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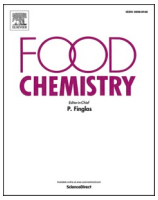

Impact of manufacturing processes on glycerolipid and polar lipid composition and ultrastructure in infant formula

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ABSTRACT

The introduction of exogenous lipids in the production of infant formula induces significant alterations in milk lipid composition, content, and membrane structure, thus affecting the lipid digestion, absorption, and utilization. This study meticulously tracks these changes throughout the manufacturing process. Pasteurization has a significant effect on phosphatidylcholine and sphingomyelin in the outer membrane, decreasing their relative contents to total polar lipids from 12.52% and 17.34% to 7.72% and 12.59%, respectively. Subsequent processes, including bactericidal-concentration and spray-drying, demonstrate the thermal stability of sphingomyelin and ceramides, while glycerolipids with arachidonic acid/docosahexaenoic acid and glycerophospholipids, particularly phosphatidylethanolamine, diminish significantly. Polar lipids addition and freeze-drying technology significantly enhance the polar lipid content and improve microscopic morphology of infant formula. These findings reveal the diverse effects of technological processes on glycerolipid and polar lipid compositions, concentration, and ultrastructure in infant formulas, thus offering crucial insights for optimizing lipid content and structure within infant formula.

1. Introduction

Milk lipids are crucial for providing approximately half of the energy required by rapidly growing infants. Glycerolipids and polar lipids (PLs) play a pivotal role in the healthy development of the brain, vision, and immune system, serving as effective carriers for the absorption of vitamins A, D, E, and K (Traves, 2019; Wang et al., 2020). In human and other mammalian milk, lipids typically exist as dispersed fat globules, each comprising a triacylglycerol (TAG) core surrounded by a three-layer PL membrane (Arranz & Corredig, 2017). This structure stabilizes the lipid core of the milk fat globule (MFG), prevents the flocculation and coalescence of fat globules, and protects the core from degradation by lipase (Hoffmann et al., 2021; Lu et al., 2021). These dispersed fat globules ensure the effective delivery and utilization of lipid nutrients and bioactive substances within the body (Lu et al., 2021). In cases where human milk is not available, infant formula becomes a vital nutritional source. The processing involved in infant formula production alters the size, zeta potential, interfacial composition, and structure of MFGs. This processing may also reduce the PL content in milk and disrupt the natural structure of MFGs, consequently modifying lipid digestion, affecting the functional properties of milk lipids, and potentially decreasing the nutritional value (Liang et al., 2017; Mou et al., 2022; Sun et al., 2023).

Essential technologies in infant formula production, such as

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; BCM, bactericidal-concentrated milk; Cer, ceramides; CM, clean milk; DHA, docosahexaenoic acid; DAG, diacylglycerol; EPA, eicosapentaenoic acid; FDP, freeze-dried powder; HM, homogenized milk; LA, linoleic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPLs, lysophospholipids; MFG, milk fat globule; MFGM, milk fat globule membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, polar lipid; PLS-DA, partial least squares-discriminant analysis; PM, pasteurized milk; SDM, standardized milk; SDP, spray-dried powder; SFA, saturated fatty acid; SIM, structured illumination microscopy; SM, sphingomyelin; TAG, triacylglycerol; VIP, variable importance projection.

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homogenization and heat treatment, have a significant impact on the MFG membrane (MFGM) structure and composition. Homogenization disrupts the original MFGM, thus leading to the formation of a new interfacial layer comprising milk proteins (caseins, whey proteins) and/or added phospholipids on the surface of fat globules (Lu et al., 2021; Zhao et al., 2022). This process results in smaller fat globules as the specific surface areas increase, yet decreases the natural MFGM protein and phospholipid on the recombined interfacial layer (Zhao et al., 2022). Heat treatment induces whey protein denaturation and its interaction with casein micelle on lipid droplets surface (Garcia et al., 2014). Furthermore, it promotes the binding of causes casein and whey protein to the MFGM surface via disulfide-interchange reactions, leading to MFGM structural changes (Zhang et al., 2022a). Additionally, heat treatment facilitates the hydrolysis and oxidation of milk lipids, and thus alters their composition and concentration (Zhang et al., 2022a). Freeze-dried powder contains a higher proportion of large fat globules and heat-sensitive nutrients compared to spray-dried powder and demonstrates intact ring-shaped layers or porous sheet-like structures at the fat globule interface, suggesting better retention of the original structure and morphology of fat globules and their membranes (Deshwal et al., 2020; Zhang et al., 2022b). The processing technology employed in milk processing significantly influences the characteristics of MFGs and their membranes, potentially impacting the nutritional quality of milk (Zhang et al, 2022b). While previous studies have compared the effects of processing technologies on lipids' physical properties, chemical changes, and digestion characteristics at the laboratory scale (Deshwal et al., 2020; Jia et al., 2022; Zhao et al., 2019), comprehensive studies on specific component changes in glycerolipids and PLs throughout infant formula production are limited. Analyzing infant formula samples from the production chain can provide deeper insights into the impact of process technology on glycerolipids and PLs (Tang et al., 2021). The addition of PLs during homogenization can participate in the recombination of MFGM, thus enhancing the stability of newly formed MFG and preserving more original membrane protein and structure (Lu et al., 2021). Furthermore, PL addition influences the physical structure of the new interface, as well as the chemical composition and content of lipids (Lu et al., 2021). Therefore, samples with and without the added PLs were selected from the production chain for analysis to understand these compositional and content differences.

Conventional light microscopy is inadequate for elucidating the structural and morphological features of MFGs throughout the entire production process of infant formula mainly due to its limitation in resolving structures at the nanometer scale. This limitation becomes particularly evident post-homogenization, where the size of MFGs is reduced to the order of nanometer, thus falling below the resolution capabilities of these techniques (Arizono et al., 2023). Super-resolution microscopy, which is exemplified by structured illumination microscopy (SIM), overcomes these constraints by enabling the observation of structures and morphologies at the nanoscale level, including dynamic and living tissue (Arizono et al., 2023). To date, SIM has not been employed to visualize the ultrastructure of MFGs and their membranes. The application of SIM shows significant promise to enhance our understanding of the structural and morphological transformations occurring at the nanoscale. Consequently, samples with and without PLs, excluding freeze-dried powder, were selected from production lines for detailed analysis. This analysis encompasses the composition and content of lipid subclasses and molecular species, as well as the super-resolved structure of MFGs.

This study investigates the effects of various technological processes—including pasteurization, standardization, homogenization, bactericidal concentration, spray drying, and lab-scale freeze drying—on the composition and concentration of milk glycerolipids and PLs. This analysis is conducted using the quadrupole time-of-flight mass spectrometry (AB Sciex, 6600). Furthermore, to the best of our knowledge, this is the first study to employ SIM to elucidate the impact of PLs and processing methods on the interfacial composition and structure of these components. Additionally, this study examines the impact of the addition of PLs on the content, composition, and structure of lipids. Our findings contribute to a deeper understanding on the effect of processing technology on lipid composition and content and clarify the role of exogenous PLs in enhancing and modifying lipids in infant formula. We anticipate that these insights will serve as a valuable reference for refining processing technology and improving the lipid quality in infant formulas.

2. Material and methods

2.1. Chemicals and reagents

All reagents utilized in this study were of mass spectroscopy grade and were acquired from Thermo Fisher Scientific, USA. The solid-phase extraction column, ProElut Silica (1 g/6 mL, CAT NO: 63006), was sourced from Dikma Scientific and Technical Corporation, USA. Standards, specifically TAG (16:0/18:1/18:1) and TAG (16:0/18:1/18:1 (d5)), were procured from Larodan AB, Sweden and Shanghai ZZBIO Co., Ltd., China, respectively. PL standards, including phosphatidylethanolamine (PE, 18:0/18:2 and 14:1/17:0), phosphatidylcholine (PC, 18:0/18:2 and 14:1/17:0), phosphatidylinositol (PI 16:0/18:1 and 14:1/17:0), phosphatidylglycerol (PG, 16:0/18:1 and 14:1/17:0), sphingomyelin (SM, d18:1/24:0 and d18:1/17:0), and ceramides (Cer, d18:1/24:1 and d18:2/24:0), were obtained from Avanti Polar Lipids Inc., USA. The organic molecular probes LIPI-QA, STARRED-DOPE, and Rhodamine B were sourced from Standard Imaging (Beijing) Biotechnology Co. Ltd., Beijing, China; Abberior, Germany; and Sigma-Aldrich, Germany, respectively.

