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(54) Title: COMPOSITION COMPRISING GROWTH FACTORS

(57) Abstract: The invention relates to a composition obtained from goat milk comprising growth factors. This composition is characterised because these growth factor are in concentrations to be physiologically active and because these factors are selected from a list that comprises: Epidermal growth factor (EGF), transforming growth factor ß 2(TGFß2), insulin like growth factor type 1 (IGF-1) and anchored glycoprotein CD-14. The invention also relates to a process for obtaining the compositions of the invention, as well as a food product, a dietetic supplement or a nutritional supplement and their use in the prevention or treatment of diseases, as well as their use in cosmetics.

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# COMPOSITION COMPRISING GROWTH FACTORS

## FIELD OF THE INVENTION

The present invention relates to a composition of growth factors and oligosaccharides from goat milk, to nutritional products containing these oligosaccharides, to a process to obtain that composition, and also to the use of this composition in the preparation of nutritional products and products to be used in the prevention of infections and intestinal disorders.

## BACKGROUND OF THE INVENTION

Milk is nature's designer food to fulfil the nutritional needs for the growth and development of the neonate. Milk, along with lactose (the most important sugar), contains other carbohydrates, such as nucleotide sugars, glycolipids, glycoproteins, and oligosaccharides. Milk oligosaccharides are thought to be beneficial for the breastfed infant with regard to their prebiotic and anti-infective properties. The interest showed by researchers for oligosaccharides started recently after observing that these could promote bifidogenic flora in children fed with breast milk. Since these oligosaccharides are not digested in the gastrointestinal tract (they constitute the "soluble fibre" of breast milk), they provide nutrients for all colonic bacteria in children fed with breast milk, therefore contributing to a beneficial increase in the PH and bacterial flora differences in breastfed children compared to those fed with infant formulae (McVeagh P, Miller JB, 1997, *J Paediatr Child Health* 33: 281-286).

Moreover, there is currently a great interest in the role of these oligosaccharides as pathogen receptors. Oligosaccharides may resemble cellular receptors for pathogenic micro organisms, producing specific interactions between oligosaccharides and pathogens which indicates oligosaccharides play a role as intestinal mucosa cell protectors against pathogens. They may constitute an additional defence mechanism for newborn children, whose gastric pH is less acidic than in adults, and whose immunity system is not yet mature. In fact, among breastfed children there is a lower incidence of diarrhoea, otitis and respiratory diseases compared to non-breastfed children (Kunz C et al, 2000, *Ann Rev Nutr* 20: 699-702).

Several examples prove this fact, including inhibition of *Escherichia coli* stable toxin, inhibition of *Campylobacter jejune* infections, inhibition of *Streptococcus pneumoniae* and enteropathogenic *Escherichia coli* interaction with their respective receptors (Newburg SN, 1999, *Curr Med Chem*; 6: 117-127).

In particular, sialyl oligosaccharides (especially 3- and 6- sialyl-lactose) have been reported to have special importance and play a major role in prevention of infections (Hirmo S et al., 1998, *Anal Biochem*; 257: 63-66). Furthermore, galactose is a major component of some very important brain lipids including myelin and it has been suggested that galactose derived oligosaccharides may play a role in neonatal brain development (Kunz C et al. 1999, *Br J Nutr* ; 82: 391-399).

The US6045854 patent is the first appeared patent that relates the use of human milk oligosaccharides in nutritional products. This patent describes the use of 3 fucosyl-lactose, lacto-N-fucopentaose III, lacto-N-fucopentaose II, difucosyl-lactose, lacto-N-fucopentaose I, lacto-N-neotetraose, lacto-N-fucopentaose V and lacto-N-tetraose, all of them structures that are present in human milk. However, since isolation of these oligosaccharides from human milk is so difficult, the authors used preferably chemically synthesised oligosaccharides, instead of natural sources.

Likewise, it has been claimed (WO 01/42263 patent) compositions based on oligosaccharides coming from one or more ruminant milks to be used for infant formula, dietetic products or pharmaceutical preparations to treat gastrointestinal impairments caused by viral or bacterial colonisation or even to preserve the gastrointestinal function. The authors describe oligosaccharide mixtures that produce health benefits that are close to those produced by human milk oligosaccharides, although the patent does not describe any specific proof or data of this activity for the described mixtures.

It is a fact that colostrums and breast milk contain multiple hormones, bioactive peptides and growth factors. These hormones have direct influence on a wide range of biological systems. Among these systems effected are: the hypothalamic-hypophyseal system (since milk contains prolactin, somatostatin, oxytocin, etc.), the thyroid gland (since milk contains the thyroid stimulant hormone, thyroxine and calcitonin), the sexual glands (since milk contains estrogens and

progesterone), and the pancreatic and adrenal glands (Koldovsky O and Strbak V, 1995, In: Handbook of Milk Composition; Jensen, RG, Ed Academic Press, New York).

These compounds seem to have a low influence on adults because the limited permeability of the intestine epithelium inhibits the actions of the most of the factors. However, it is important to state that the high intestinal permeability related to these pathologies, when these compounds are administered to patients suffering from an intestine condition (for example, Crohn's disease), may allow these compounds to reach their receptors and, therefore, exert their physiological effects. Newborn children, unlike healthy adults, quickly absorb these peptides since their gastrointestinal tract is more permeable. In addition, the resistance offered by these peptides and hormones against the proteolytic action of the gastrointestinal tract, (an essential condition to take effect), is larger and stronger than the resistance offered by these peptides in adults. Therefore, these compounds seem to play a functional role in newborn children (Playford RJ et al, 2000, *Am J Clin Nutr*; 72: 5-14). Numerous researches have been carried out to demonstrate the positive effect of all these compounds on the development and immunity of newborn children. We pay special interest to the presence of these bioactive substances in breast milk due to the fact that these are practically absent in almost every infant formula. Some of these growth factors are:

- The *Epidermal Growth Factor (EGF)* could collaborate in the regulation of hepatic and intestinal development. The oral ingestion of EGF causes an increase of DNA synthesis, RNA transcription, and as a consequence, an increase of protein synthesis, as well stimulating the transmission of glucose, water, and electrolytes (Playford RJ et al, 2000, *Am J Clin Nutr*; 72: 5-14), and it may play an important role in the prevention of the bacteria shift phenomenon.

- The *Transforming Growth Factor α (TGF-α)*, which, according to most of the research, has an important physiological role maintaining regular functionality (Playford RJ et al, 2000, *Am J Clin Nutr*; 72: 5-14).

