applied sciences

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Development and Chemico-Physical Characterization of Ovine Milk-Based Ingredients for Infant Formulae

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Abstract: The great majority of infant formula (FM) for neonate's nutrition are produced using ingredients from cow milk. Recently, some countries, such as China and New Zealand, are turning their attention to the use of ovine milk ingredients for FM production. In this study, a pilot plant process has been set up to produce infant formula ingredients from Sarda sheep milk. To meet the nutritional needs of neonates (0–6 and 6–12 months of age) two different liquid milk-derived formulations (IF1 and IF2, respectively) obtained mixing whole milk, skimmed milk, and whey milk ultrafiltration concentrate (retentate) were produced. Compositional analysis of milk, retentate, and the final IFs showed that the two formulations contain elements of nutritional interest, such as well-balanced content of high biological value proteins (casein:whey proteins ratio of 30:70 and 60:40 for IF1 and IF2, respectively), vitamin A, E and B5, cholesterol, minerals, nucleotides, free amino acids and essential fatty acids (n–6:n–3 ~1), compatible with the growth and development needs of neonates. Therefore, the obtained IF1 and IF2 can be proposed as valuable ovine dairy ingredients for FM manufacturing. Further studies will be necessary to verify the adaptability of the developed process from laboratory to industrial scale application.

Keywords: infant milk formula; breast milk; sheep milk; chemico-physical properties; NMR metabolites; ultrafiltration; retentate

Citation: Lai, G.; Caboni, P.; Piras, C.; Pes, M.; Sitzia, M.; Addis, M.; Pirisi, A.; Scano, P. Development and Chemico-Physical Characterization of Ovine Milk-Based Ingredients for Infant Formulae. *Appl. Sci.* **2023**, *13*, 653. https://doi.org/10.3390/app13010653

Academic Editor: Maria Kanellaki

Received: 28 November 2022
Revised: 21 December 2022
Accepted: 28 December 2022
Published: 3 January 2023

1. Introduction

Breast milk is the first choice for neonate's nutrition. However, when breastfeeding is not possible or is insufficient, infant formula (FM) is the main alternative for providing infants with all the nutrients they need for optimal growth and development. The study of FM composition for neonates' nutrition is an active research area due to continuing innovation in the formulations and to its primary importance in neonatal nutrition and health. Infant formula is a mixture of several ingredients, and the complexities of formula design are due to the attempt to mimic the composition and the nutritional profile of human milk and its changes according to the lactation stage [1,2]. To this aim, the market offers different FMs depending on the neonate's age: infant formula first stage, designed for neonates 0–6 months of age, and follow-on formula for age > 6 months. Most current-generation infant formulae consist of cow milk-derived ingredients, vegetable oils, and micronutrients blended in proportions such as to reproduce the composition of human milk. Milk-derived ingredients are the main source of proteins and most FMs currently available on the market are based on proteins from cow's milk [3], where the casein:whey proteins (CN:WP) ratio of bovine milk (80:20) must be adjusted to that of human milk by adding WP-based ingredients, such as whey ultrafiltration retentate, to reach a CN:WP of ~30:70 for first stage and ~50:50 for follow-on formulae [2,4,5]. Although the majority of FMs are based on bovine milk, currently, there is great interest in the study of alternative

sources of protein, especially to prevent cow's milk allergy. Recently, FMs based on ovine milk are marketed in some countries, such as China and New Zealand [3]. Sheep milk exhibits, compared to other ruminant's milk, interesting peculiarities [6–8]. Compared to cow's milk, sheep's milk shows a higher content of short and medium chain fatty acids, and a reduced size of fat globules, a characteristic that makes it easier to digest [6,9], and a higher content of conjugated linoleic acid (CLA), which has beneficial effects on health demonstrated by numerous studies [7,10]. Sheep's milk shows other nutritional characteristics, such as a higher content of nucleotides [11], vitamins A, and E, calcium and other minerals [9,10]. Caseins and whey proteins from ovine milk show health promoting properties and are important source of bioactive peptides [7,8]. Upon digestion, sheep milk beta-casein does not release the beta-casomorphin-7 (BCM7). Although still a matter of debate, adverse effects of this peptide on human health, particularly in babies, have been suggested [12]. To the best of our knowledge, there are no studies that have deeply investigated the composition of sheep's milk and its derivates to be used in the production of infant formulae. In this work, a technological process was developed, on a pilot scale, for manufacturing liquid milk-derived formulations (IF) from sheep's milk to be used in the preparation of FM intended for feeding infants from 0–6 months and from 6–12 months (IF1 and IF2, respectively). Chemico-physical analyses of dairy ingredients used and of IF samples were carried out by different analytical techniques.

2. Materials and Methods

2.1. Milk Samples

Milk samples were obtained from forty-six Sarda sheep between April–May 2021, on six different days (evening and morning milkings), from the "Bonassai" experimental farm of Agris Sardegna (Olmedo, Italy). Animals were managed in a semi-extensive farming system characterized by grazing for 4 h a day in artificial pastures based on Mediterranean grass and legume forage species. Every day 55 ± 6 kg of milk were produced (daily milk production per head: 1.2 ± 0.3 kg day^{-1}), and from this batch, for each trial, 15 kg of raw whole bulk milk was collected. Part of the collected milk (10 kg) was processed (cheese making) to obtain the sweet whey to produce retentate, the rest was immediately filtered, thermized at 60 °C for 5 s, then cooled at 44 °C, as shown in Figure 1. An aliquot (2 kg) of the thermized milk was submitted to a skimming procedure using a centrifugal separator (Sartore, Vigone, Italy) to obtain skim milk, while another aliquot (3 kg) was utilized without further processing (whole milk) in the production of the liquid milk-derived formulations (IF1 and IF2), as shown in Figure 1. The technological processes to produce skimmed milk, whey, and retentate, from ovine milk, were carried out in the pilot plant at the dairy technology laboratories of Agris Sardegna.

