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FORTIFIED MILK COMPOSITIONS AND THEIR PROCESSES OF PREPARATION

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ABSTRACT

The present invention provides a novel process for preparing fortified milk compositions from whole mammalian milk.

The process of the present invention generally involves converting dried mammalian milk into a fats-enriched fraction, a protein-enriched fraction, and a carbohydrate-enriched fraction. The process comprises treating the dried milk with a fats extracting agent to provide a fats-enriched fraction, and a residual fraction comprising proteins and carbohydrates; separating said fats-enriched fraction from the residual fraction; and removing the fats extracting agent from the separated fats-enriched fraction to produce a dry fraction of CA oily, fats-enriched material and a fraction enriched in proteins and carbohydrates.

Fortified Milk Compositions and their Processes of Preparation

FIELD OF INVENTION

[001]. The present invention pertains to fortified milk compositions and methods for preparing same, in particular fortified human milk compositions made from human milk.

BACKGROUND

[002]. Human milk is well established and widely recognized as the best source of nutrition for growth and well-being of babies. There is a voluminous literature about human milk, including a number of thorough reviews (e.g., Prentice, 1996; Ballard and Morrow, 2013; Medo *et al.*, 2013).

[003]. It is also recognized that premature infants, with only partial development at best of virtually all of their organ systems, need special attention to their nutrition to achieve steady and optimal early growth toward a healthy infancy. In other words, mother's milk alone often is insufficient to their nutritional needs (Davies *et al.*, 1997; Adamkin and Radmacher, 2014; Li *et al.*, 2016; Abrams *et al.*, American Academy of Pediatrics, 2017; Bertino *et al.* 2017; Koo and Tice, 2018).

[004]. For these reasons, especially in neonatal units of hospitals, babies are fed fortified formulas of human milk. In these formulas, the emphasis is on levels of protein that are provided at elevated amounts that better fill the needs of the infants. Protein is typically increased to about 3 grams per 100 ml. For comparison, whole mother's milk – unfortified – contains only about 1 gram of protein per 100 ml.

[005]. Fats typically fall within a range of roughly 4 grams per 100 ml of human milk, with carbohydrates at around 7 grams per 100 milliliters. In fortified milk formulas, fats and carbohydrates are typically held to approximately to these levels. Fats overall are regarded as potentially not helpful if levels are elevated too much. Carbohydrate levels do not attract the same concerns, but they are kept under control as well in the fortified formulas.

[006]. **Macronutrients:** Studies sponsored by the FDA resulted in recommendations about

the composition of milk formulas (Food and Drug Administration. 21 CFR Part 107. Infant formula. Volume 80, 120, p. 35834-35841, June 23, 2015; updated April 1, 2018, see eCFR www.ecfr.gov). At the time, the focus was on formulas comprised of bovine milk, but the recommendations are applicable to human milk formulas. The recommended levels of protein and fat were (minimum/maximum grams per 100 calories of formula): protein – 1.8 to 4.5 g/100 cal; fat – 3.3 to 6.0 g/100 cal.

- [007]. The upper and lower limits of carbohydrates were not specified, but are meant to be an amount in keeping with the recommended energy provision at present of 100 calories when added to the levels of proteins and fats. In general, there are roughly 4 calories per gram of carbohydrates and thus the amount of carbohydrates to provide 100 calories of formula would be in the range of 7 grams, again depending on the amounts of proteins and fats.
- [008]. Two other components of fortified human milk that receive careful attention are 1) inclusion of health-promoting vitamins and minerals and 2) osmolality of the fortified formulation.
- [009]. **Vitamins and minerals:** The FDA has also issued guidelines as above for the inclusion of vitamins and minerals in milk formulas. Although the list of these is lengthy and the units for designating dosages are somewhat variable and complicated, providing the desired mixture in appropriate amounts to a milk formula is straightforward. That is, in practice, an order for a list of vitamins and minerals in designated amounts can be filled by a variety of commercial sources. This resource can be added as a supplement to the 3 essential fractions of proteins, fats, and carbohydrates to complete a fortified milk formula.
- [010]. **Notation about Cellular Components:** In addition to nutrients, vitamins, minerals, and the multitude of beneficial molecules in human milk, there is a cellular population of whole milk. This is comprised mainly of beneficial maternal cells such as lymphocytes, leucocytes, macrophages, and neutrophils.
- [011]. There is even a robust population of pluripotent stem cells whose contributions to infant development and well-being are thought to be many-fold (Hassiotou et al,

2012; Ayden *et al.* 2018). If provided by direct nursing to an infant, these cells can survive and migrate from the digestive tract of infants, enter the blood stream, and find their way to various organs including the brain. Here they can differentiate into the appropriate cell types and function as integral parts of the body. They may persist throughout life. However, stem cells do not live long after the milk is freshly expressed by the woman and are not contemplated as part of the fortified human milk compositions of the present invention.

- [012]. **Probiotics:** Also resident in human milk are health-promoting bacteria such as Bifidobacterium and Lactobacillus, which are identified as useful probiotics. As in the case of fortifying human milk in particular with proteins, vitamins, and minerals, there are studies that demonstrate the benefits of probiotic supplements. For example, the addition of Bifidobacterium to human milk is exemplified in US patents and patent applications (e.g., US patent 8,197,872 to Mills *et al.*, 2012; US patent 9,808,474 to Buck *et al.*, 2017; US patent 9,808,475 to German *et al.*, 2017; US patent application 15/521,502 by Kyle *et al.*, 2017).
- [013]. **C-section babies:** Contemplated therein are benefits regarding establishment of healthy gut flora in premature infants, as well as all infants delivered by C-section. All of these infants are at risk of digestive tract infection and impairment. In the USA, late preterm (34–36 weeks) deliveries occur in over 7% of births, with early preterm births (less than 34 weeks) at 2.75% in 2018. Over 30% of infants are delivered by Cesarean section (Martin *et al.*, 2019).
- [014]. Digestive tract issues occur if there is a lack of Bifidobacterium and its ability to digest and process milk oligosaccharides that block attachment and proliferation of harmful bacterial and viral pathogens in these new-born babies. Early preterm infants are most at risk, of course, having even more incomplete development of their organ systems.
- [015]. But those infants delivered by C-section may be at risk as well, even if delivered full-term, apparently not having received the proper inoculation of their digestive tracts that accompanies a vaginal delivery. Hence, these babies can also benefit from a probiotic supplement in mother's milk (see also, Sangild, US patent applications

15/036854 and 15/036855, 2016).

- [016]. The workers in this field do not stop there, however, also contemplating Bifidobacterial supplements to other types of milk as well. Contemplated are supplemented milk not only for all human infants, but also for farm animals, pets, and basically any mammalian animal that may be provided care by humans.
- [017]. Other workers contemplate benefits to brain development pursuant to infants feeding on supplemented human milk with probiotics including Lactobacterium and Bifidobacterium (US patent 9,609,888 to Berg *et al.*, 2017; US patent applications, Chichlowski and Berg, 2018 a,b). Emphasis in this work was on the salutary effects related to ganglioside production, as well as enhancement of the immune response, antimicrobial effects, and proper development of the digestive tract.
- [018]. Another utility of probiotic supplemented human milk is reported to be production of anti-inflammatory peptides (US patent 9,457,058 to Hondmann et al, 2016). Both adult and pediatric populations were also considered for the benefits of the instant formulas, with specific discussion of reduction of the inflammatory response during diseases of neonatal infants and children in general. Anti-inflammatory action of probiotic supplement to human milk through other mediated reactions of the beneficial bacteria with various biochemical components including long-chain fatty acids and oligosaccharides along with specific proteins was also taught by Rosales *et al.*, US patent 9,439,448 (2016). Such supplements were again contemplated for use not only in promoting health of premature infants, but also children and adults as well.
- [019]. Safe supplies of probiotics, free of other microbes, are available from a variety of sources. These may be accessed if desired for use in accord with the present invention.
- [020]. **Osmolality:** Adjustment of the osmolality of the liquid milk formula is another feature that may be need some attention. The osmolality (defined as moles of solute per kilogram of solution) is a function of the molar amounts of soluble ions and dissolved molecules in the aqueous portion of the milk, assigned in this case as

moles of these solutes per kilogram of milk. For clarity, osmolality is defined as moles of solute per liter of solution. The terms osmolality and osmolarity are nearly interchangeable in many cases, but they are not the same.

- [021]. Of importance in milk, molecules of high molecular weight that may be emulsified rather than solubilized, or molecules that are not water soluble, would not contribute to osmolality. This is the case of much of the protein and fat fractions. Therefore, most of the osmolality is contributed by the soluble, low molecular weight carbohydrates (e.g., lactose) and, of course, by the soluble ions like sodium, chloride, calcium, phosphate, and the like.
- [022]. Again, in the guidelines above, the FDA has issued a rule for the minimum and maximum levels of a selection of soluble mineral components of milk formulas. However, there is no statement about osmolality as such.
- [023]. The current state of the professional literature about the effects of osmolality on health of premature infants is not very extensive and is unclear to some extent (Pearson *et al.*, 2013, Singh *et al.*, 2017). There are suggestions that higher osmolality of fortified milk can harm an infant's digestive tract, lead to infections, and even to life-threatening conditions. Suppliers of human milk fortifiers are aware of this and are able to keep the osmolalities and recommended uses of their products within a safe range (De Curtis *et al.*, 1999; Janjindamai and Chotsampancharoen, 2006; Sauret *et al.*, 2018).
- [024]. However, rather than owing to the natural content of human milk, there are other reports that in the cases of hyperosmotic-related pathologies, it was the doses of antibiotics, vitamins, and medicines that led to significantly elevated osmolalities in the fluids being provided to the distressed infants (Srinivisan *et al.*, 2004; Rigourd *et al.*, 2016).
- [025]. Although not well understood, this is a serious issue such that producers of milk formulas and providers of milk to hospitals have adopted cautionary standards for osmolality in milk for infants. The current voluntary guideline is 400 milli-osmoles per kilogram (mOsm), which is supported by the American Academy of Pediatrics

(Abrams *et al.*, 2017), hospitals and other customers of infant formulas. Other studies support an upper limit of 600 mOsm per kilogram of formula (Hossain *et al.*, 2014) with an understanding that upwards of 1,000 mOsm per kilogram is likely still a safe level. This conclusion was also made by Choi *et al.* (2016) in a thorough review and analysis of the contributing factors. As a point of comparison, an earlier study (Schanler, 1995) had shown that the osmolality of the preterm gastrointestinal tract itself is approximately 600 mOsm.