- The *Transforming Growth Factor β (TGF-β)*, implicated in numerous processes, such as the development and differentiation of the intestinal epithelium; the growth, carcinogenesis and regulation of the immune response system. With respect to the immune system, it has an essential effect in two important parts of the

intestinal mucus immune system: e.g., a production and induction of oral tolerance (Kalliomaki M et al, 2000, *J Allergy Clin Immunol*; 104: 1251-1257).

- The *Insulin Growth Factor I and II (IGF-I, IGF-II)*, expressed in large amounts in the stomach and the fetal intestine, reaches its highest level after birth. It seems to promote cellular spread and differentiation (Playford RJ et al, 2000, *Clin Nutr*; 72: 5-14).

- *Growth Hormone (GH)* is present in the colostrum and in breast milk and bovine milk. It has an important effect on intestine development and functionality (Playford RJ et al, 2000, *Am J Clin Nutr*; 72: 5-14).

- *Neuropeptides,* such as: neurotensine, P substance, somatostatin, and vasoactive peptide. These seem to foster immunity response by increasing the production of IgA (vasoactive peptide), stimulating T cells, activating macrophages and producing IL-12 by P substance (Goldman AS et al, 2000, *J Nutr*; 130: 426S-431S).

- *CD14,* an anchor glycoprotein of 53-55 kDa present on the surface of monocytes and polymorphonuclear leukocytes that is able to be linked to the bacterial LPS initiating the cellular response to infections through mediators and cytokines. They have not been detected in goat milk, but in human milk (Jarvinen KM, et al, 1999, *Pediatr Res*; 45: 76-81).

The isolation or production processes of some of these growth factors present in human milk have been patented, not only for dietetic products, but also for pharmaceutical preparations. Thus, the NZ518217 patent describes the extraction process of TGFß2 and IGF-1 of a dairy product by chromatographic techniques. The patent application WO01/24813 describes the use of TGFß2 e IGF-1, isolated from mammal milk in products to treat and/or prevent intestinal mucosa damage as a result of chemotherapy, radiotherapy or gastrointestinal diseases. The use of TGFß2, coming from mammal milks or colostrums, in products for enteral nutrition or pharmaceutical preparations for the treatment of inflammatory bowel diseases, diarrhoeas and allergies have been claimed in the E0527283. Finally, it has been submitted a patent application (WO022945) that describes the use of sCD14 coming from human, bovine or buffalo milk in the treatment of gastrointestinal tract disorders.

Therefore, these minor components present in human milk are extremely important in the prevention of infections and in the optimal development of the newborn infant. However, no animal milk has been found to be a good natural source of oligosaccharides and growth factors, and there are no reports available describing milk from any source containing an oligosaccharide fraction that resemble human milk both in quantity and quality, including the milk of rhesus monkeys (Kunz C, et al, 1996, *J Chromatog B Biomed Appl*; 685: 211-2221).

## BRIEF DESCRIPTION OF THE INVENTION

The first aspect of the invention provides a composition comprising growth factors coming from goat milk.

Likewise, the invention provides a process to obtain the compositions of the invention.

A third aspect of the inventions relates to a food product, dietetic supplement or nutritional supplement comprising a composition of the invention, as well as the use of that composition for the preparation of these products.

The invention also provides the use of this food product, dietetic supplement or nutritional supplement in the prevention or treatment of diseases, as well as its cosmetic use.

## DESCRIPTION OF FIGURES

Figure 1. It illustrates the strategy used to isolate the goat milk oligosaccharides and growth factors described in the invention.

Figure 2. It shows a Western blot of bovine and goat milk samples, samples of the concentrate of the invention and positive controls using polyclonal antibodies to TGFß2, EGF, IGF-1 and CD-14 (GM: goat milk; GC: goat milk concentrate).

Figure 3. It illustrates the effect (inhibition) of growing concentrations of the composition of the invention on the bacterial adhesion of intestinal epithelial Caco 2 cells.

Figure 4. It shows the results of the RT-PCR related to the gene expression of MUC2, MUC3 and rRNA (control) of caco 2 cells incubated in the absence (-) or presence (+) of the composition of the invention.

## DETAILED DESCRIPTION OF THE INVENTION

The first aspect of the invention relates to a composition obtained from goat milk comprising growth factors. This composition is characterised because these growth factor are in concentrations to be physiologically active and because these factors are selected from a list that comprises: Epidermal growth factor (EGF), transforming growth factor ß 2(TGFß2), insulin like growth factor type 1 (IGF-1) and anchored glycoprotein CD-14.

The patent WO01/42263 describes oligosaccharide mixtures coming from one or more ruminant milks, claiming an effect which is close to that of human milk oligosaccharides, in particular, anti-infective properties. However, the present invention describes a method to obtain and use a mixture of compounds coming from goat milk with biological activity, including not only the above mentioned oligosaccharides, but also growth factors, also present in human milk. The ratios found among the growth factors described in the invention are similar to those found in human milk

**Table 1.** Growth factor concentrations described in human milk and in the composition of the invention

| | Human milk | Composition of the invention |
|---|---|---|
| TGFß2 | 0.1-13.9 ng/mL | 1.8-25.7 ng/mL |
| EGF | 3-107 ng/mL | 20-500 pg/mL |
| IGF-1 | 7.1-19.1 mg/mL | 1-170 ng/mL |
| CD14 | 14-84 mg/mL | 0.1-0.5 mg/mL |

Some of these growth factors, such as TGFß2, IGF-1 or CD14 are claimed in several patents or patent applications (NZ518217, WO01/24813, E0524283 or WO022945). These documents claim the extraction process from different milks and/or the use in the prevention or treatment of gastrointestinal diseases. The present invention describes a method to obtain a mixture that include these four growth factors TGFß2, IGF-1, EGF and CD14, apart from the oligosaccharides, as it is described later.

Preferably, the growth factors are in the composition of the invention in the following ratios (by weight): IGF-1/TGFß2: 10-30000/1, preferably, 10-2000/1, more preferably, 1-20/1; CD14/TGFß2 ratio: 2-5000/1, preferably, 10-50/1; and EGF/TGFß2: 0.1-2000/1, preferably 1-20/1.

In a particular realisation, the composition of the invention contains in addition oligosaccharides , in weight % of at least 0.5% and these oligosaccharides are selected from a list that comprises: 3-sialyl-lactose, 6- sialyl-lactose, 3-galactosyl-lactose, 6-galactosyl-lactose, lacto-N-tetraose (LNT), lacto-N-hexaose (LNH), sialyl-LNH, N-acetyl-glucosaminyl (NAcG)-LNH, disialyl-lactose, di-NAcG lactose, sialyl-NAcG lactose, sialyl-hexosyl-lactose, NAcG-hexosyl-lactose, sialyl-NAcG-hexosyl lactose, disialyl-hexosyl-lactose, di-NAcG- hexosyl-lactose, sialyl-dihexosyl-lactose and NAcG-dihexosyl-lactose.

