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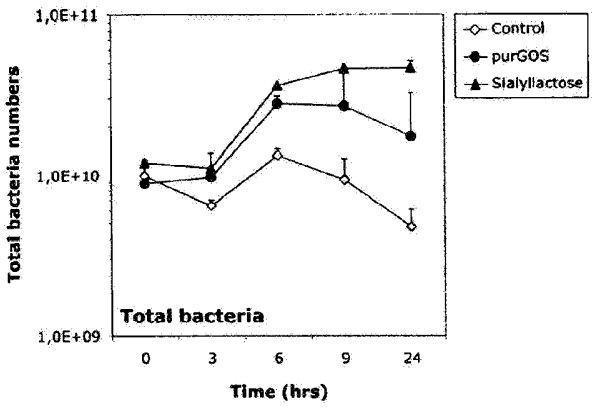
(54) **Use of sialyl oligosaccharides to modulate the immune system.**

(57) The invention relates to prebiotics and the use thereof for promoting human health. In particular, it relates to milk-, whey- or egg-derived oligosaccharides capable of enhancing the relative abundance of beneficial micro-organisms. Provided is the use of a sialyl-oligosaccharide in the manufacture of a dietetic, nutraceutical, nutritional or pharmaceutical composition for supporting or enhancing the mammalian immune system, in particular enhancing the maturation of the developing human neonatal immune system.



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Title: Use of sialyl oligosaccharides to modulate the immune system

The invention relates to prebiotics and the use thereof for promoting human health. In particular, it relates to milk-derived oligosaccharides capable of enhancing the relative abundance of beneficial micro-organisms in the gastrointestinal tract.

The gastrointestinal tract (GIT) is one of the largest organs of the body serving as an important barrier between ingested elements from the external environment and the internal milieu of the human body. The GIT is inhabited by the intestinal microflora, composed of a large diversity of bacteria that perform important functions for the host and can be modulated by environmental factors, such as nutrition. This microbiota can be viewed as the first line of defence, which competes with invading pathogens for space, nutrients and receptors on intestinal cells, and has an important function in health and disease. The human gut microbiota plays an important role in maintaining immune homeostasis in the intestine. A normal commensal microflora is required to protect against intestinal damage (Rakoff-Nahoum et al., Cell. 2004;118:229-41). This is mediated by signalling through Toll-like receptors.

Studies from germ-free mice have demonstrated that the anatomical structure of their gut associated lymphoid tissues is compromised in the absence of stimulation by intestinal bacteria (reviewed by Round and Mazmanian, Nat Rev Immunol. 2009;9:313-23). They have fewer and smaller organized lymphoid tissues in their small intestine, as well as fewer T cells in their lamina propria and fewer regulatory T cells in their mesenteric lymph nodes, and have much lower levels of sIgA in their intestines. As a result, germ free mice are more susceptible to infections by Listeria, Shigella and Salmonella (Sprinz et al., Am. J. Pathol. 1961;39:681–95, Maier, et al., Infect. Immun. 1972;6:168–173, Zachar and Savage, Infect. Immun. 1979;23, 168–74). Regulatory T cells (Treg) are important for the maintenance of immune homeostasis in the intestine. They exert their effect via the production of anti-inflammatory cytokines such as IL-10, IL-35 and TGF-β.

Colonization of the gut (both lumen and epithelium) by microorganisms is initiated at birth when newborns become exposed to the vaginal flora. The

development of the intestinal microflora is influenced by various factors such as gestational age, mode of delivery, local environment, type of feeding, and antibiotic treatment. The earliest microbial settlers are facultative anaerobes, which render the gut lumen anaerobic by depleting final traces of oxygen. This initial microbial community comprises only a few species and is dominated by members of the genus *Bifidobacterium* and *Lactobacillus*. In breast milk fed babies, this community remains rather stable. In formula- fed term infants the intestinal microflora is more diverse, with lower numbers of both genera mentioned above. The shift to a more complex diet after weaning strongly increases the complexity of the microbial community in the gut.

A number of commensal bacteria have been suggested to have beneficial effects on the homeostasis of immune responses in the intestine. There is increasing awareness that the human gut microflora plays a critical role in maintaining host health, both within the gastrointestinal tract and, through the systemic absorption of metabolites. An "optimal" gut microflora establishes an efficient barrier to the invasion and colonisation of the gut by pathogenic bacteria, produces a range of metabolic substrates which in turn are utilized by the host (e.g. vitamins and short chain fatty acids) and stimulates the immune system in a non-inflammatory manner. Although little is known about the individual species of bacteria responsible for these beneficial activities, it is generally accepted that the bifidobacteria and lactobacilli constitute important components of the beneficial gut microflora. Bifidobacteria and lactobacilli are abundant in the intestines of breast fed but not bottle-fed children, and are well recognized as beneficial commensal bacteria. Their presence correlates with fewer GI tract and airway infections, and many Lactobacilli and Bifidobacteria species have to date been used as probiotic bacteria. The outgrowth of these bacteria is promoted by complex indigestible carbohydrates (prebiotic oligosaccharides) that are present in breast milk. Prebiotic oligosaccharides are now well established as an ingredient for infant formulas to promote the outgrowth of bifidobacteria in the intestine of infants.

A number of diet-based microflora management tools have been developed and refined over recent decades including probiotic, prebiotic and synbiotic approaches. Each aims to stimulate numbers and/or activities of the bifidobacteria and lactobacilli within the gut microflora. Some commensal bacteria are used as dietary supplement.

Micro-organisms which confer a health benefit on the host when administered in adequate amounts are called probiotics. Strains of the genera Bifidobacterium and Lactobacillus are the most widely used probiotic bacteria and are associated with several beneficial effects on human health. Some of the beneficial effects of Bifidobacteria are the production of vitamins, lowering blood cholesterol levels and inhibition of the growth of potential pathogens (Gibson and Robertfroid, J Nutr. 1995; 125:1401-12). Lactobacillus improves microflora balance in the large intestine by inhibiting the growth of harmful bacteria (Cats et al., Aliment Pharmacol Ther. 2003; 17:429-35). Some studies have proposed that this is a result of local pH reduction due to lactic acid production (Aiba et al., Am J Gastroenterol. 1998; 93:2097-101). Others question this and suggest that the secretion of antibacterial substances by Lactobacillus can inhibit the growth of pathogenic bacteria (Coconnier et al., Appl Environ Microbiol. 1998; 64:4573-80).

Prebiotics have a number of advantages over probiotics; principally the fact that they are not alive means that they can be processed into a much wider range of foods than can the fragile probiotics. Furthermore, prebiotics do not share the problem of probiotic survival upon ingestion by the consumer. Since prebiotics stimulate growth of bacteria that are already present in the gut, they can be seen as more "natural" additives or ingredients than probiotics, which necessitate administration of extraneous bacteria.

Critical to the prebiotic concept is their selective fermentation within the gut microflora by what are seen as beneficial genera. Various preferred relationships between prebiotics and beneficial bacteria have been reported. For example, oligofructose can be utilized efficiently by Lactobacilli and Bifidobacteria. WO2007/101675 relates to preparations comprising a probiotic and a prebiotic mixture which is specifically designed to enhance the efficiency and the efficacy of the probiotic and to food products comprising said preparation. Galacto-oligosaccharides (GOS) are an example of a prebiotic. GOS consist of short chains of galactose molecules which can only be partially digested by humans. They are not hydrolysed in the stomach or small intestine and should therefore reach the colon intact were they can be selectively fermented by Bifidobacteria and other bacteria. Studies have shown that GOS increases the number of Bifidobacteria and Lactobacillus as well as the

amount of short chain fatty acids (SCFAs) produced in the colon (Rycroft et al., Lett Appl Microbiol. 2001; 32:156-61).

SCFAs are fatty acids with short aliphatic tails consisting of 1 to 6 carbon atoms. They are produced when dietary fiber, non-starch polysaccharides, like GOS are fermented in the colon. Various population survey data show that especially acetate, propionate and butyrate are produced as end products (Cummings et al., Gut. 1987;28:1221-7). SCFAs have been associated with reduced risk of diseases, including cardiovascular disease, cancer and inflammatory bowel disease (Wong et al., J Biol Chem. 2001;276:44641-6). Studies have shown that butyrate has anti-inflammatory properties in several human epithelial cell lines (Yin et al., J Clin Gastroenterol. 2006;40:235-43, Lührs et al., Dig Dis Sci. 2001;46:1968-73).

Thus, it is known in the art that prebiotics are suitably used to modulate the relative abundance of micro-organisms of interest in the GIT, in particular Lactobacilli and Bifidobacteria. However, other commensal bacteria have recently been identified as having anti-inflammatory properties and being capable of supporting maturation of the intestinal immune system. These include *Bacteroides fragilis, Bacteroides thetaiotamicron* and *Faecalibacterium prausnitzii.*

Bacteroides fragilis has a direct interaction with the immune system through an extracellular polysaccharide, polysaccharide A (PSA), expressed by *B. fragilis* that can directly activate CD4+ T cells. PSA expressing *B. fragilis*, but also PSA alone, can restore the decreased peripheral CD4+ T cell numbers when given to germ-free mice, and have a promoting effect on lymphoid organogenesis (Mazmanian et al., Cell. 2005; 122:107-18). PSA further balances the Th2-biases responses seen in GF mice (IL-4 down, IFNγ up), and also restores immune balance and maturation of the immune system (Mazmanian et al., Nature. 2008;453:620-5). The latter was shown in a study in which experimentally induced colitis is prevented by *Bacteroides fragilis*, as well as by the *B. fragilis* polysaccharide PSA alone, probably as a result of the inhibition of the production of the proinflammatory cytokine IL-17 and induction of the anti-inflammatory cytokine IL-10.

Faecalibacterium prausnitzii (previously referred to as *Fusobacterium prausnitzii*) is an obligate anaerobic bacterium that is present in human faeces. It produces high levels of butyrate, an anti-inflammatory short chain fatty acid (SCFA). Recent evidence indicates that *F. prausnitzii* is an anti-inflammatory commensal

bacterium. *F. prausnitzii* is decreased in the microbiota of Crohns disease patients (Sokol et al., Inflamm Bowel Dis. 2009; 15:1183-9) and the presence of *F. prausnitzii* correlates with improved clinical outcome (Sokol et al., Proc Natl Acad Sci U S A. 2008;105:16731-6). *F. prausnitzii* reduces the inflammatory activation of intestinal epithelium as evidenced by IL-8 production through excretion of metabolites, inhibits the production of the proinflammatory cytokines IL-12 and IFN-γ, but stimulates the production of anti-inflammatory cytokine IL-10 in human peripheral blood cells (Sokol et al., Proc Natl Acad Sci U S A. 2008;105:16731-6). *F. prausnitzii* has an anti-inflammatory effect in a murine TNBS-induced colitis model (Sokol et al., Proc Natl Acad Sci U S A. 2008;105:16731-6). In addition, *Faecalibacterium prausnitzii* reduces the inflammatory activation of intestinal epithelium as evidenced by IL-8 production through the excretion of metabolites.

Bacteroides thetaiotaomicron has been shown to exhibit anti-inflammatory activity on epithelial cells (Kelly et al., Nat Immunol. 2004;5:104-12). This commensal has been shown to suppress proinflammatory cytokine production by intestinal epithelial cells.

Recognizing the therapeutic potential of beneficial microorganisms to modulate the immune system, e.g. in the treatment or prevention of immune-related disorders, the present inventors set out to identify further prebiotic(s) capable of enriching the gut microbiota for bacteria having anti-inflammatory properties and/or being capable of supporting maturation of the intestinal immune system, in particular enhancing the maturation of the developing the (neonatal) immune system.

Among others, they aimed at providing a dietetic, nutritional, nutraceutical or pharmaceutical composition, comprising at least one prebiotic in an amount effective to increase the relative abundance of *Bacteroides fragilis, Bacteroides thetaiotamicron* and/or *Faecalibacterium prausnitzii* in the gastrointestinal tract of an animal.

It was surprisingly observed that a class of milk-derived oligosaccharides is particularly useful to selectively promote the growth of commensal bacteria having anti-inflammatory properties and/or the capacity to support maturation of the intestinal immune system. More specifically, the inventors found that the growth of *B. fragilis* is strongly enhanced by a sialyl-containing oligosaccharide (and not by GOS).

In addition, prebiotic effects were also observed for the selective outgrowth of *Faecalibacterium prausnitzii* and *Bacteroides thetaiotamicron*. Furthermore, sialyl-containing oligosaccharide induced the production of the anti-inflammatory SCFAs propionate, butyrate and isobutyrate.

Accordingly, the invention provides in one aspect the use of a sialyl-oligosaccharide in the manufacture of a dietetic, nutraceutical, nutritional or pharmaceutical composition for supporting or enhancing the mammalian immune system.

The expression "supporting or enhancing the mammalian immune system" is meant to encompass any effect which is desirable or beneficial to maintain or improve the healthy functioning of the innate and/or adaptive immune system. Microorganisms or toxins that successfully enter an organism will encounter the cells and mechanisms of the innate immune system. The innate response is usually triggered when microbes are identified by pattern recognition receptors, which recognize components that are conserved among broad groups of microorganisms, or when damaged, injured or stressed cells send out alarm signals, many of which (but not all) are recognized by the same receptors as those that recognize pathogens. Innate immune defences are non-specific, meaning these systems respond to pathogens in a generic way. This system does not confer long-lasting immunity against a pathogen. The innate immune system is the dominant system of host defense in most organisms. The adaptive immune system evolved in early vertebrates and allows for a stronger immune response as well as immunological memory, where each pathogen is "remembered" by a signature antigen. The adaptive immune response is antigen-specific and requires the recognition of specific "non-self" antigens during a process called antigen presentation. Antigen specificity allows for the generation of responses that are tailored to specific pathogens or pathogen-infected cells. The ability to mount these tailored responses is maintained in the body by "memory cells".

In one embodiment, supporting or enhancing the mammalian immune system comprises enhancing the maturation of the developing immune system, in particular maturation of the neonatal immune system. Immune system maturation as used herein refers to development of an immune system characterized by a TH1/TH2 balance that is associated with a healthy adult phenotype. A healthy adult phenotype

is to be contrasted with a default TH2 phenotype that is characteristic of atopy, allergic asthma, or certain types of autoimmune disease. Immune system maturation can be assessed by measuring, either at a single point in time or serially over a relevant span of time, relative contributions of at least one TH1 marker and at least one TH2 marker. A T helper 1 marker (TH1 marker) as used herein refers to an objectively measurable manifestation of a TH1 immune phenotype. TH1 markers include, without limitation, certain cytokines including interferon gamma (IFN-γ) and interleukin 2 (IL-2), : transcription factors specific for Th1 cells including T-bet, Stat1, and Stat4, as well as certain immunoglobulin isotypes, e.g., IgG1 in humans and IgG2a in mice. Methods for measuring TH1 cytokines and immunoglobulin isotypes and transcription and/or differentiation factors are well known in the art and can include, without limitation, appropriate cytokine-specific or isotype-specific enzyme-lined immunosorbent assay (ELISA), bioassay, Western blotting, EMSA, quantitative reverse transcriptase-polymerase chain reaction, and the like.

A T helper 2 marker (TH2 marker) as used herein refers to an objectively measurable manifestation of a TH2 immune phenotype. TH2 markers include, without limitation, certain cytokines including interleukin 4 (IL-4) and interleukin 5 (IL-5), transcription factors specific for Th2 cells including Stat6, GATA-3, c-Maf, NFATs, as well as certain immunoglobulin isotypes, e.g., IgE in humans and in mice. Methods for measuring TH2 cytokines and immunoglobulin isotypes, and transcription and/or differentiation factors are well known in the art and can include, without limitation, appropriate cytokine-specific or isotype-specific ELISA, bioassay, Western blotting, EMSA, quantitative reverse transcriptase-polymerase chain reaction, and the like.

In another embodiment, supporting or enhancing the mammalian immune system comprises balancing the immune system to a status associated with the absence of an inflammatory response. Regulatory T cells (Treg) are another subset of CD4+ T helper lymphocytes. These Tregs can be CD25+, Foxp3+ cells, but also other subtypes of Tregs have been described. Tregs produce anti-inflammatory cytokines like IL-10 and TGF-β and are instrumental in inhibiting inflammatory responses. TGF-β, but also IL-10 can also promote immunoglobulin class-switching to induce the production of IgA by B cells and plasma cells. IgA is a non-complement binding

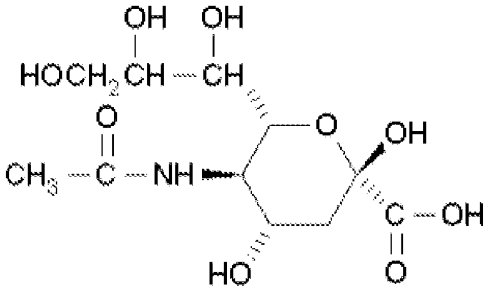
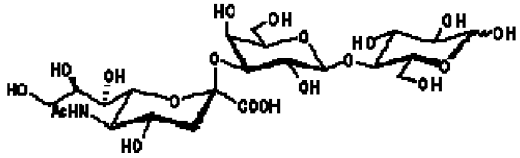
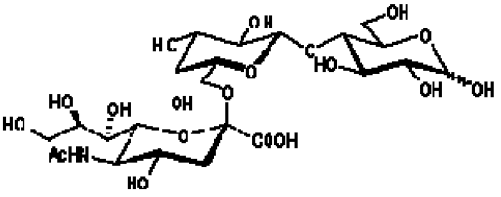
immunoglobulin isotype, and is considered a non-inflammatory immunoglobulin. A regulatory T cell (Treg marker) as used herein refers to an objectively measurable manifestation of a Treg immune phenotype. Treg markers include, without limitation, certain cytokines including interleukin 10 (IL-10) and transforming growth factor-β (TGF-β), transcription or differentiation factors specific for Treg cells including for example Foxp3 and CD25, as well as certain immunoglobulin isotypes, e.g., IgA in humans and in mice. Methods for measuring Treg cytokines, transcription and/or differentiation factors, and immunoglobulin isotypes are well known in the art and can include, without limitation, appropriate cytokine-specific or isotype-specific ELISA, bioassay, Western blotting, EMSA, quantitative reverse transcriptase-polymerase chain reaction, and the like

As used herein, the term "sialyl oligosaccharide" means an oligosaccharide having at least one sialic acid moiety with associated charge. Sialic acids comprise a family of about 40 derivates of the nine carbon sugar neuramic acid. Sialic acid is the generic term for the N- or O-substituted derivatives of neuraminic acid. Milk contains sialic acid moieties in different forms. It can be present as a constituent of water soluble free oligosaccharides (e.g. sialyllactose), or bound to either glycoproteins such as lactoferrin and κ-casein and/or glycolipids (GD3 and GM3). The present invention is preferably practiced using a sialyl oligosaccharide selected from the group consisting of sialyloligosaccharides derived from milk (e.g. cow, goat, camel, human milk), whey or egg. The sialyloligosaccharides can for instance be directly separated from milk, whey or egg or produced from components derived here from. Examples of components belonging to the group of sialyloligosaccharides are disialyllacto-*N*-tetraose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyllactosamine, 6'-sialyllactosamine, 3'-sialyl-3-fucosyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, disialyllactose, 3'-sialyl Lewis A, 3'-sialyl Lewis X, disialyllacto-N-hexaose I, disialyllacto-N-hexaose II, sialyl Lea tetra, sialyllacto-N-neotetraose c, disialyllacto-N-fucopentaose II. An exemplary commercial preparation comprising egg yolk sialyl oligosaccharides is Sunsial E from Tayio Kaguku Co, Japan. Another exemplary preparation is Vivinal SL, a product comprising sialyllactose (FrieslandCampina Domo).