- [026]. **Patents.** Fortifiers for addition to human milk historically were made from bovine milk feedstock and other non-human sources of the main components. For example, there are well-known products having tradenames of Similac (Abbott Laboratories) and Enfamil (Mead Johnson and Company) that exemplify this type of offering (Barrett-Reis *et al.*, US patent 6,472,003, 2002; Euber *et al.*, US patent 8,147,894, 2012; Rosales *et al.*, US patents 8,287,931 and 9,439,448, 2012 and 2016; Thoene *et al.*, 2014).
- [027]. More recently, human milk fortifiers made from human-milk feedstock have become available (Medo *et al.*, US patent 8,545,920, 2013; Fournell *et al.*, US patent 8,927,027, 2015 and US patent application 15/726,232, 2018). These fortifiers provide biochemical isolates of human milk that are prepared via standard biochemical methods such as ultrafiltration, reverse osmosis and high-performance, liquid chromatography. Included are isolates of the permeate fraction that are enriched in milk oligosaccharides - and likely other beneficial, small molecules such as peptides - that were at first believed to be concentrated in the retentate fraction. These oligosaccharides are potentially valuable to infants regarding development of healthy digestive tracts, and exhibiting other desirable features as well such as anti-inflammatory activity and promotion of immunity.
- [028]. Presently known methods/processes for preparing milk fortifying components involving advanced biochemical and engineering treatments of liquid milk, such as ultrafiltration, reverse osmosis, centrifugation, and a variety of high-performance chromatographic techniques, result in losses of various desirable compounds.
- [029]. Therefore, there is a need for simple and improved methods of isolating desired

fractions from milk, while conserving its health promoting components/constituents.

[030]. This background information is provided to reveal information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

[031]. An object of the present invention is to provide methods for the preparation of fortified milk using dried or lyophilized milk as feedstock.

[032]. Another object of the present invention is to extract and isolate a fats-enriched fraction, a protein-enriched fraction, and/or a carbohydrate-enriched fraction from dry whole milk.

[033]. In accordance with an aspect of the present invention, there is provided a process for converting dried whole mammalian milk into a fats-enriched fraction, a protein-enriched fraction, and a carbohydrate-enriched fraction, the process comprising the steps of: a) treating the dry milk with a fats extracting agent to provide a fats-enriched fraction, and a residual fraction comprising proteins and carbohydrates; b) separating said fats-enriched fraction from the residual fraction; and c) removing the fats extracting agent from the separated fats-enriched fraction to produce a dry fraction of oily, fats-enriched material and a fraction enriched in proteins and carbohydrates.

[034]. In accordance with another aspect of the invention, there is provided a fats-enriched fraction, a protein-enriched fraction, and/or a carbohydrate-enriched fraction isolated from the dry whole milk by the process described herein.

[035]. In accordance with another aspect of the invention, there is provided a composition comprising one or more of the fractions isolated from the dry whole milk by the process described herein.

- [036]. In accordance with another aspect, the present invention provides use of one or more of the fat-enriched fraction, protein-enriched fraction, and/or carbohydrate-enriched fractions obtained by the process described herein for mixing with human or bovine milk or water to produce a fortified milk composition.
- [037]. Another object of the present invention is to provide a premixed, liquid form of fortified human milk or bovine milk, ready-to-feed to human infants.
- [038]. Another object of the present invention is to supplement the fortified milk with vitamins and minerals.
- [039]. Another object of the present invention is to supplement the fortified milk with probiotics.
- [040]. Another object of the present invention is to maintain the osmolality of the fortified milk at a health-sustaining level in the range of 300 milliosmoles to 1000 milliosmoles per kg.
- [041]. Another object of the present invention is to use bovine and other animal milks as feedstocks for production of fortified milk for feeding new-borne or young farm animals and domestic animals like cows, horses, and dogs.
- [042]. Numerous other features, objects and advantages of the invention will become apparent from the following description when read in conjunction with the accompanying figures.

BRIEF DESCRIPTION OF THE Figures

- [043]. FIG. 1 illustrates density of CO₂ versus temperature and pressure.
- [044]. FIG. 2 illustrates results of treatments of 10% by weight lyophilized whole human milk and 10 % by weight of the protein/carbohydrate fraction of human milk at pH 4 in distilled water and in distilled water and ethanol at 50/50% by weight.
- [045]. FIG. 3 illustrates results of treatment of lyophilized human milk and a protein-

carbohydrate fraction in a 50/50 weight% solution of distilled water and ethanol.

[046]. FIG. 4 illustrates results of protein, carbohydrates, and milk oil (fats) fractions of lyophilized human milk in representative experiments as measured by FTIR.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[047]. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[048]. As used herein, the term “about” refers to approximately a +/-10% variation from a given value. It is to be understood that such a variation is always included in any given value provided herein, whether or not it is specifically referred to.

[049]. The term “mildly acidic” used in the context of the present invention mean a solution having a pH of about 2.5 to about 5, preferably, the pH of the mildly acidic solution is from 3 to 4.8.

[050]. The terms “freeze dried” and “lyophilized” have been used interchangeably in the present application, which mean a substance dried via a low temperature dehydration process that involves freezing the product, lowering the pressure, then removing the ice by sublimation.

[051]. The present invention provides novel methods for preparation of fortified mammalian milk, in particular bovine and human milk (for feeding infants), wherein a dried form of mammalian milk is used as feedstock. The present invention also provides novel compositions of fortified milk based on blends of fractions of milk obtained from dried or lyophilized whole milk as feedstock.

[052]. Non limiting examples of mammalian milk used in the context of the present invention are bovine milk, sheep milk, goat milk, camel milk, buffalo milk, and human milk.

- [053]. The present invention also provides for a process to extract and isolate the fatty acid, lipids, and fat-soluble components from the dry powder of milk by simple and benign methods, yielding a fraction enriched in fats as an oily isolate, and/or to isolate the proteins of milk by other simple and benign methods involving aqueous treatments and precipitation, yielding a fraction enriched in proteins.
- [054]. The extraction process of the present invention involves converting dried whole/mother's milk into a fats-enriched fraction, a protein-enriched fraction, and a carbohydrate-enriched fraction. The process comprises the steps of a) treating the dry milk with a fats extracting agent to provide a fats-enriched fraction, and a residual fraction comprising proteins and carbohydrates, b) separating said fats-enriched fraction from the residual fraction, and c) removing the fats extracting agent from the separated fats-enriched fraction to produce a dry fraction of oily, fats-enriched material and a fraction enriched in proteins and carbohydrates.
- [055]. The process can involve human milk, bovine milk or other mammalian milk. The preferred milk is human milk, freshly expressed and quickly frozen.
- [056]. The dried milk can be freeze-dried/lyophilized milk, spray-dried, or forced-air dried milk. Preferably, the milk is freeze dried/lyophilized. By using dried-milk as the feedstock in the extraction process, the components of the many thousands of health-promoting constituents of milk are conserved almost in their entirety, including each of the 3 macro-components of protein, fats, and carbohydrates.
- [057]. There are significant advantages to use the dried form of milk, preferably lyophilized milk relative to liquid milk as feedstock for production of human milk fortifiers.
- [058]. For one thing, as a dry powder, the human milk is more readily processed for isolation and production of the macro-components via simple and benign methods. These methods permit quantitative recovery of milk components. This leads to maximal utility of another significant feature, namely that lyophilization conserves essentially all of the multitude of beneficial molecules of human milk.

- [059]. In some embodiments, the lyophilized human milk is comprised of cream-colored to white flakes that contain essentially all of the components of fresh human milk. It retains essentially all of the nutritional and health attributes of fresh, whole human milk. It is a purified, dry powdery material
- [060]. The fresh and/or the frozen milk is first certified as safe by analytical screening for undesirable and potentially harmful drugs, microbes, and non-milk diluents (such as plant “milks”) prior to fractionation. Milk that does not pass the safety screens and human-authenticity tests is rejected and not included in further processing.
- [061]. The fats extracting agent of step a) can be supercritical carbon dioxide, or a fats solubilizing solvent. Non limiting examples of fats solubilizing solvents are ethanol, ethyl acetate, butanol, hexane or a combination thereof, as disclosed in Cohn *et al.*, 1946, 1950; Denizil, 2011; Raoufinia *et al.*, 2016.
- [062]. In some embodiments, when the fats extracting agent is supercritical carbon dioxide, step a) is carried out at a pressure range of 2500-4500 psi, and temperature range of 40 to 75 °C.
- [063]. In some embodiments, when the fats extracting agent of step a) is a fat solubilizing solvent, then step c) of the process further comprises drying the fats enriched fraction to produce the dry fraction of oily, fats-enriched material.
- [064]. In some embodiments, the process further comprises treating the proteins and carbohydrates enriched fraction obtained in step c) with:
- i. a mildly acidic aqueous solution, optionally supplemented with an organic solvent, or
 - ii. a water and an organic solvent mixture, or
 - iii. carbonated water having a pH as low as pH 3,
- to provide a precipitated protein-enriched fraction, and a soluble carbohydrate-enriched supernatant fraction. The soluble carbohydrate-enriched supernatant fraction is then separated from the protein-enriched fraction, and the protein-enriched fraction is dried.

