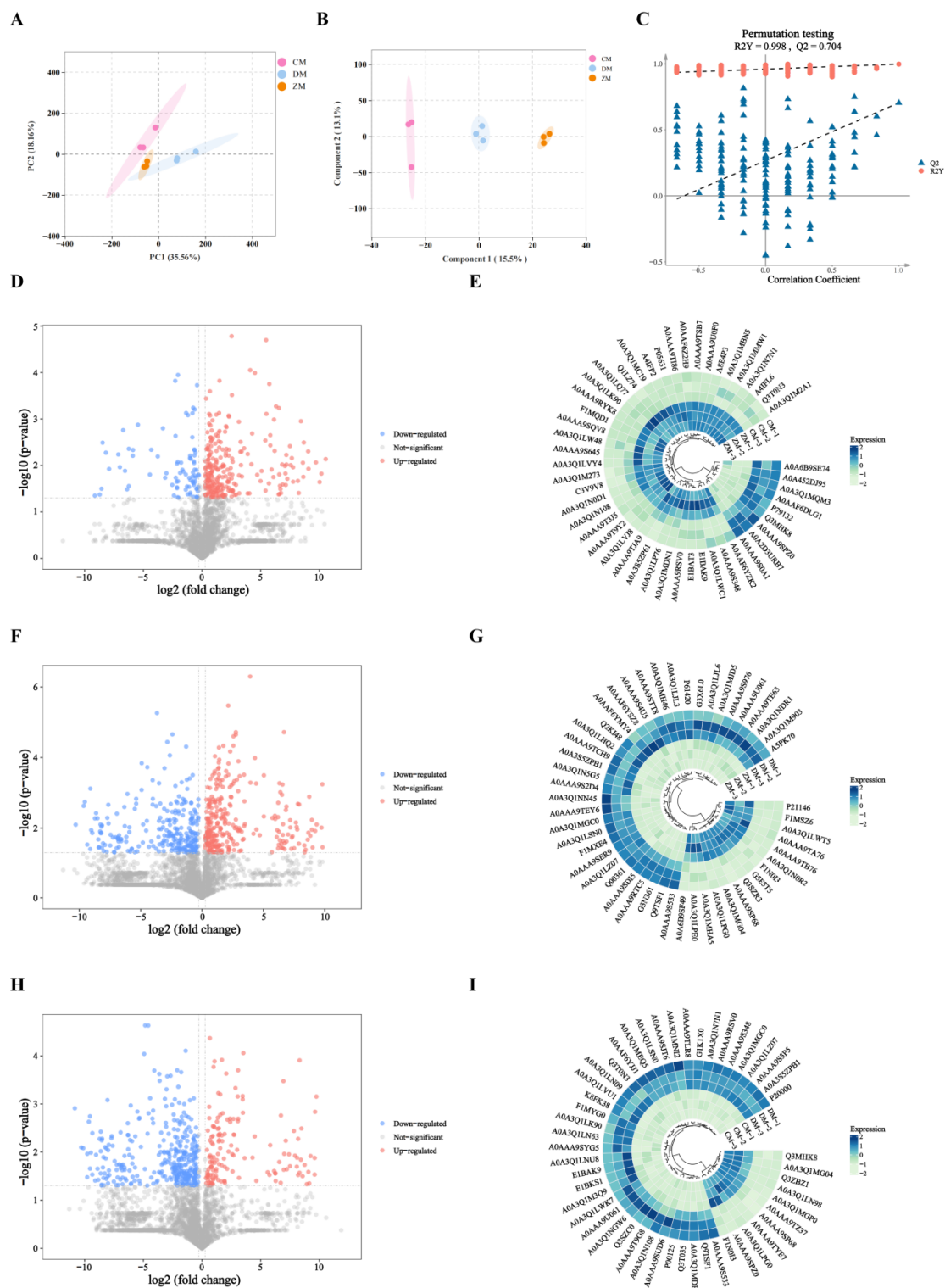


Figure 2. Differential expression of milk-fat-globule-membrane (MFGM) proteins across groups. Principal component analysis (PCA) score plots (A) and orthogonal partial least squares-discriminant analysis (OPLS-DA) (B) of MFGM proteins in Zhongdian-yak milk (ZM), Dqing cattle milk (DM), and cattle-yak milk (CM); OPLS-DA substitution test plot for total MFGM (C); Volcano plot of the identified MFGM proteins in CM vs. ZM (D); The cluster heat map of the top 50 MFGM proteins in CM vs. ZM (E); Volcano plot of the identified MFGM proteins in DM vs. ZM (F); The cluster heat map of the top 50 differential proteins in DM vs. ZM (G); Volcano plot of the identified MFGM proteins in DM vs. CM (H); The cluster heat map of the top 50 differential proteins in DM vs. CM (I).