2.2. Ultrafiltered Whey Retentate Production

The whole sheep's milk was processed applying the cheese making technology provided for uncooked cheese. The whey obtained (~7 kg) by rennet coagulation was firstly thermized (60 °C × 5 s), then cooled at 44 °C and submitted to a defatting procedure using a centrifugal separator (Sartore, Vigone, Italy). The skimmed whey was cooled to 25 °C and subjected to the ultrafiltration process using a Sartocon® Slice Crossflow Filtration System (Sartorius, Gottinga, Germany) equipped with 5 Hydrosart® stabilized cellulose-based membrane (cut-off: 30 kDa; nominal area: 0.1 m^2; Sartorius, Gottinga, Germany). The transmembrane pressure, process temperature and flow rate were 0.1 MPa, 25 °C and 18.6 L h^{-1}, respectively. The ultrafiltration process was stopped when the pre-established volumetric concentration ratio (VCR: ~7) was reached (corresponding to a protein content in the retentate between 7–9%, depending on the protein content in the obtained whey). The resulting retentate fraction (~1 kg) was then used in the production of the liquid milk-derived formulations (IF1 and IF2).

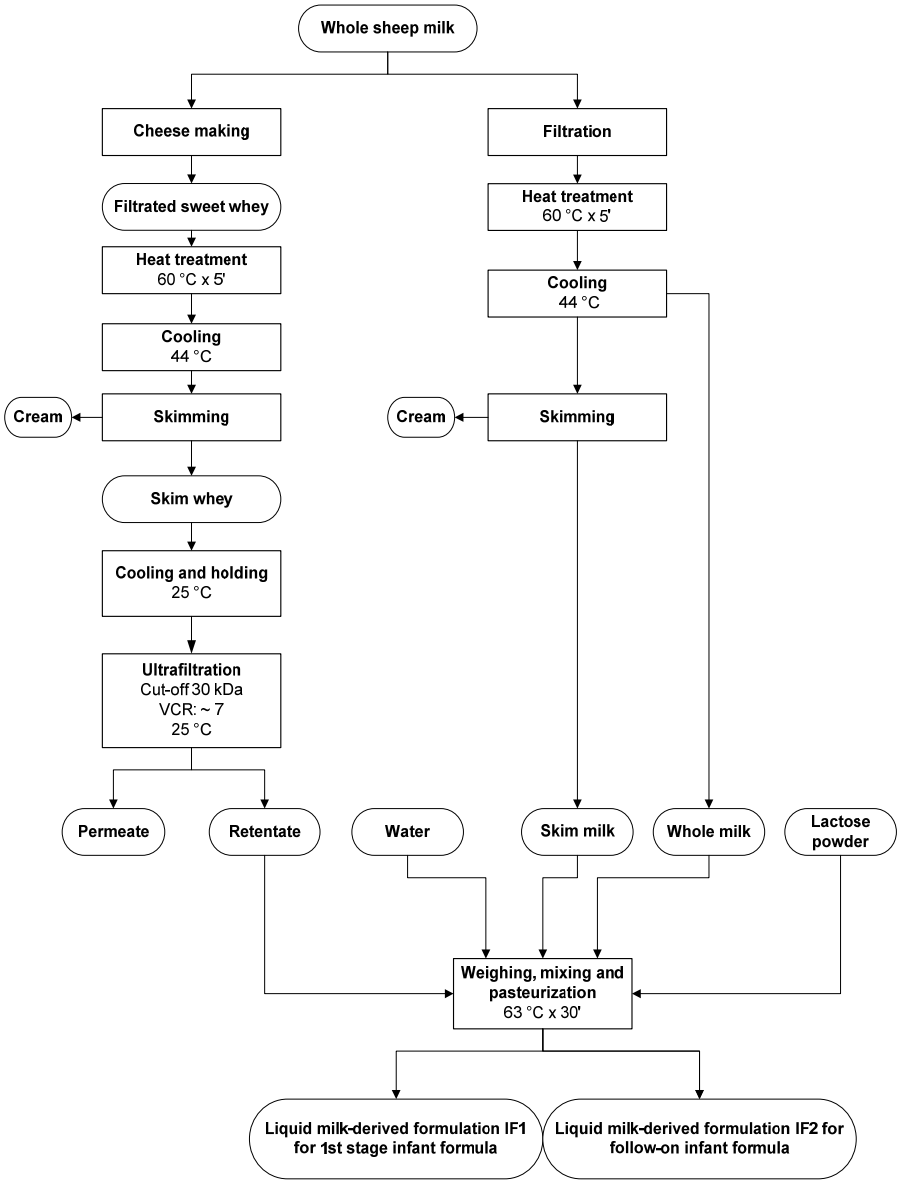

Figure 1. Flow chart of the production process of liquid milk-derived formulations IF1 and IF2 to be used in the production of first stage (from 0 to 6 months of age) and follow-on (from 6 to 12 months of age) infant formulae, respectively. VCR = volumetric concentration ratio.

2.3. Liquid Milk-Derived Formulations (IF) Manufacturing

Two liquid milk-derived formulations IF1 and IF2 (2 kg each), to be used in the production of FM, were prepared each time for six trials by mixing predetermined amounts of dairy ingredients (whole milk, skim milk and retentate) with water and lactose powder (Oxoid, Thermo Fisher Scientific, Basingstoke, UK), as shown in Figure 1. Two mixtures were obtained, one intended for the production of first stage FM (IF1) and the second one for the production of follow-on FM (IF2). The relative presence of sheep-derived ingredients in commercial FM was taken as the standard of reference. The composition of the two blends reflected the changes that occur in breast milk during lactation, particularly with respect to the different CN:WP ratio. In fact, the two liquid milk-derived formulations represented only part of the complete infant formulae but provided the entire protein content. The aim was to achieve a CN:WP ratio of approximately 30:70 in IF1 and 60:40 in IF2. Furthermore, IF1 and IF2 were also standardized for fat and lactose content. The target, in this case, was to obtain two formulations that provided the final infant formulae with a fat content of approximately 20% for IF1 and 50% for IF2 and a lactose content close to 50%

for both formulations. To achieve the desired compositions, the exact amount of the single components (whole and skim milk, retentate, lactose powder and water) was determined to avoid the marked sheep's milk composition variability which naturally occurs during the production season [13]. Therefore, the final IF1 and IF2 products had defined and constant chemical and nutritional properties.

