Rafael Jiménez-Flores, PhD

Applying **MFGM** and **Human Milk Science** to Improve Nutrition for Infants and Toddlers

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Disclosures

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Consultant	Mead Johnson Nutrition
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Learning Objectives

- Describe how dairy-processing advances have isolated MFGM for supplementation to infant formula

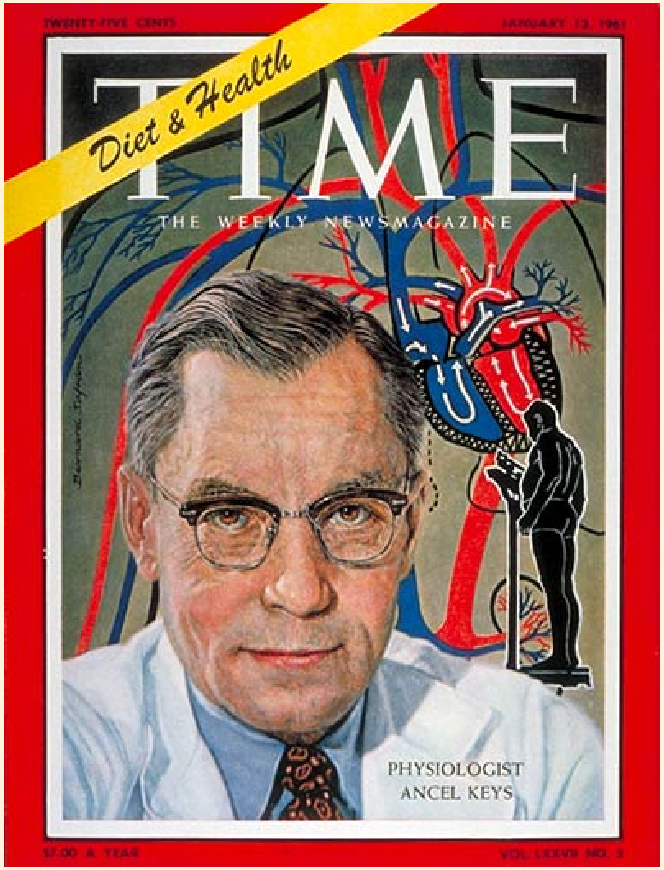
- Examine how purified MFGM from cow's milk added to formula is similar in composition and bioactivity to human milk

MFGM, milk fat globule membrane.

Underutilized Dairy Components

- Lactose
- Minerals and minor components in milk, whey, and permeate
- Milk fat and phospholipids
  - Today, MFGM is what WHEY was in the ’80s
- To learn about processing advances, we need to know about MFGM origin, structure and composition

Lipids and Phospholipids



Ancel Keys on the cover of *TIME* magazine in 1961. He claimed that saturated fats in the US diet damaged arteries and led to coronary disease.

TIME magazine cover story in 2014. Scientists were wrong about saturated fats. They are not linked to higher risk for heart disease.

Left: Diet & Health. Cover credit: Bernard Safran. *Time*. 1961.
Right: Cover photograph: Mitchell Feinberg. Cover story: Walsh B. Eat Butter. *Time*. 2014;183(24)30-35.

So… What Does MFGM Have to Do With Nutrition?

- Is MFGM similar to human and cows' milk?
- What is the contribution of phospholipids to MFGM structure?
- How do we obtain MFGM for use in foods?
- What about inclusion in infant formula?

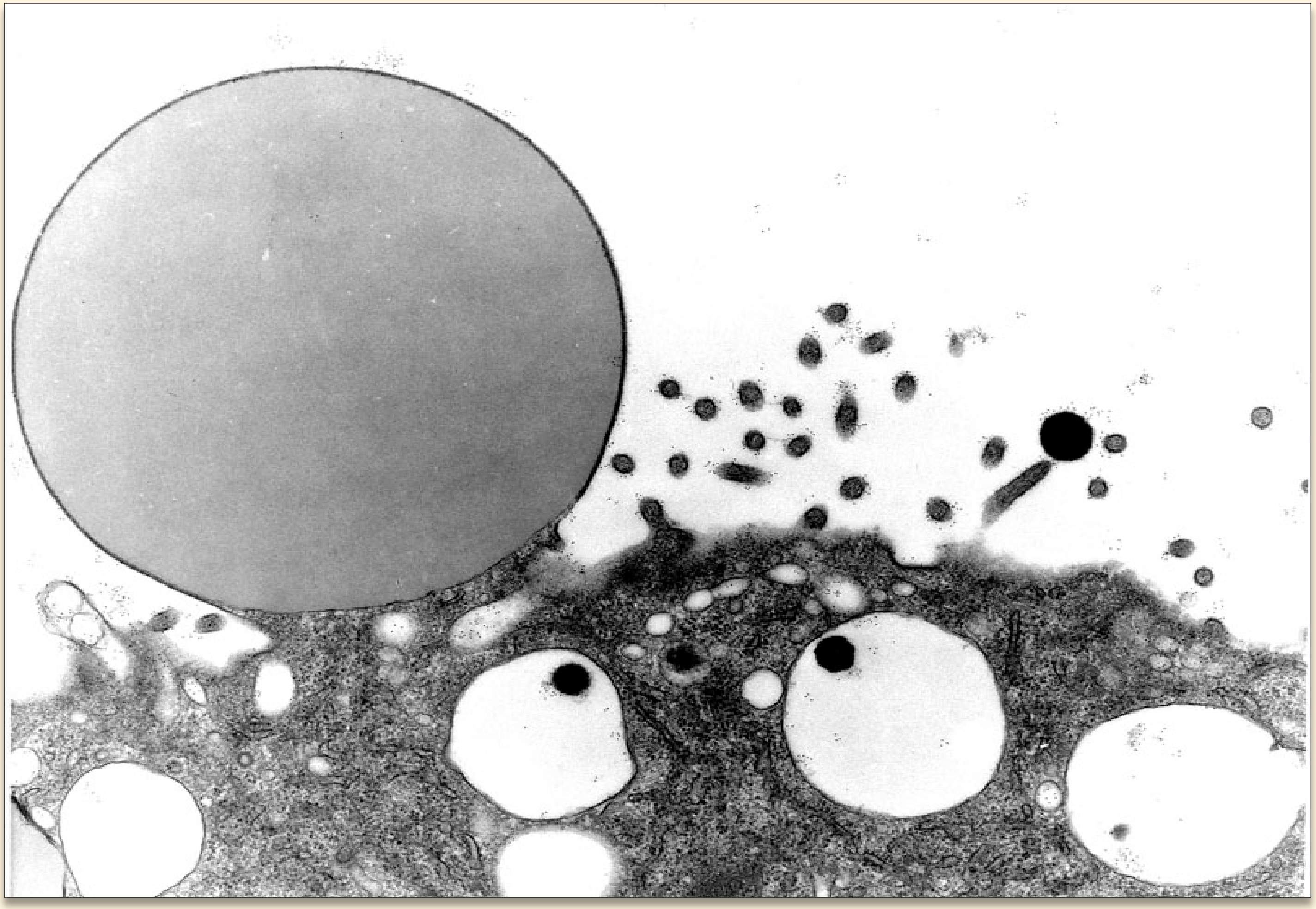
MFGM Components Found in Human and Bovine Milk and Their Potential Impact

Brain Function	Immune Defense
Choline	Mucins
Sphingomyelin	Butyrophilin
Gangliosides	Lactadherin
Cholesterol	CD14
Sialic acid	TLR1
Inositol	TLR4
Cerebrosides	Xanthine oxidase

CD14, cluster of differentiation 14; TLR, toll-like receptor.

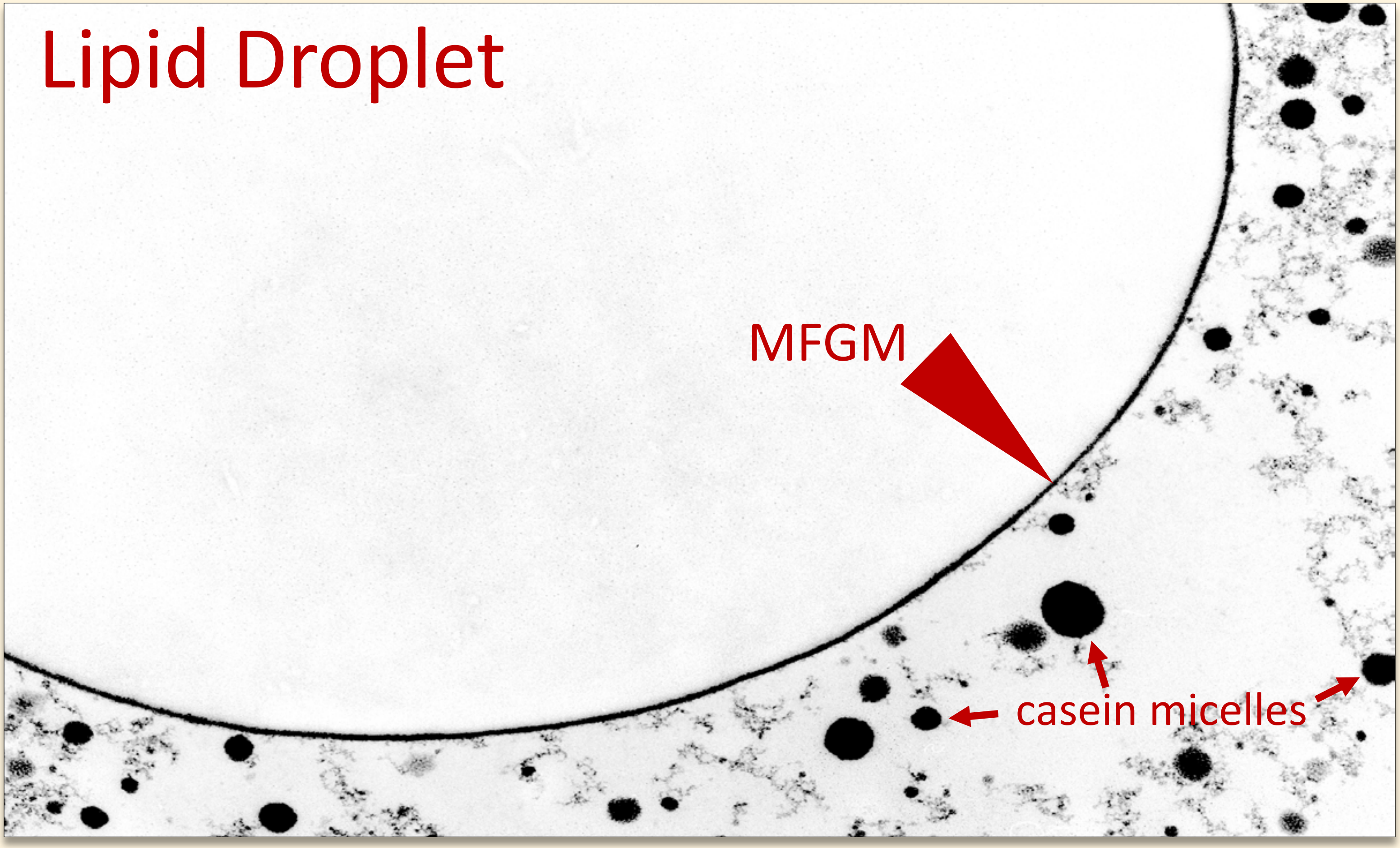
Hernell O, et al. *J Pediatr.* 2016;173 Suppl:S60-5.

Origin of MFGM



Mather ID, In: *Encyclopedia of Dairy Sciences, Second Edition*. Springer. 2011:680-690.

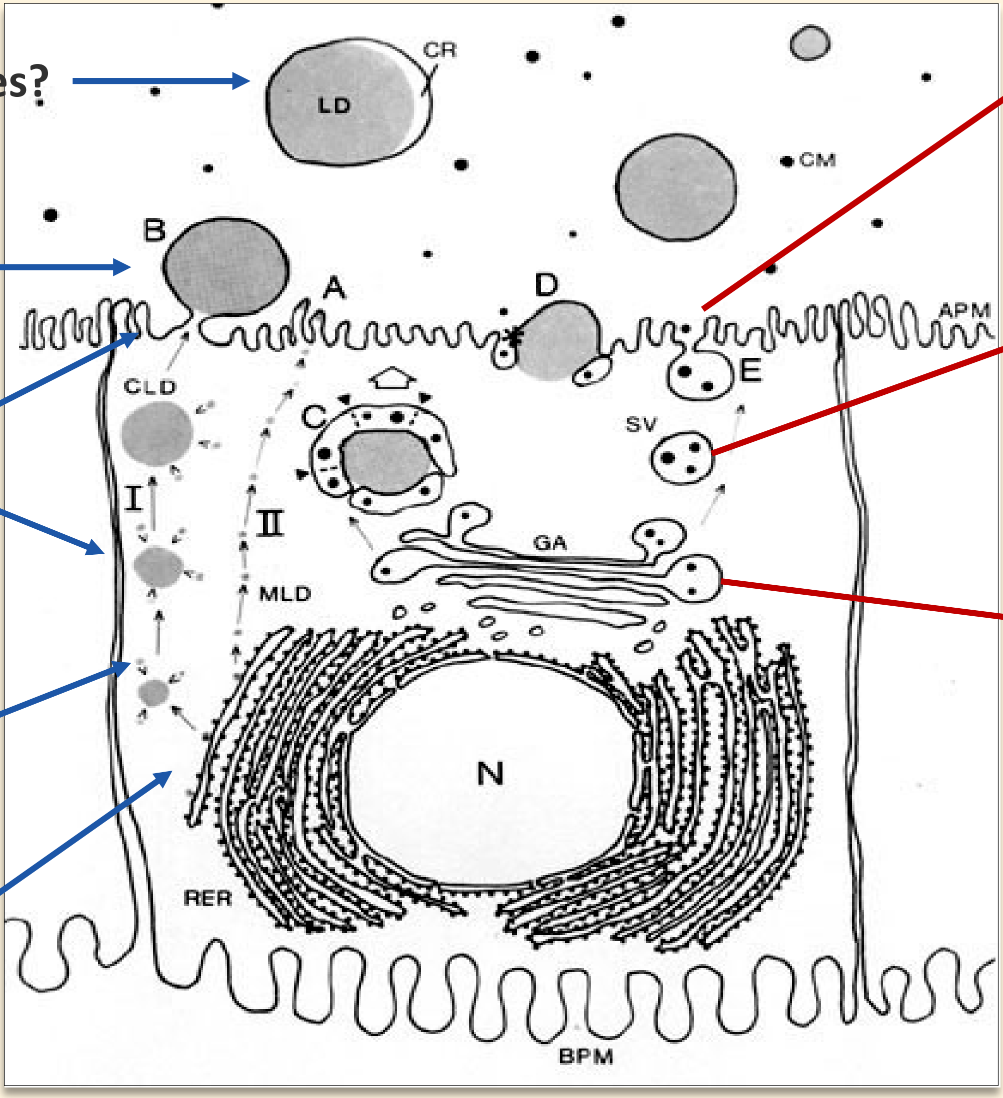
What is the Milk Fat Globule Membrane?



MFGM, milk fat globule membrane.

Mather IH. *J Dairy Sci.* 2000;83:203–247.

How is the MFGM Formed?



Post-secretion changes?

Secretion

Fusion of micro-lipid droplets

Transit to the apical membrane

Origin in the RER

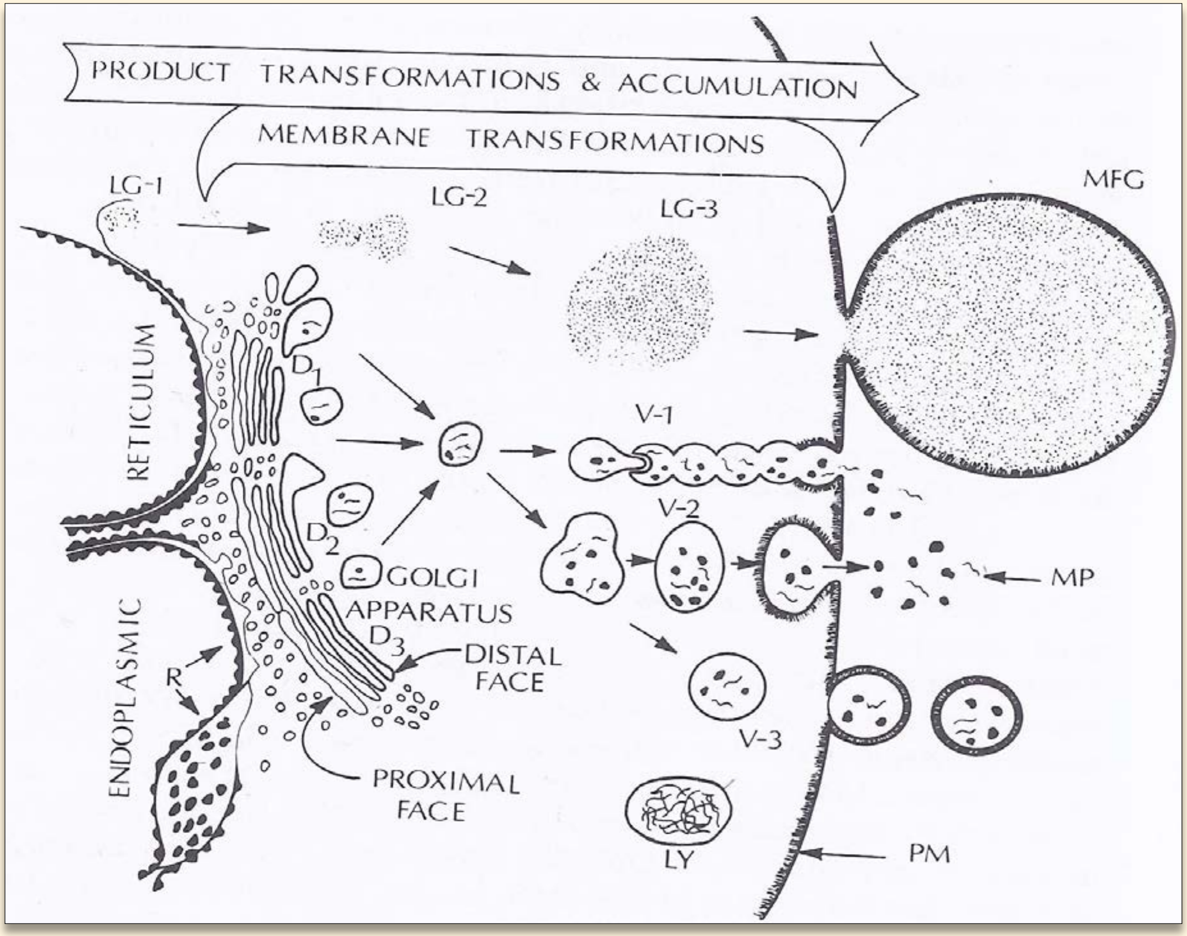
Turnover of apical membrane

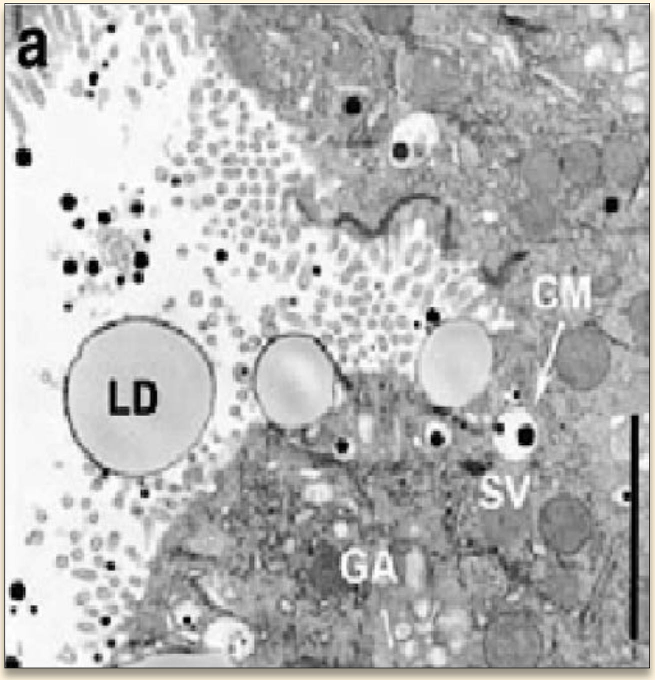
Transit of vesicles and regulation of exocytosis

Dynamics of secretory vesicle formation

APM, apical plasma membrane; BPM, basal plasma membrane; CLD, cytoplasmic lipid droplet; CR, cytoplasm residue; CM, casein micelle; GA, golgi apparatus; LD, lipid droplets; MLD, micro lipid droplets; RER, rough endoplasmic reticulum; SV, secretory vesicle.
Adapted from Mather and Keenan (1998).