- [065]. In some embodiments, the organic solvent used in the above described treatment step is an alcohol solvent, preferably ethanol.
- [066]. In some embodiments, the mildly acidic aqueous solution can be obtained by adding CO₂ gas, liquid CO₂ or supercritical CO₂ to water, preferably by sparging or micro bubbling CO₂ gas or supercritical CO₂ into water. In such embodiments, upon drying of the protein-enriched fraction CO₂ is evacuated to the atmosphere, rendering these materials pH neutral.
- [067]. In some embodiments, the mildly acidic aqueous solution is obtained by adding one or more inorganic acids and/or organic acids to water. Non limiting examples of inorganic acids include H₂CO₃, HCl, H₂SO₄, H₃PO₄, HNO₃, and combinations thereof, preferably, HCl. Non limiting examples of organic acids include acetic acid, citric acid, or a combination thereof. In such embodiments, the protein-enriched precipitates are separated from the soluble carbohydrate-enriched supernatant fraction by filtration or centrifugation (preferably by centrifugation), to isolate the protein-enriched fraction fraction. The protein-enriched fraction can then be dried by lyophilization, or by drying under conditions of forced air at 80 °C or less, preferably at about 60 °C. If desired, the pH of the separated carbohydrate-enriched supernatant fraction is restored to neutrality, by using common bases such as NaOH, KOH, or ammonia.
- [068]. In some embodiments, the proteins and carbohydrates enriched fraction obtained in step c) is dried before subjecting this fraction to the treatment step described in the preceding paragraphs.
- [069]. The soluble carbohydrate-enriched supernatant fraction may be used as-is in subsequent steps. Alternatively, it may itself be lyophilized or otherwise dried to yield a dry, carbohydrate-enriched powdered fraction of milk. There are also approaches that employ pH and solvent transients, along with heating, for precipitating carbohydrates from the aqueous phase.

- [070]. In some embodiments, the remaining carbohydrate-enriched supernatant fraction of milk is provided for use as an aqueous fluid to which the concentrates of milk fats and dried protein may be added in desired amounts.
- [071]. The aqueous carbohydrate-enriched supernatant fraction contains the components that largely contribute to osmolality – namely the low-molecular-weight carbohydrates which are present in large amounts and to a lesser extent the inorganic salts which are present but not so prevalent.
- [072]. The salts are ionic whereas the carbohydrates are neutrally charged. Therefore, the dissolved salts can be removed or their levels reduced as needed by a simple ion-exchange treatment, analogous to those commonly used in homes for ultra-purification of drinking water. In this approach, insoluble beads of mixed ion-exchange resin are added to the fluid in the appropriate stoichiometric amounts for removing or decreasing the amounts of both the soluble anions and cations. Alternatively, the carbohydrate-enriched fluid can be deionized by flowing over a bed of mixed ion-exchange materials.
- [073]. In some embodiments, the separated carbohydrate-enriched supernatant fraction is dried to render the carbohydrate fraction of milk as a dry powder for blending with the fractions of milk fats and proteins as a dry formula for mixing with mother's milk for feeding to infants. The carbohydrate-enriched supernatant fraction can be dried by lyophilization or under conditions of forced air at 80 °C or less, preferably at about 60 °C,
- [074]. In some embodiments, the process further comprises a step of adjusting the pH of the soluble carbohydrate-enriched supernatant fraction to precipitate a solid carbohydrate-enriched fraction, which is separated and optionally dried. This step may optionally further include adding a solvent before, during and/or after the pH adjustment step. The solvent can be an alcohol solvent, such as ethanol. In some embodiments, the pH adjusting step further comprises heating the soluble carbohydrate-enriched fraction at 90 °C or higher.

- [075]. In some embodiments of the processes described just above, the pH of the soluble carbohydrate-enriched fraction is adjusted to pH 10 to 11. In some embodiments, the pH of the soluble carbohydrate-enriched fraction is first adjusted to be in a range of pH 10 to 11, heated, and then readjusted to be in a range of 3 to 4.8.
- [076]. In some embodiments of the processes described herein, the pH is adjusted by use of one or more of bases elected from the group consisting of NH_4OH , NaOH , KOH , gaseous NH_3 , or an alkanolamine, such as methanolamine and ethanolamine. In this step, NH_4OH or gaseous NH_3 are preferred for use because, as gases dissolved in water, they are readily evacuated to the atmosphere, or converted to vapor phase, then collected, during the drying step.
- [077]. In another aspect of the present invention, there is provided a fats-enriched fraction obtained by the processes herein.
- [078]. In another aspect of the present invention, there is provided protein-enriched fraction obtained by the processes herein.
- [079]. In another aspect of the present invention, there is provided carbohydrate-enriched fraction obtained by the processes herein.
- [080]. In another aspect of the present invention, there is provided a composition comprising one or more of the fractions obtained by the processes herein.
- [081]. In another aspect of the present invention, there is provided use of one or more of the fat- enriched, protein-enriched, and carbohydrate-enriched fractions obtained by the processes described herein, in appropriate amounts for mixing with a whole or skimmed mammalian milk (preferably bovine milk, goat milk, sheep milk or human milk) or water to produce a fortified milk composition.
- [082]. In another aspect of the present invention, there is provided a composition comprising the aqueous carbohydrate-enriched fraction, dry fat-enriched fraction, and dry protein-enriched fraction, obtained by the processes described herein, for addition in appropriate amounts to a whole or skimmed mammalian milk (preferably

bovine milk, goat milk, sheep milk or human milk) or water to produce a fortified milk composition.

[083]. In another aspect of the present invention, there is provided a blend of one or more of the dry fat-enriched fraction, dry protein-enriched fraction, and dry carbohydrate-enriched fraction obtained by the processes described herein, in appropriate amounts as a dry material for mixing with a whole or skimmed mammalian milk (preferably bovine milk, goat milk, sheep milk, or human milk) or water to produce a fortified milk composition.

[084]. In another aspect of the present invention, there is provided an aqueous concentrate comprising one or more of the fractions obtained by the processes described herein, for mixing with a mammalian milk (preferably bovine milk, goat milk, sheep milk, or human milk) or water to produce a fortified milk composition.

[085]. In another aspect of the present invention, there is provided an aqueous concentrate comprising the aqueous carbohydrate-enriched fraction, the dry fat-enriched fraction, and the dry protein-enriched fraction, obtained by the processes described herein, for mixing with a whole or skimmed mammalian milk (preferably bovine milk, goat milk, sheep milk or human milk) or water to produce a fortified milk composition.

[086]. In another aspect of the present invention, there is provided a method of fortifying a whole or skimmed mammalian milk (preferably bovine milk, goat milk, sheep milk or human milk) comprising mixing one or more of the fractions obtained by the processes described herein. In some embodiments, the method further comprises adding vitamins and minerals. In some embodiments, the method further comprises adding probiotics as a supplement.

[087]. In another aspect of the present invention, there is provided a fortified milk composition comprising one or more of the fractions obtained by the processes described herein. In some embodiments, the fortified milk comprises 10 to 85 mg/ml of protein, 60 to 125 mg/ml of carbohydrates, and 38 to 105 mg/ml of fats. In some embodiments, the fortified milk has a caloric content of 20 to 26 calories per ounce.

- [088]. In some embodiments, the fortified milk has an osmolality in the range of 300 to 1000 milliosmoles (mOsm), preferably in the range of 300 to 600 milliosmoles per kg.
- [089]. In another aspect of the present invention, there is provided use of one or more of the fractions obtained by the processes described herein, as supplements to bovine or other mammalian milk.
- [090]. In another aspect of the present invention, there is provided use of one or more of the fractions obtained by the processes described herein, as supplements to human milk.
- [091]. In one embodiment of the present invention, the protein-enriched fraction and the fat-enriched fraction are combined appropriately with the aqueous carbohydrate phase, along with more water if needed, to produce a reconstituted human milk formula having desired or recommended levels of proteins, fats, and carbohydrates, as well as an enriched supply of the full complement of the many thousands of beneficial components of whole human milk.
- [092]. In another embodiment of the present invention, one or more of the fats-enriched fraction, carbohydrate-enriched fraction, and the protein enriched fraction may be added to freshly expressed human milk or freshly thawed frozen human milk as needed or desired.
- [093]. In another embodiment of the present invention, the milk may be further supplemented with a formulation of select vitamins and minerals, either in dry or liquid forms.
- [094]. In another embodiment of the present invention, the reconstituted, fortified, and supplemented human milk may then be homogenized, pasteurized, or both. Pasteurization or retorting may be conducted as part of a packaging process.
- [095]. In another embodiment of the present invention, a shelf-stable product of fortified

human milk, ready-to-feed to babies is produced.

- [096]. In another embodiment of the present invention, vials, syringes, or packets are produced containing blends of the isolated components of human milk, as well as vitamins and minerals, as combined concentrates (“fortifiers”) in aqueous or powdered forms. These fortifiers are appropriately prepared for adding to mothers’ milk at the hospitals or homes for provision of enriched levels of proteins, fats, carbohydrates, and many other beneficial components.
- [097]. In another embodiment of the present invention, the fortified human milk is further treated to adjust its osmolality into a preferred range.