2.4. Chemico-Physical Composition

Samples of whole milk, skim milk, retentate and liquid milk-derived formulations (IF1 and IF2) were analyzed for fat, protein, casein, whey protein and lactose content using a Milkoscan FT+ (Foss, Hillerød, Denmark), pH using a Crison Basic 20+ pH meter (Crison Instruments S.A., Alella, Spain), dry matter (DM) according to the ISO reference method [14], ash according to the AOAC method [15]. For minerals (Ca, Mg, Na, K, P, S) and trace elements (B, Zn, Cu, Mn, Fe) determination, 5 g of the samples (whole and skim milk, retentate, IF1 and IF2) were previously dried at 102 °C for 24 h and then calcined at 550 °C in a muffle furnace (Gelman Instrument, Opera, Italy). The operating conditions were set to reach 550 °C in 8 h and hold 550 °C for another 8 h. The resulting ashes were solubilized with HCl (18.5%, *w*/*w*), transferred to a 100 mL volumetric flask and made up to the mark with ultra pure water. The solution was filtered using paper filters (ashless, Grade 40, Whatman®, Merk, Darmstadt, Germany). The filtrate was used for subsequent analysis by the inductively coupled plasma optical emission spectrometry (ICP-OES) method, according to the ISO reference procedure [16]. The concentration of the elements Calcium (Ca, λ = 317.93 nm), Magnesium (Mg, λ = 279.07 nm), Potassium (K, λ = 766.49 nm), Sodium (Na, λ = 589.61 nm), Phosphorus (P, λ = 178.22 nm), Sulfur (S, λ = 180.67 nm), Boron (B, λ = 249.77 nm), Zinc (Zn, λ = 213.85 nm), Copper (Cu, λ = 324.75 nm), Manganese (Mn, λ = 257.61 nm), and Iron (Fe, λ = 238.20 nm) was determined using an ICP-OES spectrometer (OPTIMA 7300 DV, Perkin Elmer, Waltham, MA, USA) equipped with a GemTip Cross-Flow II nebulizer (Perkin Elmer) and an autosampler (SC-2 DX, Elemental Scientific Inc., Omaha, NE, USA). Argon flows to generate plasma, auxiliary argon flow and argon flow for the nebulizer were 15 L min^{-1}, 0.2 L min^{-1} and 0.8 mL min^{-1}, respectively. Sample flow was set at 0.8 mL min^{-1}.

2.5. Retinol, α-Tocopherol and Cholesterol Determination

In order to simultaneously determine the concentrations of retinol, α-tocopherol, and cholesterol in milk and liquid milk-derived formulations, a reverse-phase HPLC method was applied, as described by Panfili et al. [17] and Manzi et al. [18]. 2 mL of sample was transferred into a saponification tube. 2 mL of potassium hydroxide solution (60% aqueous solution, *w*/*v*) was added to the sample together with 2 mL of 95% ethanol, 1 mL of NaCl (1% aqueous solution, *w*/*v*), and 5 mL of an ethanolic solution of pyrogallol (6%, *w*/*v*) added as an antioxidant. After the tubes were flushed with nitrogen and sealed, the saponification process was carried out in a water bath at 70 °C for 30 min in the dark. The tubes were then cooled in cold water and 5 mL of an NaCl solution (1%, *w*/*v*) was added. A first extraction of the suspension was made by adding 10 mL of n-hexane/ethyl acetate (9:1, *v*/*v*) and shaking the tubes. The extraction procedure was repeated three more times by adding 5 mL of n-hexane/ethyl acetate (9:1, *v*/*v*) to the aqueous phase, each time. The collected organic phases were evaporated with a rotary evaporator at 30 °C, until dry. The resulting sample was suspended in 3 mL of methanol for HPLC and an aliquot was filtered using a 0.20 µm PTFE filter. A sample volume of 20 µL was injected into the HPLC equipment. The separation was performed using a liquid chromatography Agilent Series 1100 (Agilent Technologies, Palo Alto, CA, USA) equipped with a 4.6 mm ID × 150 mm Zorbax ODS column (Agilent Technologies). Two detectors were connected in series: a UV/Vis spectrophotometer (set to λ = 208 nm) and a spectrofluorometer (excitation set to λ = 293 nm; emission set to λ = 326 nm). A methanol/ultra pure water (98:2, *v*/*v*) mixture was used as mobile phase set at 1 mL min^{-1}. The temperature of the column was kept at 25 °C. The determinations were made in duplicate for all samples.

2.6. Fatty Acids Composition

Fat extractions of whole milk and liquid milk-derived formulations was carried out according to the ISO reference procedure [19], first adding an ethanol and ammonium hydroxide solution and then using a mixture of diethyl ether and n-pentane to obtain fat separation. The diethyl ether/n-pentane layer was evaporated with a rotary evaporator at 30 °C. The extracted fat was stored at −20 °C until further analysis. Fatty acids methyl esters (FAMEs) were prepared from 50 mg of milk and liquid milk-derived formulations fat with a base-catalyzed trans-methylation, using KOH in methanol, according to ISO reference procedure [20]. The obtained FAMEs were then analyzed using a Gas Chromatograph Agilent Technologies 8890 (Agilent Technologies, Palo Alto, CA, USA), equipped with a split/splitless injector, a Flame Ionization Detector (FID), a capillary column SP2560 (100 m × 0.25 mm i.d., 0.20 mm thickness, Merck Life Science, Milan, Italy). The operating chromatographic conditions were: carrier gas, helium; flow: 1 mL min^{-1}; injector split ratio: 1:100; injector temperature, 290 °C. The oven temperature program was 4 min at 45 °C, then increasing the temperature from 45 °C to 175 °C at a rate of 13 °C min^{-1}, remaining at 175 °C isotherm for 27 min, then increasing temperature from 175 °C to 215 °C at a rate of 4 °C min^{-1}, and remaining at the 215 °C isotherm for 35 min; FID temperature: 290 °C. Individual FAMEs were identified based on retention time and the comparison with a standard mixture of 37 pure compounds (Supelco 37 Component FAME Mix, Merck Life Science, Milan, Italy). In addition, a comparison of the chromatographic profile obtained with those described in the literature [21] was considered for confirmatory purpose. Each FAME was quantified with a calibration curve using the following internal standards: Me-C5:0 (C4:0 to C6:0), Me-C9:0 (C8:0 to C10:0), Me-C13:0 (C11:0 to C17:0), and Me-C19:0 (C18:0 to C26:0). Each internal standard was added to the sample at a concentration of 100 mg g^{-1} of fat. The linear ranges of the calibration curves used to quantify the fatty acids are reported in Table S1.

