(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 September 2006 (14.09.2006)

PCT

(10) International Publication Number
WO 2006/096073 A1

(51) International Patent Classification:

A23L 3/015 (2006.01)	*A23L 1/025* (2006.01)
A01N 1/00 (2006.01)	*A61K 38/40* (2006.01)
A23C 3/00 (2006.01)	*A61L 2/02* (2006.01)
A23C 9/00 (2006.01)	*A61P 7/06* (2006.01)
A23C 21/00 (2006.01)	*A61P 19/00* (2006.01)
A23C 23/00 (2006.01)	*A61P 35/00* (2006.01)
A23J 3/08 (2006.01)	*C07K 14/79* (2006.01)

(21) International Application Number: PCT/NZ2006/000038

(22) International Filing Date: 8 March 2006 (08.03.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

538671	8 March 2005 (08.03.2005)	NZ
544408	23 December 2005 (23.12.2005)	NZ

(71) Applicant *(for all designated States except US)*: **FONTERRA CO-OPERATIVE GROUP LIMITED** [NZ/NZ]; Dairy Farm Road, Palmerston North (NZ).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PALMANO, Kay, Patricia** [GB/NZ]; c/o Fonterra Co-Operative Group Limited, Dairy Farm Road, Palmerston North (NZ). **PATEL, Hasmukh, Ambalal** [IN/NZ]; c/o Fonterra Co-Operative Group Limited, Dairy Farm Road, Palmerston North (NZ). **CARROLL, Timothy, Joseph** [IE/NZ]; c/o Fonterra Co-Operative Group Limited, Dairy Farm Road, Palmerston North (NZ). **ELGAR, David, Francis** [NZ/NZ]; c/o Fonterra Co-Operative Group Limited, Dairy Farm Road, Palmerston North (NZ). **GONALEZ-MARTIN, Miguel, Alejandro** [MX/NZ]; c/o Fonterra Co-Operative Group Limited, Dairy Farm Road, Palmerston North (NZ).

(74) Agents: **ADAMS, Matthew, D** et al.; A J Park, 6th Floor Huddart Parker Building, Post Office Square, P O Box 949, Wellington, 6015 (NZ).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: HIGH PRESSURE PROCESSING OF METAL ION LACTOFERRIN

(57) Abstract: The present invention relates to a method of treating a composition comprising metal ion lactoferrin and in particular to a method of pressure treating a composition comprising metal ion lactoferrin to prevent the growth of at least one unwanted microorganism while retaining a desired level of metal ion binding.

HIGH PRESSURE PROCESSING OF METAL ION LACTOFERRIN

FIELD OF INVENTION

[0001] The present invention relates to a high pressure processing of metal ion lactoferrin compositions and in particular to a method of pressure treating a composition comprising metal ion lactoferrin to maintain or increase its keeping quality while retaining a desired level of metal ion binding.

BACKGROUND

[0002] The delivery of bioactive components (proteins, lipids or hydrolysates thereof, for example) in food or other ingestible products is constrained by the need to provide a safe product with a useful shelf life while retaining bioactivity. Products with a useful shelf life are said to have a good keeping quality and are less prone to spoilage.

[0003] Delivery of bioactive components is desirable at least because such components are physiologically active when ingested and can have positive health benefits, including but not limited to bone health, immune benefits, anti-inflammatory activity, heart health and efficacy in cancer treatment.

[0004] Traditional means of ensuring a useful keeping quality have a negative impact on the bioactivity of food products and the like. In particular, thermal processing is not generally suitable for the production of commercially sterile bioactive products. For example, an analysis of immunoglobulin proteins in commercial dairy products revealed that although between 60% and 75% of the immunoglobulins are retained through pasteurisation, levels in UHT or canned (evaporated) milk are negligible (Li-Chan et al, 1995). Commercial sterility in acid foods may be achieved by employing a lower-temperature heating than that used in canning, but the sensitivity of immunoglobulins to denaturing under heating is exacerbated by acidification (Dominguez et al, 2001).

[0005] There are many processes in the manufacture of bioactive products, ingredients and foods that may result in a partial or complete loss of bioactivity. In the case of dairy-based ingredients and foods, processes that involve heating steps that may affect bioactivity include thermal pasteurisation, homogenisation, thermalisation, evaporation and drying. In the case of food processing, examples of heating steps that may affect bioactivity include heat treatments preceding fermentation, UHT-treatments, retorting, hot filling and hot packing. A bioactive component will typically be subjected to one or more of these heating steps during the manufacture of a food. This is particularly true of dairy-based products where processing always includes an initial pasteurisation step and typically includes further heating steps prior to packaging and sale. Korhonen et al (1998) report that heating to temperatures in the range 60°C to 90°C denatures proteins therefore reducing the activity of bioactive proteins.

[0006] Drying of products produced using pasteurised milk may be used to improve keeping quality with losses of up to 40% of immunoglobulins (Li-Chan, 1995), but commercial applications are then limited to direct consumption (for example, tablets) or fresh products (for example, yogurt) where the dried bioactive ingredient is not subsequently heated again. Losses due to drying and heating may be compensated for by supplementing intermediate or final products with the bioactive component of interest but this can increase the cost to the end consumer.

[0007] Pressure treatment with pressures above about 350 MPa has been employed to achieve commercially-useful improvements in keeping quality for meat, vegetable and fruit-based products (such as cooked ham, avocado products and juices respectively). However, Huppertz et al (2002) report that high pressure denatures whey proteins in milk. Additionally, Korhonen et al (1998) report that pressure treatments at pressures of about 500 MPa and above irreversibly denature proteins in most cases. Felipe et al (1997) report that appreciable levels of immunoglobulin denaturation occur in goat's milk at pressures of 500 MPa.

[0008] Masuda et al (2001) report that pressures of 400 MPa and above may not be used to improve the keeping quality of bovine colostrum because such pressures denature the immunoglobulin protein.

[0009] Tonello et al (1992) report that pressures of 200 MPa applied for 2 hours may be used to retain at least 85% of the immunoglobulin activity, although the microbial load of colostrum is reduced by less than 2-log cycles. The same process at 63°C can reduce the microbial load below limits of detection (more than 7-log cycles), but at least 50% of the immunoglobulin activity is lost.

[0010] Thermal treatment is the currently recognised method for commercial sterilisation of lactoferrin solutions for various applications, at specific pH (Tomita et al., 1994) and for specific purposes (for example, as a drink or beverage; see Tanaka, et al., 1991). Although lactoferrin naturally loses some iron at lower (acidic) pH values by virtue of a decrease in affinity for iron (Baker et al., 2002), this loss does not become significant until below pH 4.0 (Groves, 1960; Bluard-Deconinck et al.,1978; Tweedie et al., 1994; Parry & Brown, 1974). However, recommended heat treatments for various important applications such as beverages (pH 3.8-4.0, 85° C for 10 min) and yoghurts (pH 4.6–5.0, 90° C for 10 min) result in loss of iron (or oriented iron binding) above and beyond that incurred by pH lowering alone. Moreover, the ability of lactoferrin to re-bind iron may be significantly reduced. At higher pH values that favour stoichiometric iron-binding, lactoferrin is less thermally stable (Steijns & van Hooijdonk, 2000) and at these values iron is also lost upon heat treatment, with accompanying loss of iron-binding ability. Thus, thermal processing may limit the use of iron lactoferrin in various applications, not only those at lower pH (for example, yoghurt, jellies, acid beverages) but also those across a broader pH range (for example, smoothies and other applications at neutral pH).

[0011] The need exists for a process that can provide a commercially useful keeping quality for a bioactive product such as a food or other ingestible product while retaining the bioactivity of at least one bioactive component.

[0012] Therefore it is an object of this invention to provide an improved or alternative method of preventing the growth of unwanted microorganisms in a composition comprising metal ion lactoferrin while retaining a desired level of metal ion binding or to at least provide the public with a useful choice.

SUMMARY OF INVENTION

[0013] Accordingly, in one aspect the present invention relates to a method of treating a composition to maintain or increase its keeping quality, the method comprising

(a) providing a composition comprising metal ion lactoferrin or a metal ion functional variant or fragment thereof, and

(b) subjecting the composition to a pressure treatment at a treatment pressure that will prevent the growth of at least one unwanted microorganism.

[0014] In another aspect the present invention relates to a method of treating a composition to maintain or increase its keeping quality, the method comprising

(a) providing a composition comprising metal ion lactoferrin or a metal ion functional variant or fragment thereof,

(b) selecting a treatment pressure that will prevent the growth of at least one unwanted microorganism, and

(c) subjecting the composition to a pressure treatment at the treatment pressure.

[0015] In another aspect the present invention relates to a method of treating a composition to maintain or increase its keeping quality comprising

(a) providing a composition comprising metal ion lactoferrin or a metal ion functional variant or fragment thereof and having a pH of from about 3.0 to about 8.0 or higher, and

(b) subjecting the composition to a pressure treatment at a treatment pressure of from about 350 MPa to about 1000 MPa.

[0016] In another aspect the present invention relates to a method of treating a composition to maintain or increase its keeping quality, the method comprising

(a) providing a composition comprising metal ion lactoferrin or a metal ion functional variant or fragment thereof and one or more unwanted microorganisms and having a pH of from about 3.0 to about 8.0 or higher and,

(b) selecting a treatment pressure of from about 350 MPa to about 1000 MPa that will prevent the growth of at least one unwanted microorganism and retain at least a desired level of metal ions bound to the lactoferrin or functional variant or fragment thereof, and

(c) subjecting the composition to a pressure treatment at the treatment pressure.

[0017] The following embodiments may relate to any of the aspects described above.

[0018] In one embodiment the composition comprises at least about 0.1 mg/ml, preferably about 0.1, 0.5, 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 200, 400, 500, 600, 700, 800, 900, or 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof, and useful ranges may be selected between any of these values (for example, from about 0.1 to 1000 mg/ml, about 1 to 1000 mg/ml, about 2 to 1000 mg/ml, about 3 to 1000 mg/ml, about 4 to 1000 mg/ml, about 5 to 1000 mg/ml, about 10 to 1000 mg/ml, about 0.1 to 100 mg/ml, about 1 to 100 mg/ml, about 2 to 100 mg/ml, about 3 to 100 mg/ml, about 4 to 100 mg/ml, about 5 to 100 mg/ml, and about 10 to 100 mg/ml).

[0019] In another embodiment the composition or product consists essentially of or consists of metal ion lactoferrin or a metal ion functional variant or fragment thereof.

[0020] In a further embodiment the composition or product comprises, consists essentially of or consists of at least about 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99, 99.5 or 100% by weight metal ion lactoferrin or a metal ion functional variant or fragment thereof and useful ranges may be selected between any of these values (for example, from about 0.01 to about 100%, about 0.05 to about 100%, about 0.1 to about 100%, about 0.5 to about 100%, about 1 to about 100%, about 5 to about 100%, about 10 to about 100%, about 15 to about 100%, about 20 to about 100%, about 25 to about 100%, about 30 to about 100%, about 35 to about 100%, about 40 to about 100%, about 45 to about 100%, about 50 to about 100%, about 55 to about 100%, about 60 to about 100%, about 65 to about 100%, about 70 to about 100%, about 75 to about 100%, about 80 to about 100%, about 85 to about 100%, about 90 to about 100%, about 95 to about 100%, about 99 to about 100%, from about 0.01 to about 0.05%, from about 0.01 to about 0.1%, from about 0.01 to about 0.5%, from about 0.01 to about 1%, from about 0.01 to about 5%, from about 0.01 to about 10%, from about 0.01 to about 15%, from about 0.01 to about 20%, from about 0.01 to about 25%, from about 0.01 to about 30%, from about 0.01 to about 35%, from about 0.01 to about 40%, from about 0.01 to about 45%, from about 0.01 to about 50%, from about 0.01 to about 55%, from about 0.01 to about 60%, from about 0.01 to about 65%, from about 0.01 to about 70%, from about 0.01 to about 75%, from about 0.01 to about 80%, from about 0.01 to about 85%, from about 0.01 to about 90%, from about 0.01 to about 95%, from about 0.01 to about 99%, and from about 0.01 to about 99.9%).

[0021] In one embodiment the composition or product has been enriched with a composition consisting essentially of or consisting of metal ion lactoferrin or a metal ion functional variant or fragment thereof or a mixture thereof. That is, a composition consisting essentially of or consisting of metal ion lactoferrin or a metal ion functional variant or fragment thereof or a mixture thereof is added to the composition or product to increase the concentration of metal ion lactoferrin or a metal ion functional variant or fragment thereof or a mixture thereof.

[0022] In one embodiment the composition comprises one or more unwanted microorganisms, such as one or more bacteria (including one or more probiotic bacteria), one or more fungi, one or more molds, one or more yeasts, or one or more algae, or a mixture thereof. It should be understood that in one embodiment the methods useful herein are preferably intended to prevent the growth of at least one unwanted microorganism. However, often the method may be carried out prophylactically and there may not actually be any unwanted organisms present. In such cases, the method is carried out to maintain or improve keeping quality or to comply with food safety requirements, current good manufacturing practice or regulatory requirements. Regardless of the presence or absence of an unwanted microorganism, a pressure treatment method useful herein retains at least a desired level of activity of at least one bioactive component and is useful to maintain or increase keeping quality.

[0023] In one embodiment the method prevents the growth of at least one unwanted microorganism while retaining at least a desired level of metal ion saturation. That is, the method prevents the growth of an unwanted microorganism without substantially affecting the degree of metal ion saturation of any lactoferrin polypeptides or functional variants or fragments thereof that are present.

[0024] In one embodiment the metal ion is an ion selected from the group comprising aluminium, calcium, copper, chromium, cobalt, gold, iron, manganese, magnesium, platinum, ruthenium, selenium and zinc ions. Preferably the metal ion is an iron ion.

[0025] In one embodiment the lactoferrin is selected from the group comprising sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, bovine and human lactoferrin, and mixtures thereof. Preferably the lactoferrin is bovine lactoferrin.

[0026] In one embodiment the lactoferrin is recombinant, synthetic or natural lactoferrin, or mixtures thereof.

[0027] In one embodiment the metal ion lactoferrin or a metal ion saturated functional variant or fragment thereof is at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5 or 100% metal ion saturated and useful ranges may be selected between any of the foregoing values (for example, from about 5 to about 100%, about 10 to about 100%, about 15 to about 100%, about 20 to about 100%, about 25 to about 100%, about 30 to about 100%, about 35 to about 100%, about 40 to about 100%, about 45 to about 100%, about 50 to about 100%, about 55 to about 100%, about 60 to about 100%, about 65 to about 100%, about 70 to about 100%, about 75 to about 100%, about 80 to about 100%, about 85 to about 100%, about 90 to about 100%, about 95 to about 100% and about 99 to about 100%).

[0028] In one embodiment the metal ion lactoferrin or a metal ion functional variant or fragment thereof is at least about 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195 or 200% metal ion saturated and useful ranges may be selected between any of the foregoing values (for example, about 105 to about 150%).

[0029] In one embodiment a composition or product provides a population of lactoferrin polypeptides or functional variants or fragments thereof wherein at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5 or 100% of the available metal ion binding pockets in the population are bound to a metal ion, preferably an iron ion, and useful ranges may be selected between any of the foregoing values (for example, about 5 to about 100%, about 10 to about 100%, about 15 to about 100%, about 20 to about 100%, about 25 to about 100%, about 30 to about 100%, about 35 to about 100%, about 40 to about 100%, about 45 to about 100%, about 50 to about 100%, about 55 to about 100%, about 60 to about 100%, about 65 to about 100%, about 70 to about 100%, about 75 to about 100%, about 80 to about 100%, about 85 to about 100%, about 90 to about 100%, about 95 to about 100% and about 99 to about 100%).

[0030] In one embodiment a composition or product provides a population of lactoferrin polypeptides or functional variants or fragments thereof wherein about 100% of the available metal ion binding pockets in the population are bound to a metal ion, preferably an iron ion, and additional metal ions are bound to the lactoferrin molecules in non specific binding sites so that the lactoferrin is 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195 or 200% metal ion on a stoichiometric basis.

[0031] Useful metal ion saturation ranges include about 25 to about 200%, about 30 to about 200%, about 35 to about 200%, about 40 to about 200%, about 45 to about 200%, about 50 to about 200%, about 55 to about 200%, about 60 to about 200%, about 65 to about 200%, about 70 to about 200%, about 75 to about 200%, about 80 to about 200%, about 85 to about 200%, about 90 to about 200%, about 95 to about 200% and about 100 to about 200% metal ion saturation.

[0032] In one embodiment, the post-treatment metal ion saturation value is selected from a value or range listed above.

[0033] In one embodiment the metal ion lactoferrin or metal ion functional fragment of variant thereof retains at least about 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99 or 100% of the metal ions bound.

[0034] In one embodiment the method comprises administration of a mixture of metal ion lactoferrin and at least one metal ion functional variant or fragment thereof.

[0035] In one embodiment the treatment pressure is selected from at least about 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 750, 800, 850, 900, 950 and 1000 MPa or greater, and useful ranges may be selected between any of these values (for example, from about 350 to about 400 MPa, about 350 to about 450 MPa, about 350 to about 500 MPa, about 350 to about 550 MPa, about 350 to about 600 MPa, about 350 to about 650 MPa, about 350 to about 700 MPa, about 350 to about 750 MPa, about 350 to about 800 MPa, about 350 to about 850 MPa, about 350 to about 900 MPa, about 350 to about 950 MPa and about 350 to about 1000 MPa, about 400 to about 1000 MPa, about 450 to about 1000 MPa, about 500 to about 1000 MPa, about 550 to about 1000 MPa, about 600 to about 1000 MPa, about 650 to about 1000 MPa, about 700 to about 1000 MPa, about 750 to about 1000 MPa, about 800 to about 1000 MPa, about 850 to about 1000 MPa, about 900 to about 1000 MPa, about 950 to about 1000 MPa, about 500 to about 550 MPa, about 500 to about 600 MPa, about 500 to about 650 MPa, about 500 to about 700 MPa, about 500 to about 750 MPa, about 500 to about 800 MPa, about 550 to about 800 MPa, about 600 to about 800 MPa, about 650 to about 800 MPa, about 700 to about 800 MPa, about 750 to about 800 MPa, about 400 to about 800 MPa, about 400 to about 750 MPa, about 400 to about 700 MPa, about 400 to about 650 MPa, about 400 to about 600 MPa, about 450 to about 800 MPa, about 450 to about 750 MPa, about 450 to about 700 MPa, about 450 to about 650 MPa, about 450 to about 600 MPa, about 500 to about 800 MPa, about 500 to about 750 MPa, about 500 to about 700 MPa, about 500 to about 650 MPa, about 500 to about 600 MPa, about 525 to about 675 MPa, about 550 to about 650 MPa and about 575 to about 625 MPa). Preferably the treatment pressure is at least about 350, 400, 450, 500 or 600 MPa.