Diagram of Synthesis and Secretion of Milk Constituents From Apical Membrane of the Epithelial Cell of a Mammary Gland





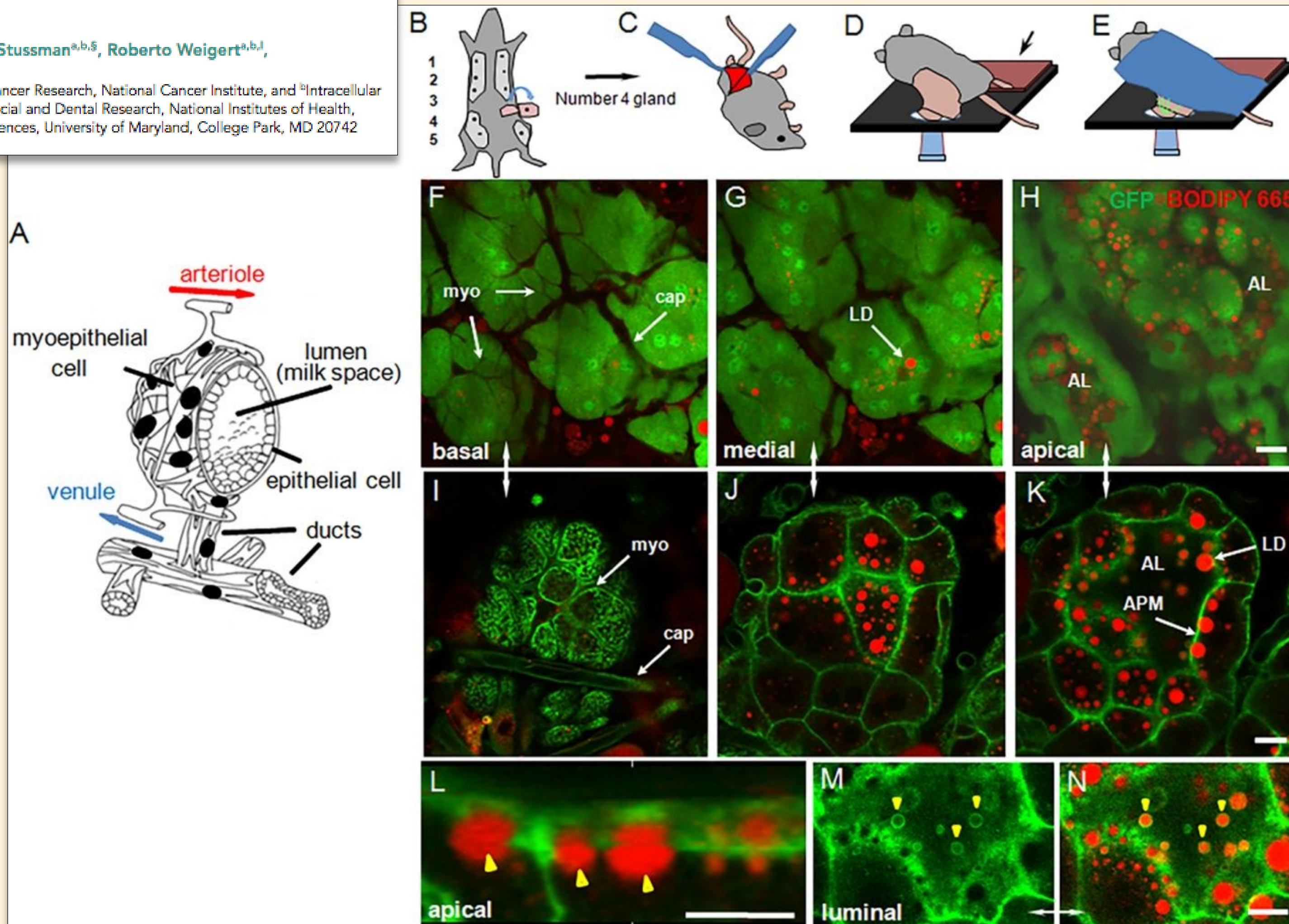
LD, lipid droplets; LG, lipid globules; MFG, milk fat globules; MP, milk protein, GA, golgi apparatus; GM, globule membrane.

Adapted from Keenan et al. 1988. Insert from Masedunskas A, et al. *Mol Biol Cell.* 2017;28:935-946.

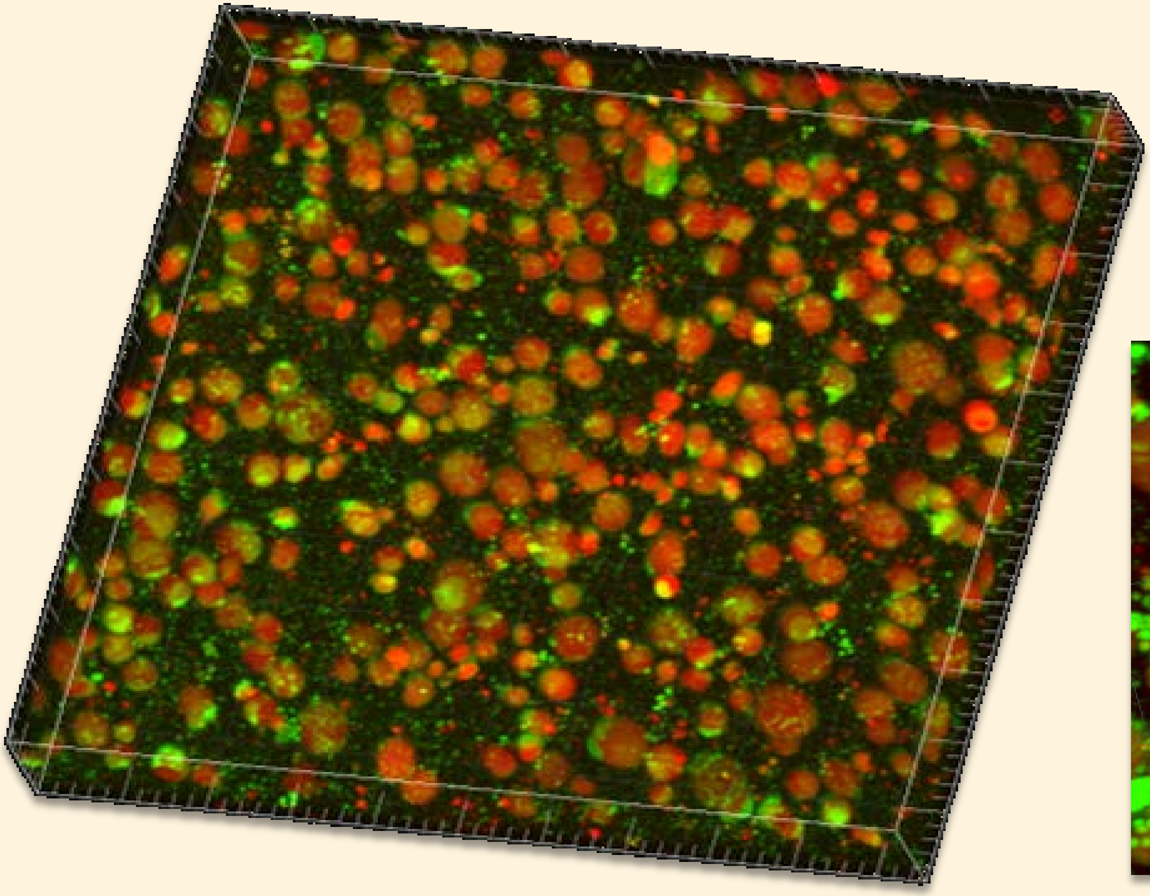
Kinetics of milk lipid droplet transport, growth, and secretion revealed by intravital imaging: lipid droplet release is intermittently stimulated by oxytocin

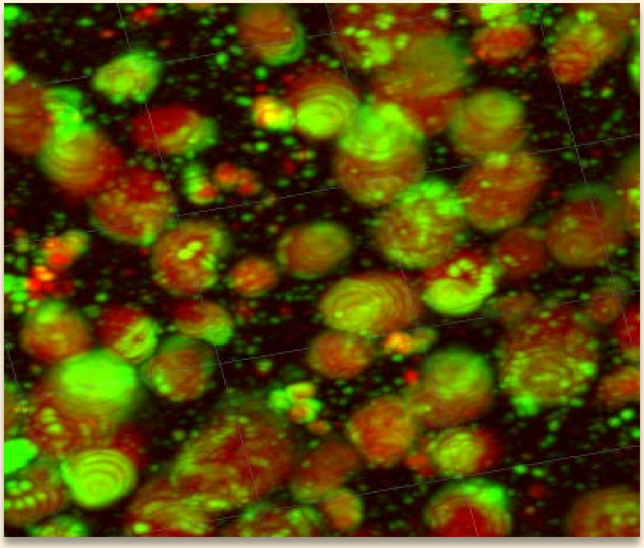
Andrius Masedunskas[a,b,†], Yun Chen[a,b,‡], Rebecca Stussman[a,b,§], Roberto Weigert[a,b,‖], and Ian H. Mather[a,b,c,‖,*]

[a]Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, and [b]Intracellular Membrane Trafficking Section, National Institute of Craniofacial and Dental Research, National Institutes of Health, Bethesda, MD 20892; [c]Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742



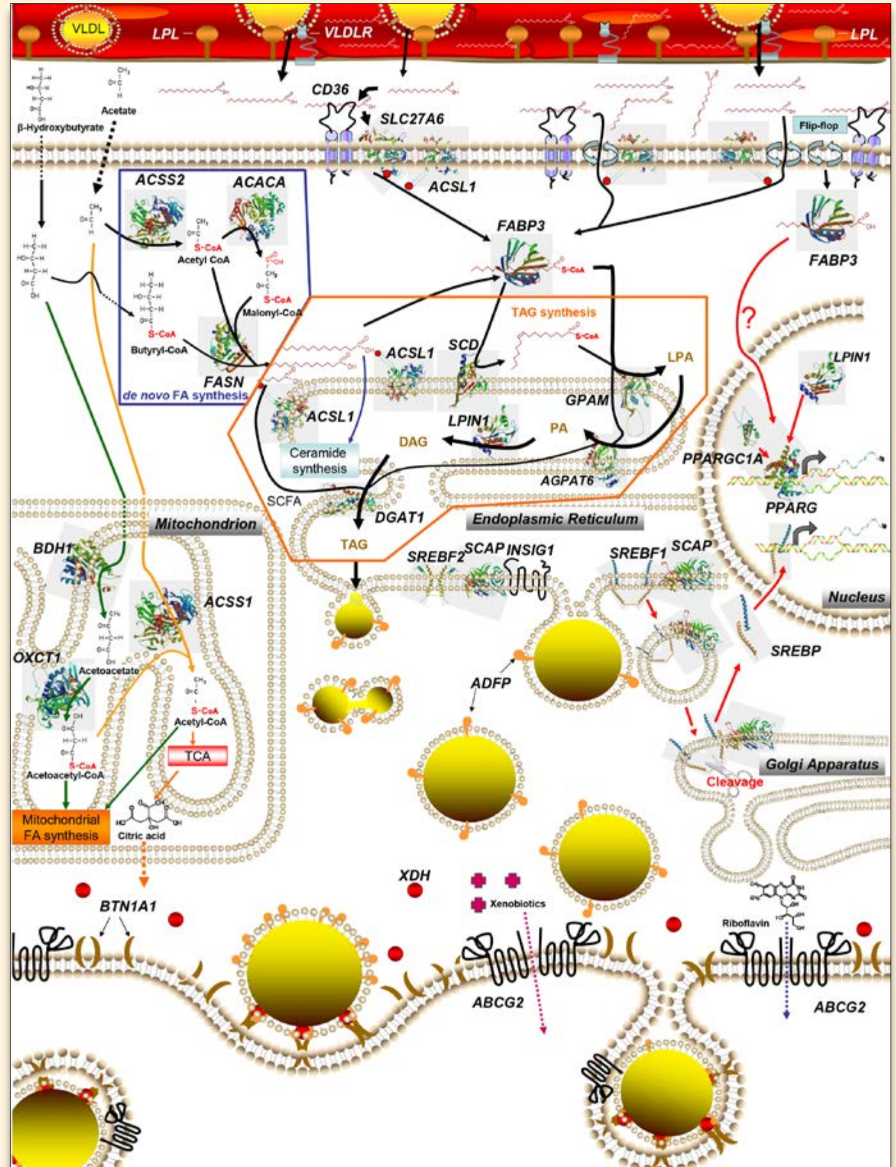
Kinetics of Milk Lipid Droplet Transport, Growth, and Secretion Revealed by Intravital Imaging *(cont)*





Masedunskas A, et al. *Mol Biol Cell.* 2017;28:935-946.

The GO indicate that most conserved genes belong to the metabolic pathway for fat globules and MFGM production.



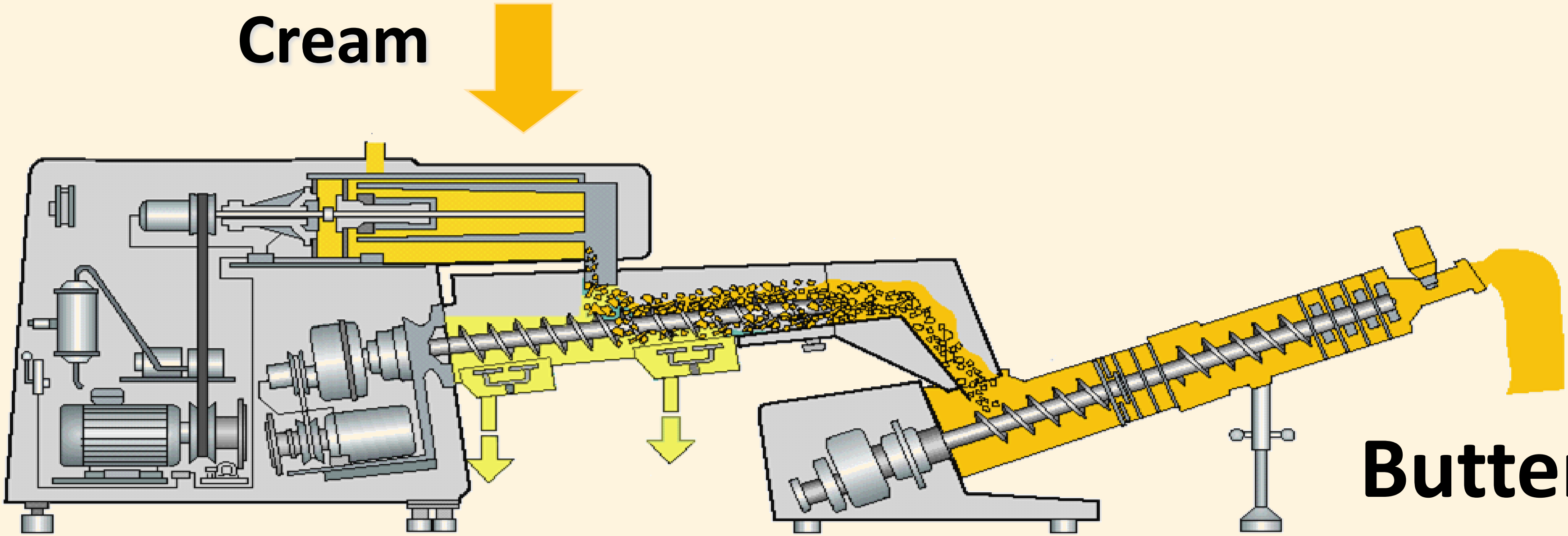
GO, gene ontology.

Industrial Processing to Obtain MFGM

- From cream
- From butter and buttermilk
- From whey
- Thermal treatments

Buttermilk Process

Cream



Butter

1.8 billion lb

Buttermilk

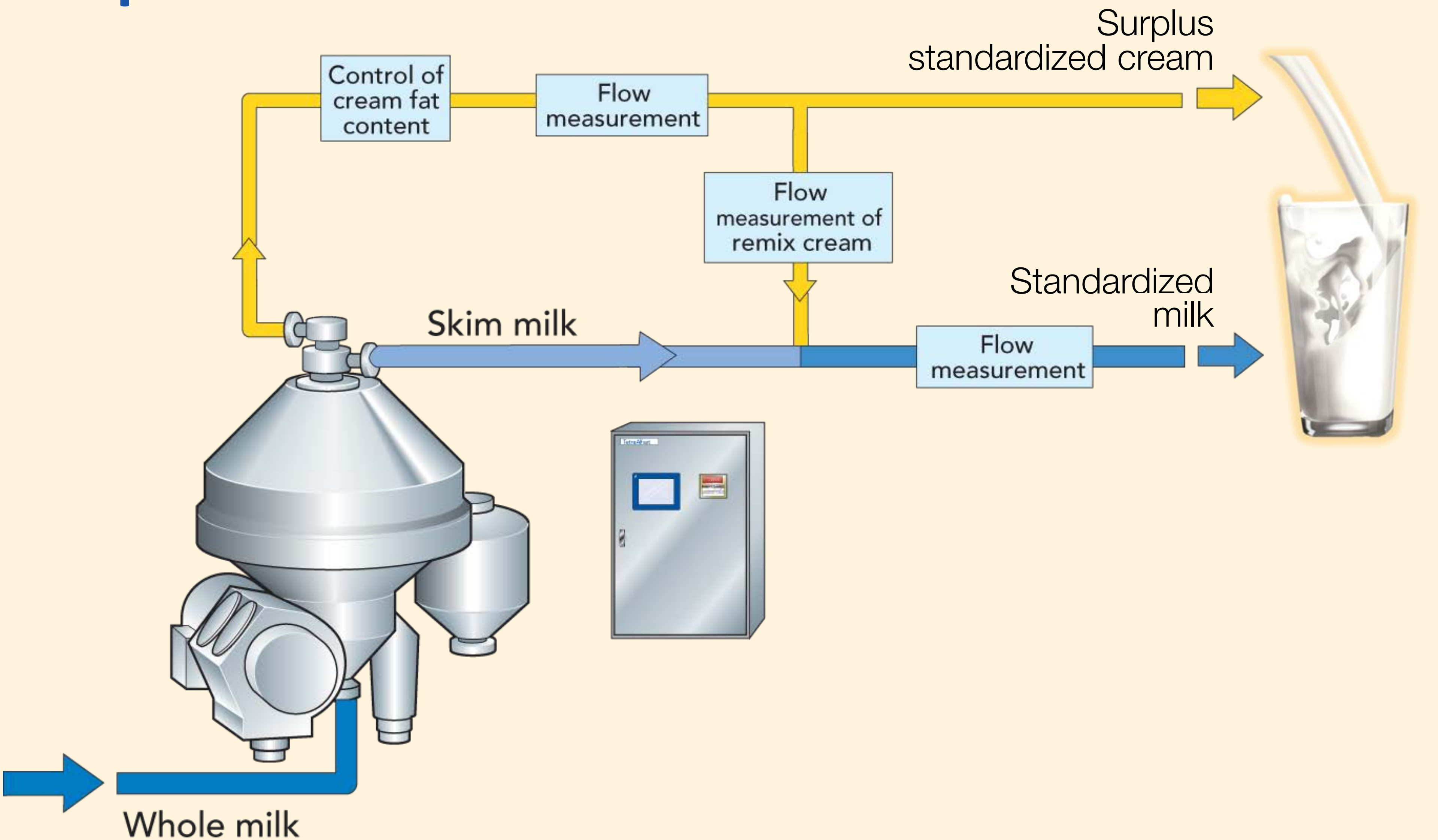
31 million lb condensed/evap.