2.7. NMR Spectroscopy

The aqueous extracts of IF1 and IF2 were studied by ^{1}H NMR spectroscopy. Samples were submitted to the Folch extraction method. An aliquot of 200 µL of the aqueous phase was dried under a gentle stream of nitrogen and dissolved in 600 µL of D_2O. ^{1}H NMR spectra were acquired at 600 MHz, with a 14.09 T NMR spectrometer (Bruker Avance III HD, Bruker Biospin GmbH Rheinstetten, Karslruhe, Germany). ^{1}H NMR spectra were recorded by applying the noesyprd1 (noesy 1D presat) sequence as implemented in the standard Bruker experiments, with a spectral width of 9600 Hz, 32K data points, an acquisition time of 1.7 s, and 64 transients. ^{1}H NMR spectra were referenced to TSP external standard at 0.00 ppm. Assignment of ^{1}H NMR signals was performed with the aid of literature reports, an in-house database, and by performing two-dimensional experiments (^{1}H-^{1}H total correlation spectroscopy, TOCSY). Metabolites were quantified using Chenomx NMR Suite 8.1.2 (Chenomx Inc., Edmonton, AB, Canada) and normalized to the known lactose concentration.

2.8. Statistical Analysis

The data obtained were submitted to analysis of variance and multiple comparison tests (Tukey), using the general linear model (GLM) procedure with the statistical package Minitab 16 (Minitab Inc., State College, PA, USA). Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Milk, Retentate, and IF Chemical-Physical Composition

As shown in Figure 1, three ingredients derived from sheep's milk were utilized in the production of the liquid milk-derived formulations (whole milk, skimmed milk and retentate from skimmed whey). Retentate is an ingredient of primary importance to raise the WP content of FM up to that of human milk, this latter being richer in WP and poorer

of CN protein, especially in early lactation stage [2]. The chemico-physical composition of milk and milk-derived ingredients is reported in Table 1.

Table 1. Chemico-physical composition of the dairy ingredients used to produce the liquid milk-derived formulations (mean ± SD).

	Whole Milk (n = 6)	Skimmed Milk (n = 6)	Retentate * (n = 6)
pH (UpH)	6.61 ± 0.03	6.63 ± 0.04	6.57 ± 0.13
Dry matter (%)	16.8 ± 0.4 ^{a}	11.7 ± 0.2 ^{c}	13.9 ± 1.7 ^{b}
Fat (%)	5.9 ± 0.2 ^{a}	0.4 ± 0.1 ^{b}	0.6 ± 0.1 ^{b}
Protein (%)	5.4 ± 0.2 ^{b}	5.7 ± 0.2 ^{b}	8.0 ± 1.9 ^{a}
Fat/Protein (-)	1.10 ± 0.04 ^{a}	0.06 ± 0.01 ^{b}	0.08 ± 0.01 ^{b}
Whey protein (%)	0.9 ± 0.1 ^{b}	0.9 ± 0.1 ^{b}	7.7 ± 1.8 ^{a}
Lactose (%)	4.4 ± 0.1 ^{b}	4.8 ± 0.1 ^{a}	4.5 ± 0.1 ^{ab}
Ash (%)	0.88 ± 0.15 ^{ab}	0.96 ± 0.08 ^{a}	0.70 ± 0.01 ^{b}

* Retentate from ultrafiltration of skimmed whey. Different letters in a row indicate mean differences statistically significant at $p < 0.05$.

The three milk ingredients showed similar pH values (6.57–6.63; $p > 0.05$) indicating that the processing conditions, applied in the whey pre-treatment stages and during ultrafiltration, limit the possible alteration of pH parameters, essentially due to spontaneous acidification processes. As expected, the compositional differences between whole and skimmed milk were mainly due to the lowering of the fat content in the latter which leads to a slightly increased percentage of all the other macro-components. Retentate, whole and skim milk showed statistically different DM values (13.9%, 16.8% and 11.7%, respectively $p < 0.05$). Since retentate was produced from skim whey concentrated by ultrafiltration, it showed by far the highest value of whey protein compared to whole and skim milk (7.7%, 0.9% and 0.9%, respectively) accounting for more than half of the DM. This whey protein content is in agreement with that expected for the VCR (~7) selected during the whey concentration process. Retentate and skimmed milk showed similar fat content (0.6% and 0.4%, respectively; $p > 0.05$), while the lactose content showed comparable values for all samples. The ash percentage, indicative of the mineral content, was lower in retentate with respect to skim milk (0.70% vs. 0.96%; $p < 0.05$) but did not differ significantly compared to whole milk (0.88%; $p > 0.05$).

The above-described ingredients were then mixed with water and lactose powder to obtain two liquid milk-derived formulations, one intended for infants of 0–6 months of age (first stage, IF1) and the second one for follow-on infant formulae (from 6 months on, IF2). It has been reported that the CN:WP ratio, in human milk, fluctuates between 30:70 and 20:80 in early lactation and decreases in late lactation [2]; therefore, the exact amount of the dairy ingredients was determined to obtain a final CN:WP ratio of approximately 30:70 for IF1 and of 60:40 for IF2, respectively. The IF1 and IF2 samples were obtained by mixing different percentages of the three ingredients with water and lactose, as shown in Table 2.

Table 2. Relative incidence of the ingredients utilized in the preparation of the liquid milk-derived formulations IF1 and IF2, to be used in the production of first stage (from 0 to 6 months of age) and follow-on (from 6 to 12 months of age) infant formulae, respectively (mean ± SD).

	IF1 (n = 6)	IF2 (n = 6)
Retentate * (%)	31 ± 6	9 ± 1
Whole milk (%)	23 ± 1	52 ± 1
Skim milk (%)	3 ± 1	13 ± 1
Lactose powder (%)	8.1 ± 0.3	4.8 ± 0.2
Water (%)	34 ± 5	21 ± 2

* Retentate from ultrafiltration of skimmed whey.

The composition of IF1 and IF2 is reported in Table 3.

Table 3. Chemico-physical composition of liquid milk-derived formulations IF1 and IF2, to be used in the production of first stage (from 0 to 6 months of age) and follow-on (from 6 to 12 months of age) infant formulae, respectively (mean ± SD).