In the sialyl oligosaccharides of human milk, sialic acid is attached to a penultimate galactose residue or N-acetylglucosamine residue via α2-3 or α2-6 linkage formed by the action of sialyltransferase (Kunz et al., Annu Rev Nutr. 2000;20:699-722, Asakuma et al., Biosci Biotechnol Biochem. 2007; 71:1447-51). Sialyllactose represents the main part of the sialyl-oligosaccharide fraction in human and cow's milk. In one embodiment, the invention provides a dietetic, nutritional or pharmaceutical composition comprising sialyllactose in an amount effective to increase the relative abundance of *Bacteroides* ssp. in the gastrointestinal tract. For example, (partially) purified 3'-sialyllactose, 6'-sialyllactose or a mixture thereof may be used. Sialyl oligosaccharide, such as sialyllactose, may be enzymatically produced. In a specific aspect, the product produced by FrieslandCampina Domo (The Netherlands) under the tradename Vivinal SL, comprising sialyllactose, is used as source of the prebiotic sialyl-oligosaccharide. The structures of sialic acid, 3'-sialylactose and 6'-sialyllactose are presented in Table 1.

The sialyl oligosaccharides 3'sialyllactose and /or 6'sialyllactose may be isolated e.g. by chromatographic or filtration technology from a natural source such as animal milks, preferably cow's milk. The concentration of oligosaccharides in cow's milk is between the 30 and 60 mg/litre. In this fraction, sialyllactose is the most abundant oligosaccharide. In a preferred embodiment, the sialyl-oligosaccharide is obtained by a method comprising ion exchange technology, as for example in WO 2009/113861. For example, whey permeate streams are used as source of sialyllactose, which is captured selectively from these streams via ion exchange technology. Subsequent downstream processing may include removal of remaining minerals and water, and a drying step.

Table 1

	Formula	Mw	pK	Structure
Sialic acid - Lactaminic acid - NAN - NANA - 5-acetamido-3,5-dideoxy-D-glycero-D-galactonoiunosonic acid	C11H19O9	309.3		
3'-Sialyllactose	C23H39NO19	633.5	1.92	
6'-Sialyllactose	C23H39NO19	633.5	2.01	

To achieve the desired health effect through enriching the gut microflora for bacteria having a positive effect on the immune system, e.g. *Bacteroides fragilis, Bacteroides thetaiotamicron* and/or *Faecalibacterium prausnitzii*, the composition comprises the at least one sialyl oligosaccharide in an amount sufficient or effective to increase the relative abundance of at least one of said bacteria in the gastrointestinal tract. In one aspect, the composition comprises at least one sialyl oligosaccharide in an amount of 0.005 – 20 grams per dose unit, for example a daily dose unit. A dose unit may consist of 0,2-500 grams, like 0,5, 15, 100, 150, 200 , 250 or 300 grams, depending among others on the type of composition (solid, liquid), intended use (complete food, pharmaceutical composition, food supplement) and/or the intended consumer (adult, infant).

For example, sialyl oligosaccharide may be present in an amount of 0.005 – 20 gram per 100 gram or 100 ml of composition, preferably 0.1-2 gram per 100 gram or 100 ml composition.

The composition may contain one or more further prebiotic ingredients known in the art. Examples of suitable prebiotics are fructo and/or galacto-oligosaccharides, with short or long chains, inulin, fucose-containing oligosaccharides, beta glycans, carob flour, gums, pectins, galactans with short or long chains, glucosaminegalactans and other glucaosamine containing oligosaccharides and nucleotides. In a preferred embodiment, a composition comprises sialyllactose and GOS. The composition may also contain one or more milk- or whey- derived components. Examples are sialic acid, alpha-Lactalbumin, lactoferrin, glycoproteins, casein macropeptide, gangliosides, phospholipids, colostrum, immunoglobulins, cytokines, milk calcium and nucleotides.

In addition to the sialyloligosaccharide having a prebiotic effect on (commensal) bacterial species resident in the GI-tract that can support or enhance the mammalian immune system, a composition according to the invention may also be supplemented with one or more of these species such that their relative abundance in the intestinal tract is even further enhanced. Therefore, a composition may further comprise *Bacteroides fragilis, Bacteroides thetaiotamicron, Faecalibacterium prausnitzii* or other *Bacteroides ssp.*, or any combination thereof. Of course, other beneficial probiotic strains may also be included, including specific strains of the genera Lactobacilli and Bifidobacteria that have been found to be able to colonise at least transiently the intestinal mucosa, to reduce the capability of pathogenic bacteria to adhere to the intestinal epithelium, to have immunomodulatory effects and/or to assist in the maintenance of well-being. Typically a minimum of 10^{7} cfu/g of formula is added although generally larger amounts are preferred, for example up to 10^{12} cfu/g of formula. The probiotic bacterial strain is preferably a lactobacillus or a bifidobacterium. Preferably strains which produce only L (+) lactic acid are used. Exemplary Lactobacillus (L.) species are *L. rhamnosus, L. paracasei, L. amylovorus, L. ultunensis, L. acidophilus, L. kalixensis, L. delbrueckii* subsp. *Lactis, L. oris, L. fermentum, L. gastricus, L. antri, L. fermentum, L. parabuchneri, L. brevis, L. plantarum, L. curvatus, L. sakei, L. gasseri, L. vaginalis, L. acidophilus, L. johnsonii, L. casei, L. salivarius* and *Lactobacillus reuteri.*. Particularly preferred strains are *Lactobacillus rhamnosus* ATCC 53103, *Lactobacillus rhamnosus* CGMCC 1.3724,

Lactobacillus reuteri ATCC 55730, *L. amylovorus* DSM20552, *L. ultunensis* DSM16047, *L. acidophilus* ATCC4356, *L. kalixensis* DSM16043, *L. delbrueckii* subsp. *lactis* DSM20073, *L. oris* DSM4864, *L. reuteri* DSM20016, *L. reuteri* DSM20053, *L. fermentum* DSM20055, *L. gastricus* DSM16045, *L. antri* DSM16041, *L. paracasei* subsp. *paracasei* 43362, *L. paracasei* subsp. *paracasei* 43332, *L. paracasei* subsp. *paracasei* 43338, *L. parabuchneri* DSM5707, *L. brevis* DSM20054, *L. plantarum* NCDO326, *L. plantarum* NCIMB8826, *L. curvatus* NCDO2739, *L. sakei* DSM20100, *L. ruminis* L5, *L. gasseri* SR21, *L. vaginalis* SR213, *L. fermentum* SR22, *L. gasseri* CR159, *L. acidophilus* NCFM, *L. acidophilus* LA5, *L. johnsonii* Lj1, *L. rhamnosus* GG, *L. casei* DN-144 001, *L. paracasei* CRL431, *L. reuteri* ATCC 55730, *L. reuteri* DSM17938, *L. gasseri* FC, *L. rhamnosus* LB21, *L. rhamnosus* 271, *L. reuteri* RC-14, *L. rhamnosis* GR-1, *L. plantarum* 299V and *Lactobacillus paracasei* CNCM I-2116.. Examples of preferred Bifidobacterium (B.) species are *B. lactis*, *B. breve B. lactis Bb-1, B. lactis Bb12, B. animalis, B. digestivus, B. infantis, B. adolescentis* and *B. longum*. Particularly preferred strains are the strain of *B. lactis* sold by the Christian Hansen company of Denmark under the trade mark Bb12, *Bifidobacterium longum* ATCC BAA-999 obtainable from Morinaga Milk Industry Co. Ltd. of Japan under the trade mark BB536, *B. Adolescentis* CIP 64.61, *B. Adolescentis* DSM 20083, *B. Adolescentis* NCFB 2229, *B. Adolescentis* NCFB 2204, *B. Adolescentis* LMG 10502, *B. animalis* JCM 20097, *B. animalis* DSM 20105, *B. bifidum* NCIMB 8810, *B. bifidum* LMG 11041, *B. breve* UCC2003, *B. breve* JCM 7017, *B. breve* JCM 7019, *B. breve* CCUG 43878, *B. breve* NCIMB 8815, *B. breve* NCFB 2258, *B. breve* NCFB 2257, *B. breve* NCFB 11815, *B. dentium* NCFB 2843, *B. longum* JCM 7050, *B. longum* JCM 7052, *B. longum* JCM 7056, *B. longum* CIP 64.63, *B. longum* CCUG 30698, *B. longum* NCIMB 8809, *B. longum* CCUG 15137, *B. longum* JCM 7053, *B. longum/infantis* CCUG 18157, *B. infantis* NCDO 2205, *B. pseudocatenulatum* LMG 10505, *B. pseudocatenulatum* NCIMB 8811, *B. pseudolongum* NCIMB 2244, *B. pseudolongum* DSM 20095, *B. globosum* JCM 5820, *B. globosum* JCM 7092, *B. thermophilum* JCM 7027. In a specific embodiment, the combination of *B. lactis* strain deposited under ATCC number 27536 and the *L. casei* strain deposited under ATCC number 55544 is used (see WO2008/056983 in the name of the applicant). In another specific embodiment, the combination of *B. lactis* BB12 and *L. paracasei* CRL431is used.

Alternatively, or additionally the composition further comprises at least one immunomodulatory substance originating from a beneficial microorganism such as from *Bacteroides fragilis*, *Bacteroides thetaiotamicron* and/or *Faecalibacterium prausnitzii*. The immunomodulatory substance is for instance a zwitterionic polysaccharide (ZPA) capable of activating CD4+ T cells, preferably it is polysaccharide A (PSA). In this respect, see also WO2009/062132.

Also provided is the use of at least one sialyloligosaccharide for the manufacture of a dietetic, nutraceutical, nutritional or pharmaceutical composition for treating or preventing a condition associated with a reduced amount of *Bacteroides fragilis, Bacteroides thetaiotamicron* and/or *Faecalibacterium prausnitzii* ssp. in the gastrointestinal tract. The sialyloligosaccharide is preferably selected from the group consisting of disialyllacto-N-tetraose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyllactosamine, 6'-sialyllactosamine, 3'-sialyl-3-fucosyllactose, sialyllacto-N-tetraose a, sialyllacto-N-tetraose b, sialyllacto-N-tetraose c, disialyllactose, 3'-sialyl Lewis A, 3'-sialyl Lewis X, disialyllacto-N-hexaose I, disialyllacto-N-hexaose II, sialyl Lea tetra, sialyllacto-N-neotetraose c, disialyllacto-N-fucopentaose II. In a specific aspect, the invention provides the use of 3'-sialyllactose, 6'-sialyllactose, or a mixture thereof, for the manufacture of a dietetic, nutraceutical, nutritional or pharmaceutical composition for treating or preventing a condition associated with a reduced amount of *Bacteroides fragilis, Bacteroides thetaiotamicron* and/or *Faecalibacterium prausnitzii* ssp. in the gastrointestinal tract. The invention further relates to the use of a sialyloligosaccharide to enhance the *in vivo* or *ex vivo* growth of a *Bacteroides fragilis, Bacteroides thetaiotamicron* and/or *Faecalibacterium prausnitzii*.

A composition of the invention may be in any form suitable for human administration, and in particular for administration in any part of the gastrointestinal tract. Enteral administration of the compositions of the invention, and preferably oral administration, and administration through a tube or catheter, are all covered by the present invention. It can be a solid, semi-solid or liquid nutritional formulation, such as a nutraceutical, nutritional or dietary supplement, functional food, beverage product, meal replacement, or food additive. Such nutritional compositions may be nutritionally complete, i.e. may include vitamins, minerals, trace elements as well as nitrogen, carbohydrate and fatty acid sources so that they may be used as the sole source of nutrition supplying essentially all the

required daily amounts of vitamins, minerals, carbohydrates, fatty acids, proteins and the like. Accordingly, the compositions of the invention may be provided in the form of a nutritionally balanced composition, e. g. a complete formula diet or a complete meal, e. g. suited for oral or tube feeding. Alternatively, the composition of the invention may be provided as part of a meal, i.e. a nutritional or dietary supplement, e. g. in the form of a health drink. It may be desirable to provide the composition of the invention in the form of a low calorie composition, e. g. meal replacement. In this case the nutritional composition, e.g. meal replacement, is preferably low fat, i.e. less than about 10 en%, or substantially fat-free, i.e. less than about 2.5 en% contributed by fat, such as about 2 en% fat, based on the total caloric content of the composition. Suitably, a single serving of a low calorie nutritional composition, e. g. meal replacement, will have a caloric value of less than about 1000 kcal, and preferably between about 200 kcal and about 500 kcal. Suitable low calorie nutritional composition may include soft drink, such as juice, smoothie or soy-based drink, or dispersed in foods of any sort, such as, dairy bars, soups, cereals, e. g. breakfast cereals, muesli, candies, tabs, cookies, biscuits, crackers, such as a rice crackers, and dairy products, such as milk-shake, yoghurt drink, yoghurts and fruit drinks. In one embodiment, the composition is a non-fermented composition. Alternatively, the compositions of the invention may be provided as high calorie compositions, e. g. high calorie dietary supplement or meal replacement, for instance with a caloric value of more than about 400 kcal, preferably more than about 600 kcal, more preferably more than about 800 kcal.

In a specific aspect, the composition is an infant formula including follow on formula or growing up milk. The skilled person will however appreciate that the concept of stimulating or enhancing the immune system by virtue of enhancing beneficial (commensal) bacteria by the approach herein disclosed is applicable to any type of liquid, solid or semi-solid composition for oral or enteral administration, be it for babies, infants, teenagers, adolescents, adults, pregnant women, elderly subjects, and the like.

Preferred compositions include infant formulas. An infant formula provided herein is especially suitable for supporting or enhancing the neonatal immune system. The infant formula is for example formulated to be administered to an infant of between 0 and 3 months of age, between 0 and 6 months of age, between 3 and 6

months of age, between 6 and 9 months of age, between 9 and 12 months of age (infant formula for infants between 6 and 12 months age are often called follow on formula). Also provided is an infant formula for infants older than 12 months and even older than 24 months, and up to an age of 6 years (often called growing up milk).. A preferred infant formula is formulated for infants of between 0 and 6 months of age.

Preferred compositions also include products for pregnant women, for maturation of the developing immune system of the unborn infant via the umbilical cord.

The bacterial composition in the infant digestive tract (also called the intestinal microflora) follows a pattern of change starting in the newborn, and varies depending on the infant diet. Development of the infant's intestinal microflora is initiated at birth. The aseptic, or sterile, digestive tract of the foetus is inoculated with bacteria during birth by the mother's intestinal and vaginal microflora. During the first week of life, enterobacteria and enterococci predominate in the gut of both breastfed and formula-fed infants. After this, the microflora changes rapidly. Some of the changes that occur depend on whether the infant is breastfed or formula-fed. An infant formula according to the present invention comprising a sialyl oligosaccharide, preferably a sialyllactose, can support, enhance, or balance the neonatal immune system. It can promote maturation of the immune system of a neonatal infant in the first weeks of the life of the infant, in particular by promoting in the first weeks of the life of the infant the development of a beneficial intestinal microbiota resembling the microbiota found in breast fed babies. Furthermore, it can balance the (infants') immune system to a status associated with the absence of an inflammatory response.

Infant formulas are commonly used today to provide supplemental or sole source nutrition early in life. These formulas contain protein, carbohydrate, fat, vitamins, minerals, and other nutrients. They are commercially available as powders, ready-to-feed liquids, and liquid concentrates. There are currently a variety of commercially available infant formulas, each one designed to meet the specific nutritional needs of a particular infant group. Milk-based infant formulas, for example, represent the majority of commercially available infant formulas. Soy- based formulas also represent a large portion of the infant formula market by offering an

alternative to milk-based formulas, especially in milk-intolerant infants. Lactose-free formulas are also available and can be useful in those infants with lactose sensitivity. Infant formulas with amino acids or partially hydrolyzed proteins are also available for certain infants.

The base infant formula comprises fat, protein, carbohydrate, vitamins and minerals, all of which are selected in kind and amount to provide a sole source of nutrition for the targeted infant or defined infant population. For example, the infant formula may be formulated for an infant of between 0 and 6 months of age, between 3 and 6 months of age, 6 and 9 months of age or 9 and 12 months of age. Infant formulas for use as base formulas include any known ready-to-feed infant formula, or any nutritional formula suitable for use in infants, provided that such a formula is a sole source nutritional having caloric density and osmolality values within the ranges defined herein. Many different sources and types of carbohydrates, fats, proteins, minerals and vitamins are known and can be used in the base formulas herein, provided that such nutrients are compatible with the added ingredients in the selected formulation and are otherwise suitable for use in an infant formula. Carbohydrates suitable for use in the base formulas herein may be simple or complex, lactose-containing or lactose-free, or combinations thereof, non-limiting examples of which include hydrolyzed, intact, naturally and/or chemically modified cornstarch, maltodextrin, glucose polymers, sucrose, corn syrup, corn syrup solids, rice or potato derived carbohydrate, glucose, fructose, lactose, high fructose corn syrup and indigestible oligosaccharides such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), and combinations thereof. Particularly preferred is an infant formula comprising the combination of sialyllactose and GOS.