49 million lb dry

Bylund G. Dairy Processing Handbook. Tetra Pak. 2015. Available at http://dairyprocessinghandbook.com.

Buttermilk From Cream or Whey

Separation of Cream From Skim Milk



Continuous Butter and Buttermilk Production

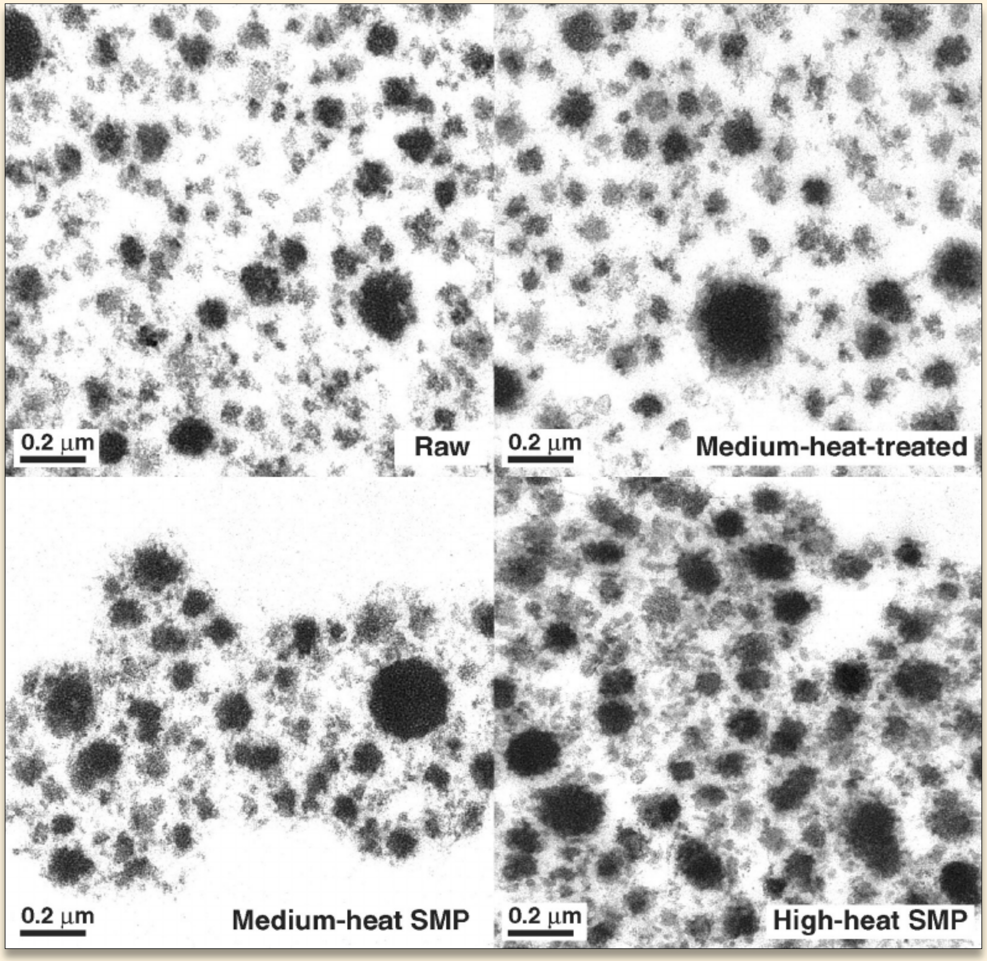
Buttermilk

Spray Drier

To assay

Products

Transmission Electron Micrograph of Buttermilk



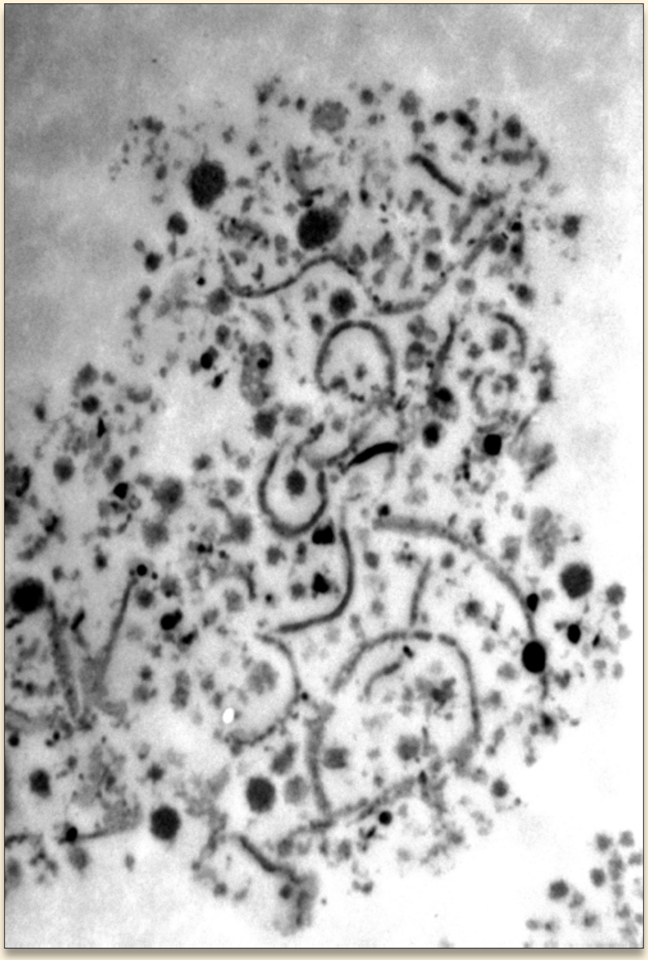
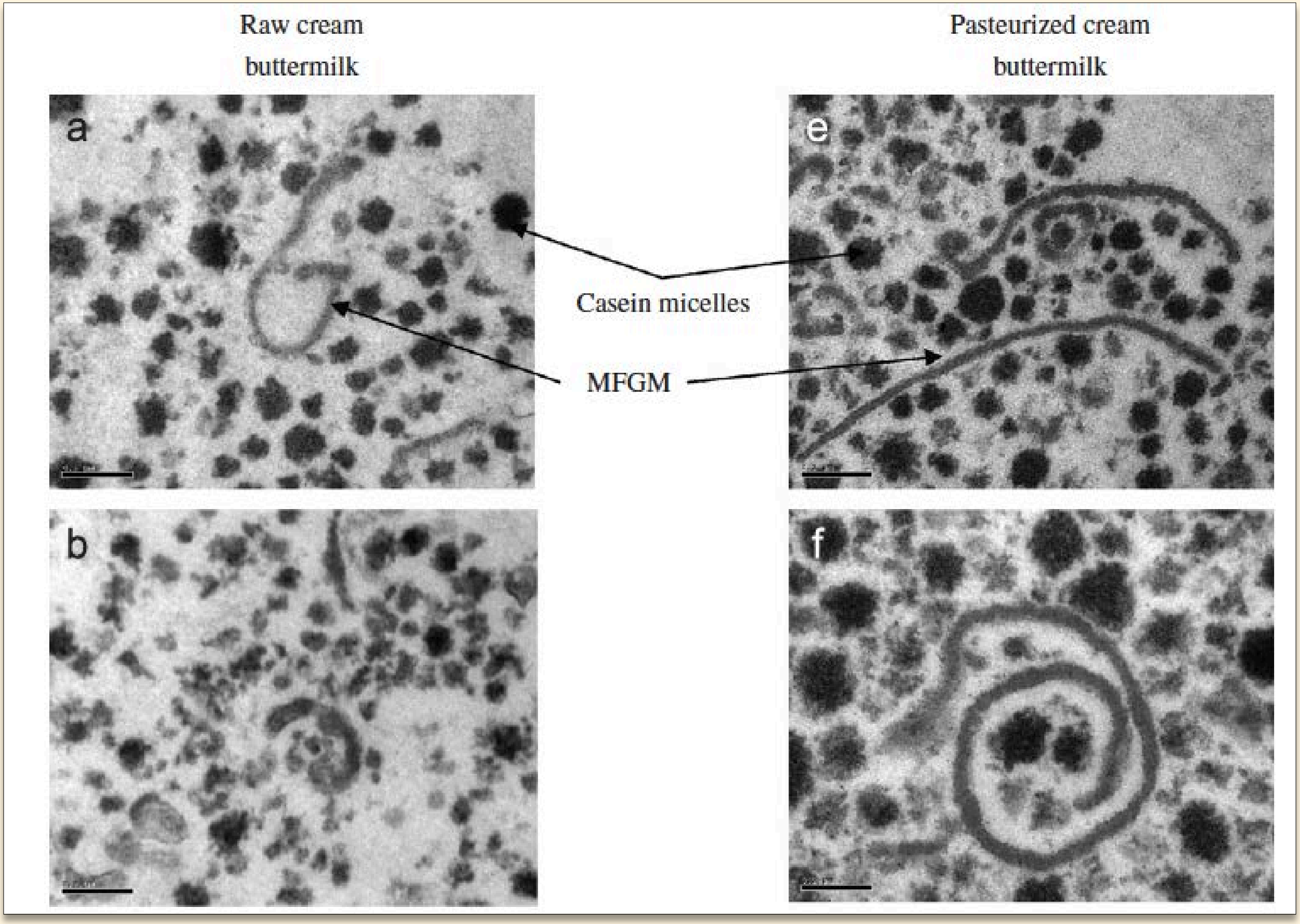


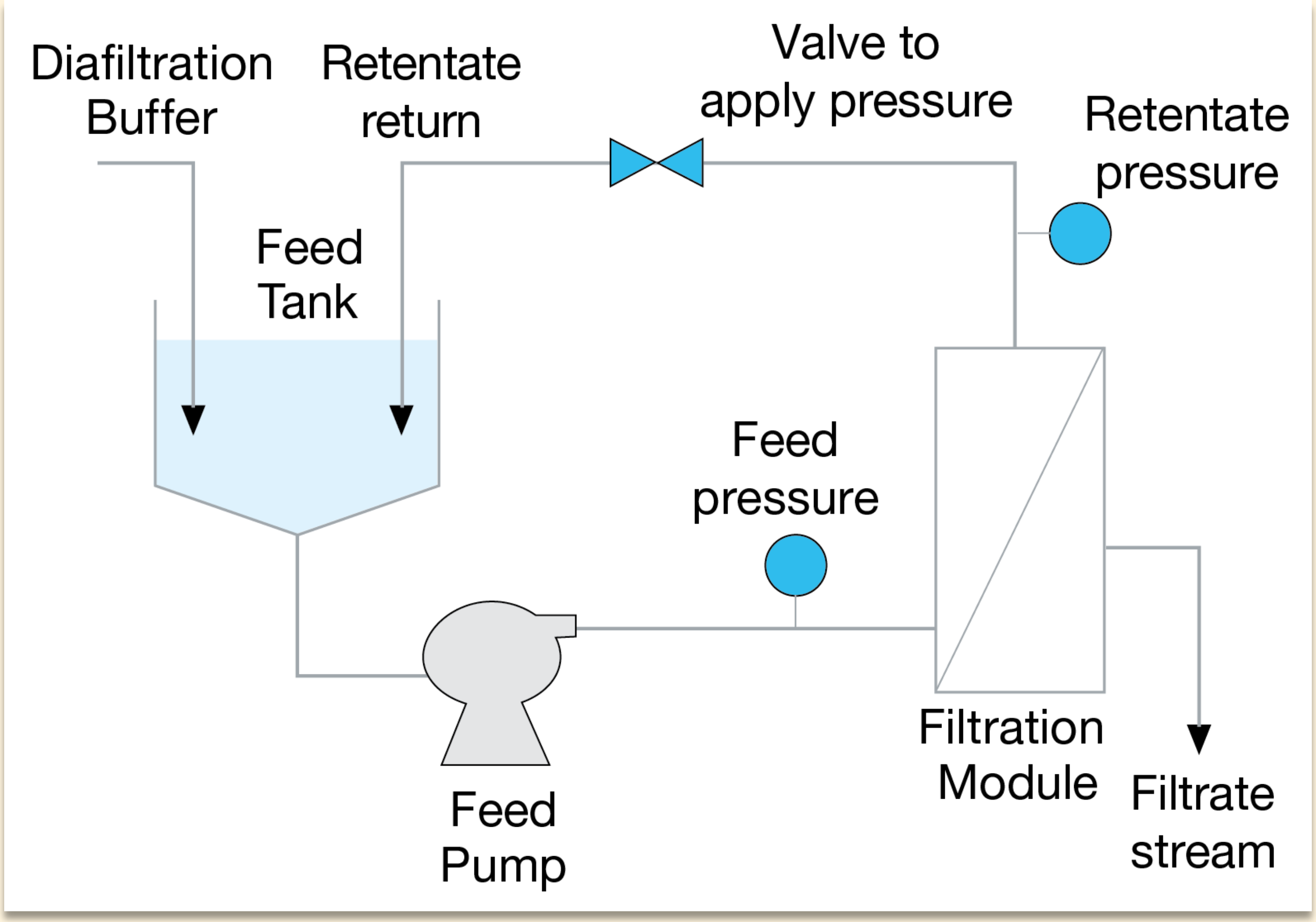
Photo: Beth Fryksdale and R. Jiménez-Flores, 1998



Morin P, et al. *Int Dairy Journal.* 2007;17(10):1179–1187.

MFGM From Whey

Ultrafiltration

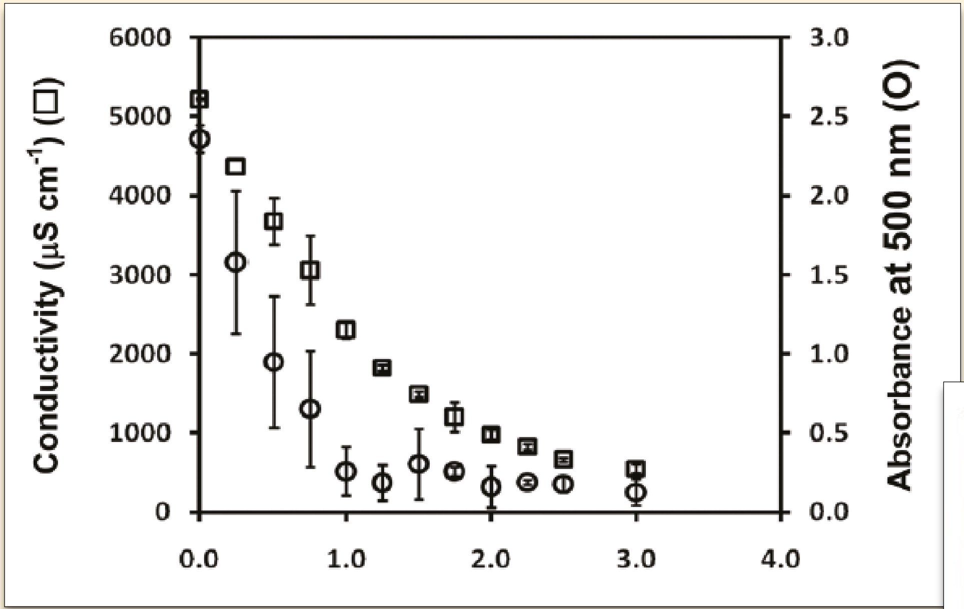


See https://www.youtube.com/watch?v=qEJVK0hhA0w

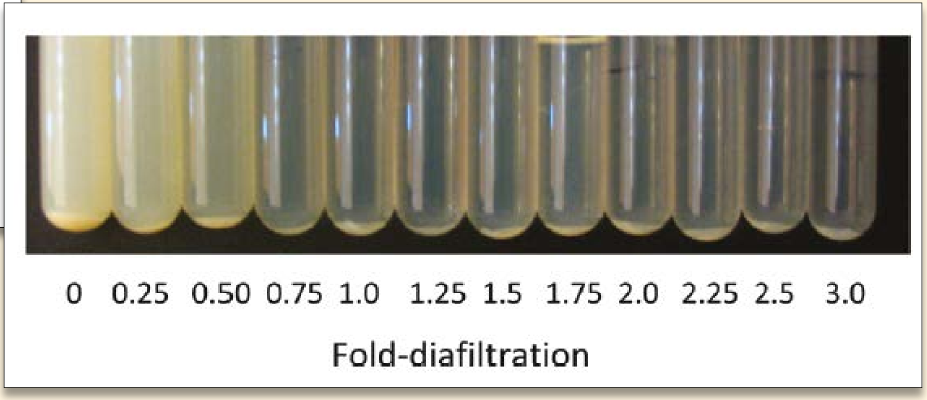
Adapted from EMD Millipore Technical Brief. No. TB032.

MFGM From Whey

A



B



(A) Effect of continuous diafiltration on the conductivity of 5 x whey (□) and the extent of precipitation of MFGM at pH 4.2 (❍). Other experimental conditions were as described in publication. Absorbance values were measured without dilution. The bars represent standard deviation (n=2). (B) Clarity of the supernatants as function of fold-diafiltration.

Damodaran S, *J Agric Food Chem.* 2011;59:10271–10276. Reprinted with permission from *Journal of Agricultural and Food Chemistry*.