The chemical composition and structure of oligosaccharides isolated from goat milk is given in Table 2. About 70% of the oligosaccharides described in the goat milk of the invention have been found for the first time in this milk, including 18 new oligosaccharide structures and, furthermore, about 80% of the oligosaccharides described in the invention were found to be present in human milk, which is especially rich in them. The oligosaccharide level found in goat milk was about 250-300 mg per litre of milk which represents 4 to 10 times more the amount of oligosaccharides reported for other commercial milks (sheep or cow). The present invention uses a natural source of oligosaccharides which is a significant difference to the above mentioned patent (US6045854) which claims the use of oligosaccharide structures that are present in human milk, but produced, preferably, by chemical synthesis.

The most abundant oligosaccharide structures present in goat milk we found were 1) N-acetyl-glucosyl lactose (70 mg/l), 2) Galactosyl-lactose (20 mg/l) and 3) N-acetyl-neuraminyl lactose (65 mg/L). These three represent about 77% of the total goat milk oligosaccharide mixture. We also found a 1:4 ratio of acidic vs. neutral oligosaccharides.

In another particular realisation, the composition of the invention contains lactose.

Another particular realisation of the invention relates to the fact that the fraction of oligosaccharides and growth factors accounts for an amount in weight that is higher than 0.5% up to 90% of the total of the composition.

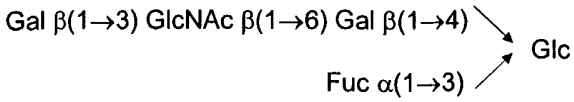
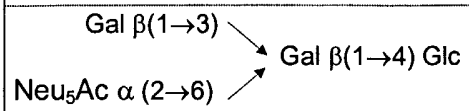
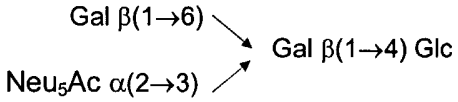
Another aspect of the invention relates to the amount of sialyl oligosaccharides found in goat milk which is especially rich. Thus, they are preferred compositions those in which the amount of sialyl oligosaccharides are between 5 and 85% in weight of the total of the composition, especially those in which this amount are between 40 and 45% in weight of the total of the composition.

Goat milk oligosaccharides are specially enriched in galactose which as mentioned above may have very important implications in neonatal brain development.

Finally, within the first aspect of the invention, a preferred realisation of the invention consists of sialyl-lactose, galactosyl-lactose and N-acetyl-glucosaminyl-lactose ratios (in weight) of 2-7/1/1-5, preferably 2.9/1/2.6.

**Table 2.** Structure of Oligosaccharides of mature goat milk.

| |
|---|
| GlcNAc β(1→6) Gal β(1→4) Glc |
| Gal β(1→4) GlcNAc β(1→6) Gal β(1→4) Glc |
| Gal β(1→4) ↘<br>GlcNAc β(1→3) Gal β(1→4) Glc<br>Gal β(1→3) ↗ |
| Gal β(1→4) ↘<br>GlcNAc β(1→6) Gal β(1→4) Glc<br>Fuc α(1→3) ↗ |

| |
|---|
|  |
|  |
|  |
| * $Neu_5Ac$ α(2→3) Gal β(1→4) Glc (3'-SL) |
| * $Neu_5Ac$ α(2→6) Gal β(1→4) Glc (6'-SL) |
| * Gal β(1→3) Gal β(1→4) Glc (3'-GL) |
| * Gal β(1→6) Gal β(1→4) Glc (6'-GL) |
| * Gal β(1→3) GlcNAc β(1→3) Gal β(1→4) Glc (LNT) |
| * Gal β(1→4) GlcNAc β(1→6)<br>Gal β(1→3) GlcNAc β(1→3)<br>Gal β(1→4) Glc (LNH) |
| * $Neu_5Ac$ – LNH |
| * GlcNAc – LNH |
| * $Neu_5Ac$<br>$Neu_5Ac$<br>Gal β(1→4) Glc |
| * GlcNAc<br>GlcNAc<br>Gal β(1→4) Glc |
| * $Neu_5Ac$ – GlcNAc – Gal β(1→4) Glc |
| * $Neu_5Ac$ – Hex – Gal β(1→4) Glc |
| * GlcNAc – Hex – Gal β(1→4) Glc |
| * $Neu_5Ac$ – GlcNAc – Hex – Gal β(1→4) Glc |
| * $Neu_5Ac$<br>$Neu_5Ac$<br>Hex – Gal β(1→4) Glc |
| * GlcNAc<br>GlcNAc<br>Hex – Gal β(1→4) Glc |
| * $Neu_5Ac$ – $Hex_2$ – Gal β(1→4) Glc |
| * GlcNAc – $Hex_2$ – Gal β(1→4) Glc |
| ** Oligosaccharides not previously described in goat milk* |

Where GlcNAc: N-acetyl glucosamine; Gal: galactose; Fuc: fucose; Glu: glucose; Neu5Ac: neuraminic acid; LNH: lacto-N-hexaose; Hex: hexose; GL:galactosyl lactose.

In its second aspect, the invention provides a process to obtain the compositions above mentioned, based on the application of membrane ultra-filtration technology to goat milk. The process is made in such conditions that it is assured the presence of growth factors and oligosaccharides in their biologically active form and in adequate concentrations for maximum functionality.

In a preferred realisation of the process, this comprises the following steps:

a. to make an initial fractionation of skimmed goat milk at slightly acid pH using a 15 to 50 kDa cut off membrane, preferably a 50 kDa cut off membrane.
b. using the permeate of the step a), a new ultrafiltration step using a 1 to 5 kDa cut off membrane, preferably a 1 to 3 kDa cut off membrane, and ideally a 1 kDa cut off membrane.
c. Optionally, a final purification step can be made with the retentate obtained in step b) to remove lactose and salts including one or more of the following processes: active charcoal chromatography, ion-exchange chromatography and electrodialysis.

Finally, the mentioned process can use goat milk whey as raw material.

A third aspect of the invention relates to a nutritional product to which the composition described in the invention above has been added. The nutritional products of the invention preferably comprise from 0.1 mg to 10g of the added goat milk oligosaccharide mixture per 100 ml or 100 g of nutritional product, more preferably, they comprise from 1 mg to 500 mg of the composition of the invention. Nutritional products containing this amount of the composition can be milk, dairy products, yoghurts, fermented milks, fruit and vegetable juices, biscuits, cakes, infant food and dehydrated food. The addition of the composition of the invention to the nutritional products is effected by mixture and homogenisation according to the technological procedure of each product.