2.2. Sample collection

Milk samples and milk powder for this study were sourced from Hebei Sanyuan Food Co., LTD, located in Hebei Province, China. The infant formula was produced using fresh raw bovine milk. Detailed parameters and procedures involved in the production of the infant formula are illustrated in Supplementary Fig. 1. To accurately represent the lipid changes during production, various samples were collected from the infant formula production line. These included clean milk (CM, N = 10), pasteurized milk (PM, N = 7), standardized milk (SDM, N = 10), homogenized milk (HM, N = 10), bactericidal-concentrated milk (BCM, N = 10), and spray-dried powder (SDP, N = 10). Additionally, freeze-dried powder (FDP, N = 10) was produced by freeze-drying bactericidal-concentrated milk at a laboratory scale. Notably, freeze-drying is not commonly used in industrial infant formula production due to high costly and time-intensive nature. The symbol N represents the number of sampling times. Liquid milk samples were divided into 2 mL aliquots, which were stored in frozen tubes or mixed with 2 mL frozen storage solution (PB180438, Procell) for lipid profiling and super-resolution imaging, respectively. In contrast, milk powder samples were only packaged in 2 mL frozen tubes for subsequent use. All samples were preserved in a − 80 °C refrigerator.

To ensure consistency and avoid the influence of milk source variability, raw milk batches were obtained from the same. The clean milk for both infant formulas (one with added PLs and the other without) underwent identical processing, therefore their differences were not compared in this study, similar to the pasteurized milk. Diverse nutrients, such as vegetable oils, structural lipids, or PLs, were added during standardization to adjust the nutritional composition of the infant formula. The samples named SDM I, HM I, BCM I, SDP I, and FDP I were enhanced with PLs, while the samples SDM II, HM II, BCM II, SDP II, and FDP II did not receive PL supplementation. Subsequently, the impact of adding PLs on lipid alterations across different processing stages was analyzed.

2.3. Extraction of glycerolipids and PLs from milk samples

The extraction of glycerolipids and PLs was performed according to a methodology established by Zhao et al. (2021). Standard substances were prepared at concentrations that were analogous to those found in human milk, using a methanol-dichloromethane solution (1:1, v/v) containing ammonium acetate (10 mM). For the procedure, 200 μL of milk or milk powder solution (1.3 g of milk powder dissolved in 9 mL of ultrapure water at approximately 45 °C, as per infant formula dilution guidelines) was combined with internal standards outlined in Supplementary Table 1. The mixture was then treated with 900 μL of dichloromethane, 2 mL of methanol, and 200 μL of ultrapure water, and shaken for 10 s. Following this, 900 μL of dichloromethane and 200 μL of ultrapure water were added to the mixture, which was then shaken for 10 s and centrifuged at 3,300 × g for 15 min to separate the aqueous and organic phases. This organic phase was then mixed with 600 μL of dichloromethane, 2.2 mL of methanol, and 1 mL of ultrapure water. The resulting mixture was centrifuged at 3,000 × g for 10 min to obtain the organic phase again. To the aqueous phase, 1.8 mL of dichloromethane was added, followed by centrifugation at 3,300 × g for 15 min to obtain the organic phase. The two organic phase extracts were mixed and subsequently dried under a nitrogen gas flow. The dried extract was reconstituted to a volume of 1 mL using a methanol/dichloromethane (1:1, v/v) solution with ammonium acetate (10 mM). A 50 μL aliquot of this extract was further diluted 200-fold with a methanol-dichloromethane (1:1, v/v) solution containing ammonium acetate (10 mM) to align the concentration within the detection range of the instrument for glycerolipid analysis.

For PL purification, the ProElut Silica (1 g/6 mL) solid-phase extraction column was activated with 3 mL of *n*-hexane. The activated column was loaded with 950 μL of the diluted sample and allowed to stand for 5 min. Non PLs were then eluted using *n*-hexane and diethyl ether mixtures in volume concentrations of 8:2 and 1:1 (3 mL each). PLs were eluted with 4 mL of methanol, allowed to stand for 5 min, followed by an addition of 2 mL of methanol and a dichloromethane-methanol–water mixture (2 mL total volume, 3:5:2, v/v/v) for another 5 min (Ali et al., 2017). The collected PL fractions were dried under a gentle nitrogen stream and reconstituted in 190 μL of a methanol-dichloromethane (1:1, v/v) solution containing ammonium acetate (10 mM) for PL analysis.

2.4. Milk glycerolipid and PL detection conditions

The separation of glycerolipids and PLs was conducted using ultra-performance liquid chromatography with Phenomenex liquid chromatography columns of dimensions 50 × 3 mm and 150 × 4.6 mm (Kinetex® 2.6 μm C18 100 Å), respectively. The chromatographic conditions were meticulously set for optimal analysis. The injection volume for both types of lipids was 2 μL. The column temperatures were maintained at 50 °C for glycerolipids and 30 °C for PLs, with flow rates of 0.3 mL/min and 0.8 mL/min, respectively. The mobile phase for the analysis consisted of two components: mobile phase A comprising a solution of ammonium acetate (5 mM) in a methanol/acetonitrile/water mixture (1:1:1, v/v/v), and mobile phase B containing a solution of ammonium acetate (5 mM) in isopropanol. The detailed elution gradient for this analysis is provided in Supplementary Table 2 (Liu et al., 2022).

For the detection of glycerolipids and PLs, quadrupole time-of-flight mass spectrometry (AB Sciex, 6600) equipped with electrospray ionization was utilized. The detection modalities varied based on the lipid class: glycerolipids and sphingolipids were detected in positive ionization mode, while glycerophospholipids were detected in negative ionization mode. The mass spectrometry conditions were carefully optimized within a scan range of 50–1300 Da, ion source gas pressure of 60 psi, curtain gas pressure of 35 psi, ion source temperature of 650 °C, declustering potential set to + 80 V and –80 V for positive and negative modes, respectively, collision energy of + 10 V and –10 V, and ion source spray voltage of + 5500 V for positive mode and –4500 V for negative mode.

2.5. Qualitative and quantitative analysis of milk glycerolipids and PLs

For qualitative and quantitative analysis of glycerolipid fragmentation ion data, the software MSDIAL was employed. The analysis parameters set in MSDIAL included: soft ionization for the ionization type, conventional LC/MS or data dependent MS/MS for the method type, profile data as the data type, positive ion mode for the ion mode, and lipidomics as the target omics. The identification parameters were meticulously defined as follows: retention time tolerance was set at 0.5 min, accurate mass tolerances for MS^1 and MS^2 were set at 0.01 Da and 0.05 Da, respectively, and the threshold for identification score was fixed at 80 %. In positive mode, glycerolipids were detected with NH_4^+ adduct ion. Neutral-loss fragments of fatty acyl compounds were detected as $[diacylglycerol]^+$(DAG) fragments, with TAGs identified from the positively charged molecular ions $[M + NH_4]^+$ and $[DAG]^+$ (denoted as $[M - RCOO + H]^+$). DAGs were similarly detected using the positively charged molecular ions $[M + NH_4]^+$ and $[monoacylglycerol]^+$.

PL data were qualitatively and quantitatively analyzed using the software Pearkview (version 2.2) and Multiquant (version 3.0.2), respectively. The database for all molecular species of PC, PE, PS, PI, SM, and Cer was established using Lipidview software. The detection data from quadrupole time-of-flight mass spectrometry were screened against the database. The customizable review settings in Pearkview included a mass error (ppm) of $\leq$ 10.0 and an isotope ratio % difference of $\leq$ 20.0. Glycerophospholipids with an "H^-" adduct and PC, with an adduct ion of "CH_3COO^-," were identified by the masses of the parent ion, two fatty acyl residues, and head group fragments in negative ion mode. The qualitative rule for lysophospholipids (LPLs) followed the same pattern as glycerophospholipids. Sphingolipids were detected by their parent ion and characteristic fragment ion masses at *m/z* 184, resulting from the neutral loss of collision-activated sphingolipids. Ceramides were identified by their parent ion mass and specific product ions at *m/z* 264 or 266, produced by the neutral loss of fatty acyl groups and dissociation of the amide bond. The regression equations derived from internal and external standards for quantifying concentrations are detailed in Supplementary Table 3 (Liu et al., 2022).