	IF1 (*n* = 6)	**IF2 (*n* = 6)**
pH (UpH)	6.74 ± 0.10	6.71 ± 0.03
Dry matter (%)	16.2 ± 0.1	16.1 ± 0.1
Fat (%)	1.5 ± 0.1 ^{b}	3.1 ± 0.1 ^{a}
Protein (%)	3.82 ± 0.08 ^{b}	4.32 ± 0.02 ^{a}
Fat/Protein (-)	0.38 ± 0.01 ^{b}	0.72 ± 0.01 ^{a}
Casein (%)	1.3 ± 0.1 ^{b}	2.8 ± 0.1 ^{a}
Whey protein (%)	2.6 ± 0.1 ^{a}	1.6 ± 0.1 ^{b}
Casein/Whey protein (-)	0.49 ± 0.06 ^{b}	1.67 ± 0.05 ^{a}
Lactose (%)	10.16 ± 0.03 ^{a}	7.88 ± 0.04 ^{b}
Ash (%)	0.46 ± 0.03 ^{b}	0.69 ± 0.11 ^{a}

Different letters in a row indicate mean differences statistically significant at $p < 0.05$.

Values of pH and dry matter did not differ between the two formulations, while all the other parameters were significantly different from each other ($p < 0.05$). It is worth remembering that, in designing the final FM, IF1 and IF2 are meant to be the only sources of proteins, while the content of fat, lactose and other components must be adjusted by adding other ingredients. As reported in Table 3, the qualitative changes in the protein fraction in IF1 and IF2 reflected those that occur naturally in breast milk during the growth period of the new-born [2]. As expected, IF1 had a significantly lower casein level than IF2 (1.3% and 2.8%, respectively; $p < 0.05$), while the whey protein value was higher in IF1 compared to IF2 (2.6% and 1.6%, respectively; $p < 0.05$). This aspect is consistent with the use of a larger amount of retentate to obtain IF1, whereas more milk (whole and skim) was used to obtain IF2 (Table 2). Therefore, the CN:WP ratio (0.49 and 1.67, respectively in IF1 and IF2) was close to the predetermined target (30:70 in IF1 and 60:40 in IF2). Whey proteins, particularly β-lactoglobulin and α-lactoglobulin, are most useful in the first few months of the neonate's growth. Since whey proteins remain soluble in the stomach, they are digested more quickly, pass more rapidly to the intestines, and are readily used in the building of muscle mass. Caseins, on the other hand, form a coagulated mass during gastric digestion and thus remain longer in the stomach; they are absorbed slowly, making them more suitable for a later stage of growth [22,23]. Table 3 also shows the fat, lactose, and ash content of the two formulations. IF2 showed about twice the fat content of IF1 (3.1 vs. 1.5; $p < 0.05$). Lactose content was significantly higher in IF1 than in IF2 (10.16% and 7.88%, respectively; $p < 0.05$), while ash percentage was lower in IF1 compared to IF2 (0.46% and 0.69%, respectively; $p < 0.05$).

3.2. Milk, Retentate, and IF Mineral Composition

In breast milk, minerals contribute to a variety of physiological functions, forming essential parts of many enzymes and are of biological importance to molecules and structures [2]. Ruminant milks contain a great amount of minerals, but data regarding their bioavailability are scanty. Minerals such as iron, zinc and copper of ruminant milks are mainly associated with casein, in contrast to human milk (linked to soluble proteins), implying lower assimilation [24]. The mineral composition of milk ingredients and liquid milk-derived formulations IF1 and IF2 is reported in Table 4.

Table 4. Mineral composition (mg 100 g^{-1}) of milk ingredients and of the two resulting liquid milk-derived formulations IF1 and IF2, to be used in the production of first stage (from 0 to 6 months of age) and follow-on (from 6 to 12 months of age) infant formulae, respectively (mean ± SD).

	Retentate * (*n* = 6)	**Whole Milk (*n* = 6)**	**Skimmed Milk (*n* = 6)**	**IF1 (*n* = 6)**	**IF2 (*n* = 6)**
Ca	44.1 ± 0.3 ^{e}	180.6 ± 6.6 ^{b}	192.3 ± 7.8 ^{a}	64.0 ± 1.6 ^{d}	122.8 ± 7.9 ^{c}
Mg	11 ± 2 ^{b}	17 ± 1 ^{a}	19 ± 1 ^{a}	8 ± 1 ^{c}	12 ± 1 ^{b}
Na	57 ± 9 ^{ab}	60 ± 8 ^{a}	60 ± 3 ^{a}	47 ± 4 ^{b}	49 ± 5 ^{b}
K	137 ± 16 ^{a}	114 ± 4 ^{b}	119 ± 5 ^{b}	79 ± 7 ^{c}	86 ± 6 ^{c}
P	57.6 ± 0.3 ^{c}	123.2 ± 8.3 ^{a}	128.6 ± 7.7 ^{a}	49.3 ± 4.8 ^{c}	86.7 ± 5.4 ^{b}
S	22 ± 2	21 ± 7	23 ± 6	17 ± 4	19 ± 4
B	<0.05	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Zn	0.30 ± 0.07 ^{bc}	0.50 ± 0.13 ^{ab}	0.64 ± 0.14 ^{a}	0.28 ± 0.07 ^{c}	0.44 ± 0.14 ^{bc}
Cu	0.05 ± 0.00	0.08 ± 0.06	0.09 ± 0.06	0.10 ± 0.04	0.09 ± 0.06
Mn	0.06 ± 0.08	0.06 ± 0.05	0.05 ± 0.06	<0.05	0.09 ± 0.10
Fe	0.09 ± 0.04	0.14 ± 0.06	0.18 ± 0.06	0.12 ± 0.11	0.15 ± 0.06

* Retentate from ultrafiltration of skimmed whey. Different letters in a row indicate mean differences statistically significant at $p < 0.05$.