Other components such as vitamins can be added to the nutritional products. Thus, vitamins A, $B_6$, $B_{12}$, C, D, E or folic acid or a mixture of one or more thereof can be added to nutritional products according to the present invention. The addition of these compounds can be effected either previously or after the heat treatment according to the specific technological procedure of each nutritional product.

An additional aspect of the invention relates to the use of a composition of the invention for the preparation of food products, dietetic supplements or nutritional supplements.

A final aspect of the invention is related to the use of these food products, dietetic supplements or nutritional supplements in the prevention of bacterial or viral infections, in gastrointestinal infections treatment and neonatal brain development. The composition of the invention can be also used for the preparation of cosmetic products.

The following examples illustrate the invention:

**Example 1**

**Isolation of goat milk oligosaccharides by membrane technology**

The process for recovery of biologically active oligosaccharides and growth factors from goat milk has been developed using membrane technology is based in ultrafiltration and nanofiltration processes, followed by active charcoal and ion-exchange chromatography and electrodialysis. Since desirable goat milk oligosaccharides and growth factor fraction is in molecular size smaller than proteins and others components, a careful selection of membrane and operating conditions (temperature, pH, circulation velocity and transmembrane pressure) make possible to selectively remove protein fraction, salts and lactose to obtain a purified oligosaccharide fraction with the functional properties described in the invention. The total removal of lactose and salts is made by active charcoal chromatography, followed by ion-exchange chromatography and electrodialysis.

The process for preparing the goat milk oligosaccharides mixture described above comprises three stages:

1. Initial fractionation of skimmed goat milk which is performed at a slightly acidic pH (6-7) using a multichannel ceramic tubular membrane Céram Inside® (TAMI Industries, France) made by zirconium and titanium with a molecular weight cut-off between 50-15 kDa, preferably 50 kDa. Temperature is kept below 50 °C to avoid thermal aggregation of α-lactalbumin. Under these conditions caseins, whey proteins and large peptides are retained by the membrane due to both steric and electrostatic interactions. The smaller components including oligosaccharides, growth factors, salts and lactose pass through the membrane and are collected in the filtrate. A process of concentration and subsequent discontinuous diafiltration with a concentration factor of 2 and a minimum number of 5 cycles is carried out to obtain an oligosaccharide fraction with a yield of about 95%. To eliminate the polarisation layer and decrease the fouling index, a channel circulation velocity of 4 m/s is used.

2. The filtrate product from the previous stage is fractionated again using a similar membrane with molecular weight cut-off of 5 to 1- kDa, preferably 3- and 1-kDa, most preferably 1-kDa. An optimal separation of salts and lactose is achieved using a working pH of 6 to 8, preferably 7 to 7.5, and a temperature below 50 °C. A process of concentration and subsequent discontinuous diafiltration with a concentration factor between 1 and 3, preferably 2 or 3, most preferably 3, and a number of cycles from 2 to 7, preferably 4 is carried out to obtain a retentate containing almost all goat milk oligosaccharides and growth factors.

3. To remove lactose and salts, a process of active charcoal chromatography, ion exchange chromatography and electrodialysis is made to obtain the fraction enriched in goat milk oligosaccharides and growth factors. This fraction is concentrated either by reverse osmosis or multiple-effect evaporator, and further dried by lyophilization or spray-drying processes. This process helps to increase the concentration of oligosaccharides and bioactive peptides in the concentrate compared to the initial goat milk, and the qualitative composition is similar to that found in human milk.

**Identification of oligosaccharide fraction.**

Milk samples (50-100 ml) from five goats (Murciano-Granadina breed) were donated from an experimental farm (County council of Granada, Spain). The

samples were taken, without prior oxytocin injection, by hand-milking immediately after the young had stopped sucking. Oligosaccharides standards were obtained from Oxford Glycosystems (Cambridge, UK) or Glyko (Oxford, UK).

Bio-liquid chromatography was performed on a Dionex-System (Sunnyvale, CA, USA) consisting of a CarboPac PA-100 column (250 x 4.6 mm i.d.) equipped with a guard column and a Model ED50A Electrochemical detector. NaOH solution (19 mol/l; low in carbonate) was purchased from Baker (Philadelphia, PA, USA). Sodium acetate of analytical grade was from Merck (Darmstadt, Germany). Sephadex G25 was obtained from Pharmacia (Uppsala, Sweden). Thin-layer plates (Silica-gel 60, 100x100 mm) were purchased from Merck. All other reagents were of analytical grade.

**Isolation of oligosaccharides**

Two ml of whole goat milk were defatted by centrifugation at 3 000 x g for 20 min at 4°C (Kunz et al. 1996aKunz C, et al., 1996 *J. Chromatogr. B Biomed. Appl.* 685:211-221). After the lipid layer had been removed and the aqueous phase decanted and filtered through glass wool, precooled ethanol 66% was added to precipitate proteins with stirring for 2 h. Then, ethanol was extracted in a refrigerated vapour trap with vacuum before direct analysis of the carbohydrate fraction by HPTLC and HPAEC-PAD (see below).

Carbohydrate-containing fraction was applied to a Sephadex G-25 column (900 x 25 mm i.d.) connected to a FPLC system and eluted with water to reduce levels of lactose and salts. Then, salt-free but carbohydrate-positive fractions were lyophilized and resuspended in 500 µl of water before further analysis. These fractions sometimes contained small amounts of lactose which is difficult to separate completely from the oligosaccharide fraction as lactose is present in milk in very large amounts and its molecular mass is very similar to the rest of the other small oligosaccharides occurring in milk.

**High-performance TLC**

Lyophilised fractions after Sephadex G-25 gel filtration chromatography were dissolved in 4 ml water; then 5 µl was analysed by silica-HPTLC in butanol – acetic

acid – water (2.5:1:1, by vol.) (two subsequent runs). The plates were sprayed with orcinol for carbohydrate detection (1 ml orcinol/l in 2.3 M-$H_2SO_4$).

**High-pH anion-exchange chromatography with pulsed amperometric detection**

Neutral and acidic oligosaccharides were analysed using the following conditions: eluent A, 100 mM-NaOH; eluent B, 100 mM-NaOH and 250 mM-sodium acetate. The elution programme began with 3 ml eluent A, followed by a gradient of up to 100% eluent B in 30 min and finally 4 ml eluent B. A re-equilibration volume of 3 ml eluent A was chosen. The flow rate of 1.0 ml/min was used and 20 µl from the last fraction was injected.

**Growth Factor Identificación.**

Several polyclonal antibodies raised against the growth factors EGF, TGF-ß2, IGF-I y CD-14 have been used for the identification. The identification was carried out using protein separation techniques (SDS-polyacrylamide gel electrophoresis) and further Western-Blotting with the antibodies indicated above (Fig. 2). Quantification of immuno-reactive bands was performed by densitometry using control peptides (growth factors) of known concentrations.