2.6. Structured illumination microscopy

The preparation of milk samples for super-resolution imaging involved specific labeling of glycerolipids, PLs, and proteins using organic molecular probes: LIPI-QA (Zhou et al., 2023), STARRED-DOPE, and Rhodamine B, respectively. These probes were dissolved in dimethyl sulfoxide to create stock solutions with a concentration of 1 mmol/L, which were stored at − 20 °C for subsequent use. The liquid milk and infant formula samples were first diluted in phosphate-buffered saline to achieve concentrations suitable for observation. The unhomogenized milk samples underwent a fourfold dilution, homogenized milk samples were diluted 100-fold, and milk powder (20 mg) was dissolved in 1 mL of phosphate-buffered saline and then further diluted 20-fold. For labeling, 19 μL of these diluted milk samples were mixed with 1 μL of stock solutions. The reaction was allowed to proceed at 25 °C with a relative humidity of 40–60 % for 15 min. Following the reaction, the samples were carefully transferred to a 3.5 cm bottom glass dish (STGBD-035–1) using a pipette for the preparation of super-resolution imaging.

Super-resolution images were acquired using a Polar-SIM super-resolution microscope (Airy Technologies) outfitted with an APO TIRF 100 × 1.49 numerical aperture oil-immersion objective lens. Polar SIM, characterized by its multidimensional polarization capability, surpasses deep SIM in resolving the molecular arrangement angle in super-resolution imaging. Lasers with wavelengths of 488 nm (180 mW), 561 nm (200 mW), and 647 nm (200 mW) were employed to efficiently

excite the LIPI-QA, STARRED-DOPE, and Rhodamine B probes, respectively. The structured illumination microscopy imagers were scaled and marked using Image-J software.

2.7. Statistical analysis

The concentration of glycerolipids and PLs is presented in Fig. 1 and Fig. 4A to illustrate specific changes in various lipid contents throughout the process of infant formula production. Concurrently, relative contents are utilized in Figs. 2–3, Fig. 4B–4F, and Fig. 5 to facilitate the comparison of relative changes in different lipid components under various treatments. The analysis of lipid species, concentration, and relative content is conducted using IBM SPSS Statistics software (version 25.0). The statistical methods employed include one-way ANOVA with Duncan's multiple tests for instances of equal variance, Tamhane's T2 for cases of unequal variance, and a nonparametric test when the distribution is not normal. The results are presented either as mean ± standard deviation or as a percentage. A threshold of $P < 0.05$ is set for statistical significance.

Partial least squares-discriminant analysis (PLS-DA) is a supervised classification technique that is widely used to classify samples based on multiple observed or measured variables. In this context, the PLS-DA is applied to classify bovine milk samples that underwent various processing steps, such as pasteurization, standardization, homogenization, bactericidal concentration, spray drying, and freeze drying. This classification is performed using MetaboAnalyst (https://www.metaboanalyst.ca/), a comprehensive tool for metabolomic data analysis and interpretation. PLS-DA and variable importance projection (VIP) scores are specifically utilized to categorize the glycerolipid and PL molecules in bovine milk processed differently, providing a detailed insight into how these processes affect the lipid profile of the milk.

3. Results and discussion

3.1. Variation of glycerolipids in differing processes

3.1.1. Molecular species number and subclass concentration of glycerolipids

Glycerolipids are crucial for the nutritional profile of milk, and they account for 98–99 % of lipid components in bovine milk, including TAGs and DAGs (Michalski & Januel, 2006). Fig. 1 displays the number and concentration of glycerolipid subclasses and the percentage of DAG in bovine milk processed through different stages. During the production of two infant formulas, there were no significant differences in the molecular species number, concentration of glycerolipid subclasses and the percentage of DAG in clean and pasteurized milk. This suggests that short-term pasteurization has a negligible effect on glycerolipid subclasses. The increase in glycerolipid species and content in standardized milk may be attributed to the addition of nutrients such as vegetable oil and structural lipids during standardization. The number of TAG molecular species significantly increased and then decreased with the treatment of bactericidal-concentration and drying, respectively. Bactericidal-concentration reduced the concentration of water in the milk, enabling the detection of TAGs originally below detection limits. High temperature or prolonged drying time may lead to oxidation or decomposition of several TAG molecules, thus reducing the number of TAG molecular species. Despite similar concentrations of glycerolipid subclasses in standardized and homogenized milk, the percentage of DAG in homogenized milk is significantly higher than in standardized milk. Moreover, the molecular species, concentration, and DAG percentage in bactericidal-concentrated milk are significantly higher than in homogenized milk. The increased DAG content and higher number of DAG species may be linked to the reduction of water during bactericidal concentration. However, the most likely cause for the rise in DAG percentage is the oxidation and hydrolysis of TAG. Lipid oxidation and hydrolysis are known phenomena during infant formula preparation, particularly when undergoing heat treatment (Dias et al., 2020; Zhang et al., 2022). Homogenization decreases the size of MFGs, enhance their specific surface area, disrupt their membrane structure, and expose the TAGs in the core to oxygen, thus facilitating their oxidation and hydrolysis. The process of bactericidal concentration further promotes the oxidation and hydrolysis of TAGs due to the prolonged duration and high temperature. Lipid oxidation typically occurs in the unsaturated fatty acid moiety of TAGs, leading to the formation of oxidized TAGs or other primary oxidation products (Dias et al., 2020; Phillips et al., 2023; Zeb, 2015). The hydrolysis of TAGs and oxidized TAGs produces of DAGs, free fatty acids, and oxidized free fatty acids (Phillips et al., 2023). Both homogenization and bactericidal concentration facilitated the oxidation and hydrolysis of TAGs, which, in turn, increases the DAG percentage.

Regarding the drying processes, both the freeze- and spray-drying did not significantly alter the molecular species, concentration of glycerolipid subclasses, or the DAG percentage. However, the molecular species and content of glycerolipid subclasses in freeze-dried powder were observed to be higher than those of the spray-dried powder. This suggests that while freeze-drying is effective in preserving glycerolipid species and their concentrations, the impact is not significant.

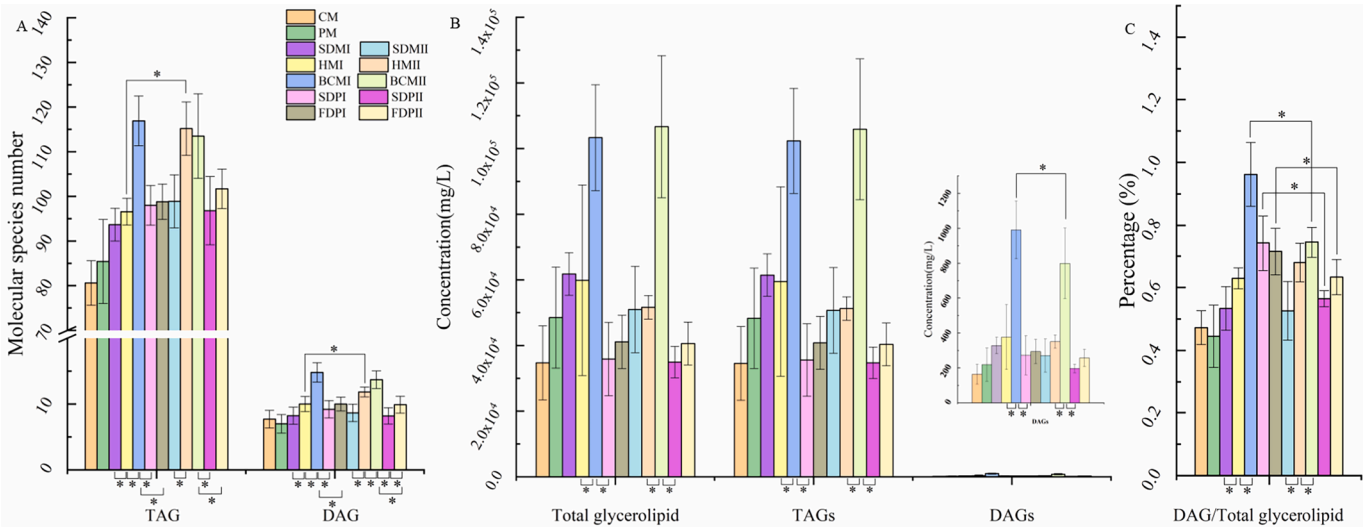

Fig. 1. Molecular species number (A) and concentration (B) of glycerolipid subclasses in bovine milk processed differently; DAG percentage of total glycerolipids (C). Error bars represent standard deviation; * represents significant difference ($P < 0.05$).