Proteins suitable for use in the base formulas herein include hydrolyzed, partially hydrolyzed, and non-hydrolyzed or intact proteins or protein sources, and can be derived from any known or otherwise suitable source such as milk (e.g., casein, whey, human milk protein), animal (e.g., meat, fish), cereal (e.g., rice, corn), vegetable (e.g., soy), or combinations thereof. In one embodiment, the composition of the invention comprises a whey fraction comprising the whey proteins a-lactalbumin (a-LA) and casein macropeptide (CMP), wherein the weight ratio between a-LA and CMP is < 2. Proteins for use herein can also include, or be entirely or partially replaced by, free amino acids known for or otherwise suitable for use in infant

formulas, non-limiting examples of which include alanine, arginine, asparagine, carnitine, aspartic acid, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, taurine, threonine, tryptophan, taurine, tyrosine, valine, and combinations thereof. These amino acids are most typically used in their L-forms, although the corresponding D-isomers may also be used when nutritionally equivalent. Racemic or isomeric mixtures may also be used.

Fats suitable for use in the base formulas herein include coconut oil, soy oil, corn oil, olive oil, safflower oil, high oleic safflower oil, algal oil, MCT oil (medium chain triglycerides), sunflower oil, high oleic sunflower oil, palm and palm kernel oils, palm olein, canola oil, marine oils, cottonseed oils, and combinations thereof.

Vitamins and similar other ingredients suitable for use in the base formulas include vitamin A, vitamin D, vitamin E, vitamin K, thiamine, riboflavin, pyridoxine, vitamin B12, niacin, folic acid, pantothenic acid, biotin, vitamin C, choline, inositol, salts and derivatives thereof, and combinations thereof. Minerals suitable for use in the base formulas include calcium, phosphorus, magnesium, iron, zinc, manganese, copper, chromium, iodine, sodium, potassium, chloride, and combinations thereof.

Preferred probiotics for use in an infant formula include those capable of promoting the development of an early bifidogenic intestinal microbiota, e.g. the strains disclosed in EP 1974734.

A pharmaceutical composition as provided herein, e.g. clinical product, and nutritional compositions of the invention, e. g. dietary supplements, may be provided in the form of soft gel, sachets, powders, syrups, liquid suspensions, emulsions and solutions in convenient dosage forms. Oral pharmaceutical or dietary supplement forms may be made by conventional compounding procedures known in the pharmaceutical art, that is, by mixing the active substances together with edible pharmaceutical acceptable solid or liquid carriers and/or excipients, e. g. fillers such as cellulose, lactose, sucrose, mannitol, sorbitol, and calcium phosphates and binders, such as starch, gelatin, tragacanth, methylcellulose and/or polyvinylpyrrolidone (PVP). Optional additives include lubricants and flow conditioners, e. g. silicic acid, silicon dioxide, talc, stearic acid, magnesium/calcium stearates, polyethylene glycol (PEG) diluents, disintegrating agents, e. g. starch, carboxymethyl starch, cross-linked PVP, agar, alginic acid and alginates, colouring agents, flavouring agents, and

melting agents. Dyes or pigments may be added to the tablets or dragee coatings, for example for identification purposes or to indicate different doses of active ingredient.

In a further aspect, the invention also provides a method for enhancing the maturation of the immune system in a mammalian subject, preferably a human subject, comprising administering to the subject a composition comprising at least one sialyl oligosaccharide. The method preferably comprises enhancing the maturation of the developing immune system, more preferably the human neonatal immune system. Said enhancement for example comprises an increase in level of one or more of *Bacteroides fragilis, Bacteroides thetaiotamicron* and *Faecalibacterium prausnitzii.*

Also within the scope of the present invention is the use of a sialyl oligosaccharide to enhance the growth of *Bacteroides fragilis, Bacteroides thetaiotamicron* and/or *Faecalibacterium prausnitzii,* preferably at least *Bacteroides fragilis.* Growth can be *in vitro* or *in vivo.* Suitable growth promoting sialyloligosaccharides include disialyllacto-*N*-tetraose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyllactosamine, 6'-sialyllactosamine, 3'-sialyl-3-fucosyllactose, sialyllacto-*N*-tetraose a, sialyllacto-*N*-tetraose b, sialyllacto-*N*-tetraose c, disialyllactose, 3'-sialyl Lewis A, 3'-sialyl Lewis X, disialyllacto-N-hexaose I, disialyllacto-N-hexaose II, sialyl Lea tetra, sialyllacto-N-neotetraose c and disialyllacto-N-fucopentaose II**.** A preferred sialyl oligosaccharide for use as prebiotic for the above mentioned beneficial bacteria is a (partially) purified sialyllactose, such as 3'-sialyllactose, 6'-sialyllactose or a mixture thereof.

A healthy, or balanced, microbiota has been considered to be one that is predominantly carbohydrate- fermenting (saccharolytic) and comprises significant numbers of bifidobacteria and lactobacilli. The latter is based on a number of observations: The products of carbohydrate (saccharolytic) fermentation, principally short-chain fatty acids (SCFA) have a great impact on colon physiology and are beneficial to host health (Topping et al., Physiol Rev 2001; 81:1031-64). They either directly or indirectly affect the proliferation of enterocytes, inflammation,colorectal carcinogenesis, mineral availability, colonisation by pathogens, enzyme activities and the product ion of nitrogenous metabolites.

An important metabolic function of the microbiota is the fermentation of non-digestible carbohydrates and endogenous mucus produced by the epithelia. The

overall outcomes of this complex metabolic activity are the recovery of metabolic energy and absorbable substrates for the host , and the supply of energy and nutritive products for bacterial growth and proliferation. The metabolic endpoint is the generation of the SCFA's acetate, propionate and butyrate, having important functions in host physiology.

Another highly important role of the intestinal microbiota in colon physiology is its trophic effect on the intestinal epithelium. Differentiation of the epithelial cell is considerably influenced by the interaction with resident microorganisms and their metabolic products, mainly SCFA; stimulating epithelial cell proliferation and differentiation of the small and large bowel. Intestinal bacteria also form a barrier against non-indigenous microorganisms, including pathogens: a phenomenon called colonization resistance. Lactic acid-producing bacteria, such as bifidobacteria and lactobacilli are believed to play a significant role in the maintenance of colonization resistance.

Finally, as an example of indirect effects of prebiotics on the immune system, short chain fatty acids have been shown to exert anti-inflammatory effects, which may in part provide a mechanism for the effects of prebiotics. Butyrate has been shown to inhibit NF-kB activation in intestinal epithelial cells under proinflammatory conditions (Inan et al., Gastroenterology. 2000;118:724-34, Yin et al., J Clin Gastroenterol. 2006;40:235-43), and has also been shown to inhibit T-cell activation (Cavaglieri et al., Life Sci. 2003;73:1683-90). Similar findings have also been reported for acetate and propionate (Tedelind et al., World J Gastroenterol. 2007;13:2826-32), suggesting that the production of short chain fatty acids induced by prebiotics may contribute to the maintenance of a non-inflammatory environment in the intestine.

There are four different receptors that bind, and are activated by fatty acids, GPR40, GPR41, GPR42 and GPR43. They form a cluster of four orphan G-protein coupled receptors, tandemly located on chromosome 19q13.1. These receptors exhibit 30-40% homology to one another. Agonists of GRP40 are medium to long-chain fatty acids and those for GPR41 and GPR43 are short chain fatty acids. GPR42 is closely related to GPR41, differing in only seven nucleotide positions. Due to lack of detectable expression in tissue and lack of functional ligand responses following recombinant expression, human GPR42 is currently described as an open reading frame pseudo gene. (Brown et al., DNA Cell Biol. 2005; 24:54-61).

GPR43 and GPR41 activation by SCFAs can be determined using a cAMP accumulation assay and a fluorescent-based calcium mobilization assay. Agonist activity can be characterized by the amount of potency. For GPR43, the rank order was propionate > acetate = butyrate > isobutyrate > caproate > isovalerate > valerate > formate > pivalate. For GPR41, the rank order of potency showed notable differences. The order was propionate > isobutyrate > butyrate > valerate > isovalerate > caproate > pivalate > acetate. Formate was totally inactive on this receptor (Le Poul 2003). The activation properties of lactate are unknown. GPR43 is mainly expressed on leukocyte populations, particularly neutrophils, whereas GPR41 expression is more widely distributed in tissues, including in intestinal epithelium (Brown et al., J Biol Chem. 2003; 278:11312-9).

Propionate, isobutyrate and butyrate thus have the highest potency for effects mediated through GPR41 of all the short chain fatty acids. The present inventors found a high production of all three of these SCFA induced by sialyllactose. As it is known that butyrate and propionate have anti-inflammatory effects on intestinal epithelium that expresses GPR4, induction of high propionate, butyrate, and isobutyrate levels by sialyllactose is expected to have a positive, anti-inflammatory effect on intestinal epithelium.

LEGEND TO THE FIGURES

Figure 1: Effects of sialyllactose and GOS on micoflora composition of batch cultures inoculated with a pool of adult faeces. The panels show the total amount of bacteria per vessel (A), bacteroides (B), and bifidobacteria (C). numbers quantified by QPCR. Values shown are averages of two separate runs, with error bars indicating the deviation of the mean of the two measurements.

Figure 2: Effects of sialyllactose and GOS on micoflora composition of batch cultures inoculated with a pool of adult faeces. The panels show the amount of bacteroides (A), and bifidobacteria (B) numbers quantified by QPCR. Values shown are values corrected for the growth in each vessel relative to t=0, and are averages of two

separate runs, with error bars indicating the bacterial counts of the two separate runs.

Figure 3: Effects of sialyllactose and GOS on micoflora composition of batch cultures inoculated with a pool of infant faeces as described in example 2. The panels show the total amount of bacteria per vessel (A), bacteroides (B), and bifidobacteria (C) numbers quantified by QPCR. Values shown are averages of two separate runs, with error bars indicating the deviation of the mean of the two measurements..

Figure 4 : Effects of sialyllactose and GOS on microflora composition of batch cultures inoculated with a pool of infant faeces as described in example 2. The panels show the amount of bacteroides (A), and bifidobacteria (B) numbers quantified by QPCR. Values shown are values corrected for the growth in each vessel relative to t=0, and are averages of two separate runs, with error bars indicating the bacterial counts of the two separate runs.

Figure 5: Effects of purified sialyllactose and purified GOS on the cumulative production of SCFA in batch cultures inoculated with a pool of adult faeces as described in example 1. The panels show the production of total SCFA levels (A), acetate (B), butyrate (C), lactate (D), propionate (E), and formiate (F), all expressed as concentration in mM.

Figure 6: Effects of sialyllactose and GOS on the cumulative production of SCFA in batch cultures inoculated with two different pools of infant faeces, essentially as described in example 2. The panels show the production of total SCFA levels (including lactate), acetate, butyrate, lactate, propionate, and formiate, and iso-butyrate, all expressed as concentration in mM. Figure 6A and figure 6B show results with two independent infant faeces pools.

EXPERIMENTAL SECTION

Example 1: Effect of sialyl oligosaccharide on adult faeces microflora composition.

Improvement of the health of human can be accomplished by selective stimulation of the growth and/or activity of groups of bacteria in the colon. This stimulation can be induced by prebiotics like oligo- and polysaccharides. This example describes a comparison of the selective fermentation of GOS and sialyl-containing oligosaccharides by adult human microflora. GOS (Vivinal GOS, FrieslandCampina Domo) and sialyllactose (Vivinal SL, FrieslandCampina Domo) were purified in order to make them suitable for use in the in vitro model and to exclude interference with other components like lactose and glucose. A pH-controlled batch fermentation model, which represents the distal part of the colon, was used to measure the effect of the oligosaccharides on the composition of the microflora, and the production of short chain fatty acids and lactate.

Methods

Production of oligosaccharides

Both GOS (Vivinal GOS) and sialyllactose (Vivinal SL) were purified by means of chromatography into purified GOS and purified SL

Faecal Sample Preparation

Faecal samples were obtained from 16 adult volunteers (average BMI 23,4, range 18,0 - 27,8) free of known gastrointestinal disorders. None of the volunteers had taken prebiotic products within a three-month period prior to sampling. Individual faecal samples were diluted to a 1 in 10 weight/weight mixture using 0.1M, pH7, phosphate buffered saline (PBS) (Sigma laboratories, Gillingham, Dorset, UK) at pH7.4 and pooled. The PBS was reduced overnight in an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2). This mixture was then homogenised for 120 seconds (Seward Stomacher 80 Biomaster), at normal speed, in the stomacher. The faecal slurry was aliquotted and

frozen. The faecal slurry was thawed just prior to the start of the experiment and used to inoculate the batch cultures.

pH Controlled, Stirred batch cultures

Chemostat nutrient medium was prepared containing the following ingredients: Peptone water 2g/l (Oxoid, Basingstoke, UK), yeast extract 2g/l (Oxoid), NaCl 0.1g/l (Fisher, Loughborough, UK), $K_2HPO_4.3H_2O$ 0.05g/l (BDH, Poole, UK), KH_2PO_4 0.04g/l (BDH), $MgSO_4.7H_2O$ 0.01g/l (Sigma), $CaCl_2.2H_2O$ 0.005g/l (BDH), $NaHCO_3$ 2g/l (Sigma), Tween 80 (BDH) 2ml, Hemin 0.02g/l, Vitamin K_1 10µl (Sigma), Cysteine HCl 0.5g/l (Oxoid), Bile Salts 0.5g/l (Oxoid), Resazarin 4ml/l (Sigma), pH7.

The chemostat nutrient medium autoclaved, then aseptically poured into the sterile batch culture vessels. This was left overnight with nitrogen pumping through the vessel to provide an anaerobic environment. 0.5ml of 10% cysteine-HCl was added to aid reduction of the medium. pH meters (Electrolab pH controller, Tewksbury, UK) were set to regulate the vessels between pH6.8 and pH7 by the addition of 0.5M HCl or 0.5M NaOH. The vessels were maintained at 37°C, and were stirred using magnetic stirrers. The test material (purified GOS or purified SL were added to the vessel to a final concentration of 10 g/l oligosaccharide just prior to the addition of 15 ml of faecal slurry of a pool of adult faecal samples (10% w/w). The vessels were left for 24h, with samples taken at 0, 3, 6, 9 and 24h. The samples were centrifuged for 5 min at 13 200 rpm and supernatants and pellet fractions were stored at –20 C until SCFA measurement (supernatants) or DNA preparation for QPCR analysis (pellet). The pH-controlled fermentation experiments were conducted in two independent experiments using samples from the same faeces pool as indicated.

QPCR analyses

DNA was isolated from frozen pellets of fermented faecal samples using the QIAamp DNA Stool Mini Kit (Qiagen). The number of total bacteria and bacteria of specific groups were quantified by quantitative PCR essentially as described in EP1997907. The primers and probes for the detection of total bacteria, Bifidobacteria, and *Bacteroides* were based on 16S rDNA gene sequences, retrieved by http://greengenes.lbl.gov website. From these sequences forward primers, reverse primers and TaqMan probes were designed using the Primer Express software. To

check specificity, the selected primers and probes were compared to all available 16S rDNA gene sequences using the BLAST database search program. Applied Biosystems manufactured primers and probes.

Results

Batch culture fermentation experiments were set up as described above. The results shown in Figure 1 show that SL strongly enhances the growth of Bacteroides, and GOS strongly enhances the growth of Bifidobacteria.

During the batch fermentation only a small increase in total bacteria counts was seen, especially in the carbohydrate groups (Figure 1A). As the total number of bacteria increased in each of the vessels, the results are also shown as values corrected for the total bacteria number in each vessel relative to t=0 (Figure 2). SL was the only compound tested that clearly promoted the growth of bacteroides (Figure 1B). This was also the case after correction for growth of bacteria in the vessel, but the effect was less prominent (Figures 1B and 2A).

A clear stimulatory effect on bifidobacteria was seen in the vessels containing GOS (Figure 1C). This was also clear after correction for the total growth of bacteria in the vessels compared to t=0 (Figure 2B). SL had only a minor effect on bifidobacteria (Figure 1C and 2B) compared to the control. These results demonstrate that SL can selectively promote the growth of bacteroides in faeces from human adults.

Example 2: Effect of sialyl oligosaccharide on baby faeces microflora composition.

This example demonstrates the effect of oligo- and polysaccharides on the microbial population of baby faeces by *in vitro* fermentation. The experiments were essentially carried out as described in Example 1, only now with a batch of *in vitro* prefermented infant faeces. The test groups included a control vessel, a vessel containing purified GOS (10 g/L oligosaccharide), and a vessel containing purified SL (10g/L oligosaccharide).

Results

After DNA isolation of the fermented samples the amount of bacteria were determined and displayed in Figure 3, and as displayed as values corrected for total bacteria growth compared to t=0 in Figure 4.

Figure 3B shows the amounts of Bacteroides present during batch fermentation. Bacteroides were stimulated by SL and GOS when compared to the control fermentation. When corrected for the total bacteria numbers a stimulation of Bacteroides was most consistent and prominent for SL (Figure 4A). Figure 3A shows that the total growth of bacteria was stimulated by GOS and SL up to a 2-5 fold increase during 24 h. The total bacteria count of control was stable. Figure 3C illustrates that the Bifidobacteria were stimulated by GOS and SL compared to the Bifidobacteria in the control vessel present. After correction for the total bacteria count (Figure 4B), there is an increase of the amount of Bifidobacteria during the first part of the fermentation (till 9 h), whereas in the second part of the fermentation the amount of Bifidobacteria decreased.

These results demonstrate that SL can also increase the amount of Bacteroides in infant faeces.

Example 3: Species analysis using DNA-Chip

The prebiotic effect of Sialyllactose was further investigated using DNA micro array technology (TNO, Zeist, the Netherlands). DNA obtained from the in vitro batch cultures of Examples 1 and 2 (T=24 h) were subjected to a DNA chip designed for the detection of more than 400 bacterial species. For each species represented on the microarray one or more unique short oligonucleotide sequences from within the 16S rDNA gene were selected. Criteria for sequence selection, apart from being unique, included length and melting temperature. Short oligonucleotide sequences (approx 20 nt) were used with a melting temperature of 60°C according to the Wallace rule for which a one nucleotide mismatch already resulted in an absence (or very strong decrease) of signal after hybridization.

Results on the effect of Sialyllactose (SL) or GOS on the preferential growth of beneficial bacteria are shown in Tables 2A (adult) and 2B (infant). The numbers are based on duplicate analysis, and represent the fold increase over background level.