**Example 2**

**PREPARATION OF A LIQUID NUTRITIONALLY BALANCED DIET FORMULATION**

A liquid nutritionally balanced diet formula was prepared using the following ingredients:

| Ingredient | Amount per litre | |
|---|---|---|
| Skimmed milk | 814.30 | g |
| Whey protein concentrate | 11.80 | g |
| Oil blend | 32.50 | g |
| Sacarose | 27.00 | g |
| Maltodextrine | 71.00 | g |
| Soluble Fiber | 10.00 | g |
| Vitamin A | 1200 | mg |
| Vitamin D | 7.5 | mcg |
| Vitamin E | 15 | mg |
| Vitamin K | 120 | mcg |
| Vitamin C | 90 | mg |
| Thiamin | 2.25 | mg |
| Riboflavin | 2.55 | mg |
| Pyridoxine | 3 | mg |
| Vitamin B12 | 3 | mcg |
| Niacin | 28.5 | mg |
| Folic acid | 30 | mcg |
| Panthotenic acid | 4 | mg |
| Biotin | 40 | mcg |

| | | |
|---|---|---|
| Calcium | 1200 | mg |
| Phosporus | 970 | mg |
| Magnesium | 47 | mg |
| Sodium | 480 | mg |
| Potassium | 1000 | mg |
| Chloride | 700 | mg |
| Iron | 43 | mg |
| Zinc | 24.5 | mg |
| Iodine | 150 | mcg |
| Manganese | 100 | mcg |
| Selenium | 10 | mcg |
| Mono- and diglycerides | 1.50 | g |
| Bisodium phosphate (Na2HPO4 5H2O) | 0.60 | g |
| Carrageenan | 4.00 | g |
| Desmineralised Water | 70.00 | g |
| Flavour | 2.75 | g |
| Composition including Goat milk oligosaccharides and growth factors | 1.6 | g |

**Processing technology**

To an appropriately sized blend tank with agitation and heating all solid ingredients were mixed with the liquid milk and water. Then, the oil blend (obtained by the procedure described above) was admixed. The mixture was then heated at 60-70° C and emulsified through a single stage homogenizer at 6 to 7 MPa in absence of oxygen. After emulsification the mixture was heated to 140-150° C, during 4-6 seconds, and was then passed through a two stages homogenizer (27-29 MPa and 3-4 MPa). Finally the mixture was packaged in absence of oxygen.

**Example 3**

**PREPARATION IN POWDER FORM OF AN INFANT FORMULA**

An infant formula in powder form was prepared using the following formula:

| Ingredients | Percentage of dry matter |
|---|---|
| Demineralised whey | 51.2 |
| Palm olein | 13.5 |
| Lactose | 9.2 |
| Milk skimmed | 9.5 |
| Rapeseed oil | 5.2 |
| Coconut oil | 4.9 |
| Sunflower oil | 2.8 |
| Water | 3.1 |
| Vitamin premix | 0.2 |
| Mineral premix | 0.4 |
| Composition including Goat milk oligosaccharides and growth factors | 0.16 |

**Processing technology**

To an appropriately sized blend tank with agitation and heating all solid ingredients were mixed with the liquid milk and water in the absence of any vitamins. Then, the vegetable oils were admixed. The mixture was then heated at 60-70° C and emulsified through a single stage homogenizer at 6 to 7 MPa in absence of oxygen. After emulsification the mixture was standardized by addition of vitamins and the pH was adjusted in the range of about 6.7 to 7.2. The mixture was reheated to between about 65°C and 70°C and finally was dried in a spray drier to obtain a final dry powder product that was packaged.

**Example 4**

**PREPARATION OF A LIQUID INFANT FORMULA**

A liquid infant formula was prepared using the following formula:

| Ingredients | Percentage of dry matter |
|---|---|
| Demineralised whey | 51.2 |
| Palm olein | 13.5 |
| Lactose | 9.2 |
| Milk skimmed | 9.5 |
| Soya oil | 5.2 |
| Coconut oil | 4.9 |
| High oleic sunflower oil | 2.8 |
| Water | 3.1 |
| Vitamin premix | 0.2 |
| Premix of minerals | 0.4 |
| Composition including Goat milk oligosaccharides and growth factors | 0.16 |

**Processing technology**

To an appropriately sized blend tank with agitation and heating all solid ingredients were mixed with the liquid milk and water in the absence of any vitamins. Then, the vegetable oils were admixed. The mixture was then heated at 60-70° C and emulsified through a single stage homogenizer at 6 to 7 MPa in absence of oxygen. After emulsification the mixture was standardized by addition of vitamins

and the pH was adjusted in the range of about 6.7 to 7.2. Then, the mixture was heated to 140-150° C during 4-6 seconds, and was then passed through a two stages homogenizer (27-29 MPa and 3-4 MPa). Finally the mixture was packaged in absence of oxygen.

**Example 5**

**PREPARATION OF A YOGHURT**

An infant formula in powder form was prepared using the following formula:

- Milk 3,1 % fat; 3,2 % protein.....987 g/Kg
- Skimmed milk powder.....13 g/Kg
- Starter.....0.1g/Kg
- Composition including Goat milk oligosaccharides and growth factors ..... ...1.6 g/Kg

**Processing technology**

The first step for yoghurt production is the milk standardisation, the milk intended must be of the highest bacteriological quality and must not contain antibiotics, bacteriophages, residues of CIP solution or sterilising agents.

The fat and dry solids contents of the milk are normally standardised according to the formulation. A sample is described below:

After standardisation the milk continues to homogenisation to prevent creaming during incubation period and to assure uniform distribution of the milk fat. Homogenisation also improves the stability and consistency of cultured milks, even those with low fat contents. As a general recommendation, the milk should be homogenised at 20-25 Mpa and 65-70 °C to obtain optimum physical properties in the product.

The homogenised milk flows now to the heat treatment before being inoculated with the starter in order to improve the properties of the milk as a substrate for the bacteria culture, ensure that the coagulum of the finished yoghurt

will be firm and reduce the risk of whey separation in the end product. Optimum results are achieved by heat treatment at 90-95 °C and a holding time of about 5 minutes. That temperature/time combination denatures about 70-80 % of whey proteins which interacts with the casein, helping to give the yoghurt a stable "body".

After pasteurisation the milk is cooled to the desired inoculation temperature, typically 40-45 °C. The handling of the starter for production of yoghurt demands maximum precision and hygiene. Concentrated, frozen and freeze-dried cultures are now available on the market and are being more and more widely used. The greatest advantage, however, is that direct inoculation of milk with a concentrated culture minimised the risk of contamination, as the intermediate stages of propagation are excluded.