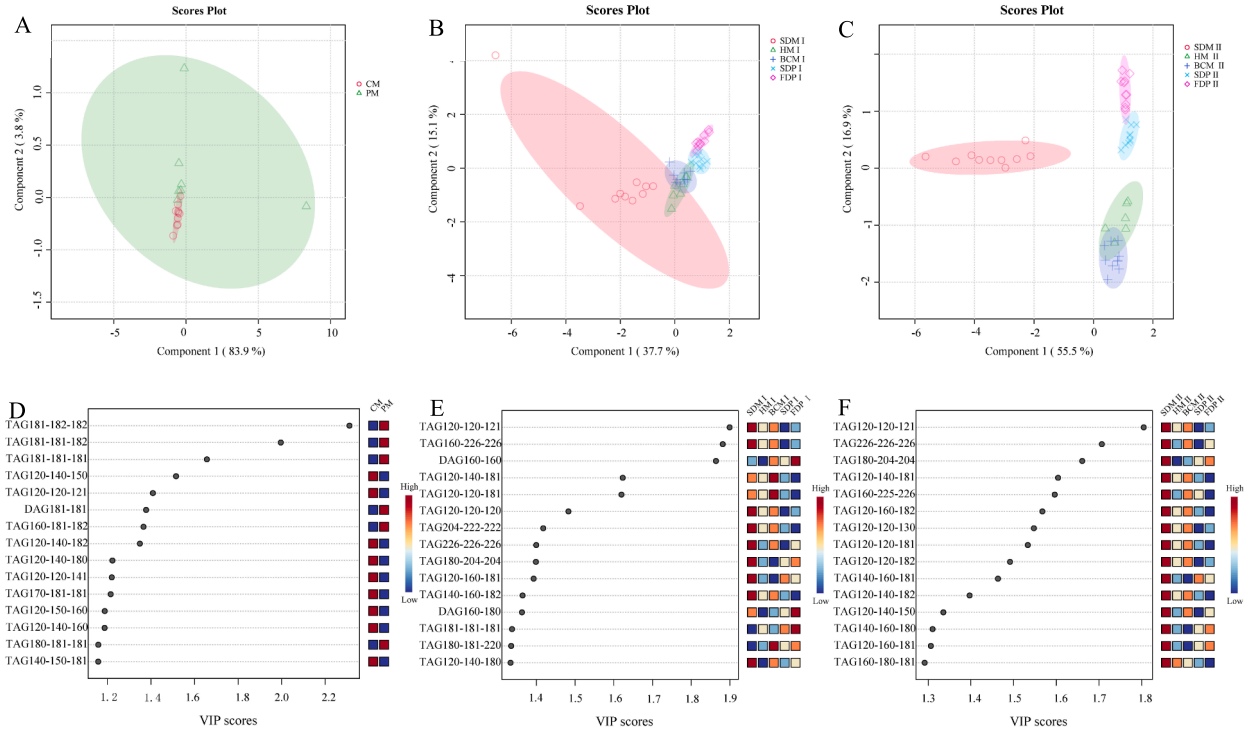

Fig. 2. PLS-DA plots and VIP scores for glycerolipid molecules in bovine milk processed differently. Plots for clean and pasteurized milk (A, D); for standardized, homogenized, bactericidal-concentrated milk, and spray-dried and freeze-dried powder of infant formula I (B, E); for standardized, homogenized, bactericidal-concentrated milk, and spray-dried and freeze-dried powder of infant formula II (C, F).

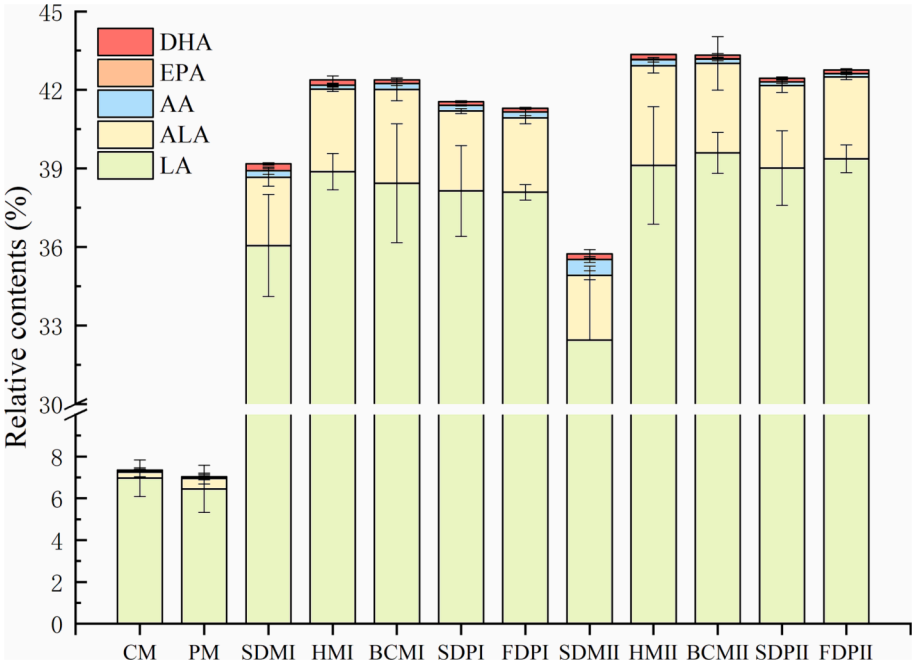

Fig. 3. Relative contents of glycerolipids containing LA, ALA, AA, EPA, and DHA in bovine milk processed differently.

Furthermore, we found that the addition of PL did not have a significant effect on the molecular species and concentration of glycerolipid subclasses in bovine milk subjected to homogenization, bactericidal concentration, spray-drying, or freeze-drying. This indicates that while PLs are an important component of MFGM, their addition does not have a significant impact on the glycerolipid profile of the milk during these processing stages.

3.1.2. *Composition and concentration of glycerolipid molecular*

The concentration of total glycerolipids and TAG in processed milk varies depending on the employed treatment (Fig. 1B). This variation can be attributed to the destruction of nutrients during processing and the reduction in moisture content caused by the processing methods. To more effectively differentiate the changes in the molecular distribution of glycerolipids, the relative content of different glycerolipid molecules