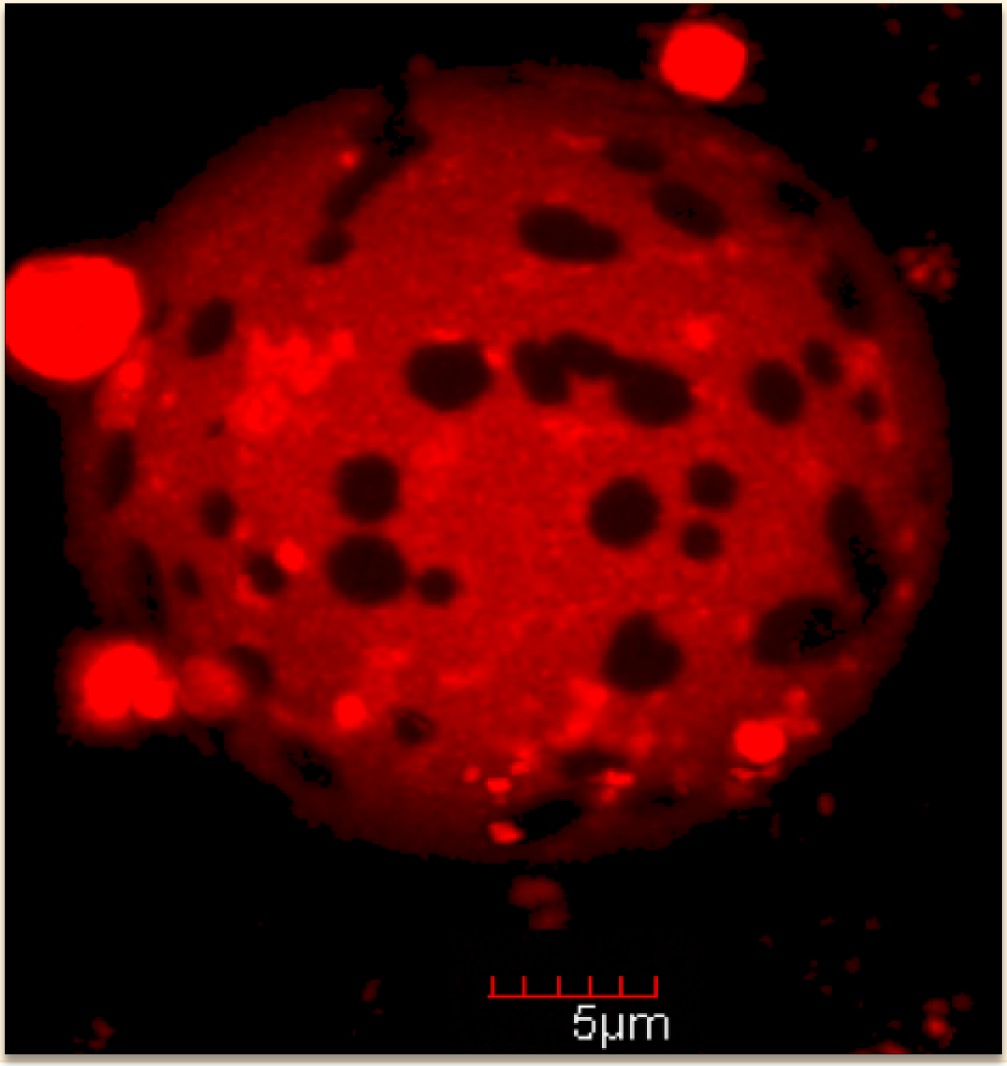
Laboratory and Industrial Scale Ultrafiltration

Laboratory

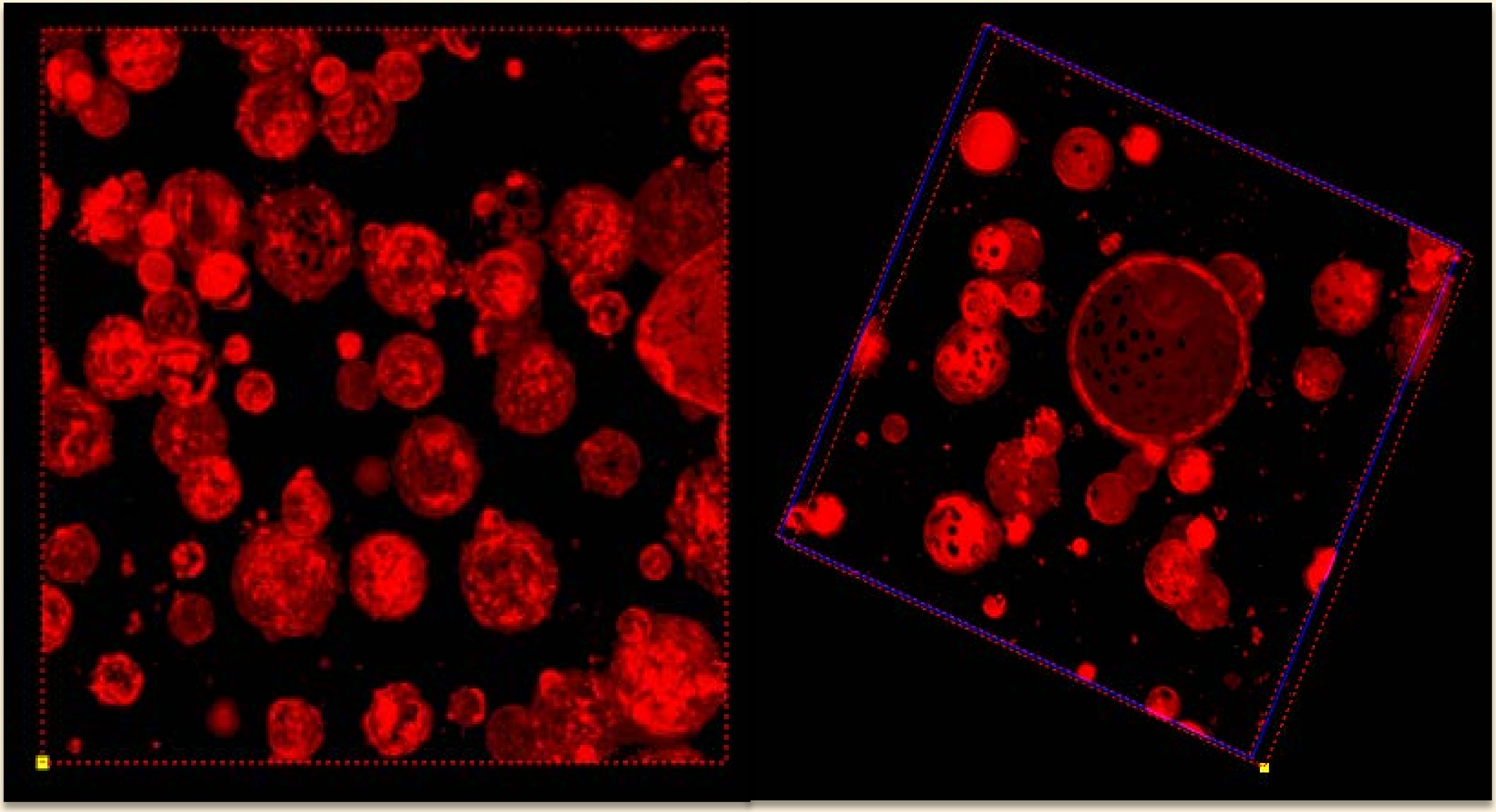
Pilot Plant

Industrial Scale

Confocal Laser Fluorescence Microscopy

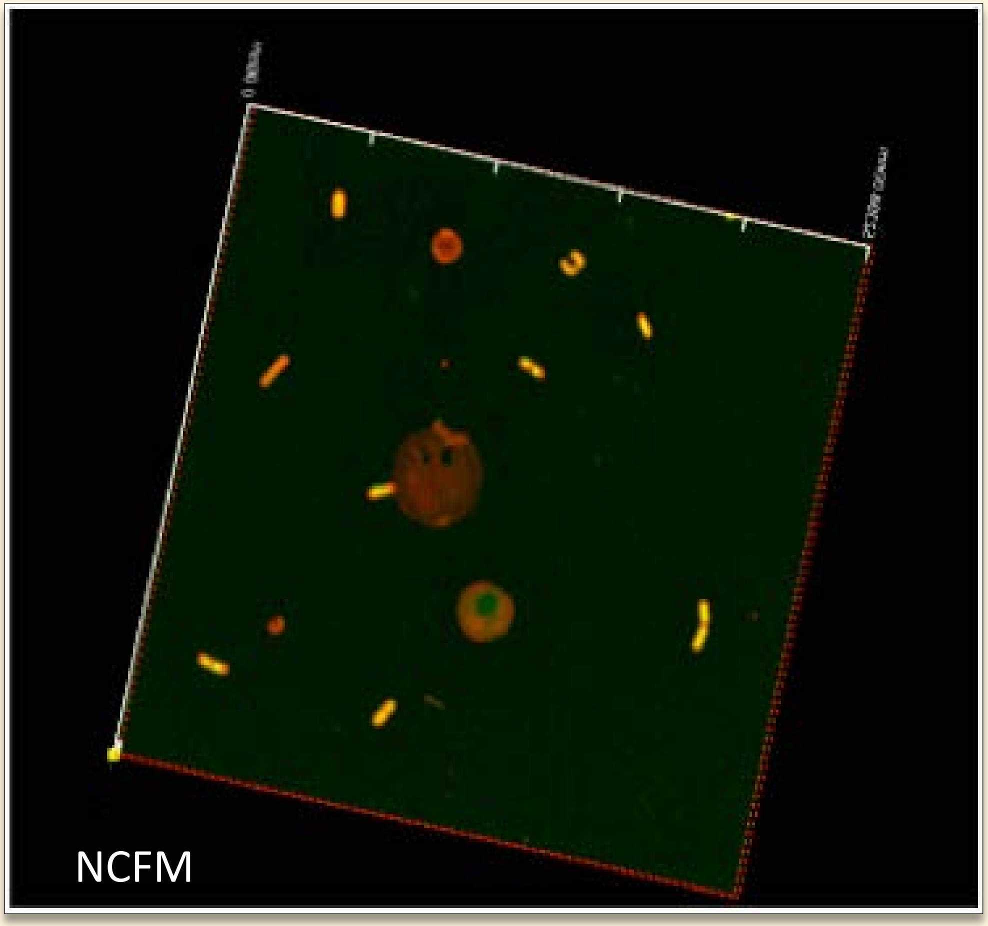


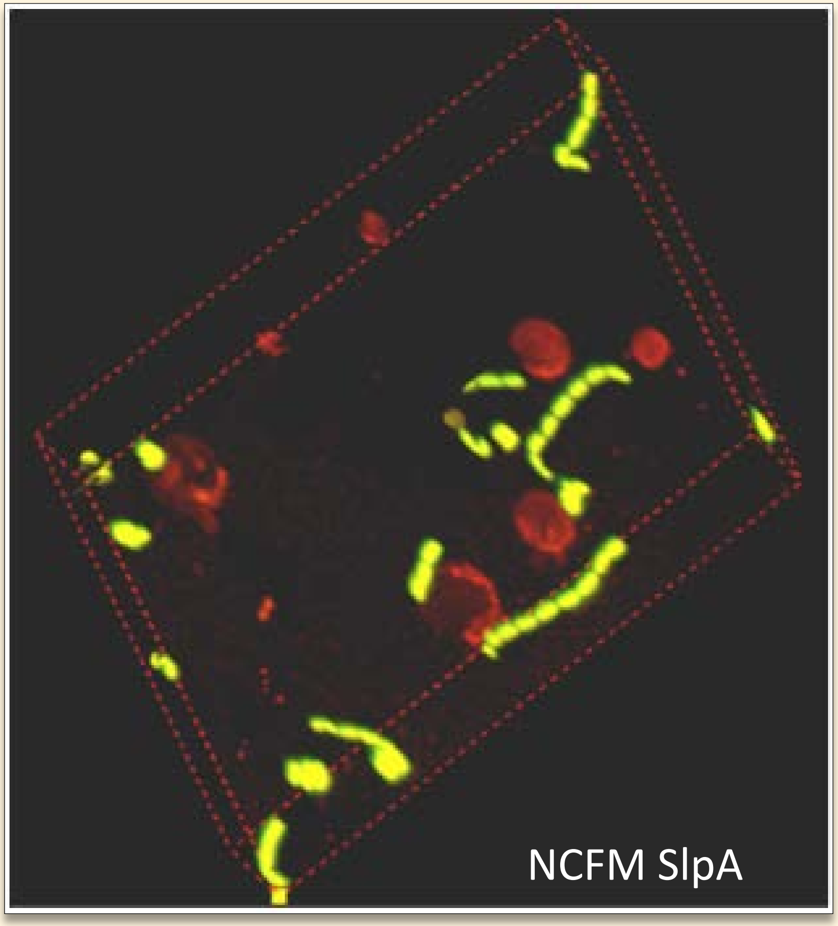
CLSM to Probe Temperature Effects



Figures: Confocal laser scanning microscopy (CLSM) images of raw cream fat globules stained with Rd-DOPE and cooled at 4°C (left) or heated at 45°C for 10min (right)

Confocal Microscopy of MFGM and Bacterial Binding



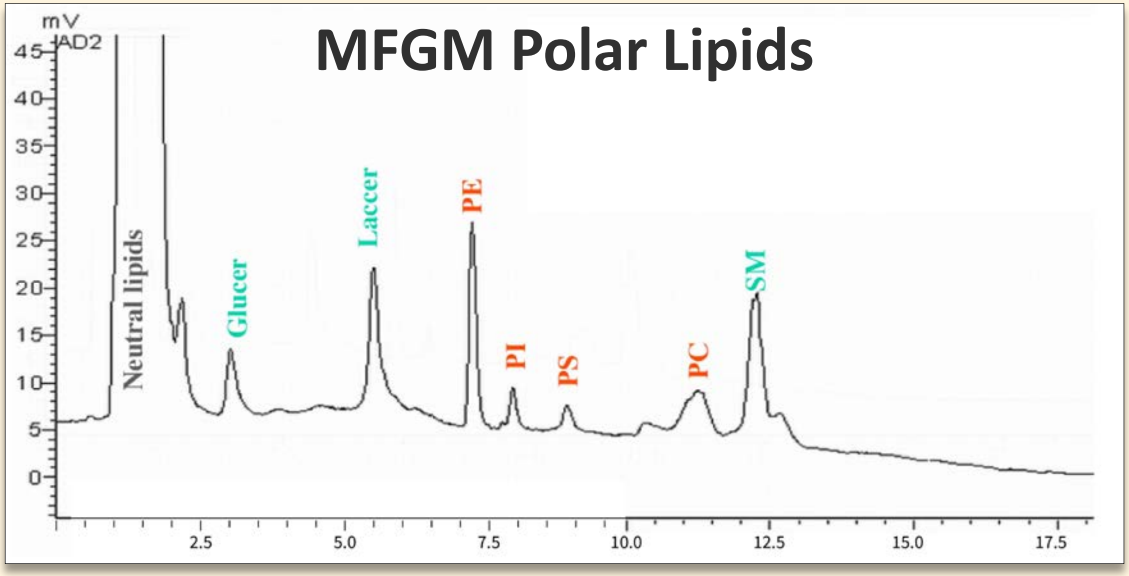


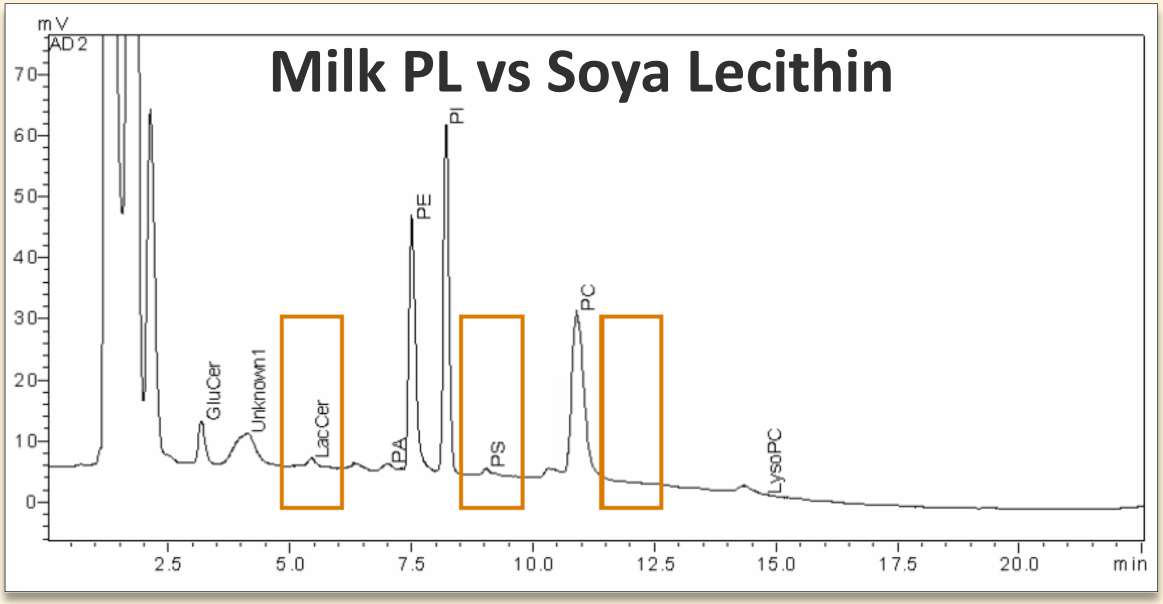
Brisson G, et al. *J Agric Food Chem.* 2010;58:5612-9. Reprinted with permission from *Journal of Agricultural and Food Chemistry*.

Comparison

Sphingolipids (30%)
- Glucosylceramide (Glucer)
- Lactosylceramide (Laccer)
- Sphingomyelin (SM)

Phospholipids (70%)
- PE – Phosphatidylethanolamine
- PI – Phosphatidylinositol
- PS – Phosphatidylserine
- PC - Phosphatidylcholine



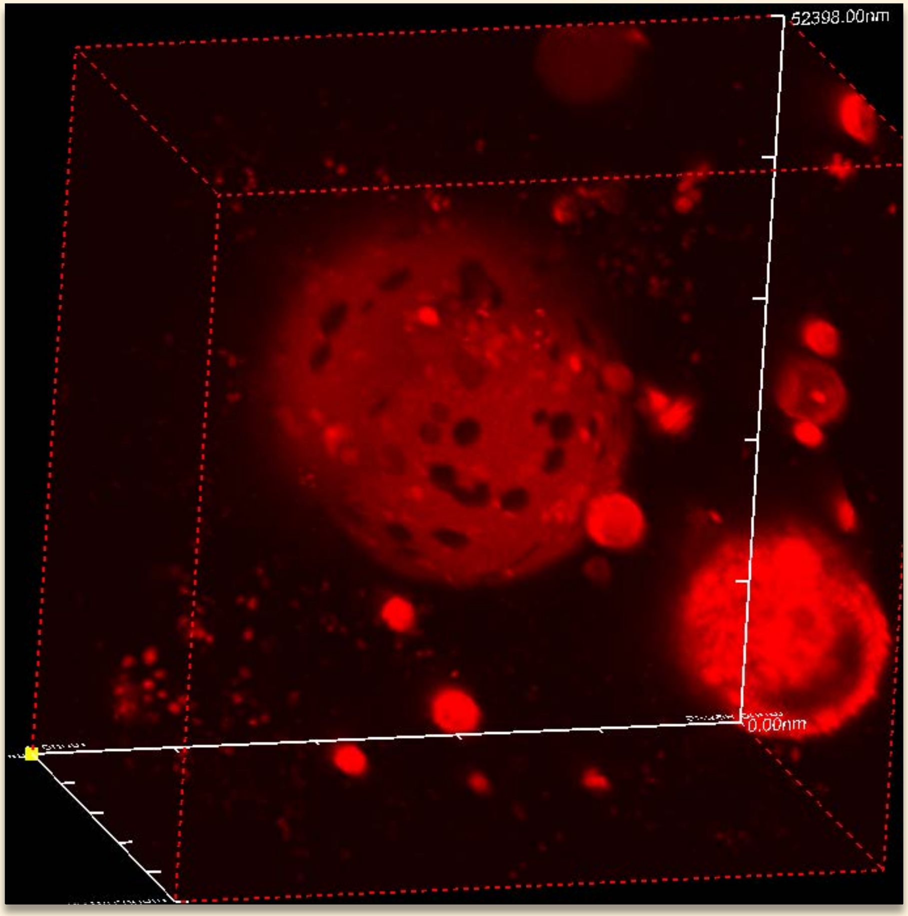


Soya Lecithin Profile

Unpublished results.

Microdomains Form in the Outer Leaflets of Biological Membranes

- Formation of disordered (Ld) and ordered (Lo) lipid phases
- Sphingomyelin (SM) and cholesterol rich
- Localization of glycated lipids likely in outer leaflet, but still debated



Gallier S, et al. *J Agric Food Chem.* 2010; 58:4250–4257. Unpublished image, used with permission.