[0036] In another embodiment the pH of the composition when it is subjected to the pressure treatment is at least about 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8.0 or greater, and useful ranges may be selected between any of these values (for example, from about pH 3.0 to about 4.5, about pH 3.0 to about 5.0, about pH 3.0 to about 5.5, about pH 3.0 to about 6.0, about pH 3.0 to about 6.5, about pH 3.0 to about 7.0, about pH 3.0 to about 7.5, about pH 3.0 to about 8.0, about pH 3.1 to about 4.9, about pH 3.2 to about 4.8, about pH 3.3 to about 4.7, about pH 3.4 to about 4.6, about pH 3.5 to about 4.5, about pH 3.6 to about 4.4, about pH 3.7 to about 4.3, about pH 3.8 to about 4.2, about pH 3.9 to about 4.1, about pH 3.1 to about 8.0, about pH 3.2 to about 8.0, about pH 3.3 to about 8.0, about pH 3.4 to about 8.0, about pH 3.5 to about 8.0, about pH 3.6 to about 8.0, about pH 3.7 to about 8.0, about pH 3.8 to about 8.0, about pH 3.9 to about 8.0, about pH 4.0 to about 8.0, about pH 4.5 to about 8.0, about pH 5.0 to about 8.0, about pH 5.5 to about 8.0, about pH 6.0 to about 8.0, about pH 6.5 to about 8.0, and about pH 7.0 to about 8.0). Alternatively, in still another embodiment, the pH of the composition is adjusted before pressure treatment to a pH or within a pH range listed above.

[0037] In one embodiment the method is conducted at ambient temperature. Preferably the pressure treatment is conducted at a temperature of at least about 0, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 degrees Celsius, and useful ranges may be selected between any of these forgoing values (for example, from about 5 to about 40 degrees Celsius).

[0038] In one embodiment the treatment pressure may be applied for a treatment time of about 1 second to about 30 minutes. Preferably the treatment time is selected from about 1, 5, 10, 20, 30, 60, 90, 120, 150, 180, 210, 240, 270 or 300 seconds or about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 minutes, and useful ranges may be selected between any of these values (for example, from about 1 to about 10 minutes, from about 1 to about 5 minutes or from about 2 to about 4 minutes).

[0039] In another embodiment the treatment pressure is held substantially constant for the treatment time. In another embodiment the pressure is increased from ambient pressure (usually atmospheric pressure) to the treatment pressure and then returned to ambient pressure within the treatment time. Ambient pressure will usually be atmospheric pressure.

[0040] It should be understood that in one embodiment a treatment time of a period listed above is the time taken to increase the pressure from atmospheric pressure to the treatment pressure and then return the pressure to atmospheric pressure. Accordingly, in one embodiment a treatment time of 1 minute means that the pressure is increased from atmospheric pressure to the treatment pressure and then returned to atmospheric pressure within 1 minute.

[0041] It should be understood that in another embodiment a treatment time of a period listed above is the time that the pressure is held at the treatment pressure (the "hold time"). Accordingly, in one embodiment a treatment time of 1 minute means that the pressure is held at the treatment pressure for 1 minute. Therefore, a treatment time of 0 (or "no hold") in this embodiment means that the pressure is raised to the treatment pressure but not held, and the pressure is then returned to atmospheric pressure. In this embodiment, preferred treatment times include 0 (no hold), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10 minutes. Preferably the pressure is held for about 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 or 5 minutes, or for about 0, 0.5, 1, 1.5, 2, 2.5 or 3 minutes, or for about 0 minutes.

[0042] In one alternative embodiment the total treatment time is less than about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 minutes. That is, the time taken for the pressure to be raised from and returned to ambient pressure (usually atmospheric pressure) is less than about 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 or 10 minutes. Preferably less than about 8 minutes, preferably less than about 7 minutes, preferably less than about 6 minutes or preferably less than about 5 minutes.

[0043] In another embodiment the composition may be subjected to additional pressure treatments. The treatment pressure may be changed from one treatment pressure to another, without first returning to atmospheric pressure. Each pressure treatment may be conducted for a separate treatment time. Accordingly, in one embodiment the pressure is increased from ambient pressure to a first treatment pressure for a first treatment time and then the pressure is changed to a second treatment pressure for a second treatment time. Preferably the first treatment time is longer than the second treatment time. Preferably the first treatment is shorter than the second treatment time. In yet another embodiment the pressure is increased to a first treatment pressure and then changed to a second treatment pressure within the treatment time.

[0044] In one embodiment the composition is subjected to the treatment pressure before incorporation into a product. In another embodiment the composition is subjected to the treatment pressure after incorporation into a product. Accordingly, the method further comprises subjecting the composition to the treatment pressure before or after incorporating the composition into a product.

[0045] In another embodiment the composition is subjected to the treatment pressure before packaging. In another embodiment the composition is subjected to the treatment pressure after packaging. Accordingly, the method further comprises subjecting the composition to the treatment pressure before or after packaging.

[0046] In one embodiment the treatment pressure is about 600 MPa and the hold time is about 0, 1, 2 or 3 minutes. In another preferred embodiment the treatment pressure is about 400 MPa and the hold time is less than about 30 minutes.

[0047] In one preferred embodiment the treatment pressure is about 350 to about 650 MPa and the hold time is about 0 minutes to about 5 minutes. Preferably the composition is yoghurt.

[0048] In one preferred embodiment the treatment pressure is about 350 to about 650 MPa and the hold time is about 0 minutes to about 5 minutes. Preferably the composition a beverage.

[0049] In one preferred embodiment the treatment pressure is about 350 to about 650 MPa and the hold time is about 0 minutes to about 5 minutes. Preferably the composition a fermented drink.

[0050] In one preferred embodiment the treatment pressure is about 350 to about 650 MPa and the hold time is about 0 minutes to about 5 minutes. Preferably the composition an acid drink, a liquid concentrate (including gels) or yoghurt.

[0051] In one preferred embodiment the treatment pressure is about 350 to about 700 MPa, the hold time is about 0 minutes to about 30 minutes, the pH of the composition is about pH 3.0 to about pH 8.0 and the composition is a beverage (including an acidified beverage, a neutral beverage or a carbonated beverage), a yoghurt or a jelly.

[0052] In one embodiment the composition comprises dairy protein or a dairy ingredient. Preferably the dairy protein composition or the dairy ingredient is recombined or fresh whole milk, recombined or fresh skim milk, reconstituted whole or skim milk powder, skim milk concentrate, skim milk isolate, whole or skim milk powder, skim milk retentate, concentrated milk, buttermilk, ultrafiltered milk retentate, milk protein concentrate (MPC), milk protein isolate (MPI), calcium depleted milk protein concentrate, calcium depleted milk protein isolate, low fat milk, low fat milk protein concentrate, low fat milk protein isolate, colostrum, a colostrum fraction, colostrum protein concentrate (CPC), colostrum milk protein concentrate, colostrum milk protein isolate, colostrum whey, colostrum whey protein concentrate, colostrum whey protein isolate, an immunoglobulin fraction from colostrum, whey, whey protein concentrate (WPC), whey protein isolate (WPI), sweet whey, lactic acid whey, mineral acid whey, reconstituted whey powder, hyperimmune milk, hyperimmune milk protein concentrate, hyperimmune milk protein isolate, hyperimmune whey, hyperimmune whey protein concentrate, hyperimmune whey protein isolate, hyperimmune colostrum, hyperimmune colostrum milk protein concentrate, hyperimmune colostrum milk protein isolate, hyperimmune colostrum whey, hyperimmune colostrum whey protein concentrate, hyperimmune colostrum whey protein isolate, a composition derived from any milk or colostrum processing stream, a composition derived from the retentate or permeate obtained by ultrafiltration or microfiltration of any milk or colostrum processing stream, or a composition derived from the breakthrough or adsorbed fractions obtained by chromatographic separation of any milk or colostrum processing stream, or a full or partial hydrolysates of any of these compositions, or a mixture thereof.

[0053] Preferably the dairy ingredient is from a cow, sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama or human source or mixtures thereof.

[0054] In another embodiment the composition or product further comprises a component selected from lysozyme, immunoglobulins, glycomacropeptide, a composition comprising at least about 1% w/w immunoglobulins, a product made by inoculating an animal to increase antibody levels, immune milk, and mixtures thereof.

[0055] In another embodiment the composition or product further comprises a component selected from non-polar lipids and polar lipids such as phospholipids, sphingolipids, gangliosides and ceramides, and mixtures thereof.

[0056] In another embodiment the composition or product further comprises at least about 1% w/w immunoglobulins wherein the immunoglobulins comprise one or more of IgA, IgD, IgE, IgG or IgM.

[0057] In one embodiment the composition or product is a food product. Preferably the food product is an acidic beverage or a carbonated beverage. Preferably the food product is a yogurt including set, stirred, flavoured, fruit and probiotic yoghurts, fromage frais, petit suisse, quarg, fermented food or drink, acidified drink or milk product. Preferably the food product is a jelly.

[0058] In one embodiment the composition further comprises a probiotic microorganism selected from the group consisting of *Lactobacillus, Streptococcus, Lactococcus, Leuconostoc, Pediococcus, Bifidobacterium, Propionibacterium, Enterococcus* or *Bacillus*, or a mixture thereof.

[0059] In one embodiment the composition further comprises a probiotic microorganism selected from the group consisting of *Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus johnsonii, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus salivarius, Bifidiobacterium bifidum, Bifidiobacterium breve, Bifidobacterium infantis, Bifidiobacterium animalis* subsp. *lactis, Bifidobacterium longum*, or *Streptococcus thermophilus*, or a mixture thereof.

[0060] In one embodiment the composition further comprises a probiotic microorganism selected from the group consisting of *Lactobacillus rhamnosus* HN001 (AGAL NM 97/09514), *Bifidiobacterium animalis* subsp. *lactis* HN019 (AGAL NM 97/09513), *Lactobacillus acidophilus* HN017 (AGAL NM 97/09515), *Lactobacillus rhamnosus* HN067 (AGAL NM 97/01925) (all of which are described in US 6,379,663), *Lactobacillus johnsonii* NCC533 (La1) (CNCM I-1225), *Lactobacillus rhamnosus* GG (ATCC 53103), *Lactobacillus casei* Shirota (FERM-P4751), *Lactobacillus acidophilus* NCFM (ATCC 700396), *Lactobacillus plantarum* 299v (DSMZ 9843), *Lactobacillus casei* DN114001 (CNCM I-1518), *Lactobacillus salivarius* UCC4331 (NCIMB 40829), *Bifidiobacterium animalis* subsp. *lactis* BB12 (ATCC 27536 and DSMZ 10140), or *Bifidobacterium infantis* 35624 (NCIMB 41003), or a mixture thereof. Preferred unwanted organisms or probiotic microorganisms include *Lactobacillus rhamnosus* HN001, *Bifidiobacterium animalis* subsp. *lactis* HN019, *Lactobacillus acidophilus* HN017, or *Lactobacillus rhamnosus* HN067, or a mixture thereof.

[0061] In one embodiment the probiotic microorganism is an inactivated probiotic microorganism. An inactivated probiotic microorganism may be non-viable, non-viable but still metabolically active, or dead.

[0062] In another aspect the present invention relates to a composition or product treated or produced according to a method of the invention.

[0063] In another aspect the present invention relates to use of a composition or product treated or produced according to a method of the invention in the manufacture of a food, drink, food additive, drink additive, dietary supplement, nutritional product, medical food, nutraceutical, medicament or pharmaceutical.

[0064] In one embodiment the use is for stimulating skeletal growth, inhibiting bone resorption, stimulating chondrocyte proliferation, stimulating osteoblast proliferation, inhibiting osteoclast development, or treating or preventing a skeletal, joint or cartilage disorder.

[0065] In one embodiment the use is for inhibiting tumour formation in a subject, inducing apoptosis in a subject, inducing apoptosis of tumour cells in a subject, inhibiting angiogenesis in a subject, inhibiting tumour angiogenesis in a subject, maintaining or improving one or both of the white blood cell count and red blood cell count in a subject, stimulating the immune system in a subject, increasing the production of Th1 and Th2 cytokines within a tumor in a subject, increasing the production of Th1 and Th2 cytokines within the intestine of a subject, increasing the level of Th1 and Th2 cytokines in the systemic circulation of a subject, increasing an anti-tumour immune response in a subject, increasing the responsiveness of a subject to a cancer therapy, or increasing the responsiveness of a tumour in a subject to a cancer therapy.

[0066] In one embodiment the use is for treating or preventing iron-deficiency anaemia or the iron-deficiency associated with pregnancy, for increasing haemoglobin count in anaemia sufferers or for gut renewal therapies for malabsorption.

[0067] In another aspect the present invention relates to a method of stimulating skeletal growth, inhibiting bone resorption, stimulating chondrocyte proliferation, stimulating osteoblast proliferation, inhibiting osteoclast development, or treating or preventing a skeletal, joint or cartilage disorder, the method comprising administration of a composition or product of the invention to a subject in need thereof.

[0068] In another aspect the present invention relates to a method of inhibiting tumour formation in a subject, inducing apoptosis in a subject, inducing apoptosis of tumour cells in a subject, inhibiting angiogenesis in a subject, inhibiting tumour angiogenesis in a subject, maintaining or improving one or both of the white blood cell count and red blood cell count in a subject, stimulating the immune system in a subject, increasing the production of Th1 and Th2 cytokines within a tumor in a subject, increasing the production of Th1 and Th2 cytokines within the intestine of a subject, increasing the level of Th1 and Th2 cytokines in the systemic circulation of a subject, increasing an anti-tumour immune response in a subject, increasing the responsiveness of a subject to a cancer therapy, or increasing the responsiveness of a tumour in a subject to a cancer therapy, the method comprising administration of a composition or product of the invention to a subject in need thereof.

[0069] In another aspect the present invention relates to a method of treating or preventing iron-deficiency anaemia or the iron-deficiency associated with pregnancy, for increasing haemoglobin count in anaemia sufferers or for gut renewal therapies for malabsorption, the method comprising administration of a composition or product of the invention to a subject in need thereof.

[0070] Other aspects relate to a pressure-treated composition comprising about 0.1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms; a pressure-treated jelly comprising about 0.1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms; a pressure-treated yoghurt comprising about 0.1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms; a pressure-treated beverage comprising about 0.1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms; a pressure-treated composition comprising about 1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms; a pressure-treated jelly comprising about 1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms; a pressure-treated yoghurt comprising about 1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms; or a pressure-treated beverage comprising about 1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms.

[0071] The entire disclosures of all applications, patents and publications, cited above and below, if any, are hereby incorporated by reference.

[0072] It is intended that reference to a range of numbers disclosed herein (for example, 1 to 10) also incorporates reference to all rational numbers within that range (for example, 1, 1.1, 2, 3, 3.9, 4, 5, 6, 6.5, 7, 8, 9 and 10) and also any range of rational numbers within that range (for example, 2 to 8, 1.5 to 5.5 and 3.1 to 4.7) and, therefore, all sub-ranges of all ranges expressly disclosed herein are hereby expressly disclosed. These are only examples of what is specifically intended and all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application in a similar manner.

[0073] The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, in any or all combinations of two or more of said parts, elements or features, and where specific integers are mentioned herein that have known equivalents in the art to which the invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

BRIEF DESCRIPTION OF THE DRAWINGS

[0074] Figure 1 is a flow chart summarising the manufacture of control iron lactoferrin (FeLF) yoghurt, heat treated FeLF yoghurt and pressure treated (HPP) FeLF yoghurt.

[0075] Figure 2 is a flow chart summarising the manufacture of a standard (heat treated) acidic drink, an unprocessed (control) acidic drink and a pressure treated acidic drink.

[0076] Figure 3 is a flow chart summarising the manufacture of control (untreated) FeLF jelly and pressure treated FeLF jelly.

[0077] Figure 4 is a flow chart summarising the manufacture of a control (untreated) FeLF carbonated beverage and pressure treated LF carbonated beverage.

DETAILED DESCRIPTION OF THE INVENTION

1. Definitions

[0078] The term “comprising” as used in this specification and claims means “consisting at least in part of”. When interpreting statements in this specification and claims that include that term, the features, prefaced by that term in each statement, all need to be present but other features can also be present. Related terms such as “comprise” and “comprised” are to be interpreted in the same manner.

[0079] The terms "enhance the immune system" and "stimulate the immune system" (and different tenses of these terms) refer to the ability of metal ion lactoferrin to stimulate the generation of antigen-specific cytolytic activity (the activity of immune cells, particularly cytotoxic T-lymphocytes) and/or NK cell activity, improve the cellular immune response to antigens (through the activity of at least cytotoxic T-lymphocytes), improve immune protection (by at least restoring the activity of cytotoxic T-lymphocytes and/or NK cells and enhancing cytokine production), restore immune protection (by at least restoring or stimulating the activity of cytotoxic T-lymphocytes and/or NK cell activity and enhancing cytokine production) or generate pro-inflammatory and immunoregulatory mediators (Th1 and Th2 cytokines).

[0080] The term "functional fragment" is intended to mean a naturally occurring or non-naturally occurring portion of a lactoferrin polypeptide that has one or two metal ion binding pockets and is able to bind a metal ion in at least one pocket. Useful lactoferrin fragments include truncated lactoferrin polypeptides, metal ion binding hydrolysates of lactoferrin, fragments that comprise the N-lobe binding pocket (including but not limited to N-lobe sequences), fragments that comprise the C-lobe binding pocket (including but not limited to C-lobe sequences), and metal ion binding fragments generated (by artificial or natural processes) and identified by known techniques as discussed below.

[0081] The term "functional variant" is intended to mean a variant of a lactoferrin polypeptide that is able to bind two metal ions per lactoferrin molecule.

[0082] The term "glycosylated" when used in relation to a lactoferrin polypeptide, functional variant or fragment is intended to mean that the lactoferrin is fully or partially glycosylated with naturally occurring or non-naturally occurring human or bovine glycosyl groups. Glycosylated and aglycosyl forms of lactoferrin are known (see Pierce, et al. (1991); Metz-Boutigue, et al. (1984); van Veen, et al. (2004)).