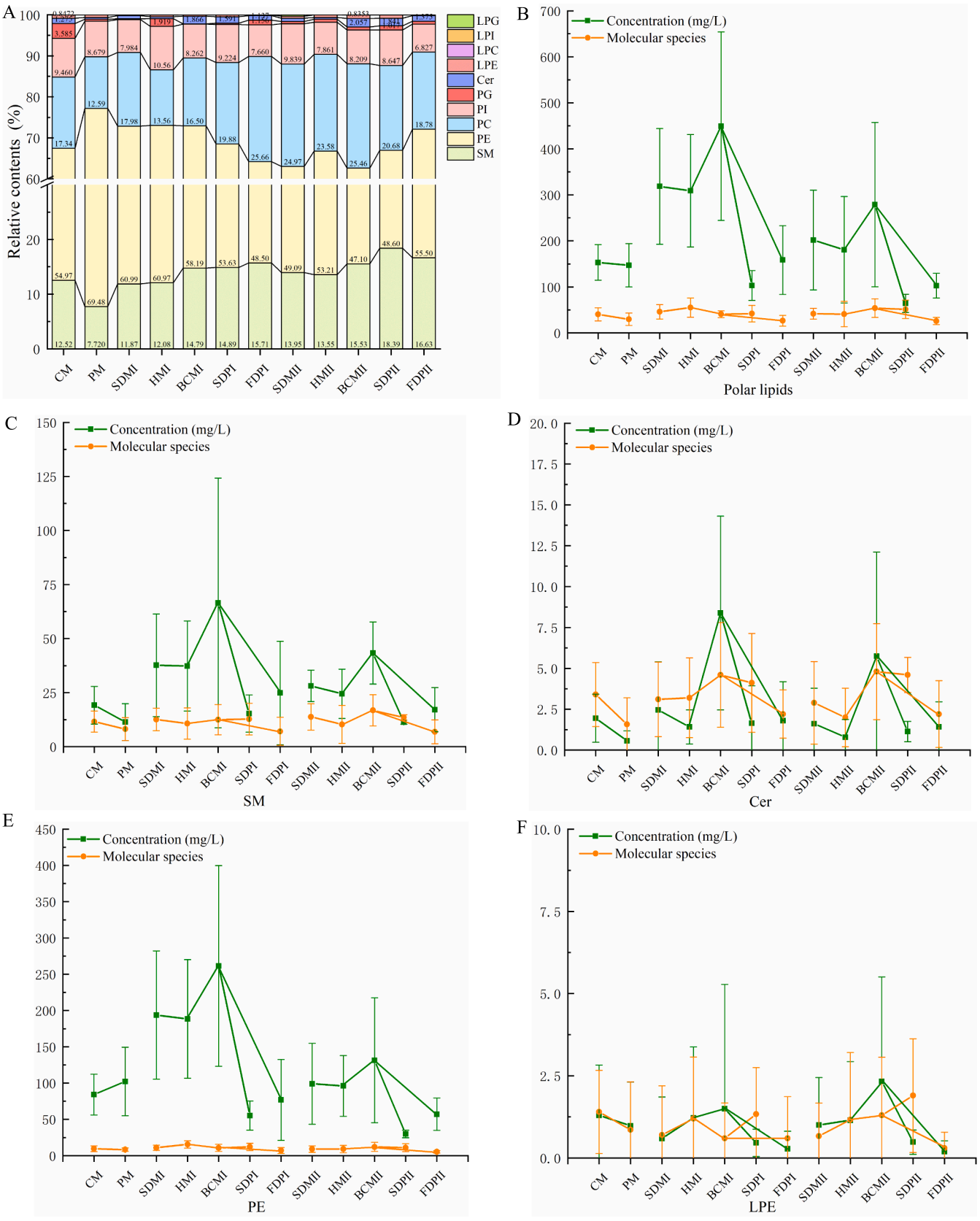

Fig. 4. Changes in relative contents of PL subclasses (A). Number of molecular species and concentration of PLs (B), SM (C), Cer (D), PE (E), and LPE (F) in bovine milk processed differently.

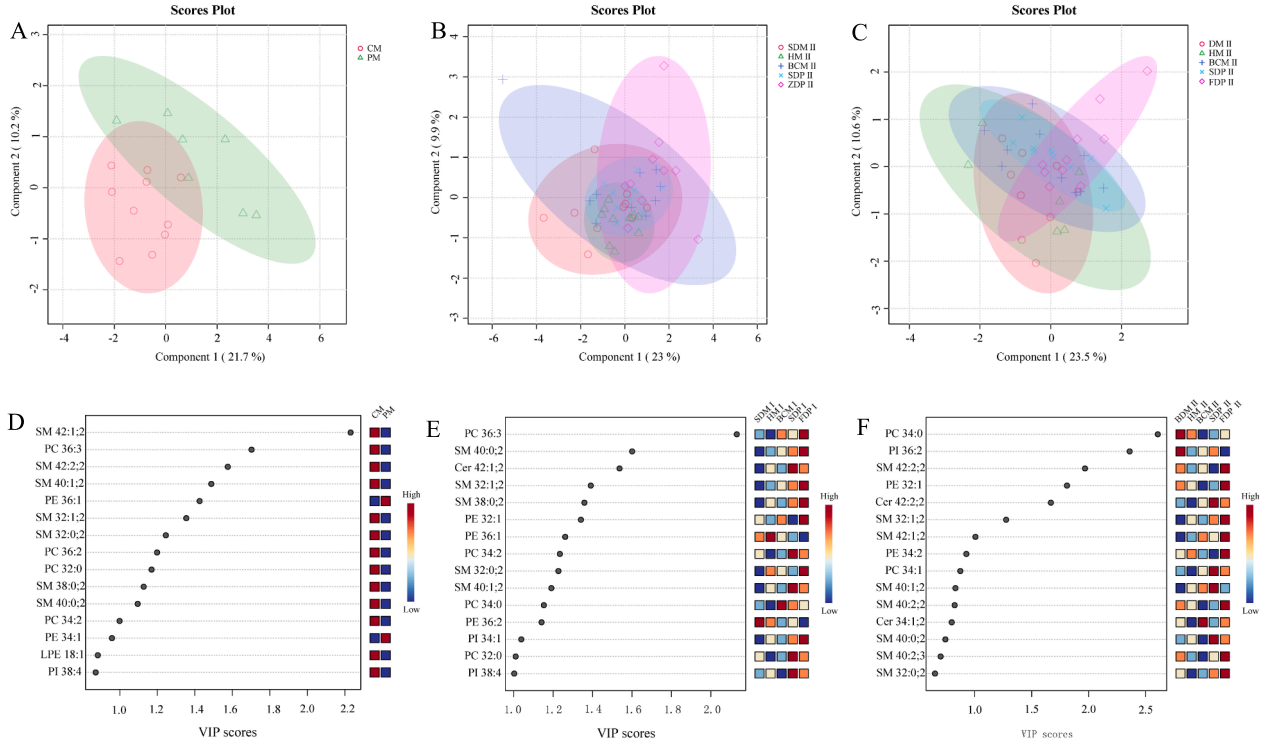

Fig. 5. PLS-DA plots and VIP scores for PL molecules in bovine milk processed differently. Plots for clean and pasteurized milk (A, D); for standardized, homogenized, bactericidal-concentrated milk, and spray-dried and freeze-dried powder of infant formula I (B, E); for standardized, homogenized, bactericidal-concentrated milk, and spray-dried and freeze-dried powder of infant formula II (C, F).

(with an identification frequency greater than 1 %) were analyzed. Fig. 2 displays the PLS-DA classification plots and VIP scores for bovine milk subjected to various processing steps. Notably, the model parameters R^2 and Q^2 in these analyses were above 0.4. The distribution patterns in clean and pasteurized milk overlapped, suggesting that pasteurization did not significantly alter the distribution of glycerolipid molecules in bovine milk. However, the distributions in homogenized and bactericidal-concentrated milk demonstrated relatively minor differences (Fig. 2B–2C). In contrast, significant differences in distribution were observed between bactericidal-concentrated milk and both spray- and freeze-dried powders (Fig. 2B–2C). This indicates that the drying processes have a more pronounced impact on the composition and concentration of glycerolipid molecules compared to bactericidal concentration. The top lipids with the greatest differences between these processed milk/powders, based on VIP scores, are listed in Fig. 2D–2F. These lipids were identified and used as markers for subsequent discussion. The glycerolipid molecules in infant formula II were more significantly affected by homogenization, spray-drying, and freeze-drying compared to those in infant formula I. Despite identical production methods for infant formulas with or without PLs, differences in the changes of glycerolipid molecules during processing were observed. This suggests that the molecular composition of the lipid component influences the alterations in glycerolipid components during the preparation of infant formula.