Experimental Protocols and Methods

- [098]. *Human-milk supply chain and safety screening*
Freshly expressed human milk was collected into containers, typically 4 ounces each, provided to participant mothers by a company that deals in mother’s milk. The milk is stored at approximately -20 °C for up to 1 month while a sufficient number of containers are accumulated (typically 40, 4 oz. containers), then sent still-frozen in specialty packaging provided for that purpose to the company’s storage and analytical facilities.
- [099]. Samples of milk were cold-thawed (e.g., at room temperature) and subsamples taken for screening purposes. Alternatively, the container coverings are removed and the milk, still mostly frozen, is combined into larger vessels, typically 5 gallon pails or 3 gallon trays, and fully re-frozen to make an integrated frozen block. These are then subsampled by coring or shaving cryo-methods for screening purposes so that the bulk of the milk remains frozen prior to lyophilization.
- [100]. Screening includes microbiological assessments via standard culture techniques. Presences of both beneficial and potential pathogenic microbes are quantified. Assays by microbiological plate kits (3M petrifilms) include counts of aerobics, E. coli, yeast, mold, and Staphylococcus aureus. Molecular detection (3M MDS kits) are also used to screen for Salmonella, Listeria, and Cronobacter.

- [101]. ***Detection of Presence or absence of viral pathogens and/or Toxins***
Presence or absence of viral pathogens including HIV and Zika was assessed using enzyme-linked immunosorbent (ELISA) assays. Presence or absence of drugs and toxins was assessed by enzyme-linked ELISA methods. In some cases, liquid chromatography linked to mass spectrometry (LCMS) is used to confirm or better quantify the measurements. Substances assessed include but are not limited to benzodiazepines, cocaine metabolite benzoylecgonine, cotinine, ethyl alcohol, lysergic acid diethylamide (LSD), methamphetamine, opiates, phencyclidine (PCP), and tetrahydrocannabinol (THC).
- [102]. Verification of sole source to a pre-screened and approved participant is done via nucleic acid methods. Such methods were also used for verification of human source versus bovine (or other animal) and verification of human source versus inclusion of plant “milk”.
- [103]. ***Freeze-drying/Lyophilization:***
In the lyophilization of larger amounts of frozen milk (e.g., 1 ton to several tons), it is preferable that the milk is not completely thawed in this process, rather the bulk frozen masses of milk are rendered into frozen flakes or shavings by a cryo-processor prior to filling of commercial freeze-driers. This obviates possible microbial contamination that can occur during an extended thawing process. Alternatively, the milk is cold-thawed sufficiently to allow pouring or pumping into the lyophilizer trays, then frozen solid and lyophilized.
- [104]. ***Extraction of fats:***
The dry, lyophilized powder of human milk is extracted using food-approved solvents (Belitz *et al.*, 2009; Schmid *et al.*, 2016). This results in a separate phase of concentrated fats, fatty acids, lipids, and fat-soluble components such as some vitamins.
- [105]. The preferred method is to use supercritical carbon dioxide as “solvent”. The supercritical CO₂ is optionally supplemented with small amounts of a co-solvent, ethanol in particular, to optimize the interaction with partially polar molecules, like fatty acids (Machado *et al.*, 2016; Idrus *et al.*, 2018).

- [106]. Supercritical CO₂ is a special phase of carbon dioxide that exists in a specific range of temperature and pressure (e.g., 31 °C and higher, 1075 psi and higher). In this form, the carbon dioxide has properties of both a gas and a liquid, and can function very effectively as a solvent (McHugh and Krukoni, 2013). At the end of the treatment, the CO₂ may be released to the atmosphere, or recycled to the process.
- [107]. In either case, the original sample is rendered into two phases: 1) the remaining, dry, lyophilized fraction of mainly proteins and carbohydrates, and 2) the extracted fraction of milk fats or oils. The details of the phases and conditions of carbon dioxide are shown in Figure 1.
- [108]. ***Isolation of proteins:***
The dry, lyophilized proteins and carbohydrates are treated with a minimal amount of water adjusted in the range of 2.5 to 5, preferably pH 3 to 4.8 or pH 4 to 4.8. The water at this step serves as a processing aid in that many of the milk proteins are insoluble in water in the selected pH range, whereas the milk carbohydrates are soluble in this aqueous phase.
- [109]. Milk caseins are readily precipitated by lowering the pH in the range of 3 to 4.8 (Northrop, 1923; O’Kennedy, 2011). This is accomplished by use of acids selected from mineral acids or food-grade materials like acetic acid or citric acid. These caseins are collected by filtration or centrifugation and dried, for example, at low temperatures in a forced-air oven or under partial vacuum. The remaining aqueous phase of milk carbohydrates, which at this stage also includes some of the whey proteins (albumin, globulins, and related molecules), is neutralized by addition of harmless amounts of food-grade bases like sodium hydroxide or ammonia.
- [110]. Use of carbon dioxide again is the preferred method in the present invention for isolation of the milk proteins. Simply bubbling or pressurizing CO₂ into water lowers the pH into the desired range. Treating the lyophilized milk solids, after removal of the fats, with the mildly acidified water acts to solubilize the carbohydrates and some of the whey proteins, whereas the caseins are insoluble under these conditions (Jablonka *et al.*, 1985, 1986; Jordan *et al.*, 1987; Hofland *et*

al., 1999, 2003). The insoluble protein fraction is collected by filtration or centrifugation. The remaining aqueous phase is outgassed by mild vacuum treatment to remove the CO₂, which also has the effect of restoring the pH to neutrality.

[111]. Milk albumins and globulins are also readily precipitated from the aqueous phase with some extra steps of manipulating the pH and temperature (Connolly, 1983, 1985). Another preferred method for quantitative precipitation of milk albumins involves adding an amount (up to 40%) of ethanol to the aqueous phase of milk carbohydrates and “whey” proteins, with or without mild pH shifts and mild heating (40 to 50 oC). These are then collected by filtration or centrifugation.

[112]. Isolation of both caseins and globulin/albumins can be accomplished simply by effecting a mildly acidic pH in water along with the ethanol addition/mild heating as above (Cohn *et al.*, 1946, 19450; Ottenhof, 1985; Denizil, 2011; Raoufinia *et al.*, 2016). In all cases, the yield of the proteins is preferred to be quantitative (in the range of 95% and higher).

[113]. ***Isolation of carbohydrates:***

Aqueous phase - Precipitation of carbohydrates requires more steps. An aqueous solution of carbohydrates is heated to 90 to 95 °C and higher, plus the pH is alkalized by addition of sodium hydroxide (Linhardt and Bazin, 2001; Odaka *et al.*, 2018). This denatures and hydrolyzes the carbohydrates partially, making them insoluble. Although the carbohydrates so produced retain food value, the heat treatment would be sacrificial to a sizable fraction of the desired molecules. In addition, the utility as a health-promoting component of milk for infants is compromised.

[114]. The preferred method for rendering the carbohydrate component as a dry solid is to re-lyophilize the remaining aqueous fraction of milk carbohydrates. This can remove the water, and the ethanol as well, if present. In so doing, essentially all of the beneficial components are preserved and the end result of the separation schemes are the dry, enriched products of milk proteins, milk fats, and milk carbohydrates.

[115]. ***Use of the aqueous phase of milk carbohydrates “as is”:***

It is also an option to supplement the remaining aqueous phase of milk carbohydrates, provided it is otherwise solvent free, directly with the appropriate amounts of the dry powders of isolated milk proteins and milk lipids. The result is a liquid, fortified milk product.

[116]. ***Measurement of macro-components***

Nutritive content of the milk is established by measurements of protein, lipids, carbohydrates, and caloric content by standard methods (Brandao *et al.*, 2010; Choi *et al.*, 2015; Adamkin and Radmacher, 2014; Fusch *et al.*, 2015). Total solids and total dissolved solids of the original thawed samples and treated samples are measured by standard gravimetric techniques.

Other additives

[117]. Vitamins, minerals, and probiotics are obtained as sterile product offerings from commercial suppliers. These supplements are added as needed or desired in amounts according to recommended protocols.

[118]. ***Adjustment of osmolality***

The aqueous carbohydrate fraction, along with the other additives if present, contains the components that largely contribute to osmolality – namely the inorganic salts and the low- molecular-weight carbohydrates. The salts are ionic whereas the carbohydrates are neutrally charged. Therefore, the dissolved salts can be removed, or their levels reduced if needed, by use of food-approved ion-exchange resins (Singh, I. *et al.*, 2007; Mahore *et al.*, 2010; FDA Code of Regulations 21.173 section 25, 2013). Such treatments are analogous to those commonly used in homes for ultra-purification of drinking water.

[119]. To gain a better understanding of the invention described herein, the following examples are set forth. It will be understood that these examples are intended to describe illustrative embodiments of the invention and are not intended to limit the scope of the invention in any way.

Examples

[120]. ***Example 1 - Lyophilization and drying of human milk:***

Frozen human milk, safety screened for drugs, microbes, and non-milk diluents, was cold thawed and/or macerated to produce a partially frozen slurry. This was poured while weighing into 8 pyrex dishes, each 12 by 16 by 3.5 cm, to a depth of approximately 1 cm. Each dish contained 300 g of milk. These were then frozen solid at -20 °C in a commercial upright freezer, and then placed on three racks within the lyophilizer (HarvestRight scientific freeze dryer), precooled to -20 °C.

[121]. The vacuum pump was turned on, and internal pressure equilibrated, ranging about 100 to 200 (and lower) milliTorr (microns of mercury). The plate temperature on which the dishes rested was 40 °C. The freeze-drying proceeded over the next 24 to 48 hours. At 17 hours, 3 sample dishes were removed from the lyophilizer, having equilibrated the pressure to atmospheric, quickly weighed to assess the progress of the drying, immediately returned to their places in the racks, and the freeze-drying recommenced. When the samples had reached a constant weight, the drying was considered to be complete. This was the case typically after roughly 24 hours. In some runs, the lyophilization was continued for another 24 hours, to confirm completion of the drying step.