Table 2A

	Control	SL	GOS
Bacteroides fragilis	10	120	25
Bacteroides thetaiotaomicron	200	100	160
Faecalibacterium prausnitzii	2	70	3

Table 2B

	Control	SL	GOS	SL+GOS
Bacteroides fragilis	25	190	125	190
Bacteroides thetaiotaomicron	40	180	90	150
Faecalibacterium prausnitzii	<2	<2	<2	<2

The results shown in Tables 2A and 2B demonstrate that SL strongly enhances the growth of *B. fragilis*, *B. thetaiotaomicron* and *F. prausnitzii*. There are some differences between the findings in adult batch culture experiments and infant batch

culture experiments. As seen in Table 2A, in adults *B. thetaiotamicron* is already abundantly present, and cannot be further upregulated by SL or GOS. In fact, the addition of these oligosaccharides results in a 1-2 fold decrease in *B. thetaiotamicron*. On the contrary, *B. fragilis* and *F. prausnitzii* are upregulated by more than 10-fold. In infant batch culture experiments, no *F. prausnitzii* was present in the infant faeces pool used for the experiment shown here, hence no upregulation was seen under the influence of SL or GOS. *B. fragilis* was strongly upregulated by SL, as was *B. thetaiotamicron*. The prebiotic effect of SL on these bacteria was stronger than that exerted by GOS and remained intact when SL was used in combination with the bifidogenic GOS (data shown for baby faeces only). Taken together, the results in this example demonstrate that SL can selectively promote the outgrowth of the beneficial bacteria *B. fragilis, B. thetaiotamicron*, and *F. prausnitzii*.

Example 4 : Sialyllactose induces production of SCFA with anti-inflammatory potential

Batch culture vessels were inoculated with adult or infant faeces pools, and fermentations were performed in the presence of purified GOS and purified sialyllactose as described in examples 1 and 2. Samples were thawed at room temperature. Twenty µL $K_4Fe(CN)_6.3H_2O$, 20 µL $ZnSO_4.7\ H_2O$ and 10 µL 0.25 mM NaOH were added to 450 µL supernatant. The samples were mixed after addition of every reagent and centrifuged again (5 min, 16 110 x g); supernatants were transferred into HPLC vials. The HPLC method, according to Guerrant (Guerrant GO, Lambert MA, Moss CW. Analysis of short-chain acids and lactate from anaerobic bacteria by high-performance liquid chromatography. J Clin Microbiol. 1982;16:355-60), was used with an ion-exchange column (HPX-87H), 0.006 M H_2SO_4 as eluent, and UV-detection at 205 nm. A SCFA mixture, containing succinate, lactate, formate, acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate, was employed in making a calibration curve. On the chromatogram, the baseline was adjusted manually, as appropriate.

Figure 5 shows the effect of GOS and Sialyllactose on SCFA and lactate production in batch cultures inoculated with adult faeces. In general purified GOS and sialyllactose induced high levels of SCFA, whereas the SCFA production in the control vessel was very low. Whereas GOS induced the production of high levels of lactate, Sialyllactose induced no lactate. Sialyllactose on the other hand, induced the formation of much higher propionate levels than GOS, and comparable but slightly higher levels of formiate, acetate, and butyrate. The maximum production of isobutyrate did not exceed a concentration of 5 mM (not shown).

Similar results were obtained in batch culture experiments performed with two different pools of infant faeces (Figure 6A and 6B). In these experiments also different batches of purified Sialyllactose were used. Sialyllactose induced the production of much less lactate and formiate, but much higher levels of isobutyrate than GOS (Figure 6A). Production levels of propionate, butyrate, and acetate were similar but slightly higher in the vessels containing Sialyllactose than those containing GOS. Figure 6B shows similar results in another batch culture experiment performed with a different infant faeces pool and a different batch of Sialyllactose. Again, the production levels of propionate, butyrate, and acetate induced by Sialyllactose were comparable to GOS, but were slightly lower in this experiment than in the experiment shown in Figure 6A. As in the previous experiment, the production of isobutyrate was much higher, and the production of formiate was much lower with Sialyllactose compared to GOS. As the Sialyllactose batch used in this experiment contained lactate that was consumed during the batch fermentation, no conclusion can be drawn about the lactate production, even though as the lactate is consumer quickly in the Sialyllactose vessels it appears that no new lactate is produced, whereas lactate is produced in the presence of GOS.

When comparing the levels of SCFA having anti-inflammatory potential (butyrate, propionate, and isobutyrate, all ligands for GPR41), it is clear from Table 3 that sialyllactose induces higher absolute as well as relative amounts of GPR41 ligand SCFA. The results described in this example indicate that sialyllactose induces a SCFA production profile that can have anti-inflammatory potential e.g. in the intestine.

SCFA	Control	GOS	Sialyllactose
Formiate	13,1 +/- 11,3	22,9 +/- 12,9	10,9 +/- 10,3
Acetate	44,7 +/- 2,7	65,1 +/- 21,1	76,9 +/- 17,6
Propionate	4,6 +/- 1,0	12,8 +/- 6,6	17,5 +/- 2,2
Butyrate	7,6 +/- 13,1	16,9 +/- 6,6	17,3 +/- 11,5
i-Butyrate	4,4 +/- 3,0	3,4 +/- 3,3	14,0 +/- 13,9
Total SCFA	74,4 +/- 29,2	121,1 +/- 42,1	136,6 +/- 20,4
Anti inflammatory SCFA	16,6 +/- 16,2	33,2 +/- 13,9	48,8 +/- 13,7
Anti inflammatory SCFA (expressed as % of total)	19,1 +/- 12,1	27,3 +/- 9,1	35,8 +/- 9,5

Table 3. Average SCFA production of the three batch culture experiments performed shown in Figure 5 and Figure 6

Example 5 : Infant formulas

Table 4 shows the composition of three exemplary nutritional formulas according to the invention, e.g. infant formulas for the age group between 0-6 months, for supporting or enhancing the infant's immune system.

Table 4: Composition of the formulas (per 100 ml)

	Basic	Formula A: SL	Formula B: SL+GOS	Formula C: SL+ probiotics
Energy, kcal	67	67	67	67
Protein (g)	1.4	1.4	1.4	1.4
Carbohydrates (g)	7.6	7.4	6.7	6.4
Fat (g)	3.5	3.5	3.5	3.5
Sialyllactose (g)		0.25 (range 0.005 – 2.0 g/100ml)	0.2	0.2
Vivinal GOS (g)			0.5 (range 0.1 – 2.0 g/100 ml)	0.5
Bacteroides (cfu)				10exp12

1. De toepassing van een sialyloligosaccharide bij de bereiding van een van een dieet-, nutraceutische, voedings-, of farmaceutische samenstelling voor het bevorderen van de rijping van het in ontwikkeling zijnde immuunsysteem.

2. Toepassing volgens conclusie 1, waarbij genoemde samenstelling een kindervoeding, een opvolgformule, een opgroeimelk of een product voor zwangere vrouwen is.

3. Toepassing volgens conclusie 2, waarbij genoemde samenstelling een kindervoeding is welke dient te worden toegediend aan een kind in de leeftijd tussen 0 en 6 maanden.

4. Toepassing volgens conclusie 2 of 3, voor de bereiding van een kindervoeding voor het bevorderen van de rijping van het immuunsysteem van een te vroeg geboren kind gedurende de eerste levensweken van het kind.

5. Toepassing volgens conclusie 4, om gedurende de eerste levensweken van het kind de ontwikkeling van een gunstige darmflora zoals gevonden in borstgevoede baby's te bevorderen.

6. De toepassing van een sialyloligosaccharide bij de bereiding van een dieet, nutraceutische of farmaceutische samenstelling voor het in balans brengen van het immuunsysteem tot een toestand die is geassocieerd met de afwezigheid van een ontstekingsrespons.

7. Toepassing volgens conclusie 6, voor de behandeling van chronische darmontsteking.

8. Toepassing volgens een der voorgaande conclusies, waarbij het genoemde sialyloligosaccharide 3'-sialyllactose, 6'-sialyllactose, of een mengsel daarvan is.

9. Toepassing volgens een der conclusies 1 tot 8, waarbij de samenstelling verder een of meer aanvullende prebiotica omvat, bij voorkeur gekozen uit de groep bestaande uit fructooligosacchariden (FOS), galactooligosacchariden (GOS), inuline, fucose-bevattende oligosaccharide, glucosamine-bevattende oligosaccharide, beta glycanen, carobmeel, gommen, pectines, galactanen met korte of lange ketens en nucleotiden, bij grotere voorkeur GOS.

10. Toepassing volgens een der conclusies 1 tot 9, waarbij genoemde samenstelling verder *Bacteroides fragilis, Bacteroides thetaiotamicron* en/of *Faecalibacterium prausnitzii* of andere *Bacteroides* ssp. omvat.

11. Toepassing volgens een der conclusies 1 tot 10, waarbij genoemde samenstelling verder ten minste één immuunmodulerende substantie verkregen uit *Bacteroides fragilis, Bacteroides thetaiotamicron* en/of *Faecalibacterium prausnitzii* omvat.

12. Toepassing volgens conclusie 11, waarbij genoemde immuunmodulerende substantie een zwitterionisch polysaccharide (ZPA) is, welke in staat is om $CD4^+$ T cellen te activeren, bij voorkeur polysaccharide A (PSA).

13. Toepassing van ten minste één sialyloligosaccharide voor de bereiding van een dieet-, nutraceutische, voedings-, of farmaceutische samenstelling voor de behandeling of ter voorkoming van een conditie die is geassocieerd met een verlaagde hoeveelheid *Bacteroides fragilis, Bacteroides thetaiotamicron* en/of *Faecalibacterium prausnitzii* ssp. in het maagdarmkanaal.

14. Toepassing volgens een der voorgaande conclusies, waarin genoemde ten minste ene sialyloligosaccharide wordt toegepast in een hoeveelheid van 0.005 – 20 gram per doseringseenheid van de samenstelling.

15. Een werkwijze voor het ondersteunen of bevorderen van het immuunsysteem in een zoogdierindividu, bij voorkeur een mens, omvattende het

toedienen van een samenstelling omvattende ten minste één sialyloligosaccharide aan het individu.

16. Werkwijze volgens conclusie 15, waarin het ondersteunen of bevorderen van het immuunsysteem het bevorderen van de rijping van het zich ontwikkelende immuunsysteem omvat.

17. Werkwijze volgens conclusie 16, waarin het zich ontwikkelende immuunsysteem het in ontwikkeling zijnde immuunsysteem van een te vroeg geboren mens is.

18. Toepassing van een sialyloligosaccharide, bij voorkeur 3'-sialyllactose, 6'-sialyllactose of een mengsel daarvan, ter bevordering van de groei van *Faecalibacterium prausnitzii*.

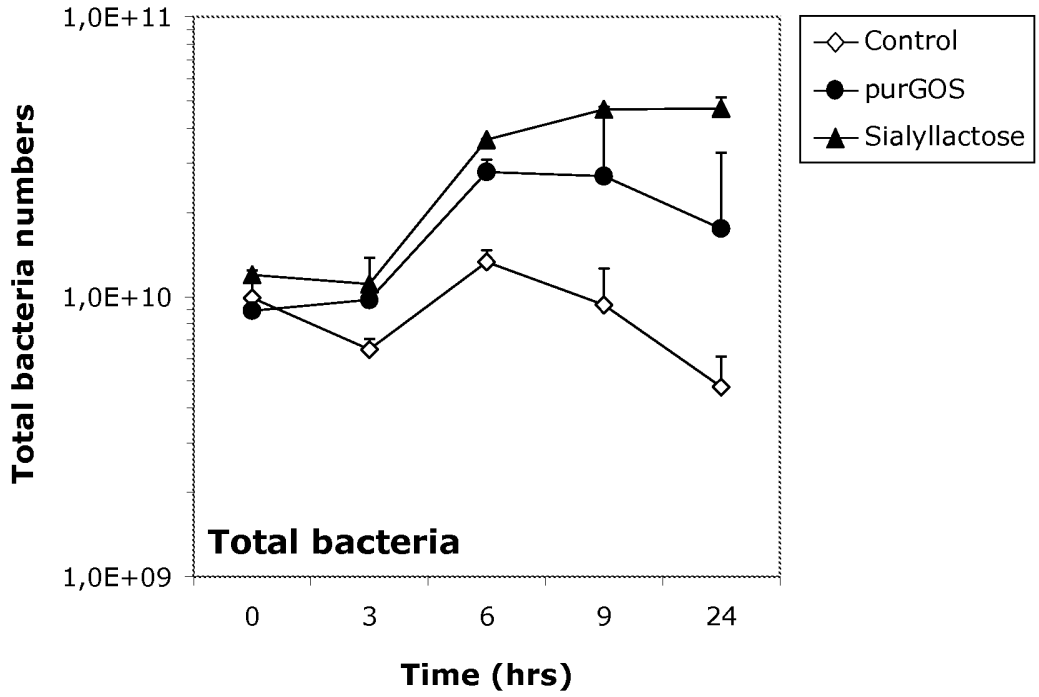

1,0E+11
1,0E+10
1,0E+09
Total bacteria numbers
Total bacteria
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 1A

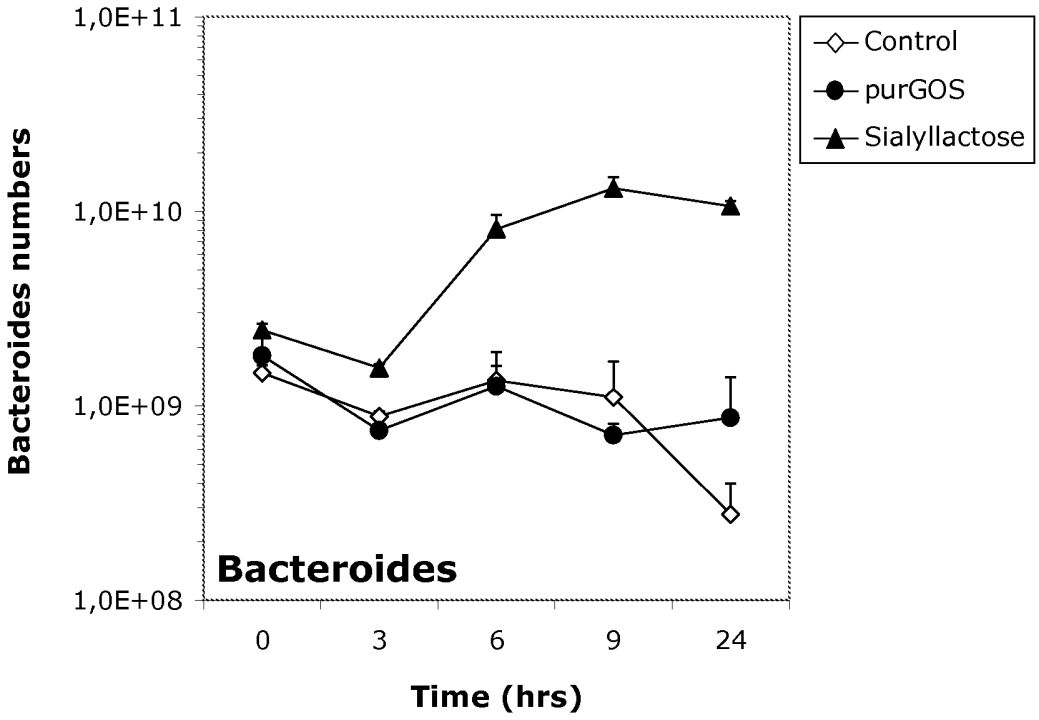

1,0E+11
1,0E+10
1,0E+09
1,0E+08
Bacteroides numbers
Bacteroides
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 1B

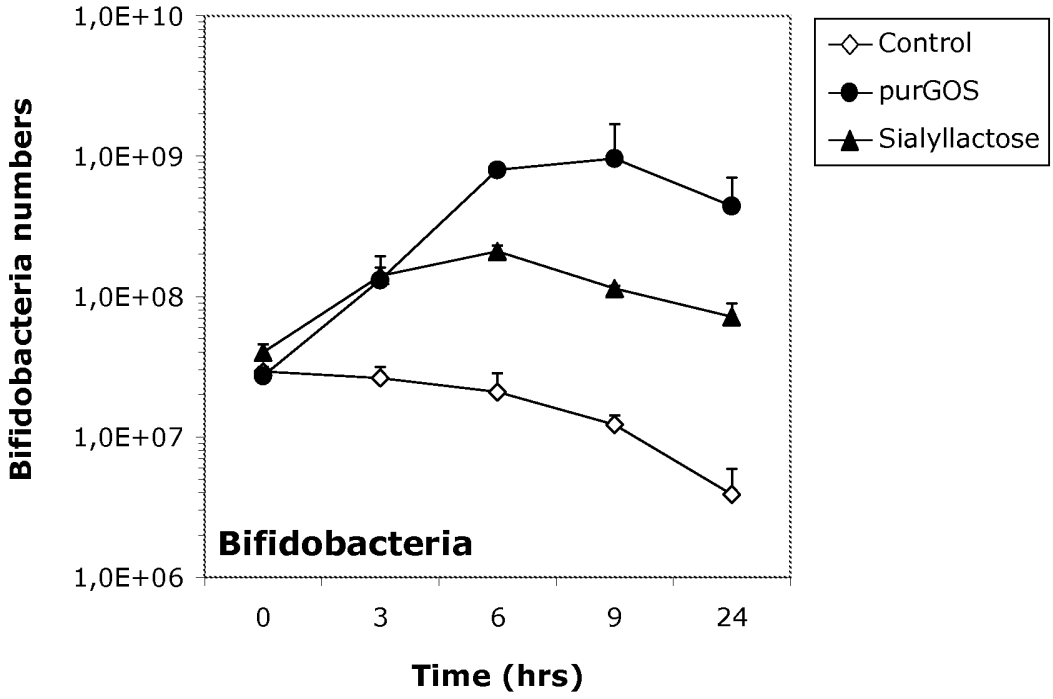

1,0E+10
1,0E+09
1,0E+08
1,0E+07
1,0E+06
Bifidobacteria numbers
Bifidobacteria
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 1C