As shown in Figure 2D, a total of 343 differential MFGM proteins were identified in ZM vs. CM. Among them, 257 and 86 were significantly upregulated and downregulated, respectively. Figure 2E shows the

clustering heat map of the first 50 proteins with significant differences between ZM and CM. Among them, A0A3Q1MC19 is involved in the regulation of osteoarthritis and protects cartilage [25]. A0A3Q1M2A1 can be recognized by CD4⁺ as an autoantigen and plays an immunoregulatory role [26]. AMPK is involved in the regulation of metabolic pathways underlying alcohol-induced liver injury via A0AAA9RSV0, and its upregulation prevents such injuries [27]. A0A3Q1MQM3 is activated in acinar cells, regulating the arachidonic acid metabolic pathway to prevent chronic pancreatitis [4]. Among the differential proteins between ZM vs. CM, the A0A3Q1MQM3 level in CM was significantly higher than that in ZM, indicating that the dietary supplementation of CM mediates better prevention of chronic pancreatitis than ZM. The A0A3Q1MC19, A0A3Q1M2A1, and A0AAA9RSV0 levels in ZM were significantly higher than those in CM, indicating that the MFGM proteins in ZM might exhibit better immune regulation and disease prevention activities.

As shown in Figure 2F, a total of 554 differential MFGM proteins were identified in ZM vs. DM. Among them, 291 and 263 were significantly upregulated and downregulated, respectively. Figure 2G shows the clustering heat map of the first 50 proteins with significant differences between DM and ZM. Butyrophilin (Q9TSF1) exhibits functional diversity and is involved in regulating T-cell response after antigen presentation [28]. Ig-like domain-containing proteins (G5E5T5, A0A3Q1LPG0, and A0AAA9SP68) belong to a novel B7-like protein family. They inhibit the production of proinflammatory cytokines in arthritis models and play an important immune regulatory role in inflammation models [29]. Coagulation factor V (F1N0I3) is a glycoprotein that plays an important role in the formation of prothrombinase complexes. Coagulation factor V deficiency might also lead to hemorrhagic diseases [30, 31]. F1N0I3, G5E5T5, A0A3Q1LPG0, and A0AAA9SP68 were highly expressed in ZM than in DM, indicating that the MFGM proteins in ZM might exhibit better immunomodulatory activity.

As shown in Figure 2H, a total of 487 differential MFGM proteins were identified in CM vs. DM. Among them, 147 and 340 were significantly upregulated and downregulated, respectively. Figure 2I shows the clustering heat map of the first 50 proteins with significant differences between CM vs. DM. Superoxide dismutase (A0AAA9U061) is an antioxidant enzyme that plays an important role in detoxification and maintaining redox balance. It is the first line of defense against reactive nitrogen species (RNS), reactive oxygen species (ROS), and other such potentially dangerous molecules [32]. Ubiquinol-cytochrome c reductase core protein 1 (G1K1X0) exhibits cardioprotective function [33]. The levels of A0AAA9U061 and G1K1X0 proteins in DM were significantly higher than those in CM, indicating that MFGM proteins in DM exhibit anti-oxidative and cardioprotective activities. In addition, the levels of proteins with immunomodulatory activity, such as coagulation factor V and Ig-like domain-containing protein, were higher in CM than in DM, indicating a better immunomodulatory activity of CM.

We also analyzed the functions of the differentially expressed proteins. Our results showed that MFGM proteins with varying functional activities existed in the three kinds of milk; however, their levels varied across the samples. The levels of immunoregulatory proteins in ZM were higher than those in CM and DM. In

addition, the immunoregulatory activity of the MFGM proteins in CM was higher than that of the proteins in DM.

3.3. Functional classification of differentially expressed MFGM proteins

The differentially expressed MFGM proteins were annotated to the related metabolic pathways using KEGG. The differential MFGM proteins between CM and ZM were mainly involved in Ribosome, Endocytosis, and Proteasome (Figure 3A). Among them, Endocytosis; Fc gamma R-mediated phagocytosis; Phagosome, Complement, and coagulation cascades; Chemokine signaling pathway; AMPK signaling pathway; and six pathways were related to immune regulation. As shown in Figure 3B, a total of 40 MFGM proteins were involved in these five immune-related pathways in CM and ZM. Among them, 28 immune-related proteins were upregulated in ZM.