[122]. For comparison, further drying of the milk was also checked by placement of the pyrex dishes post-lyophilization, and other samples of lyophilized human milk e.g. in small aluminum weighing boats, in a forced-air drying oven at temperatures ranging from 60 °C to 80 °C to 120 °C. At 60 °C overnight, the dry weights so obtained matched closely with the dry weights as measured after lyophilization. The same was the case in the drying treatments at 80 °C. In both of these cases, the color of the dried milk post treatment was like that of the lyophilized milk: white to cream-colored.

[123]. On the other hand, at 120 °C, even after 2 to 3 hours, the milk began to darken to a tan color. Upon overnight drying at 120 °C, the dry weight of the lyophilized powder was upwards of 10% less than that of an equivalent sample of lyophilized, 60 °C dried, or 80 °C dried human milk. Evidently, the lyophilized milk was not completely stable at 120 °C even though dry, presumably owing to degradation or loss of some components.

- [124]. Consequently, dry weights of human milk as reported herein were recorded as lyophilized dry weights, which were equivalent to forced-air dry weights at ≤ 80 °C.
- [125]. Conversely, the lyophilized human milk was relatively stable in weight and color even if samples were left open to the atmosphere overnight at ambient temperatures (circa 20 to 25 °C). Samples at room temperatures also remained dry with stable weights if sealed in zip-lock bags for a month or more, but began to gain weight and became sticky after several months in zip-locks. After 8 months like this, a sample like this could increase in weight by up to 30%, presumably from uptake of humidity from the air. It could be redried at 60 or 80 °C to its original dry weight, with no apparent negative effects.
- [126]. To avoid this problem, samples were not only sealed in zip-lock bags, but water-absorbing silica packets were added within the bags. The bags were then placed in sealed jars, themselves containing silica packets. So treated, the samples were stable in weight and color for 2 years or more.
- [127]. In experiments as reported below, stable samples such as these having reliable dry weights were used.
- [128]. **Example 2 - Assessment of macronutrients of human milk samples:**
Measurements of fats, proteins, carbohydrates, total solids, and freezing point depression were made by use of an in-house, analytical instrument specific for that purpose (FOSS MARS Milkoscan). The instrument was calibrated by the manufacturer from thousands of quantitative measures of these macronutrients made using bovine milk. Some human milk components are also available in sufficient amounts (10 g and more) and were used for this purpose. For example, two of the major human milk oligosaccharides were obtained from a commercial source (Glycom A/S).
- [129]. Infrared spectra (FTIR) were scanned by use of the instrument, the peak heights and volumes of the relevant components having been calibrated and differentiated based

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- [134]. **Example 4 - Extraction of 12.5 g lyophilized human milk at 50 °C; 2,800 psi; 75 minutes reaction time:**

Yield of milk oil (fats and fat soluble materials) = 3.3 g.

Yield of protein plus carbohydrates fraction = 8.9 g.

Theoretical yield of milk oil based on average value of 4 parts fats, 1 part protein, 7 parts carbohydrates in human milk, or 33% of initial weight of 12.5 g as fats = 4.17 g.

Actual yield of milk oils: $(3.3\text{g}/4.17\text{g})100 = 79\%$ of theoretical.

Note: Actual total yield of 3.3 g milk oils + 8.9 g protein & carbohydrate (prot-carb) fraction = 12.2 grams.

This is $(12.2\text{ g recovered}/12.5\text{ g initial weight})100 = 98\%$ total yield.

- [135]. **Example 5 - Extraction of 20.5 g lyophilized human milk at 55 °C; 2,500 psi; 70 minutes reaction time:**

Yield of milk oils (fats and fat soluble materials) = 5.7 g.

Yield of protein plus carbohydrates (prot-carb) fraction = 14.0 g.

Theoretical yield of milk oils at 33% of 20.5 g = 6.77 g. Actual yield $(5.7\text{g}/6.77\text{g})100 = 84\%$ of theoretical.

Total actual yield of both fractions (milk oils and prot-carb) = 19.7. This is $(19.7\text{g}/20.5\text{g})100 = 96\%$ of theoretical.

- [136]. **Example 6 - Extraction of 15.5 g lyophilized human milk at 50 °C; 2,000 psi; 100 minutes reaction time:**

Yield of milk oils (fats and fat soluble materials) = 2.3 g.

Yield of prot-carb protein fraction = 12.8 g.

Theoretical yield of milk oils at 33% of 15.5 g = 5.12 g. Actual yield $(2.3\text{g}/5.12\text{g})100 = 45\%$ of theoretical.

Total actual yield of both fractions (milk oils and prot-carb) = 15.1. This is $(15.1\text{g}/15.5\text{g})100 = 97\%$ of theoretical.

- [137]. **Example 7 - Extraction of 10 g lyophilized human milk at 50 °C; 4,000 psi; 100 minutes reaction time:**

Yield of milk oils (fats and fat soluble materials) = 3.3 g.

Yield of prot-carb fraction = 6.5 g.

Theoretical yield of milk oils at 33% of 10 g = 3.3 g. Actual yield $(3.3\text{g}/3.3\text{g})100 = 100\%$ of theoretical.

Total actual yield of both fractions (milk oils and prot-carb) = 9.8g. This is $(9.8\text{g}/10\text{g})100 = 98\%$ of theoretical.

[138]. **Example 8 - Extraction of 10 g lyophilized human milk plus 10 g of glass beads (3 mm diameter) at 50 °C; 4,000 psi; 90 minutes reaction time:**

Yield of milk oils (fats and fat soluble materials) = 2.6 g.

Yield of prot-carb fraction = 7.2 g

Theoretical yield of milk oils at 33% of 10 g = 3.3 g. Actual yield $(2.6\text{g}/3.3\text{g})100 = 79\%$ of theoretical.

Total actual yield of both fractions (milk oils and prot-carb) = 9.8g. This is $(9.8\text{g}/10\text{g})100 = 98\%$ of theoretical.

[139]. **Example 9 - Extraction of 16 g lyophilized human milk at 40 °C; 3,200 psi; 90 minutes reaction time:**

Yield of milk oils (fats and fat soluble materials) = 2.1 g.

Yield of prot-carb fraction = 13.4 g.

Theoretical yield of milk oils at 33% of 16 g = 5.28 g. Actual yield $(2.1\text{g}/5.28\text{g})100 = 40\%$ of theoretical.

Total actual yield of both fractions (milk oils and prot-carb) = 15.5g. This is $(15.5\text{g}/16\text{g})100 = 97\%$ of theoretical.

[140]. **Example 10 - Extraction of 15 g lyophilized human milk at 60 °C; 3,200 psi; 120 minutes reaction time:**

Yield of milk oils (fats and fat soluble materials) = 3.8 g.

Yield of prot-carb fraction = 11.2 g.

Theoretical yield of milk oils at 33% of 15 g = 4.95 g. Actual yield $(3.8\text{g}/4.95\text{g})100 = 77\%$ of theoretical.

Total actual yield of both fractions (milk oils and prot-carb) = 15 g. This is $(15\text{g}/15\text{g})100 = 100\%$ of theoretical.

[141]. **Example 11 - Comparison of precipitation of milk proteins at pH 4 from lyophilized human milk and from the prot-carb fraction in water and in a 50/50**

weight% of water and ethanol:

Precipitation of milk proteins from lyophilized human milk was observed by addition of 5 g lyophilized human milk into 50 g of aqueous solution having pH 4 into a 4-oz glass vessel. For comparison, 5 g of lyophilized human milk was also added to a 4-oz glass vessel containing 25 g of distilled water and 25 g of ethanol (100%). For further comparison, parallel experiments were conducted using 2.5 g of the prot-carb fraction of example 7: treatment in 25 g distilled water and treatment in 12.4g water plus 12.5 g ethanol as 50/50 wt % water/ethanol in similar 4 oz vessels. The vessels were set on the lab bench unstirred and the precipitates allowed to settle.

[142]. A clearly defined precipitated layer formed in the prot-carb fraction almost immediately. A clear precipitated layer also formed in the lyophilized, whole human milk treatment, but the precipitate formed more slowly over approximately 30 minutes, and was less compact. The results are seen in Figure 2, with images taken after settling on the lab bench overnight.

[143]. The pH was adjusted to pH 4 by addition of 1 N HCl and measured using a combination pH electrode and meter. Milk components were seen to influence the pH readings of pH standard solutions of significantly higher molarities, shifting the readings upward to some extent. Therefore efforts were undertaken to standardize the electrode and the meter via standard addition of lyophilized milk and prot-carb fractions to the pH standard buffers.

[144]. **Example 12 - Comparison of precipitation of milk proteins at pH 4 from lyophilized human milk and from the prot-carb fraction in water without ethanol addition:**

Precipitates also formed in lyophilized, whole human milk and in the protein/carbohydrate fraction when treated in distilled water with pH adjustment without ethanol addition, but only slowly, to an observable lesser extent, and without clear definition as a discrete, settled layer. The pH of lyophilized, whole human milk samples in distilled water ranged about pH 5.8 whereas the pH of the protein/carbohydrate fraction of samples in distilled water ranged about pH 6.5. Numerous prior studies have shown that the pH treatment alone can result in precipitation of casein proteins but not whey proteins or carbohydrates.

[145]. **Example 13 - Precipitation of milk proteins from lyophilized human milk and from the prot-carb fraction in 50/50 weight% water and ethanol without pH adjustment:**

Proteinaceous precipitates were obtained from lyophilized whole human milk, as well as the protein/carbohydrate fraction, when each were treated in the 50/50 weight% solution of distilled water and ethanol. The protein-carbohydrate fraction was extracted from the lyophilized whole, human milk using supercritical CO₂ at 3,000 psi, 60 °C, for 90 minutes.

[146]. Samples were prepared as 10% by weight solutions in water to which ethanol was added. As shown in Figure 3, a significant precipitate settled from the treated solutions. The precipitate began to form more quickly and consolidated better in the prot-carb treatment. Settling was evident within minutes and well-formed layers separated over the next 30 minutes in both treatments.