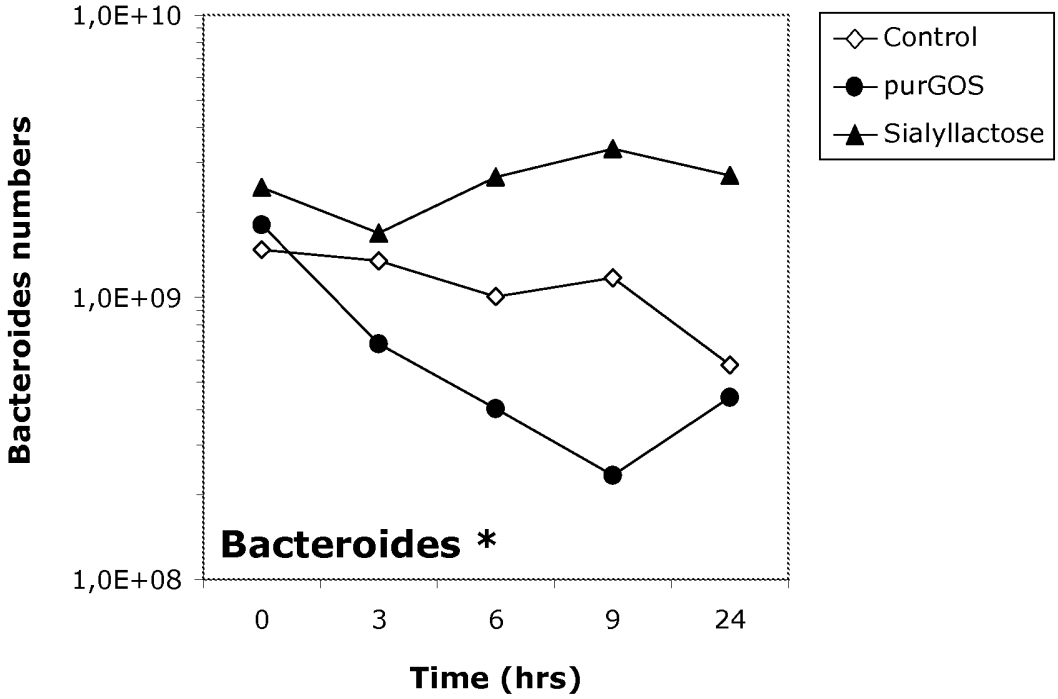

1,0E+10
1,0E+09
1,0E+08
Bacteroides numbers
Bacteroides *
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 2A

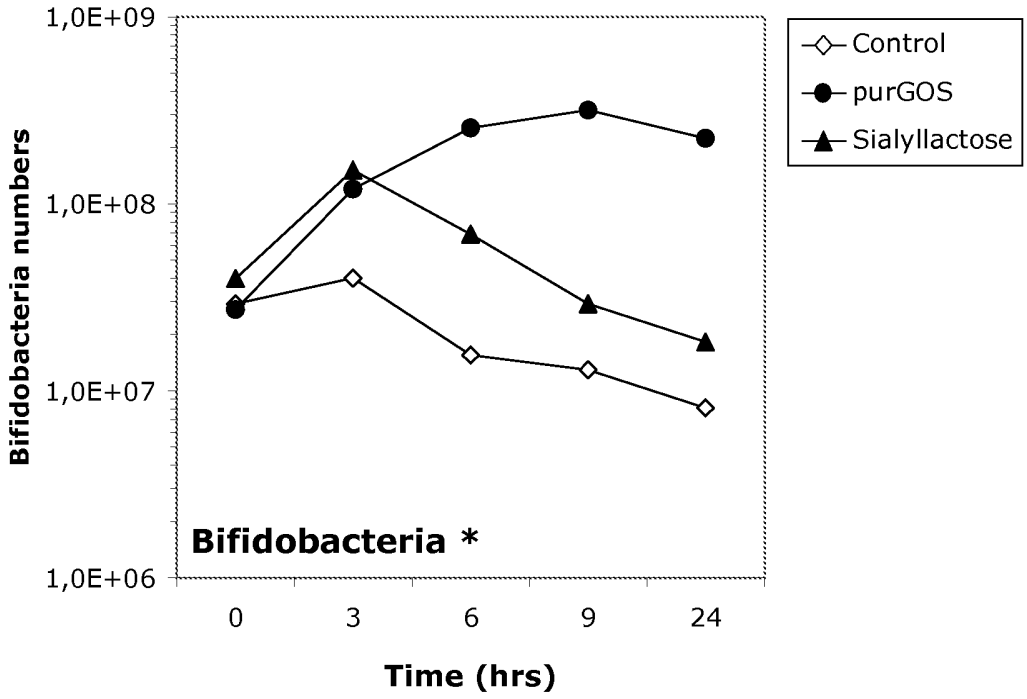

1,0E+09
1,0E+08
1,0E+07
1,0E+06
Bifidobacteria numbers
Bifidobacteria *
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 2B

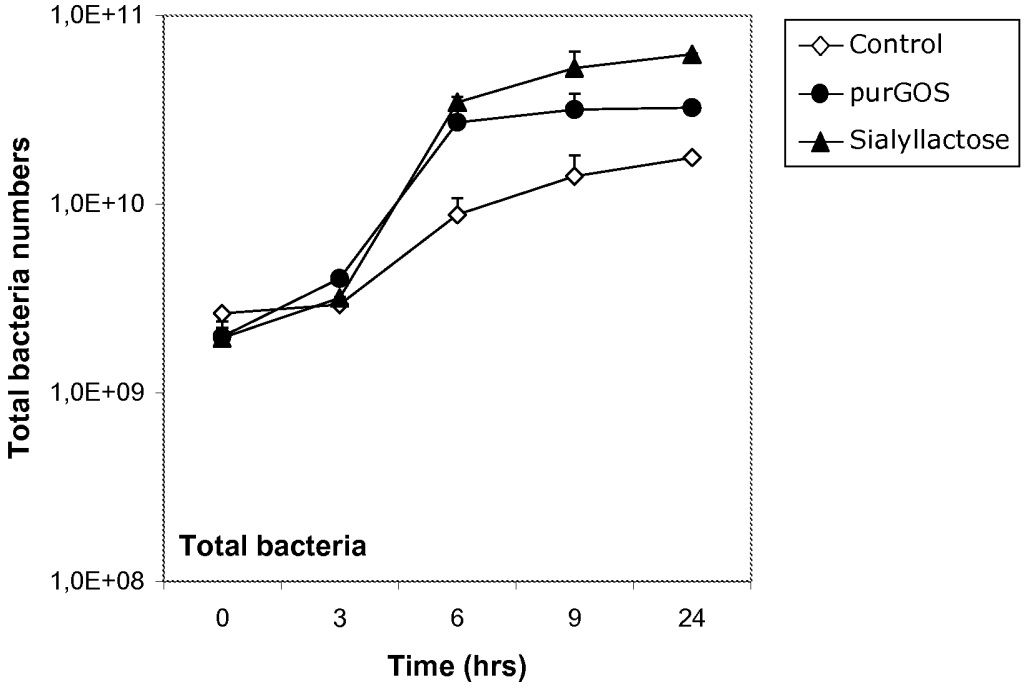

1,0E+11
1,0E+10
1,0E+09
1,0E+08
Total bacteria numbers
Total bacteria
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 3A

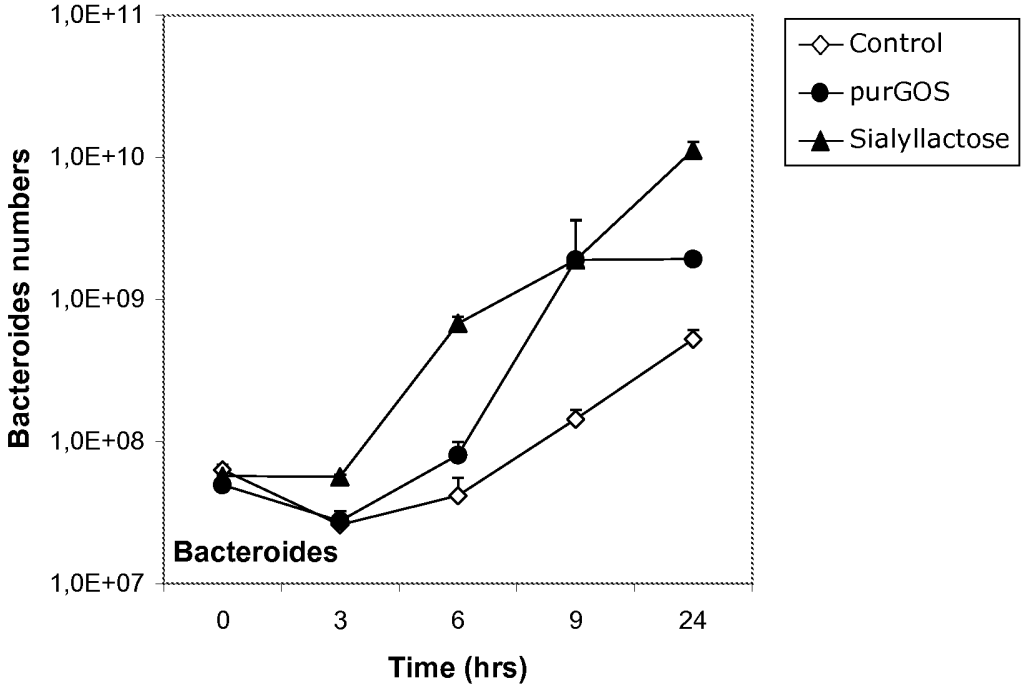

1,0E+11
1,0E+10
1,0E+09
1,0E+08
1,0E+07
Bacteroides numbers
Bacteroides
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 3B

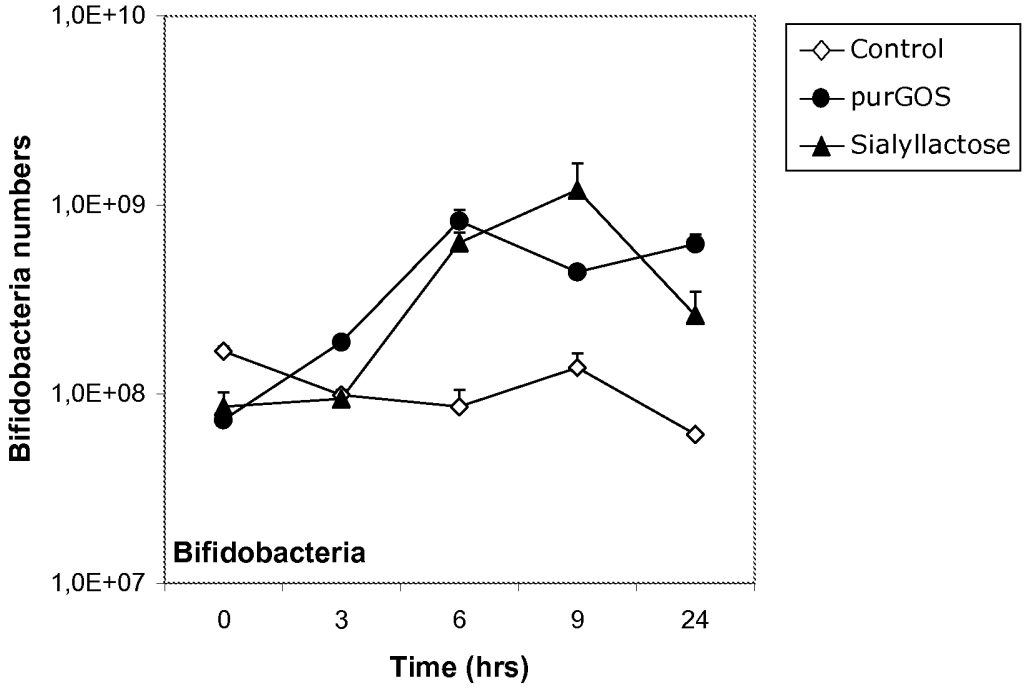

1,0E+10
1,0E+09
1,0E+08
1,0E+07
Bifidobacteria numbers
Bifidobacteria
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 3C

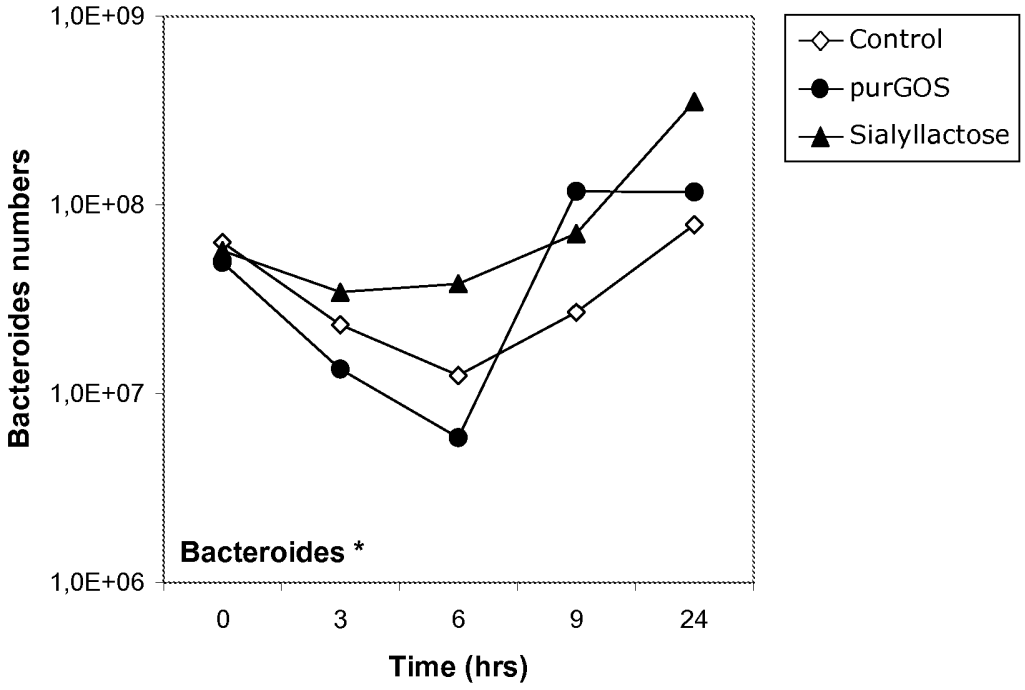

1,0E+09
1,0E+08
1,0E+07
1,0E+06
Bacteroides numbers
Bacteroides *
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 4A

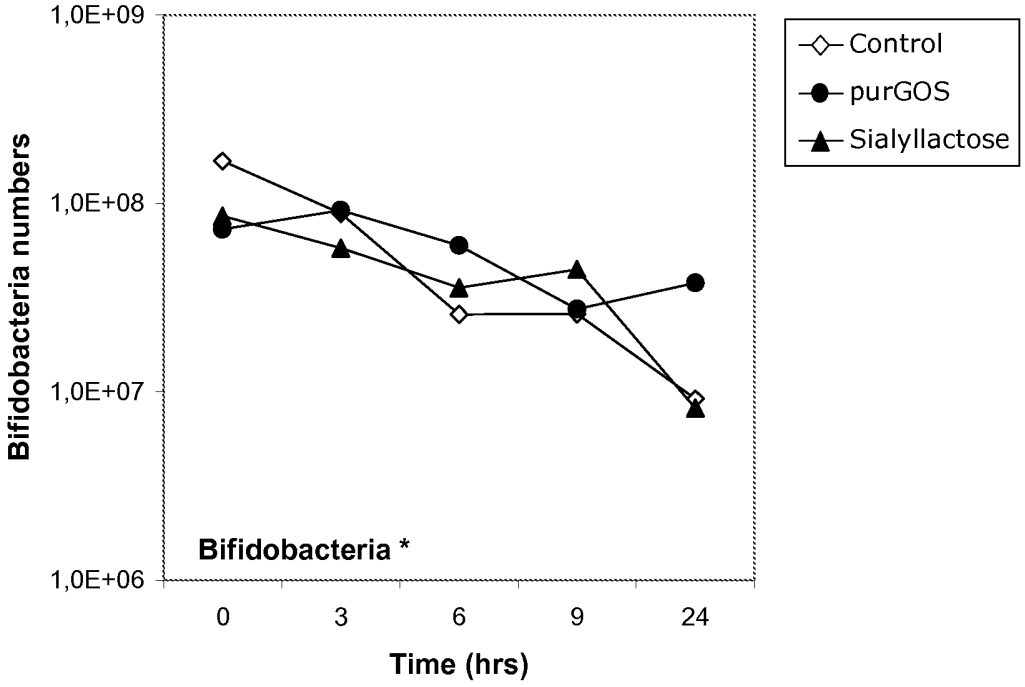

1,0E+09
1,0E+08
1,0E+07
1,0E+06
Bifidobacteria numbers
Bifidobacteria *
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 4B

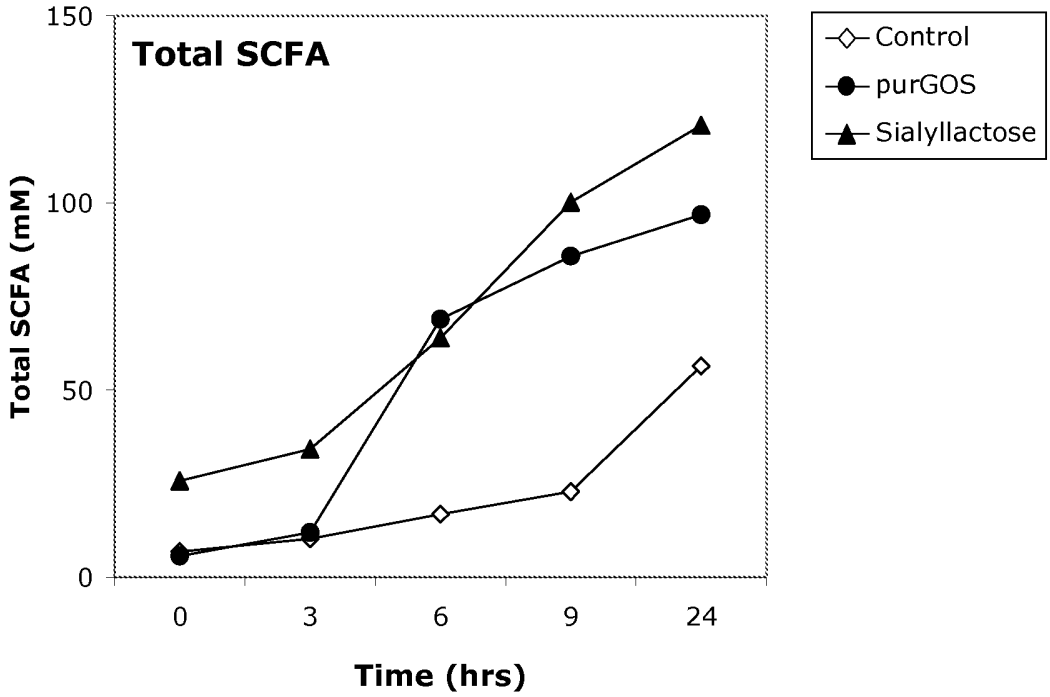

Total SCFA
Total SCFA (mM)
150
100
50
0
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 5A