Lipid Composition of the MFGM

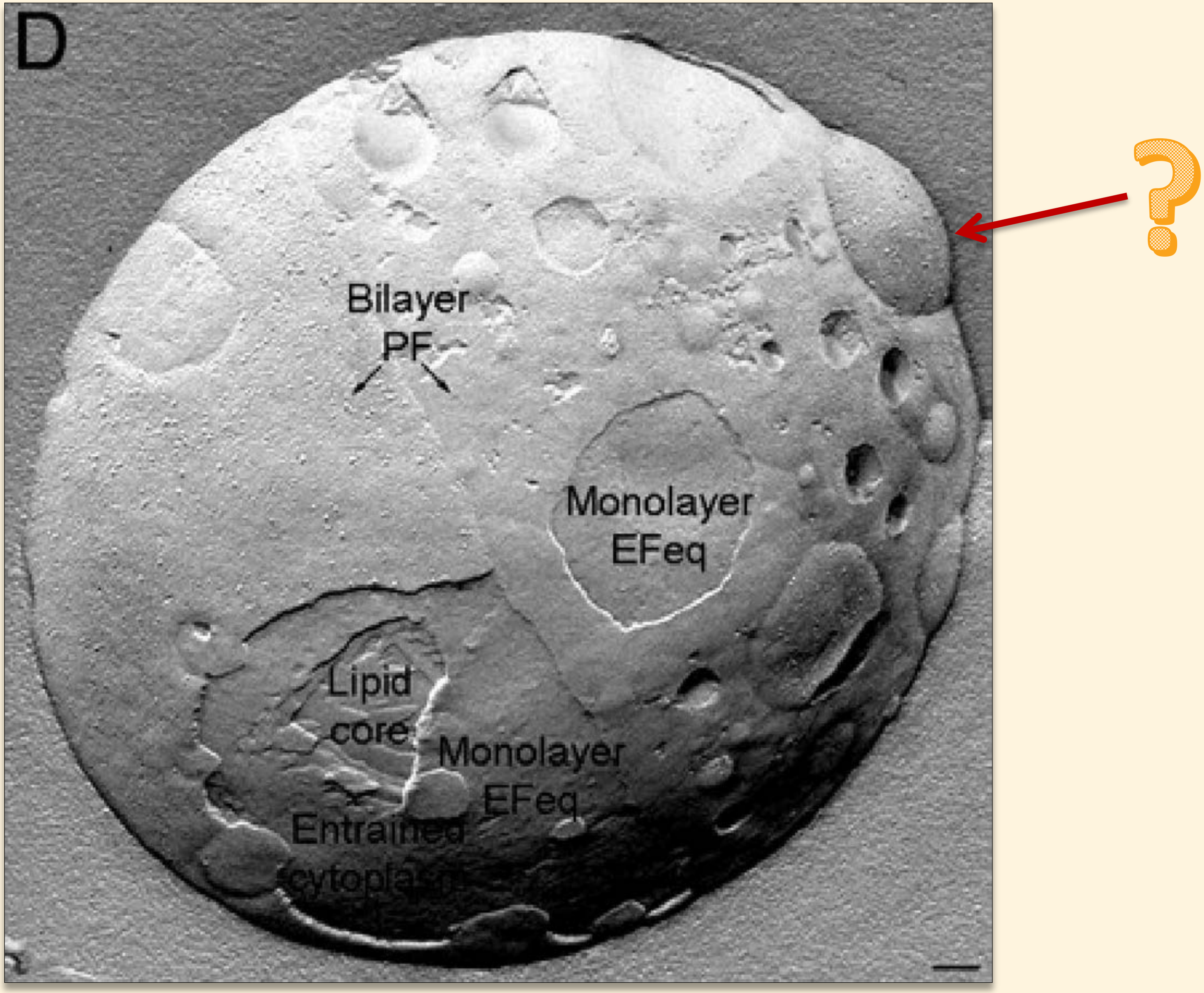
Table 2. Content of main PLs in milk fat of different mammalian species. Values are expressed as percentage of total PLs

Species	PE	PI	PS	PC	SM
buffalo	24.5	19.7	6.6	24.3	24.9
goat	31.7	6.3	8.3	28.5	25.2
ewe	34.4	4.4	5.2	28.6	27.4
human	21.3	16.4		19	43.3
donkey	60.2	2.4	11.2	17.3	8.8
mare	24.3	8.5	10.6	27.8	28.9
human	21.7	4.5	9.6	29	35.2
camel	34.3	4.9	10.5	22.1	28.1

Table 3. Polar lipid content (g/100 g of fat) and phospholipids composition (percentage of total PLs) in milk fat of dairy products and by-products of butter-making process.

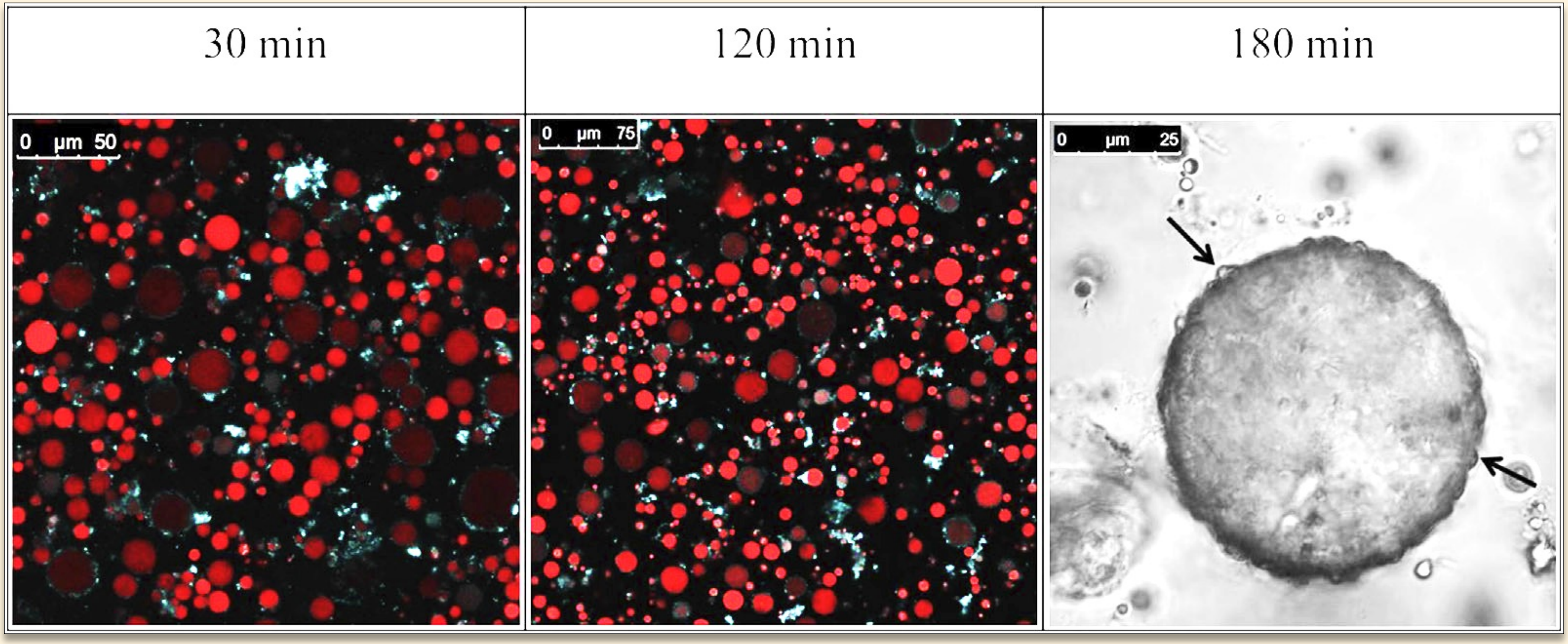
Matrix	Polar lipids	PE	PI	PS	PC	SM
cream	0.86	42.7	6.8	7.2	14.6	28.6
butter	0.2	31	11.9	15.3	24.7	17.1
buttermilk	4.49	33.5	2.4	10.3	35.5	18.3
cow cream	0.17					
cow buttermilk	0.17	38.7	9.3	9.1	23.9	18.9
cow butter serum	0.88	27.2	10.8	7.2	29.8	24.9
goat cream	0.2					
goat buttermilk	0.19	35.2	9.8	9.9	24.8	20.3
goat butter serum	1.01	27.1	11.7	8.2	26.2	26.8
cream		26.7	7.5	11.7	26.5	20.8
cream	5.65	17.7	15.4	11.3	33.7	21.8
butter	5.31	17.7	15.8	11.5	33.3	21.8
buttermilk	12.4	17	7.1	8.1	46.1	21.7
buttermilk		8.4	8.2	4.6	51.2	27.6

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.



PF, P-face; Efeq, E-face equivalent.

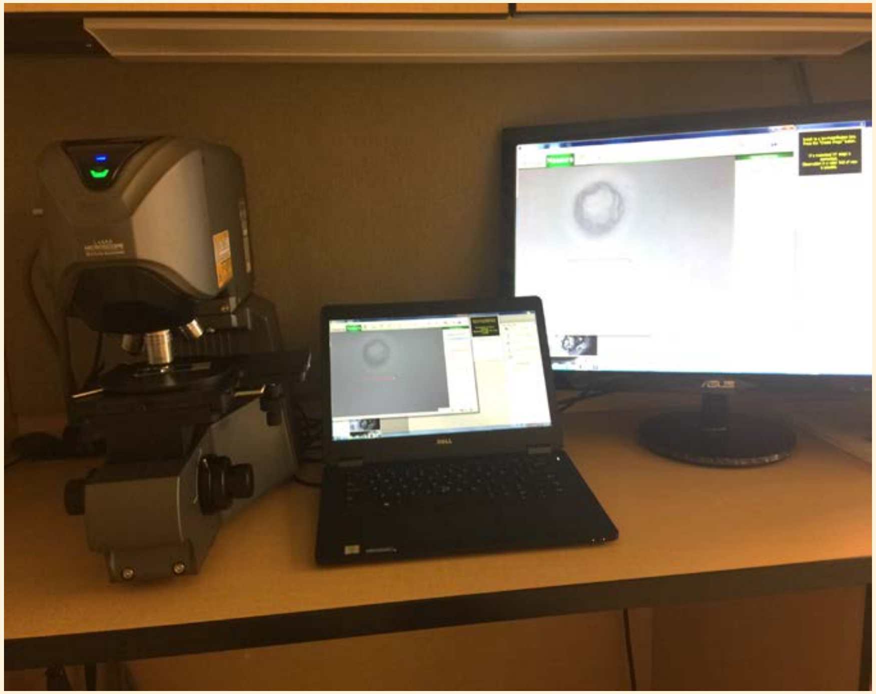
Phospholipid and SM Digestion/Absorption

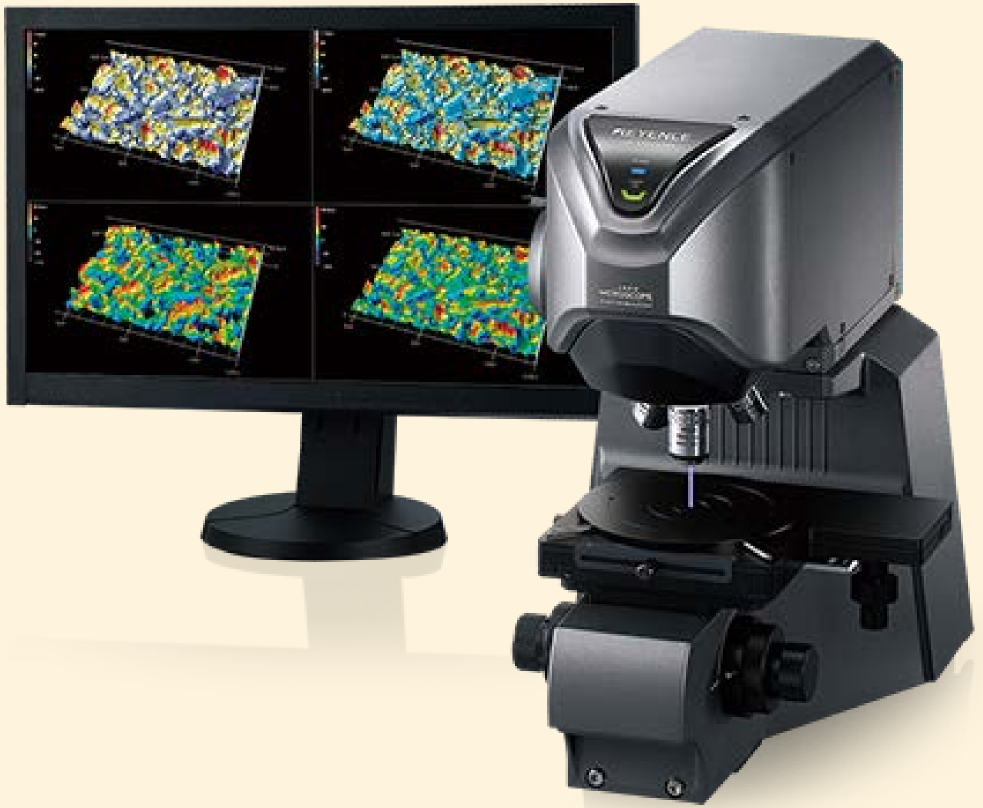


SM, sphingomyelin.

Gallier S, et al. *Food Structure.* 2016.

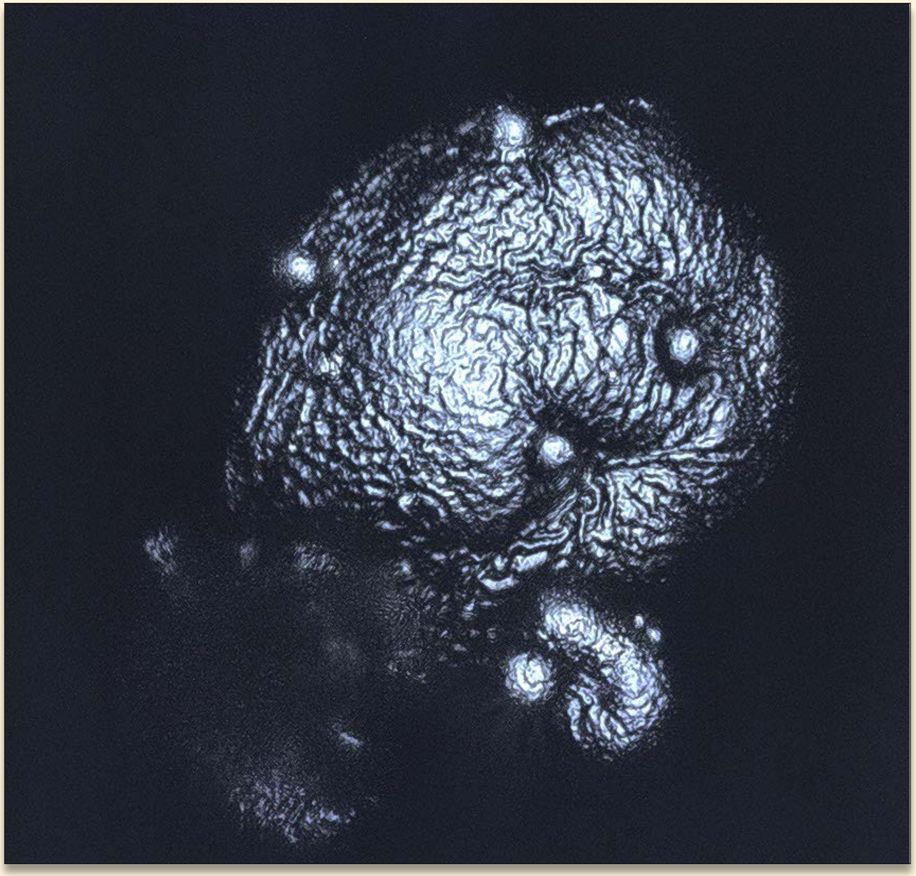
- Capture Images of MFGM
- Observe the distribution of Phospholipids and Glycoproteins