[147]. The precipitates were separated from the supernatants by centrifuging at 13,200 rpm, 16.1 relative centrifugal force for 5 minutes. Well-formed pellets resulted. These were washed with 100 % ethanol, then resuspended in distilled water.

[148]. The pelleted materials were white and soft although well integrated. They could be picked up with a spatula and manipulated without falling apart. When resuspended in water, they were resistant to solubilization at first, but upon addition of 1 N NaOH to initial pH 10 with warming to 60C and smooth but vigorous magnetic stirring, it dissolved to a milk-like appearance.

[149]. The sample prepared from lyophilized whole, human milk still contained residual fats, which did not dissolve, but remained as suspended materials. The pH of the redissolved samples ranged from pH 8.3 to 8.5.

[150]. The FTIR analysis of the pelleted material from the protein-carbohydrate fraction indicated a composition of 67 g protein, 20 g carbohydrates (protein/carbohydrate ratio = 3.35), and 13 g fats per 100 g of material.

[151]. The FTIR analysis of the pelleted material from the treatment of lyophilized, whole human milk indicated 63 g protein, 10 g carbohydrates, and 27 g fats per 100 g of material.

[152]. **Example 14. Precipitation in water of milk proteins from the protein-carbohydrate fraction by treatment with carbon dioxide gas, liquid carbon dioxide, or supercritical CO₂.**

A 10% by weight solution of the protein-carbohydrate fraction of lyophilized human milk was treated with carbon dioxide gas at 30 °C, 800 psi for 1 hour (see Figure 1). The CO₂ was provided in the reaction cylinder of the CO₂ instrument above the solution as an atmosphere and/or provided from below to pass through the liquid phase. In both cases, the pH as measured in the outflow at the end of the treatment ranged around pH 5.2, but did not reach levels in the lower range of pH 3 to 4.8. Consequently, the precipitation was incomplete. When the CO₂ was provided via microbubbling on a recycled basis, the pH does reach these preferred levels, and the precipitation becomes complete.

[153]. The prot-carb solution was also treated with liquid CO₂ at 30 °C, 2,200 psi for 1 hour. Similarly, the prot-carb solution was treated with supercritical CO₂, now again in a non-liquid, gas phase, for an hour at 50 °C, 3,000 psi. In both cases, the pH as measured at the outflow ranged around pH 5.2. It was thus possible to obtain a partial precipitate of milk proteins by these treatments, but the preferred delivery method for the CO₂ is by microbubbling or otherwise from below on a recycled basis.

[154]. **Example 15 - Precipitation in water of milk proteins from the protein-carbohydrate fraction by acid-treatment in carbonated water:**

Another way to precipitate milk proteins from the protein-carbohydrate fraction is to dissolve the protein-carbohydrate fraction of lyophilized human milk in carbonated water, i.e. water already treated with CO₂ under pressure to a pH of 3 and lower. With this treatment, the precipitation of the milk proteins was observed to be more complete.

[155]. **Example 16 - Precipitation in water of milk proteins from the protein-**

carbohydrate fraction by acid-treatment to lower pH and enhance precipitation of milk proteins:

Mild addition of 1 N HCl or 1 N H₂SO₄ was used to adjust the pH of the protein-carbohydrate fraction into the range of pH 3 to 4.8. As previously discussed, milk proteins, in particular the caseins, are insolubilized by these treatments and form a precipitate. These proteins were observed to form out of solutions, but as a gel-like suspension. As such, they were not separable by filtration and required centrifugation for removal from the liquid phase.

[156]. An advantage of using HCl for such treatment is that the HCl can be removed under vacuum, restoring the solution or the lyophilisate to the original pH if the solution is so treated. The other mineral acids like H₂SO₄ become concentrated in the solid phase upon vacuum treatment or drying, rendering the product into an acidic form.

[157]. **Example 17 - Isolation of milk proteins from the supernatant after precipitation:**

Not being filterable, the precipitate was instead separated from the supernatant by centrifugation (Eppendorf model 5415D). Treatment at 3,500 rpm (rotor radius = 15 cm) for 5 minutes was sufficient to bring down the proteins as a pellet. The pellet was firmer after 10, then 15 minutes, or by increasing the rpm upwards stepwise to 13,200 (rcf = 16.1 g forces). In any case, formation of a pellet was straightforward.

[158]. The nominal volumes of supernatant were removed by hand-pipette and the pellets washed with equivalent volumes of ethanol three times. The pellets were combined, resuspended in a total volume of 20 ml distilled water, and redissolved by vortexing. This resuspended and redissolved solution was next subjected to protein analysis as well as the other macronutrient components and milk parameters.

[159]. As shown in Figure 4, it was possible to extract samples of a wide variety of fractional amounts of protein, carbohydrates, and oils from lyophilized human milk via supercritical CO₂ treatments. In turn, these samples could be used “as is” or combined in the appropriate relative amounts to yield desired fractional amounts of the macronutrients. Fortified human milk products can be made this way.

[160]. **Example 18. Fortified human milk formulations.**

Recommendations for fortified human milk emphasize increasing the amount of protein per ounce while keeping the overall energy levels around 100 calories per feeding (typically 4 oz.; e.g., Adamkin and Radmacher, 2014). By way of exemplification, 3.4 g of sample #6 above would contain 3 g protein and 0.4 g of carbohydrate. If this is added to 10 g of the lyophilized whole human milk of sample #8, the formulation would now contain 4 g of protein, 7.2 g of carbohydrate, and 2.2 g of fats. Adding this (13.4 g) to 4 ounces (118 ml) of water would yield a fortified human milk at 11% by weight total solids, not inclusive of inorganic salts and other minor components.

[161]. Given 4 calories per gram protein, 4 calories per gram carbohydrates, and 9 calories per gram fats (on average in human milk), the total caloric value for 13.4 g of this formulation would be 64.6 calories. The formula could be supplemented as needed, if desired, to increase the caloric value.

[162]. That is, if an exact caloric value of 100 calories per 100 ml feeding is desired, it is an easy matter to add more of one component or another, or in combination, to fix the caloric level at 100 calories. For example, the milk oils are isolated at purity in the supercritical CO₂ step whereas the carbohydrates are highly concentrated into the supernatant during the protein precipitation step.

[163]. By way of another example of a fortifier composition, 4.42 g of the pelleted material of Example 13 would contain 3 g of protein, 0.9 g of carbohydrates, and 0.52 g of fats. This would comprise a total of 20.28 calories at 4 calories/g protein, 4 calories/g carbohydrates, and 9 calories/g fats.

[164]. This could be added and stirred into 100 ml of whole, liquid, human milk as a powder. If the human milk sample contained the average amounts of 1 g protein, 7 g carbohydrates, and 4 g fats per 100 ml, translating to 68 calories, the fortified milk would then contain 88.3 calories per 100 ml.

[165]. The overall composition of the milk in this example per 100 ml would be 4 g protein (16 cal), 7.9 g carbohydrates (31.6 cal), and 4.52 g fats (40.68 cal).

- [166]. Alternatively, the fortifier composition could be prepared as a concentrated liquid in, for example 10 or 20 ml of water, and pipetted or otherwise added to the whole, liquid, human milk to fortify it. In this case, the overall caloric value per 100 ml would be adjusted to account for the added volume.
- [167]. It is evident that a formulation of fortified human milk of virtually any fractional composition of the macronutrients can be made by appropriately mixing samples of the present invention such as prepared and exemplified above.
- [168]. Moreover, it is further possible by adjusting the temperature, pressure, and duration of the supercritical CO₂ step, to achieve partial removal of the milk oils, with concomitant proportional increases in proteins and carbohydrates in the extracted material. This is shown in Examples 4 through 10 in which the yield of milk oils ranged from roughly 20% to 100% of theoretical, depending on the reaction conditions.
- [169]. **Example 19 - Combined supercritical CO₂ and conventional methods for separation of milk fractions:**
It is also desirable to be able to further separate and optimize the macronutrient levels of the prot-carb fractions, without the pH precipitation, with or without the solvent (e.g., ethanol) treatment, or without the combination pH/solvent treatment.
- [170]. A recommended approach to this is to bring a prot-carb fraction into solution “as is”, then subject it to conventional separation techniques. Skimming or centrifugation of residual milk fats may not be needed, as the supercritical CO₂ treatment can remove this fraction entirely. Conventional ultrafiltration, and reverse osmosis, if desired, of the redissolved protein-carbohydrate fraction would separate the proteins from the carbohydrates. The carbohydrate fraction could be further treated by ultrafiltration to separate lactose from the oligosaccharides, if desired.
- [171]. The resulting fractions, now in an aqueous state, or partly aqueous state if some of the emulsion phase (the milk fats) as well as some suspended solids are carried over,

can be dried if desired, preferably by lyophilization.

- [172]. Supercritical CO₂ treatment is preferred for removal of fats when the feedstock is lyophilized powder, because the extraction routinely can be $\geq 99\%$ efficient. By comparison, separation of milk fats from whole, liquid, human milk by conventional skimming and/or centrifugation steps results in a cream that typically is approximately 25% fats and the rest as an aqueous fraction which still contains the other macronutrients and water-soluble materials.
- [173]. The solids of the resulting aqueous phase exclusive of the cream are primarily comprised of the proteins and carbohydrates. This liquid can be lyophilized or otherwise dried to produce a dry protein-carbohydrate fraction. This dry fraction could then be further processed by retaking it into an aqueous state for conventional ultrafiltration and reverse osmosis, if desired. This seems counterintuitive, as the protein-carbohydrate fraction was already in an aqueous state via conventional methods. In addition, this conventional protein-carbohydrate fraction does not contain those macronutrients that were removed as “cream”.
- [174]. However, the protein-carbohydrate fractions containing essentially all of the milk proteins and carbohydrates are directly available via supercritical CO₂ extraction of lyophilized, whole human milk. Use of this kind of fraction or fractions as feedstock, including a dissolution step, for conventional processing to further separate and concentrate the components in aqueous solutions thus provides novel advantages. That is, this combination of supercritical CO₂ and conventional processing would then result in a fuller complement of the milk proteins and carbohydrates than the conventional processing alone.
- [175]. **Example 20. Supercritical CO₂ as Co-solvent.**
After the extraction of the milk fats, as in examples 11 through 17, other solvents can be added to the prot-carb fraction. As exemplified, additional water, ethanol, carbonated water, and dilute acids or bases are useful in this regard. Lyophilized, whole human milk can also be treated from the outset with supercritical CO₂ as a co-solvent. The supercritical CO₂ and other solvent can be deployed statically within the

reactor, or preferably set to recycle through the solid phase of the prot-carb fraction or the lyophilized whole human milk.