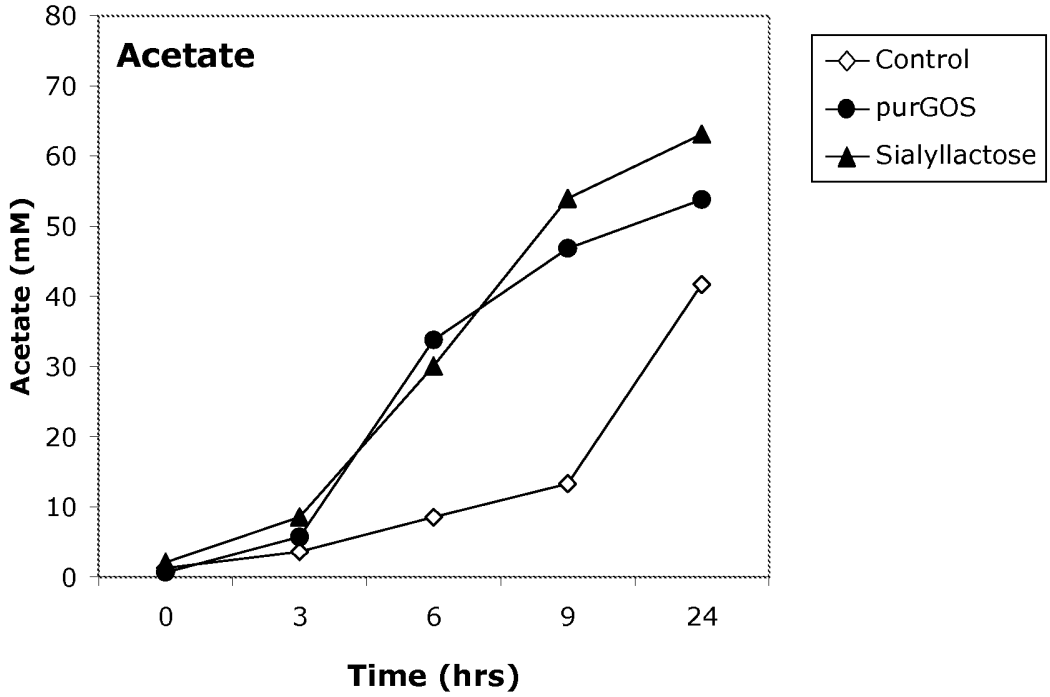

Acetate
Acetate (mM)
80
70
60
50
40
30
20
10
0
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 5B

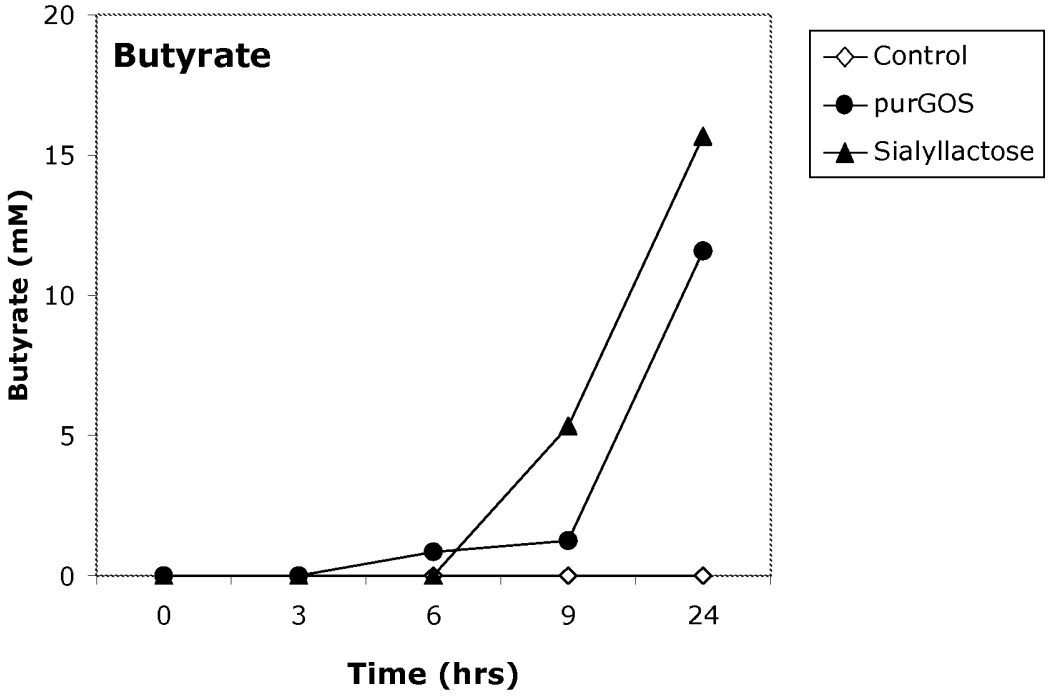

Butyrate
Butyrate (mM)
20
15
10
5
0
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 5C

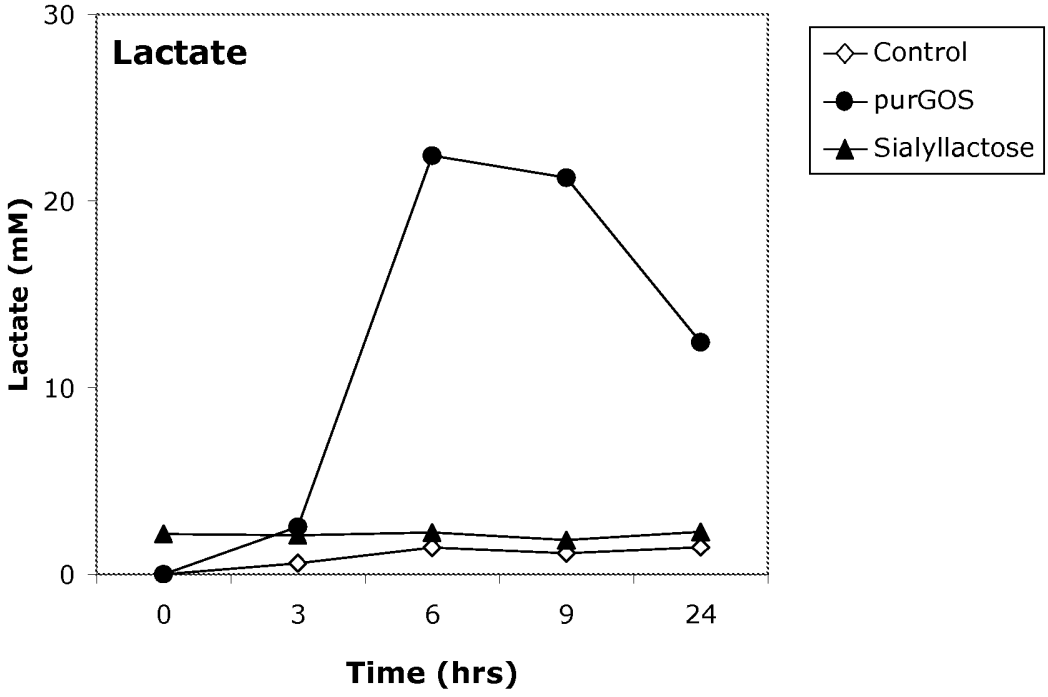

Lactate
Lactate (mM)
30
20
10
0
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 5D

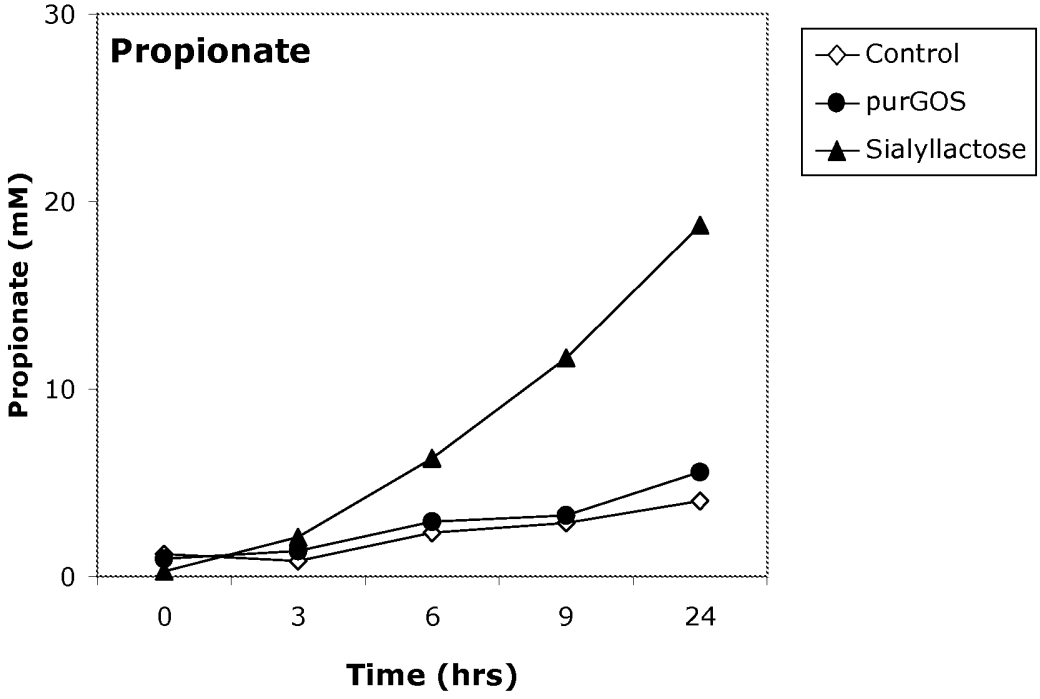

Propionate
Propionate (mM)
30
20
10
0
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 5E

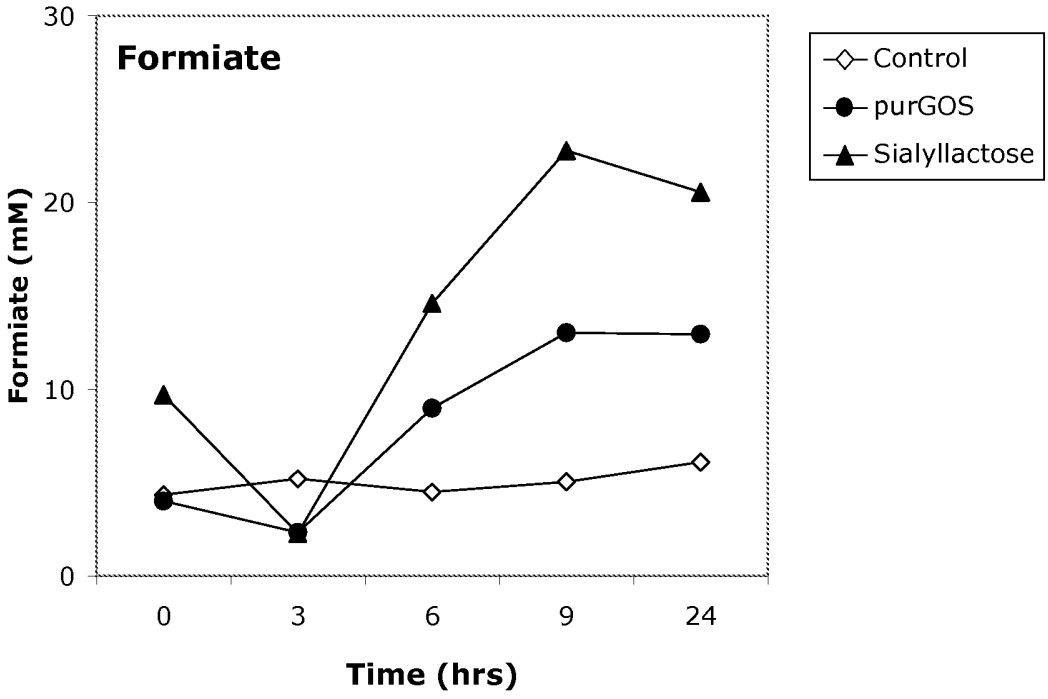

Formiate
Formiate (mM)
30
20
10
0
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 5F

Fig. 6A

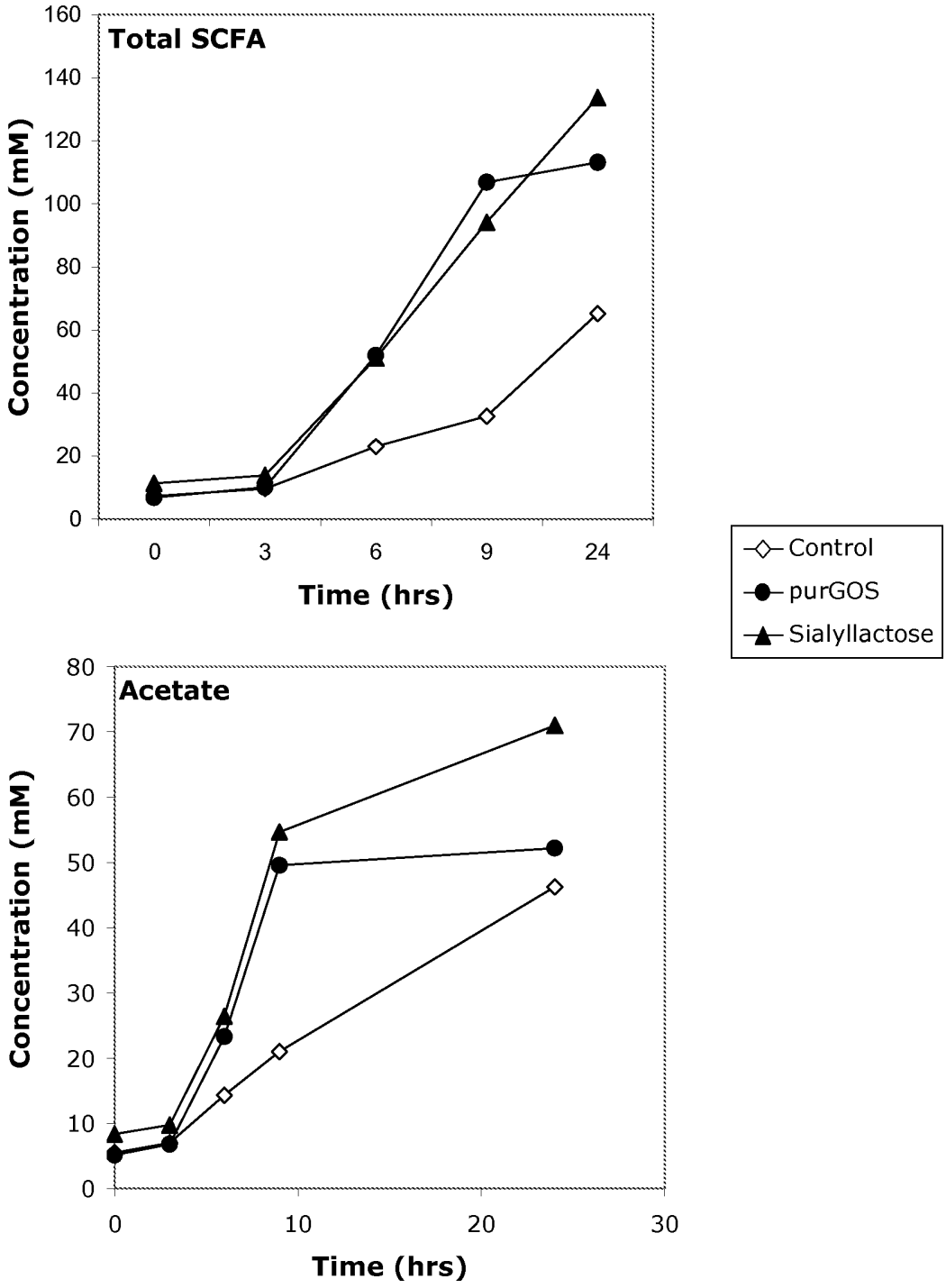

Total SCFA
Concentration (mM)
160
140
120
100
80
60
40
20
0
0
3
6
9
24
Time (hrs)
Control
purGOS
Sialyllactose
Acetate
Concentration (mM)
80
70
60
50
40
30
20
10
0
0
10
20
30
Time (hrs)

Fig. 6A, cont'd

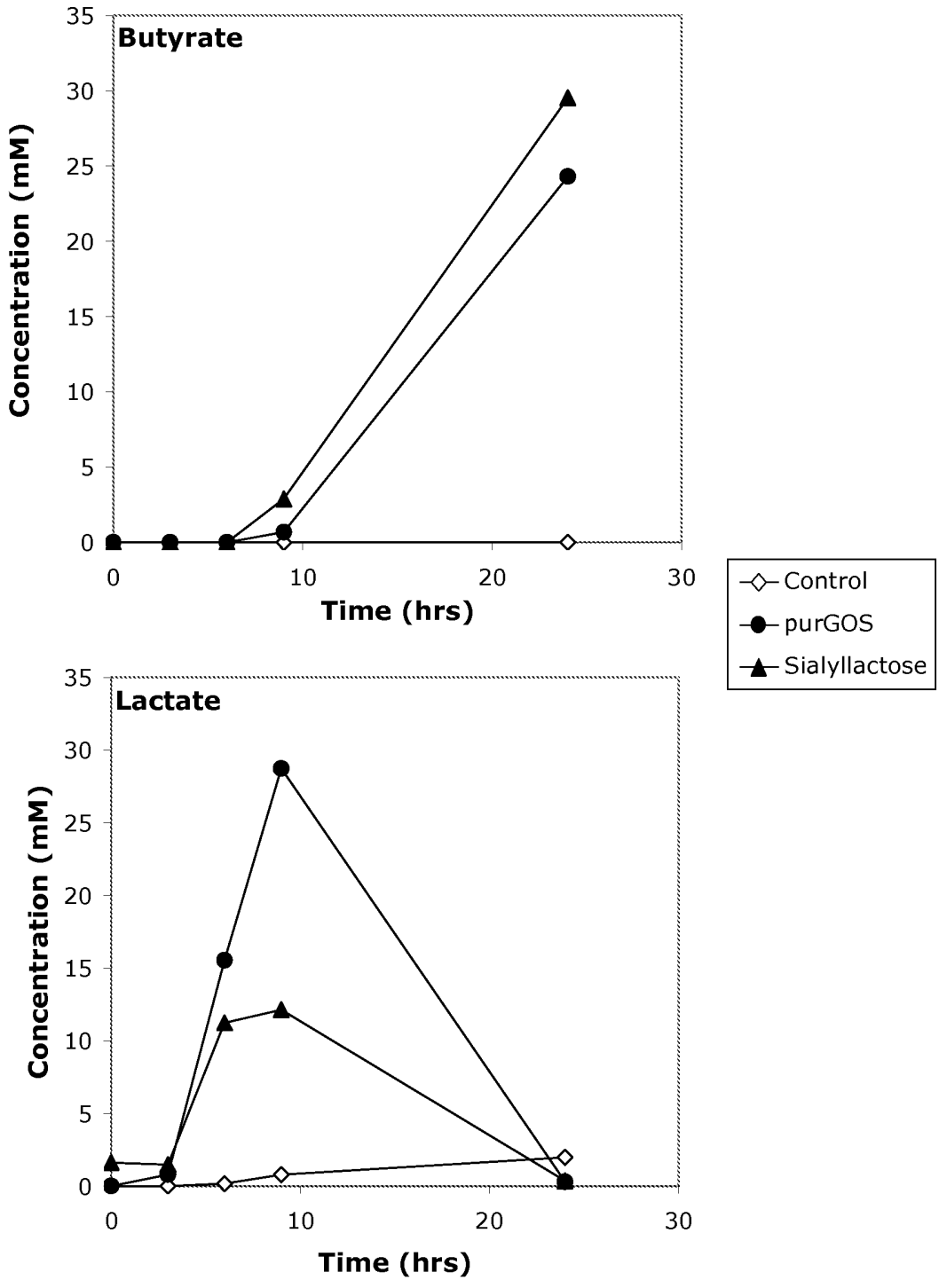

Butyrate
Concentration (mM)
Time (hrs)
Lactate
Concentration (mM)
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 6A, cont'd

