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A novel infant milk formula concept: Mimicking the human milk fat globule structure

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ABSTRACT

Human milk (HM) provides all nutrients to support an optimal growth and development of the neonate. The composition and structure of HM lipids, the most important energy provider, have an impact on the digestion, uptake and metabolism of lipids. In HM, the lipids are present in the form of dispersed fat globules: large fat droplets enveloped by a phospholipid membrane. Currently, infant milk formula (Control IMF) contains small fat droplets primarily coated by proteins. Recently, a novel IMF concept (Concept IMF) was developed with a different lipid architecture, Nuturis®, comprising large fat droplets with a phospholipid coating. Confocal laser scanning microscopy (CLSM), with appropriate fluorescent probes, and transmission electron microscopy were used to determine and compare the interfacial composition and structure of HM fat globules, Concept IMF fat droplets and Control IMF fat droplets. The presence of a trilayer-structured HM fat globule membrane, composed of phospholipids, proteins, glycoproteins and cholesterol, was confirmed; in addition exosome-like vesicles are observed within cytoplasmic crescents. The Control IMF fat droplets had a thick protein-only interface. The Concept IMF fat droplets showed a very thin interface composed of a mixture of phospholipids, proteins and cholesterol. Furthermore, the Concept IMF contained fragments of milk fat globule membrane, which has been suggested to have potential biological functions in infants. By mimicking more closely the structure and composition of HM fat globules, this novel IMF concept with Nuturis® may have metabolic and digestive properties that are more similar to HM compared to Control IMF.

1. Introduction

Human milk (HM) is the sole source of nutrients and energy in the form of lipids, proteins, carbohydrates, vitamins and minerals, and is considered to be the gold standard to support optimal growth and development of the newborn. Lipids in HM produced by the epithelial cells of the mammary gland represent up to 55% of the total caloric intake of the newborn [1]. The World Health Organization recommends to exclusively breast-feed during the first 6 months of life and to complement breastfeeding with appropriate infant foods up to 2 years of age or beyond. Breast-fed infants have a reduced risk of obesity and metabolic disease later in life compared to infants fed infant milk formula (IMF) [2,3]. Breastfeeding also has a positive effect on cognitive and immune functions [2,3].

HM (and mammalian raw milk in general) has a distinct lipid architecture as a result of the way the fat globules are produced and secreted from the mammary gland cells. The milk fat globule is composed of a triglyceride core enveloped by a trilayer, the milk fat globule membrane (MFGM), composed mainly of phospholipids, specific proteins and also cholesterol [4,5]. The size of the fat globules in mature HM varies from 0.1 μm up to 15 μm with a mode diameter between 3 and 5 μm, based on volume [6]. Approximately 98–99% of HM lipids are in the form of triglycerides [7]. HM contains 3–4.5 g fat/100 mL of which 0.4–0.5% are in the form of phospholipids and composed of sphingomyelin (36–42%), phosphatidylcholine (25–28%), phosphatidylethanolamine

Abbreviations: HM, human milk; IMF, infant milk formula; MFGM, milk fat globule membrane; WGA, wheat germ agglutinin; Rd-DOPE, rhodamine-dioleoyl-phosphatidylethanolamine; CLSM, confocal laser scanning microscopy; TEM, transmission electron microscopy; MUC, mucin; l_o, liquid-ordered; l_d, liquid-disordered.

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(17–23%), phosphatidylserine (7–10%) and phosphatidylinositol (4–7%) [8]. The main proteins in MFGM are xanthine oxidase, adipophilin, fatty acid binding proteins and glycoproteins such as mucins, lactadherin, butyrophilin and CD36 [9]. MFGM glycosphingolipids comprise cerebrosides and gangliosides, which have been shown to contribute to the development of the brain and gut immune system of infants [1]. The glycoproteins and glycolipids, with their carbohydrate residues protruding into the aqueous phase, form the MFGM glycocalyx, a rich source of bacterial and viral ligands [10]. The MFGM lipids and proteins mentioned have been shown to have important biological functions such as antimicrobial, antibacterial and antiviral activities [1,10–12]. Sphingomyelin and its metabolites play a role in gut maturation as well as myelination of the developing central nervous system in the newborn [11]. The supply of long chain fatty acids from HM phospholipids is crucial for neural membrane synthesis [8].

Currently, the IMF design is based on the nutrient composition of mature HM to deliver adequate levels of macronutrients (carbohydrates, lipids and proteins) and micronutrients (vitamins and minerals) to support growth. In current IMF (Control IMF), fat droplets have a mode diameter of 0.4 μm, based on volume, and proteins are the main emulsifier of fat droplets. This results in a stable and reproducible product with a long shelf life but with a fat droplet architecture strongly different from that in HM. Studies showed that the type of emulsifier as well as the size of emulsion droplets affect lipolysis [13–15]. In preterm infants, hydrolysis of human milk triglycerides was higher than that of triglycerides of a standard IMF with small fat droplets coated by proteins [16]. This effect was attributed to the difference in size and interfacial coating of the fat droplets in addition to a different fatty acid profile between human milk and standard IMF. Given the emerging interest in the bioactivity of MFGM compounds and the potential effect of food structure on digestion, absorption of lipids and metabolic fate, we have developed a novel IMF concept (Concept IMF) mimicking more closely the composition and structure of HM fat globules. To this end, the production process was modified and adapted and a phospholipid-enriched dairy source was used. Our Concept IMF (patent EP2825062A1) contains Nuturis®, large fat droplets coated by added phospholipids that are present in similar amount and profile as in HM [17]. In previous rodent studies, mouse pups were fed this Concept IMF or Control IMF from infancy until postnatal Day 42. Mice fed Concept IMF until early adulthood showed reduced adiposity when subsequently challenged by a moderately high-fat Western-style diet in adolescence and adulthood despite similar food intake [18,19]. These nutritional programming models using rodent pups [18,19] suggested that the altered lipid structure of the Concept IMF in early life affected adult adipocyte functionality but not adipocyte number. Also, the metabolic response to this diet was improved in Concept IMF-fed mice, with lower body weight and lower plasma insulin despite similar food intake. In the present study, we characterized the structure of the fat droplets of the Concept IMF (Nuturis®) in more detail and compared it with the structure of HM fat globules as well as Control IMF fat droplets using advanced microscopic techniques.

2. Materials and methods

2.1. Milk samples

Expressed mature HM (less than 15 mL) was collected in the morning using a breast milk pump from healthy donors (four for particle size measurement, three for confocal laser scanning microscopy (CLSM) and two for transmission electron microscopy (TEM)) between 2 and 12 months postpartum. Each freshly expressed milk sample was kept at room temperature until analysis. The particle size measurement was carried out maximum 2 h after collection and the sample preparation for CLSM and TEM was carried out just after collection.

The Control IMF (powder) was produced according to current standard stage I IMF recipe and processing procedures (Danone Nutricia Research, Utrecht, The Netherlands). The Concept IMF (powder) was produced by adding bovine milk phospholipids (as beta-serum, Fonterra Co-operative Group Ltd., New Zealand) to an amount of 1.5% of total fat (corresponding to 0.5 g of milk phospholipid in 1 L of reconstituted Concept IMF), and processing procedures were modified as to yield larger fat droplets than in Control IMF. The beta-serum was added to the aqueous phase along with proteins, lactose, vitamins and minerals. The aqueous phase was pasteurized at 85 °C for 6 min and then homogenized with the vegetable oil blend (i.e., the lipid phase) with an inline mixer to obtain large and phospholipid-coated fat droplets [17]. Concept and Control IMF powders were freshly reconstituted as follows: 13.7 g of powder was mixed with 90 g of tap water at 40 °C. The reconstituted Concept and Control IMFs contained 33.5 g/L of fat, 13.2 g/L of proteins and 70.0 g/L of lactose. As an additional control, 2 g of beta-serum, also commonly called butter serum, was reconstituted in 10 mL of water at room temperature for imaging purpose.