In ruminant's milk the two most abundant mineral components are calcium and phosphorus; they are 1/3 and 1/2 in the colloidal form, respectively, linked to caseins. Approximately 1/3 of magnesium is also found associated with caseins [25]. In this regard, in Table 4 it is interesting to note that the mineral profile of retentate differed significantly from that of the milk in terms of calcium, phosphorus and magnesium contents ($p < 0.05$). In fact, the retentate is produced by ultrafiltration of rennet whey, deriving from cheese making; therefore, since caseins are almost absent in retentate, the mineral component associated with them is also missing (calcium, phosphorus and magnesium). The minerals and trace elements composition of sheep's milk appeared to be in line with data reported in the literature [24,26], whereas no information regarding sheep whey retentate is available. IF1, compared to IF2, showed significantly lower levels of calcium, magnesium and phosphorus, ($p < 0.05$). This aspect was consistent with the fact that the IF2 formulation was obtained by blending higher quantities of milk (whole and skimmed), compared to IF1 formulation, and therefore, the level of these minerals increased with the casein content of the IF2 formulation. To compensate for the lower bioavailability of the minerals present in the liquid milk-derived formulations compared to human milk, a mix of minerals and trace elements is usually added to the final FM, so that the content of these chemical elements in the final infant formulae is higher than the levels present in human milk [27,28]. In commercial infant formulae the content of calcium and magnesium ranges between 250–600 mg 100 g^{-1} and 40–150 mg 100 g^{-1}, respectively, while that of trace elements zinc and iron ranges between 5–15 mg 100 g^{-1} and 8–15 mg 100 g^{-1}, respectively [27].

3.3. Milk and IF Fatty Fraction

The contents of the liposoluble vitamins A (retinol) and E (α-tocopherol), and of cholesterol (expressed as mg kg^{-1}) in the ovine milk and IF1 and IF2 samples are reported in Table 5.

The retinol, α-tocopherol, and cholesterol contents found in whole milk samples (Table 5) are in agreement with those reported by other authors [7,9,10]. With respect to skimmed milk and retentate, whole milk is the IF component that provides the higher quantities of all molecules related to milk fat. Compared to IF1, IF2 obtained using higher amounts of whole milk, showed significantly higher values for these three parameters ($p < 0.05$). Retinol and α-tocopherol are two liposoluble vitamins essential for human health. Retinol is the principal and most biologically active form of vitamin A and plays a central role in several biological processes during the growth and developmental stages of neonates, such as the functioning of the visual process and the immune system, and in maintaining

the integrity of epithelial tissues. It also acts as an antioxidant molecule. α-Tocopherol is the prevalent and most biologically active form of vitamin E in milk. It acts as an antioxidant molecule and can prevent the formation of free radicals, protecting the lipids of various biological structures, such as cell membranes, from peroxidation processes [29,30]. Usually, in commercial FMs, the vitamin content of milk derived ingredients is further integrated with blends of fat- and water-soluble vitamins. On the other hand, IF1 and IF2 can be considered the only sources of cholesterol in the final infant formulae. Human milk contains cholesterol [1] which is a functional and structural constituent of the cell membrane and is needed by the infant in challenging the development of cholesterol metabolizing enzymes. Cholesterol contributes to synthesis of nerve tissue, bile salts, steroid hormones, and vitamin D [31,32]. Moreover, it should be considered that the addition of vegetable oils to the final FM brings, as by-products, phytosterols, the effect of which on the cholesterol homeostasis and enzymatic systems in infants is not known.

Table 5. Retinol, α-tocopherol, cholesterol content (expressed as mg kg^{-1}) and fatty acid profile expressed as % of total fatty acid methyl esters (FAMEs) in the ovine milk and liquid milk-derived formulations IF1 and IF2, to be used in the production of first stage (from 0 to 6 months of age) and follow-on (from 6 to 12 months of age) infant formulae, respectively (mean ± SD).

	Whole Milk (*n* = 6)	**IF1 (*n* = 6)**	**IF2 (*n* = 6)**
Retinol	0.84 ± 0.21 [a]	0.17 ± 0.02 [c]	0.42 ± 0.10 [b]
α-Tocopherol	1.72 ± 0.17 [a]	0.47 ± 0.05 [c]	1.01 ± 0.07 [b]
Cholesterol	202 ± 13 [a]	112 ± 9 [c]	139 ± 8 [b]
C4:0	4.0 ± 0.2	3.9 ± 0.2	4.0 ± 0.2
C6:0	3.1 ± 0.1	3.1 ± 0.1	3.2 ± 0.1
C8:0	2.8 ± 0.1	2.8 ± 0.1	2.8 ± 0.1
C10:0	9.1 ± 0.3	9.1 ± 0.4	9.0 ± 0. 2
C12:0	5.2 ± 0.2	5.3 ± 0.2	5.2 ± 0.2
C14:0	14.0 ± 0.4	14.0 ± 0.3	13.7 ± 0.5
C16:0	30 ± 1	30 ± 1	30 ± 1
C18:0	5.5 ± 0.2	5.7 ± 0.2	5.5 ± 0.3
C18:1 11 *trans*	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.2
C18:1 9 *cis*	10.7 ± 0.7	11.1 ± 0.9	10.7 ± 0.3
C18:2 9 *cis* 12 *cis*	1.73 ± 0.15	1.77 ± 0.15	1.81 ± 0.11
C18:3 9 *cis* 12 *cis* 15 *cis*	1.6 ± 0.4	1.5 ± 0.3	1.8 ± 0.6
CLA 9 *cis* 11 *trans*	0.56 ± 0.05	0.60 ± 0.05	0.58 ± 0.06
SFAs	79 ± 1	78 ± 1	78 ± 1
MUFAs	15.6 ± 0.6	16.1 ± 0.9	15.9 ± 0.1
PUFAs	5.6 ± 0.7	5.5 ± 0.3	5.9 ± 0.9
Omega-6	2.3 ± 0.2	2.3 ± 0.2	2.4 ± 0.1
Omega-3	2.3 ± 0.6	2.1 ± 0.4	2.5 ± 0.7
TFAs	3.2 ± 0.5	3.3 ± 0.3	3.4 ± 0.6

CLA 9 *cis* 11 *trans*, conjugated linoleic acid—rumenic acid; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; TFAs, *trans* fatty acids. Different letters in a row indicate mean differences statistically significant at $p < 0.05$.

The fatty acid profile of milk and liquid milk-derived formulations IF1 and IF2 was also studied. The results are reported in Table 5, together with the main categories of fatty acids of nutritional interest. The fatty acid composition of the whole milk (Table 5) is in agreement to reports from other authors for sheep milk [9,26,33] and, in particular, for milk from Sarda sheep fed typical Mediterranean forages [34,35]. As expected, the fatty acid profiles were similar between whole milk, IF1, and IF2 formulations ($p > 0.05$). In fact, the fat content of the two formulations was derived completely from the utilized whole milk.

