PRODUCT, SUITABLE TO BE GIVEN TO THE HUMAN BODY FOR ITS PROTECTIVE EFFECTS AND HEALTH BENEFITS, BASED ON COW BUFFALO'S MILK SELF-ENRICHED WITH LACTOFERRIN, OR PURE LACTOFERRIN, OBTAINED THROUGH MEMBRANE SEPARATIVE PROCESSES
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OBTAINED THROUGH MEMBRANE SEPARATIVE PROCESSES.

TECHNICAL FIELD

The present invention concerns a nutraceutical having a high content of
lactoferrin that, if ingested, results particularly effective for a protective and benefit use
respect to functions of the human body. This product is obtained starting from cow
buffalo's milk through membrane separative processes.

In fact, it is known that milk and milk-dairy products are essential for the
human nutrition. In the constituents of milk there are proteins, peptides, enzymes and
other substances that are normally given from mother to child during the nursing
period, and that are considered to be essential for health of the newborn baby.

One of these proteins, the lactoferrin, is actually subject of many studies, according to
its important characteristic of being a iron-carrier from intestine to the blood
haemoglobin and to its antimicrobial, antiviral, antinflammatory, and prebiotic action.
Therefore, lactoferrin is a iron-carrier glycoprotein, belonging to the group of
transferrins, and it is contained in the human milk. Furthermore, this molecule is also
contained in milk of other mammals, like in example: catties, pigs, buffalos, goats,
rodents and equines.

The lactoferrin, besides the iron-carrier employed function, is a natural antioxidant and
a powerful booster of natural-killer cells, it has an important role in the defence against
tumours, it controls the granulepoiesis, the cellular cytotoxicity antibody- dependant,
the production of cytokines, and the in vitro growth of some specific cells. It employes
an additional function stimulating the immune defence system, it inhibits the in vivo and in vitro release of histamine from mastcells. The digestion with pepsin produces peptides, having antimicrobial action, so-called lactoferricin.

BACKGROUND ART

In the prior art, some processes are well-known to isolate, from the cattle's milk, proteins from fat and lactose, and therefore to produce on a large industrial scale food supplements and/or pharmacological products. Basically, the actual products on the market belong to three main classes: whey proteins isolated through ionic exchange; concentrated whey proteins; and whey proteins isolated through microfiltration. For each class, an index of quality is defined, essentially in direct ratio to quantity of contained proteins, and in inverse ratio to quantity of fat and lactose content beyond a certain limit. Another important parameter is the cost for protein extraction that defines the final product's price to the market.

Indeed, the highest quality products, having more than 90% of proteins and less than 1% of fat and lactose content, have either a typical high cost as drawback.

DISCLOSURE OF INVENTION

The above situation leads to the nutraceutical product, subject of the present invention, that, besides the high protein content, particularly of lactoferrin, is characterized by a particularly simple, effective and cheap method of production.

Therefore, the main objective of the present invention, is to provide a nutritious drink, having some considerable organoleptic characteristics and a high level of lactoferrin, able to stimulate the immune defence system, to increase the absorption of iron through the intestine, and to represent an alternative to the cow's milk, because it doesn't create phoenomena of intolerance to food.
Another objective is to provide a nutritious drink having the additional characteristic of a lower fat content respect to the cow buffalo’s milk and the same antioxidant power of the cow buffalo’s milk.

A further objective is to provide a method suitable for large-scale industrial production, employing available and cheap components, characterized by reasonable time and costs, and limiting the waste of raw materials.

Therefore, it is specific subject of the present invention a product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo’s milk self-enriched with lactoferrin, or pure lactoferrin, obtained through membrane separative processes, according to two methods of production:

1. cycles of dialysis with different external solutions, keeping the same membrane: wherein the starting solution is always the same inside the membrane and the external solution is renewed in each cycle of dialysis;

2. cycles of dialysis with different membranes, keeping the same external solution: wherein the internal solution, instead, changes time by time in the various cycles of dialysis.

BRIEF DESCRIPTION OF DRAWINGS

The present invention will now be described for illustrative but not limiting purposes, according to its preferred embodiments, with particular reference to the figures of the enclosed drawings, wherein:

figure 1 is a schematic perspective view of a particular, concerning a semi-permeable membrane for dialysis;

figure 2 is a lateral sectioned view of a device for dialysis, enclosing a semi-permeable membrane allowing the transfer of particles from one solution to the other.
figures 3, 4, 5 and 6 are respective lateral sectioned views of a device for dialysis, corresponding to a specific state, related to a first method of lactoferrin extraction;

figure 7 is a graphical view of lactoferrin concentration as a function of the number of cycles, related to a first method of lactoferrin extraction;

figure 8 is a schematic view representing the final phase of mixing, related to a first method of lactoferrin extraction;

figures 9, 10, 11 and 12 are respective lateral sectioned views of a device for dialysis, corresponding to a specific state, related to a second method of lactoferrin extraction;

figure 13 is a lateral sectioned view of a device in the final phase of mixing, related to a second method of lactoferrin extraction;

figure 14 is a graphical view of lactoferrin concentration as a function of the number of cycles, related to a second method of lactoferrin extraction.