2.2. Particle size

The particle size distribution of HM, Concept IMF and Control IMF was determined using a laser light scattering instrument (Mastersizer 2000—Hydro 2000G, Malvern Instruments, UK). The refractive index for the dispersed phase was 1.460 and the absorbance was 0.001; the parameters used for the continuous phase were those of water (i.e., viscosity 0.8872 cP; refractive index 1.330). Measurements were done in duplicate.

2.3. Confocal laser scanning microscopy

Nile Red (Sigma–Aldrich, Zwijndrecht, The Netherlands), a lipophilic fluorescent probe, was diluted at 1 mg/mL in acetone (Fisher Scientific, Landsmeer, The Netherlands). Fast Green FCF (Sigma–Aldrich) was diluted at 1 mg/mL in deionized water. The fluorescent phospholipid analogue 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rd-DOPE, Avanti Polar Lipids, Alabaster, AL, USA) was diluted at 1 mg/mL in chloroform (Merck, Houten, The Netherlands). Filipin complex from *Streptomyces filipinensis* (Sigma–Aldrich) was diluted at 2 mg/mL in ethanol (VWR International, Amsterdam, The Netherlands). The Alexa Fluor 488 conjugate of wheat germ agglutinin (WGA, 1 mg/mL in 0.2 M PBS, pH 7.4) was purchased from Invitrogen (Carlsbad, CA, USA). All solvents were of analytical grade.

The samples were single- or dual-stained with Nile Red (1:100 v/v), Fast Green FCF (1:100 v/v), Rd-DOPE (1:100 v/v) and WGA (5:100 v/v) as described previously in Gallier et al. [20]. The samples were allowed to react for 20 min in the dark at room temperature. The staining with filipin (1:10 v/v) was carried out at 40 °C for 1 h in the dark. 40 μL of stained sample was quickly mixed with 70 μL of 1% melted agarose (Sigma–Aldrich) solution. A coverslip was placed rapidly on top of the sample before gelling.

An inverted confocal laser scanning microscope LSM 700 Axio Observer Z1 (Zeiss, Oberkochen, Germany) was used to image the HM and reconstituted IMF samples with a Plan-Apochromat 63×/1.40 Oil DIC (WD = 0.19 mm) objective. The images were analyzed with the proprietary Zeiss Zen 2011 software.

Nile Red and WGA were each excited with the 488 nm laser line and the filters were set to collect the emitted light between 493 and 550 nm. Dual-stained samples with Nile Red and WGA were excited with the 555 nm laser line (for Nile Red) and the emitted light was collected between 560 and 800 nm. The same latter parameters

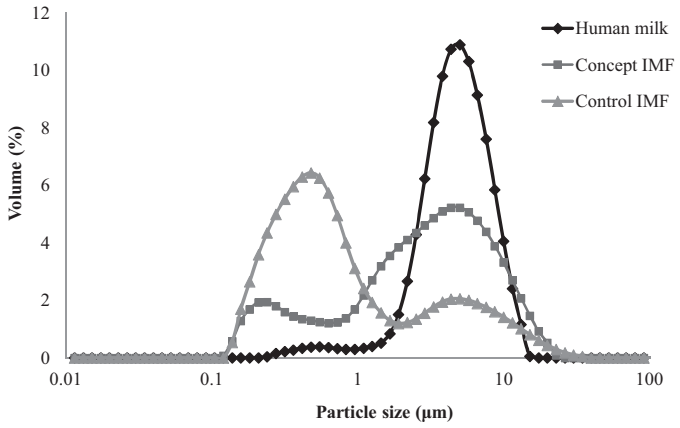

Fig. 1. Droplet size distribution of HM (♦), Concept IMF (■) and Control IMF (▲).

were used for samples stained with Rd-DOPE. Excitation of Fast Green FCF was achieved with the 639 nm laser line and the emitted light was collected between 644 and 800 nm. The 405 nm laser line was used for the excitation of filipin and the emitted light between 300 and 483 nm was collected.

Autofluorescence of the unstained samples was also checked for with all laser lines. 3D imaging was obtained by scanning the sample in the *z*-axis in steps of 0.25 µm.

2.4. Transmission electron microscopy

The sample preparation for TEM imaging was similar to the one described in Gallier et al. [21] with some modifications. The samples (HM, Concept IMF, Control IMF and beta-serum) were mixed with 3% low temperature gelling agarose (kept at 50 °C) at a ratio 1:1 in a 15 mL tube. After cooling, the gelled samples were cut into 1 mm^3 blocks and transferred into 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After fixation, the blocks were washed with 0.1 M cacodylate buffer, pH 7.2. Post-fixation was carried out with 1% osmium tetraoxide in distilled water for 2 h. The blocks were washed with distilled water. Staining en-bloc was carried out with 1% uranyl acetate. Subsequently, the blocks were dehydrated in an ethanol series (50–100%) and embedded in Epon resin. Sections (60 nm) were prepared with a Leica ultramicrotome (Wetzlar, Germany). Post-staining on grids (Cu 100M–H coated with Formvar film and carbon coat) was carried out with 7% uranyl acetate and with Reynolds Pb–citrate.

The imaging was carried out with a Tecnai 10 transmission electron microsope (FEI, Hillsboro, OR, USA) equipped with a SIS CCD camera MegaView II, a single tilt specimen holder and the proprietary software AnalySISPro.