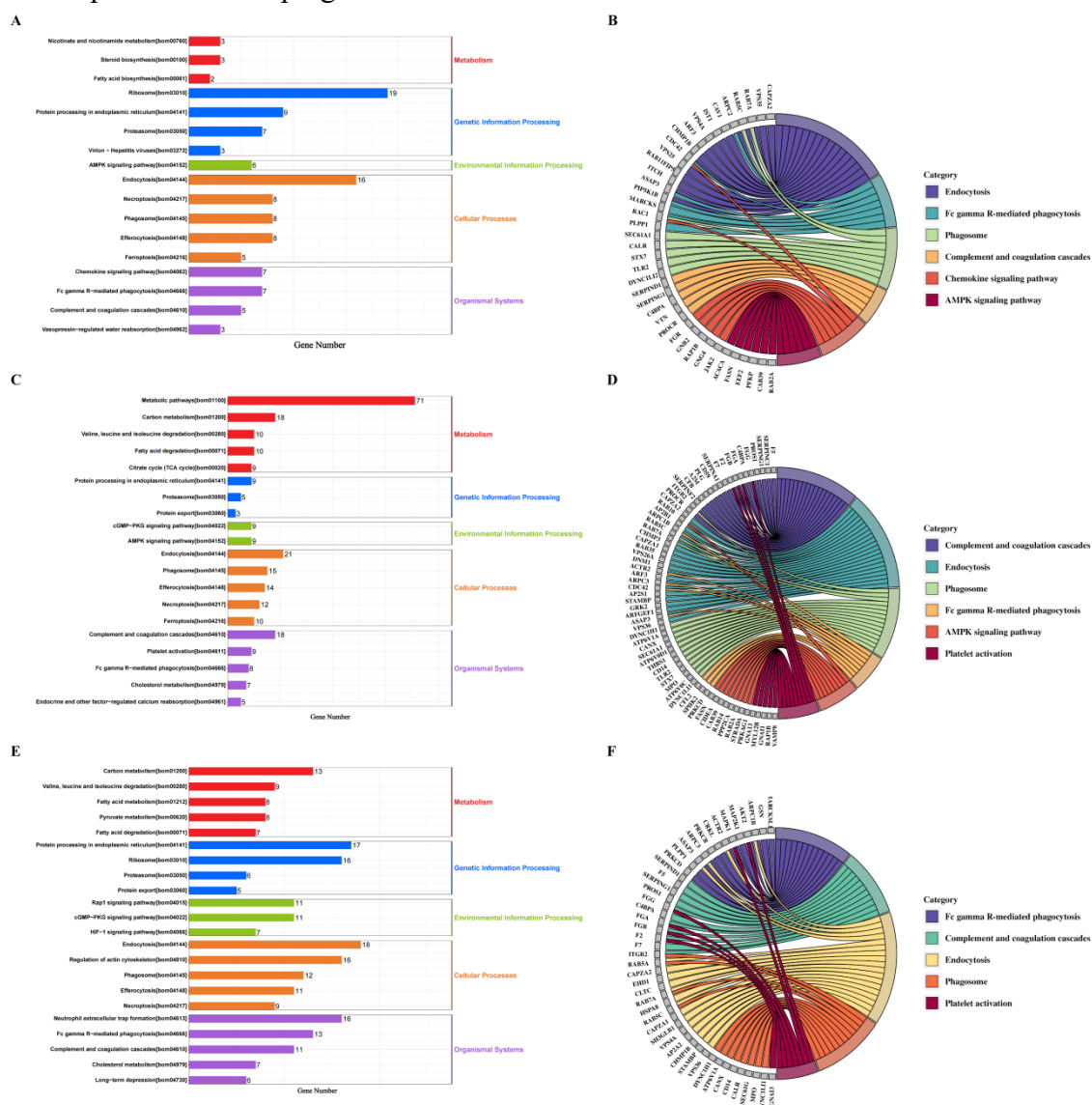


Figure 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed milk-fat-globule-membrane (MFGM) proteins in cattle-yak milk (CM) vs. Zhongdian-yak milk (ZM) (A); Immune-related proteins and pathways in CM vs. ZM (B); KEGG pathway analysis of differentially expressed milk-fat-globule-membrane (MFGM) proteins in Diqing cattle milk (DM) vs. Zhongdian-yak milk (ZM) (C); Immune-related proteins and pathways in DM vs. ZM (D); KEGG pathway analysis of differentially expressed milk-fat-globule-membrane (MFGM) proteins in Diqing cattle milk (DM) vs. cattle-yak milk (CM) (E); Immune-related proteins and pathways in DM vs. CM (F).

The differential MFGM proteins between DM and ZM were primarily involved in metabolic pathways such as Endocytosis, and Metabolic pathways (Figure 3C). As depicted in Figure 3D, 67 proteins that showed differential expression were mapped to pathways associated with the immune system. These include the Complement and coagulation cascades, Endocytosis, Phagosome formation, Fc gamma R-mediated phagocytosis, AMPK signaling pathway, and activation of Platelets. Among them, 40 immune-related proteins were upregulated in ZM.