BEST MODES FOR CARRYING OUT THE INVENTION

It is herein underlined that, in the following, only some of the many conceivable embodiments of the present invention will be described, and they are just particular examples that do not introduce any limitations, having the possibility to describe many other embodiments based on the disclosed technical solution of the present invention. More in particular, two main methods for extraction of lactoferrin from cow buffalo's milk will be described, the same methods leading to the synthesis of products based on cow buffalo's milk enriched with lactoferrin, or pure lactoferrin. Both methods employ membrane separative processes and, more in particular, extraction methods through dialysis.
The dialysis is a process providing the separation of molecules according to their
dimension, using semi-permeable membranes with pores having a smaller size than
macromolecules. This concept has been useful to separate molecules with different
dimension in the cow buffalo's whey, using a dialysis membrane having pores with a
proper diameter.

In figure 1, an example of cylindrical semi-permeable membrane 100 is illustrated. It
allows some molecules 102, 103 to pass through and, at the same time, prevents
other molecules 101 to do the same thing.

In figure 2, a typical example of a device for dialysis 200 is illustrated, with an external
container 201 containing a first solution 202, and an internal container 203, containing
a second solution 204. The internal container 203 includes a semi-permeable
membrane 100, able to allow the transfer of some molecules from the second solution
204 to the first solution 202.

Both the processes for lactoferrin extraction include a previous phase where the fat
content in the cow buffalo's milk is decreased. This is possible through a milk
centrifuge process at 4000 rpm for 10 minutes. In such a way, the fat concentrates
itself on the upper layer, where is easily removable using a paddle or a spoon.
This quantity of fat will be mixed again in a second time, in a specified measure, to
weigh the final composition (it will correspond to 2-3% of the total, in order to keep
organoleptic characteristics similar to the initial milk).

It is important to underline that the decreased quantity of fat does not change the
antioxidant power of the cow buffalo's milk, because experiments proved that the main
contribution to this parameter is essentially given by whey and not by fat content.

The methods for lactoferrin extraction of the prior art have some difficulties
arising from the final use in food industry. In fact, normally the purification process for
analytical purposes (HPLC analysis, electrophoresis, etc.) requires reactive precipitants and other materials that prevent any possible use for food. Therefore, the definition of an alternative extraction method is not trivial. One possibility is to make the cow buffalo's milk to evaporate at a temperature of 35°C, so that to avoid the denaturation of contained proteins, and at a lower pressure than the environmental one, in order to speed up the process. Unfortunately, this method requires a big quantity of time and energy. Therefore, the method disclosed in the present invention employs a process of dialysis, wherein the molecules having a lower diameter than size of the semi-permeable membrane's pores transfer from the solution with high concentration to the solution with low concentration. More in particular, the membrane is made of cellulose acetate and the solution where lactoferrin concentrates contains bidistilled water. In the different phases of this method, the main objective is to define the detailed optimal conditions of dialysis, in order to achieve as much as possible quantity of lactoferrin with the minimum quantity of required time and energy.

Therefore, two possible ways have been found:

1. cycles of dialysis with external solution renewed at each cycle, keeping the same membrane;
2. cycles of dialysis with different membranes, keeping the same external solution for all the time long.

In a preferred embodiment of the present invention, related to the first case illustrated in figures 3, 4, 5 and 6, the starting solution is always the same inside the membrane and the external solution is renewed at each cycle of dialysis. In the first cycle - figure 3 - one litre of cow buffalo's milk 304 is inserted inside a dialysis tube 302 and is put in communication with 50 mLitres of bidistilled water 303 at 4°C for 30 minutes. At the
end of 30 minutes - figure 4 - the external solution 308 will contain a certain quantity of lactoferrin and diffused salts and can be exchanged - figure 5 - with a new external solution containing other 50 mLitres of bidistilled water 312. At this point, a second cycle of dialysis - figure 6 - will allow the diffusion of an additional quantity of lactoferrin from the internal solution 313 to the external solution 316. In principle, the cycles of dialysis could follow each other for an indefinite time, according to the indicated scheme. However, at each cycle the quantity of extracted lactoferrin decreases logarithmically, following the graphical shape of figure 7. It is clear that the significant quantities of lactoferrin are obtained especially in the first two cycles, with respective values equal to 2.6 and 1.3 times the initial concentration in milk. This information suggests to use just the first two cycles of dialysis and to mix - figure 8 - the respective 50 mLitres of external solutions 502 and 504 in order to obtain 100 mLitres of solution 506 containing approximately two times the quantity of lactoferrin in the initial milk.