[0083] The term "increasing the responsiveness of a subject" is intended to mean that a subject exhibits a greater reduction in the rate of tumour growth, in tumour size, or in clinical symptoms of disease than a subject who is not subjected to a method of the invention.

[0084] The term "increasing the sensitivity of a tumour" is intended to mean that a tumour exhibits a greater reduction in the rate of tumour growth, in tumour size, or is eradicated whereas a tumour that is not subjected to a method of the invention will not exhibit these effects.

[0085] The term "inhibiting tumour formation" is intended to mean that tumours do not form, or that tumours form but do not establish or grow, or that tumours form but remain small, benign and do not become cancerous or metastasize, or that tumours grow more slowly. Tumour formation may be monitored through CT scans and tumor markers where available.

[0086] The term "inhibiting tumour growth" is intended to mean that tumours do not form in a subject treated according to the invention, or that one or more tumours that may be present in a subject treated according to the invention do not grow in size or become cancerous or metastasize, or that one or more tumours present in a subject treated according to the invention reduce in size (preferably by at least about 20, 30, 40, 50, 60, 70, 80, 90 or 100% by volume) or that one or more tumours present in a subject treated according to the invention are eradicated. Tumour size may be monitored through CT scans and tumor markers where available.

[0087] The terms "iron-lactoferrin" and "iron-saturated lactoferrin" (otherwise referred to as "FeLF") as used herein are intended to refer to a population of partially or fully iron saturated lactoferrin polypeptides or functional variants or fragments thereof providing a population of iron binding pockets where at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, 99.9 or 100% of the metal ion binding pockets present in the population have an iron ion bound.

[0088] The term "hold time" refers to a preferred embodiment where the treatment time is the time that the pressure is held at the treatment pressure. For example, a hold time of 1 minute means that the pressure is held at the treatment pressure for 1 minute. A hold time of 0 minutes (or "no hold") means that the pressure is raised to the treatment pressure but not held, and is then returned to ambient (usually atmospheric) pressure. In this embodiment, preferred treatment times include 0 (no hold), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10 minutes. It should be understood that although the pressure is not intentionally held at the treatment pressure, there may be a very short holding period (possibly several milliseconds) due to the nature of the equipment used. This very short holding period is unlikely to substantially affect the working of the method.

[0089] The term "keeping quality" as used herein is intended to mean the ability of a composition to resist the growth of unwanted microorganisms over time. Compositions that are not treated with heat or an acceptable alternative such as provided herein are unlikely to have a commercially acceptable keeping quality. Reference to maintaining keeping quality is intended to mean that a method of the invention is at least as effective as a heat treated control at extending the shelf-life of a pressure treated composition. Reference to increasing or increased, or improving or improved keeping quality is intended to mean that the ability of the composition to resist the growth of unwanted microorganisms over time is enhanced compared to an untreated composition. Enhanced keeping quality preferably leads to, for example, properties such as an extended shelf-life and an enhanced ability to withstand temperature variation. Temperature variation (such as removal from cold store) can induce growth of any residual bacteria.

[0090] Preferably the keeping quality is assessed with reference to the Aerobic Plate Count (APC). APC is a bacterial enumeration procedure used to estimate bacterial density in a sample and is otherwise known as Total Plate Count, Standard Plate Count or Total Viable Count. Samples are collected, blended, diluted, and plated in an agar medium suitable for detecting the bacteria studied (for example, food or dairy contaminants such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonellae*, *Shigellae*, coliforms, yeasts and molds, mesophilic spores, thermophilic spores). The APC result is the number of colony forming units in one millilitre (cfu/ml) of sample that is plated and incubated for 72 hrs at 32 °C. An APC of 50,000 cfu/ml or less is highly preferred for fresh dairy products that are not intended to contain viable cultures. Products having an APC of 50,000 cfu/ml or more are unlikely to have an acceptable keeping quality, unless the organism present is one that is particularly suited to the product – examples of this latter class of products include yoghurts or fermented products where a viable culture is desirable.

[0091] Accordingly, in one embodiment, a preferred method is one wherein the aerobic plate count (APC) after treatment is less than or equal to about 100,000, 75,000, 50,000, 25,000, 10,000, 5,000, 1,000, 100, or 10 colony forming units per millilitre (cfu/ml). Preferably the APC is less than about 50,000 cfu/ml.

[0092] The term "lactoferrin" refers to any non-glycosylated or glycosylated wild-type lactoferrin amino acid sequence including homologous lactoferrin sequences such as those described below. A lactoferrin polypeptide has two metal ion binding pockets and so can bind metal ions in a stoichiometric ratio of 2 metal ions per lactoferrin molecule. One metal ion binding pocket is present in the N-terminal lobe (N-lobe) of lactoferrin and the other pocket is present in the C-terminal lobe (C-lobe) (Moore et al, 1997). Verified sequences of bovine and human lactotransferrins (lactoferrin precursors), lactoferrins and peptides therein can be found in Swiss-Prot (http://au.expasy.org/cgi-bin/sprot-search-ful). Indicative lactoferrin polypeptides include the bovine lactotransferrin precursor accession number P24627, bovine lactoferrin, the human lactotransferrin precursor accession number P02788 and human lactoferrin.

[0093] The terms "metal ion saturation" and "metal ion binding" are intended to refer to binding of a metal ion in an iron binding pocket of a lactoferrin polypeptide or a functional variant or fragment thereof.

[0094] The terms "metal ion lactoferrin" and "metal ion saturated lactoferrin" are intended to refer to a population of partially or fully metal ion saturated lactoferrin polypeptides that provide a population of metal ion binding pockets where at least about 5%, preferably at least about 25% of the metal ion binding pockets present in the population have a metal ion bound. It should also be understood that the population may contain polypeptides of different species; for example, some molecules binding no ion and others each binding one or two ions. In cases where different metal ions are used, some molecules may bind an iron ion and others a different ion.

[0095] Equally, the terms "metal ion lactoferrin fragment" and "metal ion saturated lactoferrin fragment" are intended to refer to a population of partially or fully metal ion saturated lactoferrin polypeptide fragments that provide a population of metal ion binding pockets where at least about 5%, preferably at least about 25% of the metal ion binding pockets present in the population have a metal ion bound.

[0096] The present invention may employ a mixture of partially or fully metal ion saturated lactoferrin polypeptides and lactoferrin fragments. In such an embodiment, the population of metal ion binding pockets is made up of two pockets for every lactoferrin polypeptide and one or two pockets for every lactoferrin fragment, depending on the nature of the fragments.

[0097] The degree of saturation may be determined by spectrophotometric analysis (Brock & Arzabe, 1976; Bates et al, 1967; Bates et al, 1973). It should be understood that there may be metal ion exchange between lactoferrin polypeptides. Metal ion saturated lactoferrin may be prepared by any useful method. In one embodiment, iron saturated lactoferrin may be prepared by the method of Law, et al (1977). In another embodiment, iron saturated lactoferrin may be prepared by the method of Kawakami et al (1993). Metal ion saturated lactoferrin may be prepared by binding metal ions to the metal ion binding sites in lactoferrin, including the metal ion binding pockets such as the Fe binding pockets and other non-specific binding sites on the lactoferrin molecule or lactoferrin fragment. In a preferred embodiment, metal ions are only bound by metal ion binding pockets and minimal or no non-specific binding occurs, unless it is during metal ion exchange between binding pockets.

[0098] In one embodiment at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5, 99.9 or 100% of the metal ion binding pockets present in the population of lactoferrin molecules have a metal ion bound and useful ranges may be selected between any of the foregoing values (for example, about 5 to about 100%, about 10 to about 100%, about 15 to about 100%, about 20 to about 100%, about 25 to about 100%, about 30 to about 100%, about 35 to about 100%, about 40 to about 100%, about 45 to about 100%, about 50 to about 100%, about 55 to about 100%, about 60 to about 100%, about 65 to about 100%, about 70 to about 100%, about 75 to about 100%, about 80 to about 100%, about 85 to about 100%, about 90 to about 100%, about 95 to about 100% and about 99 to about 100%). In one embodiment the metal ion saturated lactoferrin is super-saturated lactoferrin.

[0099] The term "oral administration" includes oral, buccal, enteral and intra-gastric administration.

[0100] The terms "oriented metal ion binding" and "oriented iron binding" refer to the stereospecific nature of metal ion binding by lactoferrin or functional fragments when the metal ion (such as iron) is coordinated correctly in the iron-binding pocket, is not partially bound within the pocket, nor non-specifically surface bound. There may be conditions where the metal ion is bound in the iron binding pocket but without full coordination, and this may show in the spectral properties. Oriented iron binding is illustrated by a UV-VIS spectrum having a single iron-lactoferrin maxima at 465 nm. In one embodiment oriented metal ion binding is preferred. In another embodiment non-oriented metal ion binding is acceptable.

[0101] The term "parenteral administration" includes but is not limited to topical (including administration to any dermal, epidermal or mucosal surface), subcutaneous, intravenous, intraperitoneal, intramuscular and intratumoural (including any direct administration to a tumour) administration.

[0102] The term "pharmaceutically acceptable carrier" is intended to refer to a carrier including but not limited to an excipient, diluent or auxiliary that can be administered to a subject as a component of a composition of the invention. Preferred carriers do not reduce the activity of the composition and are not toxic when administered in doses sufficient to deliver an effective amount of a lactoferrin polypeptide or functional variant or fragment thereof. The formulations can be administered orally, nasally or parenterally.

[0103] The term "pressure treatment" refers to ultra high-pressure (UHP) treatment. Such a treatment is generally accepted as using a pressure of at least 100 MPa. This is also known as "high pressure" treatment, "high hydrostatic pressure" (HHP) or "high pressure processing" (HPP). Products that have been "pressure treated" are those that have been subjected to UHP treatment; namely, pressure treatment at a pressure of at least 100 MPa, preferably pressure treatment at a pressure of at least about 350, 400, 450, 500, 600, 700 or 800 MPa (or otherwise within this range as described above).

[0104] Reference to retaining a “desired level” of metal ion saturation or metal ion binding is intended to mean that the level of metal ion saturation of the lactoferrin polypeptides or functional variants or fragments thereof present in a composition is not substantially reduced when the composition is treated according to a method described herein. Preferably the level of metal ion saturation following treatment is at least about 40% of the metal ion saturation level before treatment. That is, preferably at least about 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99 or 100% of the metal ions bound by lactoferrin polypeptides or variants or fragments thereof are retained. For example, for a composition comprising 100% metal ion saturated lactoferrin, preferably the level of metal ion saturation is at least about 40% following treatment, preferably at least about 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99 or 100%, and preferred ranges may be selected between any of these values (for example, from about 40 to 100 %, about 45 to 100 %, about 50 to 100 %, about 55 to 100 %, about 60 to 100 %, about 65 to 100 %, about 70 to 100 %, about 75 to 100 %, about 80 to 100 %, about 85 to 100 %, about 90 to 100 %, about 95 to 100 %, and about 99 to 100 %).

[0105] The term “subject” is intended to refer to an animal, preferably a mammal, more preferably a mammalian companion animal or a human. Preferred companion animals include cats, dogs and horses.

[0106] The term “super-saturated lactoferrin” refers to a population of lactoferrin polypeptides or functional fragments providing a population of metal ion binding pockets where sufficient metal ions are available to fill 100% of the binding pockets and additional metal ions are present and bound by non-specific binding sites on the lactoferrin polypeptide or lactoferrin fragment. In other words, a stoichiometric excess of metal ions is provided. Preferably no free metal ions are present in a composition of the invention comprising super-saturated lactoferrin, although metal ion exchange between binding pockets, between non-specific binding sites and between binding pockets and non-specific binding sites may occur. Preferably super-saturated lactoferrin does not form insoluble aggregates. In one embodiment the super-saturated lactoferrin is at least about 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195 or 200% metal ion saturated, preferably iron saturated.

[0107] The term "treat" and its derivatives should be interpreted in their broadest possible context. The term should not be taken to imply that a subject is treated until total recovery. Accordingly, "treat" broadly includes amelioration and/or prevention of the onset of the symptoms or severity of a particular condition. The term "treat" also broadly includes the maintenance of good health for sensitive individuals and building stamina for disease prevention.

[0108] The term "unwanted microorganisms" refers to all microorganisms that may grow in a composition before pressure treatment. While such growth is undesirable, the presence of the microorganisms in states which do not grow, including non-viable, attenuated or dead microorganisms, may be of no consequence or may be desirable.

[0109] Reference to preventing the growth of unwanted microorganisms is intended to mean that the growth of microorganisms such as bacteria (including probiotic bacteria), fungi, molds, yeasts and algae is substantially reduced, delayed or eliminated. The microorganisms need not be responsible for spoilage or be pathogens. The growth of unwanted microorganisms can be assessed by visual inspection or by employing standard techniques that are known in the art, including but not limited to microscopy, staining, PCR, cell sorting and the like (see Lund, et al., 2000). Preferably the APC of the composition after pressure treatment is less than or equal to about 100,000, 75,000, 50,000, 25,000, 10,000, 5,000, 1,000, 100, or 10 cfu/ml, preferably less than about 50,000 cfu/ml

[0110] As indicated above, compositions that are not heat treated or pressure treated are unlikely to have a commercially acceptable keeping quality. That is, the keeping quality of untreated compositions is usually unacceptable because no steps are taken to prevent the growth of unwanted microorganisms. Where such steps are taken, the keeping quality will be improved. A post-treatment aerobic plate count (APC) less than or equal to about 100,000, 75,000, 50,000, 25,000, 10,000, 5,000, 1,000, 100, or 10 colony forming units per millilitre (cfu/ml), preferably less than about 50,000 cfu/ml, will reduce, delay or eliminate the ability of any organisms present to have impact on keeping quality. In combination with low pH or refrigeration (storage at temperatures less than about 10 °C, preferably less than about 4 °C) or both, their ability to affect keeping quality will be further reduced, delayed or eliminated.

[0111] In embodiments employing probiotic microorganisms, probiotic microorganism growth is unwanted but the presence of probiotic microorganisms is desirable. Accordingly, reference to preventing the growth of an unwanted microorganism in such embodiments is intended to mean that the growth of the probiotic microorganism is substantially reduced, delayed or eliminated. Preferably the pressure treatment will attenuate the probiotic microorganism, or more preferably, kill the probiotic microorganism, while retaining at least a desired level of probiotic activity.

[0112] In one embodiment the probiotic microorganism is an inactivated probiotic microorganism yet retains at least a desired level of probiotic activity. An inactivated probiotic microorganism may be non-viable, non-viable but still metabolically active, or dead, yet in all cases retain at least a desired level of probiotic activity.

[0113] In another embodiment the probiotic microorganism is an inactivated probiotic microorganism before pressure treatment. Pressure treatment in this embodiment prevents the growth of other unwanted microorganisms while retaining probiotic activity of the probiotic factors present.

[0114] The term “variant” refers to a naturally occurring (an allelic variant, for example) or non-naturally occurring (an artificially generated mutant, for example) lactoferrin polypeptide or lactoferrin fragment that varies from the predominant wild-type amino acid sequence of a lactoferrin polypeptide of a given species (such as those listed below) or fragment thereof by the addition, deletion or substitution of one or more amino acids.

[0115] Generally, polypeptide sequence variant possesses qualitative biological activity in common when assayed according to the examples below. Further, these polypeptide sequence variants may share at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity. Also included within the meaning of the term “variant” are homologues of lactoferrin polypeptides. A homologue is typically a polypeptide from a different species but sharing substantially the same biological function or activity as the corresponding polypeptide disclosed herein.

[0116] Preferred variant polypeptides preferably have at least about 70, 75, 80, 85, 90, 95 or 99% identity, preferably at least about 90, 95 or 99% identity to bovine or human lactoferrin. Variant fragments preferably have at least about 70, 75, 80, 85, 90, 95 or 99% identity, preferably at least about 90, 95 or 99% identity to a fragment described herein, including but not limited to fragments of human or bovine lactoferrin. Identity can be determined by comparing a candidate amino acid sequence to a sequence described herein, such as a lactoferrin polypeptide or fragment thereof using the BLAST suite of programs (version 2.2.12; 28 August 2005) (Tatusova, et al. (1999); McGinnis, et al. (2004)) that is publicly available from NCBI (ftp://ftp.ncbi.nih.gov/blast/).

[0117] Conservative substitutions of one or several amino acids of a lactoferrin polypeptide sequence without significantly altering its biological activity are also useful. A skilled artisan will be aware of methods for making phenotypically silent amino acid substitutions (see for example Bowie et al., (1990)).

2. Lactoferrin polypeptides

[0118] In addition to the useful lactoferrin polypeptides and fragments listed above, examples of lactoferrin amino acid and mRNA sequences that have been reported and are useful in methods of the invention include but are not limited to the amino acid (Accession Number NP_002334) and mRNA (Accession Number NM_002343) sequences of human lactoferrin; the amino acid (Accession Numbers NP_851341 and CAA38572) and mRNA (Accession Numbers X54801 and NM_180998) sequences of bovine lactoferrin; the amino acid (Accession Numbers JC2323, CAA55517 and AAA97958) and mRNA (Accession Number U53857) sequences of goat lactoferrin; the amino acid (Accession Number CAA09407) and mRNA (Accession Number AJ010930) sequences of horse lactoferrin; the amino acid (Accession Numbers NP_999527, AAL40161 and AAP70487) and mRNA (Accession Number NM_214362) sequences of pig lactoferrin; the amino acid (Accession Number NP_032548) and mRNA (Accession Number NM_008522) sequences of mouse lactoferrin; the amino acid (Accession Number CAA06441) and mRNA (Accession Number AJ005203) sequences of water buffalo lactoferrin; and the amino acid (Accession Number CAB53387) and mRNA (Accession Number AJ131674) sequences of camel lactoferrin. These sequences may be used according to the invention in wild type or variant form. Polypeptides encoded by these sequences may be isolated from a natural source, produced as recombinant proteins or produced by organic synthesis, using known techniques.