[176]. Particularly in the case of lowering the pH of milk by infusion of CO₂, it is preferred to recycle the solvent or co-solvents. The pH of milk can be lowered into the target range of pH 4 to 4.8, and lower, by static infusion of CO₂. However, this is a slow process and not preferred as it slows the precipitation and may make it incomplete.

[177]. The preferred method is to deliver the CO₂ from below, or otherwise directly into the solution, using a microbubbling device, so that the CO₂ flows and is recycled to the process. So arranged, the pH shift is rapid, reaching levels in the range of pH 3 (Balaban *et al.*, 1991; Hofland *et al.*, 1999; Yoshimura *et al.*, 2002), including lowering the pH of milk (Erkman, 1997).

[178]. **Example 21. Osmolality.**

The osmolality of the lyophilized human milk samples and its fractions when re-dissolved was assessed based on values of freezing point depression (FPD). These were measured via the analytical instrument (FOSS FTIR MARS Milkoscan) along with the other parameters, and converted to osmolality according to the physical chemical constant assigned to a FPD attributable 1 mole of solute per kilogram of water of minus (-) 1.86 °C. Thus, a solution having a freezing point depression of -0.186 °C has an osmolality of 0.1, or 0.1 mOsm. This corresponds to 100 millimoles of solute per kg of solution.

[179]. The osmolalities of the lyophilized human milk ranged from approximately 150 to 300 mOsm, whereas, the osmolalities of the prot-carb fractions at 10% solids on average were approximately 300 mOsm, each well within the target values of 400 to 600 mOsm.

[180]. It is evident that macronutrient fractions of human milk can be used to supplement lyophilized whole human milk to make a fortified human milk formulation that falls within the target values for osmolality. For example, even relatively high mOsm human milk can be supplemented with 2 or 3 grams of human milk protein per 4 ounce feeding, raising the mOsm minimally because the high-molecular weight

proteins do not add significantly to osmolality.

[181]. Similarly, the milk fats do not add significantly to osmolality. Hence, these can also be used to supplement lyophilized whole human milk if desired and keep within the mOsm guidelines, while also contributing to the energy values if desired at 9 calories per gram of milk fats on average.

[182]. The milk carbohydrates are the main contributors to mOsm values of human milk. However, these too can be used to supplement lyophilized whole human milk, which typically has significant margin for increasing mOsm while remaining within the recommended limits of mOsm of human milk.

[183]. In this regard, there may be reasons to supplement the amounts of human milk carbohydrates, in particular of human milk oligosaccharides, provided to infants. Promotion of digestive health is the best documented such reason at present. Hence, it may be desirable to provide elevated levels of these milk carbohydrates while controlling the osmolality to which the infant digestive tract is subjected. In this case, separate dosing of human milk carbohydrates can be provided to the infants, excluding or limiting the other macronutrients, as a specific beneficial therapy or preventative.

[184]. **Example 22 – Sterilization:**

Products may be sterilized, if desired, by standard Holder pasteurization, by high-temperature, short- time treatment (HTST), retorting, or other methods including gamma radiation and vapor phase infusion (Rutala and Weber, 2017).

[185]. **Example 23 – Packaging:**

Fortifier products of the present invention are provided as liquids, for example, in 5 to 20 ml vials or syringes for addition to milk as 1 vial or syringe per ounce of human milk, or in other suitable containers and volumes. The fortified milk is also provided in ready-to-feed, 4 ounce bottles and other packaging. Powdered forms of the products are provided in jars, bottles, packets, boxes, or other standard packaging.

[186]. Although the invention has been described with reference to certain specific embodiments, various modifications thereof will be apparent to those skilled in the art without departing from the spirit and scope of the invention. All such modifications as would be apparent to one skilled in the art are intended to be included within the scope of the following claims

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CLAIMS

1. A process for converting dried whole mammalian milk into a sterile fats-enriched fraction, a sterile protein-enriched fraction, and a sterile carbohydrate-enriched fraction, the process comprising the steps of:
 - a) treating the dried whole milk with a fats extracting agent at a pressure range of 2500-4500 psi (17237-31026 KPa), and temperature range of 40 to 75 °C to extract fats to provide a sterile fats-enriched fraction, and a sterile residual fraction comprising proteins and carbohydrates, wherein the fats extracting agent is supercritical carbon dioxide;
 - b) separating said fats-enriched fraction from the residual fraction; and
 - c) removing the fats extracting agent from the separated fats-enriched fraction to produce a dry fraction of oily, fats-enriched material and a fraction enriched in proteins and carbohydrates,wherein the dried milk is freeze-dried/lyophilized milk, and wherein the sterile fat enriched fraction has components including immunoglobulins at levels 90% by weight or more of levels prior to sterilization.
2. The process of claim 1, wherein the dried whole milk is human milk or bovine milk.
3. The process of claim 1 or 2, further comprising treating the proteins and carbohydrates enriched fraction obtained in step c), with:
 - i) a mildly acidic aqueous solution, optionally supplemented with an organic solvent, or
 - ii) a water and an organic solvent mixture, or
 - iii) carbonated water having a pH of up to 3,to provide a precipitated sterile protein-enriched fraction, and a soluble sterile carbohydrate-enriched supernatant fraction; and separating the soluble carbohydrate-enriched supernatant fraction from the protein-enriched fraction, and drying the protein-enriched fraction, preferably via lyophilization,

wherein the sterile protein-enriched fraction has components including immunoglobulins at levels 90% by weight or more of levels prior to sterilization, and

wherein the sterile carbohydrate-enriched fraction having components including immunoglobulins at levels 90% by weight or more of levels prior to sterilization.

4. The process of claim 3, wherein the mildly acidic aqueous solution is obtained by adding CO₂ gas, liquid CO₂ or supercritical CO₂ to water to achieve a pH of pH 3 to 4.8.
5. The process of claim 4, wherein the mildly acidic aqueous solution is obtained by sparging or micro bubbling CO₂ gas or supercritical CO₂ into water.
6. The process of claim 3, wherein the mildly acidic aqueous solution is obtained by adding an acid including H₂CO₃, HCl, H₂SO₄, H₃PO₄, HNO₃, acetic acid, citric acid or a combination thereof, to water to achieve a pH of 3 to 4.8.
7. The process of any one of claims 3 to 6, wherein the organic solvent is an alcohol solvent.
8. The process of any one of claims 3 to 7, further comprising drying the carbohydrate-enriched supernatant fraction.
9. The process of claim 8, wherein the carbohydrate-enriched supernatant fraction is dried via lyophilization.
10. The process of any one of claims 7 to 9, further comprising a step of adjusting the pH of the soluble carbohydrate-enriched supernatant fraction to precipitate a solid carbohydrate-enriched fraction, and separating and optionally drying the solid carbohydrate-enriched fraction.
11. The process of claim 10, wherein the pH is adjusted by use of one or more of bases selected from the group consisting of NH₄OH, NaOH, KOH, gaseous NH₃, and alkanolamine.

12. The process of claims 11, wherein the pH of the soluble carbohydrate-enriched supernatant fraction is adjusted to pH 10 to 11.
13. The process of claim 11, wherein the pH of the soluble carbohydrate-enriched supernatant fraction is adjusted to be in a range of pH 10 to 11, and then to be in a range of 4 to 4.8.
14. The process of any one of claims 10 to 13, further comprising adding a solvent before, during and/or after the pH adjustment step.
15. The process of claim 14, wherein the solvent is an alcohol solvent.
16. The process of any one of claims 10 to 15, further comprising heating the soluble carbohydrate-enriched supernatant fraction before and/or after pH adjustment.
17. A fats-enriched fraction obtained by the process of claim 1 or 2.
18. A protein-enriched fraction obtained by the process of any one of claims 3 to 7.
19. A carbohydrate-enriched fraction obtained by the process of any one of claims 3 to 16.
20. A composition comprising one or more of the fractions as defined in claims 17 to 19.
21. Use of one or more of the fat-enriched fraction of claim 17, protein-enriched fraction of claim 18, and carbohydrate-enriched fraction of claim 19 for mixing with whole or skimmed human milk, whole or skimmed bovine milk, or water to produce a fortified milk composition.
22. A composition comprising the carbohydrate-enriched fraction obtained by the process of claim 8, the dry fat-enriched fraction obtained by the process of claim 1 or 2, and the dry protein-enriched fraction obtained by the process of any one of claims 3 to 7, for mixing

- with whole or skimmed human milk whole or skimmed bovine milk, or water to produce a fortified milk composition.
23. A blend of one or more of the dry fat-enriched fraction obtained by the process of claim 1 or 2, the dry protein-enriched fraction obtained by the process of any one of claims 3 to 7, and dry carbohydrate-enriched fractions obtained by the process of any one of claims 8 to 16, as a dry material for mixing with whole or skimmed human milk, whole or skimmed bovine milk, or water to produce a fortified milk composition.
 24. An aqueous concentrate comprising one or more of the fractions as defined in any one of claims 17 to 19 for mixing with whole or skimmed human milk, whole or skimmed bovine milk, or water to produce a fortified milk composition.
 25. An aqueous concentrate comprising the carbohydrate-enriched fraction obtained by the process of claim 8, the dry fat-enriched fraction obtained by the process of claim 1 or 2, and dry protein-enriched fraction obtained by the process of any one of claims 3 to 7, for mixing with whole or skimmed human milk, whole or skimmed bovine milk, or water to produce a fortified milk composition.
 26. A method of producing a fortified milk composition, comprising adding one or more of the fractions as defined in any one of claims 17 to 19 to whole or skimmed human milk, or whole or skimmed bovine milk.
 27. The method of claim 26 further comprising adding vitamins and minerals.
 28. The method of claim 26 or 27 further comprising adding probiotics as a supplement.
 29. A fortified milk composition obtained by the method of any one of claims 26 to 28.
 30. The fortified milk composition of claim 29, comprising 10 to 85 mg/ml of protein, 60 to 125 mg/ml of carbohydrates, and 38 to 105 mg/ml of fats.