The differentially expressed MFGM proteins identified between DM and CM were predominantly associated with fundamental biological processes including metabolic pathway regulation, endocytotic mechanisms, endoplasmic reticulum-related protein maturation, along with other critical metabolic functions (Figure 3E). As shown in Figure 3F, a total of 61 differential proteins were annotated to immune-related pathways, including Fc gamma R-mediated phagocytosis, Complement and coagulation cascades, Endocytosis, Phagosome, and Platelet activation. Among them, 31 immune-related proteins were upregulated in DM, respectively.

In general, the MFGM proteins in ZM, DM, and CM were annotated to the metabolic pathways related to immune regulation. Recent studies have shown that MFGM proteins differ markedly among mammalian species and are closely related to immune regulation. Zhao et al. compared MFGM proteins between human and porcine milk and identified 3920 and 4001 MFGM proteins, respectively, with 956 differentially expressed proteins, indicating that species-specific MFGM protein profiles may contribute to differences in nutritional and immune functions^[7]. This is consistent with our finding that ZM, DM, and CM all contained immune-related MFGM proteins, but their abundance and pathway involvement differed among milk types. Through KEGG annotation, it was found that the MFGM proteins in the three milk varieties exhibited immunomodulatory activity. Among them, the MFGM proteins in ZM were more involved in the immunoregulatory metabolic pathways than those in DM and CM. In addition, ZM exhibited higher levels of immune-related proteins than DM and CM.

3.4. The immunoregulatory role of MFGM proteins

Macrophages exhibit their immunoregulatory function via the production of various cytokines and growth factors, antigen presentation, and phagocytosis. These cells play a key role in the occurrence, maintenance, and elimination of inflammation and are key to regulating the inflammatory response^[34]. The present study found that the MFGM proteins in all three kinds of milk exhibited immunomodulatory activity. Subsequently, we mechanistically investigated the immunomodulatory properties of MFGM proteins by employing a murine macrophage model. Specifically, we evaluated their effects on macrophage proliferation capacity and quantified the production of nitric oxide (NO), a key immunoregulatory mediator.

As shown in Figure 4A, after treatment with 12.5–100 µg/mL of MFGM proteins, the RAW264.7 cells could reach >80% confluence, indicating that MFGM proteins were not toxic to RAW264.7. Therefore, we conclude that trace SDS residues, if any, were present at levels too low to affect cell viability or interfere with NO production in RAW264.7 cells. NO plays a central role in several physiological processes, such as

vasodilation, neurotransmission, inflammation, apoptosis, and tumor growth. The release of NO is the second step in the resistance of macrophages to pathogen infection, and its secretion level is widely used to evaluate the immunomodulatory activity of macrophages^[35]. As shown in Figure 4B, the MFGM proteins in ZM, DM, and CM markedly promoted NO secretion in RAW264.7 cells ($P < 0.05$). Previously, Wu et al. also found that the novel immunoactive peptide ECFSTA, as an immunomodulator, can promote NO production in RAW264.7 cells^[36]. Moreover, Kramer et al. recently reported that bovine MFGM-enriched whey exhibited antiviral activity against rotavirus, respiratory syncytial virus, and SARS-CoV-2 in vitro, suggesting that MFGM components may strengthened by linking back to the specific pathways identified (e.g., complement and coagulation cascades)^[37]. MFGM proteins significantly induced NO production in RAW264.7 macrophages, and together with the TLR2/TLR4 interaction results, this suggests that MFGM proteins may activate innate immunity via TLR2/TLR4. Nevertheless, whether this response is pro-inflammatory or regulatory remains to be determined by cytokine analysis in future studies. Therefore, MFGM proteins from ZM, DM, and CM with higher immune-related activity, may serve as promising bioactive ingredients for the development of functional dairy products and nutritional formulations with immunomodulatory potential.