[0119] Methods for generating useful polypeptides and variants are known in the art and discussed below. Useful recombinant lactoferrin polypeptides and fragments and methods of producing them are reported in US patent specifications US 5,571,691, US 5,571,697, US 5,571,896, US 5,766,939, US 5,849,881, US 5,849,885, US 5,861,491, US 5,919,913, US 5,955,316, US 6,066,469, US 6,080,599, US 6,100,054, US 6,111,081, US 6,228,614, US 6,277,817, US 6,333,311, US 6,455,687, US 6,569,831, US 6,635,447, US 2005-0064546 and US 2005-0114911.

[0120] Useful variants also include bovine lactoferrin variants bLf-a and bLf-b (Tsuji, et al. (1989); Yoshida, et al. (1991)). Further useful variants include glycosylated and aglycosyl forms of lactoferrin (Pierce, et al. (1991); Metz-Boutigue, et al. (1984); van Veen, et al. (2004)) and glycosylation mutants (having variant points of glycosylation or variant glycosyl side chains).

[0121] Useful fragments include the N-lobe and C-lobe fragments (Baker, et al., 2002) and any other lactoferrin polypeptides that retain a lactoferrin binding pocket, such as truncated lactoferrin polypeptides.

[0122] Useful truncated lactoferrin polypeptides include polypeptides truncated by about 1 to about 300 amino acids, preferably about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 290, 295 or 300 amino acids or more, and including polypeptides truncated at the N-terminus, at the C-terminus or at both the N- terminus and C-terminus, provided that the truncated polypeptide retains at least one of the N-lobe or the C-lobe metal ion binding pockets. It is reported that residues Asp 60, Tyr 92, Tyr 192, His 253 of bovine lactoferrin are the amino acid metal ion ligands in the N-lobe. It is reported that residues Asp 395, Tyr 433, Tyr 526, His 595 of bovine lactoferrin are the amino acid metal ion ligands in the C-lobe (Karthikeyan, et al., 1999). Any fragment that contains these residues may be able to form a metal ion binding pocket.

[0123] Candidate variants or fragments of lactoferrin for use according to the present invention may be generated by techniques including but not limited to techniques for mutating wild type proteins (see Sambrook, et al. (1989) and elsewhere for a discussion of such techniques) such as but not limited to site-directed mutagenesis of wild type lactoferrin and expression of the resulting polynucleotides; techniques for generating expressible polynucleotide fragments such as PCR using a pool of random or selected primers; techniques for full or partial proteolysis or hydrolysis of wild type or variant lactoferrin polypeptides; and techniques for chemical synthesis of polypeptides. Variants or fragments of lactoferrin may be prepared by expression as recombinant molecules from lactoferrin DNA or RNA, or variants or fragments thereof. Nucleic acid sequences encoding variants or fragments of lactoferrin may be inserted into a suitable vector for expression in a cell, including eukaryotic cells such as but not limited to Aspergillus or bacterial cells such as but not limited to E. coli. Lactoferrin variants or fragments may be prepared using known PCR techniques including but not limited to error-prone PCR and DNA shuffling. Error-prone PCR is a process for performing PCR under conditions where the copying fidelity of the DNA polymerase is low, such that a high rate of point mutations is obtained along the entire length of the PCR product (Leung, et al. (1989); Cadwell, et al. (1992)). DNA shuffling refers to forced homologous recombination between DNA molecules of different but highly related DNA sequence in vitro, caused by random fragmentation of the DNA molecule based on sequence homology, followed by fixation of the crossover by primer extension in a PCR reaction (Stemmer (1994)). Suitable lactoferrin nucleic acid sequences for use in such methods include those listed above or may be generated by known methods including, for example, reverse transcription-PCR (RT-PCR) of tissue RNA isolates. Suitable primers for RT-PCR may be designed with reference to the mRNA sequences listed above. Commercial kits are available for RT-PCR (for example, Cells-to-cDNA™ kits from Ambion, USA).

[0124] Variants or fragments of lactoferrin may also be generated by known synthetic methods (see Kimmerlin, et al., 2005, for example). Alternatively, lactoferrin polypeptides or functional variants or fragments thereof can be produced by well established synthetic Fmoc chemistry as described for human kaliocin-1 and the lactoferricin derived peptide by Viejo-Diaz et al., (2003); and bovine lactoferricin peptide as described by Nguyen et al., (2005); and lactoferrampin and shorter fragments as described by van der Kraan et al., (2004).

[0125] Metal ion binding variants or fragments of lactoferrin may be obtained by known techniques for isolating metal binding polypeptides including but not limited to metal affinity chromatography, for example. Candidate variants or fragments of lactoferrin may be contacted with free or immobilised metal ions, such as Fe^{3+} and purified in a suitable fashion. For example, candidate variants or fragments may be contacted at neutral pH with a metal ion immobilised by chelation to a chromatography matrix comprising iminodiacetic acid or tris(carboxymethyl)ethylenediamine ligands. Bound variants or fragments may be eluted from the supporting matrix and collected by reducing the pH and ionic strength of the buffer employed. Metal bound variants or fragments may be prepared according to the methods described herein.

[0126] Functional variants, fragments and hydrolysates of lactoferrin may be obtained by selecting variants, fragments and hydrolysates of lactoferrin and assessing their efficacy assays chosen to test the desired efficacy.

[0127] In one embodiment the lactoferrin is any mammalian lactoferrin including but not limited to sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, bovine or human lactoferrin. Preferably the lactoferrin is bovine lactoferrin.

[0128] In another embodiment the lactoferrin is any recombinant mammalian lactoferrin including but not limited to recombinant sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, bovine or human lactoferrin. Preferably the lactoferrin is recombinant bovine lactoferrin. Recombinant lactoferrin may be produced by expression in cell free expression systems or in transgenic animals, plants, fungi or bacteria, or other useful species. Alternatively, lactoferrin may be produced using known organic synthetic methods.

[0129] In yet another embodiment the lactoferrin is isolated from milk, preferably sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, bovine or human milk. Preferably the lactoferrin is isolated from milk by cation exchange chromatography followed by ultrafiltration and diafiltration.

3. Isolation of lactoferrin from milk

[0130] The following is an exemplary procedure for isolating lactoferrin from bovine milk.

[0131] Fresh skim milk (7 L, pH 6.5) is passed through a 300 ml column of S Sepharose Fast Flow equilibrated in milli Q water, at a flow rate of 5 ml/min and at 4°C. Unbound protein is washed through with 2.5 bed volumes of water and bound protein eluted stepwise with approximately 2.5 bed volumes each of 0.1 M, 0.35 M, and 1.0 M sodium chloride. Lactoferrin eluting as a discreet pink band in 1 M sodium chloride is collected as a single fraction and dialysed against milli Q water followed by freeze-drying. The freeze-dried powder is dissolved in 25 mM sodium phosphate buffer, pH 6.5 and subjected to chromatography on S Sepharose Fast Flow with a sodium chloride gradient to 1 M in the above buffer and at a flow rate of 3 ml/min. Fractions containing lactoferrin of sufficient purity as determined by gel electrophoresis and reversed phase HPLC are combined, dialyzed and freeze-dried. Final purification of lactoferrin is accomplished by gel filtration on Sephacryl 300 in 80 mM dipotassium phosphate, pH 8.6, containing 0.15 M potassium chloride. Selected fractions are combined, dialyzed against milli Q water, and freeze-dried. The purity of this preparation is greater than 95% as indicated by HPLC analysis and by the spectral ratio values (280 nm/465 nm) of ~19 or less for the iron saturated form of lactoferrin.

4. Metal ion saturation or depletion of lactoferrin

[0132] Iron saturation is achieved by addition of a 2:1 molar excess of 5mM ferric nitrilotriacetate (Foley and Bates (1987)) to a 1% solution of the purified lactoferrin in 50 mM Tris, pH 7.8 containing 10 mM sodium bicarbonate. Excess ferric nitrilotriacetate is removed by dialysis against 100 volumes of milli Q water (twice renewed) for a total of 20 hours at 4°

C. The iron-loaded (holo-) lactoferrin may then be freeze-dried. Varying degrees of iron saturation may be obtained by providing less of the metal ion donor.

[0133] Iron-depleted (apo-) lactoferrin is prepared by dialysis of a 1% solution of the highly purified lactoferrin sample in water against 30 volumes of 0.1 M citric acid, pH 2.3, containing 500 mg/L disodium EDTA, for 30 h at 4° C (Masson and Heremans (1966)). Citrate and EDTA are then removed by dialysis against 30 volumes of milli Q water (once renewed) and the resulting colourless solution may be freeze-dried.

[0134] A lactoferrin polypeptide can contain an iron ion (as in a naturally occurring lactoferrin polypeptide) or a non-iron metal ion (for example, a copper ion, a chromium ion, a cobalt ion, a manganese ion, a zinc ion, or a magnesium ion). For instance, lactoferrin isolated from bovine milk can be depleted of iron and then loaded with another type of metal ion. For example, copper loading can be achieved according to the same method for iron loading described above. For loading lactoferrin with other metal ions, the method of Ainscough, et al. (1979) can be used.

[0135] In one embodiment the metal ion is an ion selected from aluminium, calcium, copper, chromium, cobalt, gold, iron, manganese, magnesium, platinum, ruthenium, selenium, or zinc ions, or a mixture thereof. Preferably the metal ion is an iron ion.

[0136] In a preparation of a composition for use according to the invention, a lactoferrin polypeptide or metal ion binding lactoferrin fragment can be of a single species, or of different species. For instance, the polypeptides or fragments can each contain a different number of metal ions or a different species of metal ions; or the lengths of the polypeptides can vary, for example, some are full-length polypeptides and some are fragments, and the fragments can each represent a particular portion of a full-length polypeptide. Such a preparation can be obtained from a natural source or by mixing different lactoferrin polypeptide species. For example, a mixture of lactoferrin polypeptides of different lengths can be prepared by proteinase digestion (complete or partial) of full-length lactoferrin polypeptides. The degree of digestion can be controlled according to methods well known in the art, for example, by manipulating the amount of proteinase or the time of incubation, and described below. A full digestion produces a mixture of various fragments of full-length lactoferrin polypeptides; a partial digestion produces a mixture of full-length lactoferrin polypeptides and various fragments.

5. Preparation of lactoferrin fragments or lactoferrin hydrolysates

[0137] Hydrolysates containing candidate functional fragments can be prepared by selecting suitable enzymes with known specificity of cleavage, such as trypsin or chymotrypsin, and controlling/limiting proteolysis by pH, temperature, time of incubation and enzyme to substrate ratio. Refinement of such isolated peptides can be made using specific endopeptidases. As an example, bovine lactoferricin can be produced by cleavage of bovine lactoferrin with pepsin at pH 2.0 for 45 min at 37°C (Facon & Skura, 1996), or at pH 2.5, 37°C for 4h using enzyme at 3% (w/w of substrate) (Tomita et al., 1994). The peptide can then be isolated by reversed phase HPLC (Tomita et al., 1994) or hydrophobic interaction chromatography (Tomita e al., 2002).

[0138] Alternatively, lactoferrin peptides can be produced by well established synthetic Fmoc chemistry as described for human kaliocin-1 and the lactoferricin derived peptide by Viejo-Diaz et al., (2003); and bovine lactoferricin peptide as described by Nguyen et al., (2005); and lactoferrampin and shorter fragments as described by van der Kraan et al., (2004).

[0139] In general, SDS-PAGE may be used to estimate the degree of hydrolysis by comparison of the hydrolysate to a molecular weight standard. Size exclusion chromatography may be used to separate various species within a hydrolysate and to estimate a molecular weight distribution profile.

[0140] In a preferred hydrolytic method, bovine lactoferrin was dissolved to 20mg/mL in 50mM Tris pH 8.0, 5mM CaCl2. Trypsin (Sigma T8642, TPCK treated, Type XII from bovine pancreas, 11700U/mg protein) was added at an enzyme substrate ratio of 1:50 w/w and the mixture incubated at 25° C for 3h. The reaction was stopped by the addition of PMSF to 1mM final concentration and extent of digestion monitored by SDS-PAGE. The tryptic digest (4mL) was applied to gel filtration on Sephacryl S300 (Amersham GE) (90cm x 2.6cm column) in 50mM Tris, 0.15M NaCl pH 8.0. Suitable fractions containing the major fragments of bovine lactoferrin (Legrand et al., 1984) were then subjected to cation exchange chromatography on S Sepharose fast Flow (Amersham GE) (15cm x 1.6 cm column) using sodium phosphate buffer pH 6.5 and a salt gradient to 1 M NaCl. Final separation of the C lobe and N+C lobes was achieved by further gel filtration on Sephacryl S300 as above but using 10% v/v acetic acid as eluent (Mata et al., 1994). The identity of the dialysed (versus milli-Q water) and freeze-dried fragments was confirmed by SDS-PAGE and Edman N-terminal sequencing.

[0141] In another method, a tryptic digest as above was separated by RP-HPLC on a Vydac C18 column as in Superti et al., (2001) and the high mass fragments corresponding to C-lobe and N-lobe fragments recovered. Identity was confirmed by MALDI MS.

[0142] In one embodiment hydrolysates useful herein contain one or more metal ion binding fragments.

6. Pressure treatment of metal ion saturated lactoferrin

[0143] The present inventors have shown that it is possible to pressure treat compositions or products comprising metal ion lactoferrin under conditions which achieve a commercially useful keeping quality while maintaining at least a desired level of metal ion binding.

[0144] The inventors have found that the use of high pressure to either commercially sterilise or extend the shelf-life of iron loaded lactoferrin solutions (or products containing iron lactoferrin) allows retention of the iron specifically bound to the protein at the treatment pH. Pressure treatment is also generally far less damaging to the protein structure, and thus the iron-binding ability of lactoferrin (and therefore capacity to rebind iron) is relatively unimpaired.

[0145] The Examples below show pressure treatment has much less of an effect than heat treatment on the iron binding of lactoferrin that is about 15% and about 100% iron saturated. The conditions assessed are accepted conditions for commercially useful pressure and heat treatment; that is, conditions that are accepted and approved for sterilisation or improving keeping quality.

[0146] Fully or partially iron saturated lactoferrin has been proposed for treating iron-deficiency anaemia (Bethell & Huang, 2004; Huang et al., 2004), the iron-deficiency associated with pregnancy (Valenti et al., 2005), and as an agent for increasing haemoglobin count in anaemia sufferers. As there may be an association between low dietary iron intake and decreased bone mineral density in postmenopausal women (Medeiros et al., 2002), and because lactoferrin has been shown to be a bone anabolic agent (Cornish et al., 2004), the iron loaded form may also be useful in treating bone disorders such as osteoporosis. Additionally, iron-lactoferrin could have application in gut renewal therapies for malabsorption since the iron loaded forms of both human and bovine lactoferrin proliferate enterocyte cell lines in culture (Oguchi et al., 1995).

[0147] Several patent specifications report the use of iron-saturated lactoferrin for iron-supplementation of beverages, food products and feeds (see Sakurai et al., 2000; Dugas et al., 2001; Dousako et al, 1991; Tomita et al., 1992; and Tanaka et al., 1991) and for use as medicaments (see Nitsche, 1991).

[0148] As well as iron-lactoferrin, use of other metal ions may be beneficial. For example, chromium-lactoferrin has been reported (Ainscough et al., 1979) as has the use of chromium-lactoferrin as a therapeutic agent in alleviating symptoms associated with diabetes (Chiang & Mao, 2002).

[0149] In one embodiment treatment according to the invention allows use of high pressure to commercially sterilise (by eliminating the growth of unwanted microorganisms) metal ion lactoferrin compositions where the pH of the composition or product is about pH 3.0 to 8.0, preferably less than or equal to pH 4.6.

[0150] In another embodiment treatment according to the invention allow use of high pressure to extend the shelf-life (by substantially reducing or delaying the growth of unwanted microorganisms) of metal ion lactoferrin compositions where the pH of the composition or product is about pH 3.0 to 8.0, preferably greater than or equal to pH 4.6 or at a neutral pH.

[0151] In yet another embodiment, it may be possible to achieve commercial sterilisation or to improve shelf-life of compositions or products containing metal ion lactoferrin at a selected pH in the range of pH 3.0 and 8.0 using a suitable combination of pH and carbon dioxide. Carbon dioxide in combination with pH may give an effect equal to that at a lower pH (i.e. effective growth prevention and retention of a desirable level of metal ion binding). Commercial sterilisation or improved shelf-life may thereof be obtained through use of carbon dioxide and pH control (e.g. in carbonated beverages).

[0152] By way of example, compositions or products processed according to the invention may be delivered in pressure-treated products or ingredients; added to products or ingredients which are not subsequently heat-treated; or added to products or ingredients which are subsequently pressure-treated.

[0153] Accordingly, the present invention relates to methods of treating or producing lactoferrin-containing compositions and products as described above.

[0154] While not intended to be limiting, a pressure treatment useful in a method according to the invention preferably comprises the following steps:

(i) placing a composition or product to be pressure treated into the chamber of a pressure vessel and sealing the chamber;

(ii) raising the pressure in the chamber to a predetermined set pressure (the "treatment pressure");

(iii) holding the chamber at this pressure for a predetermined time, including less than one minute (the "hold time");

(iv) releasing the pressure from the chamber; and

(v) removing the pressure treated composition or product.

[0155] Such a protocol may be followed using batch or continuous processing equipment.

[0156] It should be understood that the pressure treatment may result in temperature fluctuations in the composition or product during treatment. As such, references to preferred temperatures during pressure treatment refer to the temperature of the composition or product before the pressure is raised.

[0157] One method of identifying a suitable treatment pressure according to the present invention is to select a composition or product and subject it to a treatment pressure suitable for controlling an unwanted microorganism. A suitable treatment pressure is a pressure of at least about 100 MPa. If necessary, the composition or product may be inoculated with an unwanted organism for the purposes of assessment.

[0158] Another method of identifying a suitable treatment pressure according to the present invention is to select a composition or product and subject it to a treatment pressure that does not substantially affect the metal ion binding of lactoferrin or a functional variant or fragment thereof.

[0159] The growth of any unwanted microorganisms or the metal ion binding of lactoferrin or both may then be assessed as described herein. See for example, Lund, et al. (2000) for methods of assessing microorganism growth.

7. Dairy products and ingredients

[0160] A composition or product of the invention may be or may include a dairy product or ingredient.

[0161] Milk is not only a complete source of nutrients for the neonate, but also provides a rich source of physiologically bioactive components and as such has been referred to as 'natures' pharmacy'. In addition to providing complete nutrition, milk also has vital roles in the development, protection and repair of the young. Healthy adults usually only require the nutritional benefits of milk, but in conditions of chronic ailments the bioactivities derived from milk have more to offer in terms of both prevention and treatment of illness. Preferably the milk is sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, bovine or human milk, and most preferably bovine milk. Dairy protein is known to be immuno-stimulatory.