The two formulations (IF1 and IF2) were tailored to provide about 20% and 50%, respectively, of the total fat in the final infant formulae. Currently, the fatty fraction of the commercial infant formulae is mainly derived from mixtures of vegetable oils, while a progressive reduction in the use of bovine milk fat was observed over the years [36].

Recently, sheep's milk has received growing attention and it is already used for infant formula production in some countries (China and New Zealand) [3]. In fact, sheep's milk has interesting nutritional features, some of which are related to the fatty fraction [6,9,10].

As shown in Table 5, samples contained 78% of saturated fatty acids (SFAs), and 18% of these were short chain fatty acids (C4-C10), which usually have higher concentrations in sheep's milk with respect to cow's milk [9,10]. These fatty acids are easily digested as they are hydrolyzed from the triglycerides and transferred directly from the intestine to the portal vein, providing a rapid energy supply [9,10]. The most represented fatty acids were palmitic acid (C16:0; 30%), oleic acid (C18:1 9 *cis*; 11%), and myristic acid (C14:0; 14%). Polyunsaturated fatty acids (PUFAs) consisted mainly of linoleic acid (C18:2 9 *cis* 12 *cis*) and α-linolenic acid (C18:3 9 *cis* 12 *cis* 15 *cis*), both of which were just below 2%. These essential fatty acids are precursors of omega-6 (n-6) and omega-3 (n-3) PUFAs, respectively. A low n-6:n-3 ratio (between 1:1 and 4:1) is considered ideal in the prevention of cardiovascular diseases, inflammatory and autoimmune disorders, while values above 5 are considered unfavourable. Sheep's milk usually has a low n-6:n-3 ratio as shown by the optimal value close to 1 in the samples we examined [9,10].

Another interesting feature of sheep milk fat is its content of conjugated linoleic acid (CLA), particularly in rumenic acid, the main CLA isomer (CLA 9 *cis* 11 *trans*), and its precursor, the *trans*-vaccenic acid (C18:1 11 *trans*) (0.5% and 1%, respectively) for which various positive health effects have been demonstrated [7,9]. Rumenic acid is an intermediate in rumen biohydrogenation of linoleic acid, whereas *trans*-vaccenic acid is a common intermediate in the biohydrogenation of both linoleic and linolenic acids. These latter precursor fatty acids are ingested by the animal through feed intake (grass, fodder, hay and concentrated feed). It has been demonstrated that a diet mainly based on the exploitation of pasture greatly increased the CLA content in sheep's milk [37]. However, the *trans* configuration of the vaccenic acid enlists this fatty acid in the *trans* fatty acids (TFAs) category. TFAs from industrial hydrogenation processes are associated with the onset of cardiovascular diseases, while no adverse effects due to the ingestion of natural TFAs, such as the milk vaccenic acid were registered. However, recommendations are given that limit the presence of TFAs, regardless their origin (industrial or natural), even in infant formulae and baby products. In this respect, both European and Chinese legislation limits the content of TFAs for foods intended for infant nutrition to a value lower than 3% of the total fatty acids [38,39]. The data reported in Table 5 show that the level of TFAs in IF1 and IF2 were slightly over 3%. However, it is worth remembering that in the final FM to be marketed, both IF1 and IF2 will be mixed with vegetable oils (that should not contain *trans* fatty acids) so lowering the final TFAs % content to the recommended limits.

3.4. ^{1}H NMR Low Molecular Weight Water Soluble Metabolites of IF

The ^{1}H NMR spectroscopy, within a single experiment, is able to detect all the molecular components bearing a hydrogen atom in a complex mixture, given a MW < 1 kDa and a concentration >1 μM. By peak integration procedures it is possible to obtain the concentrations of the identified metabolites expressed in moles. A representative ^{1}H NMR spectrum of IF1 is shown in Figure S1. In the IF1 and IF2 samples the lactose concentrations, as previously determined (Table 3), were used to calibrate the relative concentrations of the other metabolites, and the results are shown in Table 6.

IF1 samples showed a higher overall content of low molecular weight metabolites than IF2. Besides lactose, citric acid was the most abundant metabolite in both formulations. Among organic acids, hippuric acid can be considered a biomarker of grass pasture diets [40]. No traces of lactic acid or benzoic acid were detected, which could suggest a good microbiological quality of the formulations.

Table 6. NMR metabolite concentrations expressed in mM per 100 mL for liquid milk-derived formulations IF1 and IF2, to be used in the production of first stage (from 0 to 6 months of age) and follow-on (from 6 to 12 months of age) infant formulae, respectively.

	IF1 (n = 3)		IF2 (n = 3)	
Metabolites	**Mean SD**	**Mean%**	**Mean SD**	**Mean%**
Amino acids				
Alanine	0.09 ± 0.01 [a]	2.7	0.032 ± 0.009 [b]	1.5
Anserine	0.10 ± 0.02 [a]	3.0	0.031 ± 0.002 [b]	1.5
Glutamate	0.28 ± 0.01 [a]	8.1	0.11 ± 0.04 [b]	5.3
Isoleucine	0.26 ± 0.03 [a]	7.5	0.08 ± 0.01 [b]	3.8
Leucine	0.287 ± 0.004 [a]	8.3	0.102 ± 0.041 [b]	4.9
Lysine	0.208 ± 0.007 [a]	6.0	0.087 ± 0.031 [b]	4.2
Proline	0.44 ± 0.13	12.5	0.29 ± 0.04	14.2
Threonine	0.044 ± 0.003	1.3	0.038 ± 0.012	1.8
Valine	0.030 ± 0.005 [a]	0.9	0.019 ± 0.003 [b]	0.9
Organic acids				
Acetate	0.069 ± 0.009 [a]	2.0	0.035 ± 0.003 [b]	1.7
Citrate	0.7 ± 0.1	21.6	0.6 ± 0.1	30.8
Formate	0.024 ± 0.001 [a]	0.7	0.015 ± 0.002 [b]	0.7
Hippurate	0.046 ± 0.003 [a]	1.3	0.031 ± 0.002 [b]	1.5
Nucleotides				
UDP-N-Acetylglucosamine	0.018 ± 0.001 [a]	0.5	0.025 ± 0.010 [b]	1.2
UDP-galactose	0.08 ± 0.01 [a]	2.4	0.06 ± 0.01 [b]	3.1
UDP-glucose	0.067 ± 0.007 [a]	1.9	0.062 ± 0.015 [b]	3.0
Uridine	0.040 ± 0.012 [a]	1.2	0.041 ± 0.007 [b]	2.0
Others				
Caprylate	0.113 ± 0.007 [a]	3.3	0.042 ± 0.001 [b]	2.0
Choline	0.020 ± 0.002	0.6	0.018 ± 0.004	0.9
O-Phosphocholine	0.027 ± 0.004	0.8	0.023 ± 0.006	1.1
sn-Glycero-3-phosphocholine	0.14 ± 0.02	4.1	0.12 ± 0.01	5.8
Creatine	0.035 ± 0.002 [a]	1.0	0.024 ± 0.003 [b]	1.2
Pantothenate	0.066 ± 0.008	1.9	0.055 ± 0.015	2.7
Fucose	0.223 ± 0.007 [a]	6.4	0.087 ± 0.020 [b]	4.2