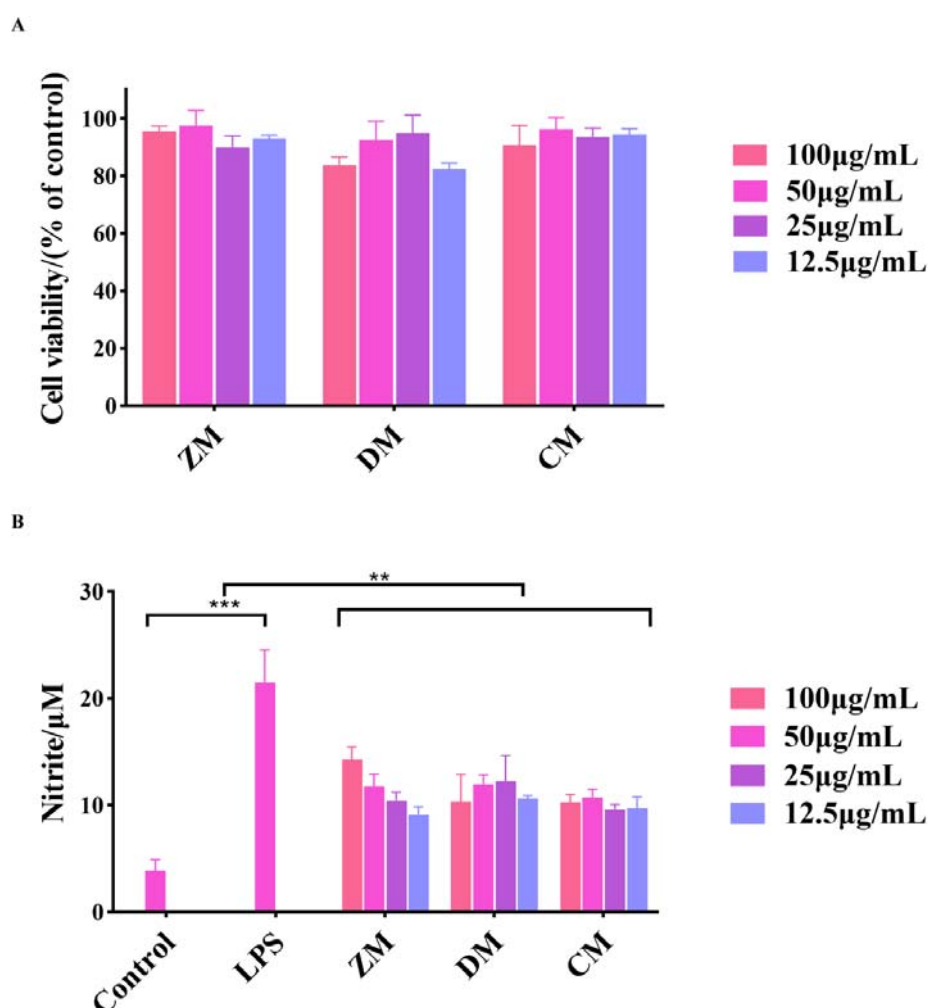


Figure 4. Effects of milk-fat-globule-membrane (MFGM) proteins on the cell viability of RAW264.7 cells; (B) Effects of MFGM proteins on nitric oxide (NO) production in RAW264.7 cells (** $P < 0.01$, *** $P < 0.001$)

3.5. The immunoregulatory Mechanism of MFGM proteins

TLR is a common receptor in macrophages. It is the first line of defense against pathogen invasion and plays an important role in immunity. Ligand binding to TLRs can activate the corresponding immune-related pathways, playing an immunoregulatory role [38]. Therefore, two MFGM proteins (MFGE8 and XDH) with high abundance in all three kinds of milk were further docked with TLR2 and TLR4 by molecular docking to explore whether MFGM proteins play an immunoregulatory role by binding to these receptors. MFGE8 and XDH docked with TLR2 (with docking energies -9.8 and -5.9 kcal/mol, respectively) and TLR4 (with docking energies -27.6 and -8.9 kcal/mol, respectively). Hydrogen bonding interactions occur between residues D42, Y66, L68, S344 on the MFGE8 protein and residues S354, D327, N379, Y376, and S42 on the TLR2 (Figure 5A). Hydrogen bonding interactions occur between residues K130, T177, R102, H148, K145, and K142 on the MFGE8 protein and residues N341, S317, Y660, S218, E951, E125, and S123 on the TLR4 (Figure 5B). Hydrogen bonding interactions occur between residues T207, and E570 on the XDH protein and residues N345, and K490 on the TLR2 (Figure 5C). Hydrogen bonding interactions occur between residues K569, E200, E199, K165, and K318 on the XDH protein and residues N293, K263, Q339, K360, H429, N529 on the TLR4 (Figure 5D). MFGE8 and XDH interacted with TLR2 and TLR4 through hydrogen bonds and salt bridges, and the hydrogen bonds serve as the primary interaction force between proteins. Consequently, it is hypothesized that TLR2 and TLR4 may be the central target in the influence of MFGM proteins (MFGE8 and XDH) on immunity.