[0162] Colostrum is the pre-milk produced immediately after birth before standard milk production begins. Prime colostrum from cows is obtained within the first six hours after calving but colostrum can be collected within the first two days following calving. Prime colostrum typically contains more than twice the milk solids and four times the protein found in milk from the same cow obtained after about forty-eight hours later. The concentrations of digestive enzymes, immunoglobulins (including IgA, IgD, IgE, IgG and IgM), cytokines, interferons, growth factors, glycoproteins, proline-rich peptides and vitamins A, D, E and K are all higher in prime colostrum compared to standard milk. Colostrum milk protein concentrates (MPC) and colostrum whey protein concentrates (WPC) may be prepared as described by Elfstrand et al., 2002.

[0163] Milk and colostrum derivatives and methods of their manufacture are known in the art. Such derivatives are generally obtained by a combination centrifugation (for fat removal), casein precipitation (with acid or enzymes), filtration (to remove lactose, minerals and water, or optionally to remove proteins), chromatography (to purify protein components) and include recombined or fresh whole milk, recombined or fresh skim milk, reconstituted whole or skim milk powder, skim milk concentrate, skim milk isolate, whole or skim milk powder, skim milk retentate, concentrated milk, buttermilk, ultrafiltered milk retentate, milk protein concentrate (MPC), milk protein isolate (MPI), calcium depleted milk protein concentrate, calcium depleted milk protein isolate, low fat milk, low fat milk protein concentrate, low fat milk protein isolate, colostrum, a colostrum fraction, colostrum protein concentrate (CPC), colostrum milk protein concentrate, colostrum milk protein isolate, colostrum whey, colostrum whey protein concentrate, colostrum whey protein isolate, an immunoglobulin fraction from colostrum, whey, whey protein concentrate (WPC), whey protein isolate (WPI), sweet whey, lactic acid whey, mineral acid whey, reconstituted whey powder, a composition derived from any milk or colostrum processing stream, a composition derived from the retentate or permeate obtained by ultrafiltration or microfiltration of any milk or colostrum processing stream, or a composition derived from the breakthrough or adsorbed fractions obtained by chromatographic separation of any milk or colostrum processing stream, or a full or partial hydrolysate of any of these compositions, and or a mixture thereof. See for example the Dairy Processing Handbook (Tetra Pak Processing Systems, Lund, Sweden, 1995). Other such derivatives include the dairy protein compositions and dairy ingredients described above.

[0164] The proteins found in milk include immunoglobulins (including IgA, IgD, IgE, IgG and IgM), growth factors, bovine serum albumin (BSA), alpha-lactalbumin, beta-lactoglobulin and a large number of caseins, all of which are phosphoproteins. These proteins, with the exception of casein, are also present in whey. Milk is known to contain a variety of mitogenic proteins and proteins which may be involved directly in bone remodeling. Growth factors (IGF – Insulin-like Growth Factor, TGF - Transforming Growth Factor etc), immunoglobulins (including IgA, IgD, IgE, IgG and IgM), BSA and some beta lactoglobulin are recovered from milk or whey by cation exchange chromatography. Some growth factors are recovered as neutral proteins. Caseinoglycomacropeptide (CGMP) is an acidic protein fraction recoverable by anion exchange. Osteopontin is a highly phosphorylated and glycosylated protein found in all body fluids (including milk).

[0165] CGMP is a peptide released from kappa-casein during the rennet-mediated casein coagulation step (through the action of chymosin) of the cheese making process and is found in the whey fraction which is known as Sweet Whey or Cheese Whey. CGMP is sometimes referred to simply as GMP (glycomacropeptide). Cheese whey proteins consist of 15% to 20% CGMP. CGMP has been put forward as one of the bone health promoting components of milk, as reported in WO 00/49885.

[0166] Lactic acid whey is produced by fermentation with lactic acid bacteria or direct addition of lactic acid during the manufacture of caseinate or cottage and ricotta cheeses. Mineral acid whey is produced by addition of mineral acids during caseinate manufacture. Lactic acid whey and mineral acid whey do not contain CGMP. The basis of these two processes is to lower pH to about 4.6 to cause casein to precipitate as opposed to using the action of chymosin to cause precipitation. Therefore any milk products that have not been exposed to chymosin will not contain CGMP.

[0167] Whey is a by-product of cheese or casein manufacture, and the protein products derived from whey may be classified on the basis of their protein content, including whey protein concentrates (WPC) containing at least 30% protein, to whey protein isolates (WPI) containing at least 90% protein (Huffman, 1996; IDF, 1998). Membrane ultrafiltration and diafiltration is typically used in the manufacture of such products to concentrate and purify the whey protein to 25-35% solids before drying, and the protein concentrate derived from the membrane filtration step is known in the art as retentate. Whey protein is a collective term encompassing several individual proteins (including but not limited to alpha-lactalbumin, beta-lactoglobulin, proteose peptones, immunoglobulins (including IgA, IgD, IgE, IgG and IgM), glycomacropeptide, growth factors (such as TGF β1 and TGF β2), bovine serum albumin, lactoferrin, and lactoperoxidase) and in the present invention may include whey protein collectively or fractions thereof. Methods suitable for the commercial production of whey are described by Zadow (1992) and Sienkiewicz et al (1990). Methods for producing WPCs and WPIs are known in the art and discussed in the US Dairy Export Council Reference Manual for U.S. Whey and Lactose Products, Chapter 7: Whey Products - Definition, Composition, Functions; Page, J., Meyer, D., Haines, B., Lagrange, V., and Kenney, A. (Eds), American Dairy Products Institute, Elmhurst, IL, USA, (June 2004) (also available on-line at http://www.usdec.org/files/pdfs/US08D_04.pdf). See also the Dairy Processing Handbook (Tetra Pak Processing Systems, Lund, Sweden, 1995). Whey protein is known to be immunostimulatory.

[0168] Hyperimmune milk and hyperimmune colostrum are made by immunising pregnant milk producing mammals with antigens from pathogens to raise specific antibodies in the colostrum and milk (see Korhonen, et al., 2000 for a review of such methods). Protein concentrates of hyperimmune products may be prepared according to the known methods referenced above. Hyperimmune milk and hyperimmune colostrum are known to be immunostimulatory (Korhonen, et al., 2000). Hyperimmune milk and hyperimmune colostrum may be processed like ordinary milk and colostrum to produce derivatives such as hyperimmune milk protein concentrate, hyperimmune milk protein isolate, hyperimmune whey, hyperimmune whey protein concentrate, hyperimmune whey protein isolate, hyperimmune colostrum, hyperimmune colostrum milk protein concentrate, hyperimmune colostrum milk protein isolate, hyperimmune colostrum whey, hyperimmune colostrum whey protein concentrate, or hyperimmune colostrum whey protein isolate, or a mixture thereof.

[0169] By way of example, compositions or products processed according to the invention may be delivered in pressure-treated products or ingredients; added to products or ingredients which are not subsequently heat-treated; or added to products or ingredients which are subsequently pressure-treated.

[0170] In order to reduce unwanted effects on proteins present in compositions treated according to the invention, it may be desirable in some embodiments where the composition is a liquid to add a stabiliser. For example, to stabilise any casein present in the composition. Accordingly, in one embodiment the composition further comprises a stabiliser such as a gum selected from locust bean gum, guar gum, xanthan gum, cassia gum, konjac flour, beta-glucan, tara gum, gum arabic, gellan gum, carboxymethylcellulose, methylcellulose, hydroxypropyl methylcellulose, tragacanth gum, karaya gum, gum acacia, chitosan, arabinoglactins, alginate, pectin, carrageenan, or psyllium or a mixture thereof. Preferably the stabiliser is pectin or carboxymethylcellulose (CMC). Preferably a composition to be treated according to the methods of the invention comprises about 0.01, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35 % w/v or more of a stabiliser, as required.

[0171] To stabilise bioactive components at about neutral pH, in some embodiments it may be desirable to include in the composition one or more hydrophobic ligands. The presence of a hydrophobic ligand allows a bioactive component to be pressure treated at a pH of about 7.0, preferably at a pH of about 5.0 to 8.0, while retaining a higher level of activity than would be readily obtained in the absence of the ligand. Without wishing to be bound by theory, it is believed these ligands bind hydrophobic pockets in the bioactive components, reducing their sensitivity to denaturation during pressure treatment. Accordingly, in some embodiments it may be desirable that the composition further comprises one or more hydrophobic ligands selected from palmitic acid, myristic acid, linoleic acid, conjugated linoleic acid (CLA), one or more phospholipids, one or more phosphatidylcholines, one or more sphingomyelins, one or more gangliosides, butyric acid, one or more omega-3 fatty acids (including but not limited to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), one or more phytosterols, one or more phytosterol esters, one or more phytosterol acetates, one or more omega-6 fatty acids (including but not limited to fish oil), fat soluble hydrophobic vitamins (including vitamin A [retinol] and vitamin D), lycopene, or sodium dodecyl sulphate, or a mixture thereof. Preferably the pH of the composition in this embodiment is from about

5.0 to 8.0.

[0172] Examples of product formulations useful herein include beverages (including acidified beverages and carbonated beverages), yoghurts and jellies. Such products may be formulated as described below and assessed as described above and in the examples.

[0173] Various aspects of the invention will now be illustrated in non-limiting ways by reference to the following examples.

EXAMPLES

1. Lactoferrin Solutions

[0174] Lactoferrin (~15% iron saturation – native saturation levels) was obtained from Fonterra Co-operative Group limited.

[0175] Iron-saturated lactoferrin (~ 100%) was made by addition of a 2:1 molar excess of 5mM ferric nitrilotriacetate (Foley and Bates, 1987) to a 1% solution of lactoferrin in 50 mM Tris-HCl, pH 7.8 containing 10 mM sodium bicarbonate. Excess reagent was removed by dialysis against 100 volumes of Milli Q water (twice renewed) for a total of 20 hours at 4° C. The iron-loaded (holo-) lactoferrin was then freeze-dried.

[0176] Lactoferrin solutions for heat and pressure treatment were made to 6% (w/v) in MilliQ water and the pH adjusted with 3M NaOH or 3 M HCl to the required value. After initial pH adjustment, the solutions were allowed to equilibrate overnight and the pH of the solutions finally re-adjusted to the required pH.

2. Pressure treatment

[0177] The solutions were transferred into Beckman Polyallomer Quick-seal™ centrifuge tubes (13 mm internal diameter, 51 mm high; Beckman Instruments, Inc., Spinco division, Palo Alto, CA 94304) and the tubes heat-sealed. The sample tubes were then treated in a high-pressure unit at 600 MPa at ambient temperature for 5, 15 and 30 min. After depressurisation, the sample tubes were cut open and the pressure-treated samples and the untreated (control) were analysed using various techniques.

3. Heat Treatment

[0178] Aliquots (2ml) of lactoferrin solution were placed in 8ml Wheaton glass screw-capped vials and the cap firmly fitted to ensure a tight seal. Vials in each sample series were preincubated in a water bath at 37°C for 5 min. The vials were quickly transferred to an oil bath set to the desired temperature and rocked and the time counted from this point. Sample vials were removed at the appropriate time points and immediately put on ice, ensuring complete immersion of the vial for rapid cooling. Samples were stored at 4° C until analysis.

4. Analyses

[0179] Qualitative and quantitative changes in the samples arising from heat or pressure processing were assessed by a range of techniques as below.

4.1 PAGE Analysis

[0180] Reducing and non-reducing polyacrylamide gel electrophoresis (PAGE) was performed as described by Manderson et al. (1998) using 15% gels.

4.2 HPLC

[0181] Samples were diluted appropriately in MilliQ water and analysed by reversed-phase HPLC as described in Palmano & Elgar (2002). Quantitation was by reference to a standard curve constructed using high purity bovine lactoferrin.

4.3 ELISA

[0182] The ELISA assay was performed on appropriately diluted samples using Bethyl kit following the manufacturer's instruction.

4.4 Immunoaffinity

[0183] Samples were appropriately diluted in Hepes buffer and analysed using a BiaCore instrument as described in Indyk & Filonzi (2005). Quantitation was by reference to a standard curve constructed using high purity bovine lactoferrin.

4.5 Iron Binding

[0184] The level of iron saturation in samples was assessed by absorbance at 465 nm. Spectrophotometric titration using ferric nitrilotriacetate (FeNTA) (Bates et al. 1967; Brock & Arzabe, 1976) was used to assess the iron binding capacity of lactoferrin in samples.

EXAMPLE 1

[0185] A 6% (w/v) native (~15% iron saturated) lactoferrin solution prepared as described above was translucent and a burnt orange colour. A solution of apo-lactoferrin (~0% iron saturated) was clear. Heat treatment of native lactoferrin over the pH range 3.8–7.0 showed that mild conditions (for example, 75°C for 5 min) induced some colour change in the test solutions indicating loss of iron or oriented iron-binding in lactoferrin, particularly at low pH (Table 1). Higher temperatures (85°C to 90°C for 5 min) resulted in virtually complete loss of colour in all solutions over the pH range. At pH 7.0, a thick precipitate formed indicating extensive denaturation of lactoferrin.

Table 1: Effect of heat treatment on native lactoferrin as a function of pH

Treatment Conditions:	**pH:**				
	3.3	**3.8**	**4.0**	**5.2**	**7.0**
Control	clear	++	++	+++	+++
75°C/5 min	clear	almost clear	+	++	++
85°C/5 min	clear	clear	clear	clear	thick ppt
90°C/5 min	clear	clear	clear	clear	thick ppt

"+" symbols indicate colour depth on visual inspection. Fewer "+" symbols indicate less iron binding.

[0186] In contrast, pressure treatment at 600 MPa for up to 45 min resulted in no colour change or turbidity in any of the samples except those at pH 3.8 and 4.0, where colour became more closely aligned with the solutions at higher pH (Table 2), indicating some re-adjustment in oriented iron binding at these lower pHs. This colour change at certain pH may have been due to pressure-induced 'solubilisation' of carbon dioxide with consequent re-equilibration to bicarbonate, which would furnish carbonate ions for iron binding.

Table 2: Effect of pressure treatment on native lactoferrin as a function of pH

Treatment Conditions:	**pH:**				
	3.3	**3.8**	**4.0**	**5.2**	**7.0**
Control	clear	++	++	+++	+++
600 MPa/5 min	clear	+++	+++	+++	+++
600 MPa/10 min	clear	+++	+++	+++	+++
600 MPa/30 min	clear	+++	+++	+++	+++
600 MPa/45 min	clear	+++	+++	+++	+++

"+" symbols indicate colour depth on visual inspection. Fewer "+" symbols indicate less iron binding or less oriented binding.

EXAMPLE 2

[0187] A 6% (w/v) 100% iron saturated lactoferrin solution prepared as described above was translucent and a dark burgundy colour. Heat treatment of fully iron-saturated lactoferrin (~ 100%) at pH 3.8, 4.8 and 7.0 (85°C for 10 min and 90°C for 10 min) resulted in some loss of colour (at pH 3.8 and 4.8) indicating a loss of bound iron and complete aggregation and loss of soluble lactoferrin in the case of the pH 7.0 sample (data not shown).

[0188] In contrast, pressure treatment at 600 MPa for up to 30 min resulted in no visible colour change for any of the solutions (Table 3).

Table 3: Effect of pressure and heat treatment on 100% iron saturated lactoferrin as a function of pH

Treatment Conditions:	**pH:**	
	3.8	**4.8**
Control	burgundy	burgundy
600 MPa/5 min	burgundy	burgundy
600 MPa/30 min	burgundy	burgundy
85°C/10 min	orange-red	orange-red
95°C/10 min	orange-red	orange-red

EXAMPLE 3

[0189] Samples of a fully iron-saturated lactoferrin solution (~ 100%) were adjusted to pH 7.0, 4.8 or 3.8 and subjected to high pressure at 600 MPa for 5 min or 30 min or heat treatment at 85°C for 10 min or 95°C for 10 min. The integrity of lactoferrin, bound iron and iron-binding capacity were determined for each sample. Untreated samples at each pH served as controls. In the case where there were precipitates or insoluble aggregates formed as a result of treatment, the samples were centrifuged at 8000 x g for 30 min, at 10°C and the supernatant taken for analysis.

[0190] Table 4 shows the results from HPLC. Quantitation was by integration of peaks, to give total soluble lactoferrin as a percentage compared to an untreated control. Peak shape was used as a qualitative index of denaturation.

Table 4: Effect of Heat and Pressure on Iron-saturated Lactoferrin assessed with RP-HPLC

	pH 3.8	**pH 4.8**	**pH 7.0**
Control	100	100	100
600 MPa x 5 min	93	98	102
600 MPa x 30 min	103	92	92
85°C x 10 min	92	82	0.3
90°C x 10 min	90	76	0.2

Results are expressed as a % of the control at each pH

[0191] For the pressure treated samples there was little change in peak shape or amount of lactoferrin over the pH range studied, whereas for the heat treated samples, there were visible changes in peak shape and greater loss of lactoferrin at each pH. This was most dramatic at pH 7.0 where there was complete denaturation of the lactoferrin.

[0192] Table 5 shows the results from BiaCore analysis.

Table 5: Effect of Heat and Pressure on Iron-saturated Lactoferrin assessed by BiaCore analysis

	pH 3.8	**pH 4.8**	**pH 7.0**
Control	100	100	100
600mPa x 5 min	104	127	99.2
600mPa x 30 min	99.7	93.5	102.9

85°C x 10 min	68.4	63.5	0.43
90°C x 10 min	65.3	65.9	0.28

Results are expressed as a % of the control at each pH

[0193] Tables 4 and 5 show pressure treatment substantially retained iron saturated lactoferrin levels at pH 3.8, 4.8 and 7.0 whereas heat treatment resulted in a loss of iron bound lactoferrin at all pHs, especially neutral pH.

[0194] Samples were analysed by non-reducing and reducing SDS-PAGE (data not shown). In all cases, lactoferrin in the pressure treated samples showed no change from the untreated controls. In contrast, the heat treated samples all showed some change in lactoferrin. For example, at lower pH values (3.8 & 4.8) the non-reducing gels show aggregated material which did not penetrate into the gel. This material was virtually absent after reduction indicating that aggregation was due to heat-induced polymerisation. At pH 7 almost no measurable lactoferrin remained in heat treated samples.