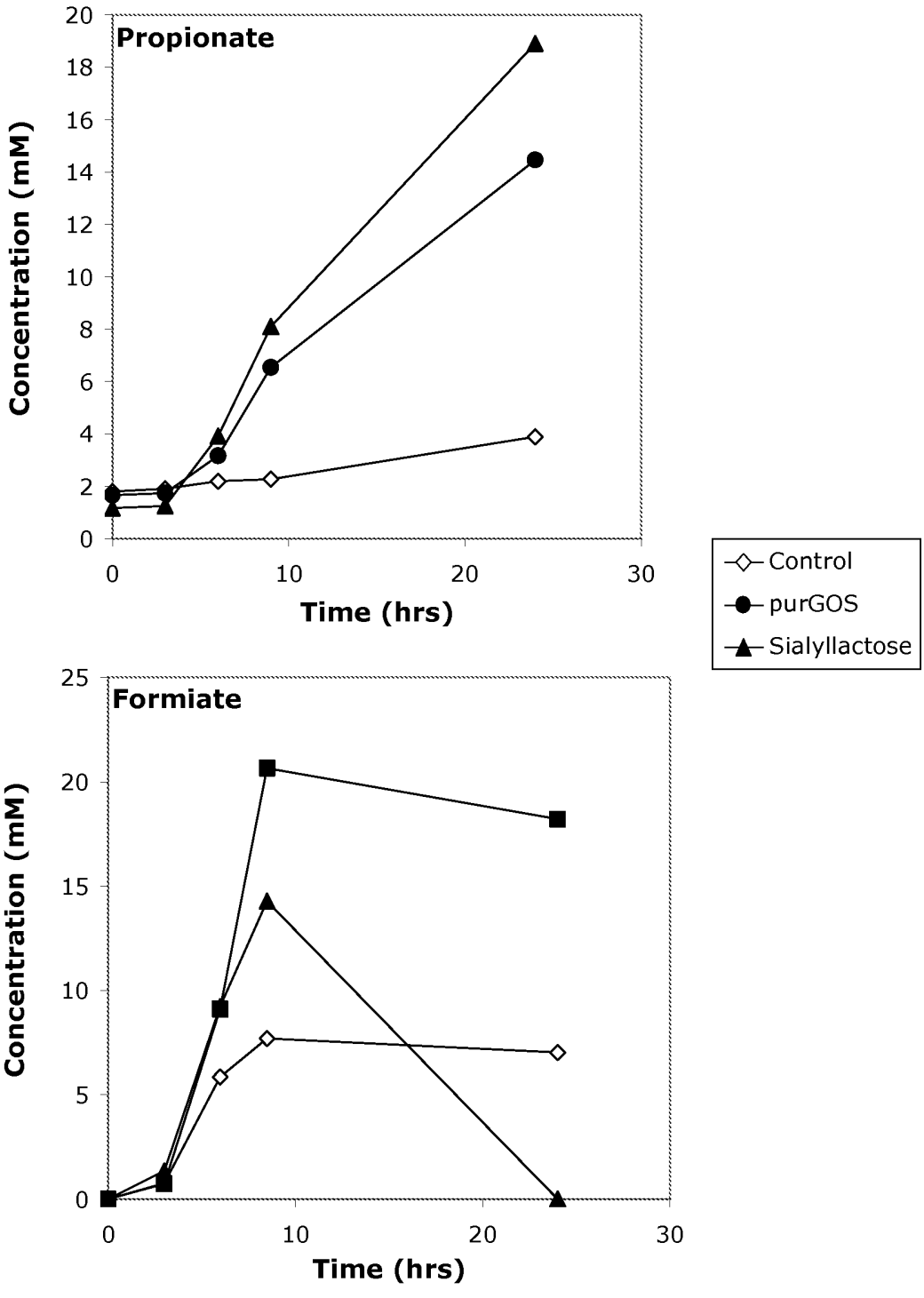

Propionate
Concentration (mM)
Time (hrs)
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
Control
purGOS
Sialyllactose
Formiate
Concentration (mM)
Time (hrs)
0
5
10
15
20
25
0
10
20
30

Fig. 6A, cont'd

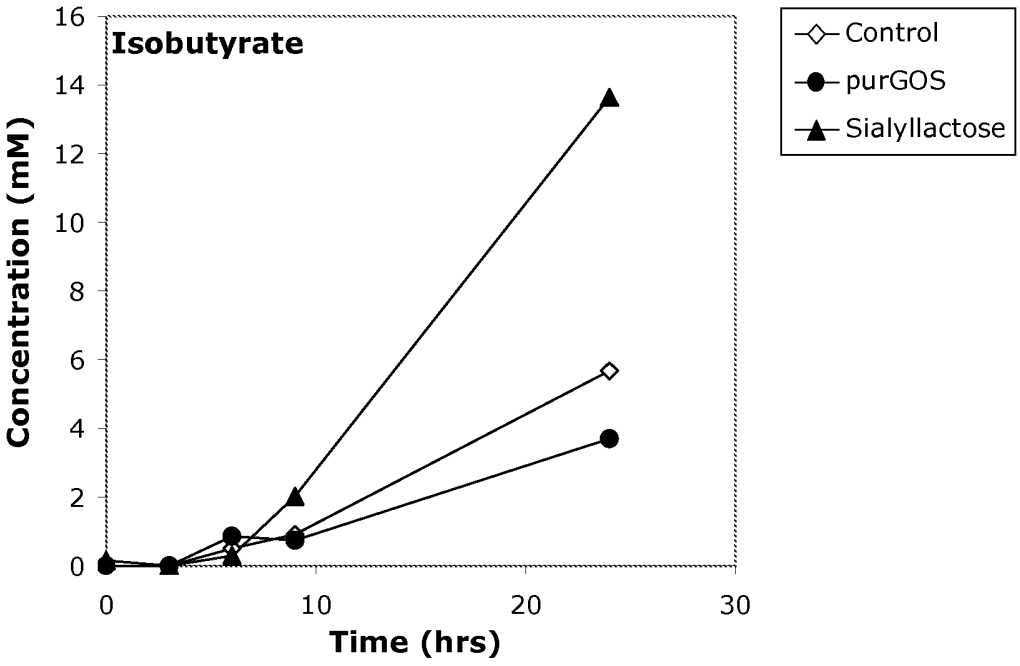

Isobutyrate
Concentration (mM)
Time (hrs)
0
2
4
6
8
10
12
14
16
0
10
20
30
Control
purGOS
Sialyllactose

Fig. 6B

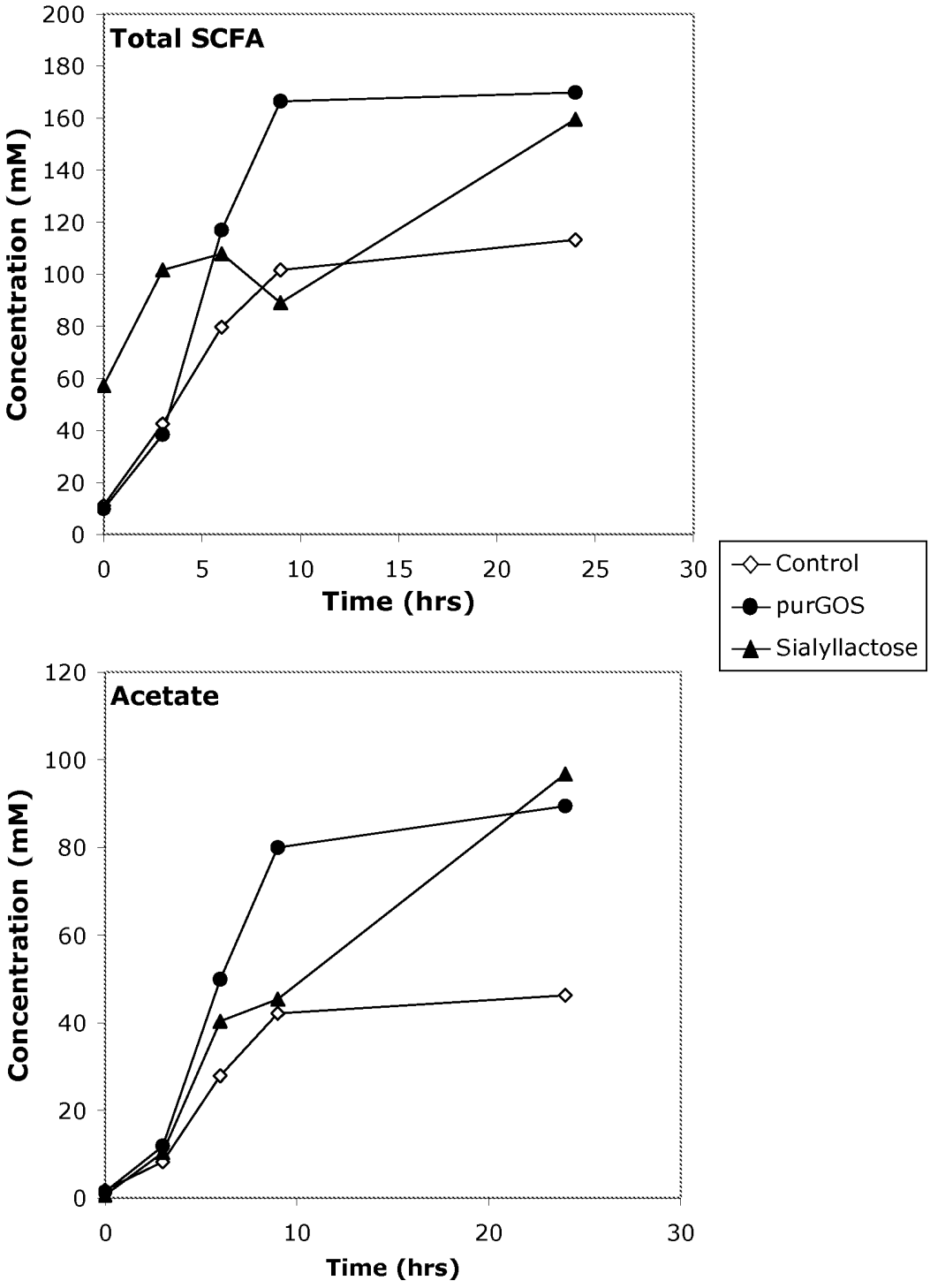

Total SCFA
Concentration (mM)
Time (hrs)
Control
purGOS
Sialyllactose
Acetate
Concentration (mM)
Time (hrs)

Fig. 6B, cont'd

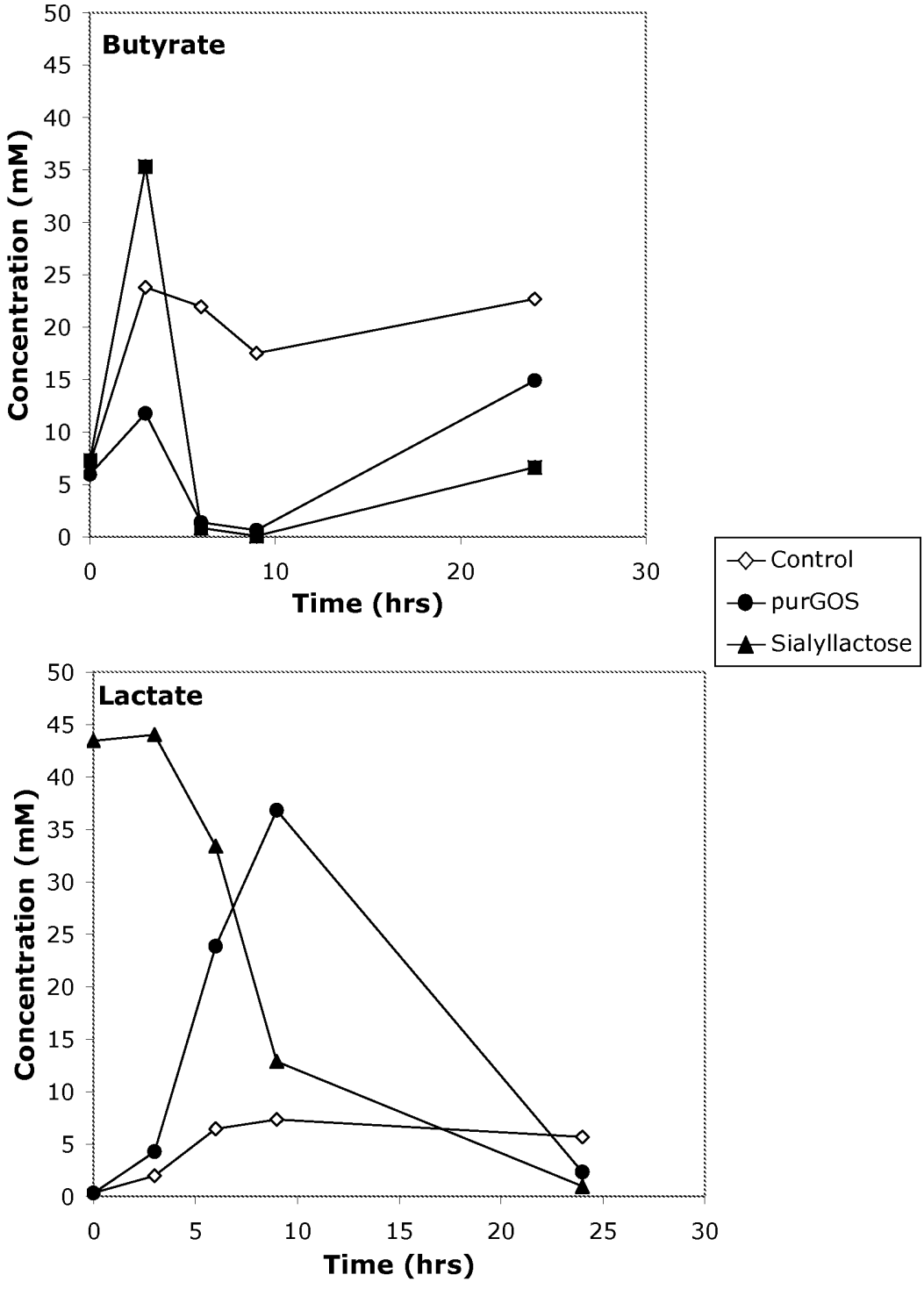

Butyrate
Concentration (mM)
Time (hrs)
0
5
10
15
20
25
30
35
40
45
50
0
10
20
30
Lactate
Concentration (mM)
Time (hrs)
0
5
10
15
20
25
30
35
40
45
50
0
5
10
15
20
25
30
Control
purGOS
Sialyllactose