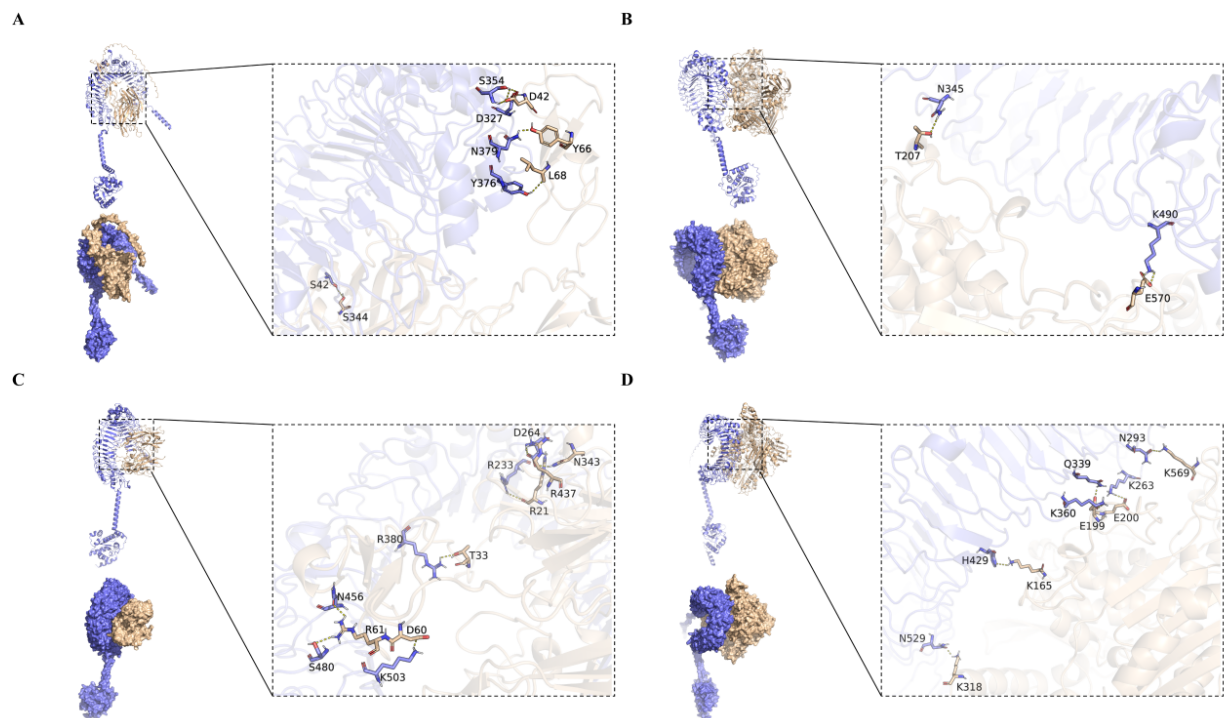


Figure 5. Molecular docking analysis of the binding mode of two milk-fat-globule-membrane (MFGM) proteins. The interactions of MFGE8 with toll-like receptor 2 (TLR2) (A); The interactions of MFGE8 with TLR-4 (B); The interactions of XDH with TLR2 (C); The interactions of XDH with TLR-4 (D).

Molecular dynamics (MD) simulations offer valuable insights into the interaction mechanisms between biomolecules, such as proteins or peptides, and their ligands, facilitating a deeper understanding of the

dynamic properties of biomolecular systems and the stability of ligand binding [39]. MD simulations can be employed to investigate the potential of milk fat globule membrane (MFGM) proteins to form stable complexes with immunoregulatory receptors and to comprehensively elucidate the intramolecular interactions at the binding sites responsible for the immunomodulatory functions of MFGM proteins.

The interaction between milk fat globule membrane proteins and key immune regulatory receptors was analyzed through molecular dynamics simulations over a 100-nanosecond timeframe. The interaction mechanisms were revealed based on RMSD, RMSF, Rg, SASA, and binding free energy (ΔG_{bind}). RMSD reflects the overall displacement of atoms in space and is commonly used to assess the stability and convergence of a complex's structure. As shown in Figure 6A, the RMSD variations of the complexes MFGE8_TLR2, MFGE8_TLR4, XDH_TLR2, and XDH_TLR4 during the molecular dynamics simulation were examined. The results indicate that initially, the RMSD values of all four complexes increased continuously with the simulation time. The RMSD fluctuation of the MFGE8_TLR2 complex was relatively large, while the MFGE8_TLR4 complex exhibited a gradual increase, stabilizing at around 12 Å after approximately 60 ns, indicating a certain level of dynamic stability. The RMSD fluctuations of the XDH_TLR2 and XDH_TLR4 complexes were smaller, stabilizing below 8 Å, suggesting that the overall conformation of the XDH-related complexes was more stable. These RMSD results indicate that the overall structure of the complexes formed by milk fat globule membrane proteins MFGE8 and XDH with TLR2 and TLR4 is stable, with XDH binding more stably to TLR2 and TLR4 compared to MFGE8.