[0195] The control solutions of iron-saturated lactoferrin showed spectral differences which were dependent on the pH (data not shown). The spectrum of the parent iron-saturated lactoferrin (pH 8.0, unadjusted) was typical of those reported for iron-bound transferrins, with the absorption maxima for iron-binding at 465 nm (Bates & Schlabach, 1973). Notably, the pressure treated samples at each pH gave spectra which were virtually identical to those for the untreated pH controls. The amount of bound iron assessed from the spectra (absorbance at 465 nm in the presence of bicarbonate and Tris buffer pH 8.0) was the same as for the parent solution. Hence pressure treatment resulted in no change to iron bound, or oriented iron-binding at each pH.

[0196] In contrast, the spectra obtained from heat-treated samples showed definite differences from controls (data not shown). At pH 3.8, the heat treated samples gave spectra showing decreased absorbance at 465 nm, indicating loss of iron-bound lactoferrin, whereas at pH 4.8 the heat treated samples gave spectral profiles which were different from the control (no spectra could be recorded for the pH 7.0 samples due to aggregation of the sample as described above). This indicates that some irreversible changes had occurred due to the heating regimes and appropriate iron-binding had been compromised. In all cases above, addition of further iron as FeNTA resulted in no change in the spectra observed.

EXAMPLE 4

[0197] Samples of a 6% w/v fully iron-saturated (~ 100%) lactoferrin solution were adjusted to pH 3.3, 3.8, 4.2, 5.0, 6.0, 7.0 or 8.0 and inoculated with coliforms and yeast and moulds. Samples were subjected to pressure treatment at 600 MPa with no hold, 3 minutes or 15 minutes, or heat treated at 85 °C/ 10 min or 90 °C/ 10 min. Microbial analysis of an untreated control showed a yeasts and moulds count of 7.0 × 10^5 cfu/ml whereas no yeasts and moulds were detectable in all pressure treated lactoferrin samples at every pH. Data is shown for samples at pH 5.0 to 8.0 in Table 6. HPLC analysis (Palmano & Elgar, 2000) on all samples showed that pressure treatment at each pH had no effect on quantifiable lactoferrin (relative to the unprocessed control) and absorbance measurement at 465 nm showed that at pH 5.0 and greater, iron saturation was essentially 100 %. RP-HPLC is a measurement of total lactoferrin content. Absorbance at 465 nm can be used to measure the amount of fully iron saturated lactoferrin (FeLF) present in the solution. Where the RP-HPLC measurement and the measurement derived from 465 nm are approximately the same (mg/ml), the concentration of approximately fully (100 %) saturated lactoferrin the in solution is indicated.

Table 6: Microbial analysis of FeLF solutions (6 % w/v) adjusted at various pH and pressure treated (600 MPa for different holding times) or heat treated (as shown)

Sample description	pH	Coliforms (cfu/ml)	Yeast & moulds (cfu/ml)	FeLF (mg/ml) RP-HPLC	FeLF (mg/ml) Absorbance at 465 nm
Unprocessed control	7.0	ND	7.0 × 10^5	53.0	52.7
No hold	5.0	ND	ND	52.6	52.5
3 min		ND	ND	54.2	51.2
15 min		ND	ND	51.4	51.5
85 °C/ 10 min		NP	NP	52.2	**
90 °C/ 10 min		NP	NP	48.8	**
No hold	6.0	ND	ND	51.7	52.1
3 min		ND	ND	54.6	50.1
15 min		ND	ND	52.8	52.3
85 °C/ 10 min		NP	NP	5.2	7.62

90 °C/ 10 min		NP	NP	1.33	**
No hold	7.0	ND	ND	52.6	51.0
3 min		ND	ND	53.5	52.8
15 min		ND	ND	51.7	52.0
85 °C/ 10 min		NP	NP	NQ	**
90 °C/ 10 min		NP	NP	NQ	**
No hold	8.0	ND	ND	52.2	49.5
3 min		ND	ND	50.5	53.2
15 min		ND	ND	52.8	55.0
85 °C/ 10 min		NP	NP	NQ	**
90 °C/ 10 min		NP	NP	NQ	**

ND = Not found or not detected; NQ = not quantifiable because peak shape indicated extensive denaturation and aggregation of lactoferrin; NP = test not performed; ** indicates absorbance not able to be assessed due to irregularities in the spectrum associated with denaturation.

EXAMPLE 5

[0198] Fully iron saturated (~100%) lactoferrin (prepared as described above) was incorporated into yoghourt (prepared according to the method outlined in Figure 1), a beverage (prepared according to the method outlined in Figure 2), or jelly (prepared according to the method outlined in Figure 3). Heat treated, pressure treated and control samples were prepared as described in the Figures. Lactoferrin was extracted from these samples for analysis of integrity, bound iron and iron-binding ability.

[0199] Briefly, samples of yoghourt (~ 100g) were centrifuged at 8000 x g, at 10°C for 30 minutes. The supernatant whey was removed, diluted 1:1 with 0.1M disodium phosphate buffer, pH 6.5, and the pH adjusted to pH 6.5 with NaOH. The precipitate formed was removed by centrifugation as above. The resulting supernatant (~ 100-110 mL) was loaded on to a column (1.6 x 20 cm) of S Sepharose Big Beads (SPBB) at 2 mL/min.

[0200] For beverage samples, 100 mL beverage was diluted to 400 mL with milliQ water and the pH adjusted to 6.5 with NaOH. A sample of diluted beverage (100 mL) was filtered through a 0.45μm PVDF membrane (Millipore) and ~ 80 mL applied to a column (1.6 x 20 cm) of S Sepharose Big Beads (SPBB) at 2 mL/min.

[0201] For jelly samples, ~ 50 g jelly was diluted to 200 mL with milliQ water, with stirrring to homogenise. A sample of diluted jelly (~ 100 mL) was filtered through a 0.45µm PVDF membrane (Millipore) and the filtrate applied to a column (1.6 x 20 cm) of SPBB at 2 mL/min.

[0202] Separate columns for each sample were treated as follows. Unbound material was washed through with 0.1M disodium phosphate buffer, pH 6.5. Bound protein was firstly eluted with 0.45 M NaCl (until absorbance at 280 nm & 214 nm had reached baseline) and then with 1.5 M NaCl. Analysis of the fractions by reversed-phase HPLC (Palmano & Elgar, 2000) showed that virtually all the lactoferrin was captured in the 1.5 M NaCl eluted fractions, and was at least 93 % purity for the yoghurt or beverage samples or 82-88% purity for the jelly samples. The 1.5 M NaCl eluted fractions for each sample were dialysed against milliQ water, freeze-dried and stored at -30°C prior to analysis.

Results – Iron Binding

[0203] The amount of lactoferrin in the yoghourts, beverages and jellies was assayed at ~ 14 days after manufacture by reversed-phase HPLC, and by immunoassay using BiaCore (Indyk & Filonzi, 2005) analysis. Degree of iron saturation in the extracted lactoferrins was measured by spectrophotometric titration. Results are given in Table 7.

Table 7

Sample	Lf (mg/g) (BiaCore)	Lf (mg/g) (RP-HPLC)	Fe Saturation (%)
Yoghourt control	3.38	3.91	12
Yoghourt HPP	2.99	4.05	17
Yoghourt Heat treated	0.0004	0.079	ND
Beverage control	2.45	3.42	78
Beverage HPP	1.9	3.10	83
Beverage Heat treated	0.23	ND	ND
Jelly control	3.72	4.701	23
Jelly HPP	3.36	4.069	61

ND = Not determined; HPP = pressure treated.

[0204] The above results show that high pressure treatment of the application samples maintained the integrity of iron-lactoferrin, whereas heat treatment was severely detrimental to the protein. In all cases measured, iron saturation in the pressure treated samples was approximately equal to or greater than that in the corresponding control, indicating that pressure treatment can maintain iron saturation status and even improve iron binding status in particular applications. Lactoferrin concentration and integrity in the heat treated samples was too residual to warrant extraction of lactoferrin. The low saturation observed in the yoghurts may be due to iron-scavenging by microorganisms combined with low pH. In all cases, the extracted lactoferrin retained full iron binding bioactivity, being able to bind iron in excess of 90% saturation (data not shown).

Results – Microbiology

[0205] Microbial analysis of the beverages, jellies and yoghurts was conducted within 1 to 2 weeks and is shown in Tables 8 to 10. The results indicate that pressure treatment was effective in reducing microbial counts.

Table 8: Microbiology of FeLF yoghurts

Test Description	Control (cfu/ml)	Heat treated (cfu/ml)	Pressure treated (cfu/ml)
Total count acid tolerant (°C)	60	<10	10
B. Cereus Confirmed count	<10 EST	<10 EST	<10 EST
Coliform counts by direct plating	< 1 EST	< 1 EST	< 1 EST
Confirmed coagulase +ve staphylococci	< 10 EST	< 10 EST	< 10 EST
Yeast and molds counts	<1 EST	<1 EST	<1 EST
Aerobic Plate count	130	<10 EST	60 EST
Clostridia perfringens counts	<10 EST	<10 EST	<10 EST
Mesophilic spores	20 EST	10 EST	10 EST
Thermophiles	50 EST	30 EST	30 EST
E. Coli detect LSTMUG	ND	ND	ND
E. Coli detect LSTMUG	ND	ND	ND
Listeria	ABSENT	ABSENT	ABSENT
Salmonella	ABSENT	ABSENT	ABSENT

ND = not detected.

Table 9: Microbiology of FeLF acid drinks

Test Description	Control (cfu/ml)	Heat treated (cfu/ml)	Pressure treated (cfu/ml)
Total count acid tolerant (°C)	2.8×10^3	<10	10
B. Cereus Confirmed count	<10 EST	<10 EST	<10 EST
Coliform counts by direct plating	< 1 EST	< 1 EST	< 1 EST

Confirmed coagulase +ve staphylococci	< 10 EST	< 10 EST	< 10 EST
Yeast and molds counts	<1 EST	<1 EST	<1 EST
Aerobic Plate count	640	<10 EST	60 EST
Clostridia perfringens counts	<10 EST	<10 EST	<10 EST
Mesophilic spores	<10 EST	<10 EST	<10 EST
Thermophiles	10 EST	<10 EST	<10 EST
E. Coli detect LSTMUG	ND	ND	ND
E. Coli detect LSTMUG	ND	ND	ND
Listeria	ABSENT	ABSENT	ABSENT
Salmonella	ABSENT	ABSENT	ABSENT

ND = not detected.

Table 10: Microbiology of FeLF jellies

Test description	**Control (cfu/ml)**	**Pressure treated (cfu/ml)**
Total count acid tolerant (°C)	500 molds	<10
B. Cereus Confirmed count	<10 EST	<10 EST
Coliform counts by direct plating	< 1 EST	< 1 EST
Confirmed coagulase +ve staphylococci	< 10 EST	< 10 EST
Yeast and molds counts	<1 EST	<1 EST
Aerobic Plate count	<10 EST	<10 EST
Clostridia perfringens counts	<10 EST	<10 EST
Mesophilic spores	<10 EST	<10 EST
Thermophiles	<10 EST	<10 EST
E. Coli detect LSTMUG	ND	ND
E. Coli detect LSTMUG	ND	ND
Listeria	ABSENT	ABSENT
Salmonella	ABSENT	ABSENT

ND = not detected.

EXAMPLE 6

[0206] Carbonated beverages prepared as outlined in Figure 4 containing iron-saturated freeze-dried lactoferrin powder at 4 mg/ml. The beverages were challenged with coliforms and yeast and moulds before pressure treatment. The beverages were left untreated or were pressure treated. Lactoferrin content in the beverages was analysed by HPLC (Palmano & Elgar, 2000) and ELISA. Results show that pressure treatment had little effect on quantifiable lactoferrin but it reduced coliform and yeast and mould and APC counts by more than 5 logs (Table 11).

Table 11: Microbial analysis of FeLF carbonated drink formulated at different pH and challenged with coliforms and yeast and moulds

Sample Description	**Coliforms (cfu/mL)**	**Yeast & mould (cfu/mL)**	**APC (cfu/mL)**	**FeLF (mg/mL) (HPLC)**	**FeLF (mg/mL) (ELISA)**
pH 4.5 Unprocessed	8.0×10^5	$< 10^5$	1.5×10^6	3.74	2.49
pH 4.5 600 MPa/3 min	ND	ND	ND	3.81	1.83
pH 6.0 Unprocessed	1.6×10^6	1.0×10^5	1.7×10^5	3.79	2.36
pH 6.0 600 MPa/3 min	ND	ND	ND	3.74	2.12

ND =Not detected

INDUSTRIAL APPLICATION

[0207] The methods of the present invention have utility in preparing metal ion lactoferrin compositions for the food and health industries. The compositions and products produced according to the invention have a number of health benefits and therapeutic applications, as described above.

[0208] Those persons skilled in the art will understand that the above description is provided by way of illustration only and that the invention is not limited thereto.

REFERENCES

Ainscough E W, Brodie A M, & Plowman J E. The chromium, manganese, cobalt and copper complexes of human lactoferrin. Inorganica Chimica Acta (1979) 149-153.

Baker E, Baker H M, Kidd R D. Functional variations on a common structural framework Biochem.Cell Biol. (2002) 80, 27-34.

Bates G W & Schlabach M R. The reaction of ferric salts with transferrin. J. Biological Chem. (1973) 248 (9) 3228-3232.

Bates G W, Billups C, Saltman P. The kinetics and mechanism of iron (III) exchange between chelates and transferrin. I. The complexes of citrate and nitrilotriacetic acid. J. Biological Chem. (1967). 242 (12) 2810-2815.

Bethell D R, Huang J. Recombinant human lactoferrin treatment for global health issues: iron deficiency and acute diarrhea. Biometals (2004)17 (3) 337-42.

Bluard-Deconinck J-M, Williams J, Evans RW, Van Snick J, Osinski PA, Masson PL. Iron-binding fragments from the N-terminal and C-terminal regions of human lactoferrin. Biochem J. (1978) 171, 321-327.

Bowie J U, Reidhaar-Olson JF, Lim WA, Sauer RT. Deciphering the message in protein sequences: tolerance to amino acid substitutions. Science. (1990) 247(4948):1306-10.

Brock JH, Arzabe FR (1976), Cleavage of diferric bovine transferrin into two monoferric fragments. FEBS Lett 69, 63-66.

Cadwell, R.C. and G.F. Joyce. Randomization of genes by PCR mutagenesis. PCR Methods Appl. . 1992;2: 28-33.

Chiang L-HC & Mao FCH (2002). Production of trivalent chromium complex compound useful for preparing milk product for controlling blood sugar involves addition of lactoferrin or whey protein to a trivalent chromium compound. GB 2366799 A.

Cornish J, Callon K E, Naot D, Palmano K P, Banovic T, Bava U, Watson M, Lin J-M, Tong, PC, Chen Q, Chan VA, Reid HE, Fazzalari N, Baker HM, Baker EN, Haggarty NW, Grey,AB, Reid IR.

Lactoferrin is a potent regulator of bone cell activity and increase bone formation in vivo. Endocrinology (2004) 145, 4366-4374.

Dominguez E, Perez M D, Puyol P, Sanchez L, Calvo M, Effect of pH on antigen-binding activity of IgG from bovine colostrum upon heating, *Journal of Dairy Research* 2001 (68) 511-518.

Dousako S, Tanaka M, Tatsumi K, Tojima T (1991). Preparation of iron fortified beverages containing lactoferrin. EP 454084.

Dugas B, Goulard S et al.(2001). Composition containing an iron complexing protein and a precursor of nitrogen monoxide metabolism and/or a chemical donor of nitrogen monoxide and uses thereof. EP 1303296 A1 030423.

Facon MJ, Skura BJ. Antibacterial activity of lactoferricin, lysozyme and EDTA against Salmonella enteritidis. Int.Dairy J. 1996, 6 (3) 303-13.

Felipe X, Capellas M, Law A J R, Comparison of the effects of high-pressure treatment and heat pasteurization on the whey proteins in goat's milk, *Journal of Agricultural and Food Chemistry* 1997, 45, 627.

Foley A A, Bates G W. The purification of lactoferrin from human whey to batch extraction Analytica lBiochemistry 1987, 162 (1) 296-300.

Groves M L. The isolation of a red protein from milk. J. American Chem. Soc. (1960) 82, 3345-3350.

Huang N, Huang J, Bethall D, Deeter SE, Berman RJ (2004). Lactoferrin protein with iron for treating iron deficiency and anaemia. WO 200460392 A1.

Huffman L M, Processing whey protein for use as a food ingredient, *Food Technology* 1996, 50(2), 49-52.

Huppertz T, Kelly A L, P.F. Fox, Effects of high pressure on constituents and properties of milk., *International Dairy Journal*, 2002, 12, 561-572.

Indyk H E, and Filonzi E L, Determination of Lactoferrin in Bovine Milk, Colostrum and Infant Formula by Optical Biosensor Analysis, *International Dairy Journal*, May 2005, 15, in press, available on-line at http://www.sciencedirect.com from 28 January 2005.

International Dairy Federation, “Whey. Proceedings of the Second International Whey Conference”, 1998, Chicago, Illinois, USA.

Karthikeyan S, Sujata Sharma, Ashwani K. Sharma, M. Paramasivam, Savita Yadav, A. Srinivasan and Tej P. Singh, Structural variability and functional convergence in lactoferrins, Current Science (1999) 77(2):241

Kawakami H et al. Effect of lactoferrin on iron solubility under neutral conditions. BiosScience Biotech Biochem 1993; 57(8) 1376-1377.

Kimmerlin T, Seebach D. 100 years of peptide synthesis: ligation methods for peptide and protein synthesis with applications to beta-peptide assemblies. J Pept Res. 2005 Feb;65(2):229-60.

Korhonen H, Pihlanto-Leppala A, Rantamaki P, Tupasela T, Impact of processing on bioactive proteins and peptides, *Trends in Food Science and Technology* 1998, 9, 307-319.

Law, B. A. and Reiter, B., The isolation and bacteriostatic properties of lactoferrin from bovine milk whey. J. Dairy Res. (1977) 44:595-599.

Legrand D, Mazurier J, Metz-Boutigue M-H, Jolles J, Jo;;es P, Montreuil J & Spik G (1984). Characterization and localization of an iron-binding 18-kDa glycopeptide isolated from the N-terminal half of human lactotransferrin. Biochimica et Biophysica acta 787, 90-96.