Infant formula is the primary source of nutrition for infants who are not breastfed, and it provides a comprehensive range of nutrients. Among these nutrients, glycerolipids play a crucial role. They not only supply energy for the rapid growth and development of infants but also serve as the primary carriers of functional fatty acids. These fatty acids are integral to the development of the infant's nervous system and immune regulation, and they provide antibacterial and anti-infective effects. Therefore, this study focused on analyzing the changes in glycerolipids containing functional fatty acids, such as linoleic (LA), α-linolenic (ALA), arachidonic (AA), eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids, during various processing stages of infant formula production. The relative contents of these functional fatty acid-containing glycerolipids in bovine milk subjected to different processing steps are depicted in Fig. 3. It was observed that the relative content of functional fatty acid-containing glycerolipids in pasteurized milk was significantly lower by 0.51 % compared to clean milk. The addition of vegetable oils or structural lipids typically increased the similarity of infant formulas to human milk in terms of glycerolipid composition and content, and were responsible for an increase of 33.08 % and 30.46 % in the relative content of functional fatty acid-containing glycerolipids in standardized milk I and II, respectively. Homogenization significantly increased the relative content of LA-containing glycerolipids. In this study, vegetable oils and structural lipids, which are glycerolipids rich in LA, were added to emulsions before homogenization. Homogenization ensures that lipids in the milk are uniformly dispersed and readily extractable, especially the newly added vegetable oils and structural lipids. This could potentially explain the higher proportion of LA-containing glycerolipids in homogenized milk compared to standardized milk. However, this hypothesis requires further experimental validation to fully confirm its accuracy.

We found that bactericidal concentration, spray drying, and freeze drying processes did not significantly affect the total relative content of functional fatty acid-containing glycerolipids (Fig. 3). However, a notable reduction was observed in the relative content of DHA-containing glycerolipids during these processes. Specifically, in infant formula I, the relative content of DHA-containing glycerolipids decreased from 0.26 % to 0.14 %, and in infant formula II, it dropped from 0.21 % to 0.13 % in (Fig. 4A). Additionally, we utilized VIP scores derived from the PLS-DA model to analyze differences in bovine milk subjected to various processing procedures. A key finding was that freeze-dried powder maintained higher levels of DHA- or AA-containing TAGs compared to spray-dried powder. For instance, in freeze-dried powder I, concentrations of TAG species such as TAG 16:0–22:6–22:6, TAG 22:6–22:6–22:6, and TAG 18:0–20:4–20:4 were higher than in

spray-dried powder I (Fig. 2E). Similarly, in freeze-dried powder II, TAG 22:6–22:6–22:6 and TAG 18:0–20:4–20:4 showed higher concentrations than in spray-dried powder II (Fig. 2F). These observations suggest that the freeze-drying process is more effective in preserving DHA- or AA-containing TAGs compared to the spray-drying process. Given the nutritional importance of DHA and AA, particularly in the context of infant formula, the loss of these TAGs during production should be carefully considered. This information is crucial for guiding the optimal addition of DHA and AA nutrients to ensure that infant formulas meet the requisite nutritional standards.

3.2. Variation of PLs in different processes

3.2.1. Molecular species number and subclass concentrations of PLs

PLs, comprising glycerophospholipids and sphingolipids, are primarily located on the MFGM, and they play a significant role in promoting neurological and cognitive development, regulating gastrointestinal lipid metabolism, and modulating intercellular signaling. They are essential for constructing a fat globule structure and ensuring emulsion stability (Brink & Lonnerdal, 2020). We found that standardization resulted in a significant increase in the total number of PLs in infant formula I. Although the total number of PLs in infant formula II also increased, this increase did not reach statistical significance (Fig. 4B), suggesting that the supplementation of PLs is beneficial for enhancing the PL species in infant formula. Despite the minimal impact of homogenization, bactericidal-concentration, and drying processes on the total number of PLs and PL subclasses, a gradual decrease in these numbers was observed following these treatments. Understanding changes in PL content during processing is crucial as it affects the physical and chemical properties of milk, thereby altering its physiological function. This information can further reveal that the added amount and proportion of PL in infant formula, and even provide ideas for the improvement of processing technology. Pasteurization reduced the concentration of PLs in bovine milk (Fig. 4B), while the glycerolipid content increased under the same treatment (Fig. 1B). This indicates that pasteurization has a more pronounced effect on PLs compared to glycerolipids, aligning with findings from previous studies on goat milk (Tan et al., 2023). Bactericidal concentration, by reducing the moisture content, led to increase in both glycerolipid and PL contents in bactericidal-concentrated milk (Fig. 1B and 4B). Glycerolipids increased by 72.55 % and 106.76 % in bactericidal-concentrated milk I and II, respectively, while PLs increased by 45.42 % and 54.30 % in the same samples. The relatively smaller increase in PLs indicates that they were more adversely affected by the bactericidal concentration treatment than glycerolipids. Additionally, the concentration of PLs in bactericidal-concentrated milk was significantly higher than in spray-dried and freeze-dried powder. The decline in PLs during drying was more pronounced (76.98–77.08 % in spray-dried powder and 63.14–64.72 % in freeze-dried powder) than that of glycerolipids (65.28–67.26 % in spray-dried powder and 60.23–61.94 % in freeze-dried powder). These findings highlight the differential impact of processing steps on glycerolipids and PLs in infant formula production. The observation that the PL content in freeze-dried powder was higher than in spray-dried powder (Fig. 4B) suggests that the drying process, which causes significant damage to PLs, results in better PL retention through freeze-drying compared to spray drying. Despite the destruction of PLs through oxidation and hydrolysis during processing, the species number and content of PLs and their subclasses in processed milk supplemented with PLs were found to be superior to those without PLs (Fig. 4B–4E). Consequently, it is recommended that infant formulas are supplemented with PLs to adequately meet the nutritional requirements of infants.

PE emerged as the most abundant PL in processed milk, both with and without additional PLs. Other PLs such as SM, PC, and PI were also present in significant amounts, whereas Cer, PG, lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), and lysophosphatidylglycerol (LPG) constituted only a small proportion of the processed milk (Fig. 4A). Pasteurization led to a reduction in the relative contents of SM (from 12.52 % to 7.72 %) and PC (from 17.34 % to 12.59 %) to the total PL, while increasing that of PE (from 54.97 % to 69.48 %) (Fig. 4A). This indicates that SM and PC are more sensitive to pasteurization than PE. Before pasteurization, the membrane structure of the MFG is intact, with SM and PC located in the outer bilayer of MFGM. Pasteurization, which is a high-temperature short-duration treatment, primarily destroys the outer membrane, thus resulting in the decrease of SM and PC (Huang et al., 2023). Post-bactericidal concentration treatment, the relative contents of SM and Cer increased significantly and were not substantially reduced by spray drying or freeze-drying (Fig. 4A). Compared to standardized milk, the concentrations of SM in spray-dried and freeze-dried powder decreased by 59.31–59.83 % and 33.99–39.27 %, respectively (Fig. 4C); meanwhile, the concentrations of Cer decreased by 29.71–33.31 % and 12.28–26.71 %, respectively (Fig. 4D). In contrast, the concentration of PLs decreased by 67.64–68.20 % and 49.09–50.25 %, respectively (Fig. 4B). These findings illustrate the varying degrees of sensitivity of different PLs to processing treatments and underscore the importance of supplementing infant formulas with PLs to maintain their nutritional quality.

Sphingosine is the primary sphingoid base in mammalian sphingolipids and binds to saturated fatty acids through amide linkage to form Cer. Here, SM is the predominant sphingolipid characterized by a phosphorylcholine head group linked to Cer (Contarini & Povolo, 2013). SM usually has high levels of saturated fatty acids (SFAs) such as C16:0 and longer-chain SFAs including C22:0, C23:0, and C24:0. The degree of saturation in sphingolipids is a key factor in the thermal stability. PLs containing polyunsaturated fatty acids undergo significant changes during production (Tang et al., 2021). Consequently, bovine milk treated with bactericidal concentration, spray drying, and freeze-drying displays a higher sphingolipid and lower glycerophospholipid content.