Then, the so obtained solution 506 is mixed with new cow buffalo’s milk, where the fat has been previously removed, and finally the resulting solution is reintegrated with fat, up to a quantity nearly to 2-3%.

In such a way a product has been obtained, that is cow buffalo’s milk enriched with lactoferrin and having a quantity of fat extremely lower than the initial milk. The same method can be employed to realize different types of food supplements containing high quantities of lactoferrin.

In case it is required a higher level of enrichment in the previous solution, an alternative method is that illustrated in figures 9, 10, 11 and 12, related to a second preferred embodiment of the present invention, where, in the different cycles of dialysis, the external solution is kept always the same, and the internal solution
changes from time to time. In such a way, the necessary time for lactoferrin extraction is longer respect to the previous method, but the consumption of raw material is approximately 50% less, and it is possible to achieve a higher level of enrichment respect to the previous method.

In the first cycle - figure 9 - 100 mL of cow buffalo's milk 601, with previously fat content removed, through a centrifuge process at 4000 rpm for 10 minutes, are inserted inside a dialysis tube 603 and are put in communication with 100 mL of bidistilled water 604 at 4°C for 30 minutes. At the end of 30 minutes - figure 10 - the external solution 608 will contain a certain quantity of lactoferrin and diffused salts. For cycles following the first one - figure 11 - the external solution is kept the same, and the dialysis membrane 607 is changed with a new one 611, containing 100 mL of new milk with previously removed fat. This process is repeated for 6 different cycles, measuring from time to time the quantity of lactoferrin contained in the external solution. The quantity of extracted lactoferrin increases linearly for each following cycle, according to the graphical shape of figure 14, up to obtain a solution having a high concentration like the one illustrated in figure 13.

The described method requires 6 cycles to enrich the external solution up to factor 2, requiring 3 hours respect to just 1 hour of the previous method, and with a raw material consumption equal to 600 mL of milk. Therefore, the first way allows to achieve quick results, but requiring a higher consumption of raw material, instead the second way allows a limitation of waste and the achievement of a higher level of lactoferrin enrichment in the external solution, but it requires a longer time for process.

Therefore, all the above examples show that the present invention achieves the proposed objectives. In particular, it provides a nutritious drink, having some considerable organoleptic characteristics and a high level of lactoferrin, able to
stimulate the immune defence system, to increase the absorption of iron through the
intestine, and to represent an alternative to the cow's milk, because it doesn't create
phoenomena of intolerance to food.

Then, the present invention provides a nutritious drink having the additional
characteristic of a lower fat content respect to the cow buffalo's milk and the same
antioxidant power of the cow buffalo's milk.

Furthermore, the present invention provides a method suitable for large-scale
industrial production, employing available and cheap components, characterized by
reasonable time and costs, and limiting the waste of raw materials.

The present invention has been described for illustrative but not limitative
purposes, according to its preferred embodiments, but it is clear that modifications
and/or changes can be introduced by those skilled in the art without departing from
the relevant scope, as defined in the enclosed claims.
CLAIMS

1. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to the method characterized by the following steps:

- in a first cycle, a defined quantity of cow buffalo's milk is inserted in a dialysis tube and put in communication with a definite quantity of bidistilled water at a defined temperature and for a defined quantity of time;
- at the end of the abovesaid quantity of time, a defined quantity of lactoferrin and diffused salts have diffused from said cow buffalo's milk to said bidistilled water, constituting outside a first solution concentrated of lactoferrin, that is exchanged with a new and identical quantity of bidistilled water;
- in a second cycle, at a defined temperature and for a defined quantity of time, an additional quantity of lactoferrin and diffused salts have diffused from said cow buffalo's milk to said new bidistilled water, constituting outside a second solution concentrated of lactoferrin;
- a series of cycles, identical to the previous ones, follow each other for a defined number of times, up to achieve N solutions concentrated of lactoferrin obtained from the method (one for each cycle);
- the abovesaid N solutions concentrated of lactoferrin are mixed in a new container in order to obtain a unique final solution, having a higher level of lactoferrin concentration respect to the initial concentration in the cow buffalo's milk, so that is possible to obtain a nutritious drink, having some considerable organoleptic characteristics and a high level of lactoferrin, having the same antioxidant power of the cow buffalo's milk, able to stimulate the immune defence system, to increase the
absorption of iron through the intestine, and to represent an alternative to the cow's milk, because it doesn't create phenomena of intolerance to food.

2. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to the method of previous claim 1, characterized by the following additional steps:

- a new defined quantity of cow buffalo's milk is submitted to a centrifuge process and the upper layer of fat content is removed;
- said new defined quantity of cow buffalo's milk with fat content removed, and said unique final solution are mixed in a new container in order to obtain cow buffalo's milk self-enriched with lactoferrin;
- a defined quantity of fat is mixed again with said cow buffalo's milk self-enriched with lactoferrin,

so that is possible to obtain a nutritious drink, like the above described one, having the additional characteristic of a lower fat content respect to the cow buffalo's milk.

3. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims, characterized in that said dialysis tube has a semi-permeable membrane composed of cellulose acetate.

4. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims, characterized in that said number of cycles of dialysis N is equal to 2.

5. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin,
obtained according to one or more of the previous claims, characterized in that said
definited quantity of cow buffalo's milk is equal to 1 Litre.

6. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin,
obtained according to one or more of the previous claims, characterized in that said
definite quantity of bidistilled water is equal to 50 mLitres.

7. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin,
obtained according to one or more of the previous claims, characterized in that said
definited temperature is equal to 4°C and that definited quantity of time is equal to 30 minutes.

8. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin,
obtained according to one or more of the previous claims, characterized in that said
definited quantity of fat is equal to 2-3%.

9. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin,
obtained according to one or more of the previous claims, characterized in that said
centrifuge process is realized at 4000 rpm for 10 minutes.

10. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to the method characterized by the following steps:

- a definited quantity of cow buffalo's milk is submitted to a centrifuge process and the upper layer of fat content is removed;
- in a first cycle, said defined quantity of cow buffalo's milk is inserted in a dialysis tube and put in communication with a definite quantity of bidistilled water at a defined temperature and for a defined quantity of time;
- at the end of the abovesaid quantity of time, a defined quantity of lactoferrin and diffused salts have diffused from said cow buffalo's milk to said bidistilled water, constituting outside a solution at a first level of concentration of lactoferrin, then said dialysis tube is exchanged with a new one, containing an identical quantity of cow buffalo's milk with fat content removed;
- a series of cycles, identical to the previous ones, follow each other for a defined number of times, up to achieve a solution obtained from the method, having a level N (number of cycles) of concentration of lactoferrin; so that is possible to obtain a nutritious drink, having some considerable organoleptic characteristics and a high level of lactoferrin, having the same antioxidant power of the cow buffalo's milk, able to stimulate the immune defence system, to increase the absorption of iron through the intestine, and to represent an alternative to the cow's milk, because it doesn't create phoenomena of intolerance to food.

11. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to the method of previous claim 10, characterized by the following additional steps:
- a new defined quantity of cow buffalo's milk is submitted to a centrifuge process and the upper layer of fat content is removed;
- said new defined quantity of cow buffalo's milk with fat content removed, and said solution obtained from the method are mixed in a new container in order to obtain cow buffalo's milk self-enriched with lactoferrin;
- a defined quantity of fat is mixed again with said cow buffalo's milk self-enriched with lactoferrin, so that is possible to obtain a nutritious drink, like the above described one, having the additional characteristic of a lower fat content respect to the cow buffalo's milk.

12. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims from 10 to 11, characterized in that said dialysis tube has a semi-permeable membrane composed of cellulose acetate.

13. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims from 10 to 12, characterized in that said number of cycles of dialysis N is equal to 6.

14. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims from 10 to 13, characterized in that said defined quantity of cow buffalo's milk is equal to 100 mLitres.

15. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims from 10 to 14, characterized in that said definite quantity of bidistilled water is equal to 100 mLitres.

16. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims from 10 to 15,
characterized in that said defined temperature is equal to 4°C and that defined quantity of time is equal to 30 minutes.

17. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims from 10 to 16, characterized in that said defined quantity of fat is equal to 2-3%.

18. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims from 10 to 17, characterized in that said centrifuge process is realized at 4000 rpm for 10 minutes.

19. Product, suitable to be given to the human body for its protective effects and health benefits, based on cow buffalo's milk self-enriched with lactoferrin, or pure lactoferrin, obtained according to one or more of the previous claims and as substantially described and essentially illustrated in the enclosed drawings.
lactoferrin in the external solution respect to the number of cycles of dialysis

\[ y = -1.1942 \ln(x) + 2.4754 \]
\[ R^2 = 0.9312 \]

FIG. 7
FIG. 13
lactoferrin in the external solution respect to the initial milk and increasing the number of cycles of dialysis

\[ y = 0.2772x + 0.3302 \]

FIG. 14
**INTERNATIONAL SEARCH REPORT**

**International application No**
PCT/IT2007/000509

### A CLASSIFICATION OF SUBJECT MATTER

INV. A23C9/142 A61K38/00 C07K14/79

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

### B. RELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K A23C

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 practical, search terms used)

EPO-Internal, WPI Data, FSTA, BIOSIS, COMPENDEX

### C DOCUMENTS CONSIDERED TO BE RELEVANT

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