Leung, D W, Chen, E.Y., Goeddel, D.V. A Method for Random Mutagenesis of a Defined DNA Segment Using a Modified Polymerase Chain Reaction. Technique 1989; 1:11-15.

Li-Chan E, Kummer A, Losso J N, Kitts D D, Nakai S, Stability of bovine immunoglobulins to thermal treatment and processing, *Food Research International* 1995 (28) 9-16.

Lund, Barbara, M.; Baird-Parker, Tony C.; Gould, Grahame W. (Eds). Microbiological Safety and Quality of Food, Volumes 1-2, (2000) Springer – Verlag.

Manderson GA, Hardman MJ & Creamer LK. Effect of heat treatment on the conformation and aggregation of beta-lactoglobulin A, B, and C. J.Agric. Food Chem. (1998) 46, 5052-5061.

Masson PL, Heremans JF. Studies on lactoferrin, the iron-binding protein of secretions. Protides of the Biological Fluids 1966, 14, 115-24.

Masuda T, Rehinarudo H Y, Suzuki K, Sakai T, Morichi T, The effect of high hydrostatic pressure treatment on the preservability and the immunological activity of bovine colostrum, *Asian-Australasian Journal of Animal Sciences* 2000 (13) 1323-1328.

McGinnis S., & Madden T.L., (2004) "BLAST: at the core of a powerful and diverse set of sequence analysis tools." Nucleic Acids Res. 32:W20-W25.

Medeiros DM, Plattner A, Jennings D, Stoecker B. Bone morpholgy, strength and density are compromised in iron-deficient rats and excerbated by calcium restriction. J. Nutrition (2002)132, 3135-3141.

Metz-Boutigue MH, Jolles J, Mazurier J, Schoentgen F, Legrand D, Spik G, Montreuil J, Jolles P. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. Eur J Biochem. 1984;145(3):659-76.

Nguyen LT, Schibli DJ, Vogel J. Structural studies and model membrane interactions of two peptides derived from bovine lactoferricin. Journal of Peptide Science 2005, 11 (7) 379-89.

Nitsche D (1991). Use of lactoferrin, optimally containing iron - for treatment of endo-toxaemia associated with for example, sepsis, gastrointestinal disorders, antibiotic therapy and liver disorders. DE 4008033.

Oguchi S, Walker WA, Sanderson IR. Iron saturation alters the effect of lactoferrin on the proliferation and differentiation of human enterocytes (Cac0-2 cells). Biology of the Neonate (1995) 67 (5) 330-9.

Palmano K P, and Elgar D E, Detection and quantification of lactoferrin in bovine whey samples by reverse-phase high-performance liquid chromatography on polystyrene-divinylbenzene, *Journal of Chromatography* A, 2002, 947, 307-311

Parry RM, Brown EM. Lactoferrin conformation and metal binding properties. In Advances in Experimental Medicine and Biology (1974) 48, 141-160.

Pierce A, Colavizza D, Benaissa M, Maes P, Tartar A, Montreuil J, Spik G. Molecular cloning and sequence analysis of bovine lactotransferrin. Eur J Biochem. 1991; 196(1):177-84.

Sakurai T, Uchida T, Sato K (2000). Iron-enhanced beverage, food products and feeds. JP 2000014355

A.

Sambrook, J; Fritsch E F; Maniatis T, (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Lab Press, Cold Spring Harbour, New York.

Sienkiewicz T, and Riedel C (Eds) "Whey and whey utilisation" (Verlag, Germany, 1990).

Steijns JM & van Hooijdonk ACM. Occurrence, structure, biochemical properties and technological characteristics of lactoferrin. British J Nutrition (2000) 84, S11-S17.

Stemmer WP. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc Natl Acad Sci USA 1994;91:10747–10751.

Superti F, Siciliano R, Rega B, Giansanti F, Valenti P, Antonini G (2001) Involvement of bovine lactoferrin metal saturation, sialic acid and protein fragments in the inhibition of rotavirus infection. Biochim Biophya Acta 1528 (2-3) 107-15.

Tanaka M, Toshima T, Dosemari S, Tatsumi K (1991). Production of iron enriched drink. JP 3130060 A.

Tatusova, T.A., Madden, T.L., "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. (1999) 174:247-250.

Tomita M, Takase, M, Wakabayashi H & Bellamy W (1994) Antimicrobial Peptides of Lactoferrin in Lactoferrin Structure and Function, pp209-218. Eds TW Hutchens, SV Rumball, B Lonnerdal, Plenum Press, New York.

Tomita M, Tamura Y, Saito H, Nagameguri Y (1992). Iron agent, production of thereof and production of iron enriched food. JP 4141067.

Tomita M, Wakabayashi H, Yamauchi K, Teraguchi S, Hayasawa H; Bovine lactoferrin and lactoferricin derived from milk: production and applications. Biochemistry and Cell Biology 2002, 80 (1) 109-12.

Tomita MY, Tamura YY, Miyakawa HK, Saito HK, Abe HY, Nagao ES (1994). Method for heat treatment of lactoferrin without losing physiological activities thereof. US 5340924.

Tonello C, Largeteau A, Jolibert F, Deschamps A, Demazeau G, Pressure effect on microorganisms and immunoglobulins of bovine colostrum, *High Pressure and Biotechnology*, Eds. C. Balny, R. Hayashi, K. Heremans, P. Masson. 1992 (224) 249-254.

Tsuji S, Hirata Y, Matsuoka K. Two apparent molecular forms of bovine lactoferrin. J Dairy Sci. 1989;72(5):1130-6.

Tweedie J W, Bain HB, Day CL, Nicholson HH, Mead PL, Sheth B, Stowell, KM. Lactoferrin cDNA. Expression and In Vitro Mutagenesis. Advances in Experimental Medicine and Biology (1994) 357, 197-208.

Valenti P, Torcia F, Berlutti F, Pacifici E, Ebano V, Moscarini M & Paesano R. Oral administration of lactoferrin increases hemoglobin and sideremia in pregnant women. 7th International Conference on Lactoferrin: Structure, Functions and Applications. Honolulu, Hawaii, October 16-19, 2005.

van der Kraan MIA, Groenink J, Nazmi K, Veerman ECI,. Bolscher JGM, Nieuw Amerongen AV. Lactoferrampin: a novel antimicrobial peptide in the N1-domain of bovine lactoferrin. Peptides 2004, 25 (2) 177-83.

van Veen H A, Geerts M E, van Berkel P H, Nuijens J H. The role of N-linked glycosylation in the protection of human and bovine lactoferrin against tryptic proteolysis. Eur. J. Biochem. (2004) 271(4): 678-684.

Viejo-Diaz M, Andrés MT, Pérez-Gil J, Sánchez M, Fierro J F. Potassium Efflux Induced by a New Lactoferrin-Derived Peptide Mimicking the Effect of Native Human Lactoferrin on the Bacterial Cytoplasmic Membrane. Biochemistry (Moscow) 2003, 68 (2) 217 – 27.

Yoshida S, and Xiuyn, Ye. Isolation of Lactoperoxidase and Lactoferrins from Bovine Milk Acid Whey by Carboxymethyl Cation Exchange Chromatography. J Dairy Sci. 1991; 74:1439-1444.

Zadow J G (Ed) “Whey and Lactose Processing” (Elsevier Applied Science, London and New York, 1992).

WHAT WE CLAIM IS:

1. A method of treating a composition to maintain or increase its keeping quality comprising

   (a) providing a composition comprising metal ion lactoferrin or a metal ion functional variant or fragment thereof, and

   (b) subjecting the composition to a pressure treatment at a treatment pressure that will prevent the growth of unwanted microorganisms that may be present in the composition.

2. A method of treating a composition to maintain or increase its keeping quality comprising

   (a) providing a composition comprising metal ion lactoferrin or a metal ion functional variant or fragment thereof,

   (b) selecting a treatment pressure that will prevent the growth of unwanted microorganisms that may be present in the composition, and

   (c) subjecting the composition to a pressure treatment at the treatment pressure.

3. A method of treating a composition to maintain or increase its keeping quality comprising

   (a) providing a composition comprising metal ion lactoferrin or a metal ion functional variant or fragment thereof and having a pH of from about 3.0 to 8.0, and

   (b) subjecting the composition to a pressure treatment at a treatment pressure of from about 350 MPa to about 1000 MPa.

4. A method of treating a composition to maintain or increase its keeping quality comprising

(a) providing a composition comprising metal ion lactoferrin or a metal ion functional variant or fragment thereof and one or more unwanted microorganisms and having a pH of from about 3.0 to about 8.0, and

(b) selecting a treatment pressure of from about 350 MPa to 1000 MPa that will prevent the growth of the unwanted microorganisms and retain at least a desired level of metal ions bound to the lactoferrin or functional variant or fragment thereof, and

(c) subjecting the composition to a pressure treatment at the treatment pressure.

5. A method of any one of claims 1 to 4 wherein the composition consists essentially of or consists of metal ion lactoferrin or a metal ion functional variant or fragment thereof.

6. A method of any one of claims 1 to 4 wherein the composition comprises about 0.1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof.

7. A method of any one of claims 1 to 4 wherein the composition comprises about 1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof.

8. A method of any one of claims 1 to 7 wherein the metal ion is selected from the group consisting of aluminium ions, calcium ions, copper ions, chromium ions, cobalt ions, gold ions, iron ions, manganese ions, magnesium ions, platinum ions, ruthenium ions, selenium ions, or zinc ions, or a mixture thereof.

9. A method of any one of claims 1 to 7 wherein the metal ion is iron.

10. A method of any one of claims 1 to 9 wherein the lactoferrin is sheep, goat, pig, mouse, water buffalo, camel, yak, horse, donkey, llama, bovine, or human lactoferrin, or a mixture thereof.

11. A method of any one of claims 1 to 10 wherein the lactoferrin is recombinant, synthetic, or natural lactoferrin, or a mixture thereof.

12. A method of any one of claims 1 to 11 wherein the metal ion lactoferrin or a metal ion functional variant or fragment thereof is at least about 5 % metal ion saturated.

13. A method of any one of claims 1 to 11 wherein the metal ion lactoferrin or a metal ion functional variant or fragment thereof is at least about 10 % metal ion saturated.

14. A method of any one of claims 1 to 11 wherein the metal ion lactoferrin or a metal ion functional variant or fragment thereof is at least about 20 % metal ion saturated.

15. A method of any one of claims 1 to 11 wherein the metal ion lactoferrin or a metal ion functional variant or fragment thereof is at least about 50 % metal ion saturated.

16. A method of any one of claims 1 to 11 wherein the metal ion lactoferrin or a metal ion functional variant or fragment thereof is at least about 100 % metal ion saturated.

17. A method of any one of claims 1 to 11 wherein the composition comprises a mixture of metal ion lactoferrin and at least one metal ion functional variant or fragment thereof.

18. A method of any one of claims 1 to 17 wherein the pressure is from about 350 to 850 MPa.

19. A method of any one of claims 1 to 17 wherein the pressure is from about 350 to 800 MPa.

20. A method of any one of claims 1 to 17 wherein the pressure is from about 350 to 750 MPa.

21. A method of any one of claims 1 to 17 wherein the pressure is from about 350 to 700 MPa.

22. A method of any one of claims 1 to 17 wherein the pressure is from about 350 to 650 MPa.

23. A method of any one of claims 1 to 17 wherein the pressure is from about 400 to 800 MPa.

24. A method of any one of claims 1 to 17 wherein the pressure is from about 400 to 700 MPa.

25. A method of any one of claims 1 to 17 wherein the pressure is from about 400 to 600 MPa.

26. A method of any one of claims 1 to 17 wherein the pressure is from about 500 to 800 MPa.

27. A method of any one of claims 1 to 17 wherein the pressure is from about 500 to 700 MPa.

28. A method of any one of claims 1 to 17 wherein the pressure is from about 500 to 600 MPa.

29. A method of any one of claims 1 to 17 wherein the pressure is from about 550 to 650 MPa.

30. A method of any one of claims 1 to 29 wherein the pH of the composition is from about 3.3 to 8.0.

31. A method of any one of claims 1 to 29 wherein the pH of the composition is from about 4.0 to 8.0.

32. A method of any one of claims 1 to 29 wherein the pH of the composition is from about 4.5 to 8.0.

33. A method of any one of claims 1 to 29 wherein the pH of the composition is from about 5.0 to 8.0.

34. A method of any one of claims 1 to 33 wherein the composition is subjected to the treatment pressure before packaging.

35. A method of any one of claims 1 to 33 wherein the composition is subjected to the treatment pressure after packaging.

36. A method of any one of claims 1 to 35 wherein the composition comprises recombined or fresh whole milk, recombined or fresh skim milk, reconstituted whole or skim milk powder, skim milk concentrate, skim milk isolate, whole or skim milk powder, skim milk retentate, concentrated milk, buttermilk, ultrafiltered milk retentate, milk protein concentrate (MPC), milk protein isolate (MPI), calcium depleted milk protein concentrate, calcium depleted milk protein isolate, low fat milk, low fat milk protein concentrate, low fat milk protein isolate, colostrum, a colostrum fraction, colostrum protein concentrate (CPC), colostrum milk protein concentrate, colostrum milk protein isolate, colostrum whey, colostrum whey protein concentrate, colostrum whey protein isolate, an immunoglobulin fraction from colostrum, whey, whey protein concentrate (WPC), whey protein isolate (WPI), sweet whey, lactic acid whey, mineral acid whey, reconstituted whey powder, hyperimmune milk, hyperimmune milk protein concentrate, hyperimmune milk protein isolate, hyperimmune whey, hyperimmune whey protein concentrate, hyperimmune whey protein isolate, hyperimmune colostrum, hyperimmune colostrum milk protein concentrate, hyperimmune colostrum milk protein isolate, hyperimmune colostrum whey, hyperimmune colostrum whey protein concentrate, hyperimmune colostrum whey protein isolate, a composition derived from any milk or colostrum processing stream, a composition derived from the retentate or permeate obtained by ultrafiltration or microfiltration of any milk or colostrum processing stream, or a composition derived from the breakthrough or adsorbed fractions obtained by chromatographic separation of any milk or colostrum processing stream, or a full or partial hydrolysates of any of these compositions, or a mixture thereof.

37. A method of any one of claims 1 to 36 wherein the composition is a beverage, an acidified beverage, a neutral beverage, a carbonated beverage, a yogurt or a jelly.

38. A composition treated according to a method of any one of claims 1 to 37.

39. A composition of claim 38 that is a beverage.

40. A composition of claim 38 that is an acidified beverage

41. A composition of claim 38 that is a neutral beverage.

42. A composition of claim 38 that is a carbonated beverage.

43. A composition of claim 38 that is a yogurt

44. A composition of claim 38 that is a jelly.

45. A pressure-treated composition comprising about 0.1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms.

46. A pressure-treated jelly comprising about 0.1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms.

47. A pressure-treated yoghurt comprising about 0.1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms.

48. A pressure-treated beverage comprising about 0.1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms.

49. A pressure-treated composition comprising about 1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms.

50. A pressure-treated jelly comprising about 1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms.

51. A pressure-treated yoghurt comprising about 1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms.

52. A pressure-treated beverage comprising about 1 mg/ml to 1000 mg/ml of metal ion lactoferrin or a metal ion functional variant or fragment thereof and less than about 50,000 cfu/ml of microorganisms.

53. A composition of any one of claims 45 to 52 wherein the metal ion is iron.

54. A composition of any one of claims 45 to 53 wherein the metal ion lactoferrin or a metal ion functional variant or fragment thereof is at least about 20% metal ion saturated.

55. A composition of any one of claims 45 to 53 wherein the metal ion lactoferrin or a metal ion functional variant or fragment thereof is at least about 40% metal ion saturated.

56. A composition of any one of claims 45 to 53 wherein the metal ion lactoferrin or a metal ion functional variant or fragment thereof is at least about 60% metal ion saturated.

57. Use of a composition of claim 38 in the manufacture of a food, drink, food additive, drink additive, dietary supplement, nutritional product, medical food, nutraceutical, medicament or pharmaceutical.

58. A use of claim 39 for stimulating skeletal growth, inhibiting bone resorption, stimulating chondrocyte proliferation, stimulating osteoblast proliferation, inhibiting osteoclast development, or treating or preventing a skeletal, joint or cartilage disorder.

59. A use of claim 39 for inhibiting tumour formation in a subject, inducing apoptosis in a subject, inducing apoptosis of tumour cells in a subject, inhibiting angiogenesis in a subject, inhibiting tumour angiogenesis in a subject, maintaining or improving one or both of the white blood cell count and red blood cell count in a subject, stimulating the

immune system in a subject, increasing the production of Th1 and Th2 cytokines within a tumor in a subject, increasing the production of Th1 and Th2 cytokines within the intestine of a subject, increasing the level of Th1 and Th2 cytokines in the systemic circulation of a subject, increasing an anti-tumour immune response in a subject, increasing the responsiveness of a subject to a cancer therapy, or increasing the responsiveness of a tumour in a subject to a cancer therapy.

60. A use of claim 39 for treating or preventing iron-deficiency anaemia or the iron-deficiency associated with pregnancy, for increasing haemoglobin count in anaemia sufferers or for gut renewal therapies for malabsorption.

61. A method of stimulating skeletal growth, inhibiting bone resorption, stimulating chondrocyte proliferation, stimulating osteoblast proliferation, inhibiting osteoclast development, or treating or preventing a skeletal, joint or cartilage disorder comprising administration of a composition of claim 38 to a subject in need thereof.

62. A method of inhibiting tumour formation in a subject, inducing apoptosis in a subject, inducing apoptosis of tumour cells in a subject, inhibiting angiogenesis in a subject, inhibiting tumour angiogenesis in a subject, maintaining or improving one or both of the white blood cell count and red blood cell count in a subject, stimulating the immune system in a subject, increasing the production of Th1 and Th2 cytokines within a tumor in a subject, increasing the production of Th1 and Th2 cytokines within the intestine of a subject, increasing the level of Th1 and Th2 cytokines in the systemic circulation of a subject, increasing an anti-tumour immune response in a subject, increasing the responsiveness of a subject to a cancer therapy, or increasing the responsiveness of a tumour in a subject to a cancer therapy comprising administration of a composition of claim 38 to a subject in need thereof.

63. A method of treating or preventing iron-deficiency anaemia or the iron-deficiency associated with pregnancy, for increasing haemoglobin count in anaemia sufferers or for gut renewal therapies for malabsorption, the method comprising administration of a composition of claim 38 to a subject in need thereof.