The starter (0,1 g/Kg) is metered into the stream of milk as it is pumped from an intermediate storage tank to the filling machine. Following packaging in the filling machine, the packages after crating and palletising, are trucked into the system for incubation and cooling. The incubation room is able to accommodate a large number of filled pallets and maintain a temperature of about 40-45 °C during the fermentation process, about 5-6 hours.

When the empirically determined optimum pH (typically 4,5) is reached, it is time to start cooling. The pallets are trucked to a conveyor passing through the cooling sections enclosed in a tunnel. The normal target temperature is 18-20 °C; it is important to stop further growth quickly, which means that a temperature of about 35°C should be reached within 30 minutes, and 18-20 °C after another 30-40 minutes.

Final cooling, normally down to 5°C, takes place in the chill store, where the products are held to await distribution.

**Example 6**

**PREPARATION OF A COMPOSITION OF GOAT MILK OLIGOSACCHARIDES AND GROWTH FACTORS WITH PREBIOTIC EFFECTS**

The prebiotic effect of the composition of the invention was tested using an *in vitro* fermentation approach based on culture and further growth quantification of different species of *Bifidobacterium* and *Lactobacillus.* Bacteria was cultured in microtiter plates at $10^6$ cfu/ml dilution for 24 h in the presence or absence of different concentrations of purified goat milk oligosaccharides obtained as described in the invention as an unique source of carbohydrates. The culture media contains a pH indicator that allows for the quantification of the pH fall due to fermentation process, and an aliquot of the culture was plated to count the increase in bacterial cfu (colony forming units).

The results obtained clearly show goat milk oligosaccharides can be fermented and therefore used as energy source by for most of the *Lactobacillus* species and for all of the *Bifidobacterium* species tested, which indicates that the composition of the invention allows and promotes growth of beneficial *probiota* in the colon (Table 7).

**Table 3:** Effect of the composition of the invention on fermentation and proliferation of several species of *Lactobacillus* and *Bifidobacterium.*

| | Fermentation (Fold-Induction) | Proliferation (CFU, Fold-Induction) |
|---|---|---|
| *Bifidobacterium bifidum* | 4.10 | 4.95 |
| *B. adolescentis* | 3.98 | 4.81 |
| *B. breve* | 3.23 | 3.12 |
| *B. infantis* | 3.71 | 3.77 |
| *Lactobacillus acidophilus* | 5.23 | 23.6 |
| *L. casei* | 5.80 | 25.4 |
| *L. rhamnosus* | 1.92 | 9.23 |
| *L. sakei* | 7.79 | 35.2 |
| *L. salivarius* | 8.26 | 36.8 |
| *L. reuteri* | 6.85 | 24.9 |

**Example 7**

**PREPARATION OF A COMPOSITION OF GOAT MILK OLIGOSACCHARIDES AND GROWTH FACTORS TO PREVENT BACTERIAL INFECTIONS**

The preventive effect of the composition of the invention was tested in Caco 2 cells.

Intestinal Caco-2 cells were obtained from the ATCC (passages 10 to 20). Cells grown routinely at $3x10^6$ cells in T 75 $cm^2$ culture flasks in Dulbecco's modified minimum essential medium containing 25 mmol/L glucose, 30.8 mmol/L $NaHCO_3$ and supplemented with 20% heat-inactivated foetal bovine serum, 2 mmol/L glutamine, 1mg/L fungizone, $10^5$ U/L penicillin-streptomycin, 150 mg/L gentamycin and 1% non-essential amino acid solution (containing in mg/L, L-alanine, 890 ; L-asparagine, 1500; L-aspartic acid, 1330; L-glutamic acid, 1470; glycine, 750; L-proline, 150; L-serine, 1050). Cells were maintained in a 10% $CO_2$ air atmosphere at 37°C, and media were changed every other day. For experiments, cells were plated at $1x10^6$ cells in 10 cm diameter Petri dishes and media changed every other day. Under these conditions, cells reached confluence in 6-7 days.

The Caco-2 cells cultured as above were used to study the influence of the composition of the invention on in vitro adhesion of different dilutions of pathogenic bacteria (*Escherichia coli, Salmonella typhi or S. typhimurium*). We have observed that goat oligosaccharide fractions reduce binding capabilities of these pathogens up to 60% (Figure 3). Since bacterial adhesion to the intestinal epithelium is a required step for most pathogenic infections, our results clearly indicate that the composition of the invention have anti-infectious effect that may resemble the one observed for human milk in breast-fed infants. The very high amount of sialyl- and fucosyl-oligosaccharides in goat milk may also explains the inhibitory effect that the composition of the invention have on the adhesion capabilities of pathogenic bacteria to human intestinal epithelial cells.

**Example 8**

**PREPARATION OF A COMPOSITION OF GOAT MILK OLIGOSACCHARIDES AND GROWTH FACTORS TO INHIBIT BACTERIAL TOXINS**

Besides the inhibitory effect of fucosyl-oligosaccharides on bacterial adhesion we found that the composition of the invention also blocks the effect of the heat-stable enterotoxin of *E. coli* which is responsible for the diarrheic effect observed upon infection. To test the anti-diarrheic properties of the composition of the invention we treated Balb/c mice with 10 μg/ml of purified heat-stable enterotoxin from *E. coli* with or without the composition of the invention. Preliminary results showed that 75% of mice treated with enterotoxin in absence of

oligosaccharides develop a diarrheic episode before 12 hours whereas only a 32 % due it when treated with the composition of the invention.

**Example 9**

**PREPARATION OF A COMPOSITION OF GOAT MILK OLIGOSACCHARIDES AND GROWTH FACTORS THAT STIMULATES INTESTINAL EPITHELIAL CELLS DIFFERENTIATION**

To establish the effects of the composition of the invention on epithelial cell differentiation, we studied the effect of those in (a) brush border specific enzymatic activities and (b) gene expression of some mucin genes.

Caco-2 cells were grown in culture media in the presence or absence of the composition of the invention, obtained as previously described in the invention (10 mg/dish). At day 21, culture medium was aspirated a cell homogenate was prepared and centrifuged at 100 000 x g for 30 min at 4° C to obtain a membrane pellet. Crude membrane fractions were used for the specific enzyme assay.

**Table 4.** Effect of the composition of the invention on membrane enzyme activities in Caco-2 cells

| | Control | Composition of the invention |
|---|---|---|
| Lactase | 42.0 ± 1.7 | 47.5 ± 2.1* |
| Sucrase | 108.3 ± 5.7 | 122.9 ± 3.1* |
| Maltase | 258.4 ± 9.7 | 301.2 ± 8.9* |
| Alkaline phosphatase | 197.2 ± 6.7 | 232.5 ± 6.8* |
| Leucine aminopeptidase | 57.1 ± 2.7 | 65.0 ± 2.4* |

Values are means ± SEM (n=8). *p< 0.05. Results are expressed as microunits per mg protein. One unit is defined as 1μmol substrate hydrolysed/min.