Inconsistent changes were observed in the relative contents of SM, PE, PC, and PI during homogenization, bactericidal concentration, and drying of bovine milk, regardless of PL supplementation (Fig. 4A). The relative content of PE in PL supplemented milk generally decreased, while it varied in milk without PL supplementation. Standardized milk I with PLs showed PE and SM concentrations of 193.74 mg/L and 37.36 mg/L, corresponding to relative contents of 60.99 % and 11.87 %, respectively (Fig. 4A, 4C, and 4E). In contrast, standardized milk II without PLs possessed PE and SM concentrations of 99.08 and 28.16 mg/L, respectively, which translates to relative contents of 49.09 % and 13.95 % (Fig. 4A, 4C, and 4E). This indicates that milk with PL supplementation exhibited higher PE concentration and relative content. Being the most unsaturated PL, PE, rich in mono- and polyunsaturated fatty acids, is susceptible to oxidative decomposition, leading to significant losses during homogenization, bactericidal concentration, and drying processes. This susceptibility might account for the consistent reduction in PE observed in the production of infant formula I. During homogenization, PE content decreased (Fig. 4E), while LPE content increased (Fig. 4F), potentially due to PE hydrolysis(Zhang et al., 2022a). The increase in LPE, which may be attributed to PE losing a fatty acid molecule, indicates that hydrolysis may contribute to higher LPE levels.

Homogenization likely exposes PE in the inner layer of the fat globule, increasing its susceptibility to hydrolysis. In freeze-dried powder, the concentration of PE is higher than in spray-dried powder, while the LPE content is lower in freeze-dried powder compared to spray-dried powder (Fig. 4E and Fig. 4F, respectively). This indicates that high-temperature drying promoted PE hydrolysis, leading to more LPE, which aligns with findings from previous studies (Tang et al., 2021; Zhang et al., 2022a). The highly unsaturated nature of PE makes it more prone to oxidation during spray drying, which involves a higher temperature than freeze drying. Furthermore, PE undergoes glycosylation during heat processing, resulting in reduced concentrations (Kodate et al., 2018).

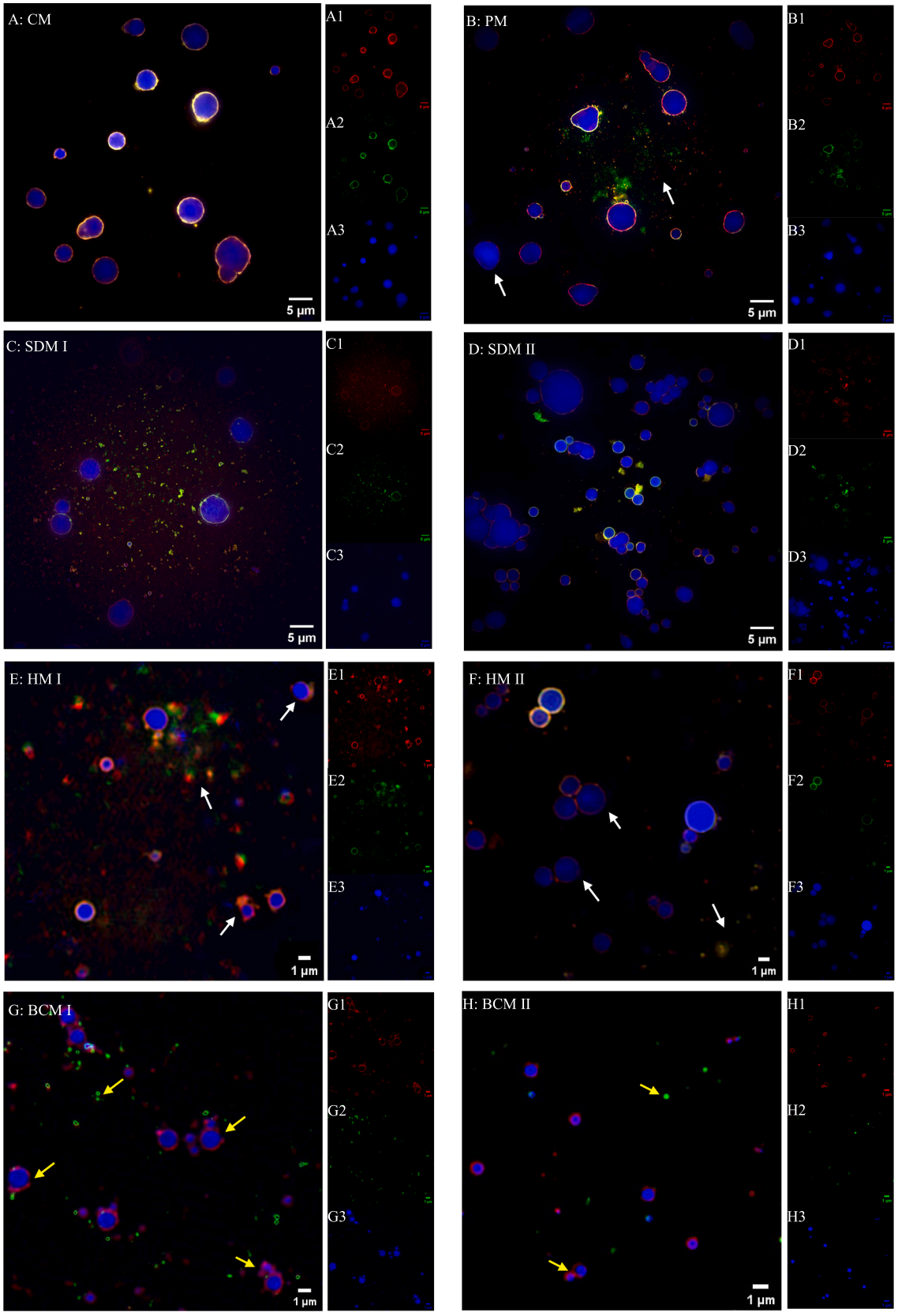

Fig. 6. SIM images of bovine milk processed differently. Merge images of PLs, protein, and glycerolipid (A-L). Split images of PL (A1-L1), protein (A2-L2), and glycerolipid (A3-L3). Stains: STARRED-DOPE for PLs (red), Rhodamine B for protein (green), and LIPI-QA for glycerolipids (blue).

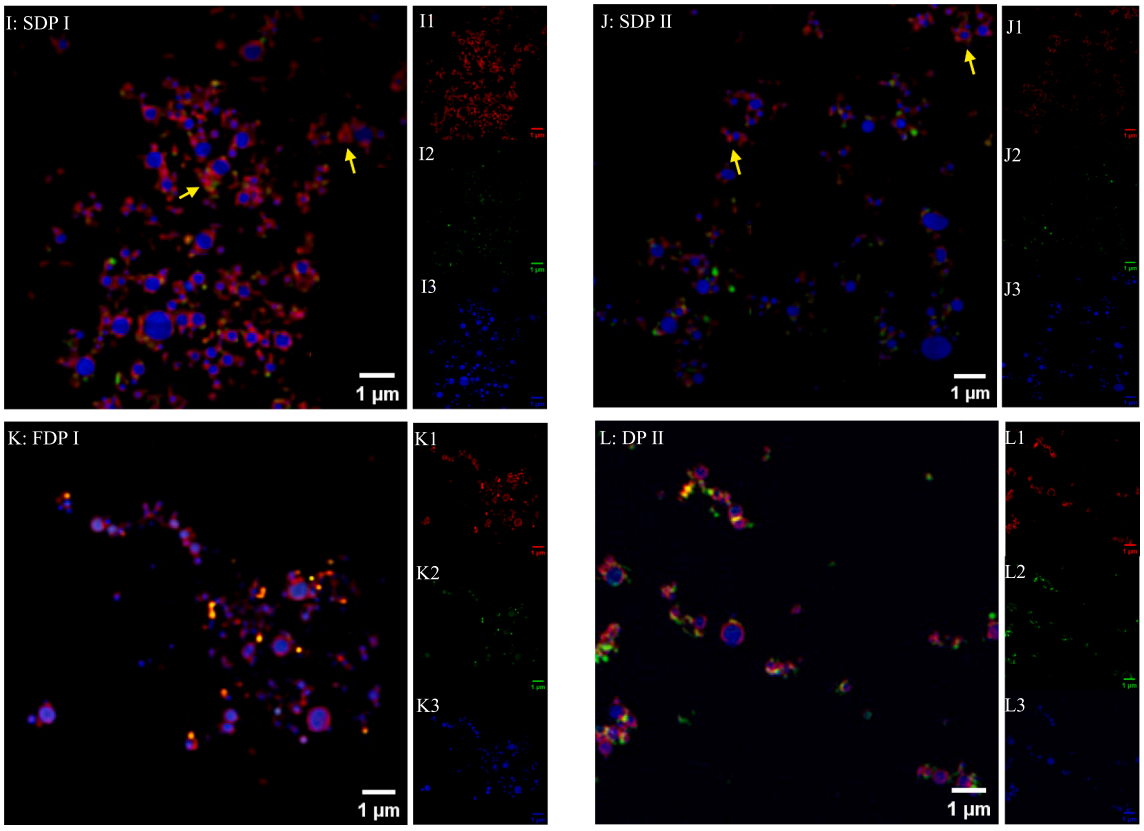

Fig. 6. (*continued*).