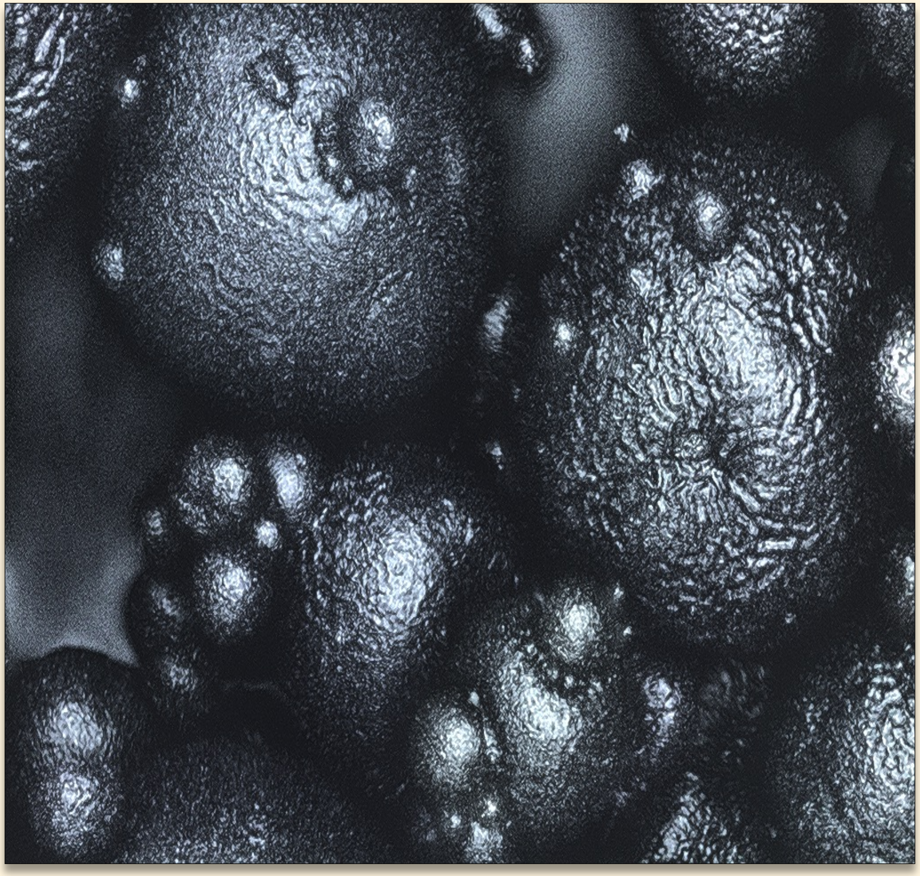


Photos courtesy of Johnny Vo

Buttermilk Powder Granule—Morphology



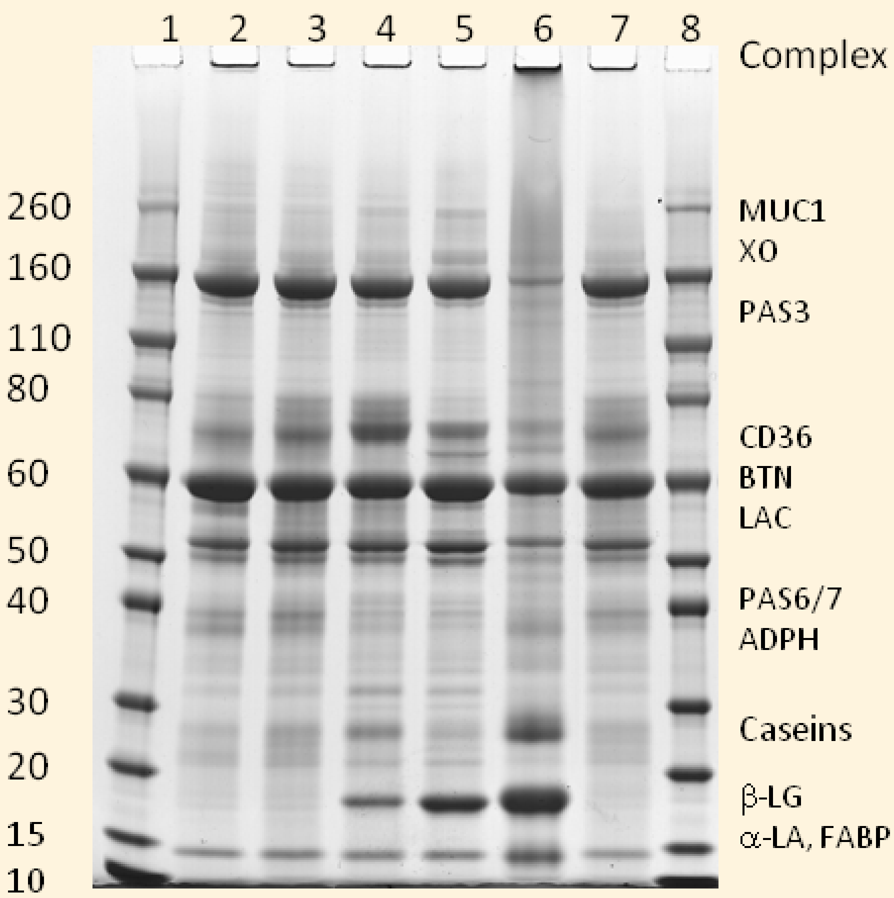
Untreated sample – 150 x confocal/laser

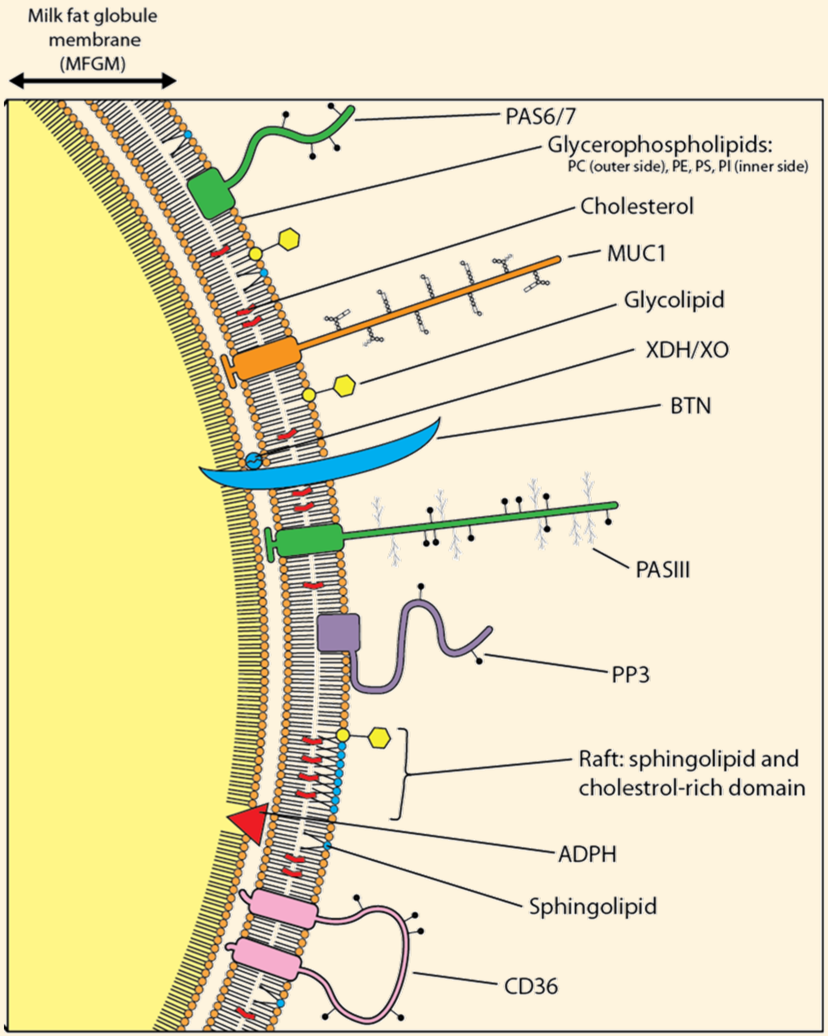


350 bar, 75^{o}C – 150 x confocal/laser

Unpublished results.

Buttermilk: More Than a Source of Milk Phospholipids





Laubscher, Elias, and Jiménez-Flores. Unpublished results.

Membrane-Rich Milk-Fat Diet Provides Protection Against Gastrointestinal Leakiness in Mice Treated With Lipopolysaccharide

	Saline Vehicle Control Injection	LPS Injection
AIN-76A	n = 6	n = 6
AIN-76A with milk fat	n = 6	n = 6

Figure 1. A 2 × 2 experimental design of treatments was used for each time point (24 and 48 h). Mice (n = 24) were randomly assigned to one of the following treatments: 1) control diet [American Institute of Nutrition (AIN)-76A], saline vehicle control injection (n = 6); 2) control diet, LPS injection (n = 6); 3) AIN-76A + milk fat diet, saline vehicle control injection (n = 6); or 4) AIN-76A + milk fat diet, LPS injection (n = 6).

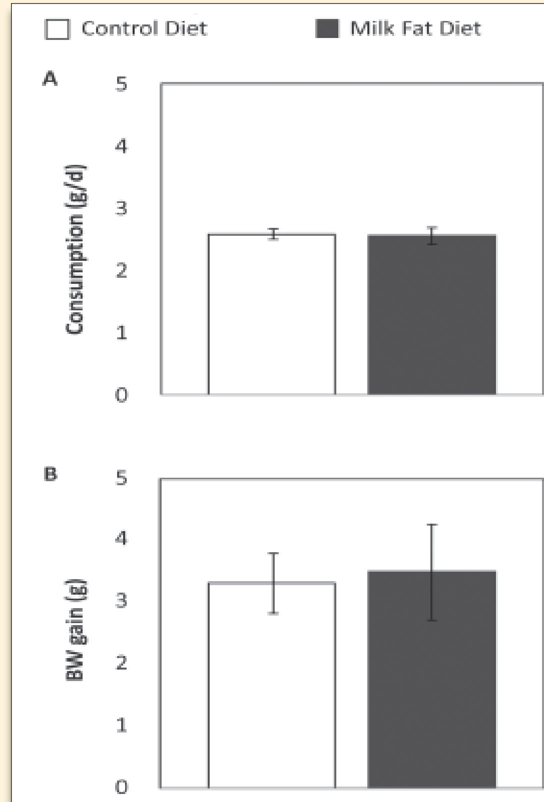
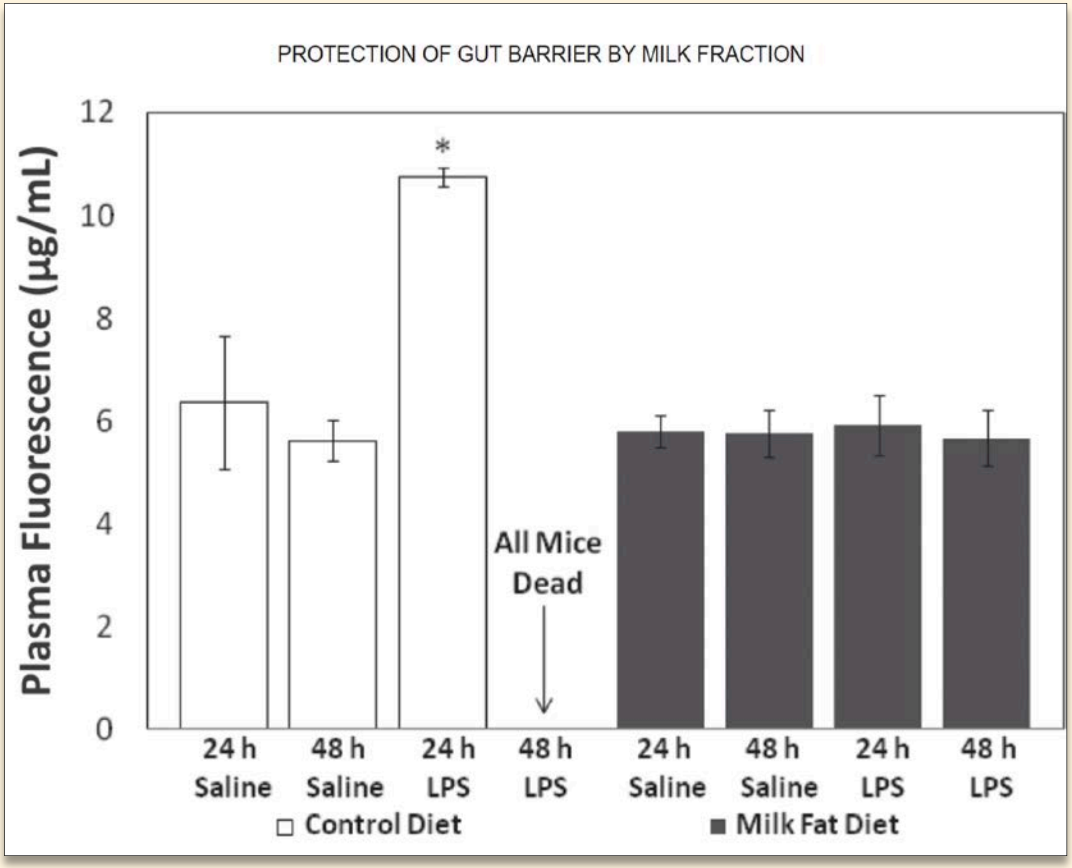


Figure 2. Effect of experimental diets on consumption (A) and total weight gain (B). Values are means (n=36 control diet, n=33 milk fat diet) ± SD. Experimental diets did not significantly affect consumption or weight gain.

Membrane-Rich Milk-Fat Diet Provides Protection Against Gastrointestinal Leakiness *(cont)*



Although the diets used in this study were formulated to differ only in the fat source, the use of a **complex material like MFGM makes it impossible to associate the stress protection with any single component.**

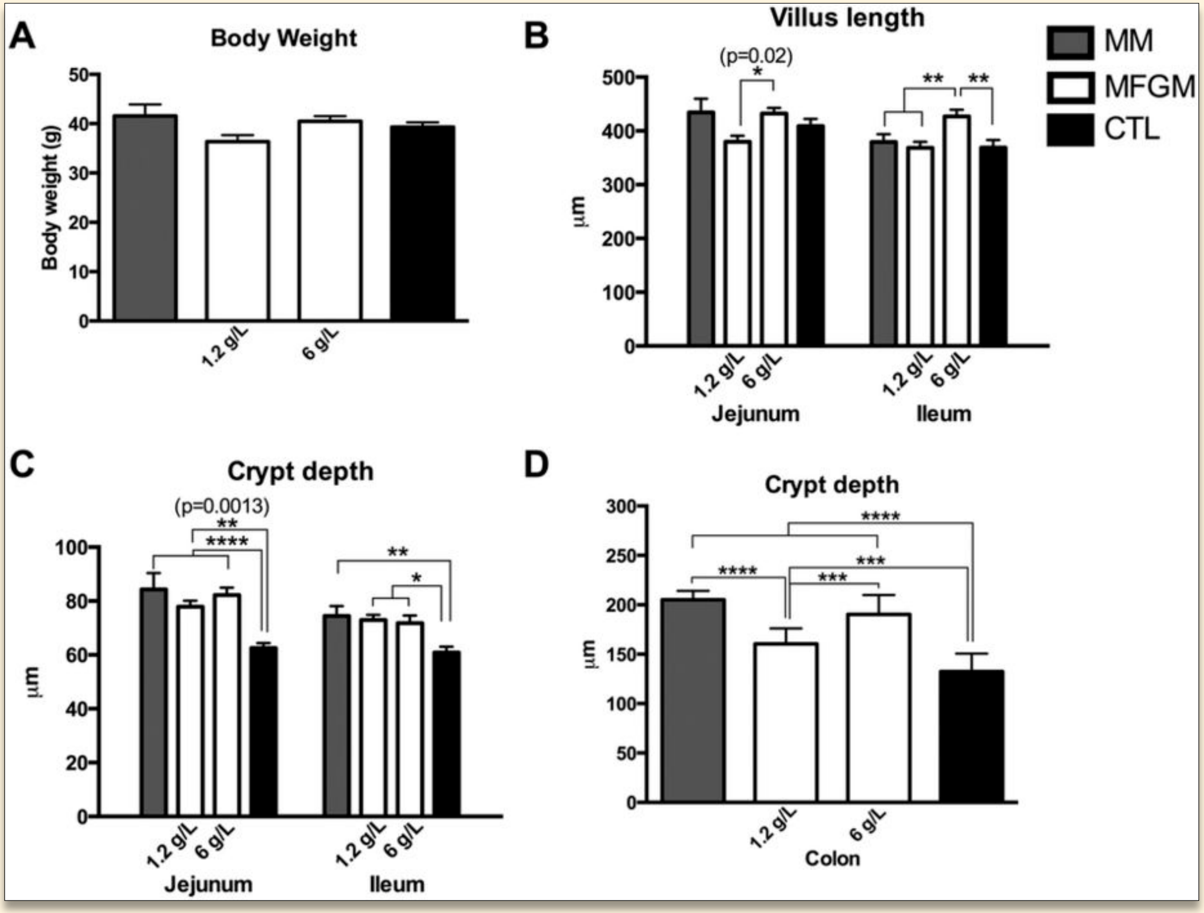
Conversely, MFGM is hypothesized to contain many constituents with beneficial bioactivity (**Spitsberg, 2005**) and is available as an ingredient to potentially modulate the barrier properties of the gut without the necessity of isolating specific components.

LPS, lipopolysaccharide.

Snow DR, et al. *J Dairy Sci.* 2011;94:2201-12; Used under terms of the Creative Commons License. Spitsberg VL. *J Dairy Sci.* 2005;88(7):2289-94.

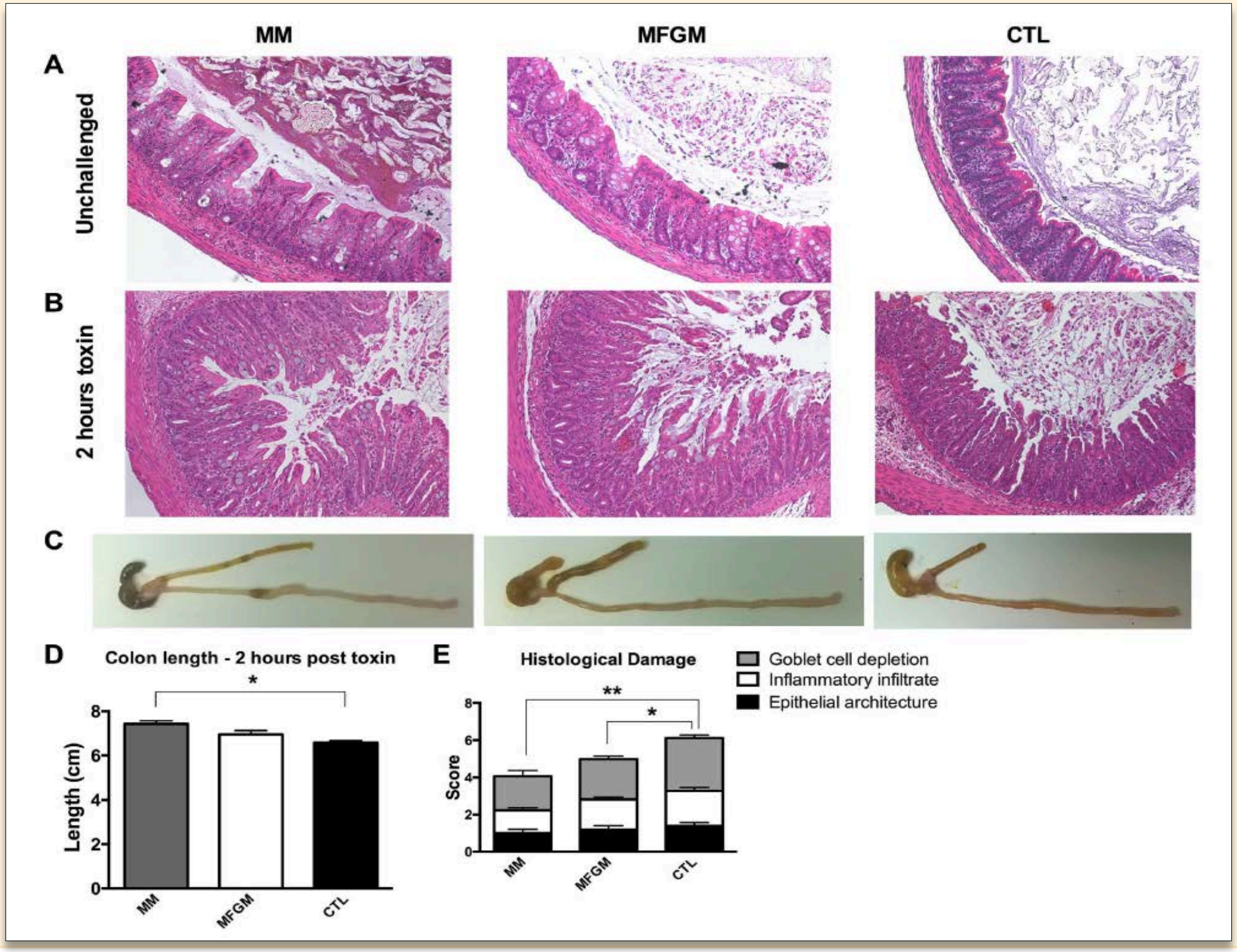
MFGM Supplementation in Formula Modulates the [Rat] Neonatal Gut Microbiome and Normalizes Intestinal Development

Figure 1. MFGM supplementation in formula normalizes intestinal architecture in a dose-dependent manner without affecting body weight at postnatal day 15.



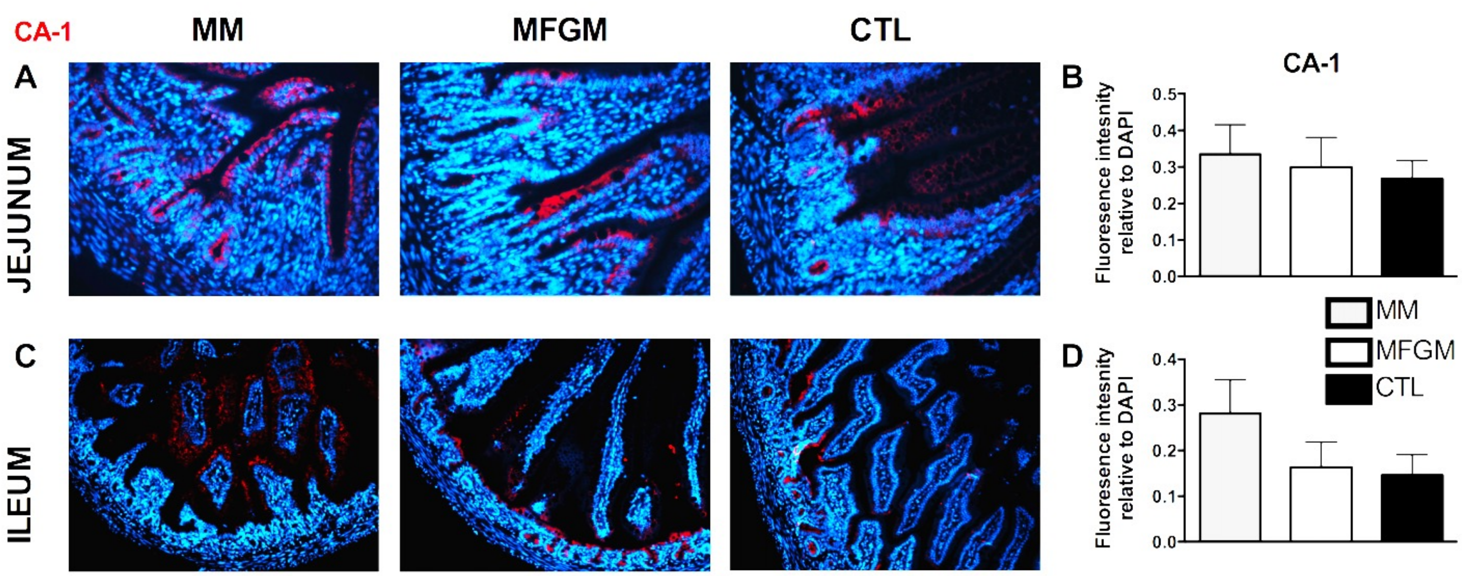
MFGM Supplementation in Formula Modulates the [Rat] Neonatal Gut Microbiome and Normalizes Intestinal Development *(cont)*

MFGM supplementation protects the formula fed neonate intestine from *C. difficile* toxin-induced damage.