Goat milk oligosaccharides and growth factors can also bind to carbohydrate receptors on intestinal epithelial cells and modulate some of their physiological functions. In this regard, we have explored the effect of the composition of the invention on the expression of mucins in Caco-2 cell lines by a semi-quantitative RT-

PCR technique. The results obtained show that human intestinal epithelial cells treated with purified goat milk oligosaccharides for 24 and 48 hours induce the expression of MUC 2 and MUC 3 genes (Figure 4).

It has been described that production of intestinal mucins is one of the first lines of protection against bacterial invasion in the intestine and therefore the MUC2 MUC3 expression induction observed here could represent another infection protection mechanism of the composition of the invention.

**Example 10**

**PREPARATION OF A COMPOSITION OF GOAT MILK OLIGOSACCHARIDES AND GROWTH FACTORS THAT MODULATES THE GUT INFLAMMATORY RESPONSE**

The modulating effect of the gut inflammatory response of the composition of the invention was studied in intestinal epithelial cells (Caco 2 and HT-29 cells).

Intestinal epithelial cells (Caco-2 and HT-29) were cultured as described above and incubated with or without the composition of the invention. The expression of inflammatory cytokines Tumor Necrosis Factor-alpha and interleukins 1 and 8 were studied and quantified upon incubation with two pathogenic bacteria *E.coli and Salmonella sp*. We found a significant reduction of these cytokines at the protein (ELISA) and expression level by RT PCR which indicates that the composition of the invention may modulate gut inflammatory response induced by pathogens.

## CLAIMS

1. A composition obtained from goat milk comprising growth factors, characterised in that these growth factors are in concentrations to be physiologically active and in that these factors are selected from a list that comprises: Epidermal growth factor (EGF), transforming growth factor ß 2(TGFß2), insulin like growth factor type 1 (IGF-1) and anchored glycoprotein CD-14

2. A composition according to claim 1, characterised in that it contains in addition oligosaccharides, in weight % of at least 0.5% and these oligosaccharides are selected from a list that comprises: 3-sialyl-lactose, 6-sialyl-lactose, 3-galactosyl-lactose, 6-galactosyl-lactose, lacto-N-tetraose (LNT), lacto-N-hexaose (LNH), sialyl-LNH, N-acetylglucosaminyl (NAcG)-LNH, disialyl-lactose, di-NAcG lactose, sialyl-NAcG lactose, sialyl-hexosyl-lactose, NAcG-hexosyl-lactose, sialyl-NAcG-hexosyl lactose, disialyl-hexosyl-lactose, di-NAcG- hexosyl-lactose, sialyl-dihexosyl-lactose and NAcG-dihexosyl-lactose

3. A composition according to claims 1 and 2, wherein lactose is present in the composition.

4. A composition according to claims 1 to 3 where the goat oligosaccharide and growth factors fraction comprises between 0.5 to 90% of the total composition.

5. A composition according to claims 1 to 4 wherein the amount of sialyl oligosaccharides is 5-80% of the oligosaccharide fraction, more preferably 40-45%.

6. A composition according to claims 1 to 5 wherein the sialyl-lactose, galactosyl-lactose andN-acetyl-glucosaminyl-lactose ratios (in weight) are 2-7/1/1-5, preferably 2.9/1/2.6

7. A composition according to claims 1 to 6 wherein the growth factors are in the following ratios (by weight): IGF/TGFß2: 10-30000/1, preferably, 10-2000/1, more preferably, 1-20/1; CD14/TGFß2: 2-5000/1, preferably, 10-50/1; and EGF/TGFß2: 0.1-2000/1, preferably 1-20/1.

8. A process to obtain any of the compositions defined in claims 1 to 7, wherein the process is based on the application of membrane ultrafiltration technology to goat milk.

9. A process according to claim 8 comprising the steps:
   a. to make an initial fractionation of skimmed goat milk at slightly acid pH using a 15 to 50 kDa cut off membrane, preferably a 50 kDa cut off membrane.
   b. using the permeate of the step a), a new ultrafiltration step using a 1 to 5 kDa cut off membrane, preferably a 1 to 3 kDa cut off membrane, and ideally a 1 kDa cut off membrane.
   c. Optionally, a final purification step can be made with the retentate obtained in step b) to remove lactose and salts including one or more of the following processes: active charcoal chromatography, ion-exchange chromatography and electrodialysis.

10. A process according to claims 8 and 9 in which the initial raw material is goat milk whey.

11. A food product, dietetic supplement or nutritional supplement comprising a composition according claims 1 to 7.

12. A product according to claim 11, wherein the compositions according claims 1 to 7 has been added to the food product, dietetic supplement or nutritional supplement.

13. A product according to claim 12, wherein it incorporates vitamins A, B6, B12, C, D, E, folic acids or a or a mixture of one or more thereof.

14. A product according to claims 11 to 13 wherein the product is for clinical nutrition, infant food, dehydrated food or animal feed.

15. A product according to claims 11 to 13 as diet supplement for infants, adolescents, elderly, pregnant women and athletes.

16. A product according to claims 11 to 15 which is milk, dairy products, fermented milks, juices, vegetables, biscuits, cakes or bakery products.

17. Use of a composition according to claims 1 to 7 for the preparation of food product, dietetic supplement or nutritional supplement according to claims 11 to 16.

18. A composition according to claims 1 to 7 for use in prevention of bacterial or viral infections, treatment of gastrointestinal infections, allergic processes and also for adequate newborn development and neonatal brain development.

19. Use of a composition according to claims 1 to 7 for the preparation of a drug to be used in the prevention or general treatment of bacterial or viral infections, treatment of gastrointestinal infections, allergic processes and also for adequate newborn development and neonatal brain development.