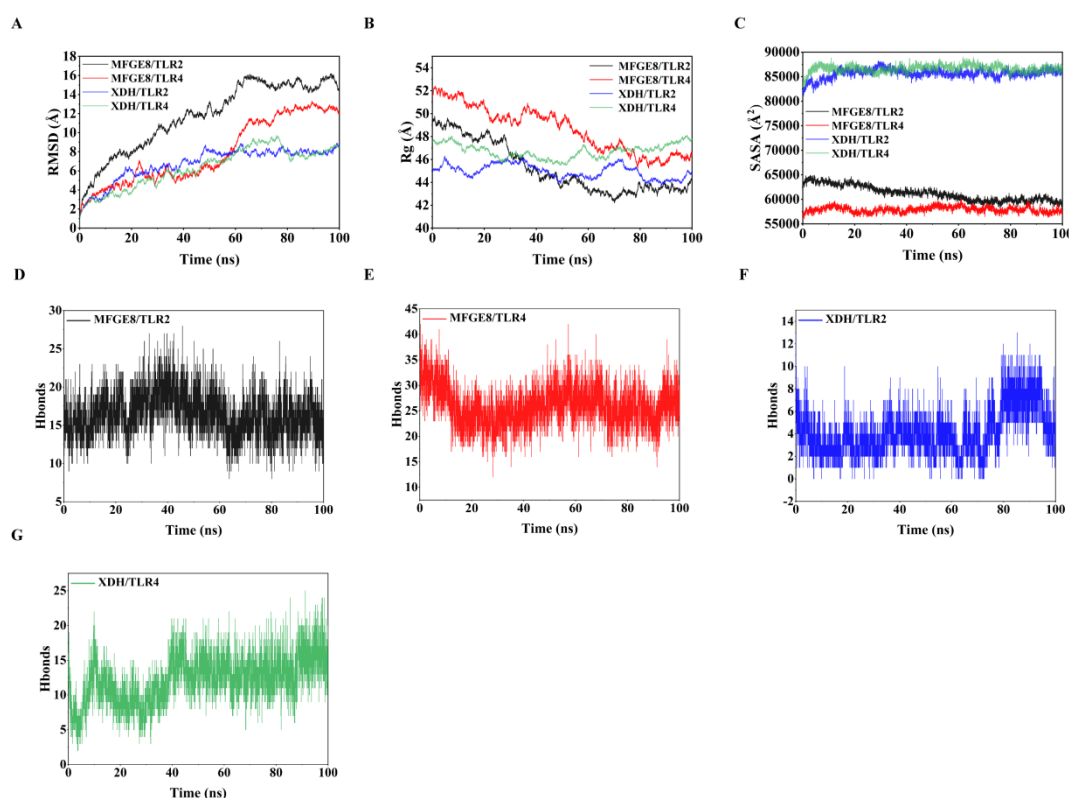


Figure 6. The molecular dynamics simulations (100 ns) of complexes between two milk-fat-globule-membrane (MFGM) proteins and toll-like receptors. Root mean square deviations (RMSD) of the complexes (A); Radius of gyration (Rg) of the complexes (B); Solvent accessible surface area (SASA) the complexes (C); Number of hydrogen bonds in MFGE8/TLR2 (D); Number of hydrogen bonds in MFGE8/TLR4 (E); Number of hydrogen bonds in XDH/TLR2 (F); Number of hydrogen bonds in XDH/TLR4 (G).

The radius of gyration (Rg) reflects the compactness of the overall structure of the complex and its variation can be used to evaluate the conformational stability of the complex. As shown in Figure 6B, the Rg value of the MFGE8_TLR4 complex continuously decreased, suggesting that its structure became increasingly compact, which implies some structural contraction during the simulation. In contrast, the Rg values of the XDH_TLR2 and XDH_TLR4 complexes were relatively low (around 43–46 Å) and fluctuated minimally, indicating that the XDH-related complexes maintained a more compact and stable conformation during the simulation.

The solvent accessible surface area (SASA) represents the extent to which the surface of the complex is exposed to the solvent and can be used to evaluate the openness and stability of its conformation. From the data, it can be observed that the SASA value of the MFGE8_TLR4 complex was significantly lower than that of the other complexes, indicating that it had a more compact structure with a smaller surface exposed to the solvent. The SASA values of MFGE8_TLR2, XDH_TLR2, and XDH_TLR4 were all higher and fluctuated less, suggesting that these complexes maintained a relatively stable open conformation during the simulation. The SASA fluctuations of the XDH-related complexes were slightly larger, which may be related to dynamic conformational changes of the proteins.