Different letters in a row indicate mean differences statistically significant at $p < 0.05$.

The IF1 samples showed a higher free amino acid content than the IF2 samples (Table 6). Amino acids in their free form are more easily absorbed than those present in proteins and may have a beneficial role during early post-natal development [41]. The following free amino acids were identified: proline, glutamic acid, lysine, leucine, isoleucine, alanine, valine, and threonine, together with the bioactive peptide anserine. The most represented amino acid in the two formulations was proline which shows neuroprotective properties [8]. No resonances ascribable to aromatic amino acids were found. The amino acid profile found in IF1 and IF2 samples is compatible with the presence in the whey from cheese making, used to obtain retentate, of the bioactive caseinomacropeptide (CMP), which is formed by chymosin hydrolysis of milk κ-casein (κ-cn) [42]. Several health beneficial effects of CMP have been reported [42].

In the ^{1}H NMR spectra of the analyzed samples, signals ascribable to nucleotides, nucleosides, and sugar nucleotides, such as UDP-galactose, UDP-glucose, Uridine, UDP-GlcNAc and UDP-GalNAc were detected [11]. These compounds belong to the non-protein-nitrogen (NPN) fraction of milk. Nucleotides are believed to be active in the maturation of the gastrointestinal tract and the development of neonatal immune function [1]. They are also assumed to play an important role in carbohydrate, lipid, protein, and nucleic acid metabolism [1]. Sugar nucleotides usually occur intracellularly and are utilised as donors for glycosyltransferases that biosynthesize glycoconjugates, but their biological functions in milk or colostrum for suckling neonates are unknown [43]. Although a heterogeneity of sugar nucleotides among breeds exists [43], it has been shown that ovine milk contained the highest concentrations of nucleosides and nucleotides compared to goat's and cows' milk [11]. Nucleotides play key roles in many biological processes and currently, the addition of 5′-CMP, 5′-UMP, 5′-AMP, 5′-GMP, 5′-IMP to infant formulae is suggested but

not mandatory [38]. No traces of orotic acid, a pyrimidine derivative, were detectable. Unlike cow's milk, orotic acid is not found in appreciable quantities in sheep milk or human milk [44]. The effect on neonates' health of orotic acid is a matter of debate [45]. Among monosaccharides we found fucose, while glucose and galactose were detected in negligible quantities. Fucose is one of the possible glycosidic residues of glycosylated proteins. Fucose in its free form is present in human breast milk and its addition has been proposed for use in infant formulae to better simulate the free saccharides present in human breast milk [46]. Fucose plays important roles in the development of the immune and nervous systems and is involved in cognitive function and memory formation. Among the low-molecular-weight NPN constituents, we detected creatine and choline analogues (O-Phosphocholine and sn-Glycero-3-phosphocholine). Choline shows several important biological functions and is considered an essential nutrient in human nutrition, from the fetus to old age [47]. Creatine, being a component of human breast milk, should be present in infant formulae [48]. It has been reported that sheep's milk contains a high content of B vitamins, which are responsible for the proper functioning of the nervous system [8]. In agreement with this, we detected pantothenic acid (vitamin B5), which is also present in human milk [49].

4. Conclusions

In this study, a pilot-scale process was developed for manufacturing two liquid sheep's milk-derived formulations as ingredients for FM (first stage and follow-on) production. Whey proteins were obtained by ultrafiltration of whey from cheese making, thus valorizing a by-product of ovine cheese production. The two formulations showed a proper CN:WP ratio for neonate's growth and can provide the complete protein content of FM. In addition, they provided other interesting nutrients for infants (short chain, essential and rumenic fatty acids, free amino acids, nucleotides, vitamins, minerals, etc.), which are directly related to the specific features of sheep's milk. In further studies the influence of different sheep farm systems and diets on the quality of the derived IFs will be addressed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13010653/s1, Table S1. Linear range (LR) of the calibration curves used to quantify the investigated fatty acids; Figure S1. ^{1}H NMR representative spectrum of milk-based formulation IF1.

Author Contributions: Conceptualization, A.P., M.P., G.L., M.A. and P.S.; methodology, M.P., G.L., M.A., P.S. and P.C.; software, G.L., P.S. and C.P.; validation, M.A., M.P., P.S. and C.P.; formal analysis, G.L., M.P., P.S. and C.P.; investigation, M.P., G.L., M.A. and P.S.; data curation, M.A., G.L., M.P. and P.S.; writing—original draft preparation, G.L. and P.S.; writing—review and editing, G.L., M.A., P.S. and P.C.; visualization, P.S.; supervision, M.A., M.P., P.S. and P.C.; project administration, P.C., A.P., M.A. and M.S.; funding acquisition, P.C., A.P. and M.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Regione Autonoma della Sardegna (Italy) POR FESR 2014–2020 Azione 1.2.2. Programma di Ricerca e Sviluppo Agroindustria—Research project "BiomilkChina"—CUP F26C18000480002.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We wish to thank Alimenta S.r.l. for helpful discussion and Francesco Sanna and the all staff of the Chemical Laboratory of the Servizio Ricerca Studi Ambientali, Difesa delle Colture e Qualità delle Produzioni of Agris Sardegna for help in determining the milk, retentate, and IF mineral composition. We acknowledge the CeSAR (Centro Servizi d'Ateneo per la Ricerca) of the University of Cagliari, Italy, for the ^{1}H NMR experiments.

Conflicts of Interest: The authors declare no conflict of interest.

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