20. A cosmetic product comprising a composition according to claims 1 to 7.

21. Use of a composition according to claims 1 to 7 for the preparation of a cosmetic product.

FIGURE 1:

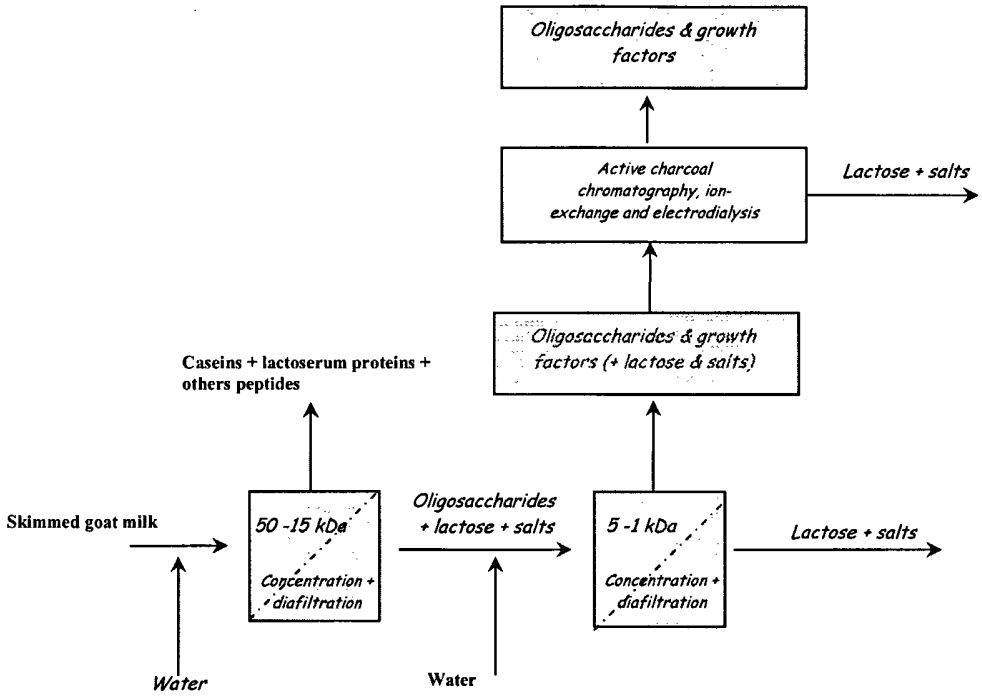

Oligosaccharides & growth factors
Active charcoal chromatography, ion-exchange and electrodialysis
Lactose + salts
Oligosaccharides & growth factors (+ lactose & salts)
Caseins + lactoserum proteins + others peptides
Skimmed goat milk
50 -15 kDa
Concentration + diafiltration
Oligosaccharides + lactose + salts
5 -1 kDa
Concentration + diafiltration
Lactose + salts
Water
Water

FIGURE 2

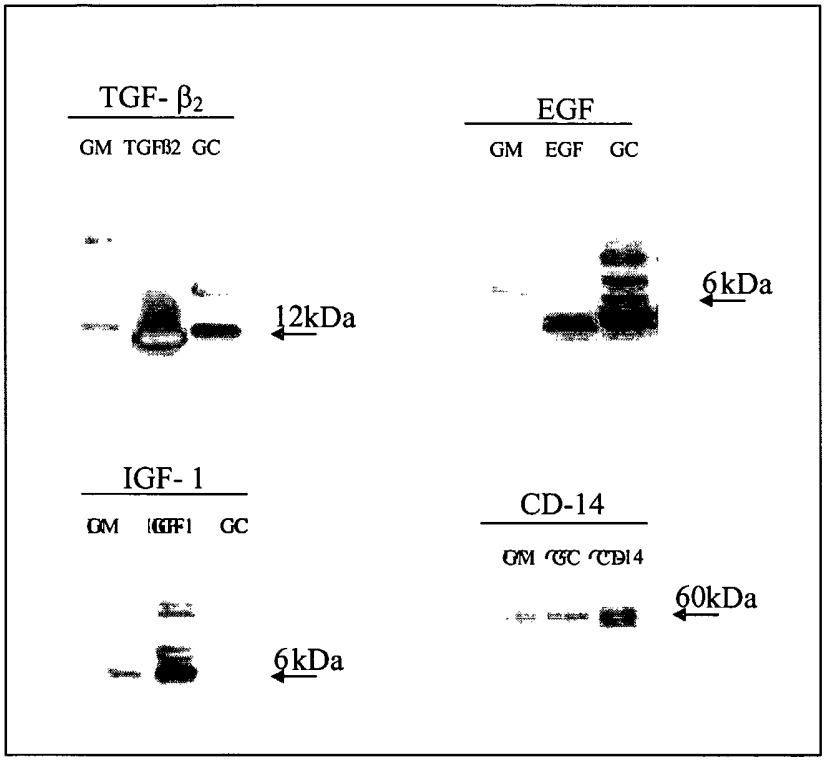

TGF- β2
GM TGFß2 GC
12kDa
EGF
GM EGF GC
6kDa
IGF- 1
GM IGF1 GC
6kDa
CD-14
GM GC CD14
60kDa

FIGURE 3

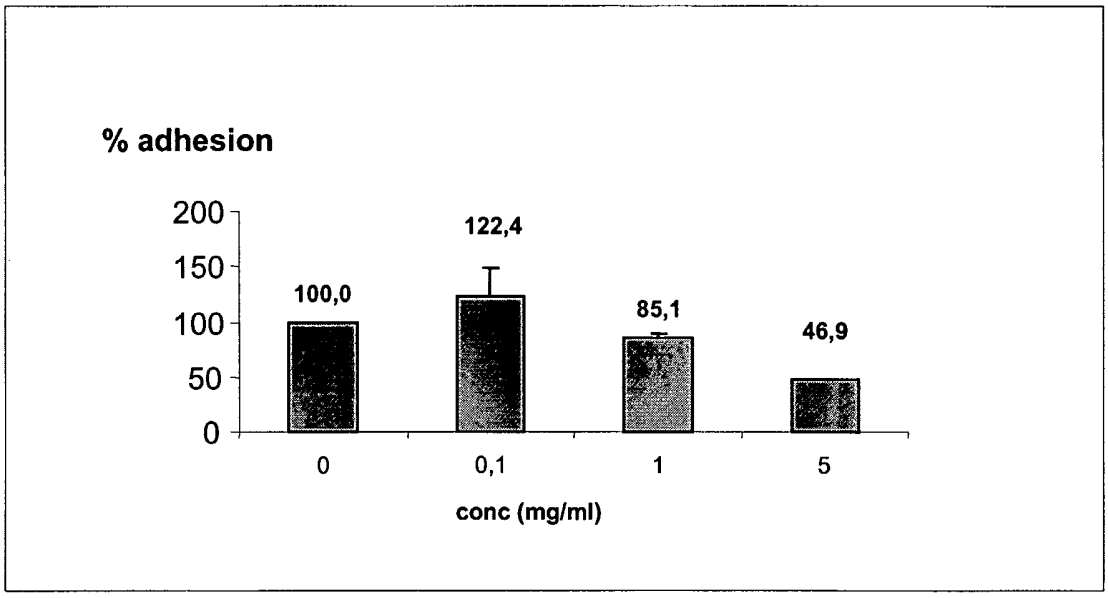


FIGURE 4