Binding energy is an important metric for assessing the stability of protein-ligand complex interactions. It is calculated as the sum of van der Waals forces, electrostatic forces, solvent-accessible surface area energy, and polar solvation energy. The binding energies of the MFGE8_TLR2, MFGE8_TLR4, XDH_TLR2, and XDH_TLR4 complexes were -78.39 ± 12.38 , -171.81 ± 20.41 , -25.35 ± 8.54 , and -92.97 ± 7.32 kcal/mol, respectively. The negative values indicate that these complexes exhibit binding affinity, with lower values indicating stronger binding. The RMSD, Rg, SASA, and binding energy results all suggest that both MFGE8 and XDH bind stably with TLR2 and TLR4, with XDH binding more stably than MFGE8.

Hydrogen bonds play a critical role in maintaining the structural stability of complexes and mediating intermolecular interactions. Therefore, in this study, the number of hydrogen bonds and the key amino acid residues involved in the interactions within the MFGE8_TLR2, MFGE8_TLR4, XDH_TLR2, and XDH_TLR4 complexes were further analyzed. As shown in Figures 6D – G, the trends in the number of hydrogen bonds formed during molecular dynamics simulations for each of the four complexes are presented. Among the complexes, MFGE8_TLR4 formed the highest number of hydrogen bonds throughout the simulation, consistently ranging between 20 and 35 with relatively low fluctuation, indicating a stable and sustained interaction network. The number of hydrogen bonds in the XDH_TLR4 complex was initially lower but gradually increased over time, eventually stabilizing between 10 and 20. This trend suggests the presence of adaptive conformational adjustments that facilitate protein-protein recognition and binding. For the XDH_TLR2 complex, the number of hydrogen bonds ranged between 2 and 10 and exhibited relatively large fluctuations, suggesting a more flexible but loosely associated interaction. The MFGE8_TLR2 complex formed 14 to 22 hydrogen bonds, with significant fluctuations observed during the simulation, indicating weaker intermolecular interactions and limited binding stability. Overall, the MFGE8_TLR4 complex

demonstrated the highest and most stable number of hydrogen bonds among all complexes, supporting the presence of a robust and persistent interaction interface.

These findings highlight the specific residue-level interactions that underpin the stable association between milk fat globule membrane proteins and immune receptors TLR2 and TLR4. Hou et al. found that rutin plays a key role with TLR-4 receptor and regulates downstream signaling pathways, thereby reducing inflammatory responses^[40]. Previously, Wu et al. found that TLR2 and TLR4 are the main receptors of ECFSTA, and they activate macrophages to produce NO, IL-6, and TNF- α , exerting immunomodulatory activity^[36]. Bai et al. further reported that proteomic and phosphoproteomic differences in human and bovine MFGM were associated with immune-related biological processes, indicating that MFGM proteins may play important roles in immune regulation^[41]. Thus, these results showed that the high-abundance MFGM proteins MFGE8 and XDH in milk could bind to TLR2 and TLR4, mediating downstream signaling pathways, and playing immunoregulatory roles. Therefore, the interaction between MFGE8/XDH and TLR2/TLR4 may contribute to macrophage activation and downstream immune responses, the stronger and more stable binding of XDH and MFGE8 to TLRs provides a mechanistic basis for the observed NO release. Further studies using receptor inhibitors or related approaches are needed to further clarify the underlying immunomodulatory mechanisms. These findings not only support the immunomodulatory potential of MFGM proteins but also provide a theoretical basis for the development of MFGM-enriched functional dairy products or nutritional ingredients.

4. Conclusions

In summary, 3085 MFGM proteins were identified in ZM, DM, and CM. All three kinds of milk were rich in MFGM proteins with functional activities such as immunoregulatory activity, improvement of sarcopenia, and regulation of neonatal intestinal immune system. The differential proteins were mainly annotated in immune regulation-related pathways, such as Complement and coagulation cascades, Endocytosis, and Phagosome. The MFGM proteins in ZM have more immunoregulatory activities compared to DM and CM. In addition, *in vitro* cell experiments, molecular docking, and molecular dynamics suggested that the MFGM proteins bound to TLR2 and TLR4, stimulating RAW264.7 cells to produce NO and exert immunomodulatory activity. The MFGM protein from the three kinds have the potential to develop immunoregulatory products. Our results provided valuable insights into the functions of MFGM proteins in ZM, DM, and CM and provided a theoretical basis for the optimal deep processing of the three kinds of milk during production.

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Ethical statements

In this study, the researchers did not contact the animals directly, and the milk was collected through a milking device. The experimental protocol involving milk collection was in accordant with the national law of China. Animal-related experiments strictly abide by the guidelines for animal experiments of Yunnan Agricultural University and approved by the Ethics Committee of Life Science of Yunnan Agricultural University.

Conflict of interest form

We declare that we do not have any personal, financial, commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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