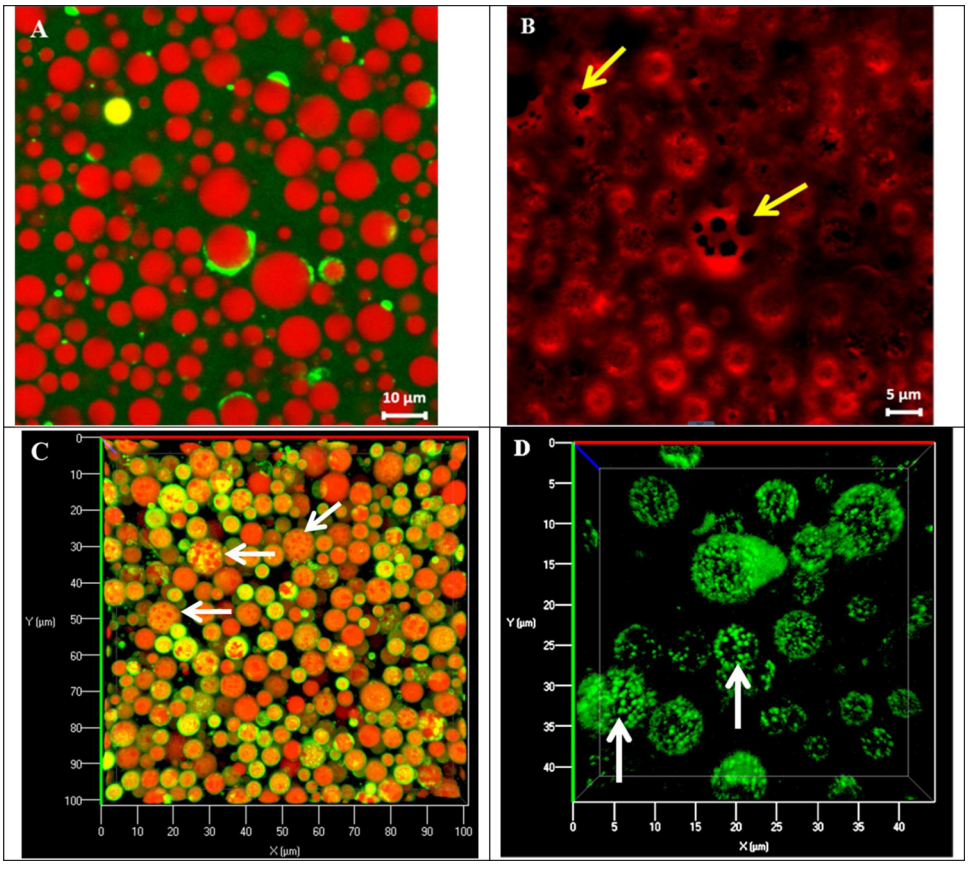

Fig. 2. 2D (A and B) and 3D (C and D) CLSM images of HM. (A) The milk fat globules were stained with Nile Red (Red) and Fast Green FCF (green). (B) The MFGM was stained with Rd-DOPE; the yellow arrows are pointing at l_o domains which are rich in sphingomyelin and cholesterol and tightly packed, preventing the insertion of the fluorescent Rd-DOPE. (C) The milk fat globules were stained with Nile Red (red) and the MFGM glycoproteins and glycolipids with WGA (green); the white arrows are pointing at globules showing fluorescence from the lectin outside of the l_o domains, indicating that glycoproteins and glycolipids are not located inside the l_o domains. (D) The milk fat globules were stained with filipin; the white arrows are pointing at round spots of filipin–cholesterol fluorescent complexes; filipin reorients cholesterol molecules in the membrane plane, which may affect the distribution of cholesterol within the MFGM. However, the filipin–cholesterol complexes followed a similar pattern to that of the l_o domains. Scale bars: (A) 10 µm; (B) 5 µm; (C) *X* and *Y* = 0–100 µm; (D) *X* and *Y* = 0–50 µm.

3. Results and discussion

3.1. Particle size

HM fat globules showed a mode diameter (4.2 ± 0.3 μm), based on volume, similar to the fat droplets in Concept IMF (4.3 ± 0.1 μm) but were approximately 10 times larger than the fat droplets in Control IMF (0.4 ± 0.0 μm). The volume-weighted diameter, $d_{4,3}$, of human milk (4.5 ± 0.3 μm) was slightly different than that of Concept IMF (3.9 ± 0.0 μm) and higher than that of Control IMF (2.3 ± 0.3 μm). The surface-weighted diameter, $d_{3,2}$, of human milk (3.0 ± 0.5 μm) was higher than that of Concept IMF (0.9 ± 0.0 μm) and Control IMF (0.5 ± 0.0 μm). Thus, the modified IMF processing conditions [17] resulted in a fat droplet size comparable to that of HM fat globules but with a higher proportion of small fat droplets (Fig. 1). The $d_{3,2}$ (more sensitive to the presence of small droplets) and $d_{4,3}$ (more sensitive to the presence of large droplets) indicated that Concept and Control IMF contained two size population of fat droplets (Fig. 1). The smaller fat droplets are likely digested to a greater extent than the larger ones, due to an increased available surface area [14]. Lactosomes (<500 nm), rich in immunomodulatory proteins and with a different protein and lipid profile than MFGM, have been detected in human milk but their role is still unclear [22]. Small particles between 200 nm and 1 μm (Fig. 1) were detected in human milk and could contain lactosomes.

3.2. CLSM imaging

CLSM was used in combination with fluorescent probes to observe the structure of fat droplets in HM (Fig. 2), Concept IMF (Fig. 3) and Control IMF (Fig. 4). The fluorescent probes were used to show the localization of the lipids (Nile Red) and proteins (Fast Green FCF) or to explore the droplet interfacial composition and structure (Rd-DOPE, WGA and filipin).

HM fat globules showed proteins at their surface either as a thin layer or occasionally as thick patches, the latter likely being cytoplasmic crescents (Fig. 2A). The literature indicates that an average of 7.2% of HM fat globules contained cytoplasmic crescents, unlike cow's milk which contains only less than 1% [23]. The formation of cytoplasmic crescents results from entrapment of cytoplasmic material when fat droplets are pinched off from the mammary epithelial cells into the alveolar lumen. Cytoplasmic crescents have been observed previously in HM using CLSM and electron microscopy [5,23,24].

The labeling of the MFGM phospholipids (Fig. 2B) showed lateral segregation of the phospholipids into micron-sized liquid-ordered (l_o) domains, rich in saturated sphingomyelin and cholesterol where Rd-DOPE could not insert itself, and a liquid-disordered (l_d) phase, rich in unsaturated glycerophospholipids into which Rd-DOPE could penetrate and emit fluorescence, as previously also observed by others [4,5]. Extrinsic phospholipids labeled with a fluorophore have been commonly used to localize phospholipids in