MM, Mother's milk; MFGM, milk fat globule membrane; CTL, control

While Control diet (CTL) formula yielded significant deficits in intestinal development as compared to Mother's Milk (MM) littermates, addition of MFGM to formula restored intestinal growth, Paneth and goblet cell numbers, and tight junction protein patterns to that of MM pups.



Supplementary Figure 2: MM, 6 g/L MFGM and CTL formula fed pups display similar CA-1 staining at small intestinal sites. Representative immunostaining of enterocyte marker CA-1 (red) and DNA (blue) in the jejunum (A) and ileum (C) of pups at pn day 15, semi-quantified for fluorescence intensity of CA-1 relative to DAPI in (B) and (D). n>5. The graphed data presented are the mean ± SEM. Original Magnification: 200X.

CTL, control; MM, Mother's milk; MFGM, milk fat globule membrane

Dietary Prebiotics and Bioactive Milk Fractions Improve NREM Sleep, Enhance REM Sleep Rebound and Attenuate the Stress-Induced Decrease in Diurnal Temperature and Gut Microbial Alpha Diversity

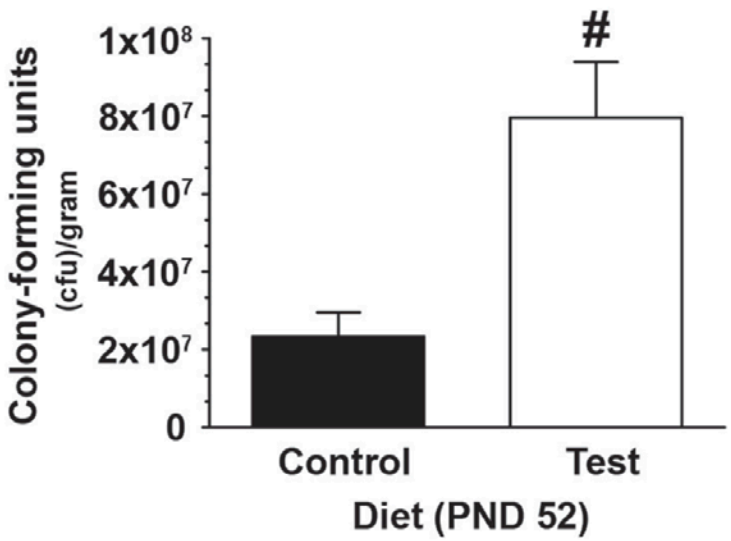


FIGURE 2 | Test diet increases *Lactobacillus rhamnosus*. Data demonstrating, in a subset of rats, that consumption of the test diet significantly increased levels of *Lactobacillus rhamnosus* in fecal cultures at PND 52, when compared with those eating the control diet ($^{\#}p < 0.05$ vs. control diet).

Dietary Prebiotics and Bioactive Milk Fractions Improve Sleep

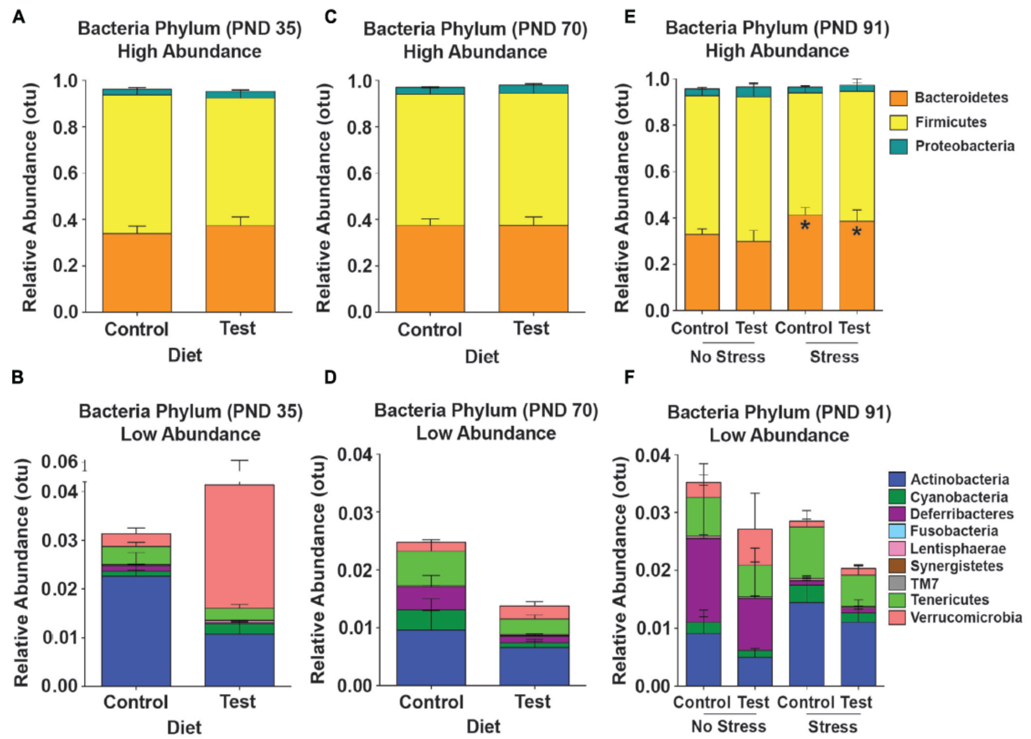


FIGURE 6 | 16S rRNA data. Taxonomic graphs depicting the relative abundance of phyla in the gut microbial communities at PND 35, PND 70 and PND 91. **(A)** The high abundance gut microbial community at PND 35 in rats consuming either the control or test diet. **(B)** The low abundance microbial communities depicted in the absence of bacteroidetes, firmicutes and proteobacteria. **(C)** The high abundance microbial community at PND 70 in rats consuming either the control or test diet. **(D)** The low abundance microbial communities depicted in the absence of bacteroidetes, firmicutes and proteobacteria. **(E)** The high abundance gut microbial community at PND 91 4 days after stress exposure in rats consuming either the control or test diet. There was a small, but significant, increase in the phylum bacteroidetes (orange bars). **(F)** The low abundance microbial communities depicted in the absence of bacteroidetes, firmicutes and proteobacteria 4 days after stress exposure. Abbreviations are as follows: OTUs, operational taxonomic units; PND, post natal days (*$p < 0.05$ vs. no stress groups).

Conclusion:
The bioactive milk fractions, Lf and MFGM, not only help promote growth of beneficial gut microbial species (Chatterton et al, 2013; Timby et al, 2015), but they may affect brain function directly.*

Thompson RS, et al. *Front Behav Neurosci.* 2016;10:240; Used under terms of the <u>Creative Commons Attribution 4.0 International</u> License.
*Chatterton DE, et al. *Int J Biochem Cell Biol.* 2013;45:1730-47; Timby N, et al. *J Pediatr Gastroenterol Nutr.* 2015;60:384-9.

Mimicking the Human MFGM Structure

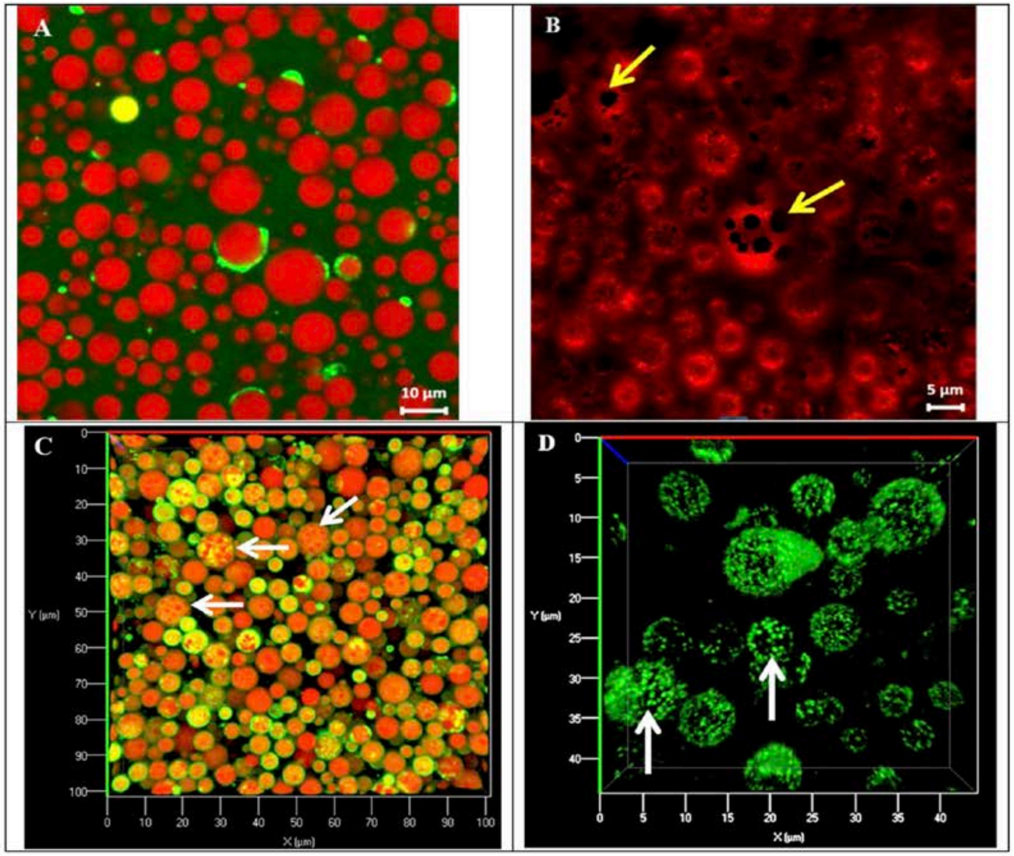


Fig. 2. 2D (A and B) and 3D (C and D) CLSM images of HM. (A) The milk fat globules were stained with Nile Red (Red) and Fast Green FCF (green). (B) The MFGM was stained with Rd-DOPE; the yellow arrows are pointing at l_o domains which are rich in sphingomyelin and cholesterol and tightly packed, preventing 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.

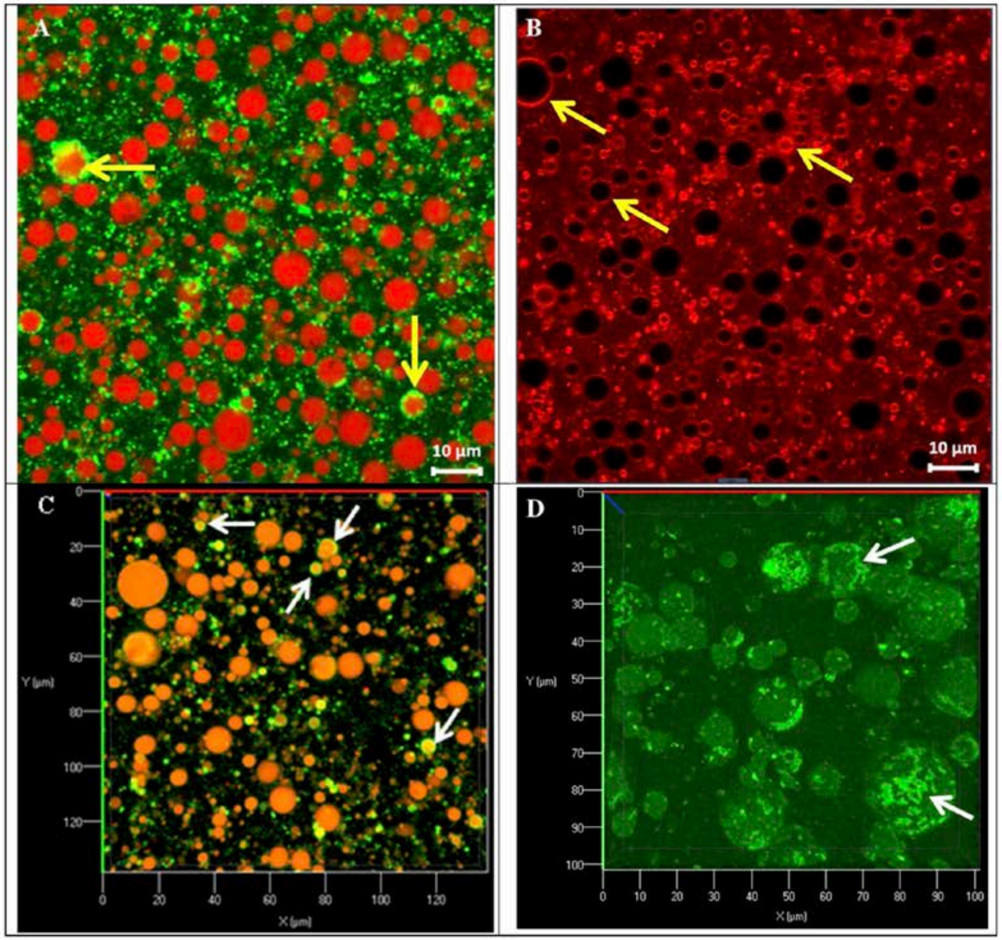


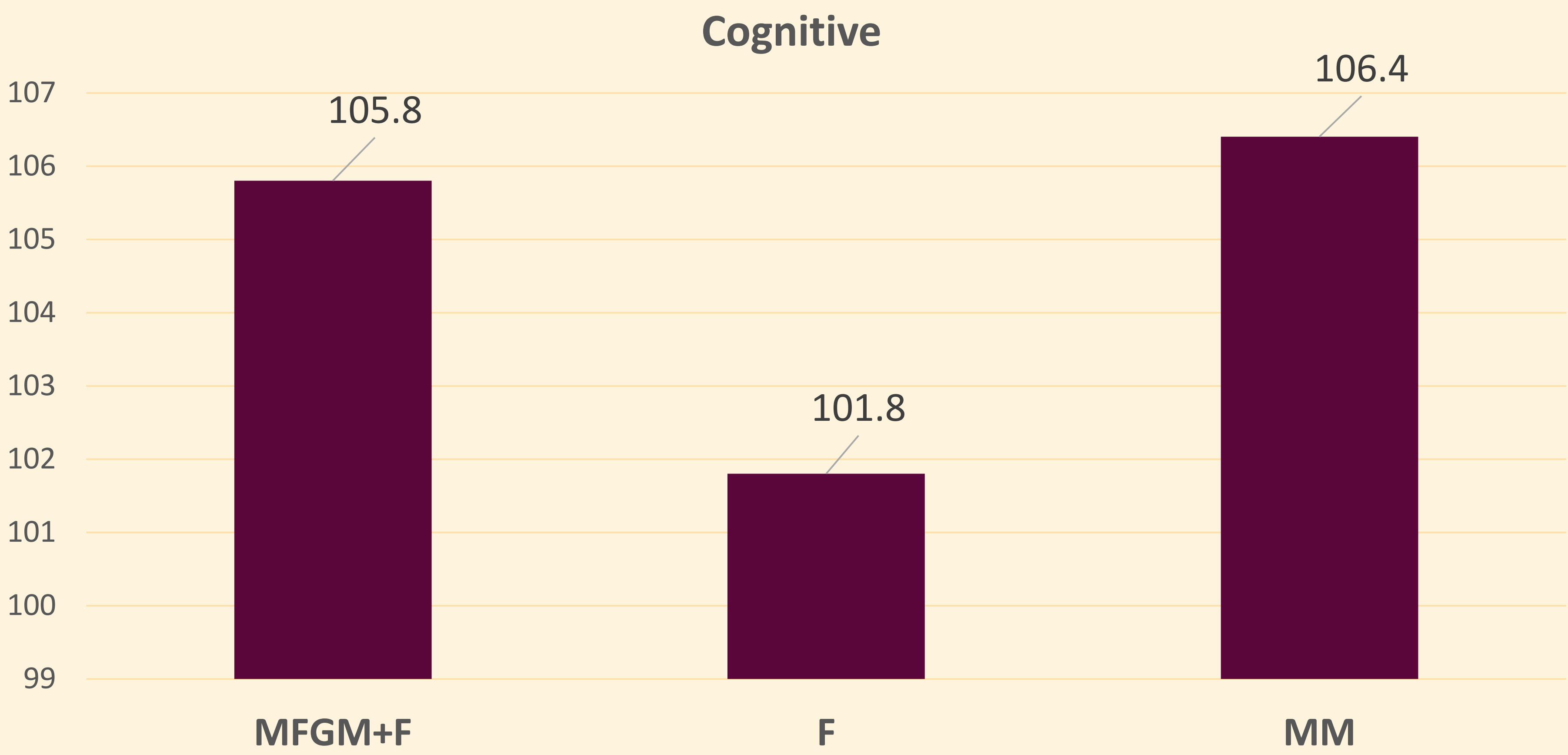
Fig. 3. 2D (A and B) and 3D (C and D) CLSM images of Concept IMF. (A) The fat droplets were stained with Nile Red (Red) and Fast Green FCF (green); the yellow arrows are pointing at dense patches of interfacial proteins. (B) The surface of the fat droplets was stained with Rd-DOPE; as a fluorescent phospholipid 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.

Clinical Trials of MFGM

- **Sweden—Timby et al study:** n=160; <2 to 6 months
- A low-energy and low-protein infant formula with bovine MFGM or a standard formula was randomized until 6 months of age. Reference group: breastfed infants
- At 12 months, MFGM-supplemented group obtained significantly higher cognitive scores in Bayley-III compared to the standard formula group (105.8 ± 9.2 vs 101.8 ± 8.0, M ± SD)
- Scores of the MGFM group do not differ from breastfed group (106.4 ± 9.5)
- Decreased incidence of otitis media in infants <6 months
- No increased risk of skin reactions in the group supplemented with MGFM

Timby N, et al. *J Pediatr Gastroenterol Nutr.* 2015;60:384-9. Timby N, et al. *Clin Med Insights Pediatr.* 2015;9:63–64. Timby N, et al. *PLOS One.* 2017;12(1):e0169831.

Study From Timby & Lönnerdal



MFGM, milk fat globule membrane formula supplemented formula; F, standard formula; MM, mother's milk.

Timby N, et al. *J Pediatr Gastroenterol Nutr.* 2015;60:384-9. Timby N, et al. *Clin Med Insights Pediatr.* 2015;9:63–64. Timby N, et al. *PLOS One.* 2017;12(1):e0169831.