These results suggest that both the overall loss of PLs during processing and the differential loss of PL subclasses due to oxidative hydrolysis and other chemical reactions should be considered when determining the appropriate amount and proportion of PLs to add during the processing of infant formula.

3.2.2. *Composition and concentration of PL molecular*

The impact of different treatments on the distribution of molecular composition of PLs was less significant compared to that on glycerolipids (Fig. 2B–2C and Fig. 5B–5C). The PLS-DA scores indicate that the composition distribution of PL molecules was affected by pasteurization, spray drying, and freeze drying, with model parameters R^2 and Q^2 exceeding 0.4. (Fig. 5B–5C). In the PLS-DA model, the VIP score signifies the contribution of a variable to the model. The PL molecules are deemed significantly different when VIP $>$ 1. In clean and pasteurized milk, twelve PL molecules were identified as significantly different. Post-pasteurization, concentrations of four SM, two PI, and one PC decreased, while three PE and two SM increased. Furthermore, fifteen significantly different PL molecules were identified in processed milk supplemented with PLs after homogenization, bactericidal concentration, spray drying, and freeze drying. In contrast, only seven significantly different PL molecules were identified in processed milk without PLs. The variation in PL molecules between the two infant formulas may be attributed to the differences in the fatty acid composition and concentration of PL molecules.

3.3. *Milk-fat globule structured illumination microscopy*

The resolution of SIM surpasses that of conventional fluorescence microscopy by two to five times, achieving an in-plane resolution of 60–80 nm (Mennella, 2023). During the production of infant formula, homogenization reduces the size of MFG from several microns to hundreds or even tens of nanometers, ensuring even dispersion of milk fat in the emulsion (Liu et al., 2023; Yao et al., 2015). However, this process also changes the interface structure and composition of MFG (Zhao et al., 2019). Driven by capabilities such as multicolor labeling and high-throughput imaging compatibility, SIM effectively reveals the changes of glycerolipids, PLs, and proteins within MFGs, their membranes, and the emulsion during processing (Mennella, 2023).

Fig. 6 presents super-resolution images of bovine milk subjected to various processes are shown in Fig. 6. Clean milk, treated to remove impurities, predominantly contains highly dispersed spherical fat globules, with a minor amount of aggregated milk fat. All MFGs exhibit a ring-shaped membrane structure, as observed in SIM imaging (Fig. 6A), with a blue core of the glycerolipid and a membrane layer of MFGM (including PLs and protein) appearing yellow or pink. Yellow results from the overlap of green (protein) and red (PLs), while pink arises from the merging of red with green and blue (glycerolipids). The MFGM structure in pasteurized milk appears thinner than in clean milk, and some fat globules lack surface PL layer, as indicated by the white arrow in the PM image (Fig. 6B). This observation aligns with the noted reduction in PL content post-pasteurization (Fig. 4B). Additionally, the emulsion in pasteurized milk contained red-labeled PL and green-labeled protein, which may be attributed to the pasteurization process. Vegetable oils, structured lipids, and/or PL supplements were added to bovine milk samples during standardization. The microstructures of standardized milk I with PLs supplement were significantly different from those of standardized milk II without PLs (Fig. 6C–6D), highlighting the impact of these additives on the milk's microstructure. In standardized milk I, the fat globules were uniformly dispersed within emulsion rich in protein and PLs fragments, while standardized milk II showed pronounced flocculation and coalescence. The addition of PL to standardized milk I resulted in an increased number of PL fragments and more obvious PL layer, contributing to the stable dispersion of the fat globules. In contrast, the PL layers in standardized milk II, which did not contain added PL, became thinner or partially disappeared during

pasteurization. These changes led to flocculation and coalescence of newly added vegetable oils, structural lipids, and the original milk glycerolipids.

Homogenization is a crucial step in infant formula production because it not only reduces the size of MFGs and enhances their specific surface area but also facilitates the reorganization of the interface structure. Post-homogenization, the emulsions contain increased amounts of PL and protein fragments, with some fat globules partially surrounded by PL layers (Fig. 6E–6F, white arrows in HM images). This suggests that homogenization disrupts the original MFGM structure, resulting in the loss of some PLs and proteins in the emulsions. Additionally, fat globule aggregation still occurs in homogenized milk II, with lower PL content both on the surface of the fat globules and in the aqueous phase compared to homogenized milk I (Fig. 6E–6F). Bactericidal concentration may lead to different structural changes. In bactericidal-concentrated milk, ring-shaped PL layers are observed with more fragmentation and aggregation (yellow arrows in BCM images), while ring-shaped protein structures decrease and become more dot-like (Fig. 6G–6H). Freeze and spray drying have significantly different effects on the structure, as observed in the SDP and FDP images. Although freeze-drying disrupts the MFGM structure, resulting in differences in membrane thickness and increased protein adsorption on the interface, MFGM was relatively smooth and intact (Fig. 6K–6L). In the spray-dried powder, there was evident degeneration and coke aggregation (Fig. 6I–6 J). Prior studies have indicated that heating milk above the thermal denaturation temperature of adsorbed whey protein may lead to the unfolding and interfacial cross-linking of whey protein, potentially causing significant changes in the interfacial structure and aggregation of fat globules (Liang et al., 2017). These observations underscore the critical impact of drying technology on the structure of MFGs and their membranes. Images of SDP and FDP demonstrate that supplementation with PLs effectively enhances the PL embedding rate in fat globules and improves their spherical membrane structure, which is consistent with the findings of Zhang et al. (2022b). Conversely, MFGs and their membranes typically lose their native biological structure and composition during homogenization, heat treatment, and drying processes.

In conclusion, the structure of MFG and their membranes undergoes significant alterations during the production of infant formula. The use of PLs and freeze-drying techniques may enhance the preservation of the natural structure of the MFGM. Furthermore, the SIM effectively visualizes the structural changes in MFGs throughout the infant formula processing, shedding light on the specific impacts of various processing technologies and PL supplementation on the membrane structure of MFGs.

4. Conclusion

In this study, we investigated the impact of process stages and the addition of exogenous lipids on the lipid profile during the manufacturing of infant formula. We found that the content of glycerolipids rich in AA or DHA, as well as glycerophospholipids, substantially decreased during thermal processing. Conversely, glycerolipids and sphingolipids with high saturation levels exhibited relative stability. These findings suggest that highly unsaturated lipids are more sensitive to heat, and conventional heat treatment impairs lipid nutrition. These results highlight the importance of considering lipid losses and variations in loss rates when formulating infant formula to ensure effective supplementation of exogenous lipids. The incorporation of PLs and the application of freeze-drying technology were found to enhance both the quantity and microscopic morphology of PL, thereby reducing the differences between infant formula lipids and human milk lipids and improving the digestion, absorption, and utilization of infant formula. The SIM enabled nanoscale visualization of the alterations in fat globules resulting from processing, which provide effective data support for process improvement. Further research is needed to elucidate the implications of these structural changes on lipid metabolism and functionality.

CRediT authorship contribution statement

Qian Liu: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Yan Liu:** Investigation, Visualization. **Junying Zhao:** Conceptualization, Writing – review & editing. **Weicang Qiao:** Project administration, Software. **Juncai Hou:** Resources. **Yaling Wang:** Investigation. **Minghui Zhang:** Investigation. **Ge Jia:** Investigation. **Xiaofei Fan:** Resources. **Ziqi Li:** Visualization. **Haidong Jia:** Resources. **Xiaojiang Zhao:** Resources. **Lijun Chen:** Conceptualization, Project administration, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.138623.

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