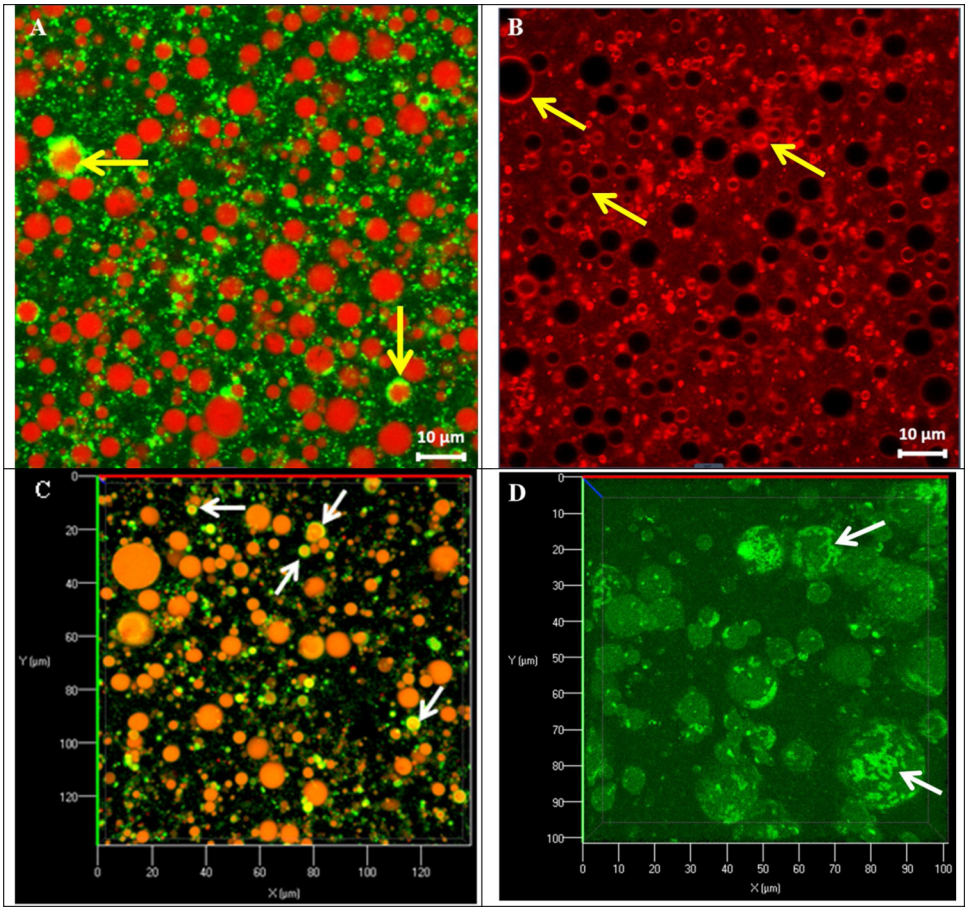

Fig. 3. 2D (A and B) and 3D (C and D) CLSM images of Concept IMF. (A) The fat droplets were stained with Nile Red (Red) and Fast Green FCF (green); the yellow arrows are pointing at dense patches of interfacial proteins. (B) The surface of the fat droplets was stained with Rd-DOPE; as a fluorescent phospholipid analogue, Rd-DOPE indicates the location of phospholipids in a sample; the yellow arrows are pointing at fat droplets with a stained surface. The surface of all droplets showed a phospholipid coating. (C) The fat droplets were stained with Nile Red (red) and the interfacial glycoproteins and glycolipids with WGA (green); the white arrows are pointing at fluorescent patches of sugar residues from glycoproteins and/or glycolipids. (D) The fat droplets were stained with filipin; the white arrows are pointing at linear networks of filipin-cholesterol fluorescent complexes, indicating the presence of cholesterol at the surface of the droplets. Scale bars: (A and B) 10 μm; (C) *X* and *Y* = 0–140 μm; (D) *X* and *Y* = 0–100 μm.

biological samples [4,5,24]. HM sphingomyelin is highly saturated [8] and can form tightly packed structures unlike glycerophospholipids that comprise unsaturated fatty acids with a kinked configuration [10].

Glycoproteins and glycolipids form the glycocalyx, which spans the surface of each HM fat globule. They are found exclusively within the l_d phase [5,20] and are distributed at the surface as dense patches as can be seen in Fig. 2C. The fluorescently labeled lectin WGA binds to the sugar moieties (*N*-acetylglucosamine and *N*-acetylneuraminic acid (sialic acid)) of glycoproteins and glycolipids, and hence WGA can be used to detect the presence of these oligosaccharide-containing molecules in the MFGM using SEM and TEM [25]. As the l_o domains are tightly packed, MFGM glycoproteins and glycolipids are not able to penetrate them and are located in the surrounding l_d phase (see Fig. 2C) [5].

HM contains 10–20 mg/dL of cholesterol; cholesterol is known to interact with sphingomyelin in biological membranes to form rigid l_o domains [10]. Filipin is a polyene antibiotic molecule, interacting specifically with 3β-hydroxysterols [26], and has been used to locate cholesterol at the surface of bovine milk fat globules by EM [27] and CLSM [28]. We clearly show the location of cholesterol at the surface of HM fat globules (Fig. 2D). Small circular protuberances (300–800 nm) are seen all around the surface of the HM fat globules comparable to those observed at the surface of raw bovine milk fat globules using electron microscopy [27]. The observed protuberances are due to the deformation of the membrane upon complexation of cholesterol with filipin and reorientation of cholesterol molecules from a parallel to a perpendicular position in the membrane plane [26]. Even though the complexation creates a deformation of the membrane, the localization of cholesterol (Fig. 2D) seems to follow a similar pattern to that of l_o domains (Fig. 2B). As filipin is a protein, it is unlikely to be able to penetrate the triglyceride core of the globules and bind to cholesterol present in the core. Consequently, only cholesterol located at the surface of the fat globules was visualized.

Breast-feeding may be associated with reduced risk of cardiovascular disease in adulthood, which is thought to be related to the early cholesterol exposure [29]. MFGM compounds, especially polar lipids and cholesterol, have indeed been associated with this protective effect related to metabolic health. Polar lipids, and in particular sphingomyelin, as present in MFGM, are known to reduce intestinal uptake of cholesterol [29]. Due to its high degree of saturation, milk sphingomyelin can form a complex with cholesterol and reach the lower part of the small intestine and colon [22,30]. The complexation reduces the cholesterol micellar solubility and subsequent absorption in the small intestine. Breast-fed infants are exposed to higher concentration of cholesterol compared with infant formula-fed infants. However, early exposure may regulate cholesterol uptake later in life, as breast-feeding is associated with higher plasma cholesterol in infancy but lower total cholesterol in adulthood [29]. The incorporation of cholesterol in the structurally complex human MFGM, rich in phospholipids, glycolipids and glycoproteins and long-chain polyunsaturated fatty acids, likely contributes to the metabolic programming of cholesterol homeostasis [31,32]. Furthermore, the polyunsaturated fatty

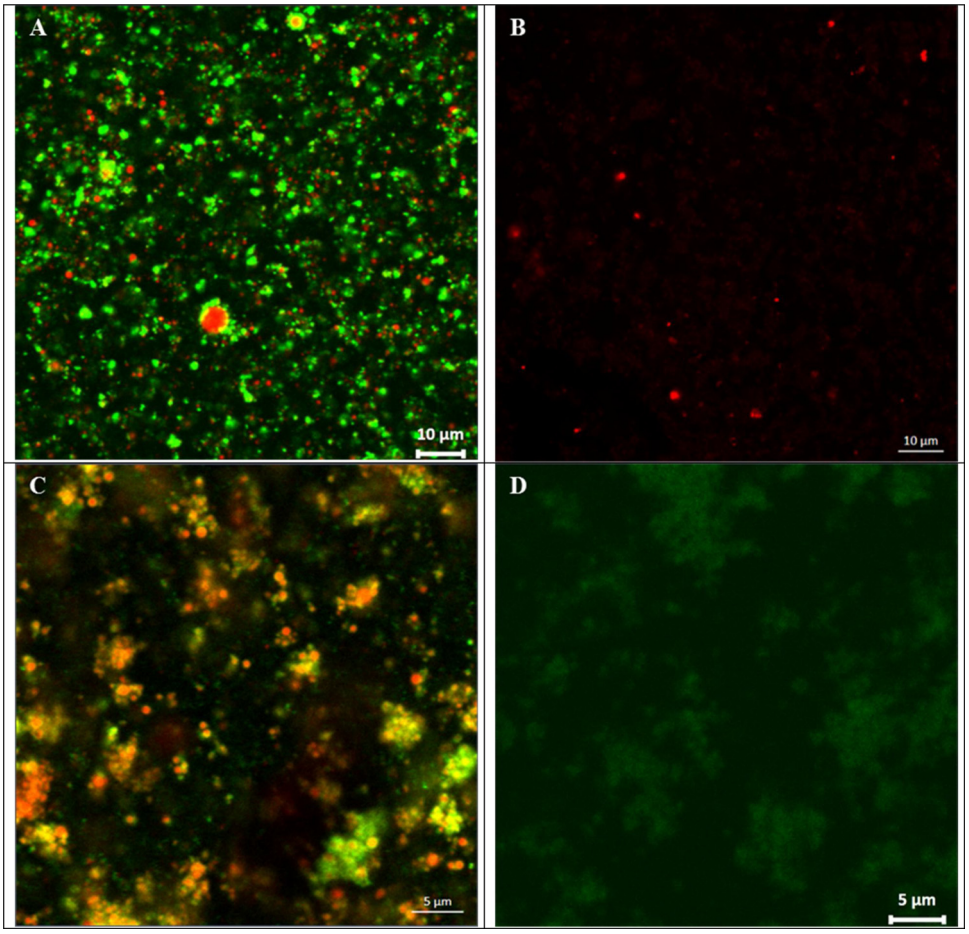

Fig. 4. 2D CLSM images of Control IMF. (A) The fat droplets were stained with Nile Red (Red) and Fast Green FCF (green). (B) The surface of the fat droplets was stained with Rd-DOPE. (C) The fat droplets were stained with Nile Red (red) and the interfacial glycoproteins and glycolipids with WGA (green). (D) The fat droplets were stained with filipin; the signal is mostly due to autofluorescence of the sample. Scale bars: (A and B) 10 μm; (C and D) 5 μm.

acid content of human milk and infant formula is key in the regulation of the rate of cholesterol synthesis [32].

The staining data confirmed the Mastersizer measurements (Fig. 1): Concept IMF fat droplets (Fig. 3A) showed a similar average size to that of HM fat globules (Fig. 2A). A few droplets presented dense patches of proteins at their interface (Fig. 3A). The addition of Rd-DOPE revealed that the fluorescent phospholipid analogue was able to locate at the surface of Concept IMF fat droplets (Fig. 3B) despite the bulky fluorophore attached to the head group of DOPE. This indicates that the phospholipids added during the manufacturing process of Concept IMF are likely to move to the surface of the fat droplets during emulsification. The fluorescence emitted by WGA was also detected at the surface of a few Concept IMF fat droplets (Fig. 3C), denoting the presence of molecules with sugar residues. Cholesterol was detected at the surface of the concept IMF fat droplets (Fig. 3D) using filipin. The fluorescence of the filipin–cholesterol complex showed a clear distribution of cholesterol molecules as interfacial linear networks (Fig. 3D); however, this distribution may be caused by the redistribution of the cholesterol molecules due to local changes in the surface pressure induced by the reorientation of the cholesterol molecules by filipin [27]. As cholesterol interacts mainly with saturated phospholipids in biomembranes, we hypothesize that the presence of cholesterol at the surface of the Concept IMF fat droplets (Fig. 3D) indicates the presence of interfacial phospholipids containing saturated fatty acids. To what extent the new concept formula may deliver comparable benefits to HM with respect to cholesterol metabolism remains to be determined.

Because of the submicron size of the Control IMF fat droplets, the evaluation was limited using CLSM (Fig. 4). In Fig. 4A and C, a few small fat droplets are observed as well as protein aggregates. Some fluorescence signal from WGA is detected, likely due to the interaction of WGA with some milk glycoproteins, such as lactoferrin and κ-casein. Low fluorescence signal from Rd-DOPE (Fig. 4B) and no fluorescence signal from filipin was detected (Fig. 4D), indicating the very low concentration of phospholipids and absence of cholesterol in the sample. Very small amounts of soy lecithin

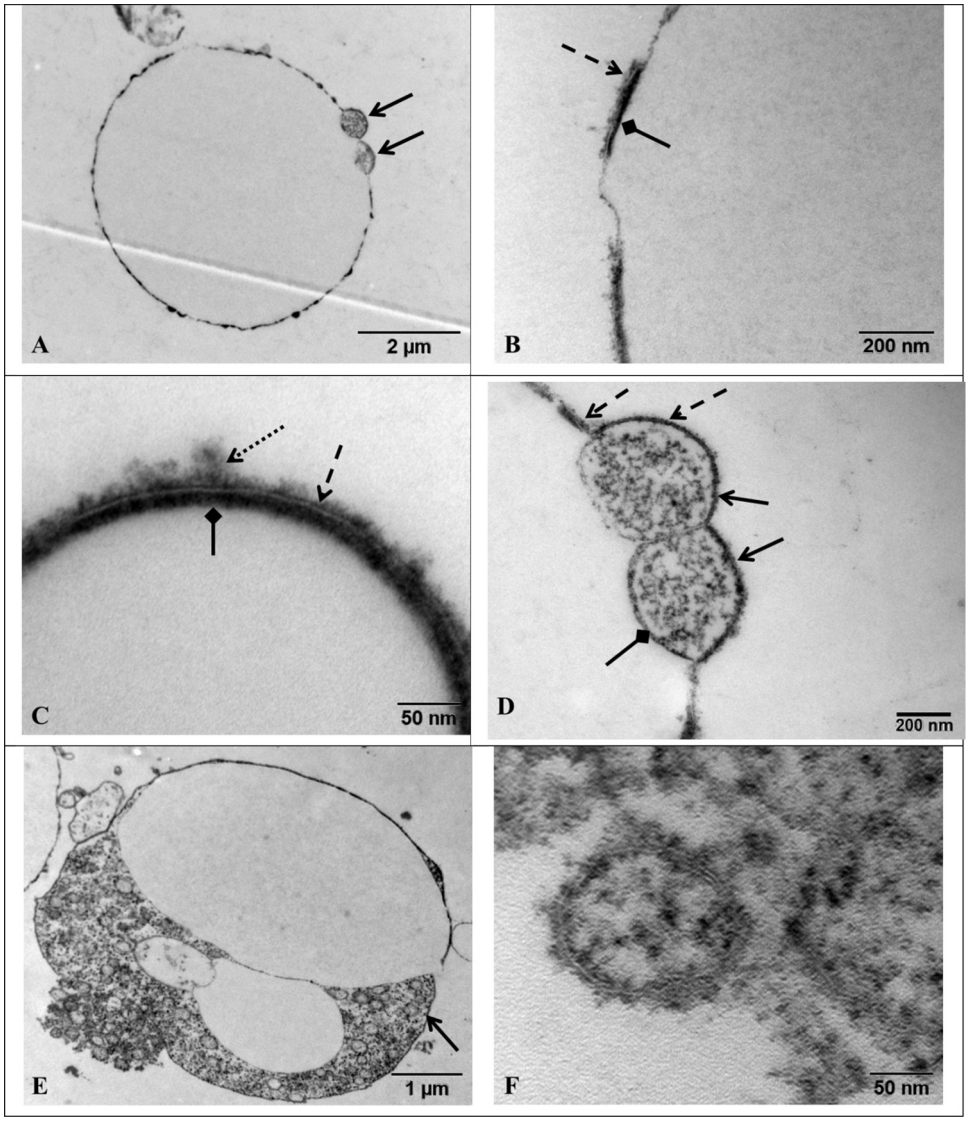

Fig. 5. TEM images of HM. (A) A milk fat globule with two cytoplasmic crescents. (B) Close-up on the MFGM where the bilayer shows dark patches rich in proteins and unsaturated phospholipids. (C) A milk fat globule with a well-defined MFGM. (D) Two cytoplasmic crescents entrapped between the MFGM bilayer and monolayer. (E) A milk fat globule with a large cytoplasmic crescent filled with vesicles (close up in (F)). The dashed arrows are pointing at the MFGM bilayer; the diamond arrows are pointing at the MFGM inner monolayer; the solid arrows are pointing at interfacial cytoplasmic crescents trapped between the MFGM inner monolayer and outer bilayer; the dotted arrow is pointing at the MFGM glycocalyx protruding in the aqueous phase. Scale bars: (A) 2 μm; (B and D) 200 nm; (C and F) 50 nm; (E) 1 μm.

are indeed added as stabilizers in the oil emulsion used to produce Control IMF.

3.3. Transmission electron microscopy

The structure of HM fat globules (Fig. 5), Concept IMF fat droplets (Fig. 6) and Control IMF fat droplets (Fig. 7) was well preserved during the sample preparation. The osmium impregnation was limited in the triglyceride core of the droplets, enhancing the contrast and resolution of the fat droplet interface.

Fig. 5A shows a typical HM fat globule of 5 μm in diameter with interfacial cytoplasmic crescents. The thickness of the MFGM varies between 5 and 20 nm (Fig. 5). The structure of the milk fat globule membrane (Fig. 5B and C) is similar to that reported previously by Wooding [33]. In Fig. 5B, a close-up of the MFGM shows the non-continuous MFGM bilayer. As osmium stains unsaturated fatty acids [34], the observed interrupted bilayer is likely due to a concentrated presence of saturated fatty acids in these unstained areas of the membrane. So Fig. 5B might reveal the presence of l_o domains, rich in saturated sphingomyelin and lacking glycolipids and glycoproteins, as also seen with CLSM (Fig. 2B and C, respectively). Indeed it is known that sphingomyelin is preferentially located on the outside surface of the MFGM [10]. However, possible shedding of MFGM material might have occurred during sample preparation. Fig. 5C shows a well-defined MFGM trilayer with its glycocalyx on a very small fat globule. The MFGM trilayer is composed of an electron-dense inner monolayer, composed of phospholipids and proteins and formed during the secretion of the droplets within the endoplasmic reticulum, and an outer bilayer originating from the apical plasma membrane of the lactating cell when the droplets are expelled and pinched off into the alveolar lumen [35].

Fig. 5D is a close-up of two cytoplasmic crescents with a diameter of about 300 nm. The crescents are filled with intracytoplasmic organelles, such as secretory vesicles and vesicles of endoplasmic reticulum origin, and membrane fragments [35]. The presence of inner protein material (Fig. 5D) confirms the protein staining observed with CLSM (Fig. 2A). The cytoplasmic crescents are entrapped between the inner monolayer and outer bilayer of the MFGM (Fig. 5D; [33]). In Fig. 4E, a cytoplasmic crescent, larger than the fat globule it is part of, is observed, filled with protein material and vesicles surrounded by a clear bilayer (Fig. 5F). Cytoplasmic

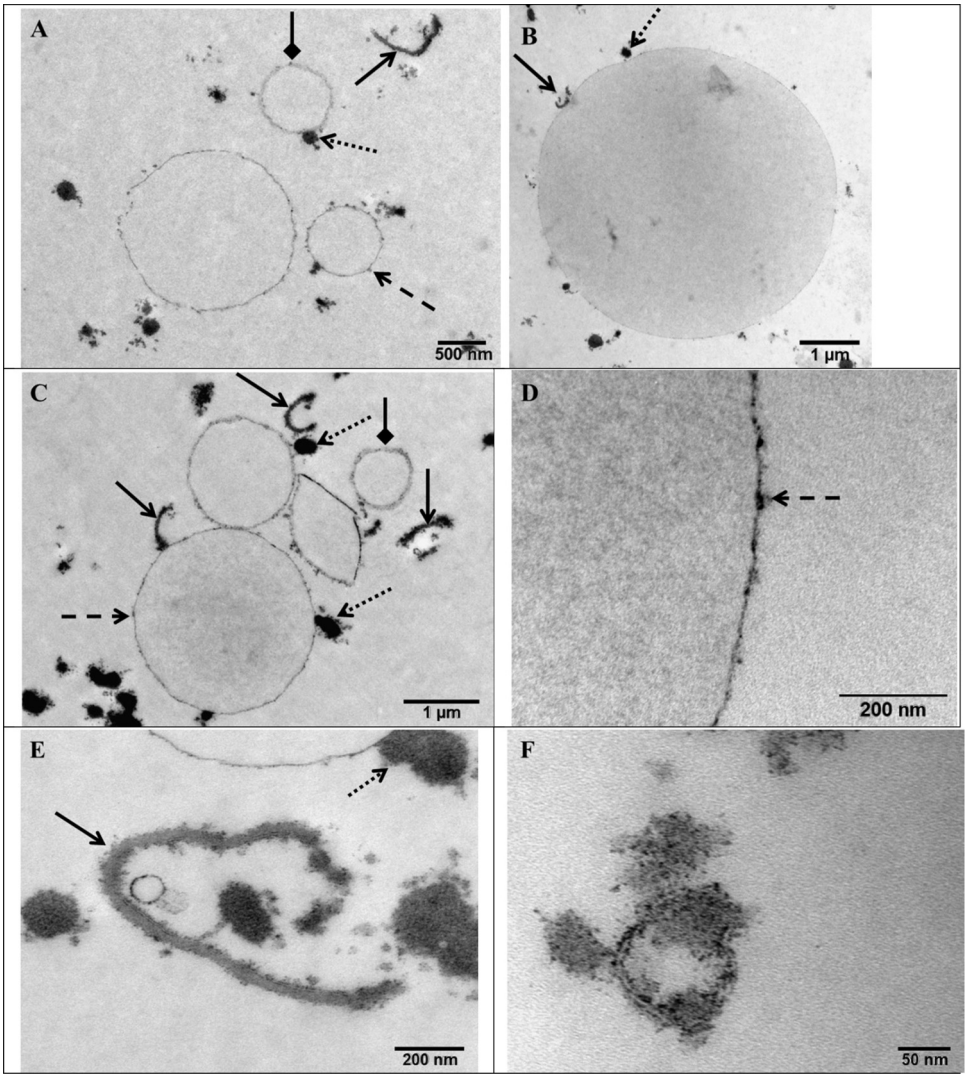

Fig. 6. TEM images of Concept IMF. (A–C) Fat droplets with different membrane thicknesses and with or without casein micelles and MFGM fragments at the interface. (D) Close-up on the interface of a fat droplet. (E) Large MFGM fragment present in the aqueous phase. (F) An MFGM fragment forms a vesicle. The dotted arrows are pointing at casein micelles interacting with the interface of the fat droplets; the solid arrows are pointing at MFGM fragments in the aqueous phase and at the interface of some droplets; the dashed arrows are pointing at small interfacial protein aggregates; the diamond arrows are pointing at droplets with a thicker membrane. Scale bars: (A) 500 nm; (B and C) 1 μm; (D and E) 200 nm; (F) 50 nm.