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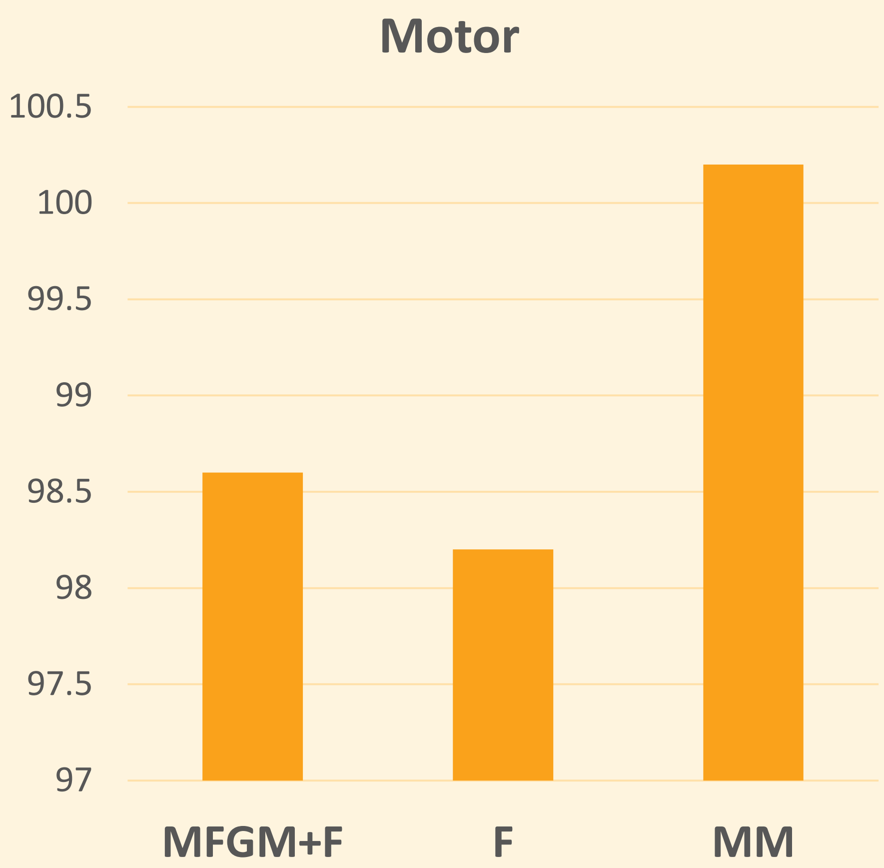
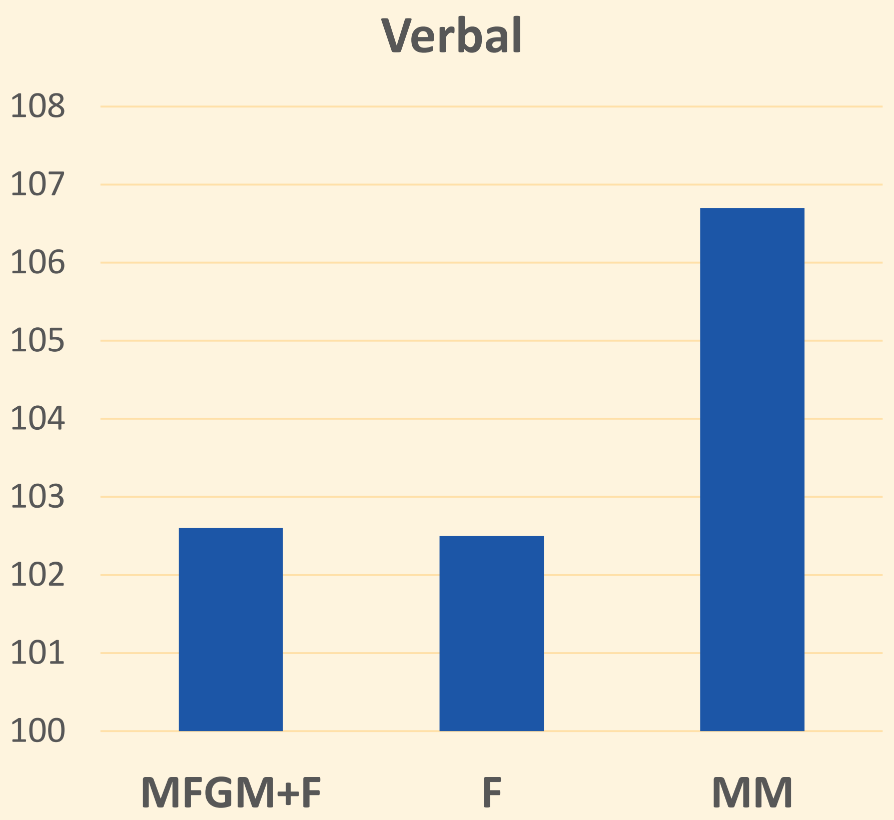
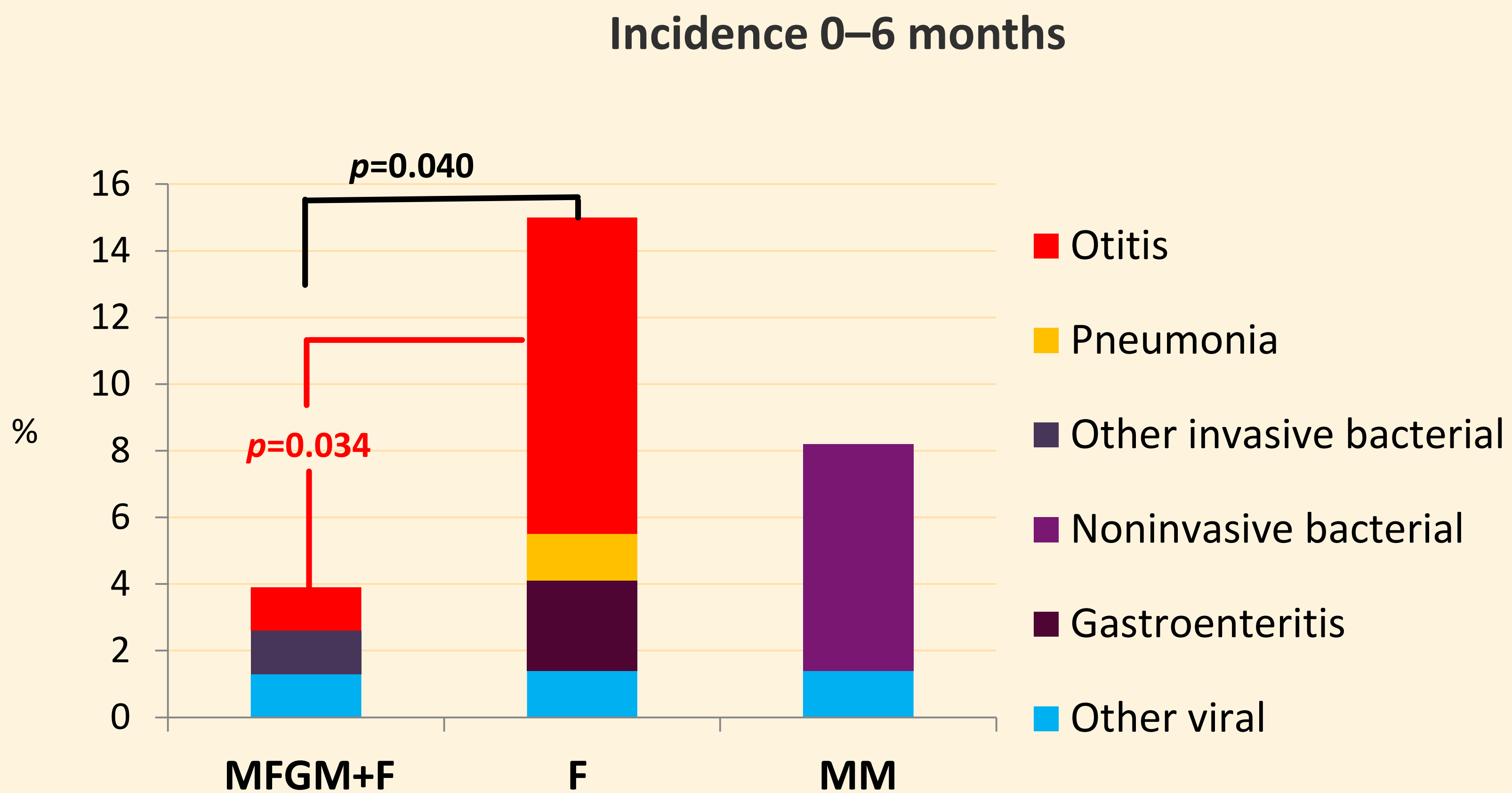




MFGM+F, milk fat globule membrane formula supplemented formula; F, standard formula; MM, mother's milk.

Timby N, et al. *J Pediatr Gastroenterol Nutr.* 2015;60:384-9. Timby N, et al. *Clin Med Insights Pediatr.* 2015;9:63–64. Timby N, et al. *PLOS One.* 2017;12(1):e0169831.

Infections Treated With Antibiotics or Hospitalization

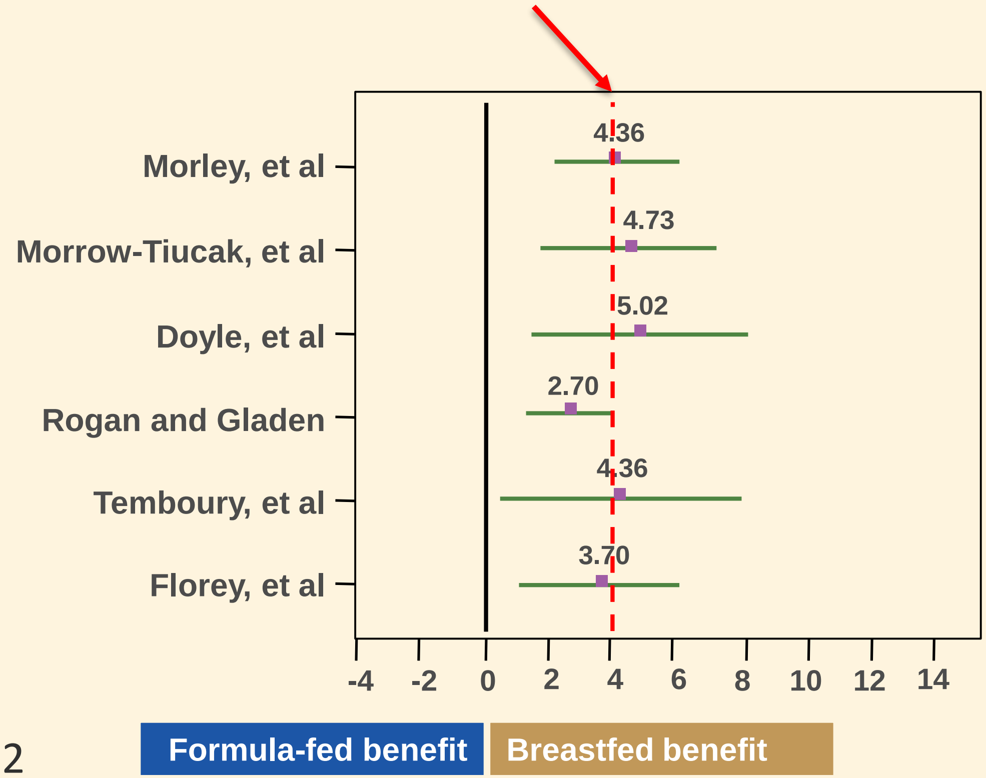


Note: Both EF and SF contained supplemental DHA.
MFGM+F, milk-fat globule membrane supplemented formula; F, standard formula; MM, mother's milk.

Timby N, et al. *J Pediatr Gastroenterol Nutr.* 2015;60:384-9.

Significance of the Bayley-III Score: What is the Gap in Past Studies?

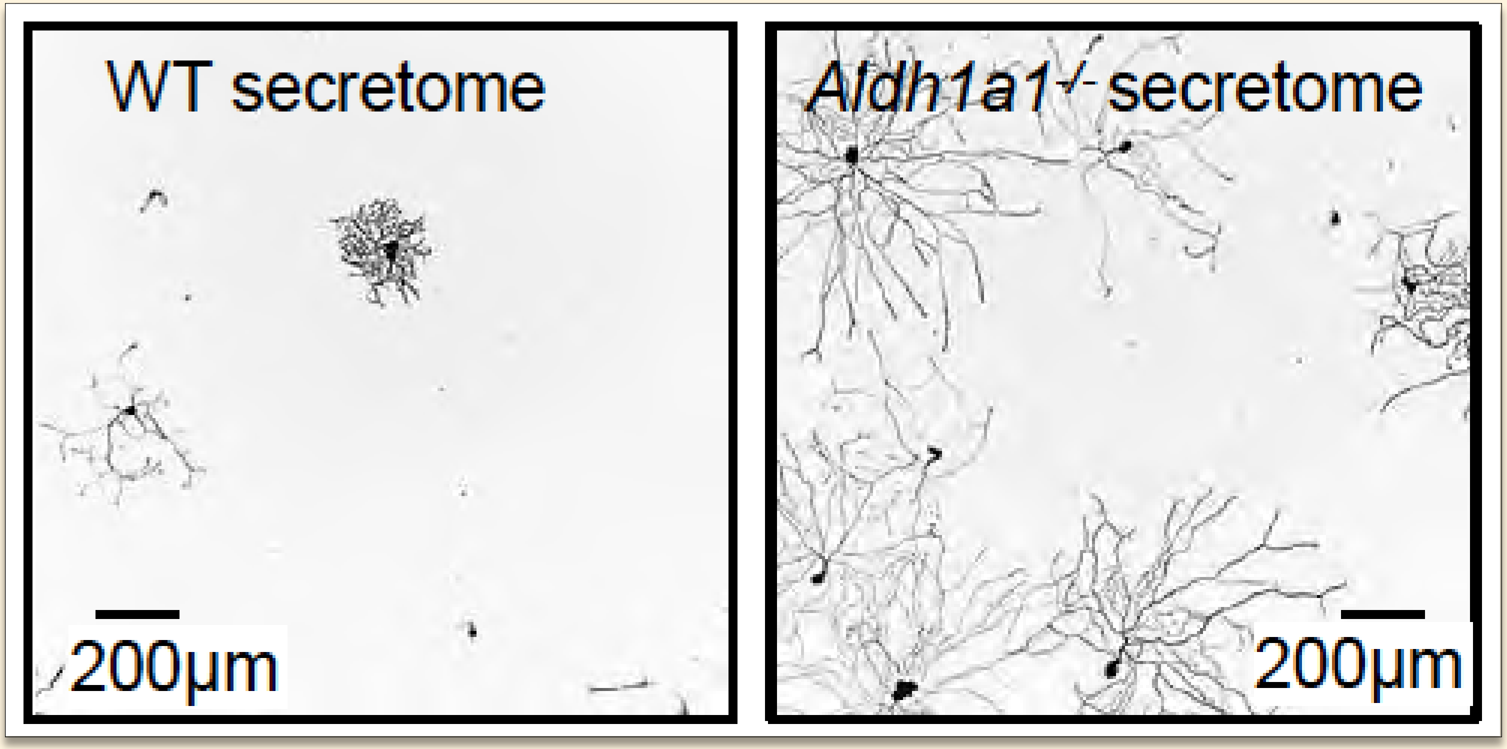
- A meta-analysis examined the mean differences between breastfed and formula-fed groups for cognitive score.[1]
- In multiple studies, the mean difference between groups was approximately 4 points—in line with the effect reported for MFGM.[2]



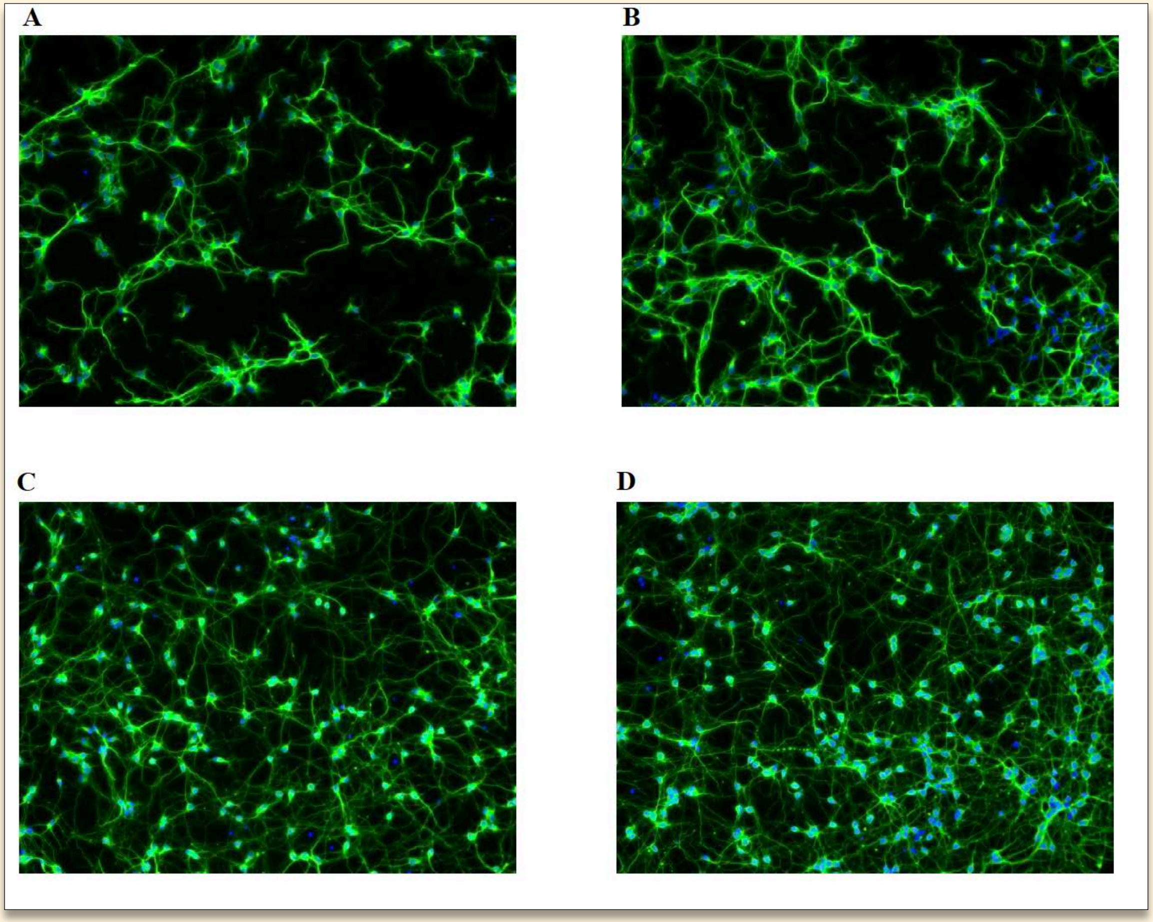
1. Anderson JW, et al. *Am J Clin Nutr.* 1999;70(4):525-535.
2. Timby N, et al. *Am J Clin Nutr.* 2014;99:860-868.

Adapted with permission of the *American Journal of Clinical Nutrition*.

Effect of Brown-Adipocyte Exosomes on Neural Growth



Effect of Dairy Phospholipids on Neuron Stimulation and Growth



A. Control cell growth
B. 1% Fetal Calf Serum
C. 75 μg PL
D. 150 μg PL

MFGM Key Points

- MFGM is a complex structure containing bioactive fats and proteins naturally produced in breast milk and bovine milk
- Human and bovine MFGM are compositionally similar and share key bioactive components
- Advances in processing technology allow isolation and concentration of MFGM from bovine milk
- MFGM may bring formula composition and function closer to those of breast milk

OPPORTUNITIES
MULTIPLY
AS THEY ARE
SEIZED

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