Fig. 6B, cont'd

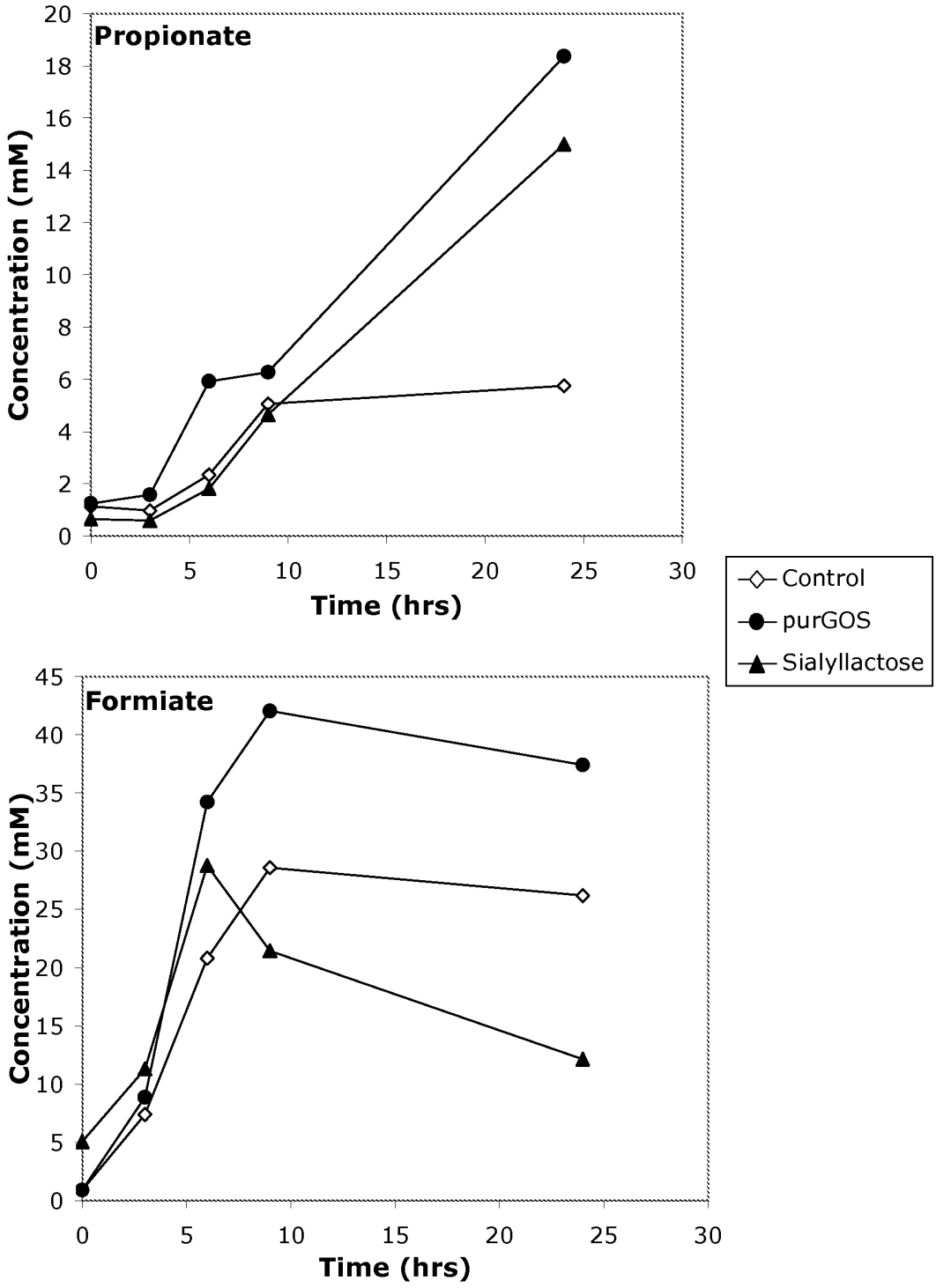
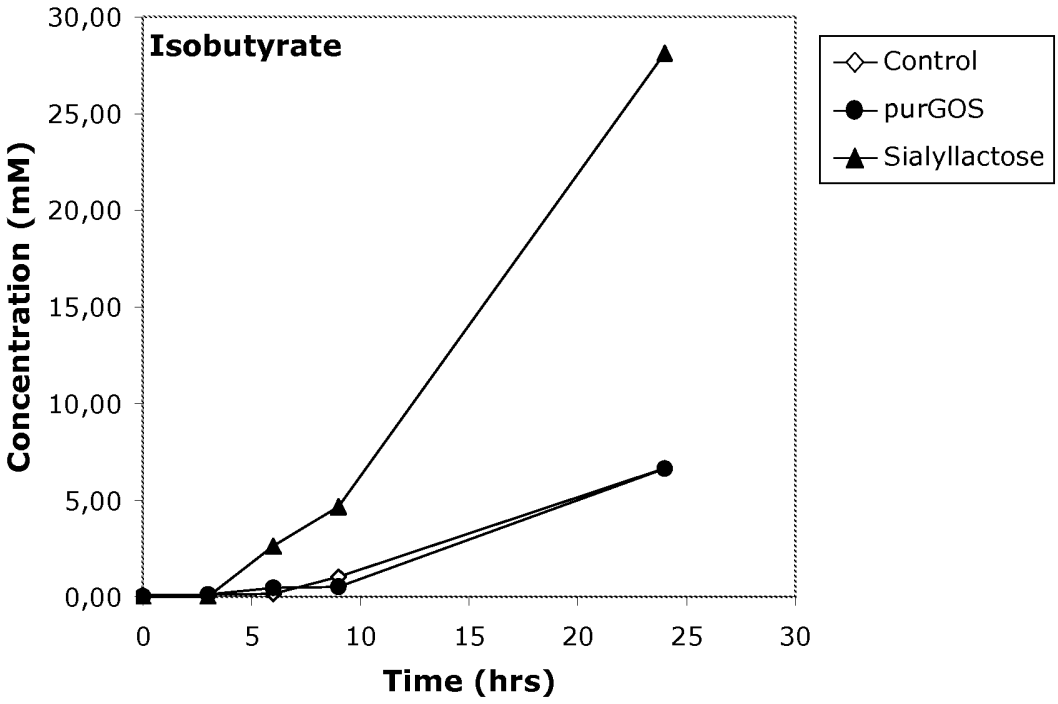

Propionate
Concentration (mM)
Time (hrs)
Formiate
Concentration (mM)
Time (hrs)
Control
purGOS
Sialyllactose

Fig. 6B, cont'd


Isobutyrate
Concentration (mM)
30,00
25,00
20,00
15,00
10,00
5,00
0,00
0
5
10
15
20
25
30
Time (hrs)
Control
purGOS
Sialyllactose

SAMENWERKINGSVERDRAG (PCT)

RAPPORT BETREFFENDE NIEUWHEIDSONDERZOEK VAN INTERNATIONAAL TYPE

IDENTIFICATIE VAN DE NATIONALE AANVRAGE	KENMERK VAN DE AANVRAGER OF VAN DE GEMACHTIGDE
	P88840NL00
Nederlands aanvraag nr. **2004201**	Indieningsdatum **05-02-2010**
	Ingeroepen voorrangsdatum
Aanvrager (Naam) **Friesland Brands B.V.**	
Datum van het verzoek voor een onderzoek van internationaal type **05-06-2010**	Door de Instantie voor Internationaal Onderzoek aan het verzoek voor een onderzoek van internationaal type toegekend nr. **SN 54289**

I. CLASSIFICATIE VAN HET ONDERWERP (bij toepassing van verschillende classificaties, alle classificatiesymbolen opgeven)

Volgens de internationale classificatie (IPC)

A23L1/29 **A23L1/308** **A61K31/7028** **A61P37/04**

II. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimumdocumentatie

Classificatiesysteem	Classificatiesymbolen		
IPC8	**A23L**	**A61K**	**A61P**

Onderzochte andere documentatie dan de minimum documentatie, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

III. ☐	**GEEN ONDERZOEK MOGELIJK VOOR BEPAALDE CONCLUSIES**	(opmerkingen op aanvullingsblad)
IV. ☐	**GEBREK AAN EENHEID VAN UITVINDING**	(opmerkingen op aanvullingsblad)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2004201

A. CLASSIFICATIE VAN HET ONDERWERP
INV. A23L1/29 A23L1/308 A61K31/7028 A61P37/04
ADD.

Volgens de Internationale Classificatie van octrooien (IPC) of zowel volgens de nationale classificatie als volgens de IPC.

B. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte miminum documentatie (classificatie gevolgd door classificatiesymbolen)
A23L A61K A61P

Onderzochte andere documentatie dan de mimimum documentatie, voor dergelijke documenten, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

Tijdens het onderzoek geraadpleegde elektronische gegevensbestanden (naam van de gegevensbestanden en, waar uitvoerbaar, gebruikte trefwoorden)
EPO-Internal, BIOSIS, FSTA, WPI Data

C. VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X Y	WO 2010/002241 A1 (NUTRICIA NV [NL]; SCHMITT JOACHIM [DE]; LECROIX FRANCIS [FR]; JESENNE) 7 januari 2010 (2010-01-07) * bladzijde 6, regels 10-25 * * bladzijde 11, regel 25 - bladzijde 12, regel 31 * * conclusies 1-17 * -----	1-17 6-14,18
X	WO 2004/112509 A2 (NESTEC SA [CH]; GARCIA-RODENAS CLARA LUCIA [CH]; BERGONZELLI GABRIELA) 29 december 2004 (2004-12-29) * bladzijde 7, regel 30 - bladzijde 8, regel 10 * * conclusies 2-5,7-14 * * bladzijde 10, regel 31 - bladzijde 11, regel 10 * ----- -/--	1-17

[X] Verdere documenten worden vermeld in het vervolg van vak C.

[X] Leden van dezelfde octrooifamilie zijn vermeld in een bijlage

° Speciale categorieën van aangehaalde documenten

"A" niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft

"D" in de octrooiaanvrage vermeld

"E" eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven

"L" om andere redenen vermelde literatuur

"O" niet-schriftelijke stand van de techniek

"P" tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur

"T" na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvrage, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding

"X" de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur

"Y" de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht

"&" lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

Datum waarop het onderzoek naar de stand van de techniek van internationaal type werd voltooid	Verzenddatum van het rapport van het onderzoek naar de stand van de techniek van internationaal type
30 juli 2010	

Naam en adres van de instantie	De bevoegde ambtenaar
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Heirbaut, Marc

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ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2004201

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	NL 1 027 262 C2 (FRIESLAND BRANDS BV [NL]) 13 oktober 2005 (2005-10-13) * bladzijde 3, regels 19-26 * * conclusies 1-10 * * bladzijde 7, regels 4-24 * -----	1-17
X	WO 01/60346 A2 (AMERICAN HOME PROD [US]) 23 augustus 2001 (2001-08-23) * conclusies 1-14 * * bladzijde 4, regels 27-37 * * voorbeeld 1 * -----	1-17
Y	MAZMANIAN SARKIS K ET AL: "A microbial symbiosis factor prevents intestinal inflammatory disease" NATURE, NATURE PUBLISHING GROUP, LONDON, GB LNKD- DOI:10.1038/NATURE07008, deel 453, nr. 7195, 1 mei 2008 (2008-05-01), bladzijden 620-625, XP002560841 ISSN: 0028-0836 * samenvatting * -----	6-14,18
A	SOKOL HARRY ET AL: "Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES (PNAS), NATIONAL ACADEMY OF SCIENCE, US LNKD-DOI:10.1073/PNAS.0804812105, deel 105, nr. 43, 28 oktober 2008 (2008-10-28), bladzijden 16731-16736, XP002579466 ISSN: 0027-8424 [gevonden op 2008-10-20] * het gehele document * -----	1-18

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Informatie over leden van dezelfde octrooifamilie

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2004201

In het rapport genoemd octrooigeschrift	Datum van publicatie	Overeenkomend(e) geschrift(en)	Datum van publicatie
WO 2010002241 A1	07-01-2010	WO 2010002244 A1	07-01-2010
WO 2004112509 A2	29-12-2004	CA 2530437 A1	29-12-2004
		CN 1863463 A	15-11-2006
		EP 1638416 A2	29-03-2006
		US 2007104700 A1	10-05-2007
NL 1027262 C2	13-10-2005	GEEN	
WO 0160346 A2	23-08-2001	AR 027450 A1	26-03-2003
		AT 357854 T	15-04-2007
		AU 3828501 A	27-08-2001
		BR 0108478 A	22-04-2003
		CA 2400737 A1	23-08-2001
		CN 1406111 A	26-03-2003
		EP 1255449 A2	13-11-2002
		JP 2003522784 T	29-07-2003
		MX PA02008016 A	29-11-2002
		PT 1255449 E	24-08-2007
		TW 265008 B	01-11-2006

OCTROOICENTRUM NEDERLAND

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
SN54289	05.02.2010		NL2004201

International Patent Classification (IPC)
INV. A23L1/29 A23L1/308 A61K31/7028 A61P37/04

Applicant
Friesland Brands B.V.

This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the application
- ☐ Box No. VIII Certain observations on the application

	Examiner
	Heirbaut, Marc

Application number

WRITTEN OPINION

NL2004201

Box No. I Basis of this opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☐ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☐ furnished subsequently for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	18
	No:	Claims	1-17
Inventive step	Yes:	Claims	
	No:	Claims	1-18
Industrial applicability	Yes:	Claims	1-17
	No:	Claims	18

2. Citations and explanations

see separate sheet

WRITTEN OPINION

Box No. VII Certain defects in the application

see separate sheet

Section V

1 Reference is made to the following prior art documents (D):

D1 WO 2010/002241 A1

D2 WO 2004/112509 A2

D3 NL 1 027 262 C2

D4 WO 01/60346 A2

D5 MAZMANIAN SARKIS K ET AL: "A microbial symbiosis factor prevents intestinal inflammatory disease"NATURE, NATURE PUBLISHING GROUP, LONDON, GB LNKD- DOI:10.1038/NATURE07008, deel 453, nr. 7195, 1 mei 2008 (2008-05-01) , bladzijden 620-625, XP002560841ISSN: 0028-0836

Document **D1** discloses a nutritional composition comprising inactivated Gram-negative bacteria and/or bacterial cell fragments of Gram-negative bacteria and optionally Gram-positive bacteria and/or bacterial cell fragments of Gram-positive bacteria, wherein the composition comprises less than 10+3 cfu Gram-negative bacteria per g dry weight of the composition for use in providing nutrition to an infant delivered via caesarean section. Preferably, it further comprises at least one, more preferably at least two, non-digestible oligosaccharide selected from the group comprising fructo-oligosaccharides, galacto-oligosaccharides, sialic acid comprising oligosaccharides. The non-digestible oligosaccharide stimulates the growth of the beneficial intestinal bacteria, particularly lactic acid producing bacteria and/or Bacteroides.

Document **D2** discloses a nutritional composition comprising a combination of a least one substance selected from the group consisting of specific fats or non-digestible oligosaccharides, associated with at least one microorganism, in an amount efficient to induce a pattern of gut barrier maturation similar to that observed with breast-feeding. Preferably, said oligosaccharides are sialyl-oligosaccharides. Said composition thus reduces the risk of developing allergy and infection.

Document **D3** discloses a composition for reducing intestinal wall permeability, used in *eg* baby food, comprises whey protein, proline and sialic acid or sialyl-oligosaccharides. A preferred sialyl-oligosaccharide is sialyllactose.

Document **D4** discloses a nutritional composition comprising oligofructose and sialyllactose, preferably a mixture of 3'-sialyllactose and 6'-sialyllactose. It can be in the form of an infant formula, and can be used for increasing the amount of Bifidobacteria and inhibiting the binding of pathogenic bacteria in a human. It is indicated that said composition does not have a positive influence on the growth of pathogenic bacteria such as Bacteroides, when compared to glucose in the medium.

Document **D5** discloses that Bacteroides fragilis has an immunoprotective effect through excretion of polysaccharide A (PSA). Particularly, it protects animals from experimental colitis induced by Helicobacter hepaticus. It is indicated that harnessing of the immunomodulatory capacity of symbiosis factors such as PSA may provide therapeutics for human inflammatory disorders, such as inflammatory bowel disease.

Particular reference is made to the passages of said prior art documents and the combination of features taught therein as indicated in the search report.

2 The subject-matter of present independent claims 1 (use) and 15 (method) does not meet the requirements of novelty in the light of any of the prior art documents **D1-D4**, which teach the combination of features indicated in said claims.

It is stressed that induction of gut barrier maturation and stimulation of the growth of beneficial intestinal bacteria enhance the maturation of the developing immune system, see present claim 5.

Reference is made to the description of the disclosure of said documents *supra*.

3 The subject-matter of present independent claim 6 (use) does not meet the requirements of novelty in the light of any of the prior art documents **D1-D4**, which teach the combination of features indicated in said claim.

It is stressed that induction of gut barrier maturation implicitly leads to a balanced immune system without in the absence of an inflammatory response, as this is precisely the functionality of a mature gut barrier.

Reference is made to the discussion of said prior art documents *supra*.

4 The subject-matter of present independent claim 13 (use) does not meet the requirements of novelty in the light of any of the prior art documents **D1-D4**, which teach the combination of features indicated in said claim.

The present description indicates that the commensal bacteria referred to in said claim are instrumental in gut maturation and immunostimulation. Consequently, gut barrier maturation is a condition associated with a reduced amount of said commensal bacteria.

Reference is made to the discussion of said prior art documents *supra*.

5 The subject-matter of present independent claim 18 (use) meets the requirements of novelty.

None of the prior art documents cited in the international search report teaches the subject-matter having the combination of features indicated in said claim.

6 The subject-matter of present independent claim 18 (use) does not meet the requirements of inventive step in the light of the teachings of prior art document **D1** in combination with document **D5**.

Document D1 is considered to represent the closest prior art, as it discloses a nutritional composition which comprises at least one, more preferably at least two, non-digestible oligosaccharide selected from the group comprising fructo-oligosaccharides, galacto-oligosaccharides, sialic acid comprising oligosaccharides. The non-digestible oligosaccharide stimulates the growth of the beneficial intestinal bacteria, particularly lactic acid producing bacteria and/or Bacteroides. Consequently, the skilled person seeking to solve the technical problem underlying the present would have considered the teachings of this document.

The subject-matter of present independent claim 18 differs from the teaching of document D1 in that it is specified that, rather than the growth of Bacteroides or lactic acid producing bacteria, the growth of another type of commensal bacteria, *ie* Faecalibacterium prausnitzii, is stimulated.

No unexpected technical effects or advantages of this difference are demonstrated in the present application. The presence of Faecalibacterium prausnitzii is correlated with improved clinical outcome of IBD patients.

Consequently, the objective technical problem could be seen as to provide a method for manufacture of a composition for treatment of IBD.

The solution provided in present claim 18, by using sialyl-oligosaccharides, was obvious in the light of a combination of the teachings of documents D1, which discloses the stimulative effect of sialyl-oligosaccharides on commensal bacteria such as Bacteroides, and document D5. The latter discloses that Bacteroides fragilis has an immunoprotective effect through excretion of polysaccharide A (PSA). Particularly,

it protects animals from experimental colitis induced by Helicobacter hepaticus. It is indicated that harnessing of the immunomodulatory capacity of symbiosis factors such as PSA may provide therapeutics for human inflammatory disorders, such as inflammatory bowel disease (IBD). The stimulation effect of sialyl-oligosaccharides on Faecalibacterium prausnitzii is considered to represent a scientific discovery, as the technical/therapeutic application thereof (treatment of IBD), is rendered obvious by a combination of documents D1 and D5.

Furthermore, it is pointed out that document D4 discloses that a composition comprising sialyl-oligosaccharides does not have a positive influence on the growth of pathogenic bacteria such as Bacteroides, when compared to glucose in the medium. Hence, it contradicts the experimental evidence provided by the Applicant in this regard.

7 The dependent claims do not appear to contain any features which, in combination with the features of the independent claims to which they refer, meet the requirements of novelty and inventive step. Reference is made to the description of the disclosure of said documents *supra*. Furthermore, it is stressed that dependent claims are only allowable if appended to (a) patentable independent claim(s).

8 Present claims 1-17 meet the requirements of industrial applicability, as their subject-matter can be applied in the food industry. However, present claim 18 and the corresponding passages of the description comprise embodiments which relate to a method for treatment of the human or animal body by therapy.

Section VII

The relevant background art disclosed in the documents **D1-D4** has not been mentioned in the description, nor have these documents been identified therein.