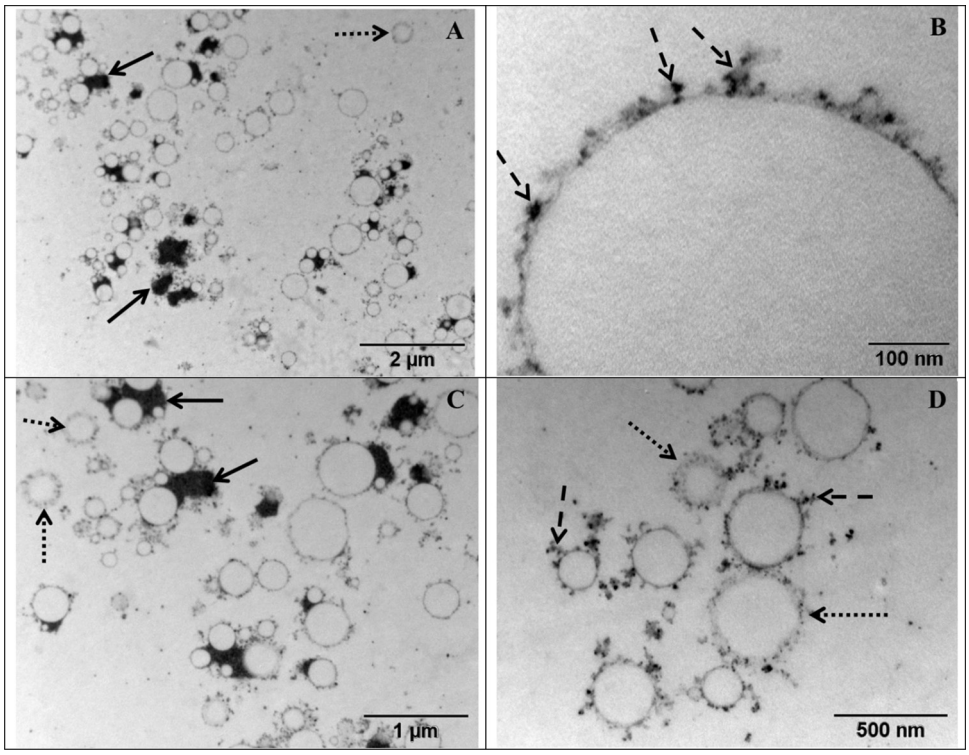

Fig. 7. TEM images of Control IMF. (A and C) Fat droplets aggregated by proteins. (B) Close-up on the interface of a fat droplet. (D) Fat droplets with different membrane thicknesses and interfacial aggregated proteins. The dashed arrows are pointing at interfacial protein aggregates; the dotted arrows are pointing at fat droplets with a thick protein coat; the solid arrows are pointing at large casein/protein aggregates, clustering fat droplets. Scale bars: (A) 2 µm; (B) 100 nm; (C) 1 µm; (D) 500 nm.

crescents vary in volume, and it is not uncommon that they exceed the size of the globule they are attached to [23].

Membrane fragments, with a composition similar to plasma membrane, can easily form vesicles [35]. Membrane vesicles in milk have been identified as "lactosomes", "exosomes" and "lipoprotein particles" [36,37]. Several types of membranes and organelles of the mammary secretory cells have been observed in cytoplasmic crescents, including secretory vesicles containing the components of the aqueous phase of milk and ribosome-studded vesicles from the endoplasmic reticulum [38]. Exosomes are nanovesicles (30–100 nm) of endocytic origin and rich in MUC1 [37], nucleic acid and proteins, as well as cholesterol and polar lipids [39]. As carrier of RNAs and microRNAs, exosomes and lactosomes also play a role in the immune response of the neonate [22,37]. We speculate that the vesicles within the cytoplasmic crescent as shown in Fig. 5E and F might be exosome-like vesicles. As exosomes are usually collected only after centrifugation of HM, they are clearly shown here to be potentially included in cytoplasmic crescents, which sheds a new light on their origin. Lemay et al. [40] showed the presence of exosomal RNA within cytoplasmic crescents or associated with the milk fat fraction. However, further research is needed to identify their nature.

Fig. 6A–D shows fat droplets of Concept IMF with a thin monolayer membrane (5–10 nm), including few protein aggregates (Fig. 6C and D). A few droplets (Fig. 6A and C) showed a thicker membrane (20–30 nm) whereas other droplets showed casein micelles interacting with the interface (Fig. 6A–C and E). MFGM fragments were detected in the aqueous phase (Fig. 6A, C and E) showing a clear bilayer structure (Fig. 6E) and interacting at the interface of the fat droplets (Fig. 6B and C), which may confirm the staining found with WGA in the confocal assessments (Fig. 3C). Some MFGM vesicles were also detected in the aqueous phase (Fig. 6F) with a diameter of about 50–100 nm.

The MFGM fragments present in Concept IMF (Fig. 6) are derived from the addition of beta-serum during the manufacturing process of Concept IMF. Indeed, beta-serum contains linear amorphous MFGM fragments of various lengths in addition to casein micelles (Fig. 8) as observed by others in buttermilk, the aqueous by-product of butter-making [41]. Beta-serum or butter-serum is the aqueous by-product of anhydrous milk fat production [42], containing ruptured MFGM fragments released from the surface of the milk fat globules during phase inversion of concentrated cream [1]. In Fig. 8B, casein micelles are seen interacting with an MFGM fragment; this is likely due to the heat treatment of the beta-serum resulting in the binding of caseins with MFGM proteins [10]. MFGM vesicles, similar to the ones in Concept IMF (Fig. 6F), were detected in beta-serum (Fig. 8D). When MFGM fragments are subjected to proteinases which can degrade butyrophilin, they tend to form vesicles [38], suggesting that the MFGM vesicles in Fig. 6F and D may have a low butyrophilin content.

Due to the increased knowledge on the nutritional and functional properties of MFGM proteins and phospholipids and the technological properties of buttermilk and beta-serum, few research groups [12,43] have investigated the emulsifying properties of buttermilk and MFGM isolates. Buttermilk is a source of MFGM but less concentrated than beta-serum [12]. MFGM fragments and MFGM-rich dairy products have proven to be good emulsifiers and are resistant to displacement by small surfactants and proteins due to the lowering of the interfacial tension by adsorbed MFGM phospholipids [44]. The adjusted processing, resulting in the Concept IMF, may allow the further breakdown of MFGM fragments or facilitate the adsorption of MFGM fragments at the interface of the fat droplets. However, clearly not all MFGM fragments were affected by processing as some remained intact in the aqueous phase (Fig. 6A, C and E). As the phospholipid-rich beta-serum was added to the protein aqueous phase before injection of the oil phase and emulsification, it is anticipated that

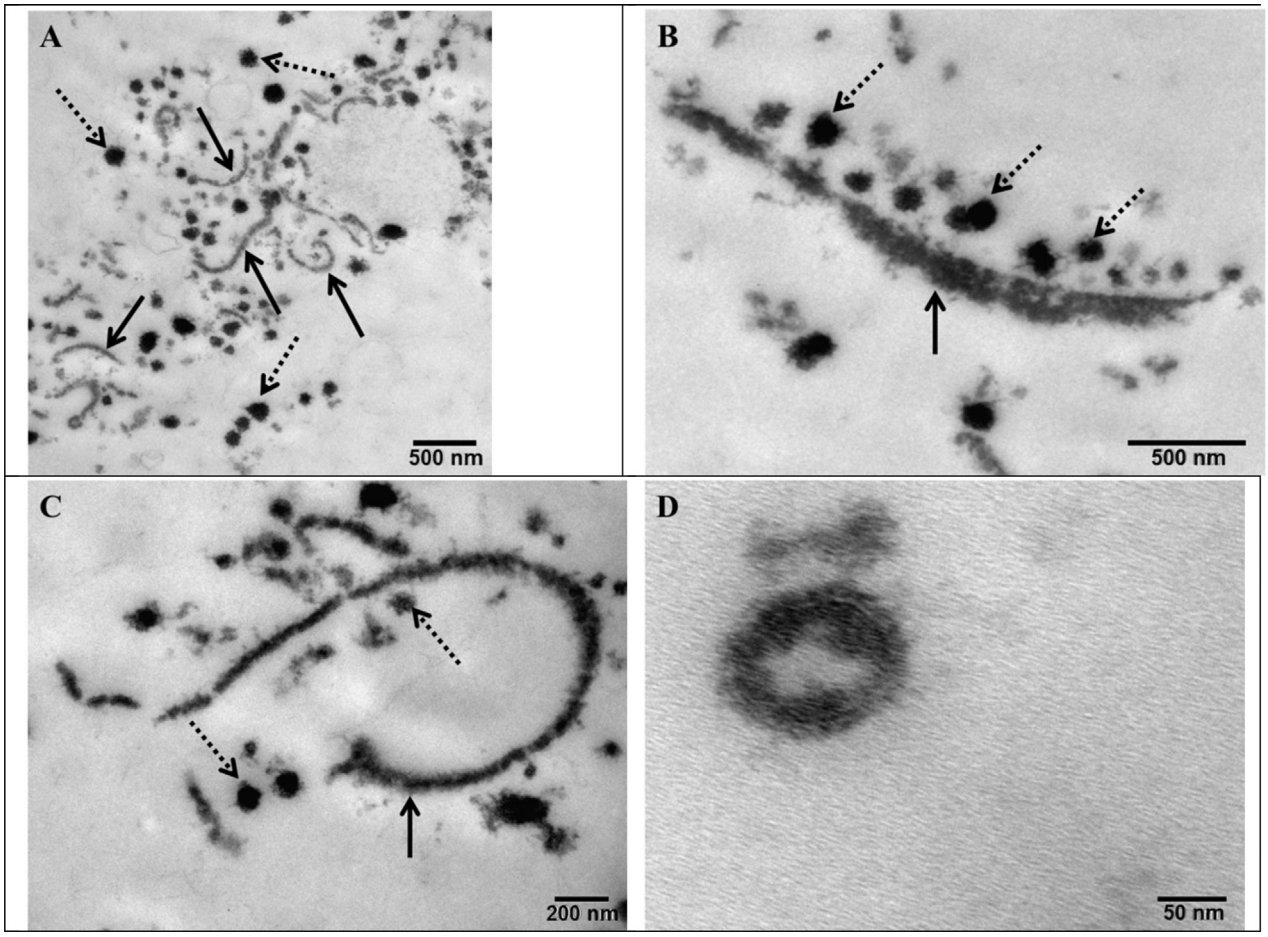

Fig. 8. TEM images of beta-serum. (A) Beta-serum contains caseins and MFGM fragments. (B) MFGM fragment with many associated casein micelles. (C) A long MFGM fragment. (D) MFGM fragment forming a vesicle. The dotted arrows are pointing at casein micelles and the solid arrows at MFGM fragments of variable length. MFGM vesicles (7D) were also observed. Scale bars: (A and B) 500 nm; (C) 200 nm; (D) 50 nm.

phospholipids and proteins are both competing and interacting at the interface of the Concept IMF fat droplets, and thus form a mixed protein–phospholipid monolayer [45].

Fig. 7 shows the fat droplets of Control IMF with a thicker (20–100 nm) and more densely stained protein membrane compared to the thin membrane observed surrounding the Concept IMF fat droplets (Fig. 6). Protein aggregates (Fig. 7B) were observed at the interface of Control IMF fat droplets, and were larger than the ones at the interface of Concept IMF fat droplets (Fig. 6C and D). Aggregation of fat droplets (Fig. 7A, C and D), as observed in Control IMF only, was likely due to protein bridges or inter-droplet protein–protein interactions [46]. MFGM fragments were confirmed to be absent from Control IMF.

In general, IMFs have a whey protein:casein ratio of 60:40 and β-lactoglobulin is the main whey protein. During heat treatment above 65 °C, β-lactoglobulin unfolds and interacts with itself and other proteins due to its free thiol group leading to the formation of disulphide bonds between molecules [47]. The aggregation of β-lactoglobulin with other proteins will produce a thicker membrane compared to the compact and thin (2–3 nm) layer formed by native β-lactoglobulin molecules [46]. As both Concept IMF and Control IMFs were heat-treated at temperatures higher than 65 °C, β-lactoglobulin is more likely to be present as an aggregated protein molecule. Unsaturated phospholipids form a very thin monolayer of about 2 nm and saturated phospholipids, like sphingomyelin, form monolayers of up to 4 nm due to straighter fatty acid chains [48]. The observations support the notion that (1) the thick interface of Control IMF fat droplets is composed of caseins and whey proteins only, mostly in the aggregated state, and that (2) the thin interface of Concept IMF fat droplets is more likely to be composed of phospholipids, MFGM fragments or adsorbed MFGM proteins and lipids, and native undenatured milk proteins as well as few casein micelles associated with the interface.

The coating of human milk fat globules is likely optimal for the digestive system of infants as it facilitates lipolysis by digestive lipases and bile salt-stimulated lipase present naturally in HM. The MFGM also provides essential nutrients such as liposoluble vitamins and cholesterol and functional components such as mucins and other oligosaccharide-containing molecules protecting the gastrointestinal tract of the infant from viruses and pathogens [9,12] and contributing to immune education [22].

Lipolysis is an interfacial process [49]. Gastric and pancreatic lipases hydrolyze fat at the oil–water interface. Thus, the composition and structure of the oil–water interface is likely to affect lipolysis in the gastrointestinal tract [22]. In infants, the pancreatic lipase and bile salt levels are low compared with levels in adults, so the products from gastric lipolysis play an important role in the digestion of milk lipids by compensating for low levels of pancreatic lipase and emulsifying lipids, respectively [7]. Processing of human milk (i.e., freezing) and bovine milk (i.e., homogenization and heat treatment) has an effect on the structure of the fat globules and thus affects their bioavailability and bioaccessibility [21,22,50]. Current infant milk formulas are highly processed, resulting in fat droplets much smaller than human milk fat globules that are

covered by a thick protein coat, possibly limiting access of the digestive lipases to the triglyceride core of the fat droplets [22]. In addition, it was recently reported that intestinal lipolysis was enhanced and postprandial lipemia was modulated by emulsification with milk polar phospholipids compared with soy phospholipids [51]. Thus, feeding the newborn an IMF produced using milder processing conditions resulting in fat droplets resembling more closely HM fat globules in composition but more importantly also in structure, as achieved in our Concept IMF, we may expect a digestion pattern that is more similar to that of HM. This adapted digestion could affect postprandial responses and the bioavailability of ingested fatty acids and have possible longer term consequences on metabolism regulation [22], which is currently under investigation.

4. Conclusion

Using confocal laser scanning microscopy and transmission electron microscopy, we have characterized the HM fat globule as well as the fat droplets of two IMFs. We evaluated a novel IMF, with fat droplets having a comparable size and a phospholipid coating mimicking structural aspects of HM. The similar location of cholesterol and the likely association of cholesterol with sphingomyelin in the MFGM fragments but also at the surface of fat droplets of the Concept IMF and human milk fat globules may suggest a similar cholesterol exposure which could lead to comparable regulation of cholesterol uptake in adulthood. The Concept IMF also contained MFGM fragments, which have been suggested to have potential benefits for infant health. The Control IMF is a milk containing smaller fat droplets than human milk and with a protein-only coating. The improved structural features of the novel IMF concept, Nuturis®, may result in an adjusted digestion of fat similar to HM to support optimal development of the neonate, which we are currently investigating. Preclinical studies may also suggest possible longer term benefits of feeding this Concept IMF in early life on body composition development. Clinical studies in infants are currently ongoing (clinical trial NCT01609634). In this so called VENUS study, a randomized controlled double-blind clinical design, the effect of Nuturis® (Concept IMF) on growth and body composition development is investigated. Primary outcomes are centered around formula tolerance in the first 4 months and growth trajectories throughout the first year of life using regular weight and height measures as well as skinfold thickness and diary recordings. At two years of life, ultrasound measures to obtain a proxy of abdominal adipose tissue distribution are performed. The VENUS study tests the assumption that the altered lipid structure in the concept IMF may result in a healthier growth and body composition development pattern. This highlights the role of lipid structure in infant nutrition.

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