31. The fortified milk composition of claim 29 or 30 that has a caloric content of 20 to 26 calories per ounce.
32. The fortified milk composition of any one claims 29 to 31 that has an osmolality in range of 300 to 1000 milliosmoles (mOsm).
33. A sterile fats-enriched fraction of whole mammalian milk, said fraction having components including immunoglobulins at levels 90% by weight or more of levels prior to sterilization.
34. A sterile protein-enriched fraction of whole mammalian milk, said fraction having components including immunoglobulins at levels 90% by weight or more of levels prior to sterilization.
35. A sterile carbohydrate-enriched fraction of whole mammalian milk, said fraction having components including immunoglobulins at levels 90% by weight or more of levels prior to sterilization.
36. The fraction of any one of claims 33 to 35 having 100 colony forming units of microbes or less per 1 ml when reconstituted at 10% weight/weight in water.
37. A fortified milk composition comprising one or more of the fractions of claims 33 to 36.

Figure 1. Density of CO₂ versus temp and pressure

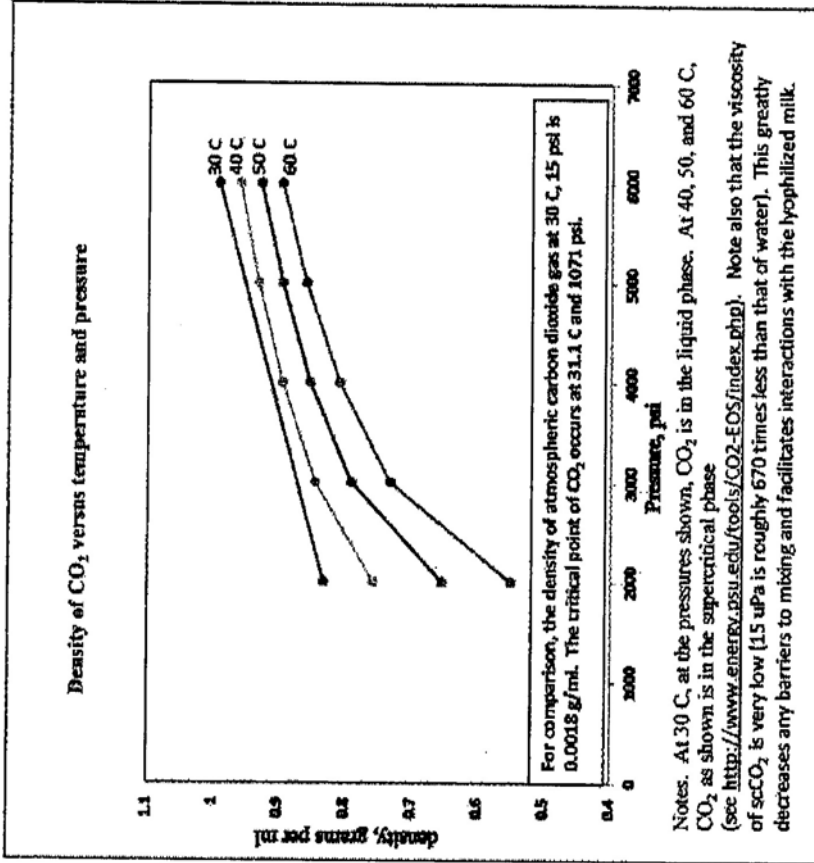
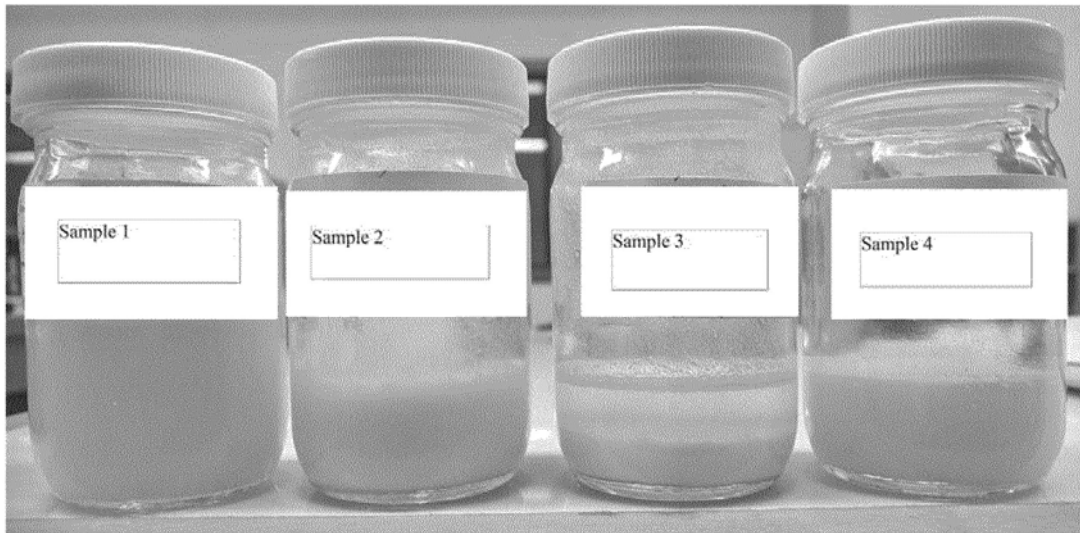
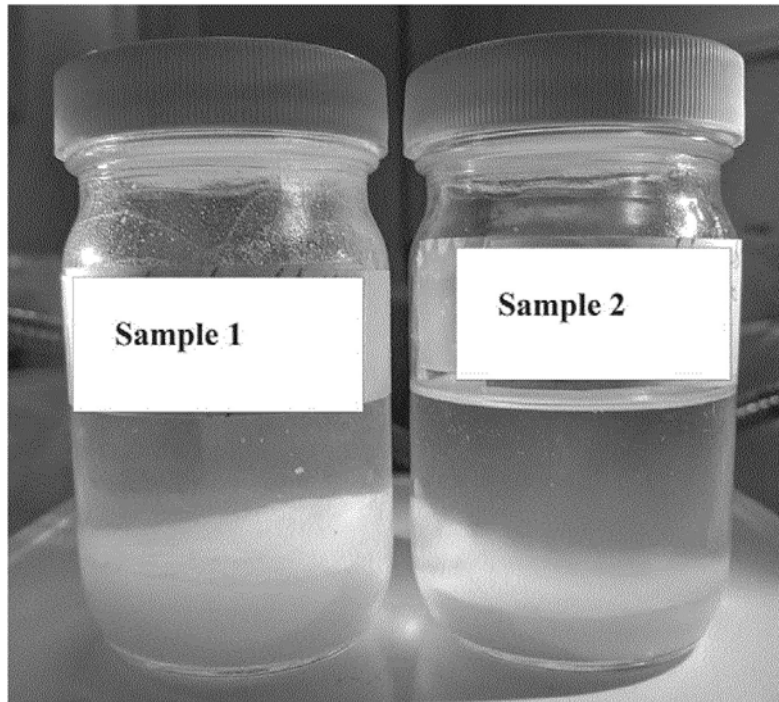


FIG. 1



Sample 1: Lyophilized, whole human milk at pH 4. **Sample 2:** Lyophilized, whole human milk at pH 4 plus addition of ethanol at 50/50 weight %. **Sample 3:** Protein-carbohydrate fraction of lyophilized human milk at pH 4 in water plus ethanol at 50/50% by weight ethanol. **Sample 4.** Protein carbohydrate fraction of human milk at pH 4 in distilled water

FIG. 2



Sample 1: lyophilized whole human milk. **Sample 2:** protein-carbohydrate fraction of lyophilized whole human milk.

FIG. 3

Results of protein, carbohydrates, and milk oil (fats) fractions of lyophilized human milk:
 FTIR measurements of macronutrients, grams per 100 grams

Number	Treatment	Protein	Carbohydrates	Milk oils (fats)	prot/carb
1	4,000 psi, 60C, 90 min: pH 4 supernatant	20	57	23	0.35
2	4,000 psi, 60C, 90 min: pH 4 pellet, 1st wash	28	25	47	1.12
3	4,000 psi 60C, 90 min: pH 4 pellet, 2nd wash	56	44	0.1	1.27
4	4,000 psi, 60C, water/EtOH 50/50 upper layer	8	88	3	0.09
5	4,000 psi, 60 C, water/EtOH50/50 pellet	28	61	11	0.46
6	4000 psi, 60C, ppt as above washed 3 times	88	13	negligible	7.33
7	Protein-carb fraction 3000 psi, 60C, 90 min.	14	81	5	0.17
8	Lyophilized Human whale milk	10	68	22	0.15

FIG. 4