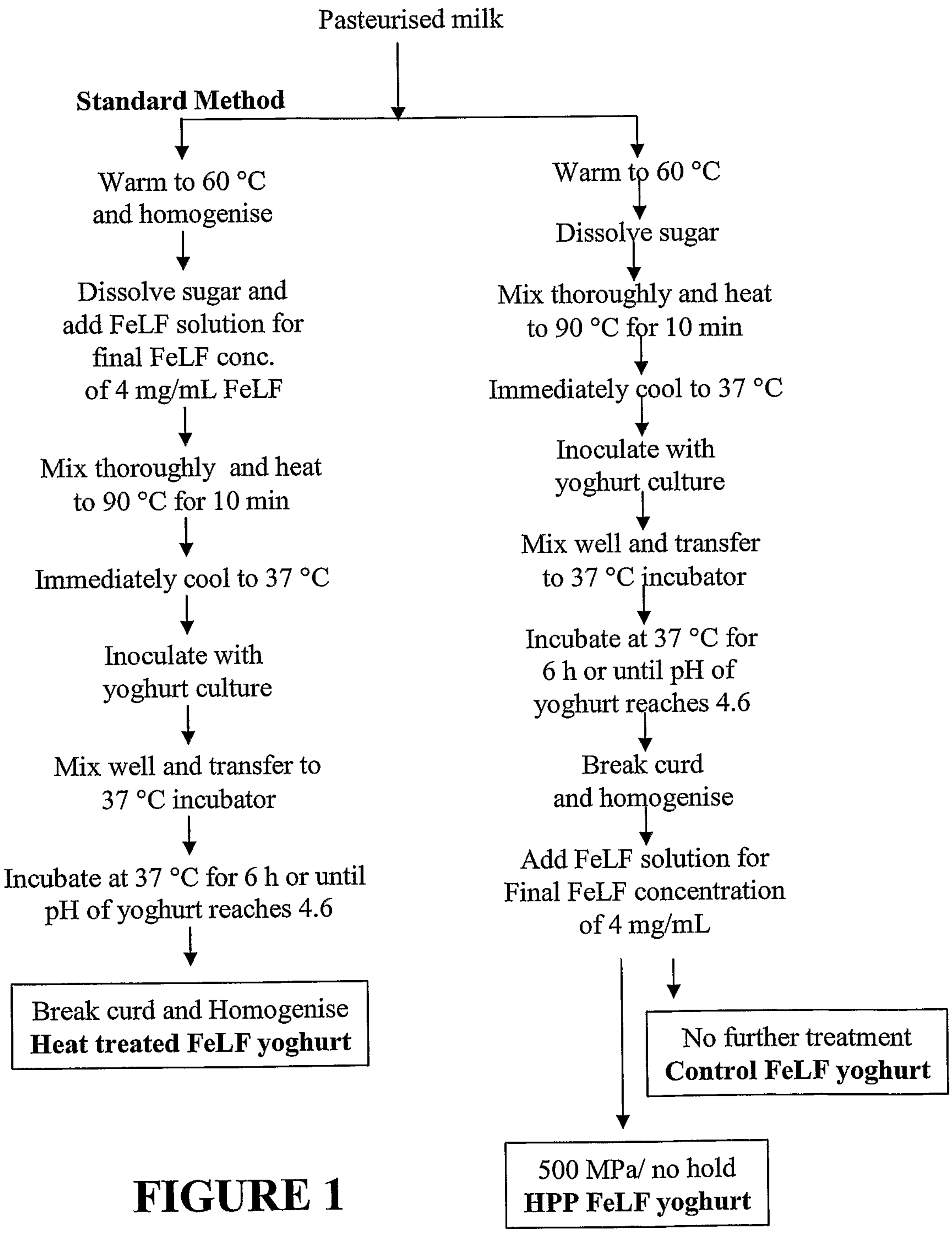
Pasteurised milk
Standard Method
Warm to 60 °C and homogenise
Dissolve sugar and add FeLF solution for final FeLF conc. of 4 mg/mL FeLF
Mix thoroughly and heat to 90 °C for 10 min
Immediately cool to 37 °C
Inoculate with yoghurt culture
Mix well and transfer to 37 °C incubator
Incubate at 37 °C for 6 h or until pH of yoghurt reaches 4.6
Break curd and Homogenise
Heat treated FeLF yoghurt
Warm to 60 °C
Dissolve sugar
Mix thoroughly and heat to 90 °C for 10 min
Immediately cool to 37 °C
Inoculate with yoghurt culture
Mix well and transfer to 37 °C incubator
Incubate at 37 °C for 6 h or until pH of yoghurt reaches 4.6
Break curd and homogenise
Add FeLF solution for Final FeLF concentration of 4 mg/mL
No further treatment
Control FeLF yoghurt
500 MPa/ no hold
HPP FeLF yoghurt

FIGURE 1

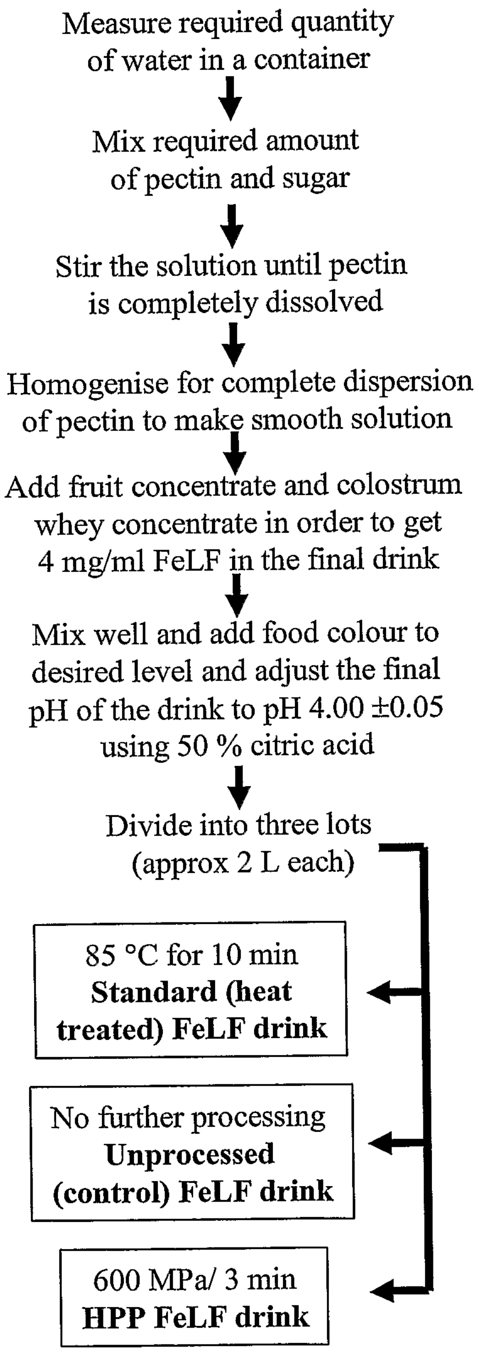

FIGURE 2

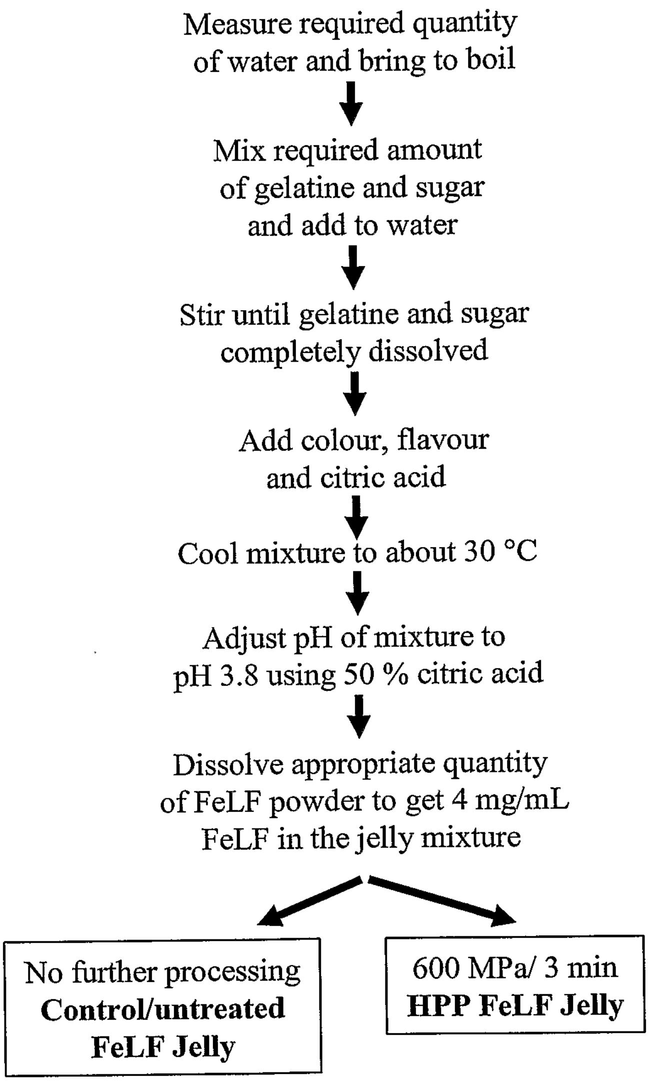

FIGURE 3

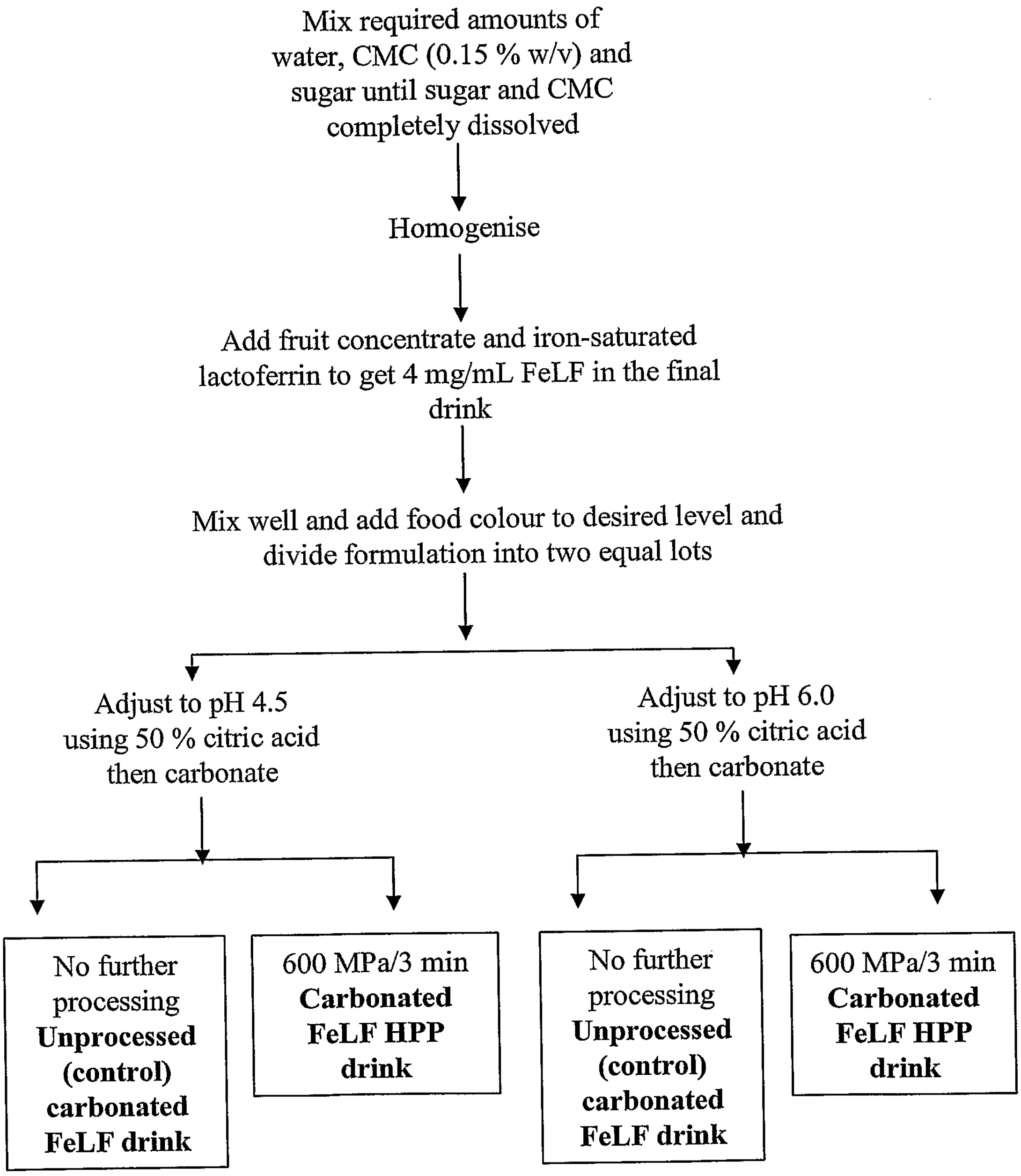
Mix required amounts of water, CMC (0.15 % w/v) and sugar until sugar and CMC completely dissolved
Homogenise
Add fruit concentrate and iron-saturated lactoferrin to get 4 mg/mL FeLF in the final drink
Mix well and add food colour to desired level and divide formulation into two equal lots
Adjust to pH 4.5 using 50 % citric acid then carbonate
Adjust to pH 6.0 using 50 % citric acid then carbonate
No further processing **Unprocessed (control) carbonated FeLF drink**
600 MPa/3 min **Carbonated FeLF HPP drink**
No further processing **Unprocessed (control) carbonated FeLF drink**
600 MPa/3 min **Carbonated FeLF HPP drink**

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2006/000038

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A23L 3/015 (2006.01)	*A23C 23/00* (2006.01)	*A61P 7/06* (2006.01)
A01N 1/00 (2006.01)	*A23J 3/08* (2006.01)	*A61P 19/00* (2006.01)
A23C 3/00 (2006.01)	*A23L 1/025* (2006.01)	*A61P 35/00* (2006.01)
A23C 9/00 (2006.01)	*A61K 38/40* (2006.01)	*C07K 14/79* (2006.01)
A23C 21/00 (2006.01)	*A61L 2/02* (2006.01)	

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, CAPLUS, MEDLINE, FSTA, JAPIO; Keywords: protein, lactoferrin, transferrin, iron binding protein, pressure, treat, sterilise, ultra high pressure, UHP, microbe, bacteria, microorganism, probiotic, preserve, disinfect, shelf life, keeping quality, activity, function, bioactivity.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 4218360 A (SNOW BRAND MILK PROD CO LTD) 07 August 1992 See abstract, pages 1-2, Examples 1-3.	1-56
X	MASSCHALCK B et.al.: 'High Pressure increases bactericidal activity and spectrum of lactoferrin, lactoferricin and nisin.' INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, 2001, Vol 64, No 3, pages 325-332. See abstract, Figure 2, pages 325-329.	1-56

☒ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:

"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 31 May 2006	Date of mailing of the international search report 9 JUN 2006
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **GAYE HOROBIN** Telephone No : (02) 6283 2069

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Derwent Abstract Accession No. 2003-407579/39, Class D13, JP 2003009760 A (NIPPON SHOKUHIN KK) 14 January 2003. See whole abstract.	1-56
X	WO 2000/048641 A1 (BBI BIOSEQ INC) 24 August 2000 See abstract, Examples 4, 6, 12, p34 last paragraph, claims 8-10.	1-56
X	US 2003/0103863 A1 (GRISLAIN ET AL) 5 June 2003 See whole document.	1-56
X	Derwent Abstract Accession No. 1999-396139/34, Class D12, DE 19801031 A1 (KORTSCHACK F) 15 July 1999. See whole abstract.	1-56
X	PUIG A ET.AL.: 'Microbiological and biochemical stabilization of wines using high-pressure technique.' BULLETIN DE L'O.I.V., 2003, Vol 76, No 869-870, pages 596-617. See abstract, page 605 paragraph 4.	1-56
A	VARDAG T & KOERNER P: 'High pressure: a real alternative in food processing.' FOOD MARKETING & TECHNOLOGY, February 1995, Vol 9, No 1, pages 42-47. See pages 1-2.	

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **57-63**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
They relate to such a large number of possible uses that a meaningful search can not be carried out, and the description lacks support for such a large number of uses.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See separate sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-56

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: III

This International Searching Authority has found that there are different inventions as follows:

1. Claims 1-37. It is considered that the method of pressure treating a composition comprising metal ion lactoferrins or variants or fragments thereof to increase keeping quality while preserving the activity of the lactoferrins comprises a first distinguishing feature.
2. Claim 37-56. It is considered that pressure treated lactoferrin compositions produced from the above method comprises a second distinguishing feature.
3. Claims 57-63. It is considered that uses of pressure treated lactoferrin compositions comprises a third distinguishing feature.

Prima facie the claims all share the feature of pressure treatment of compositions comprising lactoferrin. However the examiner was aware of the following prior art document which anticipates this feature:

JP 4218360 A (SNOW BRAND MILK PROD CO LTD) 07 August 1992.

As such, this feature cannot be considered a special technical feature. Therefore there is no special technical feature common to all the claims.

Since the abovementioned groups of claims do not share any of the special technical features identified, a "technical relationship" between the inventions, as defined in PCT rule 13.2 does not exist. Accordingly the international application does not relate to one invention or to a single inventive concept, *a posteriori.*

NOTE: It was considered that the search and examination for the second invention would not require more than a little additional search and examination effort over that for the first invention, and therefore both the first and second inventions have been fully searched. However it is considered that search and examination for the third invention would require more than a little additional search and examination effort over that for the first invention, and therefore an additional search fee is warranted.

Because no additional search fees were paid, the International Search Report and the Written Opinion of the International Searching Authority was restricted to the first and second inventions claimed in claims 1-56.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/NZ2006/000038

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
JP	4218360						
JP	2003009760						
WO	0048641	AU	46843/99	AU	2002367749	CA	2301067
		CA	2455898	EP	1112091	EP	1421185
		US	6270723	US	6696019	US	2002182107
		US	2004151620	WO	02056824	WO	03089015
US	2003103863	AU	31930/01	CA	2398337	EP	1250159
		FR	2804326	WO	0154737		
DE	19801031						

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX