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ORIGINAL SUBMISSION

Mead Johnson Nutritionals

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August 22, 2007

Paulette Gaynor, Ph.D. (HFS-255)
Consumer Safety Officer
Office of Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: GRAS Exemption Claim and Exemption Notification for the addition of a combination of Polydextrose (PDX) and Galactooligosaccharides (GOS) to Infant Formula

Dear Dr. Gaynor,

Pursuant to criteria provided in proposed 21 CFR 170.36, Mead Johnson & Company submits the attached GRAS Exemption Claim and Exemption Notification for the addition of a combination of Polydextrose (PDX) and Galactooligosaccharides (GOS) to Milk-based Infant Formula.

A panel of experts convened by MJ determined that PDX and GOS and the sources of PDX and GOS are GRAS for use in infant formula.

Pre-notification meetings were held with representatives of the Office of Food Additive Safety and the Office of Nutritional Products, Labeling and Dietary Supplements.

Sincerely,

Matias Diez, Pha~~mD~~
Associate Director, Regulatory Science North America

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I. GRAS Exemption Claim

Pursuant to the criteria provided in proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; *Substances Generally Recognized as Safe (GRAS)*; the GRAS proposal), Mead Johnson & Company (MJ) hereby notifies the Food and Drug Administration that the use of a combination of Vivinal® or Vivinal® 10 and Litesse® Two as sources of galactooligosaccharides (GOS) and polydextrose (PDX), respectively, in milk-based infant formulas at maximum addition levels of 5.0 g/L or 7.7 g/L and 2.5 g/L, respectively, is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because MJ has determined that such use is generally recognized as safe (GRAS) through scientific procedures.

08/22/07

Matias R Diez
Associate Director, Regulatory Science, North America
Mead Johnson & Company

Date

A. Name and Address of Notifier

Mead Johnson & Company
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B. Name and Identification of GRAS Substances

The names of the substances that are the subject of this GRAS determination are galactooligosaccharides (GOS) and polydextrose (PDX). The specific GOS product is Vivinal® GOS (SYRUP) or Vivinal®10 GOS (POWDER), manufactured by Friesland Foods Domo (The Netherlands) through the trans-galactosylation of lactose by the enzyme ß-galactosidase produced by *Bacillus circulans* Jordan, ATCC 4516. The specific PDX product is sold as Litesse® Two and is manufactured by Danisco Sweeteners (Terre Haute, IN) using a vacuum-melt condensation method in which glucose or glucose-containing materials are combined with a polyol such as sorbitol in the presence of low levels of a catalytic acid such as citric acid or phosphoric acid and heated under vacuum at defined temperature conditions.

C. Intended Conditions of Use and Consumer Exposure

MJ intends to add a combination of Vivinal® GOS (in the commercial forms Vivinal® or Vivinal® 10 GOS), and Litesse® Two PDX, to milk-based infant formula as prebiotic ingredients.

When defining the maximum addition levels, the manufacturer specifications were taken into consideration. In addition, according to experimental trials in our infant formula production environment, we have observed loss of the notified substances during processing of the infant formulas containing these ingredients. We have considered both factors when defining the maximum addition levels.

The maximum intended addition level of Vivinal is 5.0 g/L. Considering losses during the manufacturing of our infant formula and that Vivinal® comprises 42% GOS, its maximum intended addition level is equivalent to 2 g/L of GOS. When GOS is added as Vivinal® 10 the maximum addition level is 7.7 g/L (equivalent to 2 g GOS/L).

The maximum intended addition level of Litesse® Two is 2.5 g/L. Considering losses during the manufacturing of our infant formula and that Litesse® Two comprises approximately 86% PDX, the maximum intended addition level of Litesse® Two is equivalent to 2 g/L of PDX.

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The subpopulation of infants with the highest intake of energy per kg body weight is boys age 14–27 days; the 90th percentile energy intake by this group is 141.3 kcal/kg bw/day (Fomon, 1993). Among girls, the highest energy intake is found in the same age group, 14–27 days, and is nearly as high as boys: 138.9 kcal/kg bw/day (Fomon, 1993). MJ's Enfamil LIPIL with Iron, along with most other standard formulas, contains 0.676 kcal/mL when ready to consume. Therefore, to obtain 141.3 kcal energy/kg bw, an infant boy must consume

[redacted]

209.0 mL/kg bw of formula. To reach her 90th percentile of energy consumption, 138.9 kcal/kg bw/day, an infant girl must consume 205.5 mL/kg bw of formula. The 90th percentile of formula intake for the two sexes combined is about 207 mL/kg bw/day.

The 90th percentile daily intake of Vivinal® added at a maximum concentration of 5.0 g/L is thus estimated to be 1 g/kg bw. The 90th percentile daily intake of Vivinal® 10 added at a maximum concentration of 7.7 g/L is thus estimated to be 1.6 g/kg bw. The 90th percentile intake of GOS is estimated to be 0.4 g/kg bw/day.

The 90th percentile daily intake of Litesse® Two added at 2.5 g/L is estimated to be 0.5 g/kg bw and that of PDX is estimated to be 0.4 g/kg bw. The 90th percentile intake of GOS and PDX together is estimated to be 0.8 g/kg bw/day. Since neither GOS nor PDX are found in other foods likely to be consumed by infants, the intake of GOS and PDX from supplemented formula is the total intake of these prebiotic ingredients

D. Basis for GRAS Determination

The GRAS determination regarding the use of a combination of Vivinal® GOS and Litesse® Two PDX is based on scientific procedures as described under 21 CFR170.30(b). To demonstrate that Vivinal® and Litesse® Two are GRAS under their intended conditions of use, the safety of the intake of both GOS and the whole product, and PDX and the whole product, was established under the intended conditions of use. Following the determination of safety, the intended use of Vivinal® and Litesse® Two was determined to be GRAS by showing that the safety of the estimated levels of intake of these products is based on generally available and accepted information and is generally recognized by experts qualified by scientific training and experience to evaluate the safety of substances directly added to food. This latter demonstration is based on the conclusions of an Expert Panel specially convened to evaluate the information bearing on the safety of the intended use of Vivinal® GOS and Litesse® Two PDX. The members of the GRAS review panel were:

Dennis M. Bier, M.D.
Baylor College of Medicine
Houston, Texas, USA

Berthold V. Koletzko, M.D.
University of Munich
Munich, Germany

Michael P. Doyle, Ph.D.
University of Georgia
Griffin, Georgia, USA

Robert A. Rastall, Ph.D.
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George C. Fahey, Ph.D.
University of Illinois
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John A. Thomas, Ph.D.
University of Texas (Emeritus)
San Antonio, Texas, USA

Glenn R. Gibson, Ph.D.
University of Reading
Reading, UK

The members of the GRAS review panel individually and collectively reviewed monographs on GOS and PDX prepared by JHeimbach LLC and the Burdock Group, as well as other information they deemed pertinent. The GRAS panel evaluated the methods of manufacture of the substances, product specifications and contaminant levels, estimated dietary exposure, and information from published *in vitro*, animal, and human studies. The GRAS panel, which MJ regards as qualified by scientific training and experience to evaluate the safety of substances added to food, concluded that, based on the published literature, Vivinal® and Vivinal® 10, and Litesse® Two, meeting food grade specifications, are GRAS for infants under their intended conditions of use. Indeed, the panel concluded that use of these products at higher levels than those intended would still be both safe and GRAS. Therefore, it is concluded, based on scientific procedures, that the intended use of Vivinal® and Vivinal® 10, and Litesse® Two is safe, and generally recognized as safe (GRAS).

E. Availability of Information

The data and information that serve as the basis for the GRAS determination will be sent to the FDA upon request or are available for the FDA's review and copying at reasonable times at the offices of Mead Johnson & Co., 2400 W. Lloyd Expressway, Evansville, Indiana 47721-0001.

II. Impact of Human Milk on Infant Health and Development

A. Goal of Infant Formula Modifications: Closer Approximation to Human Milk Performance

Breastfeeding is regarded as the gold standard for infant feeding (Vandenplas 2002). As a result, multiple health organizations, including the World Health Organization (WHO 2002), the American Academy of Pediatrics (AAP 1997), and the U.S. Department of Health and Human Services (USDHHS 2000), endorse breastfeeding as the optimal form of nutrition for infants during the first year of life. In recognition of the advantages of human milk over infant formula, infant formula manufacturers have made changes in formulas to more closely simulate either human milk composition or breastfeeding performance (IOM 2003). The IOM's Committee on the Evaluation of the Addition of Ingredients New to Infant Formula noted that the attempt to match human milk composition is a "quixotic quest since

human milk is a complex body fluid that is variable not only between individuals, but within an individual over time" (IOM 2003, p3–4). IOM also suggested that the alternative to matching human milk composition is to match breastfeeding performance.

B. Role of Human Milk Components on Gastrointestinal Development

It is now well recognized that human milk is a complex physiological fluid, the components of which fulfill nutritive, developmental and protective functions in systemic infant nutrition and within the specific microenvironment of the neonatal gastrointestinal (GI) tract. Various protein, lipid and carbohydrate components of this complex fluid are involved in the stimulation of GI cell growth and maturation, the establishment of symbiotic microbiota and mucosal barrier function, and the development of gut-associated lymphoid tissue (GALT), the last being a crucial interface between the ingested contents of the intestinal environment and the immature infant immune system (Donovan, 2006). Given the general immaturity of certain elements of the neonatal immune system, it has been suggested that various bioactive components of human milk act in concert to protect the infant until a functional level of immunological and developmental maturity is achieved (Newburg and Walker, 2007). This hypothesis is supported by numerous studies in which the incidence and/or severity of infectious diseases such as diarrhea (Popkin et al., 1990), otitis media (Duncan et al., 1993) and bacteremia (Istre et al., 1985) are lower in breast-fed infants relative to formula-fed infants.

A review of the research literature on the bioactive components typically identified in human milk offers a number of insights into the physiological basis of protective and developmental functions and how they might influence neonatal GI development. Early in lactation, the whey-predominant protein fraction of human milk is reported to contain high concentrations of hormones, growth factors (e.g., transforming growth factors (TGF) α and β) and numerous antimicrobial proteins (lactoferrin, lysozyme, lactoperoxidase, etc.) (Donovan, 2006). As lactation progresses, the whey:casein ratio declines as the nutritional requirement for amino acids increases, presumably at the expense of the more bioactive protein and peptide components. While the various nutritional, developmental and protective contributions of the protein fraction have been investigated for many years, the oligosaccharide components have come under scrutiny only more recently. The importance of human milk oligosaccharides (HMO) is suggested by their relative prominence as they constitute the third largest component after lactose and lipid (Morrow et al., 2005). HMO the concentrations of which vary considerably among women and during lactation, are thought to play significant developmental and protective roles.

A current model describing the activity of human milk bioactive substances suggests that these components have evolved to fulfill a number of functions that are deemed beneficial to neonatal GI development, including: a) compensation for a lack of developmental maturity; b) enhancement of poorly expressed functions; c) promotion of

intestinal adaptation to extrauterine life; d) prevention of infection and inflammation; e) digestion resistance; and f) establishment of a beneficial microbiota population (Goldman, 2000). As with other classes of bioactive components, human milk oligosaccharides appear to have evolved to support many of these developmental and protective strategies.

C. Human Milk Oligosaccharide Properties

Human milk oligosaccharides (HMO) constitute a structurally diverse and highly variable group of bioactive carbohydrates. Though they resist digestion by human enzymes and reach the lower GI tract largely intact, HMO are minimally absorbed into the circulatory system and transported to other sites where they are thought to function systemically to block pathogen adhesion. HMO are composed of combinations of five monosaccharide building blocks: D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc) and sialic acid (N-acetyl neuraminic acid (Neu5Ac). Both Neu5Ac and N-glycolyl neuraminic acid (Neu5Gc) occur in the oligosaccharides of other mammalian species (Bode, 2006). Unbound HMO are found in mature human milk at levels ranging from 5-10 g/L, though they are present at higher levels in colostrum (Bode, 2006). They have been estimated to vary in size from 3 to 32 sugars (Morrow et al., 2005), and current analytical methods have detected approximately 200 distinct molecular species (Ninonuevo et al., 2006), though that may represent only a small fraction of their true structural diversity (Stahl et al., 1994).

The majority of HMO that have been isolated and characterized to date bear lactose at the reducing end and at least one fucose or sialic acid residue on the nonreducing terminus (Newburg et al., 2005). Elongation is carried out through successive additions of GlcNAc (in ß(1-3) or ß(1-6) linkage) to Gal, then Gal (in ß(1-3) or ß(1-4) linkage), to form a core structure, followed by further additions of lactosamine (derived from lactose and N-acetylglucosamine), Fuc, and/or NeuAc residues (Kunz et al., 2000). It is of interest to note that the addition of fucose is dependent upon at least three different fucosyltransferases, the activities of which are driven by maternal genetics in comparable fashion to the Lewis blood group determinants (Kunz et al., 2000). Multiple sialytransferases are also involved in the addition of NeuAc at different positions within the elongating oligosaccharides, clearly indicating that individual genetic factors are important determinants of the ultimate structural diversity of the oligosaccharides produced by a given individual.

Variability occurs in both the composition and concentration of HMO during the normal course of lactation (Chaturvedi et al., 2001[a]). HMO differ in size, charge, and carbohydrate composition and consist of two groups: (1) the neutral oligosaccharides containing galactose, N-acetylglucosamine, fucose and lactose core; and (2) the anionic oligosaccharides, similarly composed but with the addition of N-acetylneuraminic acid as well (Ninonuevo et al., 2006). An assessment of the neutral oligosaccharide profiles in the milk of 50 donors revealed that 2'-fucosyllactose (1.21 mg/ml) and lacto-N-fucopentaose I (0.94 mg/ml) were the predominant molecular species in almost 70% of the samples

(Chaturvedi et al., 1997) while 3-fucosyllactose (0.36 mg/ml) and lacto-N-fucopentaose-II and lacto-N-fucopentaose-III (0.64 mg/ml) predominated in the remainder (Table 1; derived from Chaturvedi et al., 1997)).

Table 1. Structures of predominant human milk oligosaccharides.

Trivial Name	Abbreviation	Structure
Lactose	Lac	Gal ß(1→4) Glc
2'-Fucosyllactose	2'-FucLac	Fuc a(1→2) Gal ß(1-4) Glc
3-Fucosyllactose	3-FucLac	Gal ß(1→4) ↓ Glc ↑ Fuc a(1→3)
Lacto-*N*-fucopentaose-I	LNF-I	Fuc a(1→2) Gal ß(1→3) GlcNAc ß(1→3) Gal ß(1→4)Glc
Lacto-N-fucopentaose-II	LNF-II	Gal ß(1→3) ↓ GlcNAc ß(1→3) Gal ß(1→4)Glc ↑ Fuc a(1→4)
Lacto-*N*-fucopentaose-III	LNF-III	Gal ß(1→4) ↓ GlcNAc ß(1→3) Gal ß(1→4)Glc ↑ Fuc a(1→3)

Breast feeding is known to afford infants significant protection from diarrheal diseases, respiratory tract infections, meningitis and necrotizing enterocolitis, and this has been attributed in part to the presence of a(1-2)-linked fucosylated oligosaccharides (conjugated and unconjugated), which are collectively termed glycans (Morrow et al., 2005). As described previously, oligosaccharides are thought to be a constituent of an innate immune system residing in human milk that affords infants additional protection from pathogens through the act of breast feeding. This is supported by observations that fucosylated oligosaccharides are capable of preventing diarrheal illness through diverse mechanisms including: 1) the inhibition of *Escherichia coli* stable toxin activity (by binding and blocking access to the target receptor guanylate cyclase); 2) the prevention of *Campylobacter* adhesion to intestinal cells (through presentation of 2-fucosyllactose as a decoy receptor); and 3) the presentation of other decoy receptor molecules containing a(1-2)-

fucose epitopes that are known to competitively inhibit the binding of Norovirus to intestinal epithelium (Newburg et al., 2005). A recent report has indicated that the capacity to block pathogen adhesion is not the sole province of HMO. Shoaf and coworkers (2006) evaluated the capacity of a range of commercially available prebiotic oligosaccharides to block the attachment of enteropathogenic *E. coli* (EPEC) to HEp-2 and Caco-2 cell lines. Purified galactooligosaccharides (GOS) exhibited the greatest activity, inhibiting the EPEC adherence to HEp-2 and Caco-2 cells by 65% and 70%, respectively.

A significant portion of HMO passes undigested to the lower gastrointestinal tract (Chaturvedi et al., 2001[b]) where it can be selectively metabolized by beneficial microorganisms such as *Bifidobacterium infantis* (Ward et al., 2006). The bifidogenic potential of HMO is frequently cited as an important determinant in the proportional dominance of bifidobacteria in gastrointestinal microbiota of breast-fed infants in comparison to formula-fed infants (Harmsen et al., 2000). In contrast to human milk, bovine milk contains only trace amounts of oligosaccharides. By default, infant formula derived from bovine milk is essentially devoid of oligosaccharides unless they are provided exogenously.

The high level of structural diversity and dynamic oligosaccharide compositions observed across human milk samples precludes the addition of chemically identical oligosaccharides to infant formula, an issue recognized by the EU's Scientific Committee on Foods.(SCF 2003). While not duplicating the chemical composition of HMO, MJ proposes to substitute food-grade prebiotic ingredients (i.e., oligosaccharides) to partially mimic the functional properties of HMOs. The intended technical effect of adding such prebiotic ingredients to infant formula is to bring the performance of formula-fed infants into closer alignment with the performance of breast-fed infants, particularly with regards to their impact on GI microbiota and stooling patterns (e.g. frequency and consistency).

III. Role of Microbiota in Gastrointestinal Development and Function

A. Impact of Colonization

The human gastrointestinal tract constitutes a complex ecosystem of food materials, host cells and as many as a thousand species of microbes (Zoetendal et al., 2006). The predominant bacterial communities in humans, as measured in mucosal and fecal populations, are highly individualized and, once established, are largely stable over time. The bacteria residing in the human GI tract, while reportedly reaching the highest cell densities recorded for any ecosystem, comprise of only 8 of some 55 known bacterial divisions

identified to date (Bäckhed et al., 2005). These select bacterial divisions are highly variable at the strain and species level and are thought to have evolved as mutalistic and commensal organisms capable of providing metabolic capacities that are not encoded in the host genome.

Numerous studies conducted in germ-free or pathogen-free mice have demonstrated that the resident microbiota has a profound influence on GI development and functionality. As an example, the colonization of germ-free mice with *Bacteroides thetaiotaomicron*, a prominent member of both the human and mouse intestinal microbiota, has been shown to significantly modulate the expression of host genes related to nutrient absorption, mucosal barrier function, xenobiotic metabolism, angiogenesis and maturation (Hooper et al., 2001). Comparable studies have confirmed the stimulatory effect of GI microbiota on angiogenesis (vascularization) (Stappenbeck et al., 2002) and extended the recognized effects to include protection against epithelical cell injury (Rakoff-Nahoum et al., 2004) and regulation of host fat storage (Bäckhed et al., 2004). Clearly, the presence of a complex microbial community is integral to many functional processes in the human GI tract.

Depending upon identity, location and other factors, the bacteria routinely encountered among the intestinal microbiota can have beneficial, neutral or negative impact on the host (Dai and Walker 1999). For example, a variety of potentially pathogenic or opportunistic bacteria or their metabolites have been associated with diarrhea, inflammation, necrosis and ulceration, liver damage, and carcinogenesis (Dai and Walker 1999). In contrast, other types of intestinal bacteria such as bifidobacteria and lactobacilli are seldom associated with disease and are believed to positively influence health (Vandenplas 2002). Beneficial gastrointestinal bacteria are thought to positively influence the health of the host through metabolic, trophic and protective effects (Guarner and Malagelada, 2003) which can be briefly described as follows:

Metabolic Effects:

Intestinal microbiota provide an important function in transforming complex dietary and host substances into simpler compounds (succinate, short-chain fatty acids [SCFA], etc.) which can be used as energy sources by other organisms and/or the host. Most notable is the role intestinal bacteria play in fermenting non-digestible dietary carbohydrates through highly adapted fermentation processes (Gibson and Roberfroid, 1995; Guarner and Malagelada, 2003). The primary end products of this metabolic activity are SCFA, which serve as energy sources for colonic epithelial cells and also lower the pH of intestinal contents; with the latter effect favoring the growth of certain bacteria that are considered beneficial at the expense of less desirable constituents of the microbiota (Gibson and Roberfroid, 1995; Guarner and Malagelada, 2003; Agostoni et al., 2004).

Trophic Effects:

Intestinal microbiota produce important growth-promoting (i.e., trophic) effects on the intestinal epithelium and associated lymphoid tissue (Abrams, 1983). As described earlier, SCFA, along with carbon dioxide, methane and hydrogen, are end products of bacterial metabolism of carbohydrates in the distal gastrointestinal tract. The amount of SCFA produced is dependent on the diet consumed and the resident microbial community. SCFA are readily absorbed by the intestinal mucosa and have been shown to increase epithelial proliferation and mucosal growth in both the large and small intestine (Kripke et al., 1988; Kripke et al., 1989; Sakata, 1989; Kripke et al., 1991; Frankel et al., 1994). In addition, daily administration of a mixture of the SCFAs acetate, propionate and butyrate increases the total colon tissue mass including the mucosa, submucosa and mucosal surface area (Kissmeyer-Nielsen et al., 1995). A three- to four-fold increase in epithelial cell proliferation is stimulated in the small and large intestine by daily or continuous administration of SCFA into the colon (Sakata, 1987). In addition, SCFA contribute caloric content, stimulate sodium and water absorption in the colon, and affect the immune function of the gastrointestinal tract. The addition of complex fermentable carbohydrates to the diet can modulate the type and function of cells from different regions of the gut-associated lymphoid tissue (Lim et al., 1997; Field et al., 1999; Nagai et al., 2000), increase immunoglobulin production (Kudoh et al., 1998), and alter the profile of inflammatory cytokines (Kanauchi et al , 1999; Segain et al., 2000; Milo et al., 2002). The intestinal microbiota also appear to play a role in enhancing the surface area of the intestinal lining, crypt-villus structure, epithelial turnover, motility, and development of the gut associated lymphoid tissue (Abrams, 1983).

Protective Effects:

One of the most important functions of the intestinal microbiota is the provision of a natural barrier which can inhibit the colonization and proliferation of opportunistic pathogens, thereby decreasing the risk of intestinal infection and disease (Rolfe 1997). Colonization resistance is a term that describes this ability of certain indigenous intestinal bacteria, such as bifidobacteria and lactobacilli, to interfere with colonization and establishment by exogenous and possibly disease-causing microorganisms (Dai and Walker, 1999).

The indigenous intestinal microbiota can inhibit the growth of exogenous pathogenic microorganisms in several ways, including: (1) competition with pathogens for available nutrients; (2) production of organic acids that lower intraluminal pH to a level that inhibits pathogen growth; (3) production of other substances that inhibit pathogen adhesion to the gut wall (Agostoni et al., 2004); and (4) compromising the virulence of potential pathogens (Guarner and Malagelada, 2003). Finally, the indigenous intestinal microbiota can play a role in preventing

systemic disease through their role in enhancing the structure and function of the intestinal epithelial layer and associated immune-related tissues, which decrease the likelihood of bacterial and antigen translocation across the gut epithelial lining.

The GI microbiota is highly variable immediately after birth up until the time when complex diets are introduced, indicating a state of suboptimal balance or development of the intestinal microbiota. It has been suggested that this unstable state may contribute to some of the intestinal disease seen in infants and young children, since the protective mechanisms of the normal microbiota are diminished or absent (Cooperstock and Zedd, 1983). For example, there are extensive data demonstrating the protective role of the intestinal microbiota against *Clostridium difficile*, the cause of pseudomembranous colitis (Borriello et al., 1988; Gorbach et al., 1988; Rolfe, 1988; Rolfe, 1997). It has been reported that as many as 90% of infants are asymptomatically colonized with *C. difficile*, populations of which fall sharply after the first year of life with the introduction of a complex diet.

B. Composition of Adult Microbiota

The human GI microbiota can be thought of as a complex organ that contributes to the host's nutritional requirements (e.g.. vitamins, SCFA) and is involved in regulating epithelial cell development and the maturation of the innate immune system. Of the estimated 500 to 1000 species of microorganisms thought to inhabit the human GI tract, most have not been cultured and 99.9% of those that have are obligate anaerobes (Kaper and Sperandio, 2005). The predominant bacteria that have been cultured via classical microbiological approaches include members of the *Bacterioides*, *Bifidobacterium*, *Eubacterium*, *Lactobacillus*, *Clostridium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*, *Escherichia* and *Veillonella* genera. Considering the evident complexity of the GI microbiota, and the difficulties associated with culturing strictly anaerobic organisms, classical microbiological approaches are being rapidly supplanted by culture-independent molecular methodologies based on the amplification and comparison of conserved genetic regions (e.g., 16S ribosomal RNA gene) in bacterial DNA that can be recovered directly from tissue or feces. As an example, a recent study utilizing such methods to randomly sample human mucosal and fecal bacteria reported that 80% of the organisms detected had not been previously cultured, 62% represented novel organisms, and populations varied in a given individual depending upon anatomical site (Eckburg et al., 2005). Methods based on 16S rRNA gene similarity have become accurate enough to identify up to 90% of the bacteria routinely encountered in human feces (Harmsen et al., 2002).

Sufficient 16S rRNA gene sequences have been generated as to allow the development of a phylogenetic tree that is representative of the major taxonomic groups most frequently detected in human adult feces. Based on these data, it appears that the typical adult gastrointestinal microbiota is likely to consist of 46-58% low GC Gram-positive bacteria consisting of the *Eubacterium cylindroides* (1.1-1.4%), *Lactobacillus-Enterococcus* (<0.1-

1.8%), *Veillonella* (<0.1-1.3%), *Phascolarctobacterium* (0.6%), *Clostridium coccoides-Eubacterium rectale* (22.7-28.0%), and *Clostridium leptum* groups (21.1-25.2%)); 8-17% high GC Gram-positive bacteria consisting of the *Atopobium* group (3.1-11.9%), and *Bifidobacterium* (4.4-4.8%)); and 10-30% Gram-negative bacteria consisting of *Enterobacteriacaea* (0.1-0.2%), and the *Bacteroides-Prevotella* (8.5-28%), and *Akkermansia* groups (1.3%)) (Zoetendal et al., 2006). Though just as individualized in composition, the infant gastrointestinal microbiota is typically less stable and less complex than that of adults.

C. Composition of Infant Microbiota

The development of normal gastrointestinal and immunological functionality in human infants depends in part on the establishment of a complex commensal microbiota (Bäckhed et al., 2005). Though essentially sterile at birth, the infant GI tract is immediately exposed to maternal and environmental bacteria and the subsequent colonization process is influenced by numerous factors including mode of delivery, gestational age, hospitalization, antibiotic use, and type of infant feeding (Penders et al., 2006). Early studies utilizing standard microbiological methods have shown that during the first days of life the intestinal microflora of the human infant comprise almost entirely enterobacteria and Gram-positive cocci which are obtained largely during the birth process (Cooperstock and Zedd 1983; Cooperstock 1987; Conway 1997). These aerotolerant bacteria are thought to reduce the redox potential of the intestinal environment, generating conditions that are more favorable for the subsequent establishment of obligate anaerobes such as *Bacteroides*, bifidobacteria, and clostridia (Dai and Walker, 1999; Mackie et al., 1999).

As mentioned above, the mode of delivery at birth may be particularly important in determining the initial composition of the intestinal microbiota since it typically represents the first group of microorganisms to which the infant's GI tract is exposed. Infants born by the vaginal route typically acquire their intestinal biota from maternal vaginal and fecal microbiota. Infants born by Caesarean section, however, show a very different pattern of GI microbiota development, which is likely due to other factors such as reduced gestational age and extensive exposure to the hospital environment and prophylactic antibiotics (Fryklund et al., 1992; Gronlund et al., 1999). Generally, the microbiota development in Caesarean-born infants is delayed for up to several months, showing slower acquisition of *Bifidobacterium* and *Bacteroides* (Fryklund et al., 1992; Gronlund et al., 1999). In preterm infants, intestinal colonization with bifidobacteria is also delayed for several weeks (Blakey et al., 1982; Sakata et al., 1985), possibly due to reduced GI maturation (Butel et al., 2007).

The next important stage in the development of the GI microbiota occurs during the processes of dietary supplementation and weaning, which result in the addition of non-milk foods and complex carbohydrates to the diet. Throughout life the host is repeatedly exposed to new microbes which continuously enter the intestinal tract with the diet. By adulthood, the intestinal microbiota are extremely diverse and complex with total numbers reaching as high

as 10^{13} bacterial cells per gram feces, representing as many as 500 to 1000 different species (Tannock et al., 2000; Zoetendal et al., 2006).

While individual components of the intestinal microbiota may change throughout life, the variability in composition appears to be greatest during the succession period following birth through the weaning process, i.e., the first two years of life. As weaning progresses, both intestinal function and fermentation capacity continue to mature with the composition of the intestinal microbiota beginning to resemble that of adults by the second year of life. Because of the complex nature of the adult microbiota, it is widely believed that new microorganisms that gain access to the intestines of the infant have a higher likelihood of becoming established compared to introducing the same microorganisms into the adult digestive tract.

As stated previously, the succession pattern for bacterial colonization of the GI tract is influenced by a number of factors, including exposure to new environmental microorganisms, treatment with medications, host physiology, and source of nutrition. It is well recognized that ecological principles apply to the impact of these influences on the GI microbiota (Savage, 1977; Conway, 1995), such that an alteration in one component of this ecosystem will result in a change in another. For example, during the first months of life the infant diet has a particularly crucial influence on the composition and metabolism of intestinal bacteria. While breast feeding is recognized as the best source of nutrition in infancy, some mothers will supplement or replace breastfeeding with infant formula. As described earlier, the intestinal microbiota of breast-fed infants is rapidly dominated by bifidobacteria, and more diverse biota only develops after dietary supplementation occurs. The intestinal microbiota of formula-fed infants shows similar quantitative levels of bifidobacteria, but contains a greater diversity and higher numbers of other bacterial genera, including *Bacteroides*, *Clostridium* and Enterobacteriaceae (Fanaro et al., 2003; Stark and Lee, 1982; Cooperstock, 1987; Edwards and Parrett, 2002). While not all studies are in complete agreement, many have confirmed these overall patterns, including more recent studies using molecular techniques (Harmsen et al., 2000; Martin et al., 2000).

D. Influence of Dietary Factors on the Development of Infant Microbiota

It is widely reported that breast milk consumption by infants can promote the growth of beneficial bacteria like *Bifidobacterium* spp. (Fanaro et al., 2003) and/or reduce colonization by less beneficial (e.g., *Escherichia coli*) or potentially harmful (e.g., *Clostridium difficile*) species (Penders et al., 2005). Selective stimulation of beneficial members of the infant GI microbiota (at the expense of harmful bacterial species) is referred to as "competitive exclusion" and has been proposed as one of the protective mechanisms whereby breast-fed infants are more resistant to infection. Numerous nutrition-related factors (parenteral nutrition, delayed feeding, reduced intestinal maturation etc.) that would be typical in the care of extremely low birth-weight infants in neonatal intensive care settings are thought to contribute to the development of an atypical microbiota characterized by the

presence of hospital-acquired facultative anaerobes and a lack of bifidobacteria and lactobacilli. In addition to nutrition related factors, a younger gestational age at birth has been shown to be a major determinant of reduced bifidobacteria colonization in such preterm infants (Butel et al., 2007).

To further evaluate the differences in microbiota patterns among breast-fed and formula-fed infants, Tannock et al. (1994) compared results from several studies to determine whether specific patterns of fecal bacteria in infants were predictive of feeding regimen. They reported no differences in the overall levels of either bifidobacteria or lactobacilli in fecal samples obtained from breast-fed and formula-fed infants (Tannock 1994). However, their study found that higher levels of clostridia in fecal samples were predictive of formula-fed infants while lower levels were more typical in breast-fed infants. While counts of enterobacteria or streptococci were typically lower in feces of breast-fed infants, these differences were not as consistent as the differences seen with clostridia. This finding may be of particular clinical relevance, since several types of clostridia have been associated with human disease, especially in infants. *Clostridium difficile,* common during the first year of life although seldom seen in healthy adults, is responsible for 20-40% of cases of antibiotic-associated diarrhea (Fekety and Shah, 1993), and has been implicated in necrotizing enterocolitis (Blakey et al., 1985). *Clostridium perfringens* has been associated with various gastrointestinal disorders, including flatulence, distended abdomen, diarrhea, and blood in stools (Ahtonen et al., 1994).

As described previously, the enumeration of fecal microbiota using classical microbiological techniques involves the attempted recovery of specific bacterial genera using partially selective artificial media containing complex blends of nutrients, growth factors, antibiotics, and other inhibitors. Given that such methods have been reported to recover as few as 10% of the total fecal bacteria (Harmsen et al., 1999), that 99% of the target populations require a strictly anaerobic growth environment (Mackie et al., 1999), and that as many as 56% represent unknown entities (Favier et al., 2002), nonculture-based molecular methods have proven essential in the development of a more accurate representation of the infant GI microbiota (Favier et al., 2003). The use of molecular methodologies has proven particularly relevant in the evaluation of beneficial members of the microbiota (e.g. bifidobacteria) at the species or strain level where classical microbiology can provide little or no information.

Using an analysis of 16S rDNA to investigate the development of bacterial and bifidobacterial communities in the feces of newborns, Favier et al. (2003) reported that despite early colonization by other genera, *Bifidobacterium* species appeared by days 5 and 6 in breast-fed and formula-fed infants, respectively. There were clear indications that even though infants were initially colonized with bifidobacteria inherited from their mothers, they went on to develop their own unique bifidobacterial patterns. During the course of the same study, no bifidobacteria appeared in the feces of a single infant that was treated with Augementin® (clavulanic acid and amoxicillin) and Bactrimel® (trimethoprim and sulfamethoxazol) despite being partially breast-fed. A study employing a combination of

classical (colony formation on selective media) and molecular (16S rDNA and terminal restriction fragment length polymorphism) methods recently indicated that approximately 30% of the fecal biota were culturable and bifidobacteria populations were comparable regardless of feeding regimen (human milk, formula, or mixed) (Sakata et al., 2005). *Bifidobacterium longum* ssp. *longum* (63.2%) *Bifidobacterium longum* ssp. *infantis* (33.8%) and *Bifidobacterium breve* (23.5%) were the species most often recovered. This species distribution was also detected in an earlier comparison of bifidobacterial populations in adults and breast-fed infants (Matasuki et al., 1999). The adults were found to harbor a more complex mix of *B. catenulatum* (92%), *B adolescentis* (60%), *B. longum* (65%) and *B bifidum* (38%) than infants whose fecal biota contained simpler combinations of *B breve* (70%), *B infantis* (41%), and *B. longum* (37%).

Changes in bifidobacterial and other microbial patterns during breast-feeding, weaning and post-weaning periods have been followed during a longitundinal study of 11 infants by Magne et al.(2006) using temporal temperature gradient gel electrophoresis (TTGE) profiles. This study confirmed that bifidobacteria represented a dominant fraction of the fecal bacteria in all infants, reaching 10.5 $\log_{10}$ cells g^{-1}, regardless of dietary stage. In the TGGE profiles, the main bands that were identified corresponded to *E coli*, *Ruminococcus* spp. and a mixed bifidobacterium population consisting primarily of *B longum*, *B infantis*, and *B breve*. Despite a high level of interindividual variability in fecal microbiota, the profiles appeared more homogeneous after weaning, indicating that breast milk factors, the introduction of other foods or both had an impact on population dynamics. The establishment of GI microbiota in two healthy infants from the first day of life have been followed in great detail over a 10 month period by Favier et al. (2002) using 16S rDNA-based denaturing gradient gel electrophoresis (DGGE) patterns. The authors estimated that even though 56% of the detectable bands were derived from unknown species, the dominant members of the GI microbiota were most closely related to *Bifidobacterium* and *Ruminococcus* in terms of both stability and population density. *Bifidobacterium* species as well as *Clostridium* species were detected within a few days, the latter of which disappeared in the breast-fed infant with weaning. Over time strong bands corresponding to *Ruminococcal* species also appeared in the feces of both infants and became a stable constituent of their resident microbiota. Real-time quantitative PCR was employed by another group to monitor the GI microbiota of 100 exclusively breast-fed or formula-fed infants (Penders et al., 2005). All infants were colonized by *Bifidobacterium* species and the mean values were comparable among the formula (10.24 $\log_{10}$ CFU g^{-1} feces) and human milk (10.56 $\log_{10}$ CFU g^{-1} feces) groups. In contrast, the *Clostridium difficile* values differed significantly with feeding regimen, appearing in 14% (at 3.28 $\log_{10}$ CFU g^{-1}) and 30% (at 7.43 $\log_{10}$ CFU g^{-1}) in the feces of the human milk and formula groups, respectively. The *E. coli* values also differed significantly with feeding regimen as they were detected at rates of 80% (9.11 $\log_{10}$ CFU g^{-1}) and 94% (9.57 $\log_{10}$ CFU g^{-1}) in the feces of the human milk and formula groups, respectively. The authors concluded that the prevalence and counts of both *C. difficile* and *E coli* were reduced in breast-fed infants though the *Bifidobacterium* populations appeared comparable.

E. Microbiota Summary

The presence of a complex gastrointestinal microbiota is integral to the normal development and function of the human GI tract as these organisms perform numerous metabolic, trophic and protective roles. Bacterial colonization begins during the birthing process and the succession of organisms is heavily influenced by mode of delivery, form of nutrition and other factors. Taken together, the results of the studies cited above indicate a predominance of bifidobacteria in the GI microbiota of infants, a species distribution unlike that of adults (i.e., consisting primarily of *B. breve*, *B longum*, and *B. infantis*), and an emerging but significant contribution of additional genera (e.g., *Ruminococcus* spp.). In general, the results also suggest that the GI microbiota of breast-fed infants contains reduced populations of potentially pathogenic species (e.g.., *C. difficile* and *E coli*). Efforts to improve the performance of infant formula may include the inclusion of ingredients with the potential to shift the GI microbiota of formula-fed infants in a manner comparable to that of those fed breast milk. To that end, one purpose of adding food-grade prebiotic oligosaccharides such as PDX and GOS to infant formula, with an overall goal of bringing the performance of formula-fed infants into closer resemblance to that of breast-fed infants, is to stimulate and maintain a high level of bifidobacteria at the expense of potential pathogens such as *C. difficile*.

IV. Functionality: Selection of Prebiotic Ingredients as HMO Substitutes

A. Rationale for the Substitution of Prebiotic Ingredients

The oligosaccharide content of human breast milk is incredibly diverse at a structural level, highly dynamic in composition across both individuals and time, and physiologically important in the normal development and health of infants. Human breast milk is widely considered the gold standard in infant nutrition, so there is a continuing effort to more closely approximate its nutritional and functional properties in infant formula. Though the addition of oligosaccharides is a logical step in that process, the diverse and variable nature of HMO precludes the use of chemically identical molecular species. In order to more closely mimic some of the functional properties of human breast milk oligosaccharides (higher levels of beneficial bacteria including bifidobacteria; improved stool characteristics, etc.), it has been proposed that appropriate levels of food-grade oligosaccharides (prebiotics) be added to infant formula (Vandenplas, 2002). A prebiotic is defined as "a selectively fermented ingredient that allows specific changes in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health," (Roberfroid, 2007). The criteria that are generally accepted for classifying a food ingredient as a prebiotic include the following: 1) resistance to gastric acidity, mammalian enzymes,

and gastrointestinal absorption; 2) fermentation by members of the intestinal microbiota; and 3) selective stimulation of the growth and/or activity of intestinal bacteria that contribute to health and well-being. These criteria are comparable to some of the biological activities of human milk oligosaccharides.

As described earlier, human breast milk is a complex fluid that fulfills numerous nutritional, developmental and protective functions in infant health. Infant formula, as a class, is designed to deliver functional properties comparable to those of human milk (nutritional and developmental support), while not being chemically identical to it, which is not technically feasible. As an example, the protein content of the majority of infant formulas is based on cow's milk or fractions thereof. The protein constituents of human and cow's milk are not chemically identical though they are functionally similar. When compared at the level of amino acid sequences, typical protein constituents of human and cow's milk, like a-lactalbumin, a-casein, ß-casein, and ?-casein, are only 73%, 31%, 53%, and 53% identical, respectively (National Center for Biotechnology Information; protein-protein Basic Local Alignment Search Tool (BLAST) search; 12-19-2006), though they have been found to deliver comparable nutritional support. Likewise, the purpose of adding prebiotic oligosaccharides to infant formula is the delivery of similar functionalities and not to achieve chemical identity.

Numerous prebiotic oligosaccharides have been evaluated for use in infant formula, including inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and polydextrose (PDX). Such evaluations are typically based on *in vitro* colonic screening methodologies. As an example, Probert and colleagues (2004) carried out *in vitro* continuous fermentations using a human colon model to evaluate the effects of polydextrose (PDX), lactitol, and fructooligosaccharides (FOS) on the growth and metabolism of intestinal bacteria. PDX was found to have a stimulatory effect upon both the population size and diversity of colonic bifidobacteria when added at 1 or 2%. An alternative but complementary *in vitro* screening method was employed by Cinquin et al.(2006) in which infant fecal bacteria were immobilized in gel beads and continuously cultured in a three-stage chemostat. When added to the base medium at a concentration of 9.8 g/L, FOS was found to significantly increase organic acid concentration and lactobacilli populations while decreasing both ammonia production and coliforms. Similar methods have been recently employed to carry out structure-function studies of novel oligosaccharides in order to determine their prebiotic potential (Sanz et al., 2006). In this study, random maltose polymers of various lengths (degrees of polymerization; DP) were generated and their impact on the growth and metabolism of human adult fecal microbiota measured. In general, oligosaccharides with a DP of 3 demonstrated the greatest stimulation of bifidobacteria, while those with a DP of 5-7 displayed a similar but reduced activity.

In these and similar studies, a number of oligosaccharides have demonstrated a capacity to stimulate the growth of select beneficial bacteria (e.g., bifidobacteria), which is typically considered one of the functional attributes of HMO. None of the commercially available prebiotic oligosaccharides is chemically identical to human breast milk

oligosaccharides, though they may deliver similar functionality (e.g., bifidogenicity, stool softening). As an example, inulin, which is present in many common foods, is industrially obtained from chicory roots or other sources by hot water extraction. It is a disperse mixture of linear molecules of the general formula $G\text{-}F_n$, where a glucosyl moiety (G) is attached to a fructosyl (P) polymer linked by ß(2-1) bonds, and a 3-60 range of polymerization (n) (Franck, 2006). FOS is derived in turn by the partial enzymatic hydrolysis of inulin and is composed of linear $G\text{-}F_n$ and $F\text{-}F_n$ chains, with a DP ranging from 2-8 and averaging 4. GOS is formed by a transgalactosylation reaction utilizing the ß-galactosidase enzyme (derived from *Bacillus circulans* ATCC 4516 in the case of Vinvinal® GOS) and pharmaceutical-grade lactose. GOS is typically described as chains of galactose with a glucose end-cap displaying a 2-8 DP and averaging 2-4. The following formula has been published for the Oligomate GOS: $a\text{-Glu-}(1\rightarrow4)\text{-}[ß\text{-Gal-}(1\rightarrow6)]_{2\text{-}4}$ (Shoaf et al., 2006). This structure is viewed by some as more closely resembling some HMO than other prebiotic oligosaccharides (Schmelzle et al., 2003). PDX, which has been utilized as a low caloric sugar and fat substitute (bulking agent), is described as a highly branched glucose polymer with sorbitol endcaps (Stumm and Baites, 1997). Litesse®-brand polydextrose is prepared by vacuum thermal polymerization of glucose, using glucitol (sorbitol) as a plasticizer and an approved food acid (citric acid) as catalyst (89:10:1 ratio) (Craig et al., 1998). This random polymerization and branching yields a mixture of structures containing various types of glycosidic bonds (though ß(1-6) predominate) and an average DP of 12, though 90% of the material is calculated to contain between 3 and 30 sugars. A combination of prebiotic materials such as GOS and PDX can deliver a broader range of linkage units that clearly fall within the range reported for breast milk oligosaccharides (DP of 3-32; Morrow et al., 2005).

B. Prebiotic Selection Process

MJ recently collaborated with two groups on preclinical studies that used *in vitro* fecal fermentation models to assess the prebiotic activity of various ingredients: 1) Dr. Maria Saarela and colleagues at VTT Biotechnology (Helsinki, Finland) and; 2) Dr. Glenn Gibson of the University of Reading (UK). Such *in vitro* fecal fermentation models are designed to mimic the action of the total colonic microbiota of infants rather than isolated strains, and are a useful tool to assess the potential prebiotic activity of various ingredients. Briefly, a minimal growth media, or growth media supplemented with infant formula, are inoculated with fresh fecal samples obtained from healthy breast-fed or formula-fed infants. Following a suitable period of incubation to stabilize the microbiota, the effect of test prebiotic ingredients on populations of intestinal microorganisms is measured using various culture-based and/or molecular methods. The consumption of carbohydrates and the production of short-chain fatty acids (SCFA), several gases, and other metabolites can also be measured as indicators of bacterial metabolism.

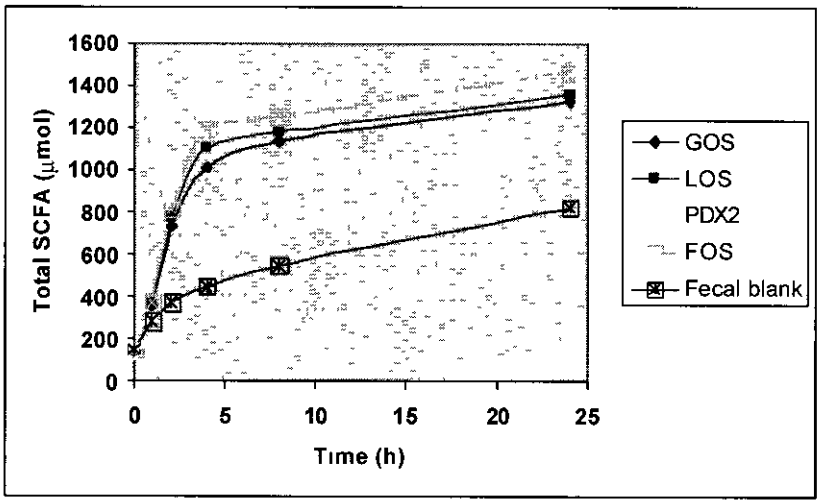
VTT Biotechnology Studies

MJ collaborated with Saarela et al. at VTT Biotechnology (Helsinki, Finland) on studies to assess the rate of fermentation and formation of metabolic end-products from prebiotic carbohydrates using a closed *in vitro* fermentation system. *In vitro* fecal fermentations were performed according to the method of Karppinen et al (2000). Fecal samples were collected from healthy infants aged 2.5-13 months, prepared as fecal slurries, and pooled to serve as the fecal inocula. Prebiotic substrates were suspended in carbonate-phosphate buffer and combined with fecal slurry samples (10% w/v) under anaerobic conditions and incubated at 37°C. Samples from the inoculated mixtures were obtained at various time points over a 24 hour period and evaluated for pH, SCFA content, and gas production during fermentation. SCFA were extracted with diethyl ether and analyzed with gas chromatography as described by Karppinen et al (2000) Gases (hydrogen, carbon dioxide, methane, hydrogen disulfide, and oxygen for validating anaerobic conditions) were analyzed isothermally at 30°C using a static headspace technique by gas chromatography (Karppinen et al., 2000). Prebiotic ingredients were also tested for their growth-promoting ability against a panel of selected samples of human fecal bacteria.

Various prebiotics were tested, both individually and in combination, in an attempt to identify a desirable rate of microbial fermentation *in vitro*. A controlled rate of fermentation may be more desirable to avoid the excessive gas formation and rapid acidification of intestinal contents that may result from rapidly fermented substrates. Such rapid accumulation of acidic metabolites (acetic acid, etc.) and gases (carbon dioxide, etc.) may lead to bloating, abdominal distention, and irritation of the intestinal lining or anal region, resulting in reduced tolerance in infants.

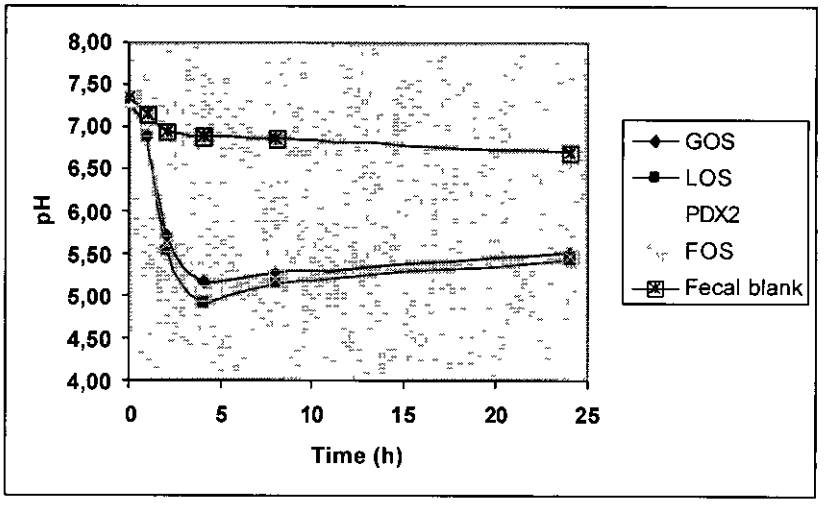
The production of total SCFA (sum of acetic, propionic and butyric acids) and the associated decrease in pH were used as indicators of the rate of prebiotic fermentation by infant fecal bacteria. The rate of fermentation by fecal bacteria was highly variable among the different prebiotics tested (Figures 1 and 2). PDX was fermented very slowly, whereas the FOS, GOS and lactulose (LOS) substrates were fermented rapidly and completely. The fermentation rate of FOS was extremely rapid as it was almost completely consumed within the first sampling time points and produced the highest amount of SCFA among the prebiotics tested. A blend of PDX and GOS in a 1:1 ratio resulted in a fermentation rate lower than that seen with GOS alone, as measured by total SCFA production (Figures 3 and 4). Similarly, the combination of PDX with a mixture of GOS and LOS, at either 1:1:1 or 3:2:1 ratios (approximately 50%:33%:17%), had a lower overall fermentation rate than the mixture of GOS and LOS alone. The addition of PDX to GOS or the combination of GOS, PDX and LOS also resulted in a more moderate decrease in pH over time, as indicated in Figure 4. The slower pH decline resulting from the PDX fermentation is consistent with reduced SCFA production and overall *in vitro* fermentation rate, when compared to GOS and LOS alone. These results demonstrate that replacing a portion of GOS, FOS or LOS with PDX can be used to produce a prebiotic combination that results in a more controlled bacterial fermentation, when compared to those prebiotics alone.

Figure 1. Total SCFA production following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



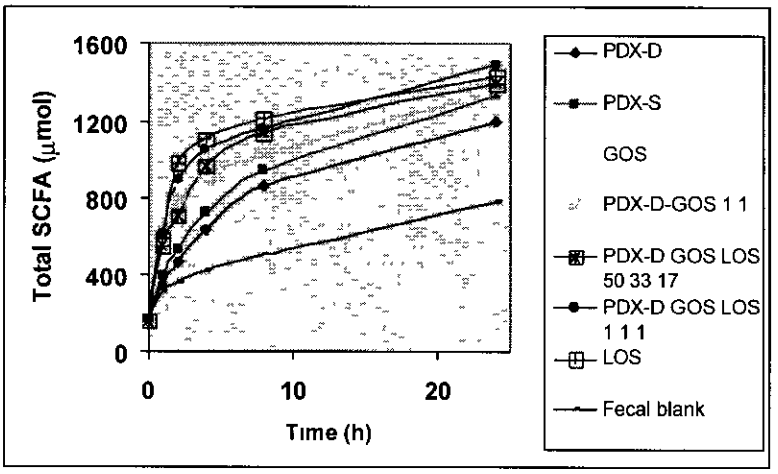
Key: GOS, galactooligosaccharides; LOS, lactulose; PDX2, polydextrose from Danisco; FOS, fructooligosaccharides.

Figure 2. Change in pH following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



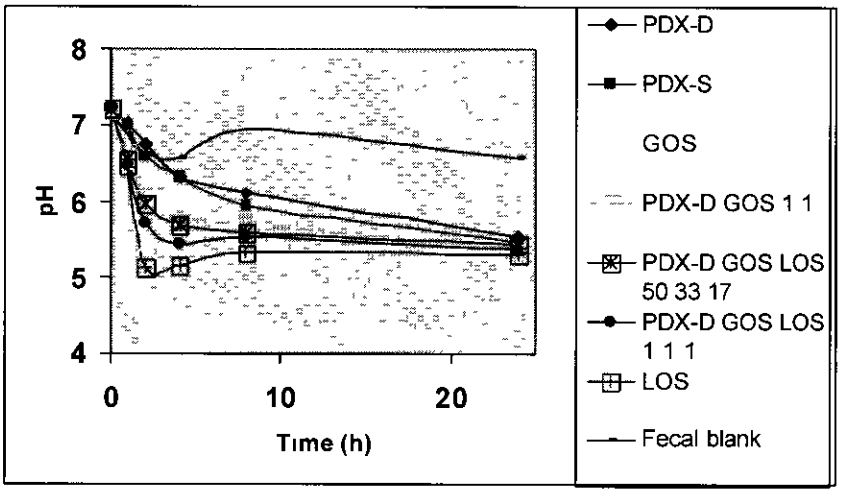
Key: GOS, galactooligosaccharides; LOS, lactulose; PDX2, polydextrose from Dansico; FOS, fructooligosaccharides.

Figure 3. Total SCFA production following fermentation of single or combinations of prebiotic ingredients by infant fecal bacteria *in vitro*.



Key: GOS, galactooligosaccharides; LOS, lactulose, PDX-D, polydextrose from Danisco; PDX-S, polydextrose from A E. Staley.

Figure 4. Change in pH following fermentation of single or combinations of prebiotic ingredients by infant fecal bacteria *in vitro*.



Key: GOS, galactooligosaccharides; LOS, lactulose, PDX-D, polydextrose from Danisco; PDX-S, polydextrose from A. E. Staley.

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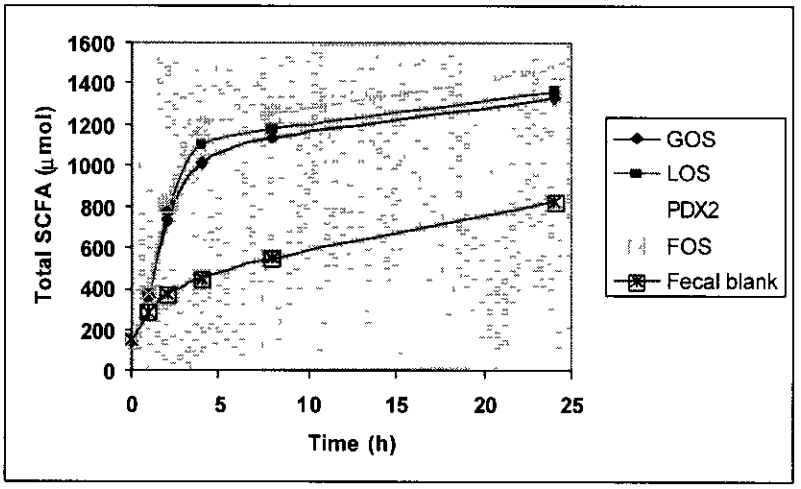
SCFA, along with carbon dioxide, methane and hydrogen, are end products of bacterial metabolism of carbohydrates in the distal gastrointestinal tract. The amount of SCFA produced is dependent on both the composition of the intestinal microbiota and the diet. Many of the beneficial effects of fiber are attributed to the SCFA fermentation byproducts, since the gastrointestinal tract is known to be highly responsive to these compounds. They are readily absorbed by the intestinal mucosa, contribute caloric content, are readily metabolized by intestinal epithelium and liver, stimulate sodium and water absorption in the colon, enhance small bowel digestion and absorption and affect the immune function of the gastrointestinal tract. Thus, SCFA have been the focus of much research investigating gastrointestinal health (Frankel et al., 1994; Tappenden et al., 1996; Schley and Field, 2002).

In adults, acetate, propionate and butyrate comprise 83% of the SCFA produced in the colon, which are distributed in a fairly consistent molar ratio of about 60:25:15, respectively (Gibson et al., 1995). Infants on the other hand, produce lesser amounts and a very different distribution of SCFA,with acetate occurring at relatively higher levels and both propionate and butyrate occurring at lower levels (Lifschitz et al., 1990; Siigur et al., 1993; Edwards et al., 1994; Parrett and Edwards, 1997). In addition, it appears that fermentation is slower in infants than adults, but by two years of age an adult SCFA profile emerges, reflecting likely changes in the infant diet and microbiota (Midtvedt and Midtvedt, 1992).

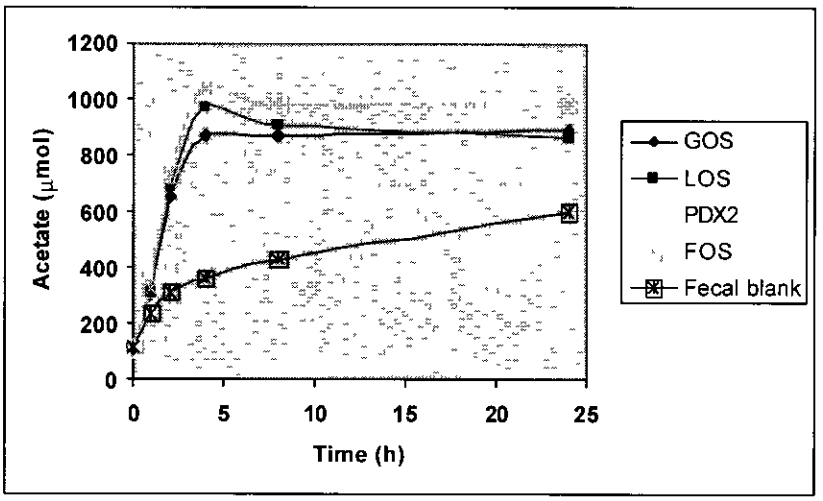
While each SCFA is thought to perform multiples roles, *in vitro* studies have indicated that propionate displays an inhibitory effect on the growth of enteric pathogens. Infant fecal fermentations using PDX as substrate resulted in the highest propionate production and the lowest butyrate production during 24 hours (Figures 5-8). Acetate remained the highest SCFA produced during PDX fermentation, though the initial rate was much lower than those of the other substrates. The initial rate of propionate production from PDX was similar to that of the other substrates, but higher levels were found at the end of fermentation. In contrast, the fermentation of FOS, GOS and LOS resulted in increased concentrations of acetate and butyrate and a decreased concentration of propionate. As a result, the combined relative proportion of acetate and propionate produced by infant fecal bacteria was much higher for PDX than for FOS, LOS or GOS. These results demonstrate that PDX was the least butyrate-yielding substrate, and the only substrate that increased the relative proportion of propionate. This is a potentially important finding as *in vitro* studies have demonstrated that propionate is much more inhibitory to the growth of enteric pathogens like *Salmonella typhimurium* than either acetate or butyrate (Kwon and Ricke, 1998), and when produced by protective bacterial cultures (e.g. *Lactobacillus crispatus* and *Clostridium lactatifermentans*) contributes to the competitive exclusion of *Salmonella enterica* (van der Wielen et al., 2002).

Figure 5. Total SCFA production following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



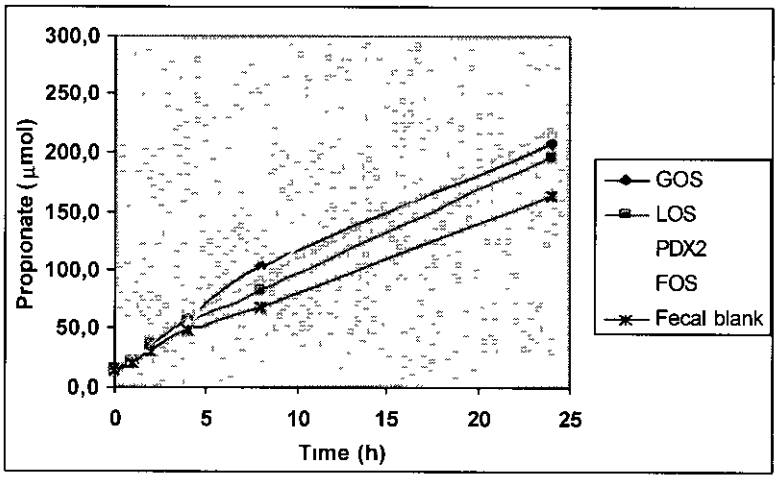
Key: GOS, galactooligosaccharides; LOS, lactulose, PDX2, polydextrose from Danisco; FOS, fructooligosaccharides.

Figure 6. Acetate production following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



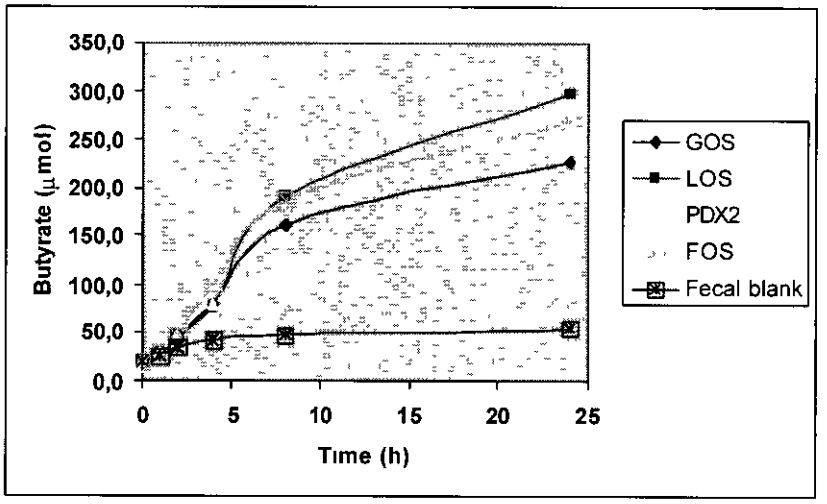
Key: GOS, galactooligosaccharides, LOS, lactulose; PDX2, polydextrose from Danisco; FOS, fructooligosaccharides.

Figure 7. Propionate production following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



Key· GOS, galactooligosaccharides; LOS, lactulose, PDX2, polydextrose from Danisco; FOS, fructooligosaccharides

Figure 8. Butyrate production following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



Key: GOS, galactooligosaccharides; LOS, lactulose, PDX2, polydextrose from Danisco; FOS, fructooligosaccharides.

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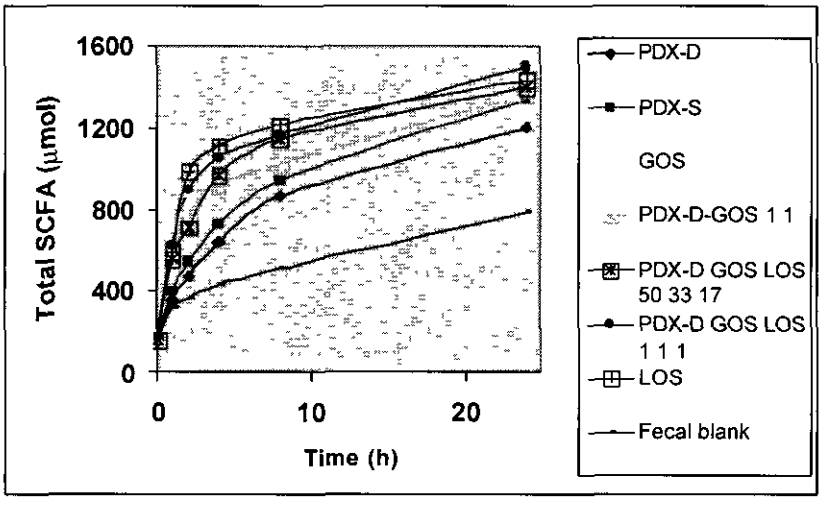
The effect of combining various prebiotic ingredients on the production of total SCFA, acetate, propionate and butyrate was also evaluated using inoculum derived from the feces of younger infants (under 6 months of age) (Figures 9-12). The addition of PDX to GOS (1:1 ratio) led to a slower overall rate of total SCFA production when compared to single fermentations of LOS or GOS, or combinations of PDX, GOS, and LOS at both 1:1:1 and 3:2:1 ratios (Figure 9). The SCFA profiles indicated that blends of PDX and GOS (1:1) and PDX, GOS, and LOS (3:2:1) led to a slower rate of acetate production and a higher rate of propionate production compared to LOS alone (Figures 10 and 11). Butyrate continued to be produced in significantly lower amounts and the addition of PDX to other prebiotics did not substantially changes the rate of butyrate production. Overall, these results demonstrate that the addition of PDX to prebiotic mixtures results in a slower rate of fermentation *in vitro* and a higher relative amount of propionate production.

One of the more interesting observations from this experimental set is the impact of infant age on the outcome of prebiotic fermentation by fecal microbiota. Production of butyrate was suppressed and the relative proportion of acetate increased when all prebiotic substrates were fermented with stool inoculum from infants younger than 6 months. Not even the presence of LOS, which resulted in the highest production of butyrate in an earlier experimental set (Figure 8), could enhance the formation of butyrate when the stool inoculum originated from infants under 6 months of age (Figure 12). These data provide support to the hypothesis that the composition of fecal microbiota, and their associated metabolic capacity, vary substantially between younger and older infants.

Figure 9. Total SCFA production following fermentation of single or combinations of prebiotic ingredients by infant fecal bacteria *in vitro*.



Key: GOS, galactooligosaccharides; LOS, lactulose, PDX-D, polydextrose from Danisco; PDX-S, polydextrose from A. E. Staley.

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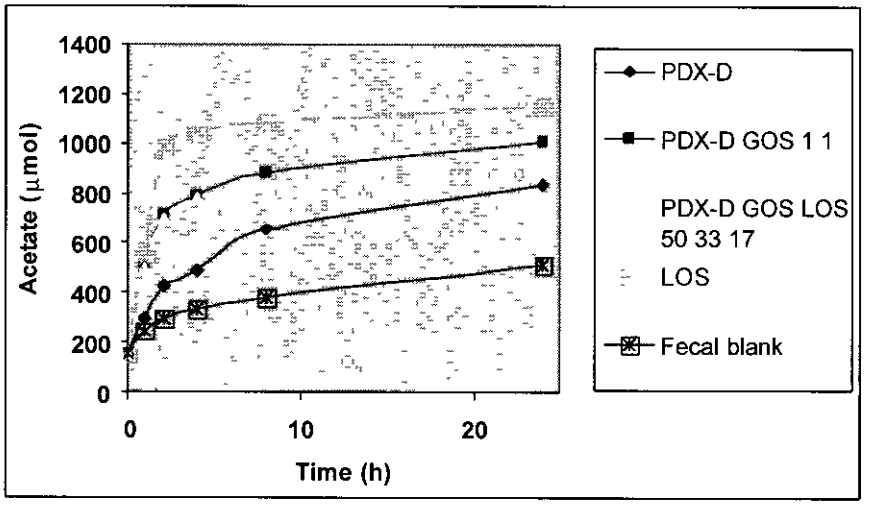
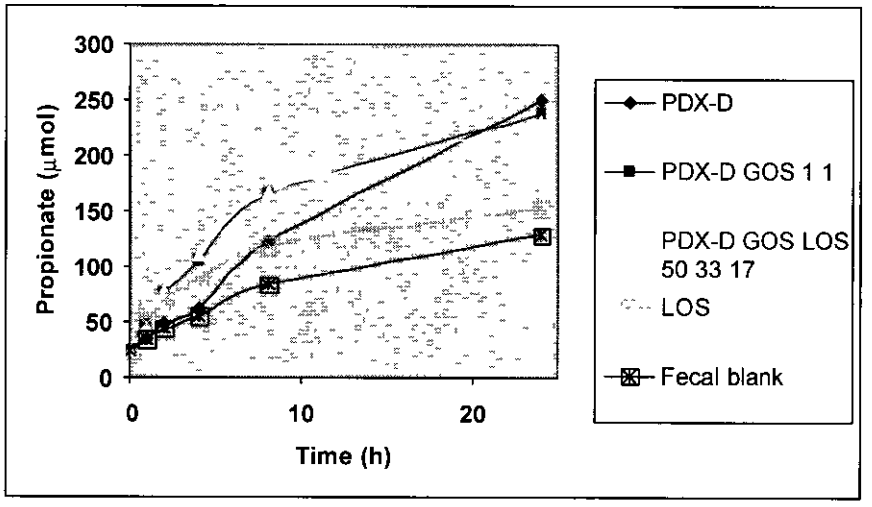
Figure 10. Acetate production following fermentation of single or combinations of prebiotic ingredients by infant fecal bacteria *in vitro*.



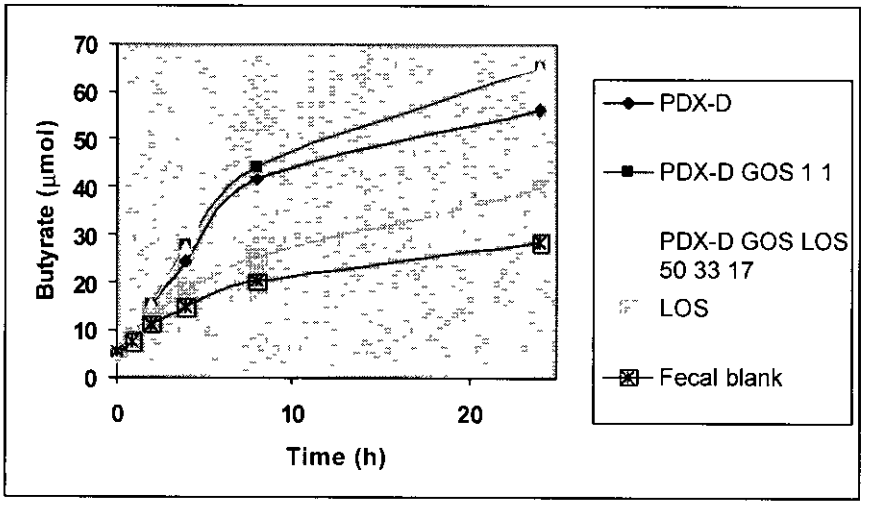
Key: GOS, galactooligosaccharides; LOS, lactulose; PDX-D, polydextrose from Danisco.

Figure 11. Propionate production following fermentation of single or combinations of prebiotic ingredients by infant fecal bacteria *in vitro*.



Key: GOS, galactooligosaccharides; LOS, lactulose; PDX-D, polydextrose from Danisco.

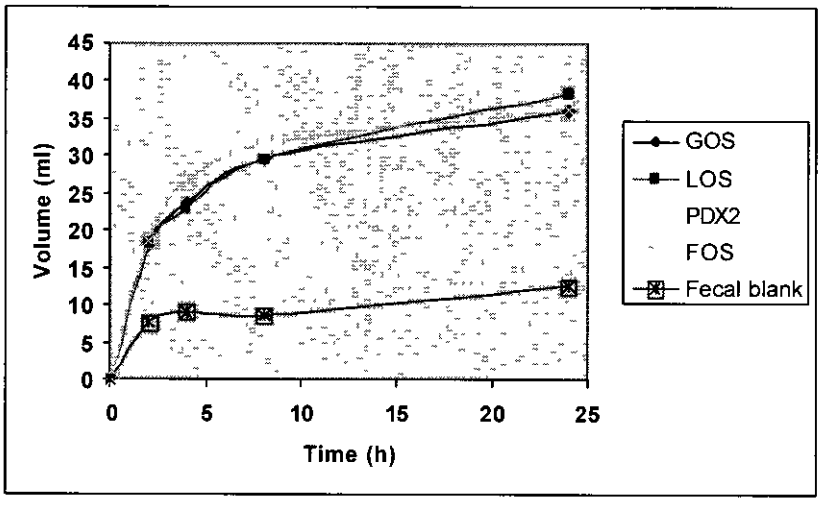
Figure 12. Butyrate production following fermentation of single or combinations of prebiotic ingredients by infant fecal bacteria *in vitro*.



Key: GOS, galactooligosaccharides, LOS, lactulose; PDX2, polydextrose from Danisco.

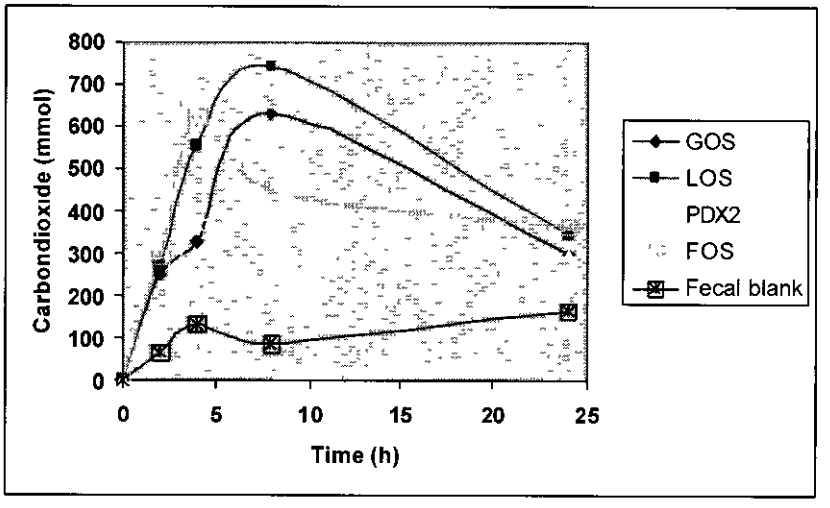
The formation of gases (total, carbon dioxide, hydrogen and hydrogen disulphide) are shown in Figures 13-16. Gas production measured as the total volume per fermentation vessel was about equal with GOS, LOS and FOS as substrates. Carbon dioxide, a primary fermentation metabolite, was the major gas product in these *in vitro* fermentations, being evolved in 1000-fold and 100- fold higher amounts than hydrogen and hydrogen disulphide, respectively. Methane, a gas normally produced in comparable studies using adult feces, was not produced in these infant fecal fermentations, indicating age-related differences in GI microbiota composition. The more gradual nature of PDX fermentation was also in evidence in comparisons of total gas volumes. The rate of carbon dioxide production was slowest with PDX, followed by GOS. FOS and LOS were equal in the initial rate of carbon dioxide formation, but LOS showed a higher maximum at 8 hours. The maximum values for FOS were visible by 4 hours.

Figure 13. Total gas production following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



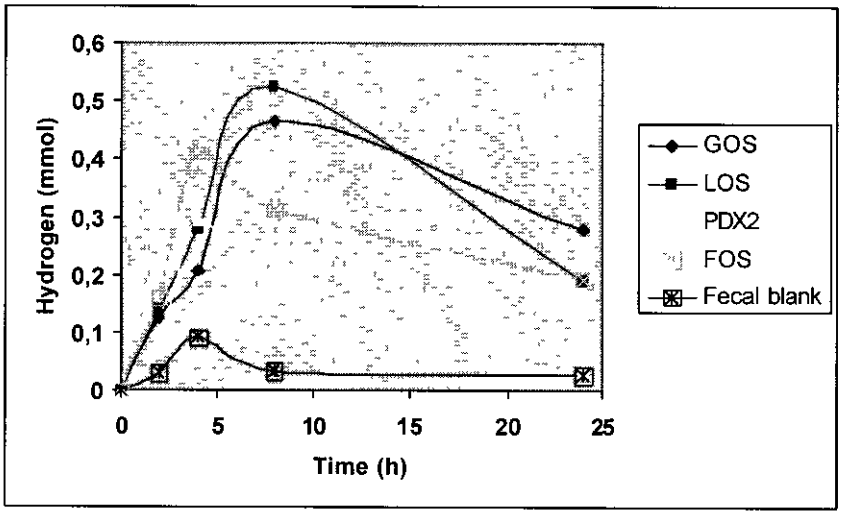
Key: GOS, galactooligosaccharides; LOS, lactulose; PDX-D, polydextrose from Danisco.

Figure 14. Carbon dioxide production following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



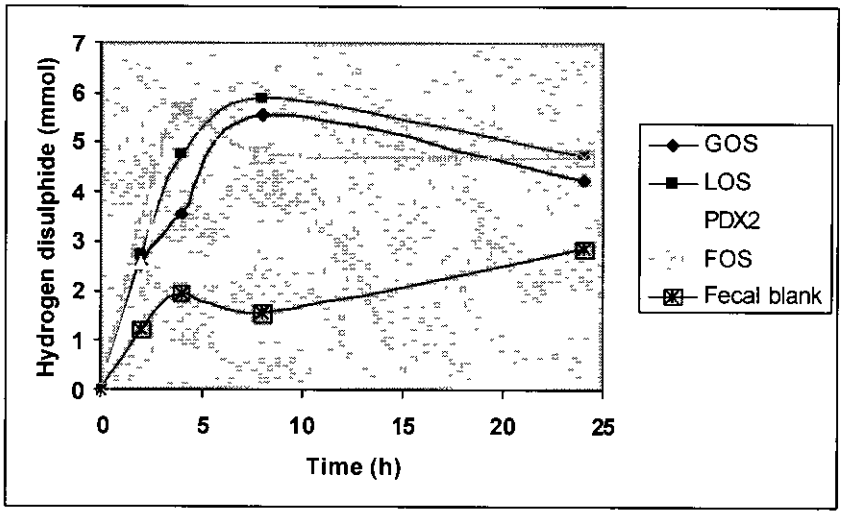
Key: GOS, galactooligosaccharides; LOS, lactulose; PDX2, polydextrose from Danisco.

Figure 15. Hydrogen production following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



Key: GOS, galactooligosaccharides; LOS, lactulose; PDX-D, polydextrose from Danisco.

Figure 16. Hydrogen disulphide production following fermentation of single prebiotic ingredients by infant fecal bacteria *in vitro*.



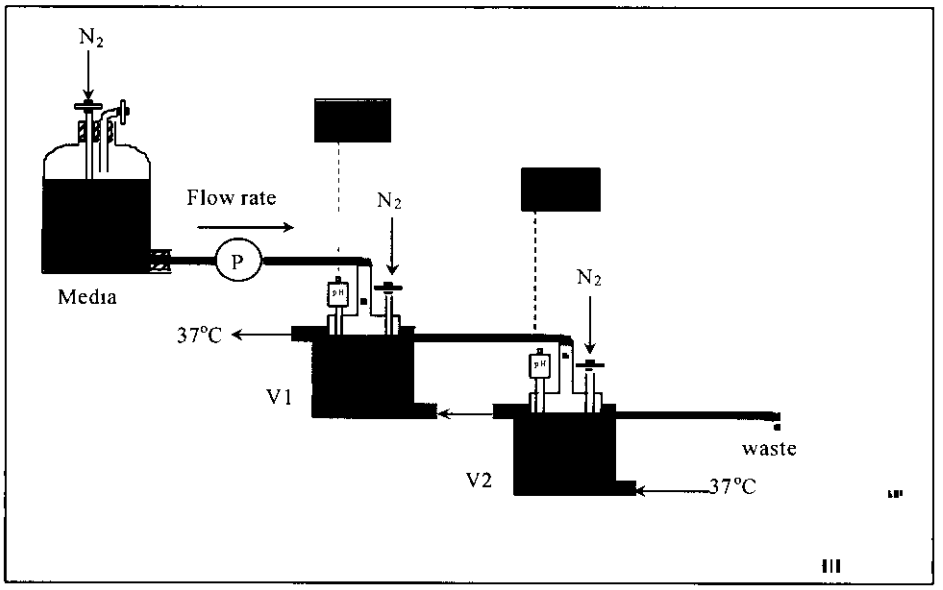
Key: GOS, galactooligosaccharides; LOS, lactulose; PDX2, polydextrose from Danisco

The primary focus of the VTT Biotechnology research was evaluation of the fermentation characteristics (pH reduction, production of SCFA and gas) of specific food-grade non-digestible carbohydrates using an *in vitro* model based on 24-hour batch fermentations by the fecal microbes of healthy infants. The non-digestible carbohydrates evaluated singly or in combination included GOS, FOS, LOS, and two PDX preparations obtained from either A. E. Staley or Danisco. GOS, LOS and FOS were rapidly fermented as indicated by all fermentation parameters—SCFA production, pH reduction, gas evolution and sugar utilization (data not shown). As expected, PDX was fermented more slowly, most likely due to its higher degree of polymerization and more complex structure. Blends of PDX and the other carbohydrates displayed intermediate fermentation characteristics suggesting that such a combination would deliver a more gentle and sustained fermentation profile when added to infant formula. Additionally, the production of specific short-chain fatty acids depended in part upon the identity of the prebiotic carbohydrate (e.g., PDX yielded the most propionate) suggesting that the various carbohydrates were metabolized by different constituents of the infant microbiota. The data suggests that blends of PDX and the other prebiotic carbohydrates would be more likely stimulate fermentation by a broader array of GI bacteria resulting in greater SCFA production, including propionate, and reduced pH, conditions that are considered unfavorable for enteric pathogens like *Salmonellla*.

University of Reading Studies

Various non-digestible carbohydrates were tested for prebiotic activity using another *in vitro* gut model developed by Gibson and Rastall at the University of Reading (United Kingdom). The infant gut model used by these investigators consisted of a 2-vessel, continuous-flow system designed to simulate the intestinal tract of the human infant. The infant model was adapted from a more extensively utilized adult gut model, a three-vessel fermentation system designed to mimic the adult colon, which has been validated against gut contents from sudden death victims (i.e., similar bacterial composition and activities at different regions of the colon). The infant gut model consists of two 100 mL glass vessels arranged in series, inoculated with infant fecal samples and maintained under specific conditions (pH, temperature, and flow rate) to represent the proximal (Vessel 1 (V1); pH 5.2) and distal (Vessel 2 (V2); pH 6.7) regions of the infant colon (see schematic below). A minimal growth medium supplemented with infant formula was continuously fed into V1 at a defined rate, which then fed into V2, with the overflow being collected as waste material. The feed and culture vessels were magnetically stirred and maintained under anaerobic conditions following inoculation with a fecal slurry obtained fresh from infants aged 2-4 months, breast fed, with or without formula supplementation, and carefully prepared under anaerobic conditions.

Figure 17. Schematic of the *in vitro* infant gut fermentation model (Gibson, 2002)



Following inoculation with the fecal slurry, the two-stage infant gut model was allowed to equilibrate for 6 days, after which samples were obtained from both vessels. After the equilibration period, PDX, GOS, FOS, LOS, or specific combinations, were added to the formula feed (7.5 g/L) and after a second 6 day equilibration period, samples were again obtained on day 12. An additional reference experiment was conducted using diluted human milk as the fermentation substrate. Microbiological evaluations of each sample were conducted using fluorescence *in situ* hybridisation (FISH) and probes for specific constituents of the infant microbiota (*Lactobacillus, Bifidobacterium, Bacteroides* and *Clostridium*), and prebiotic potential was calculated based on relative changes in their populations.

The results of 12 separate experiments are summarized in Table 2. Values followed by a superscript (+) sign are indicative of an overall positive effect (e.g. increase in bifidobacteria or lactobacilli, decrease in clostridia or *Bacteroides*) while values followed by a superscript (–) sign are indicative of an overall negative effect (opposite of above). As expected, the addition of human milk to the feed system of the *in vitro* gut model led to increases in the numbers of beneficial bacteria, both bifidobacteria and lactobacilli, and decreased levels of clostridia in both vessels 1 and 2. *Bacteroides* numbers remained at a similar level throughout the fermentation cycles. The addition to the formula feed of FOS, traditionally considered a good prebiotic ingredient, resulted in increases in bifidobacteria and clostridia with decreases in levels of lactobacilli and *Bacteroides* in vessel 1, but led to no changes in bifidobacteria and lactobacilli levels and increases in clostridia and *Bacteroides* in vessel 2. The addition of GOS to the formula feed increased bifidobacteria in

both V1 and V2, decreased clostridia and *Bacteroides* in V1 but not V2, and had little apparent effect on lactobacilli in either vessel. The addition of PDX produced favorable results, with marked increases in lactobacilli and decreases in both clostridia and *Bacteroides* evident in both vessels (Table 2).

Various combinations of test prebiotics were also evaluated in the *in vitro* infant gut system. While results frequently varied among experiments (likely due to differences in fecal inocula from different infants), largely favorable results were seen with GOS, PDX, and a 1:1 mixture of GOS and PDX. The PDX:GOS mixture increased levels of bifidobacteria and lactobacilli in both vessels, but also caused increased levels of clostridia (V1 and V2) and *Bacteroides* (V2 only). The LOS:GOS combination was effective in increasing numbers of bifidobacteria and lactobacilli, but had opposing effects on clostridia and *Bacteroides*, depending upon the vessel.

In general, bifidobacteria increased in proportion to the total bacterial population in vessel 1 (simulating conditions in the proximal colon) with human milk, GOS, FOS, PDX and the PDX:GOS combination. In vessel 2 (simulating the distal colon), human milk, GOS, the PDX:GOS combination and the LOS:GOS combination each led to an increase in bifidobacteria levels. Clostridia decreased in proportion to the total bacterial population with human milk, GOS and PDX, and the LOS:GOS combination (V2 only).

Table 2. Summary of Prebiotic Effects on Infant Fecal Microbiota

Test Prebiotic	Log_{10} change with prebiotic (from day 6 to day 12)							
	Bifidobacteria		Lactobacilli		Clostridia		Bacteroides	
	V1	V2	V1	V2	V1	V2	V1	V2
Human Milk	1 34^{+}	2 13^{+}	1 66^{+}	1 87^{+}	-1 00^{+}	-2 55^{+}	0 09	-0 12
FOS	1 55^{+}	0 05	-1 26^{-}	0 26	1 14^{-}	0 71^{-}	-0 48^{+}	0 97^{-}
LOS	-0 50^{-}	-0 28	0 70^{+}	1 19^{+}	0 11	1 22^{-}	-0 59^{+}	0 20
GOS	0 33^{+}	0 75^{+}	0 00	0 00	-1 31^{-}	-0 15	-0 85^{+}	0 03
PDX	0 23	-0 17	1 35^{+}	0 55^{+}	-1 79^{+}	-0 88^{+}	-1 64^{+}	-1 79^{+}
1 1 PDX GOS	0 58^{+}	0 47^{+}	1 21^{+}	0 81^{+}	0 91^{-}	0 43^{-}	0 00	0 74^{-}
1 1 PDX LOS	-0 32	-0 33	0 57^{+}	-0 25	-0 22	-0 10	0 12	-0 41
1 1 LOS GOS	0 14	0 53^{+}	0 21	1 53^{+}	0 48^{-}	-1 63^{+}	-1 60^{+}	2 04^{-}

Note Vessel 1 (V1) fermentation at pH 5 2; Vessel 2 (V2) fermentation at pH 6 7

Overall, the most favorable microbiological results (i.e. increased bifidobacteria and lactobacilli and decreased clostridia) were observed in the human milk trial. It would be difficult to attribute these effects to a specific constituent of human milk such as the oligosaccharides as numerous bioactives are likely to have been present in significant quantities (e.g., lactoferrin, lysozyme). In comparison to human milk, the various prebiotic ingredients displayed more modest and selective effects. As an example, PDX and GOS were most inhibitory of clostridial growth when added individually, while the blend of the two proved most effective at stimulating the growth of the beneficial bacteria bifidobacteria and lactobacilli. Together, these results provide support for the hypothesis that PDX and GOS may have the capacity to substitute for some of the physiological properties of human milk oligsaccharides.

C. Conclusions from Preclinical Testing

The goal of including prebiotic carbohydrates in infant formula is the delivery of some of the functional properties attributed to human milk oligosaccharides (improved microbial balance, SCFA production, stool characteristics, etc.) The *in vitro* studies described above demonstrate that larger, more complex carbohydrates like PDX are fermented more slowly and less completely than short-chain materials such as GOS and FOS. The combination of specific long-chain (PDX) and short-chain (GOS) carbohydrates thus allows for a slower, more controlled fermentation by fecal bacteria, a process that may translate into a more gentle and sustained effect during gastrointestinal transit. Some variation in the effects of these carbohydrates on the gastrointestinal microbiota was encountered using the *in vitro* models, most likely due to variability in the bacterial content of the fecal inocula derived from different infants. However, favorable results were obtained with PDX and GOS, both individually and in combination. Collectively, the results from *in vitro* screening of prebiotic carbohydrates were most promising for GOS and PDX, which led to the decision to evaluate them further in clinical trials.

The decision to select a combination of PDX and GOS as prebiotic carbohydrates for infant formula supplementation is consistent with both preclinical and clinical results generated in other laboratories. Experimental trials involving healthy adults have indicated that GOS preparations are completely fermented in the GI tract and slightly increase lactobacilli (Alles et al., 1999) and either stimulate (Bouhnik et al., 1997) or have no effect (Alles et al., 1999) on bifidobacteria populations. *In vitro* studies have demonstrated that GOS is fermented by a range of human and dairy bifidobacteria (Vernazza et al., 2006) and is capable of blocking the adherence of enteropathogenic *E. coli* (EPEC) to the HEp-2 and Caco-2 intestinal cell lines (Shoaf et al., 2006). GOS has also been shown to enhance the colonization of exogenous bifidobacteria in gnotobiotic mice (Morishita et al., 2002) but not in humans (Alander et al., 2001).

The microbiological and/or metabolic effects attributed to PDX fermentation have varied depending upon experimental factors including target bacteria, nutrient composition,

complexity of the *in vitro* model, and test animal. In the single culture, simplified media system employed by Vernazza et al.(2006), PDX was poorly utilized by five strains of *Bifidobacteria*. In contrast, the addition of PDX at 1% or 2% w/v in a continuous three-stage human colon model inoculated with an adult fecal slurry stimulated the growth of a wide variety of bifidobacteria, including *Bifidobacterium infantis* (Probert et al., 2004). Similar to the results reported for the *in vitro* model, a positive impact of PDX on the balance of colonic microbiota has been demonstrated in adult humans (Jie et al., 2000). In this placebo-controlled, randomized, double-blind study, 120 healthy subjects consumed 0, 4, 8 or 12 g/day of PDX for 28 days. Fecal populations of bifidobacteria and lactobacilli increased significantly in a dose-dependent manner while *Bacteroides* declined. Fecal concentrations of acetate and butyrate increased significantly in subjects that consumed 8 and 12 g/day PDX. PDX also promoted the growth of normal cecal epithelial cells (calculated as whole-crypt labeling index following [^{3}H]thymidine pulse labeling of 3 biopsies of normal cecal mucosa per subject). The subjects who consumed PDX reported improved bowel function (increased frequency and ease of defecation) without laxation problems (i.e., cramps, diarrhea). Favorable tolerance of PDX relative to laxation endpoints was generally reported in a number of clinical trials conducted in human adults and children that were discussed in a recent review article (Flood et al., 2004).

A combination of a four-stage colon model (simulating ascending, transverse, descending and sigmoid regions) and a cell-culture based human intestinal epithelial function model were employed to evaluate the impact of PDX on both intestinal microbiota and select mucosal functions related to colon cancer (Mäkivuokko et al., 2005). The authors reported that PDX supported sustained fermentation throughout the system, increasing the production of all major short-chain fatty acids in a dose-dependent manner without increasing putrefactive factors (i.e., biogenic amines and branched-chain amino acids) or increasing the density of the microbiota. The beneficial effects of PDX were further supported by the observation that the application of culture effluent also normalized COX gene expression in the colon cancer cell line in a dose dependent manner. A later study utilizing the same four-stage adult colon model confirmed both the sustained degradation of PDX (estimated 80% reduction during 48 hours) and the resulting stimulation of short chain fatty acid production (Mäkeläinen et al., 2007). The authors reported that in comparison to a control, the addition of 2% PDX increased the production of acetate (30.6 ± 3.1 to 71.3 ± 7.1 mM), propionate (8.4 ± 1.2 to 21.1 ± 3.7 mM), butyrate (10.3 ± 0.8 to 22.1 ± 1.3 mM) and total SCFA (49.3 ± 3.9 to 114.4 ± 10.4 mM) during 48 hours without affecting branched-chain amino acid concentration. A shift from putrefactive to saccharolytic metabolism has also been demonstrated in a rodent model (Peuranen et al., 2004), where the authors reported that while PDX shifted the population of cecal bacteria (as measured by changes in the guanine + cytosine percentage), a combination of PDX and lactitol was much more effective. Similarly, the combination more effectively reduced the production of biogenic amines and branched, volatile fatty acids and increased the luminal secretion of IgA in the cecum. Interestingly, an additive effect of PDX and lactitol on fermentation in the GI tract has also been demonstrated in humans where breath hydrogen values were found to increase 2-fold over their anticipated individual contributions (Livesey et al., 1993).

A recent report has indicated that when fed to 67-day-old pigs at a rate of 30 g/day, PDX is gradually fermented throughout the intestinal tract, starting at 53 mg/g digesta in the distal small intestine and declining to 35 mg/g digesta in the distal colon (Fava et al., 2007). Though PDX had little impact on the resident microbiota, it appeared to induce changes in both SCFA and biogenic amine profiles and to decrease mucosal COX-2 gene expression. Based on the total reduction during intestinal transit, it is estimated that 34% of the PDX ingested by pigs is fermented. This finding is in rough agreement with the [^{14}C]polydextrose utilization studies in both rats (Figdor and Rennhard, 1981) and humans (Figdor and Bianchine, 1983), where approximately 60% of the material was excreted (leaving 40% fermented). These estimates of PDX fermentation fall within the broad range (3-60%) that has been calculated for human milk oligosaccharide digestion in infants (Chaturvedi et al., 2001[b] and Coppa et al., 2001, respectively). Though they are not fermented at the same rate or to the same extent, both PDX and GOS are actively metabolized by constituents of the gastrointestinal microbiota and are capable of positively influencing the colonic environment through the production of short-chain fatty acids and other metabolites. By combining PDX and GOX, non-digestible carbohydrates displaying different, but complimentary fermentation and metabolic profiles, a gentler and more sustained prebiotic effect should be delivered by infant formula. In addition, the PDX:GOS blend delivers a broader range of molecular structures (predominantly 2-30 carbohydrate linkage units) than either material alone, that encompasses the range reported for HMO (DP of 3-32; Morrow et al., 2005).

V. Potential Impact of Prebiotic Ingredients on Bacterial Translocation

The individual safety of GOS and PDX is discussed in detail in the specific monographs included in this GRAS notice. However, one aspect of the safety of adding prebiotic ingredients to infant formulas that could benefit from further discussion is the alleged potential for increased translocation of pathogenic microorganisms. Several recent publications have indicated that the consumption of certain prebiotic oligosaccharides, fructooligosaccharides (FOS), inulin, and lactulose, can increase the translocation of *Salmonella* in rats. None of these findings implicates either GOS or PDX. Further, these findings, all from a single research group, conflict with a large body of research indicating that the consumption of prebiotic materials and subsequent fermentation by colonic bacteria tends to inhibit the proliferation and translocation of pathogenic bacteria through the production of lactic acid and short-chain fatty acids.

Bovee-Oudenhoven et al. (1997) reported on a study in which specific pathogen-free male Wistar rats were fed one of three purified diets including: 1) low calcium control (LCa) at 20 mmol/kg; 2) low calcium supplemented with 10% lactulose (LCa/Lact); or 3) high calcium (180 mmol/kg) supplemented with 10% lactulose (HCa/Lact). After 10 days, the animals were orally infected with 4×10^8 colony-forming units (CFU) of the pathogen

Salmonella enteritidis, whose virulence was maintained through routine passage through Wistar rats and subsequent recovery of the translocated bacteria from liver or spleen cells. After infection, total 24-hour urine was collected and assayed for total NO_x (nitrate and nitrite), a possible biomarker for inflammation (including but not exclusively that caused by bacterial translocation), as well as fecal pH, and total fecal *Salmonella* (by standard dilution plating on Brilliant Green Agar). In addition, fecal water (an aqueous extract of lyophilized feces) was evaluated for cytotoxicity against erythrocytes.

On the first day after infection, rats fed lactulose excreted 1000-fold less *Salmonella* in feces than the controls. This was interpreted as greater colonization resistance, although there were no statistically significant differences on any day thereafter. The authors reported that although lactulose did not significantly increase bacterial translocation, both low calcium groups displayed a rapid and progressive increase in daily urine NO_x, suggesting that a lack of buffering capacity, and not prebiotic fermentation *per se*, was the important factor. In contrast, lactulose consumption did increase the cytotoxicity of fecal water (arbitrarily set at 100%) relative to either the low calcium control (60%, estimated) and high calcium (30%, estimated) groups. The fact that cytotoxicity was measurably higher in the low calcium control (without lactulose) than the high calcium (with lactulose) group, suggests that the lack of buffering capacity is more biologically relevant than the presence of excess substrate.

The same laboratory evaluated the potential impact of short-chain fructooligosaccharide (FOS) on *Salmonella* translocation in rats (Ten Bruggencate et al. 2003). Again, male Wistar rats were fed restricted quantities of a low calcium diet (control), or the control diet supplemented with either 3% or 6% FOS. Following oral infection with 1.7×10^{10} CFU *Salmonella enteritidis*, fecal samples were collected and assayed for bifidobacteria (via quantitative PCR), lactobacilli, enterobacteria and *Salmonella* (via dilution plating on selective and nonselective microbiological media). The cytotoxicity of fecal water as well as urine NO_x were measured as described above as biological indicators of mucosal irritation and *Salmonella* translocation, respectively. *Salmonella* populations were also measured in both cecal contents and mucosa.

Prior to infection, 6% FOS increased fecal bifidobacteria, neither concentration affected lactobacilli, and both 3% and 6% FOS increased enterobacteria 100-fold. The cytotoxicity of fecal water was significantly increased by both FOS concentrations prior to infection, though not in a dose-dependent manner, suggesting that the buffering capacity of the low calcium diet was exhausted at 3% FOS. This is particularly relevant as the authors reported that at 6% FOS total lactic acid production in cecal contents was elevated 10-fold (76.4 μmol) over that produced at 3% FOS (7.7 μmol) and this excess could have led to mucosal irritation. The authors also reported that by 9 days post infection, the *Salmonella* populations in the cecal contents and mucosa increased in a dose-dependent manner with 3% and 6% FOS in the former and at 6% FOS in the latter. These findings are problematic for several reasons. As cecal bacteria are typically carried through in fecal material and

enterobacteria were reportedly increased in feces, it is difficult to see how the *Salmonella* estimates were generated separately due to the fact that the genus is also considered to be among the enterobacteria. Simple enumeration on Brilliant Green Agar and EMB are unlikely to completely resolve the populations.

A follow up study was published by the same group in which they evaluated the capacity of lactulose, FOS, resistant starch, cellulose and wheat fiber to influence colonization and translocation of *S enteritidis* populations in rats (Bovee-Oudenhoven et al., 2003). Again, specific pathogen-free male Wistar rats were maintained for two weeks on a low calcium (20 mmol/kg) basal diet amended to contain 4% of cellulose, wheat fiber, resistant starch, FOS, or lactulose, after which they were orally infected with $1x10^9$ CFU *S. enteritidis*. Growth was measured, as were fecal bacteria and lactic acid, pH and cytotoxicity of fecal water, and NO_x in total urine collected over 24 hours. In a second infection experiment, $1x10^8$ *S enteritidis* were given orally and translocation was measured by direct enumeration from spleen, mesenteric lymph nodes, and liver.

Resistant starch, FOS, and lactulose were found to increase lactobacilli, bifidobacteria and enterobacteria to a greater extent than cellulose and wheat fiber prior to infection. This is reasonable as fewer bacteria are capable of metabolizing the latter two and this was reflected in fecal lactic acid concentrations, which reached 1.38, 1.52, 6.96, 0.57, and 0.45 µmol, respectively. In parallel, the cytotoxicity of fecal water was significantly higher in rats fed either FOS or lactulose prior to infection. This would seem to indicate that mucosal inflammation is simply driven by the production of lactic acid from the easily fermentable substrates, which is not unexpected in such a poorly buffered system. Rats consuming the more easily fermented lactulose and FOS also shed fewer *Salmonella* in feces, possibly due to direct inhibition by lactic acid and other metabolites. As expected, the production of urinary NO_x was also greater in rats consuming lactulose and FOS and this was interpreted as indicating greater bacterial translocation (Bovee-Oudenhoven et al., 2003). Direct enumeration revealed that *Salmonella* translocation occurred in mesenteric lymph nodes, spleen, and liver with cellulose as well as FOS and lactulose. This was to be expected as *S. enteritidis* is a known invasive pathogen and in this study was passaged through rats to maintain this translocation capacity. There was no significant difference between the groups in translocation to the mesenteric lymph nodes while that of FOS was slightly higher in the spleen. Only in the liver was translocation significantly higher in both the FOS and lactulose groups where the *Salmonella* counts reached 2.32 and 2.89 CFU/g relative to 1.55 CFU/g in the cellulose group. The authors interpreted these results as indicating impaired resistance to *Salmonella* infection but it is just as likely that the measurable increase in bacterial translocation was due to mucosal damage resulting from the production of excess acidic metabolites in the poorly buffered, low calcium system typically employed by these researchers.

This hypothesis is supported by the results of a subsequent study of *Salmonella* resistance in rats fed a diet containing FOS or inulin in which calcium was also present at either a low level (30 mmol $CaHPO_4 \bullet H_2O$/kg diet) or high level (100 mmol $CaHPO_4 \bullet H_2O$/kg diet) (Ten Bruggencate et al., 2004). Fecal concentrations of bifidobacteria, lactobacilli, enterobacteria and *Salmonella* were measured, as were urine NO_x, fecal lactic acid and fecal water cytotoxicity.

No effect of inulin or FOS on fecal bifidobacteria was observed. The growth of lactobacilli was stimulated by inulin, FOS, and higher calcium. The addition of inulin and FOS increased enterobacteria populations but only in rats fed the poorly buffered low calcium diet. The lack of FOS- and inulin-stimulated growth of enterobacteria in rats fed the high calcium diet could be attributed to increased lactic acid production under the higher buffering conditions. The measurable fecal water cytotoxicity associated with inulin and FOS consumption were much higher in rats fed the low calcium diet (85% and 78% estimated, respectively) than the high calcium diet (35% and 5% estimated, respectively), further strengthening the association of low buffering capacity and the development of mucosal irritation.

In an additional rat study, Ten Bruggencate et al. (2005) attempted to define a mechanism of bacterial translocation in rats following the consumption of 6% FOS and oral *Salmonella* infection ($2x10^8$ CFU). As in previous experiments, the authors utilized a limited quantity, low calcium (30 mmol $CaHPO_4 \bullet H_2O$/kg) diet but added chromium EDTA (CrEDTA) as a potential measure of intestinal permeability.

Prior to infection, the consumption of FOS was associated with significant increases in fecal bifidobacteria, lactobacilli, and *Escherichia coli* (a commonly encountered enterobacteria) relative to a cellulose-containing control diet. As expected, both lactate concentration and fecal water cytotoxicity were also increased in the FOS fed group. The authors reported that following *Salmonella* infection, both intestinal permeability (urinary excretion of CrEDTA) and *Salmonella* translocation (urinary NO_x) were increased in rats fed FOS. Unfortunately, a normal calcium control group was not included to account for the potential impact of limited buffering capacity

A complementary study was conducted in humans to determine if the consumption of dietary FOS could increase intestinal permeability (Ten Bruggencate et al., 2006). In a double-blind, placebo-controlled, crossover study of 2 x 2 weeks (with a washout period of 2 weeks), 34 healthy men consumed 20 g FOS or placebo along with CrEDTA as a marker of intestinal permeability. FOS consumption induced increases in both bifidobacteria and lactobacilli but not *E. coli*. Even though the authors employed a low calcium diet (~300 mg/day) instead of a normal calcium diet (1000-1200 mg/day), fecal water cytotoxicity and large intestine permeability (CrEDTA excretion) were not affected by prebiotic fermentation, although a significant increase in fecal lactic acid was noted. Despite the lack of compelling

evidence, the authors suggested that FOS consumption increased mucosal irritation (based solely on increased mucin production), though moderately in comparison to rats.

This suggestion has been directly challenged by Scholtens et al. (2006), who conducted another study in humans to evaluate the effect of FOS on fecal cytotoxicity. In a randomized, double-blind, placebo controlled, crossover trial, a total of twelve subjects (6 men and 6 women) consumed 25-30 grams of Raftilose 95 FOS (experimental group) or maltodexrin (control group) per day for two weeks. After each intervention period, measurements were taken of fecal pH, SCFA, and lactate, the cytotoxicity of fecal water against human erythrocytes and fecal alkaline phosphatase activity, both considered measures of intestinal epitheliosysis, and fecal O-linked oligosaccharides (a possible indicator of mucosal irritation). Fecal pH was lower and lactate was higher following FOS consumption indicating an increase in fermentation by gastrointestinal (GI) microbiota. In contrast to the collective results published by the Bovee-Oudenhoven group, FOS consumption tended to reduce fecal water cytotoxicity relative to the control period (37.5 (SEM 6.9) versus 18 5 (SEM 6.9)). No statistically significant changes were noted in either mean fecal alkaline phosphatase activity or O-linked oligosaccharide (mucin) production. As a result, the authors concluded that the consumption of up to 25-30 g/d FOS altered the bacterial fermentation profile but did not affect fecal cytotoxicity or the fecal concentration of mucin-type oligosaccharides in human adults when consuming a regular diet (e.g. unrestricted without calcium limitation) and thus no alteration in intestinal permeability is suggested.

A summary of the research investigating the possible association between the consumption of specific prebiotic carbohydrates (i.e. FOS, inulin and lactulose) and decreased resistance to *Salmonella enteritidis* infection in rats, as well as the possible linkage of that work to intestinal barrier function in humans, is presented below through an analysis of several key points:

1. In the studies in which an association with prebiotic consumption and *S enteritidis* translocation was postulated, food consumption was frequently restricted and dietary calcium was always reduced to a low level, which most likely led to a loss in buffering capacity. The various prebiotic materials (FOS, inulin and lactulose) were added at fairly high concentrations (3%, 6%) and fermented by the cecal and/or colonic microbiota (bifidobacteria, lactobacilli, enterobacteria, etc.) and lactic acid and short chain fatty acids (SCFA) were generated by the process. This combination of acidic end products and low buffering capacity is the most likely explanation for the increase in fecal water cytotoxicity (described as a biomarker for mucosal irritation) so often cited in the studies. This hypothesis is supported by the observation that the addition of a higher level of calcium reduced or eliminated this phenomenon. This would not be problematic for the intended use of PDX and GOS, as infant formula contains appreciable amounts of calcium.

2. Measurable urine NO_x excretion following infection was described as a sensitive indicator of intestinal bacterial translocation though it is typically thought of as a measure of general inflammation. Increases in total urine NO_x (NO_2 and NO_3) were demonstrated in rats consuming the various prebiotic materials, apparently varying directly with dosage and ease of fermentation. As *S.enteritidis* is a known invasive and intracellular pathogen, it is expected to translocate at some frequency in sensitive hosts and this was supported in the few studies in which organ populations were actually measured. *S. enteritidis* was found to translocate at a basal frequency in the absence of prebiotics, suggesting that it is a generalized trait. In fact, the authors frequently described maintaining the pathogenicity of the strain by routinely infecting rats and recovering it from organ tissues. Rather than attributing the increase in urine NO_x to increased colonization and translocation, driven by prebiotic-associated intestinal permeability, a simpler explanation could suffice. Excess production of acidic metabolites in such a poorly buffered, low calcium environment could have caused minor mucosal damage and induced inflammation directly.
3. Among the studies that detected an association between prebiotic consumption and increased fecal water toxicity, all were carried out using a rat model. This could constitute a confounding variable if some species bias exists in this physiological response. This possibility is not completely without merit as both human studies reported above failed to establish a similar linkage.
4. Additional studies on the effects of prebiotic fibers on inflammation, intestinal barrier function, bacterial translocation and infection have come to opposing conclusions.
    a. The addition of dietary transgalactosylated oligosaccharides (GOS) has been shown to strengthen the anti-infectious activity of *Bifidobacterium breve* in a synbiotic manner in mice artificially infected with *Salmonella typhimurium* (Asahara et al. 2001).
    b. The efficacy of two fermentable fibers (soy polysaccharides (SPS) and FOS in reducing *Salmonella typhimurium* induced diarrheal illness has been investigated using a neonatal piglet model (Correa-Matos et al. 2003). The consumption of 7.5 g/l of SPS and FOS by 7-day-old piglets was found to increase the cecal production of SCFA, reduce recovery time, and improve infection-associated symptoms following oral infection with 10^{10} CFU *S. typhimurium*.
    c. An investigation of gut barrier function and sepsis in critically ill patients found that the consumption of a synbiotic product containing FOS and a mixture of four probiotic lactic acid bacteria had an antimicrobial effect. Though no impact on intestinal permeability or systemic inflammatory response was observed, the synbiotic product significantly reduced potentially

pathogenic bacteria and bacterial diversity in nasogastric aspirates collected from the upper gastrointestinal tract (Jain et al. 2004).

d. Another study found that the daily oral administration of *Bifidobacteria* ($3x10^6$ CFU/animal) and a combination of FOS and inulin (0.5 g/animal) had an anti-inflammatory effect in a rat model of acute colitis (Osman et al. 2006). Bacterial translocation to mesenteric lymph nodes was also decreased significantly while cecal short chain fatty acids were increased.

e. Lastly, in a comparable study conducted to evaluate the effects of FOS on disease activity and colonic damage in the distal colon of mice in which colitis was induced by sodium dextran sulphate (Winkler et al. 2006), FOS treatment significantly reduced disease activity and damage and improved recovery of crypt depth and area.

Though a small number of publications have suggested that the addition of high levels of certain prebiotic oligosaccharides (i.e. FOS, inulin and lactulose) to the diet can increase the translocation of *Salmonella* in rats, none of these findings implicates either GOS or PDX. The studies in question, all from a single research group, reported an association between the consumption of fermentable prebiotics and elevated biomarkers of mucosal irritation and/or translocation, but only in conjunction with a poorly-buffered, low calcium diet. As infant formula contains appreciable quantities of calcium, a lack of buffering capacity is not expected to influence mucosal physiology. These findings conflict with a large body of research indicating that the consumption of prebiotic materials and subsequent fermentation by colonic bacteria tends to inhibit the proliferation and translocation of pathogenic bacteria through the production of lactic acid and short-chain fatty acids. In summary, there is nothing in the research literature that suggests a likelihood that the addition of GOS and PDX to infant formula will result in increase *Salmonella* infection or in the increased translocation of any other colonic pathogen.

VI. Prebiotic Ingredients in Current Infant Formula

A. Marketed Formulas with Prebiotic Carbohydrates

Globally, infant formula manufacturers now add non-digestible prebiotic oligosaccharides to many of their products. It has been stated that, "The addition of certain mixtures of oligosaccharides in infant formula bring infant formula, the second choice infant feeding, one step closer to the gold standard, breast-feeding" (Vandenplas, 2002). Numerous formulas supplemented with prebiotic oligosaccharides have been introduced in Europe and Asia in the past 5 years and are currently in use (Table 3). Although prebiotic-supplemented infant formula is a relatively new concept in the United States, these non-digestible

carbohydrates have been added to infant formula in Japan for over 20 years. According to Ghisolfi (2003), 90% of infant formulas in Japan currently contain prebiotics. The introduction of prebiotics to formula in Europe has been more recent, occurring within the last decade (Ghisolfi 2003).

Table 3. Marketed Infant Formulas Containing Prebiotic Ingredients

Country	Manufacturer	Brand	Ingredients
Belgium	Nutricia	Nutrilon 1 - 2 – 3	Galctooligosaccharides + Fructooligosaccharides
Belgium	Nutricia	Nutrilon HA1 – 2	Galctooligosaccharides + Fructooligosaccharides
Belgium	Nutricia	Nutrilon Omneo 1 – 2	Galctooligosaccharides + Fructooligosaccharides
China	Numico	Cow & Gate Follow-On	Oligolactose
China	Numico	Cow & Gate Starter	Oligolactose
China	Numico (Nutricia)	Daily Follow-On	Oligosaccharides
China	Numico (Nutricia)	Daily Starter	Oligosaccharides
China	Dumex	Follow-On Dumex Gold	Oligolactose
China	East Asiatic Co (Dumex)	Follow-On Dumex Reg	Oligosaccharides
China	Meiji	Meiji Follow-On	Oligosaccharides
China	Meiji	Meiji Starter	Oligosaccharides
China	Dumex	Starter Dumex Gold	Oligolactose
China	East Asiatic Co (Dumex)	Starter Dumex Reg	Oligosaccharides
China	Weichung	Wei Chuan U+ Follow-On	Oligolactose
China	Weichung	Wei Chuan U+ Starter	Oligolactose
France	Milupa	Conformil 1er âge	Galactooligosaccharides, Polyfructose
France	Milupa	Conformil 2ème âge	Galactooligosaccharides, Polyfructose
France	Milupa	Milumel Premium 1er âge	Galactooligosaccharides, Polyfructose
France	Milupa	Milumel Premium 2ème âge	Galactooligosaccharides, Polyfructose
France	Nutricia	Nutricia Confort Plus 1er âge	Galactooligosaccharides, Polyfructose
France	Nutricia	Nutricia Confort Plus 2ème âge	Galactooligosaccharides, Polyfructose
Germany	Milupa	Milumil Pre	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Milumil 1	Galactooligosaccharides & Fructooligosaccharides

Country	Manufacturer	Brand	Ingredients
Germany	Milupa	Milumil HA 1	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Milumil 2	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Milumil HA 2	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Milumil 3	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Aptamil Pre	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Aptamil 1	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Aptamil HA 1	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Aptamil 2	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Aptamil HA 2	Galactooligosaccharides & Fructooligosaccharides
Germany	Milupa	Aptamil 3	Galactooligosaccharides & Fructooligosaccharides
Germany	Humana	Humana 2	Galactooligosaccharides
Germany	Humana	Humana 2 baby fit banana	Galactooligosaccharides
Germany	Humana	Humana 3 apple	Galactooligosaccharides
Germany	Humana	Humana 3 banana-vanilla	Galactooligosaccharides
Germany	Humana	Humana HN	Galactooligosaccharides
Germany	Humana	Humana HN & Electrolyte	Galactooligosaccharides
Hong Kong	Nutricia	Cow & Gate 1	Fructooligosaccharides
Hong Kong	Nutricia	Cow & Gate 2	Fructooligosaccharides
Hong Kong	Nutricia	Cow & Gate Royal 1	Fructooligosaccharides
Hong Kong	Nutricia	Cow & Gate Royal 2	Fructooligosaccharides
Hong Kong	Maeil	Excelmil	Oligosaccharide
Hong Kong	Maeil	Excelcon	Oligosaccharide
Hong Kong	Friesland	Frisolac 1	Galactooligosaccharide
Hong Kong	Feiesland	Frisomel 2	Fructooligosaccharides
Hong Kong	Snow	Smart Baby 1	Oligosaccharide
Hong Kong	Snow	Smart Baby 2	Oligosaccharide
Hong Kong	Nestle	Nan 2	Bifidus and Thermophilus culture
Hong Kong	Nestle	Nan HA 2 (Gold)	Bifidus
Indonesia	Morinaga (Shanghy Perk)	Chil-Mil Platinum	Lactulose
Indonesia	Numico (Lyempf)	Bebelac 2	Fructooligosaccharides
Indonesia	Numico (Lyempf)	Bebelac FL	Fructooligosaccharides

Country	Manufacturer	Brand	Ingredients
Indonesia	Morinaga (Shanghy Perk)	Chil-Mil Reg	Lactulose
Indonesia	Nestle	Lactogen 2	Fructooligosaccharides
Indonesia	Morinaga (Shanghy Perk)	Morignaga BMT Lactoferin	Lactulose
Indonesia	Morinaga	Morinaga BMT Platinum	Lactulosa
Indonesia	Numico (Sari Husada)	SGM 1	Fructooligosaccharides
Indonesia	Numico (Sari Husada)	SGM 2	Fructooligosaccharides
Indonesia	Numico (Sari Husada)	Vitalac 1	Fructooligosaccharides
Indonesia	Numico (Sari Husada)	Vitalac 2	Fructooligosaccharides
Italy	Milupa	Conformil 1 and 2	Galactooligosaccharides +Fructooligisacharides
Italy	Nutricia	Omneo 1 and 2	Galactooligosaccharides +Fructooligisacharides
Italy	Milupa	Aptamil 1, 2 and 3 w preb	Galactooligosaccharides +Fructooligisacharides
Italy	Nutricia	Nutrilon 1, 2 and 3 w preb	Galactooligosaccharides +Fructooligisacharides
Italy	Humana	Disanal CP	Galactooligosaccharides
Italy	Humana	Humana 1 LCP e Gos	Galactooligosaccharides
Italy	Plasmon (Heinz)	Plasmon Lenilac 1 and 2	Galactooligosaccharides
Italy	Dicofarm	Formulat 1	Fructooligisacharides
Italy	Mellin (Numico)	Mellin Progress 1 and 2	Galactooligosaccharides
Italy	Mellin (Numico)	Mellin Pantolac 1 and 2	Galactooligosaccharides
Korea	Pasteur	B&I No Sub 0-6mths	Galactooligosaccharide, Fructooligosaccharide, Galactosylactose
Korea	Namyang	Namyang Hope Allergy	Fructooligosaccharide
Korea	Ildong Foodis	True mom New Class 0-6mths	Lactulose, Galactooligosaccharide, Fructooligosaccaride, Galactosylactose
Korea	Pasteur	Organic	Galactooligosaccharide, Galactosylactose, Isomalto-oligosaccharides
Korea	Pasteur	Emerald	Lactulose, Galactosylactose, Galactooligosaccharide
Korea	Pasteur	Emerald Premium	Galactooligosaccharides, Galacotsylactose, Lactulose
Korea	Namyang	Namyang Soo	Galactosylactose
Korea	Namyang	Organic	Maltooligosaccharide
Korea	Namyang	Sanyang	Fructooligosaccharide, Galactosylactose

Country	Manufacturer	Brand	Ingredients
Korea	Namyang	Premium XO	Galactooligosaccharide, Galatosylactose, Lactulose
Korea	Ildong Foodis	Foodis S	Galactooligosaccharide Lactulose
Korea	Maeil	Premium Absolute	Galactooligosaccharide, Oligofructose
Korea	Maeil	Maeil IF	Galactooligosaccharide, Fructooligosaccharide
Malaysia	Morinaga	Chil-Mil FU	Lactulose
Malaysia	Dutch Lady	Frisolac 3 Follow-On	Fructooligosaccharide
Malaysia	Nestle	Lactogen 2	Inulin & Fructooligosaccharide
Malaysia	Meiji	Meiji FMT	Oligosaccharides
Malaysia	Meiji	Meiji FU	Oligosaccharides
Malaysia	Snow	Snow P7 Beta	Oligosaccharides
Malaysia	Snow	Snow F-Plus Beta	Oligosaccharides
Malaysia	Nutricia	Bebelac 1	Fructooligosaccharide
Malaysia	Nutricia	Bebelac 2	Fructooligosaccharide
Malaysia	Dumex	Mamex Gold Step1	Oligosaccharides
Malaysia	Dumex	Mamex Gold Step2	Oligosaccharides
Malaysia	Dumex	Mamex Routine Step 1	Oligosaccharides
Malaysia	Dumex	Mamex Routine Step 2	Oligosaccharides
Netherlands	Nutricia	Nutrilon 1 - 2 – 3	Galctooligosaccharides + Fructooligosaccharides
Netherlands	Nutricia	Nutrilon HA1 – 2	Galctooligosaccharides + Fructooligosaccharides
Netherlands	Nutricia	Nutrilon Omneo 1 – 2	Galctooligosaccharides + Fructooligosaccharides
Netherlands	Nutricia	Nutrilon Forte 1 – 2	Galctooligosaccharides + Fructooligosaccharides
Philippines	East Asiatic Co (Dumex)	Dupro 2 with DHA	Oligosaccharides
Philippines	Nestle	Nestogen 1 w/Prebio Plain	Oligofructose & Inulin
Philippines	Nestle	Nestogen 2 w/Iron DHA + Prebio	Oligofructose & Inulin
Philippines	Nestle	Nestogen 2 w/Prebio Plain	Oligofructose & Inulin
Philippines	East Asiatic Co (Dumex)	Dulac w/DHA	Oligossacharides
Poland	Nutricia	Bebiko Omneo 1	Oligosaccharides
Poland	Nutricia	Bebiko Omneo 2	Oligosaccharides
Poland	Nutricia	Bebilon 1	Oligosaccharides

Country	Manufacturer	Brand	Ingredients
Poland	Nutricia	Bebilon 2	Oligosaccharides
Poland	Nutricia	Bebilon 3	Oligosaccharides
Russia	Nutricia	Nutrilon 1	Fructooligosaccharides
Russia	Nutricia	Nutrilon 2	Fructooligosaccharides
Russia	Nutricia	Nutilon Omneo 1	Galctooligosaccharides + Fructooligosaccharides
Russia	Nutricia	Nutrilon Omneo 2	Galctooligosaccharides + Fructooligosaccharides
Russia	INC	Mamex plus	Fructooligosaccharides
Russia	INC	Mamex 2 plus	Fructooligosaccharides
Russia	INC	Mamex 2 plus Night formula	Fructooligosaccharides
Russia	Semper	Semper Bifidus	Lactulose
Russia	Letri de Craon	MD Mil Standart	Fructooligosaccharides
Russia	Letri de Craon	MD Mil Junior	Fructooligosaccharides
Russia	Frisland	Frisovom	Gluten of carob
Russia	Frisland	FrisoPre (Frosolac Premature)	Galactooligosaccharides
Russia	Humana	Humana HN +MCT	dietary fibers
Russia	Humana	Humana HN	Galactooligosaccharides, dietary fibers
Russia	Humana	Humana HA	Galactooligosaccharides
Russia	Humana	Humana Folgemilch 2	Galactooligosaccharides, dietary fibers
Russia	Humana	Humana Folgemilch 3	Galactooligosaccharides
Spain	Numico (Nutricia)	Almiron 1	Oligosaccharides
Spain	Numico (Nutricia)	Almiron 2	Oligosaccharides
Spain	Nutricia	Almirón Omneo 1	Fructooligosaccharides + Galactooligosaccharides
Spain	Nutricia	Almirón Omneo 2	Fructooligosaccharides + Galactooligosaccharides
Spain	Alter	Alter Nutriben 1 A E	Fructooligosaccharides
Spain	Alter	Alter Nutriben 2 A E	Fructooligosaccharides
Spain	Alter	Alter Simbiótico	Fructooligosaccharides
Spain	Numico (Milupa)	Aptamil 1	Oligosaccharides
Spain	Numico (Milupa)	Aptamil 2	Oligosaccharides
Spain	Numico (Milupa)	Aptamil 3	Oligosaccharides
Spain	Nutrexpa (Ordesa)	Blemil Plus Re 2	Oligosaccharides, Lactobacillus
Spain	Novartis (Sandoz)	Natur 2	Fructooligosaccharides
Spain	Alter	Nutriben Simbiotic	Oligosaccharides, Bifodobacterium, & Strep Thermophilus
Spain	Numico	Omneo 1	Oligosaccharides
Spain	Numico	Omneo 2	Oligosaccharides

Country	Manufacturer	Brand	Ingredients
Taiwan	Meiji	Meiji FP-T	Oligosaccharides
Taiwan	Morinaga	Morinaga BMT	Oligosaccharides
Taiwan	Morinaga	Morinaga Chil Mil	Lactulose
Taiwan	Snow	Snow F2 Beta Plus	Oligosaccharides
Taiwan	Meiji	Meihi Fu	Oligosaccharides
Taiwan	Snow	Snow A1 Beta Plus	Oligosaccharides
Taiwan	Dairy Goat	Karihome 1	Oligosaccharides
Taiwan	Dairy Goat	Karihome 2	Oligosaccharides
Thailand	Nestle	Bear 2 Prebio1	Fructooligosaccharides
Thailand	Dumex	Dumex HI-Q1	Oligosaccharides
Thailand	East Asiatic Co (Dumex)	Dulac	Oligosaccharides
Thailand	Dumex	Dumex HI-Q 2	Oligosaccharides
Thailand	East Asiatic Co (Dumex)	Dupro	Oligosaccharides
Thailand	Meiji	Meiji FMT	Oligosaccharides
Thailand	Meiji	Meiju FU	Oligosaccharides
Thailand	Nestle	Snow F Plus Beta	Oligosaccharides
Thailand	Snow	Snow P7L Reg	Oligosaccharides
UK	Cow & Gate (Numico)	Cow & Gate Premium (from birth whey based)	Galctooligosaccharides + Fructooligosaccharides
UK	Cow & Gate (Numico)	Cow & Gate Plus (from birth casein based)	Galctooligosaccharides + Fructooligosaccharides
UK	Cow & Gate (Numico)	Cow & Gate Step Up (from 6 months)	Galctooligosaccharides + Fructooligosaccharides
UK	Cow & Gate (Numico)	Cow & Gate Next Step (from 9 months)	Galctooligosaccharides + Fructooligosaccharides
UK	Cow & Gate (Numico)	Omneo Comfort 1 (from birth)	Galctooligosaccharides + Fructooligosaccharides
UK	Cow & Gate (Numico)	Omneo Comfort 2 (from 9 months)	Galctooligosaccharides + Fructooligosaccharides
UK	Milupa (Numico)	Aptamil First (from birth whey based)	Galctooligosaccharides + Fructooligosaccharides
UK	Milupa (Numico)	Aptamil Extra (from birth casein based)	Galctooligosaccharides + Fructooligosaccharides
UK	Milupa (Numico)	Aptamil Forward (from 6 months)	Galctooligosaccharides + Fructooligosaccharides
Vietnam	Meiji - Japan	Meiji FM-T	Oligosaccharides
Vietnam	Namyang - Korean	Star Science 1	Fructose-Oligosaccharides
Vietnam	Namyang - Korean	Star Science 2	Fructose-Oligosaccharides
Vietnam	Namyang - Korean	Star Science 3	Fructose-Oligosaccharides
Vietnam	Dumex - Denmark	Dulac 1	Oligosaccharides
Vietnam	Dumex - Denmark	Dupro 2	Oligosaccharides

Country	Manufacturer	Brand	Ingredients
Vietnam	Friesland Nutrition - Holland	Frisolac 1	Galactooligosaccharides
Vietnam	Friesland Nutrition - Holland	Friso 2	Galactooligosaccharides

B. Published Research Findings Related to Infant Formula Supplemented with Prebiotics

In order to gain a more thorough understanding of the current clinical findings relative to the supplementation of infant formula with prebiotic carbohydrates, an extensive literature search was conducted. These searches identified current research articles related to the clinical effects (i.e. intestinal microbiota, laxation, mineral absorption and immunity) of prebiotic carbohydrates (i.e. galactooligosaccharide, polydextrose, lactulose, inulin and fructooligosaccharide) on humans and other animals. When reviews, most abstracts, and research articles based on *in vitro* models, animal models, human children and adults were excluded, a subset of 35 relevant publications covering infant trials remained. Each of these articles was reviewed for information relative to manufacturer, prebiotic carbohydrate identity and concentration, experimental design and principal findings.Important information included in these articles is presented in abbreviated form in Table 4.

Table 4. Current References Related to Infant Clinical Trials Evaluating Formula Supplemented With Prebiotic Ingredients

Reference	Prebiotic/Level	Key Findings
Bakker-Zierikzee AM, Tol EA, Kroes H, Alles MS. Kok FJ, Bindels JG. 2006 Faecal SIgA secretion in infants fed on pre- or probiotic infant formula Pediatr Allergy Immunol. 17(2): 134-40	Numico, 9:1 GOS:FOS at 6 g/L or viable *Bifidobacterium animalis*	Trend toward higher fecal sIgA with prebiotics (relative to control, statistically significant only once) but not close to breast-fed values (10-fold higher at start) or profile (linear reduction over time), Study group characteristics (11% caesarean section. 54% infants born at home) very different from typical U.S. population, Post-hoc power analysis suggest 100 infants per group may be needed
Bakker-Zierikzee AM, Alles MS, Knol J, Kok FJ, Tolboom JJ, Bindels JG 2005 Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable *Bifidobacteria animalis* on the intestinal microbiota during the first 4 months of life Brit J. Nutr 94:783-790	Numico, 9.1 GOS:FOS at 6 g/L or viable *Bifidobacterium animalis*	Difference in bifidobacteria populations was not statistically significant at 16 weeks; SCFA, pH similar to breast fed reference group, Fecal acetate ratio and lactic acid concentration higher in prebiotic group
Ben X-M, Zhou X-Y, Zhao	Friesland; GOS	Bifidobacteria and Lactobacilli increased with prebiotic,

W-H, Yu W-L, Pan W, Zhang W-L, et al. 2004 Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-biota and fermentation in term infants Chin Med J (Engl) 117(6)· 927-931.		similar to breast fed reference group
Bettler J, Euler AR. 2006. An evaluation of the growth of term infants fed formula supplemented with fructo-oligosaccharide International Journal of Probiotics and Prebiotics, 1(1): 19-26	Wyeth; FOS at 1.5 and 3.0 g/L	Growth parameters were normal; Well tolerated and safe, Adverse Events were fully reported as not different among groups, additional blood chemistry as back up values
Boehm, G , Jelinek, J., Stahl, B , van Laere, K , Knol, J , Fanaro, S , Moro, G , and Vigi, V. 2004. Prebiotics in infant formula J Clin. Gastroenterol 38 (Supp. 2).S76-S79	Numico, 9:1 GOS FOS at 8 g/L	Fecal Bifidobacteria were reported as close to breast-fed reference, In vitro SCFA production from feces of infants receiving prebiotics was close to breast-fed reference
Boehm G, Fanaro S, Jelinek J, Stahl B, Marini A. 2003 Prebiotic concept for infant nutrition Acta Paediatr Suppl, 91(441)· 64-67	Numico; 9·1 GOS.FOS	Bifidobacteria closer to upper breast-fed range; stools like breast fed
Boehm, G., Lidestri, M., Casetta. P , Jelinek, J , Negretti, F , Stahl, B., and Marini, A., 2002 Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants Arch Dis Child. Fetal Neonatal Ed. 86.178-181.	Numico, 9 1 GOS FOS	Prebiotic increased Bifidobacteria in preterm infants (closer to breast-fed reference group; stool softness also increased by prebiotic,
Brunser O, Figueroa G, Gotteland M, Haschke-Becher E, Magliola C, Rochat F, et al 2006 Effects of probiotic or prebiotic supplemented milk formulas on fecal microbiota composition of infants. Asia Pac J Clin Nutr 15(3): 368-76.	Nestle, FOS or LA1 (probiotic)	No difference in URI or diarrhea, Bifidobacteria not different at end including breast-fed infants
Brunser O, Gotteland M,	Nestle; 7 3	Bifidobacteria increased by treatment; Diarrhea similar

Cruchet S, Figueroa G, Garrido D, Steenhout P, Effect of a milk formula with prebiotics on the intestinal microbiota of infants after an antibiotic treatment. Pediatr Res, 2006 59(3) 451-6.	FOS·Inulin	
Decsi T, Arato A, Balogh M, Dolinay T, Kanjo AH, Szabo E, et al.2005 [Randomised placebo controlled double blind study on the effect of prebiotic oligosaccharides on intestinal biota in healthy infants] Orv Hetil 146(48) 2445-50.	No company attribution, Numico-like; 9·1 GOS.FOS	Bifidobacteria increased, Growth, feeding, atopic manifestations similar
Duggan, C , Penny, M., Hibberd, P , Gil, A . Huapaya, A., Cooper, A., Coletta, F., Emenhiser, C , Kleinman, R. 2003. Oligofructose-supplemented infant cereal. 2 randomized, blinded, community-based trials in Peruvian infants Am. J. Clin Nutr. 77 937-942	Gerber, FOS at 0 55 g/15 g cereal	Not associated with differences in diarrhea prevalence, use of health care resources or response to immunization
Euler AR, Mitchell DK, Kline R, Pickering LK 2005 Prebiotic effect of fructo-oligosaccharide supplemented term infant formula at two concentrations compared with unsupplemented formula and human milk J Pediatr Gastroenterol Nutr. 40(2): 157-64	Wyeth; FOS at 1 5 and 3.0 g/L	More frequent and softer stools at higher dose, Minimal effect on microbiota
Fanaro S. Jelinek J, Stahl B, Boehm G, Kock R. Vigi V. 2005 Acidic oligosaccharides from pectin hydrolysate as new component for infant formulae. effect on intestinal biota, stool characteristics, and pH J Pediatr Gastroenterol Nutr	Numico; 9.1 GOS FOS (0.6 g) and/or Acidic Oligosaccharides (0 2 g)	Bifodobacteria increased with mixed oligos, not acidic alone, Softer stools with all prebiotics,

41(2) 186-90.		
Haarman M, Knol J. 2006 Quantitative real-time PCR analysis of fecal Lactobacillus species in infants receiving a prebiotic infant formula. Appl Environ Microbiol, 72(4) 2359-65.	Numico, 9 1 GOS.FOS at 8 g/L)	Microbiota (Lactobacilli) total increased and similar distribution to breast-fed with prebiotic
Haarman M, Knol J 2005 Quantitative real-time PCR assays to identify and quantify fecal Bifidobacterium species in infants receiving a prebiotic infant formula Appl Environ. Microbiol, 71(5) 2318-24	Numico, 9.1 GOS FOS at 8 g/L	Bifidobacteria increased, Bifidobacteria profile similar to breast fed
Kapiki A, Costalos C, Oikonomidou C, Triantafyllidou A, Loukatou E, Pertrohilou V 2006. The effect of a fructo-oligosaccharide supplemented formula on gut biota of preterm infants Early Hum Dev In press	No company designation, FOS at 4 g/L	Bifidobacteria increased with prebiotic, Stool frequency increased with prebiotic
Knol J, Boehm G, Lidestri M, Negretti F, Jelinek J, Agosti M, et al 2005. Increase of faecal bifidobacteria due to dietary oligosaccharides induces a reduction of clinically relevant pathogen germs in the faeces of formula-fed preterm infants. Acta Paediatr Suppl, 94(449) 31-3.	Numico, 9.1 GOS FOS at 10 g/L	Bifidobacteria increased and potential pathogens decreased with prebiotic (though latter barely changed)
Knol J, Scholtens P, Kafka C, Steenbakkers J, Gro S, Helm K, et al. 2005 Colon microbiota in infants fed formula with galacto- and fructo-oligosaccharides more like breast-fed infants J Pediatr Gastroenterol Nutr. 40(1) 36-42	Numico, 9:1 GOS FOS at 8 g/L	Bifidobacteria increased closer to breast fed with prebiotic, pH and SCFA more similar to breast fed, softer stool and slight increase in frequency with prebiotic
Kukkonen, K , Savilahti, E , Haahtel, T , Juntunen-Backman, K , Korpela, R , Poussa, T , Tuure, T , and	[Valio, GOS (0 8 g/day)and 4 Probiotics (L rhamnosus GG,	1223 pregnant women carrying high risk children, No effect of probiotic treatment on incidence of allergic disease, tended to reduce IgE-associated (atopic) diseases, Probiotic treatment reduced eczema and atopic

Kuitunen, M. 2007 Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases a randomized, double-blind, placebo-controlled trial. J Allerg Clin Immunol. 119 (1):192-198	L rhamnosus LC705, B. breve bb99, P freudenreichii ssp shermanii JS)	eczema, Incidence of some side effects (abdominal discomfort, vomiting, excessive crying) were reported
Lidestri M, Agosti M, Marini A, Boehm G. 2003 Oligosaccharides might stimulate calcium absorption in formula-fed preterm infants Acta. Paediatr Suppl 441 91-92.	Numico; 9 1 GOS.FOS	Calcium concentrations increased in urine only (not serum) with prebiotic
Mihatsch. W., Hoegel, J, and Pohlandt, F 2006 Prebiotic oligosaccharides reduce stool viscosity and accelerate gastrointestinal transport in preterm infants Acta Paediatrica 95 843-848.	Milupa, GOS FOS at 10 g/L	20 preterm infants enrolled, Prebiotic significantly reduced stool viscosity and gastrointestinal transit time
Moore N, Chao C, Yang LP, Storm H, Oliva-Hemker M, Saavedra JM 2003 Effects of fructo-oligosaccharide-supplemented infant cereal a double-blind, randomized trial Br J Nutr, 90(3)· 581-587	[Nestle; FOS at 0.75 g/serving of infant cereal	Stool frequency slightly increased and consistency decreased with prebiotic
Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, Boehm G. 2006 A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Arch Dis Child. 91(10) 814-9	Numico, 9 1 GOS.FOS at 8 g/L	Evaluated infants at high risk for atopic dermatitis, Mixed breast milk and formula allowed to 6 weeks, Stool samples handled carefully (homogenized in glycerol transport medium, frozen to -80°C, and shipped on dry ice), Significant increase in Bifidobacteria (selective media used); No difference in Lactobacilli (selective media used); During 6 months AD occurred in 10 infants (9.8%) receiving prebiotics and 24 infants (23 1% receiving the placebo (SCORAD scores were not statistically significant)
Moro GE, Stahl B, Fanaro S, Jelinek J, Boehm G, Coppa GV. 2005. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants Acta Paediatr Suppl. 94(449): 27-30.	Numico, 9:1 GOS:FOS at 8 g/L	Bifidobacteria increased with prebiotic
Moro, G , Mosca, F., Miniello, V., Fanaro, S ,	Numico; 9·1 GOS FOS at 4	Dose-dependent increase in Bifidobacteria with prebiotic;

Jelinek, J , Stahl, B , and Boehm, G. 2003. Effects of a new mixture of prebiotics on faecal biota and stools in term infants Acta Paediatr Suppl. 441.77-79.	and 8 g/L	
Moro G, Minoli I, Mosca M, Fanaro S, Jelinek J, Stahl B, et al. 2002 Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. J Pediatr Gastroenterol Nutr. 34(3). 291-295.	Numico, 9·1 GOS:FOS at 4 and 8 g/L	Dose-dependent increase in Bifidobacteria with prebiotic
Nagendra R, Viswanatha S, Arun Kumar S, Krishna Murthy B, Venkat Rao S 1995 Effect of feeding milk formula containing lactulose to infants on faecal bifidobacterial biota Nutr Res 15(1) 15-24.	No Company Designation, LOS at 0 5% and 1%	Bifidobacteria same as breast fed reference group with prebiotic
Puccio G, Cajozzo C, Meli F, Rochat F, Grathwohl D, and Steenhout P 2007. Clinical evaluation of a new starter formula for infants containing live *Bifidobacterium longum* BL999 and prebiotics Nutrition 23 1-8	Nestle; 9.1 GOS FOS at 4 g/L with *Bifidobacterium longum* BL999	Single center in Palermo, Italy 138 infants enrolled; Cesarean delivery reported at 44% and 36% in the Control and Experimental Groups, respectively,96% Caucasian (limited genetic diversity), Mean weight gain, recumbent length, and tolerability (gastrointestinal symptoms) were recorded up to 112 days of age, No difference in growth parameters, Slightly higher stool frequency in experimental group (2 2) than control (1.8), Experimental group had a lower risk of constipation and displayed trend toward lower incidence of respiratory tract infections (28%) compared to the control group (42%)
Rinne MM, Gueimonde M, Kalliomaki M, Hoppu U, Salminen SJ, Isolauri E. 2005 Similar bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and breastfeeding on infant gut microbiota. FEMS Immunol Med Microbiol. 43(1). 59-65	Nutricia-Omneo, 9 1 GOS.FOS	Bifidobacteria increased with prebiotic
Savino. F., Palumeri, E., Castagno, E , Cresi, F , Dalmasso, P , Cavallo, F.,	Numico; 9 1 GOS.FOS at 8 g/L (question	222 infants presenting with colic enrolled in Italy, 199 completed study, Reduction in colic episodes and crying with prebiotic formula

Oggero, R. 2006 Reduction of crying episodes owing to infantile colic· a randomized controlled study on the efficacy of a new infant formula. Euro J. Clin. Nutr 60 1304-1310	concerning addition of 4 g/L prebiotic to control formula)	
Savino F, Maccario S, Castagno E, Cresi F, Cavallo F, Dalmasso P, et al. 2005 Advances in the management of digestive problems during the first months of life Acta Paediatr Suppl. 94(449)· 120-4.	Numico; 9 1 GOS FOS	Positive impact on stooling pattern
Savino F, Cresi F, Maccario S, Cavallo F, Dalmasso P, Fanaro S, Oggero R, Vigi V, and Silvestro L 2003. Minor feeding problems during the first months of life: effectof partially hydrolyzed milk formula containing fructo- and galacto-oligosaccharides Acta Paediatr Suppl 441:86-90	Company not stated; 9·1 GOS:FOS	Observational study, 14 days Complete formula change, No control 932 healthy infants seen by pediatrician for colic, regurgitation or constipation were enrolled. 604 completed study, Episodes of colic, regurgitation declined Increased stool softness at day 7 (not day 14)
Scholtens PA, Alles MS, Bindels JG, van der Linde EG, Tolboom JJ, Knol J. 2006 Bifidogenic Effects of Solid Weaning Foods With Added Prebiotic Oligosaccharides A Randomised Controlled Clinical Trial J Pediatr Gastroenterol Nutr 42(5). 553-559	Numico, 9 1 GOS FO,	Significant increase in Bifidobacteria with prebiotic, No change in stool frequency or consistency
Schmelzle, H., Wirth. S., Skopnik, H . Radke, M , Knol, J , Bockler, H -M , Bronstrup, A , Wells, J , and Fusch. C. 2003. Randomized double-blind study of the nutritional efficacy and bifidogenicity of a new infant formula containing partially hydrolyzed protein, a high β-palmitic acid level, and nondigestible	Numico, 9.1 GOS·FOS at 8 g/L	Significant increase in Bifidobacteria with prebiotic, softer stools with prebiotic

oligosaccharides. J. Ped Gastroenterol. Nutr 36·343-351		
Ziegler E, Vanderhoof J, Petschow B, Mitmesser S, Stolz S, Harris C. and Berseth C. 2007. Term infants fed formula supplemented with selected blends of prebiotics grow normally and have soft stools similar to those reported for breast-fed infants. J Pediatr. Gastroenterol Nutr. 44·359-364	Mead Johnson Nutritionals; PDX·GOS (50·50) 4 g/L PDX GOS.LOS (50 33 17) 8g/L	226 healthy, formula-fed term infants received 1 of 3 formulas up to 120 days of age, Anthropmetric measures taken at 30, 60, 90 and 120 days, No statistically significant differences in weight or length growth rates, Infants on supplemented formulas had looser stools at 30, 60 and 90 days. No statistically significant difference in drop out rate

An evaluation of these 35 articles provided considerable insight into the current body of clinical experience and level of scientific support for the supplementation of infant formula with prebiotic carbohydrates. Though the recent publication dates suggest that the area of prebiotic supplementation is fairly new, the significant number of articles indicates a high level of interest and increasing application. At this time, sufficient clinical evidence indicates that when added to infant formula, prebiotic carbohydrates are safe and capable of mimicking some of the effects of human milk oligosaccharides, particularly in the areas of intestinal microbiota and stool characteristics. The principal findings from this body of clinical work are presented below. In addition, simple numerical information on individual manufacturers and potential prebiotic benefit categories is presented in matrix format in Table 5.

Principal Findings:

- Distribution of research articles by potential benefit (physiological) category
  - Intestinal microbiota related totaled 18 (51%).
  - Mineral absorption related totaled 1 (3%).
  - Laxation related totaled 10 (29%).
  - Immunity/allergy related totaled 6 (17%).
- Distribution of research articles by key research finding:
  - Bifidobacteria increased in proportion and/or in species distribution similar to breast-fed infants: 18 (51%)
  - Bifidobacteria did not change significantly: 4 (11%)
  - Lactobacilli increased in proportion and/or species distribution similar to breast-fed infants: 3 (9%)
  - Lactobacilli did not change significantly: 1 (3%)
  - Potential pathogenic bacterial decreased in stool: 1 (3%)
  - Fecal Short Chain Fatty Acids (SCFA) increased in concentration and/or in proportion to that reported for breast-fed infants: 2 (6%)

- Fecal pH was reduced: 1 (3%)
- Fecal Secretory IgA increased (minimally): 1 (3%)
- Mentioned growth parameters were normal: 3 (9%)
- Improved stool patterns or more similar to breast-fed infants: 12 (34%)
- No change in stool pattern: 1 (3%)
- Mentioned no change occurred in diarrhea or respiratory infection: 3 (9%)
- Decrease in atopic dermatitis or allergic symptoms: 2 (6%)
- Reported no change in atopic dermatitis or allergic symptoms: 1 (3%)
- Decreased constipation: 2 (6%)
- Decreased colic: 1 (3%)
- Mineral (calcium) increase in urine (not serum): 1 (3%)

Additional Findings:

- There was sufficient detail in two of the research papers to offer some insight into the typical composition and characteristics of European clinical trials and how they might differ from similar trials conducted in the U.S.
  - In a publication by Bakker-Zierikzee at al. (2006), 54% of the infants were born at home and the rate of delivery by caesarean section was 11%. An evaluation of fecal samples from 1032 infants at one month of age, recruited from the KOALA Birth Cohort Study in the Netherlands, found that term infants born vaginally at home (in addition to those receiving exclusive breast feeding) appeared to have the most "beneficial" gut microbiota (e.g. highest number of Bifidobacteria and lowest number of C. difficile and E. coli) (Penders et al., 2006).
  - In contrast, a publication by Puccio et al. (2007), reports rates delivery by caesarean section of 44% and 36% in the control and experimental groups, respectively. The study, which was carried out in Italy, included information on ethnicity where 96% of the mothers were Caucasian.
  - One estimate of caesarean delivery in the U.S. has been reported as 24%, the rate of home delivery is much lower than that cited in Bakker-Zierikzee et al. (2006), and the ethnic diversity is much higher than that reported in Puccio et al.,(2007). As each of these factors is known to contribute significantly to the development of the gastrointestinal microbiota in infants, clinical trials conducted in the U.S. and Europe are likely to diverge with respect to typical microbiota composition and responsiveness to oligosaccharides.
- Levels of fecal secretory IgA are described as playing an important role in the defense of the gastrointestinal tract and frequently used as a "marker" for immune system function.
  - In a single infant trial where the effects of prebiotic oligosaccharides (9:1 GOS:FOS) on sIgA were measured, Bakker-Zierikzee et al. (2006) reported a trend toward higher levels relative to infants consuming control formula.
  - The highest level of sIgA measured in infants consuming the GOS:FOS mixture was 1.141 mg/g wet weight feces and a significant difference occurred only once in 10 measurements [0.841 (GOS:FOS) versus 0.292

(Control)] at week 16. The low level of fecal sIgA observed in formula-fed infants is likely to have resulted from endogenous production within the immature infant gastrointestinal tract and associated lymphoid tissue.
  - In contrast, the fecal sIgA levels in the breast-fed reference group were 10-fold higher, peaking at their highest level (10.918 mg/g wet fecal weight) on Day 5 and declining in a linear fashion during 32 weeks to 0.265. This is to be expected in breast-fed infants where the majority of sIgA is likely to have originated in maternal milk where it is known to afford some passive immunity.
  - The authors reported that statistical analysis of the data was difficult and indicated that post-hoc power analysis suggests that group's sizes of more than 100 infants might be necessary to counteract the effects of large inter-individual variation.
- Oligosaccharide identity and dosage, sample collection and storage, as well as research methodologies varied among the clinical studies and this can reasonably be expected to influence microbiological results.
  - The various trials evaluated several oligosaccharides including GOS, FOS, a GOS:FOS combination, and inulin.
  - Dosage varied from 1.5 g/L to 10 g/L.
  - Fecal samples were collected differently (by parents at home or at the study site) and stored for various times (2 to 24 hours) and at different temperatures (+4°C, -20°C, -40°C, and -80°C) prior to microbiological evaluation.
  - Various analytical methods were utilized to measure microbiological populations including: 1) standard dilution plating on selective media, 2) Fluorescence In Situ Hybridization (FISH), and 3) Quantitative Real-Time PCR. A fourth technique, Denaturing Gradient Gel Electrophoresis (DGGE), was not utilized in this clinical subset.

Summary of Published Prebiotic Clinical Trial Results:

A subset of 35 research articles describing the results of infant clinical trials of prebiotics, and in a few cases synbiotics, were further evaluated for information relative to prebiotic material and dosage, potential clinical benefits, and manufacturer identification. The potential benefits (physiological outcomes) were evaluated relative to four broad categories including 1) intestinal microbiota, 2) mineral absorption, 3) laxation, and 4) immunity/allergy. The key finding reported in 51% the publications related to intestinal microbiota where bifidobacteria were shown to increase significantly in infants consuming prebiotic oligosaccharides, relative to those consuming control formulations. However, a bifidogenic response was not always demonstrated as these populations were not significantly different in at least four recent publications (11%). Beneficial changes in stooling patterns (increased frequency and softness) were the second most cited prebiotic effect reported in 29% of the publications. Relative to immune function and allergy, a minor increase in fecal secretory sIgA was reported in a single trial, and decreases in atopic dermatitis and allergy symptoms in two others. In the category of mineral absorption, a single

study reported a prebiotic-associated increase in urine calcium levels (though not in serum levels).

A complete review of the information contained in these 35 articles offered additional insights into the manner in which various differences in subject populations and scientific methodology could influence the microbiological results of infant clinical trials. Those differences included the level of genetic variation, birthing practices, stool collection and storage instructions, and enumeration methodology.

Table 5. Matrix of Manufacturer Publications and Potential Benefit Categories

Benefit Category	**Manufacturer**					**Category Subtotals**
	Numico	**Nestlé**	**Wyeth**	**MJN**	**Other**	
Intestinal Microbiota	12	2	1	0	3	18 (51%)
Mineral Absorption	1	0	0	0	0	1 (3%)
Laxation (Stools)	6	1	1	1	1	10 (29%)
Immunity Allergy	3	1	0	0	2	6 (17%)
Manufacturer Subtotals	22 (63%)	4 (11%)	2 (6%)	1 (3%)	6 (17%)	

C. Summary of Information on Prebiotic Ingredients in Current Infant Formula

Infant formula supplemented with select prebiotic ingredients has been on the global market for at least five years. The prebiotic ingredients in use outside the United States are reported to include inulin, isomaltooligosaccharide, lactulose, galactooligosaccharide and fructooligosaccharide. Prebiotic carbohydrates, have been shown to improve the balance of intestinal microbiota in infants (i.e. increased bifidobacteria at the expense of less beneficial bacteria or pathogens) and promote a softer, looser stool pattern. Both of these characteristics are typically reported for breast-fed infants, indicating that prebiotic carbohydrates can substitute for some of the physiological characteristics of human milk oligosaccharides. Through a combination of cumulative market experience with infant formulas supplemented with prebiotics, and a growing body of evidence based on clinical research, the safety of this ingredient category has been largely established.

VII. Clinical Studies of Infant Formula Containing PDX and GOS Conducted by Mead Johnson Nutritionals

MJ conducted three clinical studies with milk-based formulas containing prebiotics. The first study was investigational in nature and not published. As such, it was not considered in the GRAS determination for GOS and PDX. It is included here both to ensure that all relevant information is made available and to corroborate the findings of the published literature. The first study was designed to screen the overall tolerance of two different non-digestible carbohydrates in infants, GOS and lactulose (LOS) and since it is not available in the literature, it is presented in more detail than would ordinarily be the case.

The second study, which was published in the Journal of Pediatric Gastroenterology and Nutrition (Ziegler *et al.* 2007), was designed to evaluate the effect of two different combinations of GOS, LOS, and PDX at two different intake levels. Overall growth, tolerance, and stool patterns of healthy term infants were monitored for four months..

In the third clinical trial (28 day duration), the gastrointestinal microbiota, tolerance and stool patterns of infants fed formulas supplemented with combinations of GOS, PDX, and LOS were compared to those of breast-fed or control formula-fed infants.

The primary finding of the studies was that infants fed formulas supplemented with prebiotic mixtures containing PDX and GOS, with and without LOS, achieved normal growth. When compared to infants fed unsupplemented control formula, the stool characteristics of infants consuming formula supplemented with the prebiotics were more like those typically reported for breast-fed infants.

All MJ clinical trials were performed in accordance with applicable standards and regulations, including the requirement for IRB approval prior to subject enrolment.

A. MJ Clinical Study #1: Acute Tolerance

The principal objective of the first clinical study conducted by MJN was to evaluate the tolerance of a commercial infant formula supplemented with the non-digestible carbohydrates, GOS and LOS, with potential prebiotic activity in healthy term infants. The study assessed the tolerance of a one-time feeding and a single total daily feeding of formula containing the different levels of the prebiotic ingredients.

1. STUDY DESIGN

This study was a multi-center, randomized, double-blinded, parallel group, five-arm trial. It was designed to evaluate two levels of GOS and LOS and a combination of the lower level of the two prebiotics for tolerance in infants 30-120 days of age, by administration of a single feeding of prebiotics in marketed infant formula followed by a 24 hour of feeding of the same product. This study was conducted in 5 centers across the United States.

Healthy male and female term infants who met the following inclusion criteria were enrolled: were fed milk-based formula; 30-120 days of age, and of normal weight/length (between and inclusive of 10th and 90th percentiles) on standard growth charts.

The study was 12 days in duration and consisted of (1) a 7 day run-in period when subjects received control formula (Enfamil LIPIL with Iron), (2) a single test feeding of the study formula followed by 24 hours of control formula, (3) a 24-hour feeding period of study formula and control formula (if needed) and (4) a 48-hour period of control formula. There were four study visits. At Study Visit 1, parents/guardians were given information about the study and asked to give permission for the infant to participate prior to any study procedures specific to the study being conducted. Parents who were willing for their infants to participate in the study were asked to sign a written informed consent document and an authorization to use and/or disclose protected health information. After permission had been obtained, all participants were examined to determine current health status. Weight, length, temperature, health history, and information about the infant's daily consumption of formula were collected. Infants who qualified for the study were provided with control formula (Enfamil LIPIL with Iron) to be fed for a period of seven days to ensure all infants were on the same formula prior to receiving the test formula containing prebiotic and would not refuse the study formula. Parents/guardians completed a daily diary regarding symptoms of gastrointestinal distress including fussiness, and gas, and the number of bowel movements and their characteristics. At Study Visit 2, following the 7-day feeding period of control formula, the parent/guardian returned to the site with the infant for examination of the infant's health status. Adverse events and diary compliance were also checked. Participants continuing to meet the study entrance criteria were randomly assigned to a study product based upon a randomization list provided by MJ. Out of 137 infants enrolled in the study, 95 were randomized to one of the following 5 groups:

Enfamil LIPIL with Iron + 2g/L LOS

Enfamil LIPIL with Iron + 3g/L LOS

Enfamil LIPIL with Iron + 5g/L GOS

Enfamil LIPIL with Iron + 7g/L GOS

Enfamil LIPIL with Iron + 2g/L LOS + 5g/L GOS

The test formula of Enfamil LIPIL with Iron plus a prebiotic was fed in one feeding based on the infant's weight at the time of Study Visit 2 (25 mL/kg bw), and administered at the study site. Infants remained at the study site for a 2-hour observation period where the parent/guardian recorded symptoms of gastrointestinal distress, including fussiness, and gas, and the number of bowel movements and their characteristics. During the 2-hour observation period, additional feedings with control formula were allowed as needed. After completing the 2-hour observation period, infants were released from the study site with adequate Enfamil LIPIL with Iron (without prebiotics) for feeding until the next study visit (24 hours post single feeding). The parents/guardians were asked to observe and record symptoms of fussiness, gas, and stool characteristics until the next study visit.

At study visit 3 on Day 9, parents/guardians returned to the site with the infant at approximately the same time of day as Study Visit 2. If no episodes of watery stools or inconsolable crying were reported, additional volumes of the same study product as the test feeding were dispensed for feeding over the next 24 hours. After the 24-hour test formula feeding period, infants consumed control formula (without prebiotic) for an additional 48-hour period. The parents/guardians again observed and recorded symptoms of fussiness, and gas, and stool characteristics during this 48-hour period. At study visit 4 on Day 12, the parents/guardians returned to the site with the infant. Information relative to the 24-hour study product feeding and the following 48 hour interval was collected. Subjects were dismissed from the study if no adverse events had occurred.

2. DATA COLLECTION METHODS

Symptoms of gastrointestinal distress like fussiness and gas were evaluated during the 12-day study period by observing their occurrence and recording them in a daily diary. Any symptoms of gastrointestinal distress and/or other problems were recorded as adverse events. Stool data were collected in terms of overall frequency (number of bowel movements over the recording period) and consistency (hard, formed, soft, loose, or watery).

Subjects who experienced an episode of a watery bowel movement after the single feeding of the experimental study formula (with prebiotics) were not given the full day feeding of the study formula with prebiotics. These subjects were placed on control formula for the duration of the study and followed until the event resolved or there was a return to acceptable medical status. Subjects who experienced one or more episodes of a watery bowel movement while on the experimental study formula (with prebiotics) during the home use portion of the study were taken off the product, placed on control formula for the duration of the study, and followed until the event resolved or there was a return to

acceptable medical status. Subjects who experienced at least 2 hours of continuous inconsolable crying after the single feeding of the experimental study formula (with prebiotics) or during the home use portion of the study were discontinued from the study and followed until the event resolved or until there was a return to acceptable medical status.

Prebiotic intolerance was defined as either one or more watery stools and/or two or more hours of continuous, inconsolable crying anytime after consuming the study formula with prebiotics. The occurrence of two infants with watery stools, or five infants with continuous, inconsolable crying, or a combination of one infant with watery stool and four other infants with continuous, inconsolable crying in one arm of the study would result in discontinuation of that study arm.

Adverse events, defined as any unfavorable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with participation in the clinical study, whether or not related to the study product, were recorded at each study visit. If the investigator was not the participant's primary physician, the investigator was asked to obtain copies of the participant's medical records for the study period to determine if an adverse event had occurred during the study. If such records were not available, information received from the parents was to serve as a source document for reporting adverse events.

An adverse event was considered serious if it met one or more of the following criteria: resulted in death, was life-threatening, required inpatient hospitalization or prolongation of existing hospitalization; resulted in persistent or significant disability/incapacity, or was a congenital anomaly/birth defect.

3. RESULTS

One hundred thirty seven (137) infants were enrolled in this study, with 11 (8%) showing diarrhea before randomization. A total of 95 subjects were randomized into the study. Three of the test groups were discontinued (LOS 2 g/L; GOS 5 g/L; LOS 2 g/L + GOS 5 g/L) based on the criteria established at the onset of the study. The incidence of watery stools among the 5 test groups is shown in Table 6.

Table 6. Incidence of watery stools among infants fed milk-based formula containing non-digestible carbohydrates.

Test Prebiotic	Number of Subjects Randomized	Number of Subjects with Watery Stool(s)
LOS (2 g/L)	22 [a]	2
LOS (3 g/L)	25	1
GOS (5 g/L)	10 [a]	2
GOS (7 g/L)	24	1
LOS 2 g/L + GOS 5 g/L	14 [a]	2
	95	8

[a] Group discontinued due to subjects reaching predetermined intolerance criteria (watery diarrhea, inconsolable crying).

4. CONCLUSIONS

This initial clinical trial preceded evaluations of PDX and included only two levels of GOS (5 g/L and 7 g/L) and LOS (2 g/L and 3 g/L) and a single combination of the two at 5 g/L and 2 g/L, respectively. Of the 137 healthy infants enrolled in the study, 11 demonstrated diarrhea prior to randomization, indicating a certain level of gastrointestinal symptoms was normal for the population. Three test groups were discontinued based on the occurrence of two subjects with watery stools and not inconsolable crying (LOS 2g/L; GOS 5 g/L, and the combination of LOS 2 g/L and GOS 5 g/L). A single incident of inconsolable crying was reported in the GOS 7 g/L group. The following two observations should be noted: 1) in the discontinued LOS and GOS groups, the level of prebiotic material consumed was the lower of the two study levels; and 2) the number of subjects with watery stools reported for the combination group was no greater than LOS or GOS alone. If the occurrence of watery diarrhea were truly a response to prebiotic consumption, it is logical to assume that there would be a dose response relationship, which was lacking. Based on these data, one could argue that the discontinuation levels observed in these three groups were simply random occurrences and the discontinuation criteria were excessively stringent.

B. MJ Clinical Study #2: Four Month Feeding Study (Ziegler et al., 2007)

A second study was conducted to evaluate the impact of milk-based formula containing combinations of the prebiotic ingredients PDX, GOS and LOS on infant growth, tolerance, and stool patterns.

1. STUDY DESIGN

This study was a double-blind, randomized, controlled, parallel-group, prospective trial conducted at 14 clinical sites in the United States. Healthy term infants were randomized to one of three study formulas: 1) a marketed milk-based formula (Enfamil® LIPIL® with Iron, designated C); 2) the control formula supplemented with 4 g/L of a prebiotic blend containing PDX and GOS (1:1 ratio, designated PG4); or 3) the control formula supplemented with 8 g/L of a prebiotic blend containing PDX, GOS, and LOS (3:2:1 ratio, designated PGL8).

Weight gain from 14 to 120 days of age was the primary outcome variable. The sample size was determined so that the study would have a power of 80% to detect a clinically relevant difference of 3 grams of weight gain per day (α=0.05, one-tailed).

The study protocol called for enrollment of 218 infants in order to obtain 51 completed participants per group after accounting for study dropouts. A parent or caregiver was required to bring the participant to the study site five times for data collection: Visit 1 at 14 ± 2 days of age, Visit 2 at 30 ± 3 days of age, Visit 3 at 60 ± 3 days of age, Visit 4 at 90 ± 3 days of age, and Visit 5 at 120 ± 4 days of age.

Parents were contacted and advised of the study if their term infants met the study inclusion criteria and did not have any of the exclusion criteria. Parents who were willing for their infants to participate in the study were asked to sign a written informed consent document and an authorization to use and/or disclose protected health information. Study site personnel then verified that the infant was eligible for the study and randomized the infant into the study. Body weight, length, and head circumference were measured at this time (Visit 1), and study formula was dispensed. Weight, length, and head circumference were measured at all subsequent visits (Visits 2, 3, 4, and 5), and parents were asked to complete two questionnaires: a 24-Hour Diet Recall, and a 24-Hour Tolerance Recall covering the period immediately preceeding the visit. Parents were given enough formula at each visit to feed their infants until the next scheduled visit. Study site personnel recorded adverse events as they occurred throughout the study. All unopened cans of study formula were collected at the final study evaluation (Visit 5) or at the time of study withdrawal if the participant discontinued early from the study. A final study evaluation was made at Visit 5

or upon discontinuation from the study. Prescriptions and over-the-counter medications were permitted during this study and were not required to be documented.

2. DATA COLLECTION METHODS

Infants were weighed to the nearest gram or ounce on a standard pediatric balance, without clothing or diaper. Body length was measured in triplicate to the nearest cm or 1/4 inch using a length board. Head circumference was measured in triplicate to the nearest cm or 1/4 inch with a flexible, non-stretchable cloth or vinyl tape. Paper tapes were not allowed.

Data on dietary intake and formula tolerance were recorded by parents at Visits 2-5. Parents were asked to record the amount of study formula consumed in the prior 24 hours. Parents also recorded the number of bowel movements and general consistency of those bowel movements from the 24 hours prior to the study visits. Consistency was rated on a defined scale of 1-5, with 1 denoting hard stool and 5 denoting watery stool; sample pictures were provided for reference. Formula tolerance was assessed by parents' recall of infant fussiness and gas in the 24 hours preceding each study visit. Parents rated infants' fussiness, fussiness relative to normal, gas, and gas relative to normal by checking boxes that most closely represented their infants' tolerance. Parents were also asked to report diarrhea and constipation in the past 24 hours based on definitions provided. Diarrhea was defined as an increased number of bowel movements and/or increased amount of water in the stool resulting in loose or watery stools at least 3 times within the last 24 hours. Constipation was defined as more than one day with no bowel movement followed by a hard stool, or three or more days without a bowel movement.

Adverse events were recorded at each study visit; an adverse event was defined as any unfavorable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with participation in the clinical study, whether or not related to the study product. If the investigator was not the participant's primary physician, the investigator was asked to obtain copies of the participant's medical records for the study period to determine if an adverse event had occurred during the study. If such records were not available, information received from the parents was to serve as a source document for reporting adverse events.

An adverse event was considered serious if it met one or more of the following criteria: resulted in death, was life-threatening, required inpatient hospitalization or prolongation of existing hospitalization; resulted in persistent or significant disability/incapacity, or was a congenital anomaly/birth defect.

3. RESULTS

Two hundred and twenty-six infants (76 C, 74 PG4, 76 PGL8) enrolled in this study and consumed study formula. Of these, 164 infants (58 C, 58 PG4, 48 PGL8) completed the study. Results presented in Tables 7-8 and Figures 18-19 below are data from all participants who consumed study formula for whom data were available.

Growth. No statistically significant differences were detected between the prebiotic-supplemented formula groups and the control group in mean weight growth rate from day 14 to days 30, 60, 90, and 120, as shown in Table 7. The study had adequate power (91-93%) to detect a clinically significant difference in weight gain of 3 g/day from 14 to 120 days of age and the similarities in growth were reflected in similarities of achieved body weights (Figures 18 and 19).

Table 7. Mean Weight Growth Rate g/day) from Day 14 to Days 30, 60, 90, and 120

Age (Days)	Study Group	n	Mean	(s.e.)	Comparison	p-value
30	Control	71	36.0	(1.7)	Control vs PG4	0.843
	PG4	66	38.0	(1.7)	Control vs PGL8	0.993
	PGL8	68	40.9	(1.7)		
60	Control	62	33.2	(1.2)	Control vs PG4	0.900
	PG4	61	34.8	(1.2)	Control vs PGL8	0.892
	PGL8	54	34.8	(1.2)		
90	Control	59	31.0	(0.9)	Control vs PG4	0.840
	PG4	58	32.1	(0.9)	Control vs PGL8	0.803
	PGL8	52	31.9	(0.9)		
120	Control	58	28.4	(0.8)	Control vs PG4	0.840
	PG4	58	29.3	(0.7)	Control vs PGL8	0.806
	PGL8	48	29.3	(0.8)		

The mean weights plotted on standardized growth charts published by the U.S. Centers for Disease Control are shown in Figures 18 and 19.

Figure 18. Mean Achieved Weights of Male Participants with Reference Percentiles (3 to 97) from 14 to 120 Days of Age

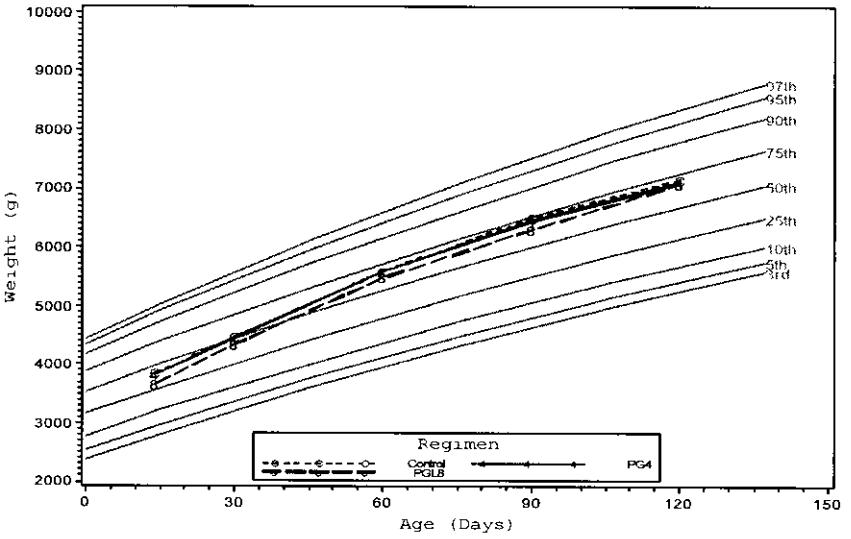
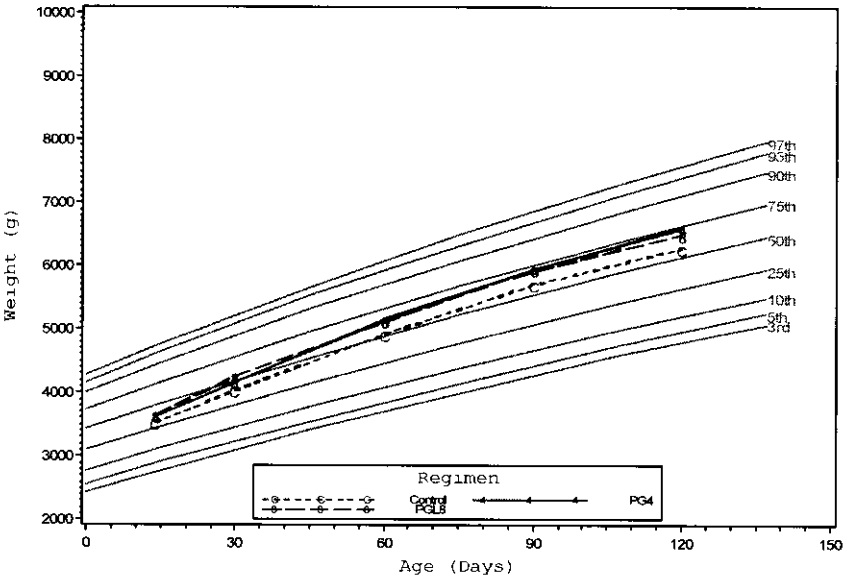


Figure 19. Mean Achieved Weights of Female Participants with Reference Percentiles (3 to 97) from 14 to120 Days of Age



No statistically significant differences were detected among the formula groups in mean length gain from day 14 to days 30, 60, 90, and 120, as shown in Table 8.

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Table 8. Mean Length Growth Rate (cm/day) from Day 14 to Days 30, 60, 90, and 120

Age (Days)	Study Group	n	Mean	(s.e.)	Comparison	p-value
30	Control	71	0.14	(0.009)	Overall	0.309
	PG4	66	0.13	(0.009)		
	PGL8	67	0.14	(0.009)		
60	Control	62	0.12	(0.004)	Overall	0.446
	PG4	61	0.11	(0.004)		
	PGL8	54	0.12	(0.004)		
90	Control	59	0.11	(0.003)	Overall	0.306
	PG4	58	0.11	(0.003)		
	PGL8	52	0.11	(0.003)		
120	Control	58	0.10	(0.002)	Overall	0.340
	PG4	58	0.11	(0.002)		
	PGL8	48	0.10	(0.002)		

A statistically significant difference among the study groups in head circumference gain was detected at 30 days, but not at 60, 90 and 120 days of age as shown in Table 9. The difference in head circumference gain from 14 to 30 days of age is not considered clinically significant.

Table 9. Mean Head Circumference Growth Rate (cm/day) from Day 14 to Days 30, 60, 90, and 120

Age (Days)	Study Group	n	Mean	(s.e.)	Comparison	p-value
30	Control	71	0.09	(0.006)	Overall	0.007*
	PG4	66	0.08	(0.006)	Control vs PG4	0.568
	PGL8	67	0.11	(0.006)	Control vs PGL8	0.014*
					PG4 vs PGL8	0.003*
60	Control	62	0.06	(0.003)	Overall	0.785
	PG4	61	0.06	(0.003)		
	PGL8	54	0.06	(0.003)		
90	Control	59	0.06	(0.002)	Overall	0.598
	PG4	58	0.06	(0.002)		
	PGL8	52	0.06	(0.002)		
120	Control	58	0.05	(0.001)	Overall	0.711
	PG4	58	0.05	(0.001)		
	PGL8	48	0.05	(0.001)		

*Statistically significant, $p < 0.05$.

Discontinuation: Of the 226 infants enrolled in the study who consumed study formula, 18 C (24%), 16 PG4 (22%), and 28 PGL8 (37%) infants discontinued from the study prior to visit 5 (p=0.084). Infants who discontinued were classified by the investigators into those who discontinued for reasons associated with study formula and those who discontinued for reasons not related to study formula, as shown in Table 10. In this table, participants who were lost to follow-up were conservatively classified as discontinued due to formula. Differences among the groups did not reach statistical significance, but did reflect a trend toward significance at the higher dosage level.

Table 10. Relationship of Study Discontinuation to Study Formula

Study Group	Completed N	Completed (%)	Discontinued Due to Formula n	Discontinued Due to Formula (%)	Discontinued Not Due to Formula N	Discontinued Not Due to Formula (%)	Comparison	p-value
Control	58	(76)	8	(11)	10	(13)	Overall	0.073
PG4	58	(78)	11	(15)	5	(7)		
PGL8	48	(63)	20	(26)	8	(11)		

Intolerance to study formula was the most frequently stated reason for study discontinuation (7 C, 9 PG4, and 16 PGL8 infants). The most frequent reasons for discontinuation due to intolerance were gas, fussiness, vomiting, and diarrhea, as shown in Table 11.

Table 11. Feeding Intolerances‡ Resulting in Study Discontinuation

	Study Group		
	Control	PG4	PGL8
Vomiting	3	3	6
Fussiness	2	5	9
Allergic Reaction	1		
Constipation	1	1	
Diarrhea	1	2	4
Gas	5	5	11
Excessive Spitting		2	1
Abdominal Pain			1
Reflux			1
Colic	2		
Rash			1

‡More than one intolerance may have been reported for a participant.

Stool Consistency. Statistically significant differences in parental recall of stool consistency were observed at 30 days of age (Control vs. PGL8; PG4 vs. PGL8) and 60 and 90 days of age (Control vs. PG4; Control vs. PGL8), when infants fed experimental formulas were reported to have more loose stools compared with control infants, as shown in Table 12.

Table 12. 24-Hour Recall of Stool Consistency

		Study Group							
	Stool	Control		PG4		PGL8			
Age (Days)	Consistency	N	(%)	n	(%)	N	(%)	Comparison	p-value
30	Formed	4	(6)	2	(3)	1	(2)	Overall	<0.001*
	Soft	44	(66)	39	(63)	29	(44)	Control vs PG4	0.546
	Loose	19	(28)	20	(32)	34	(52)	Control vs PGL8	<0.001*
	Watery	0	(0)	1	(2)	2	(3)	PG4 vs PGL8	0.004*
60	Formed	3	(5)	2	(3)	0	(0)	Overall	0.025*
	Soft	41	(68)	32	(54)	27	(52)	Control vs PG4	0.044*
	Loose	15	(25)	24	(41)	23	(44)	Control vs PGL8	0.011*
	Watery	1	(2)	1	(2)	2	(4)	PG4 vs PGL8	0.297
90	Formed	4	(7)	1	(2)	3	(6)	Overall	0.004*
	Soft	45	(79)	33	(60)	29	(58)	Control vs PG4	0.001*
	Loose	8	(14)	20	(36)	18	(36)	Control vs PGL8	0 011*
	Watery	0	(0)	1	(2)	0	(0)	PG4 vs PGL8	0.554
120	Formed	8	(14)	1	(2)	1	(2)	Overall	0.064
	Soft	40	(69)	40	(71)	27	(63)		
	Loose	9	(16)	14	(25)	14	(33)		
	Watery	1	(2)	1	(2)	1	(2)		

*Statistically significant, p <0.05.

Adverse Events· A total of 72 categories of adverse events in 10 body systems were reported during the study. Of these, statistically significant differences were detected among formula groups for 3 categories of adverse events: irritability, diarrhea, and eczema, as shown in Table 13. The statistical interpretation of the p-value is the probability of making a Type 1 error (i.e. concluding that treatments are different when they are actually the same). Setting the testing criterion at 0.05 indicates that even if two treatments are really the same we would expect to conclude a treatment difference (i.e. Type I error) 5% of the time (approximately 1 in 20). Given that 72 categories of adverse events were reported, the conclusion of a treatment difference for three events is well within the 5% Type I error rate. The statistically significant adverse events included irritability (C, 3; PG4, 6; PGL8, 12), eczema (C, 5; PG4, 13; PGL8, 3), and diarrhea (C, 3; PG4, 13; PGL8, 10). It is worth noting that the parental reports of eczema and diarrhea did not appear to be dose related as both were higher in the PG4 group than the PGL8 group. This is particularly evident in the reports of eczema where the PGL8 group value was actually lower than the control group value suggesting the event was unrelated to prebiotic consumption.

It is important to distinguish clinically relevant diarrhea from normal laxation patterns. Laxation is a general term that refers to the normal process of defecation (elimination of fecal waste through the anus). While there is no strict definition for what constitutes normal laxation, the presence of softer, looser stools falls within a continuum of normal stool patterns. As an example, Stedman's Medical Dictionary refers to laxation as a bowel movement characterized by soft stools and reduced gastrointestinal transit time (Spraycar, 1995e). The occurrence of a softer, looser stool pattern is typically reported for breast fed infants. When compared to formula fed infants, breast fed infants have been shown to pass more stools per day (median of 1.6 versus 4.4, respectively), the greatest range of stools per day (0.6-3.9 versus 0.3-8.0, respectively), as well as looser (runny, pasty) stools (Tham et al., 1996). This laxation (stooling) pattern could be attributed, in part, to the presence of significant quantities (5-10 g/L mature milk) of a large complex (> 130 structures) of human milk oligosaccharides (HMO) (Bode, 2006).

Clinically relevant diarrhea is characterized by the presence of frequent, watery bowel movements, accompanied by an excessive loss of fluid and electrolytes. The most widely accepted clinical definition of a diarrhea day is one where a subject "experiences three or more loose or watery stools in 24 hours or any number of loose or watery bloody stools," (Wright et al., 2006). Though the physiological basis for diarrhea is disturbed intestinal transport of solutes (primarily sodium, chloride and glucose) followed by passive water movement, several mechanisms, including secretory and osmotic abnormalities, have been identified as typical causative agents (Behrmen et al., 2004). Secretory diarrhea, which is characterized by watery, voluminous defecations, and is typically driven by the binding of a secretogue (e.g. cholera toxin) to a receptor on the intestinal epithelium. Osmotic diarrhea, which is usually of lesser volume and stops with fasting, is often associated with the ingestion of a poorly absorbed solute (e.g. magnesium). In infants, acute diarrhea is more commonly associated with gastroenteritis, systemic infection and/or antibiotic use while the causes of chronic diarrhea are likely to include food intolerance (e.g. cow's milk, soy protein) or other disease states (e.g. cystic fibrosis, celiac disease).

Clinically relevant diarrhea then is an abnormal condition characterized by the frequent excretion of semisolid or fluid (*i e* , watery) feces from the bowel (Spraycar, 1995b). Thus, what distinguishes diarrhea from laxation is the frequency of fecal excretion and the watery consistency of the excreted feces. Laxation, which covers a broad range of stool patterns, is generally not adverse to health, while chronic diarrhea can have deleterious consequences when a nutritional deficit develops. For the purposes of this study, diarrhea was defined as, "an increased number of bowel movements and/or increased amount of water in the stools resulting in watery stools at least three times in the last 24 hours." Two separate measures of diarrhea were collected during the course of the study. In the first, among the

data collected on 24 hour parental recall was a rating of stool characteristics. Though softer, looser stools were noted among the prebiotic groups, there were no significant differences among parental reports of infants experiencing diarrhea. In the second measure, rates of study discontinuation were monitored by the study investigator. Again, there were no statistically significant differences in rates of discontinuation due to diarrhea. It was only in the adverse events category (i.e. adverse events which were compiled from several sources) that diarrhea was reported as statistically significant, though it did not occur in a dose-dependent manner. In contrast with the two previous methods used to measure diarrhea, the AE reports were not based on a standardized scale that would allow the differentiation of laxation from diarrhea. As discussed above, diarrhea was one of only three of 72 categories of adverse events that reached the level of statistical significance, which is certainly within the type I error indicating it could have occurred by chance. Coupled with the fact that the consumption of the PDX:GOS blend shifted the stool consistency pattern closer to that typically reported for breast fed infants, what was reported as an adverse event can be explained simply as being within the normal range of laxation pattern expected to occur with the addition of prebiotic carbohydrates to infant formula.

Table 13. Statistically Significant Adverse Events

	Study Group							
	Control		PG4		PGL8			
Event	n	(%)	n	(%)	N	(%)	Comparison	p-value
Diarrhea	3	(4)	13	(18)	10	(13)	Overall	0.019*
							Control vs PG4	0.008*
							Control vs PGL8	0.078
							PG4 vs PGL8	0.502
Eczema	5	(7)	13	(18)	3	(4)	Overall	0.011*
							Control vs PG4	0.046*
							Control vs PGL8	0.719
							PG4 vs PGL8	0.008*
Irritability	3	(4)	6	(8)	12	(16)	Overall	0.047*
							Control vs PG4	0.324
							Control vs PGL8	0.027*
							PG4 vs PGL8	0.209

*Statistically significant, p-value <0.05.

There were 15 reports of serious adverse events in 12 infants (5 C, 0 PG4, 7 PGL8), none which were were judged by the investigators to be a result of the study formula.

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Serious adverse events among infants in the C group included pyloric stenosis, acute gastroenteritis, bronchiolitis, infection, and craniosynostosis. The infant with pyloric stenosis and the infant with bronchiolitis were discontinued from the study at the time of the event or subsequent to the event; all other infants continued in the study.

Serious adverse events were reported for 7 infants in the PGL8 group. Two infants had respiratory viruses: one was treated for respiratory syncytial virus and one was hospitalized with Coxsackie virus. One infant was hospitalized for gastroenteritis and dehydration. One infant was treated for bronchiolitis and was hospitalized for meningitis approximately one month later. One infant was hospitalized for bronchiolitis and one was hospitalized for fever. One infant was hospitalized for viral infection and dehydration. Only the infant hospitalized for gastroenteritis and dehydration and the infant hospitalized with Coxsackie virus were discontinued from the study at the time of the event or subsequent to the event.

4. CONCLUSIONS

The results of this study indicate that the use of prebiotic-supplemented formulas at 4 g/L and 8 g/L is safe when fed to healthy, term infants from 14 days of age to 120 days of age. Feeding the supplemented formulas resulted in normal growth and acceptable tolerance. Parents reported that infants who consumed supplemented formulas experienced softer, looser stools at 30, 60 and 90 days than infants who received the control formula. The occurrence of a softer, looser stool pattern is typically reported for breast-fed infants. When compared to formula-fed infants, breast-fed infants have been shown to pass more stools per day (median of 1.6 versus 4.4, respectively), as well as looser (runny, pasty) stools (Tham et al., 1996). This stooling pattern could be attributed, in part, to the presence of significant quantities of a large complex of human milk oligosaccharides (reviewed by Bode, 2006). Considering these observations, the addition of non-digestible, prebiotic carbohydrates to infant formula would be expected to shift the stool patterns of infants consuming them closer to those of breast-fed infants. The results of this clinical trial were published in *The Journal of Pediatric Gastroenterology and Nutrition* on March 1, 2007 (Ziegler *et al.* 2007).

C. MJ Clinical Trial #3: 28 Day Fecal Microbiota Study

The third clinical trial completed by MJN involved feeding infants milk-based formulas containing blends of PDX, GOS and LOS in order to assess their impact on fecal microbiota composition and formula tolerance. The primary objective of this study was to compare the change from baseline in the fecal populations of specific *Clostridium* species (sum of clusters I and XI) among groups of infants fed formulas containing different prebiotic blends for a period of 28 days. Cluster I (the *Clostridium botulinum* group) and

Cluster XI (the *Clostridium lituseburense* group) are known to include *Clostridium perfringens* and *Clostridium difficile*, respectively. The decision to focus on the change in *Clostridium* spp. levels was based on results from published studies indicating that levels of *Clostridium* spp. are consistently lower in breast-fed infants compared to infants fed infant formula (Conway, 1997). The secondary objectives of the study included a comparison of tolerance, and an evaluation of changes in both the total microbiota and specific groups of other fecal bacteria in infants fed formula with different blends of prebiotics (e.g. *Clostridium* spp. Clusters I, XI and XIV, bifidobacteria, lactobacilli, and enterobacteria). A human milk-fed group was also included as a reference group.

1. STUDY DESIGN

This study was a multi-center, double-blind, randomized, controlled, parallel-group, prospective trial conducted in the United States. Healthy, term, formula-fed infants who were 14 to 90 days old and had weight for length $\geq 10^{th}$ percentile and $\leq 90^{th}$ percentile on standard growth charts were randomized to one of four study formulas: 1) a marketed control formula (Enfamil LIPIL with Iron), control; 2) the control formula supplemented with 4 g/L of a prebiotic blend containing PDX and GOS (1:1 ratio), designated PG4; 3) the control formula supplemented with 4 g/L of a prebiotic blend containing PDX, GOS, and lactulose (LOS) (3:2:1 ratio), designated PGL4; 4) the control formula supplemented with 8 g/L of a prebiotic blend containing PDX, GOS, and LOS (3:2:1 ratio), designated PGL8). A group of human milk-fed infants was included as a reference group.

As stated above, the primary objective of this study was to compare the change from baseline in the fecal populations of specific *Clostridium* species (sum of clusters I and XI) among groups of infants fed formulas containing different prebiotic blends, control formula or human milk for a period of 28 days. It was determined that a sample size of 17 infants per formula group was needed for the study to have a power of 80% to detect a difference of one standard deviation unit of change from baseline of *Clostridium* (sum of clusters I and XI) (α=0.05). The study protocol called for enrollment of approximately 100 infants in order to obtain 17 completed participants per group after accounting for study dropouts. A parent or caregiver was required to bring the participant to the study site 4 times for data collection: Visit 1, enrollment, study day 1; Visit 2, randomization to study formula, study day 8±3; Visit 3, after 14 days of study formula, study day 22±3; Visit 4, after 28 days of study formula, study day 36±3, last day of study.

Parents who were willing for their infants to participate in the study were asked to sign a written informed consent document and an authorization to use and/or disclose protected health information. Study site personnel verified that the infant was eligible for the study. Weight, length, and head circumference were measured at this time (Visit 1) and formula-fed infants were placed on marketed Enfamil LIPIL infant formula and stayed on

this formula until Visit 2. Visit 2 occurred the 8th day after enrollment after infants had received marketed Enfamil LIPIL infant formula for 7 days. On Visit 2, study-site personnel randomized formula-fed infants into one of the study formula groups and parents were given enough of the assigned study formula to feed their infants for 28 days. On Visits 2, 3, and 4 parents provided completed 24 hour diet and tolerance diaries and study-site personnel collected stool samples. Anthropometric measurements were also collected on the last day of the study (Visit 4). Adverse events were recorded throughout the study as they occurred.

2. DATA COLLECTION METHODS

Data on dietary intake and formula tolerance (levels of fussiness and gas) were recorded by parents on a daily basis throughout the study. Parents were asked to record the amount of study formula consumed in the 24 hours prior to each study visit. Parents also recorded the number and general consistency of bowel movements that occurred during the 24 hours prior to each study visit. Stool consistency was rated on a defined scale of 0-5, with 0 denoting no bowel movement and 5 denoting watery stool. Parents were also asked to report incidences of diarrhea and constipation in the 24 hours preceding each study visit (based on definitions provided). Diarrhea was defined as an increased number of bowel movements and/or increased amount of water in the stool resulting in loose or watery stools for the last 24 hours. Constipation was defined as more than one day with no bowel movement followed by a hard stool, or three or more days without a bowel movement.

Infants were weighed to the nearest gram or ounce on a standard pediatric balance, without clothing or diaper. Body length was measured in triplicate to the nearest cm or 1/4 inch using a length board. Head circumference was measured in triplicate to the nearest cm or 1/4 inch with a flexible, non-stretchable cloth or vinyl tape.

Adverse events were recorded at each study visit; an adverse event was defined as any unfavorable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with participation in the clinical study, whether or not related to the study product. An adverse event was considered serious if it met one or more of the following criteria: resulted in death, was life-threatening, required inpatient hospitalization or prolongation of existing hospitalization; resulted in persistent or significant disability/incapacity, or was a congenital anomaly/birth defect. If the investigator was not the participant's primary physician, the investigator was asked to obtain copies of the participant's medical records for the study period to determine if an adverse event had occurred during the study. If such records were not available, information received from the parents was to serve as a source document for reporting adverse events.

Study personnel collected the infant stool samples from chemical-free diapers (Tushies®, TenderCare International, Inc., Eau Claire, WI) at Visits 2, 3, and 4. Samples were collected no later than 2 hours after defecation. The samples were divided into two

separate aliquots and handled as follows: 1) sample one was transferred by scoop into a 15 mL transport container and shipped under ambient conditions to a central clinical laboratory (North Coast Clinical Laboratory, Inc., Sandusky, OH) for the enumeration of *Enterobacteriaceae*; and 2) sample two was similarly transferred into a 15 mL transport container, immediately frozen at -20° C or below, and then shipped on cold packs to a second central laboratory (Institute for Genomic Biology, University of Illinois, Urbana-Champaign, IL) for evaluation.

Enterobacteriaceae were enumerated in the first (ambient) sample set by standard dilution plating to appropriate selective media (e.g. MacConkey's Agar, Eosin Methylene Blue (EMB) Agar, etc.). Quantitative Real-Time Polymerase Chain Reaction (qPCR) was used to quantify the abundance of *Clostridium* spp. (clusters I, XI and XIV), *Bifidobacterium* spp., *Lactobacillus* spp., as a percentage of total bacterial 16S rDNA in the second set of frozen fecal samples. Genomic DNA was obtained using previously described phenol-based extraction methods (Tsai and Olson, 1991). The isolated bacterial genomic DNA from the fecal samples were used as templates for PCR amplification in a GeneAmp 5700 Sequence Detection System using primers specific for each bacterial species and 2X SYBR® Green PCR Master Mix. The primary outcome measure was based on the sum of clostridia detected using primers specific for clusters I and XI (subgroups of different species of clostridia). Microbial community profile analysis was conducted using Denaturing Gradient Gel Electrophoresis (DGGE) as described previously (Muyzer and Smalla, 1998). This analysis is a genetic fingerprinting technique used to examine microbial community profiles within and across feeding groups.

3. RESULTS

One hundred and four formula-fed infants (25 Control, 27 PG4, 27 PGL4, 25 PGL8) enrolled in this study and consumed infant formula. A group of 30 human milk-fed infants were also enrolled. Of these, 117 infants (21 Control, 23 PG4, 23 PGL4, 21 PGL8, and 29 HM) completed the study.

Birth Characteristics

Two statistically significant differences in birth characteristics (i.e. weight, length, head circumference, gender, race, ethnicity) were noted among study groups at enrollment. The first occurred in the mean birthweight where that of the control group (3274.4 g/ s.e. 91.7) was significantly less than that of the PGL8 group (3649.1 g/ s.e.95.4) (p-value, 0.024). The second occurred in race where the relative proportions of the control group (white, n = 14 (56%); black, n = 11 (44%); more than one race = 0 (0%) differed from the human milk-fed reference group (white = 25 (83%); black = 1 (3%); more than one race = 4 (13%) (p-

value, 0.002). The study groups did not differ significantly in age at study entry and age at enrollment ranged from 13 days (minimum) to 92 days (maximum).

Growth

Statistically significant differences were noted among the groups in mean weight-for-age z-scores at both time points measured (visit 1, p=0.004 overall; visit 4, p=0.022 overall), as shown in Table 14. Mean weight-for-age z-scores at Visit 1 were 0.5±0.2 (HM), -0.0±0.2 (C), 0.4±0.2 (PG4), 0.0±0.2 (PGL4), and 0.8±0.2 (PGL8). Statistically significant differences were observed at Visit 1 between the HM and C groups (p=0.024), between the HM and PGL4 groups (p=0.048), between the C and PGL8 groups (p<0.001), and between the PGL4 and PGL8 groups (p=0.002).

Statistically significant differences were also noted among the groups in mean weight-for-age z-scores at Visit 4. At this visit, mean weight-for-age z-scores were 0.6±0.2 (HM), 0.2±0.2 (C), 0.7±0.2 (PG4), 0.4±0.2 (PGL4), and 1.0±0.2 (PGL8). Statistically significant differences were observed between the C and PG4 groups (p=0.022), between the C and PGL8 groups (p=0.002), and between the PGL4 and PGL8 groups (p=0.028). As statistically significant differences in mean weight-for-age z-scores were evident at both the beginning (Visit 1) and end (Visit 4) of the study, and no particular pattern was evident in the response of the treatment groups, it is unlikely that prebiotic supplementation affected growth. This is supported by the observation that all groups exhibited normal growth over the study period and the differences that were seen were not considered to be clinically significant.

Table 14. Weight-for-Age Z-Scores

Week	Study Group	n	Mean[1]	(s e)	Comparison	p-value
Visit 1	HM	30	0 5	(0 2)	Overall	0 004*
	Control	25	-0 0	(0 2)	HM vs Control	0 024*
	PG4	27	0 4	(0 2)	HM vs PG4	0 627
	PGL4	27	0 0	(0 2)	HM vs PGL4	0 048*
	PGL8	25	0 8	(0 2)	HM vs PGL8	0 254
					Control vs PG4	0 074
					Control vs PGL4	0 744
					Control vs PGL8	<0 001*
					PG4 vs PGL4	0 133
					PG4 vs PGL8	0 105
					PGL4 vs PGL8	0 002*
Visit 4	HM	29	0 6	(0 2)	Overall	0 022*
	Control	22	0 2	(0 2)	HM vs Control	0 119
	PG4	24	0 7	(0 2)	HM vs PG4	0 434
	PGL4	24	0 4	(0 2)	HM vs PGL4	0 590

Week	Study Group	n	Mean[1]	(s e)	Comparison	p-value
	PGL8	21	1 0	(0 2)	HM vs PGL8	0 091
					Control vs PG4	0 022*
					Control vs PGL4	0 305
					Control vs PGL8	0 002*
					PG4 vs PGL4	0 191
					PG4 vs PGL8	0 348
					PGL4 vs PGL8	0 028*

Repeated Measures Period*Group p-value=0 191 Period p-value=<0 001*
*Statistically significant
[1]Z-scores are based on NCHS reference data

Statistically significant differences were also noted among the groups in mean length-for-age z-scores at both time points measured (Visit 1, p=0.021 overall; Visit 4, p=0.038 overall), as shown in Table 15. Mean length-for-age z-scores at Visit 1 were 0.3±0.2 (HM), -0.0±0.2 (C), 0.5±0.2 (PG4), -0.1±0.2 (PGL4), and 0.5±0.2 (PGL8). Statistically significant differences were observed between the HM and PGL4 groups (p=0.049), between the C and PG4 groups (p=0.034), between the C and PGL8 groups (p=0.044), between the PG4 and PGL4 groups (p=0.008), and between the PGL4 and PGL8 groups (p=0.012) at Visit 1.

Statistically significant differences were also noted among the groups in mean length-for-age z-scores at Visit 4. At this visit, mean length-for-age z-scores were 0.3±0.2 (HM), 0.2±0.2 (C), 0.6±0.2 (PG4), -0.0±0.2 (PGL4), and 0.6±0.2 (PGL8). Statistically significant differences were observed between the PG4 and PGL4 groups (p=0.011) and between the PGL4 and PGL8 groups (p=0.009). As statistically significant differences in mean length-for-age z-scores were evident at both the beginning (Visit 1) and end (Visit 4) of the study, and no particular pattern was evident in treatment groups, it is unlikely that prebiotic supplementation affected growth. This is again supported by the observation that all groups exhibited normal growth over the study period and the differences that were seen were not considered to be clinically significant.

Table 15. Length-for-Age Z-Scores.

Week	Study Group	n	Mean[1]	(s e)	Comparison	p-value
Visit 1	HM	30	0 3	(0 2)	Overall	0 021*
	Control	25	-0 0	(0 2)	HM vs Control	0 139
	PG4	27	0 5	(0 2)	HM vs PG4	0 497
	PGL4	27	-0 1	(0 2)	HM vs PGL4	0 049*
	PGL8	24	0 5	(0 2)	HM vs PGL8	0 553
					Control vs PG4	0 034*
					Control vs PGL4	0 644
					Control vs PGL8	0 044*
					PG4 vs PGL4	0 008*

Week	Study Group	n	Mean[1]	(s e)	Comparison	p-value
					PG4 vs PGL8	0 949
					PGL4 vs PGL8	0 012*
Visit 4	HM	29	0 3	(0 2)	Overall	0 038*
	Control	22	0 2	(0 2)	HM vs Control	0 489
	PG4	24	0 6	(0 2)	HM vs PG4	0 263
	PGL4	24	-0 0	(0 2)	HM vs PGL4	0 140
	PGL8	21	0 6	(0 2)	HM vs PGL8	0.235
					Control vs PG4	0 078
					Control vs PGL4	0 453
					Control vs PGL8	0 068
					PG4 vs PGL4	0 011*
					PG4 vs PGL8	0 914
					PGL4 vs PGL8	0 009*

Repeated Measures Period*Group p-value=0 789 Period p-value=0 030*
*Statistically significant
[1]Z-scores are based on NCHS reference data

No statistically significant differences were noted among groups in mean head circumference-for-age z-scores at either Visit 1 (p=0.315 overall) or Visit 4 (p=0.613 overall) (Data not shown).

Stool Characteristics

There were no significant differences among study groups at any time point in the number of participants experiencing constipation (Data not shown) or diarrhea (Table16). Though of shorter duration than the 4-month growth study discussed previously, the lack of an effect of feeding regimen on the incidence of diarrhea during 28 days, supports the general safety of the prebiotic carbohydrates PDX, GOS and LOS.

Table 16. Number of Participants Experiencing Diarrhea

Period	Study Group	N	(%)	Comparison	p-value
Run-in	HM	2	(7)	HM vs Control	0 724
	Control	6	(25)	HM vs PG4	0 994
	PG4	4	(16)	HM vs PGL4	0 939
	PGL4	5	(19)	HM vs PGL8	0 920
	PGL8	5	(21)	Control vs PG4	0 999
				Control vs PGL4	1 000
				Control vs PGL8	1 000
				PG4 vs PGL4	1 000
				PG4 vs PGL8	1 000
				PGL4 vs PGL8	1 000

Period	Study Group	N	(%)	Comparison	p-value
Week 1	HM	3	(10)	HM vs Control	1 000
	Control	3	(13)	HM vs PG4	1 000
	PG4	4	(16)	HM vs PGL4	0 236
	PGL4	10	(37)	HM vs PGL8	0 957
	PGL8	6	(25)	Control vs PG4	1 000
				Control vs PGL4	0 452
				Control vs PGL8	0 998
				PG4 vs PGL4	0 726
				PG4 vs PGL8	0 999
				PGL4 vs PGL8	0 992
Week 2	HM	7	(24)	HM vs Control	1 000
	Control	6	(26)	HM vs PG4	1 000
	PG4	4	(17)	HM vs PGL4	0 999
	PGL4	4	(15)	HM vs PGL8	1 000
	PGL8	7	(32)	Control vs PG4	1 000
				Control vs PGL4	0 999
				Control vs PGL8	1 000
				PG4 vs PGL4	1 000
				PG4 vs PGL8	0 977
				PGL4 vs PGL8	0 972
Week 3	HM	4	(14)	HM vs Control	1 000
	Control	4	(17)	HM vs PG4	1 000
	PG4	3	(13)	HM vs PGL4	1 000
	PGL4	5	(21)	HM vs PGL8	1 000
	PGL8	4	(18)	Control vs PG4	1 000
				Control vs PGL4	1 000
				Control vs PGL8	1 000
				PG4 vs PGL4	1 000
				PG4 vs PGL8	1 000
				PGL4 vs PGL8	1 000
Week 4	HM	2	(7)	HM vs Control	1 000
	Control	3	(14)	HM vs PG4	1 000
	PG4	1	(4)	HM vs PGL4	1 000
	PGL4	3	(13)	HM vs PGL8	0 992
	PGL8	4	(19)	Control vs PG4	0 986
				Control vs PGL4	1 000
				Control vs PGL8	1 000
				PG4 vs PGL4	1 000
				PG4 vs PGL8	0 857
				PGL4 vs PGL8	1 000
Study	HM	11	(38)	HM vs Control	1 000
	Control	7	(29)	HM vs PG4	1 000
	PG4	8	(32)	HM vs PGL4	1 000
	PGL4	13	(48)	HM vs PGL8	1 000
	PGL8	9	(38)	Control vs PG4	1 000
				Control vs PGL4	0 944
				Control vs PGL8	1 000
				PG4 vs PGL4	0 957
				PG4 vs PGL8	1 000
				PGL4 vs PGL8	1 000

Stool frequency per day was significantly different between the human milk reference group and all formula groups during the run-in period but not during the study period as a whole (Table 17). The formula groups did not differ in daily stool frequency during either period. During the run-in period, infants in the human milk group had more stools per day in comparison to formula-fed infants. Though the average number of stools per day remained relatively constant during the study for formula-fed infants, that number declined for human milk-fed infants. Thus the differences detected among the study groups in the run-in period disappeared by the conclusion on the study.

Table 17. Stool Frequency per Day by Study Period.

Period	Study Group	N	Mean	(s e)	Comparison	p-value
Run-in	HM	29	3 6	(0 3)	HM vs Control	<0 001*
	Control	24	1 6	(0 3)	HM vs PG4	0 018*
	PG4	25	2 3	(0 3)	HM vs PGL4	<0 001*
	PGL4	27	2 0	(0 3)	HM vs PGL8	<0 001*
	PGL8	24	1 8	(0 3)	Control vs PG4	0 636
					Control vs PGL4	0 993
					Control vs PGL8	1 000
					PG4 vs PGL4	0 992
					PG4 vs PGL8	0 908
					PGL4 vs PGL8	1 000
Study	HM	29	2 8	(0 3)	HM vs Control	0 154
	Control	24	1 9	(0 3)	HM vs PG4	0 329
	PG4	25	2 0	(0 3)	HM vs PGL4	0 415
	PGL4	27	2 1	(0 3)	HM vs PGL8	0 502
	PGL8	24	2 1	(0 3)	Control vs PG4	1 000
					Control vs PGL4	1 000
					Control vs PGL8	1 000
					PG4 vs PGL4	1 000
					PG4 vs PGL8	1 000
					PGL4 vs PGL8	1 000

Repeated Measures Period*Group p-value=<0 001* Period p-value=0 424
*Statistically significant

Statistically significant differences were observed in stool consistency between the human milk group and each of the formula groups during the run-in period and the study period as a whole (Table 18). Infants fed human milk had stools that were more soft/loose compared with infants fed formula. Stool consistency among infants in the formula groups did not differ during either period. These data suggest that a softer/looser stool pattern is more typical of human milk fed infants in comparison to formula fed infants. It is informative to consider that in the preceeding 4 month feeding study, while prebiotic consumption was

associated with a shift toward a softer/looser stools, the laxation pattern of infants fed human milk described in the current study appeared even closer to the fluid side of the normal hard to watery continuum.

Table 18. Stool Consistency by Study Period.

Period	Study Group	N	Mean[1]	(s e)	Comparison	p-value
Run-in	HM	29	3 8	(0 1)	HM vs Control	0 004*
	Control	24	3 3	(0 1)	HM vs PG4	0 004*
	PG4	25	3 3	(0 1)	HM vs PGL4	<0 001*
	PGL4	27	3 2	(0 1)	HM vs PGL8	<0 001*
	PGL8	24	3 1	(0 1)	Control vs PG4	1 000
					Control vs PGL4	1 000
					Control vs PGL8	0 976
					PG4 vs PGL4	1 000
					PG4 vs PGL8	0 971
					PGL4 vs PGL8	1 000
Study	HM	29	3 8	(0 1)	HM vs Control	<0 001*
	Control	24	3 2	(0 1)	HM vs PG4	0 049*
	PG4	25	3 4	(0 1)	HM vs PGL4	0 012*
	PGL4	27	3 4	(0.1)	HM vs PGL8	0 039*
	PGL8	24	3 4	(0 1)	Control vs PG4	0 732
					Control vs PGL4	0 927
					Control vs PGL8	0 822
					PG4 vs PGL4	1 000
					PG4 vs PGL8	1 000
					PGL4 vs PGL8	1 000

Repeated Measures Period*Group p-value=0 187 Period p-value=0 019*
*Statistically significant
[1]Rating Scale 1=Hard 2=Formed 3=Soft 4=Loose 5=Watery

Tolerance

Tolerance of the prebiotic carbohydrates was assessed in this study by evaluating discontinuations, stool number and consistency, fussiness, gas, diarrhea, and constipation. Infants who discontinued the study prior to Visit 4 were classified by the investigators into those who discontinued for reasons associated with study formula and those who discontinued for reasons not related to study formula. No statistical difference was detected among the formula-fed groups in discontinuation status (Table 19) which included 2 infants in the Control group (8%), 1 (4%) in PG4 group, none in the PGL4 group, and 1 (4%) in the PGL8 group discontinuing from the study for reasons associated with study feedings.

Table 19. Relationship of Study Discontinuation to Study Formula

Study Group	Completed N	Completed %	Discontinued Due to Formula n	Discontinued Due to Formula %	Discontinued Not Due to Formula N	Discontinued Not Due to Formula %	Comparison	p-value
Control	21	(84)	2	(8)	2	(8)	Overall	0.914
PG4	23	(85)	1	(4)	3	(11)		
PGL4	23	(85)	0	(0)	4	(15)		
PGL8	21	(84)	1	(4)	3	(12)		

The reasons for study discontinuation are summarized in Table 20. Two product-related reasons for discontinuation were reported: 1) intolerance to study formula as judged by the investigator (Control, 1 infant; PG4, 1 infant; PGL4, 0 infants; and PGL8, 1 infant), and 2) parent elected to remove participant from the study (Control, 1 infant).

Table 20. Reasons for Study Discontinuation

	Reasons for Discontinuation	Control	PG4	PGL4	PGL8
Product-Related	Intolerance to study formula as judged by the investigator	1	1		1
	Parent elected to remove participant from the study	1			
Not Product-Related	Parent/care giver failed to comply with scheduled study visits	1	2	1	
	Parent elected to remove participant from the study	1		1	2
	Participant lost to follow-up after 3 unsuccessful attempts to contact parent or guardian				1
	Prohibited medications		1	2	

Table 21 summarizes the types of intolerance to formula resulting in discontinuation from the study. Vomiting and diarrhea were reported for the single Control infant who discontinued due to formula intolerance. Fussiness and diarrhea resulted in study discontinuation for one PG4 infant. Vomiting, fussiness, diarrhea and gas were formula intolerances reported for the one PGL8 infant who discontinued due to formula intolerance.

Table 21. Feeding Intolerances‡ Resulting in Study Discontinuation

	Study Group		
	Control	PG4	PGL8
Vomiting	1		1
Fussiness		1	1
Diarrhea	1	1	1
Gas			1

‡More than one intolerance may have been reported for a participant.

Adverse Events

Adverse events were monitored throughout the study. An adverse event was defined as any unfavorable and unintended symptom or disease temporally associated with the participation in a clinical study whether or not related to study product. There were no differences among study groups in the number of participants who experienced at least 1 adverse event: 12 infants (40%) in the HM group, 12 (48%) in the C group, 13 (48%) in the PG4 group, 9 (33%) in the PGL4 group, and 13 (52%) in the PGL8 group (p=0.656). Additionally, there were no differences among study groups in the number of participants who experienced adverse events when analyzed by body system or by individual event, as shown in Table 22.The medical director reviewed all adverse events and determined that there were no adverse events of particular clinical significance.

Table 22. Adverse Events.

Event	HM n	HM (%)	Control n	Control (%)	PG4 n	PG4 (%)	PGL4 N	PGL4 (%)	PGL8 N	PGL8 (%)	Overall Comparison p-value
Body As A Whole	2	(7)	1	(4)	6	(22)	3	(11)	6	(24)	0 126
Reaction To Vaccine	1	(3)	1	(4)	2	(7)	2	(7)	3	(12)	0 728
Irritability	1	(3)	0	(0)	2	(7)	0	(0)	3	(12)	0 165
Infection	0	(0)	0	(0)	2	(7)	1	(4)	0	(0)	0 342
Pain	0	(0)	0	(0)	0	(0)	1	(4)	0	(0)	0 776
Eyes, Ears, Nose And Throat	2	(7)	3	(12)	2	(7)	3	(11)	1	(4)	0 854
. Thrush	1	(3)	1	(4)	2	(7)	1	(4)	0	(0)	0 868
. Nasal Congestion	1	(3)	1	(4)	0	(0)	0	(0)	1	(4)	0 738
Conjunctivitis	0	(0)	2	(8)	0	(0)	1	(4)	0	(0)	0 158
Nasal/Tear Duct Obstructions	0	(0)	0	(0)	0	(0)	1	(4)	0	(0)	0 776
Gastrointestinal	5	(17)	6	(24)	5	(19)	4	(15)	8	(32)	0 582
Gas	2	(7)	3	(12)	3	(11)	2	(7)	5	(20)	0 608
Constipation	1	(3)	2	(8)	0	(0)	1	(4)	1	(4)	0 704
G E. Reflux	0	(0)	0	(0)	1	(4)	1	(4)	2	(8)	0 318
Diarrhea	1	(3)	1	(4)	1	(4)	0	(0)	1	(4)	0 915
Colic	1	(3)	1	(4)	0	(0)	0	(0)	0	(0)	0 818
Acute Gastroenteritis	0	(0)	1	(4)	0	(0)	0	(0)	1	(4)	0 265
. Emesis	0	(0)	1	(4)	0	(0)	0	(0)	0	(0)	0 373
. Umbilical Hernia	1	(3)	0	(0)	0	(0)	0	(0)	0	(0)	1 000
Metabolic And Nutrition	1	(3)	1	(4)	0	(0)	0	(0)	0	(0)	0 818
Abnormal Laboratory Result	1	(3)	1	(4)	0	(0)	0	(0)	0	(0)	0 818
Musculoskeletal	1	(3)	0	(0)	0	(0)	0	(0)	0	(0)	1 000
Trauma	1	(3)	0	(0)	0	(0)	0	(0)	0	(0)	1 000
Respiratory	1	(3)	2	(8)	1	(4)	2	(7)	1	(4)	0 907
URI	0	(0)	2	(8)	1	(4)	1	(4)	1	(4)	0 595
Cough	1	(3)	0	(0)	0	(0)	0	(0)	0	(0)	1 000
Bronchiolitis	0	(0)	0	(0)	0	(0)	1	(4)	0	(0)	0 776
Skin	2	(7)	4	(16)	2	(7)	1	(4)	5	(20)	0 295
Seborrhea	1	(3)	1	(4)	0	(0)	0	(0)	3	(12)	0 128
Eczema	1	(3)	1	(4)	1	(4)	0	(0)	1	(4)	0 915
Yeast Infection	0	(0)	1	(4)	1	(4)	0	(0)	0	(0)	0 568
Other Rash	0	(0)	1	(4)	0	(0)	0	(0)	1	(4)	0 265
Neonatal Acne	1	(3)	0	(0)	0	(0)	1	(4)	0	(0)	1 000
Urogenital	0	(0)	0	(0)	0	(0)	1	(4)	1	(4)	0 568
Urinary Tract Infection	0	(0)	0	(0)	0	(0)	1	(4)	0	(0)	0 776
Labial Adhesions	0	(0)	0	(0)	0	(0)	0	(0)	1	(4)	0 373

Bacterial Analysis

Variation in bacterial populations, in response to the consumption of infant formula containing two levels (4 or 8 g/L) of two or three prebiotics (PDX, GOS, or LOS) for ~28 days, was evaluated in three ways. A total of 370 fecal samples were collected and *Enterobacteriaceae* were enumerated from an unfrozen fecal aliquot by standard dilution plating to selective media. A molecular method, quantitative real-time PCR (qPCR), was utilized to quantify *Clostridium* clusters I, XI, and XIV, lactobacilli and bifidobacteria, which were calculated as a percentage of total bacterial 16S rDNA. A second molecular method, denaturing gradient gel electrophoresis (DGGE), was utilized to measure change in total fecal bacteria (microbiota). Regardless of the methodology employed, few statistically significant differences and/or changes were noted during the course of this 28 day feeding trial.

A slight but statistically significant increase ($p = 0.027$) from a mean of 7.1 to 7.3 $\log_{10}$ CFU/gram stool was observed in the fecal *Enterobacteriaceae* populations of infants fed experimental formula containing 8 g/L of a mixture of PDX, GOS and LOS (50:33:17 ratio) (PGL8) between the baseline and 28 day measurements. Repeated measures analysis revealed significantly higher levels of *Enterobacteriaceae* in the human milk reference group (7.9) when compared with all other groups at baseline (e.g. Control = 7.1, $p < 0.001$; PG4 = 7.2, $p < 0.001$; PGL4 = 7.1 $p < 0.001$; and PGL8 = 7.1 $p < 0.001$). The differences between groups were short lived however, disappearing by 4 weeks. The early predominance of Enterobacteriaceae in the breast-fed infants was unexpected as these bacteria reportedly occur at higher levels in formula fed infants (Penders et al., 2005).

No significant differences were measured in the abundance of *Clostridium* Cluster I (the *C. botulinum* group; including *C perfringens, C paraputrificum, C barati, C beijerinckii, C. novyi* etc.) at any point in the study. The abundance of *Clostridium* Cluster XI (the *C. lituseburense* group; including *C. difficile, C. felsineum, C. bifermentans,* etc) was found to be consistently lower in infants fed human milk compared to infants fed formula or prebiotic supplemented formulas. At baseline, the differences between the human milk fed group (0.0% of 16S bacterial rDNA) and the prebiotic supplemented groups were statistically significant (PG4 = 0.3%; p 0.006; PGL4 = 0.3%; $p <0.001$; and PGL8 = 0.2%, p 0.016). At day 14, the differences between the human milk fed group (0.0%) and all other groups were statistically significant (Control = 0.1%, p 0.011; PG4 = 0.4%, $p < 0.001$; PGL4 = 0.2%, p 0.003; and PGL8 = 0.1%, p 0.019). At day 28, only the differences between the human milk fed group (0.0%) and both the PG4 (0.5%, p 0.005) and PGL4 (0.2%, p 0.015) remained statistically significant. In addition, a significant decrease was detected in the fecal samples of the PGL8 group between baseline (0.2%) and day 28 (0.1%) (p 0.049), indicating that relative to *Clostridium* Cluster XI, the performance of the PGL8 group was closest to that of the human milk fed reference. No significant differences were noted in the abundance of the

Clostridium Cluster XIV (the *C coccoides* group including *C. nexile, C aminovalericum, Eubacterium eligens, E. rectale, Butyrivibrio crossotus* etc.) at any point in the study. Finally, when the abundance of *Clostridium* Clusters I and XI was evaluated together, no significant differences and/or changes occurred at any point in the 28 day feeding trial. In combination, these results suggest that while these *Clostridium* clusters were modestly lower in human milk fed infants, feeding regimen had little measurable effect on this bacterial genus.

The abundance of *Lactobacillus* detected in the fecal samples of infants consuming human milk was not statistically different from those consuming control formula or formula supplemented with any prebiotic combination. During the course of the study, the only significant change occurred in the prebiotic group supplemented with PDX, GOS and LOS at the higher level (8 g/L) where the *Lactobacillus* population declined from 3.5% of 16S bacterial rDNA to 2.0% (p 0.013). Interestingly, this genus also declined in both the human milk fed group (3.5% to 2.7%) and PGL4 group (3.1% to 2.5%), though the changes were not significant. Significant differences and/or changes in the *Bifidobacterium* group were not observed at any point in the feeding trial although the values in the human milk fed reference group were slightly higher (data not shown). This result is not without precedent as a recent evaluation of fecal bifidobacteria in 50 formula-fed and 50 breast-fed infants detected comparable levels (10.24 $\log_{10}$ and 10.56 $\log_{10}$ CFU g^{-1} wet feces, respectively) (Penders et al., 2005).

Potential relationships and change in individual and collective microbial community profiles in response to feeding regimen were evaluated by comparing 16S rDNA PCR-denaturing gradient gel electrophoresis (DGGE) profiles. The individual bands in a DGGE profile are generated at the subspecies level and the collective pattern (fingerprint) is reflective of the entire bacterial population in a given sample. The DGGE profiles of fecal samples collected from the same infant throughout the study were run together in order to minimize the influence of electrophoresis conditions and gel quality. 10 ng of template fecal DNA was amplified using bacterial universal 16S-V3 primers and the amplicons separated through a 35% to 65% linear DNA-denaturing gradient gel using the BioRad D-Code system. After electrophoresis, the DGGE gels were silver stained, scanned using a GS-710 calibrated imaging densitometer (BioRad) and the images analyzed and compared by Diversity Database (BioRad) using the Dice Coefficient method (Magurran et al., 1988). Clustering analysis was performed using dendrograms and principal component analysis (PCA) profiles created by the Diversity Database software, based on the similarity comparison. Both methods enabled the visualization of the relationship between samples, by clustering highly similar samples together and dissimilar samples further apart.

Using both methods, samples derived from the same individual tended to cluster together (data not shown). No specific clustering patterns were observed relative to feeding regimen (treatment), age, or race indicating that the individual microbial profiles were both

idiosyncratic in composition and stable through the course of the 28 day study. The DGGE profiles (fingerprints) generated for the same individual at the three sample times were compared across intervals (e.g. Baseline-14 days, 14-28 days, Baseline-28 days). The similarity index, which is taken as a measure of microbial stability over a given time period (e.g. higher index values indicates greater stability), were not significantly different although the similarity scores were slightly higher in the human milk fed and control formula fed infants (data not shown). The only statistically significant differences occurred between the Control and PGL4 groups during the baseline-14 day period ($p < 0.05$) and between the human milk and PG4 groups during the 14-28 day period ($p < 0.01$). In both cases, the greatest change in microbial profile occurred in one of the experimental groups (PGL4 or PG4) where the feeding regimen had shifted after a baseline period on control formula in comparison to a group whose nutrition had remained constant (control or human milk, respectively) throughout the study.

4. DISCUSSION

This study used molecular approaches developed in the field of microbial ecology to evaluate the response of fecal microbiota in infants consuming breast milk, standard infant formula (Control) and infant formula containing combinations of the prebiotic oligosaccharides polydextrose (PDX), galactooligosaccharides (GOS) and lactulose (LOS). The study evaluated the microbiota in a total of 134 infants, a population that was expected to be of sufficient size to allow the characterization of both intra- and interindividual variation in response to feeding regimen. Changes in the various DGGE profiles were not statistically significant and the absence of feeding-specific clustering patterns most likely reflects a large interindividual variation in microbial composition. This outcome is comparable to some reports for humans and other species. Lay and colleagues studied fecal microbiota of 91 northern Europeans by fluorescent in situ hybridization (FISH), and observed no significant grouping with respect to geographic origin, age, or gender (Lay et al., 2005)). Minamida and colleagues studied the effects of di-D-fructofranose-1,2':2,3'-dianhydride (DFA III) on human intestinal microbiota using DGGE (Minamida et al., 2004). They found no treatment-specific clustering of DGGE profiles, but observed some shift of microbiota in a few of the unhealthy subjects treated with DFA III. Extensive interindividual variation in microbiota has also been demonstrated in other mammalian species (Simpson et al., 2002; Vaahtovuo et al., 2001; Bernbom et al., 2006). Based on these collective findings, it is likely that an individual microbial profile consisting of hundreds of species is too complex for subtle changes (as might accompany a dietary change) to be observed. This could derive from the sensitivity of methodologies designed to measure complex, bacterial populations (e.g. DGGE) which generate sufficient noise (bands generated from total commensal microbiota) to mask signals of interest (bands generated from beneficial bacteria like *Bifidobacterium* spp.).

There is increasing evidence that in addition to age, delivery mode and nutrition, host genetic factors have a significant effect on composition of the microbiota. Zoetendal and colleagues assessed the DGGE profiles of homozygotic (identical) twins and found them to have a higher degree of similarity than dizygotic (fraternal) twins, with the fecal DGGE profiles of both twin groups being significantly more similar than unrelated individuals (Zoetendal et al., 2001). They also demonstrated that the fecal microbiota of spouses, sharing similar diets and environment, were not more similar than unrelated individuals living separately. Studies with inbred mice also provide clear evidence that host genetics affects the composition of the fecal microbiota. Toivanen and colleagues studied fecal samples of six inbred mouse strains congenic for the major histocompatibility complex (MHC) and demonstrated that the MHC haplotype alone had a pronounced effect on the composition of microbiota (Toivanen et al., 2001). They also demonstrated that genes outside the MHC have an effect, as the microbiota of mice with same MHC haplotype, on a different genetic background, differ significantly from each other. Considering these findings, it is likely that the large interindividual differences observed in this study reflects, at least partially, the genetic variation expected of U.S. populations.

The fecal microbiota of breast-fed infants is generally thought to be characterized by higher numbers of *Bifidobacterium* and lower numbers of diverse *Clostridium* spp. (Copperstock and Zedd, 1983; Tannock, 1994). The results of recent molecular methods-based studies have varied, finding comparable differences (Harmsen et al., 2000), or no differences in the levels of these genera between the two feeding types (Penders et al., 2005; Euler et al., 2005). Several studies have also suggested that differences in the microbiota of breast-fed and formula-fed infants are expressed at the species level (Penders et al., 2005; Rinne et al., 2005). As only group-specific primers were used in the present study, treatment-specific species effects would not have been detected._The qPCR data generated in this study revealed minimal differences between the microbiota of breast-fed and formula-fed infants, with the only significant difference being the lower level of *Clostridium* cluster XI in the former. A significant effect on microbiota was observed in infants consuming formula containing the higher level (8 g/L) of PDX, GOS and LOS (PGL8) as both *Lactobacillus* and *Clostridium* cluster XI decreased in this group. The decrease of *Clostridium* cluster XI in PGL8 group suggests that the consumption of prebiotic oligosaccharides might shift a specific subpopulation of the infant microbiota toward the pattern observed in breast-fed infants. It has been reported that the prevalence and counts of *C. difficile*, a member of *Clostridium* cluster XI, is lower in breast-fed infants than in formula-fed infants (Penders et al., 2005). It has also been reported that *Clostridium* cluster XI is significantly higher in autistic children compared to healthy children (Song et al., 2004)). Thus, a lower level of *Clostridium* cluster XI in intestinal microbiota might represent a beneficial influence on host microbiota.

Though clinically irrelevant differences were noted in anthropometric Z scores at the beginning and end of the 28 day trial, all groups exhibited normal growth over the study period. No differences occurred at any time point in discontinuation rate, intolerance or adverse events. Feeding regimen had no impact on diarrhea during the study but as expected (and as typically reported), the human milk fed reference group experienced softer/looser stools in comparison to all formula groups. Together, these data indicate that both the 4 g/L and 8 g/L concentrations of the PDX:GOS:LOS blends were well tolerated and safe during the 28 day trial.

5. CONCLUSIONS

While some statistically significant differences were observed among the feeding groups in anthropometric z-scores at both the initial and final visits, all groups exhibited normal growth over the study period and the differences seen were not considered to be clinically significant. The results of this study indicate that the consumption of infant formula containing blends of polydextrose (PDX), galactooligosaccharides (GOS) and lactulose (LOS) at 4 g/L and 8 g/L for 28 days is generally well tolerated, is safe, and supports normal growth. Statistically significant differences in stool consistency were noted between the human milk-fed reference group and all the formula groups with infants fed human milk having stools that were characterized as more soft/loose when compared with those consuming standard infant formula. One of the intended effects of PDX and GOS addition is the promotion of stool patterns more like those of typical breast fed infants. The fact that the laxation pattern displayed by the human milk fed reference group was scored as softer/looser than any of the prebiotic supplemented-formula fed groups, provides further support that the laxation effect observed at the 4 g/L and 8 g/L addition levels is well within the normal range.

Few statistically significant changes in the composition of infant microbiota in response to the consumption of prebiotic supplement formula were detected. However, due to the high level of interindividual variation that was observed in both microbial community profiles and the quantities of specific bacterial groups, definitive conclusions cannot be drawn from this study regarding the effect consuming PDX, GOS and LOS on infant colonic microbiota. Given the extent of variability in the microbial populations, this study may not have included a large enough sample size to detect potential effects attributable to prebiotic ingredients despite meeting study enrollment goals. Future studies evaluating the effects of such ingredients on infant colonic microbiota may benefit from larger sample sizes and increased uniformity of participant age at study entry. In closing it should be emphasized that the colonic microbiota of infants consuming infant formula, with and without prebiotic oligosaccharides, did not differ significantly from infants fed human milk in most measures.

D. Summary and Conclusions of MJN Cinical Trials of PDX, GOS and LOS in Infant Formula

Results from a 4-month feeding study demonstrated that infants who received milk-based formula supplemented with combinations of PDX and GOS at 4 g/L, or blends of GOS, PDX, and LOS at 8 g/L showed comparable growth to infants fed Enfamil LIPIL with Iron infant formula. Prebiotic carbohydrates at either the high or low level led to softer stools more like those of breastfed infants, indicating that the added prebiotics meet the intended effect of mimicking the performance of human milk oligossacharides. Out of 72 categories of adverse events reported by parents, statistically significant differences were detected among formula groups for 3 categories: irritability, diarrhea, and eczema. Given that 72 categories of adverse events were reported, the conclusion of a treatment difference for three events is within the 5% Type I error rate, suggesting the events were likely to have been unrelated to prebiotic consumption. The results of this clinical trial were published in *The Journal of Pediatric Gastroenterology and Nutrition* on March 1, 2007 (Ziegler *et al.* 2007).

The results of a 28 day fecal microbiota study indicated that the consumption of infant formula containing blends of polydextrose (PDX), galactooligosaccharides (GOS) and lactulose (LOS) at 4 g/L and 8 g/L for 28 days is generally well tolerated, is safe, and supports normal growth. Statistically significant differences in stool consistency were noted between the human milk-fed reference group and all the formula groups with infants fed human milk having stools that were characterized as more soft/loose when compared with those consuming standard infant formula. This finding is in keeping with frequent references that softer looser stools constitute the normal laxation pattern in breast-fed infants. It also supports the conclusion of the 4-month feeding trial that the consumption of infant formula supplemented with prebiotics yielded softer/looser stools, in effect shifting the laxation pattern toward that typically displayed by human milk fed infants. The observation that the laxation patterns displayed by infants consuming PDX, GOS and LOS for 28 days was less soft/loose than infants fed breast milk, further supports the safety and tolerability of these prebiotic ingredients at the proposed levels.

With the exception of consistently lower levels of *Clostridium* cluster XI in the human milk-fed reference group, and a reduction of this cluster and lactobacilli in the PGL8 group, the consumption of prebiotic supplemented formulas was not associated with statistically significant changes in the composition of infant microbiota. This lack of significant treatment effects is possibly due to the high level of interindividual variation that was observed in both microbial community profiles and the quantities of specific bacterial groups. As a result, definitive conclusions cannot be drawn from this study regarding the effect of consuming PDX, GOS and LOS on infant colonic microbiota.

Finally, a panel of experts independently concluded that GOS and PDX are safe for use in milk-based formulas at levels of up to 6 g/L and 4 g/L, repectively. Collectively, these results

have led Mead Johnson Nutritionals to select two prebiotic carbohydrates, GOS and PDX, for addition to milk-based formula at 2 g/L each to partially mimic the physiological impact of human milk oligosaccharides on stool characteristics and microbiota.

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A

Generally Recognized As Safe (GRAS) Determination for the Addition of Galactooligosaccharides as Vivinal® and Vivinal® GOS 10 to Infant Formula as a Prebiotic Ingredient In Combination with Polydextrose

Mead Johnson & Co.
Evansville, Indiana

August 2007

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I. DESCRIPTION OF SUBSTANCE

A. Identity

1. Common or Usual Name

The subject of this GRAS determination are Vivinal® GOS and Vivinal® GOS 10. These products contain, galacto-oligosacchrides (GOS) prepared with a β-galactosidase derived from *Bacillus circulans* (ATCC 31382). Vivinal® GOS 10 is a dried 1-1 (w/w) mixture of Vivinal® GOS and food-grade whey protein concentrate (WPC). In order to avoid confusion, henceforth the whole products are referred to as Vivinal® and Vivinal® GOS 10, and the term GOS refers only to the galactooligosaccharides.

2. Chemical Names

Chemical names for the substance, in addition to galactooligosaccharides or GOS, are β-1,4-galactooligosaccharide, 4'-galactooligosaccharide, 4'-galactosyllactose, transgalactosylated oligosaccharide, and oligogalactosyl-lactose. The systemic name of GOS is D-glucose, o-*beta*- D-galactopyranosyl-(1-4)-o-*beta*- D-galactopyranosyl-(1-4)-.

3. CAS Registry Number

The CAS Registry Number is 6587-31-1.

4. Composition

GOS are chains of galactose with a glucose end-cap ((galactose (Gal))$_n$-glucose (Glu); see the diagram of the chemical structure below). In enzymatically produced GOS, the saccharides vary in chain length from disaccharides to octasaccharides (Shin and Wang 1998; Albayark and Yang 2002). The major saccharide is the trisaccharide, accounting for about 39% by weight of the GOS, followed by the disaccharide, accounting for about 33% by weight of the GOS (*Table 1*). The linkage between the monomer units is mainly β(1-4), but other linkages such as β(1-2) and β(1-6) also occur.

a) Molecular Formula

The molecular formula for the trisaccharide, the predominant saccharide, is $C_{18}H_{32}O_{16}$. This formula and formulas for other saccharides is shown in *Table 1*.

b) Molecular Weight

As shown in *Table 1*, the molecular weights of the individual oligosaccharides range between 343 Daltons (disaccharide) and 1314 Daltons (octasaccharide). The average molecular weight of the GOS fraction is approximately 522 Daltons.

Table 1. Typical GOS Composition

Chain Length of Saccharide (Gal)nGlu		Empirical Formula	MW (Daltons)	Wt Fraction of GOS
Disaccharide (other than lactose)	n = 1	$C_{12}H_{22}O_{11}$	342.30	0 33
Trisaccharide	n = 2	$C_{18}H_{32}O_{16}$	504 44	0 39
Tetrasaccharide	n = 3	$C_{24}H_{42}O_{21}$	666 58	0 18
Pentasaccharide	n = 4	$C_{30}H_{52}O_{26}$	828 72	0 07
Hexa-, hepta-, octasaccharides	n = 5–7		Av = 1153	0 03

c) Chemical Structure

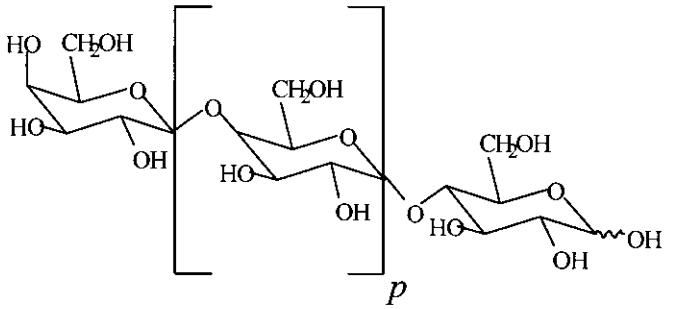

Figure 1. *Basic Structure of GOS*

5. Characterization

For determination of GOS and related saccharides, High Performance Anion Exchange Chromatography, coupled with Pulsed Amperometric Detection HPAEC-PAD is used. This method is approved by the AOAC as an official AOAC method (2001.02).

To identify the GOS fraction, HPLC with Refractive Index (RI) detection is used. For this analytical method, a silver column and an RI detector are used. The column is a Rezex Ag-column measuring 300 x 7.8 mm maintained at a temperature of 80°C; the flow rate is 0.3 mL/minute.

B. Manufacturing Process

GOS is formed by a transgalactosylation reaction when lactose is subjected to enzymatic hydrolysis by β-galactosidase. Factors such as source of the enzyme and reaction conditions determine the outcome in variables such as the types of linkage between the galactose units, the efficiency of transgalactosylation, and the components in the final product. The GOS component of Vivinal® is prepared from lactose utilizing β–galactosidase derived from *Bacillus circulans* (ATCC 31382); the glycosidic bonds produced are mainly β1-4 linkages.

000120

1. Raw Materials

Vivinal® GOS is prepared from lactose meeting the food grade specifications listed in *Table 3*, isolated from sweet whey. Lactose is a natural component of milk (milk sugar) and consists of the monomers galactose and glucose. Other materials used in the manufacturing process are listed in *Table 2*. The Product Specification Sheets are in *Appendix A*. All materials used in the production of Vivinal® and Vivinal® GOS10 are food grade.

Table 2. Materials Used to Produce Vivinal® and Vivinal® GOS 10

Raw Material	CAS No.	Use	Specifications
Lactose	63-42-3	Substrate	See Table 3
β-galactosidase	9031-11-2	Enzyme Catalyst	See Product Specification Sheet for Biolacta N5
Citric acid	77-92-9	Production Aid (pH Control)	See Product Specification Sheet for Citroenzuur
Sodium hydroxide	1310-73-2	Production Aid (pH Control)	See Product Specification Sheet for Natronloog 33%
Activated carbon	7440-44-0	Purification Aid	See Product Specification Sheet for Norit PN 2
Cellulose	9004-34-6	Purification Aid	See Product Specification Sheet for Alpha-Cel HKB200
Whey protein concentrate	9771-020-58	Texturizer	See Product Specification Sheet for 35% WPC
Water	7732-18-5	Solvent	Food use approval (21CFR scetion) N/A
Hydrochloric Acid	7647-01-0	Purification Aid	Food use approval (21CFR section) 182 1057
Perlite Filter Aid	93763-70-3	Purification Aid	Food use approval (21CFR section) NA (Not applicable, Perlite is qualified as a filtration aid in food processing under 7 CFR 205 605)

Cation Resin	N/A	Purification Aid	Food use approval (21CFR section) 173 25
Weak Anion Resin	N/A	Purification Aid	Food use approval (21CFR section) 173 25
Strong Anion Resin	N/A	Purification Aid	Food use approval (21CFR section) 173 25

The lactose that is the substrate for the production of Vivinal® GOS meets the following specifications (*Table 3*):

Table 3. Specifications for Lactose

Parameter	Specification*
Identification	**a-lactose monohydrate,**
Physical/Chemical	
Lactose H2O 99 3 wt % min 99 0 wt %	
Protein 0 15 wt % max 0 5 wt %	
Minerals 0 25 wt % max 0 3 wt %	
Appearance in solution	Nearly colorless
ICUMSA 420 nm^2 <350	Method GS 2/3-9 (1994)
Transmission 600 nm pH 6,3 >80% 20% w/w solution	
Transmission 600 nm pH 4,6 >80% 20% w/w solution	
Moisture content (%)	4 5–5 5
Loss on drying (%)	NMT 0 5
Heavy metals (mg/kg)	NMT 5
Particle Size Distribution	
< 250 micron (%)	NLT 90
< 150 micron (%)	55–95
< 75 micron (%)	5–30
Microbiological	
Total plate count (per g)	NMT 100
Enterobacteriaceae	Absent in 1 g
E coli	Absent in 10 g
Salmonella	Absent in 100 g
Yeasts (per g)	NMT 10
Molds (per g)	NMT 10

*NMT = not more than, NLT = not less than

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The enzyme is obtained from the natural, non-GMO, non-pathogenic, bacterium Bacillus circulans. This species also produces other enzymatic actions that are applied in the food industry. The ß-galactosidase that is used for the production of Vivinal® GOS is derived from the non-pathogenic Bacillus circulans strain ATCC 31382. Bacillus circulans strain ATCC 31382 is listed as a microorganism accepted as a harmless contaminant present in food by The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and The Association of Microbial Food Enzyme Producers (AMFEP).

Citric acid and sodium hydroxide are affirmed GRAS (21 CFR 184.1033 and 184.1763, respectively) for use as pH control agents in the production of foods and food ingredients. Food-grade activated carbon and powdered cellulose are unlisted GRAS substances permitted for use as purification aids.

Whey protein concentrate (WPC) is mixed 1-1 (w/w) with Vivinal® and spray-dried to convert the product from a syrup into a powder, designated Vivinal® GOS 10. WPC is affirmed GRAS (21 CFR 184.1979c) for general use in foods, subject only to good manufacturing practice.

2. Process

a) Vivinal®

A process diagram schematically illustrating the production of Vivinal® appears as *Figure 2*. The enzyme, β-galactosidase, is added to an edible lactose in suspension and the mixture is held with constant stirring. Sodium hydroxide is added as needed to adjust the pH of the mixture. The enzyme is then deactivated by heating the mixture and reducing the pH by adding citric acid. Once the enzyme is deactivated, the residues are removed from the product by adsorption and filtration processes. These processes remove the denatured enzyme. For these processes, activated carbon, cellulose, hydrochloric acid, perlite, and several resins are used. These components are removed from the product by filtration. Citric acid is again added to reduce the pH of the product, which improves its shelf life. Finally, the product is concentrated by the evaporation of water to produce a heavy syrup, Vivinal®.

b) Vivinal® GOS 10

The process diagram in *Figure* 2 also illustrates production of Vivinal® GOS 10. Production of Vivinal® GOS 10 follows the same process as that of Vivinal® up to the point at which citric acid is added to extend shelf life. The purified GOS is mixed with whey protein concentrate (WPC) in a 1-1 ratio by weight. This blend is pasteurized and then dried by evaporation; the resulting powder product is Vivinal® GOS 10.

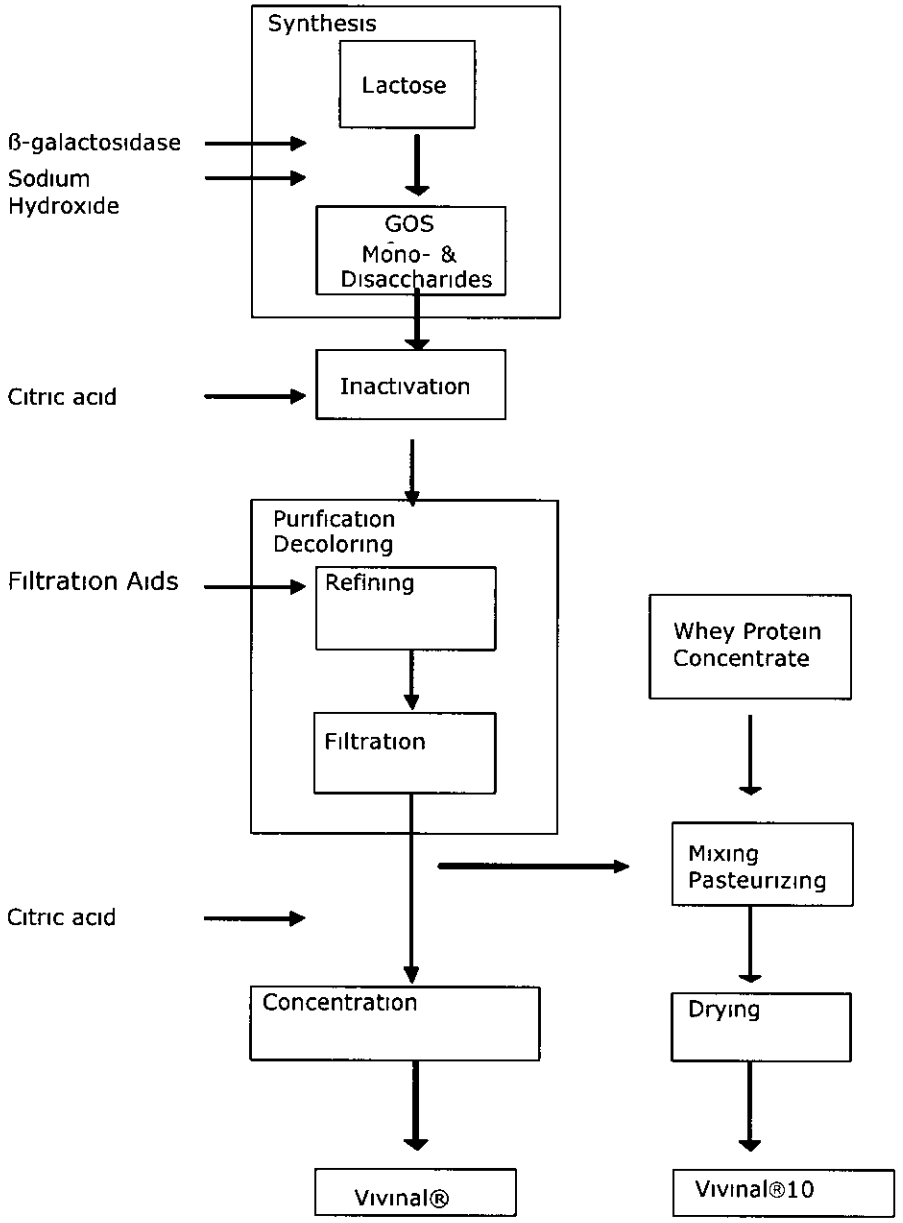

Figure 2. Process Diagram of the Production of Vivinal® and Vivinal® GOS 10

C Composition of GOS

1. Specifications for Food-Grade Vivinal®

The specifications established to assure that Vivinal® is food grade are shown in *Table 4*. Every production batch of Vivinal® is monitored for compliance with these specifications.

Table 4. Specifications for Vivinal®

Component/Impurity	Specification	Method of Analysis
Dry matter (DM)	NLT* 74%	IDF 26A (1993)
GOS	NLT 57% of DM	AOAC 2001.02 (2002)
Lactose	NMT* 23% of DM	AOAC 2001 02 (2002)
Glucose	NMT 22% of DM	AOAC 2001 02 (2002)
Galactose	NLT 0 8% of DM	AOAC 2001.02 (2002)
Sulfated Ash	NMT 0 3% of DM	AOAC 930 30 (2000)
Nitrogen	NMT 0.016% of DM	IDF 20B (1993)
Nitrite	NMT 2 µg/g on DM	IDF 97A (1984)
pH	3 2–3 8	ISO 10523 (1994)
Viscosity (cPs at 25°C)	1000–5000	HAAKE
Total Plate Count (30°C)	NMT 3000 cfu/g	IDF 100B (1991)
Enterobacteriaceae	absent in 1 g	BDI 23
E. coli	absent in 5 g	IDF 170A-1 (1999)
Yeasts	NMT 50 cfu /g	IDF 94B (1990)
Molds	NMT 50 cfu /g	IDF 94B (1990)
***Staph.* coag. Pos**	absent in 1 g	IDF 60C (1997)
Salmonella	absent in 25 g	IDF 93B (1995)

* NLT = not less than, NMT = not more than

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2. Specifications for Food-Grade Vivinal® GOS 10

The specifications established to assure that Vivinal® GOS 10 is food grade are shown in *Table 5*. Every production batch of Vivinal® GOS 10 is monitored for compliance with these specifications.

Table 5. Specifications for Vivinal® GOS 10

Component/Impurity	Specification	Method of Analysis
GOS	min 27 0 %	AOAC 2001-01, HPAEC-PAD
Protein (N x 6.38)	15 – 20 %	IDF 20B (1993), Kjeldahl
Total Moisture	max 4 5%	IDF 23A (1988), Karl Fischer
Fat	max 2 0%	IDF 9C (1987), Rose Gottlieb
Ash	max 5 0 %	AOAC 17 ed (2000) 930 30
Nitrate	max 50 ppm	IDF 97A (1994)
Nitrite	max 1 ppm	IDE 97A (1994)
pH	6 0 – 7 0	ISO 10523 (1994) 10% w/w
Insolubility index	max 0 5 mL	IDF 129A (1988)
Total Plate Count (30°C)	10000 cfu/g	IDF 100B (1991)
Enterobacteriaceae	absent in 5 g	BDI 23ª VRBG 24h 30° C
E. coli	absent in 1 g	IDE 170A-1 (1999)
Yeasts	max 50 cfu/g	IDF 94B (1990)
Molds	max 50 cfu/g	IDF 94B (1990)
***Staph.* aur**	absent in 1 g	IDF 60C (1997)
Salmonella	absent in 25 g	IDF 93B (1995)
Bacillus cereus	max 100 cfu/g	ISO 7932 (1993)

2. Product Analysis

a) Macroingredients

The macroingredient composition of five non-consecutive lots of Vivinal® were analyzed, with the results shown in *Table 6*. All five lots met all of the macroingredient specifications. Furthermore, the standard deviations across the lots are small, indicating that the production process is under control and that it results in lots of product that exhibit excellent consistency.

Table 6. Composition of Vivinal®

Component	Lot Number					Mean	Standard Deviation
	202056	208052	214050	220137	226071		
	Percent of Total Product						
Dry matter	76 8	75 7	75 5	74 9	75 2	75 6	0 73
	Percent of Dry Matter						
GOS	62 8	58 6	60 7	58 3	60 0	60 1	1 81
Lactose	19 3	20 4	20 1	20 9	21 3	20 4	0 77
Glucose	17 9	19 9	18 2	19 6	17 4	18 6	1 09
Galactose	0 9	1 2	1 0	1 2	1 3	1 1	0 16
Total	100 9	100 1	100 0	100 0	100 0		

b) Food-grade Specifications

Five non-consecutive lots of Vivinal® were analyzed to demonstrate that the product consistently meets the specifications established for food-grade material. The results of these analyses are shown in *Table 7*. Certificates of Analysis for these five lots are provided in *Appendix B* All five of the lots met all of the specifications, showing that the production process is in control and is able to consistently produce material meeting food-grade specifications. It is intended that any milk-based infant formula to which Vivinal® is added will meet all applicable microbiological and other specifications.

Table 7. Analyses of Vivinal® Against Food-grade Specifications

Component	Specification	Lot Number					Mean
		600216	340077	600233	600324	600184	
Dry matter (%)	NLT* 74	76	77	75 5	75 2	75 5	75 8
GOS (% of DM)	NLT 57	61 5	62 0	58 2	58 2	61 9	60 4
Lactose (% of DM)	NMT* 23	20 3	20 0	22 5	21 7	19 4	20 8
Glucose (% of DM)	NMT 22	17 4	17 4	20 4	19 1	17 7	18 4
Galactose (% of DM)	NLT 0 8	1 0	1 0	1 1	1 0	1 0	1 0
Sulfated ash (% of DM)	NMT 0 3	0 22	0 24	0 21	0 21	0 22	0 22
Nitrogen (% of DM)	NMT 0 16	0 0006	0 0002	0 0005	0 0002	0 0005	0 0004
Nitrite (µg/g of DM)	NMT 2	0 01	0 01	0 01	0 01	0 02	0 01
pH	3 2–3 8	3 47	3 50	3 27	3 32	3 37	3 39
Viscosity (cPs at 25°C)	1000–5000	2363	3985	2158	2145	2118	2554
Total plate count (cfu/g)	NMT 3000	<10	<10	34	10	2420	497
Enterobacteriaceae (in 1 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent
E coli (in 5 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Yeasts (cfu/g)	NMT 50	4	<1	<1	<1	<10	<2 4
Molds (cfu/g)	NMT 50	<10	<1	<1	<1	<10	<4 6
Staph coag Pos (in 1 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Salmonella (in 25 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent

* NLT = not less than, NMT = not more than

c) Impurities

Any impurities remaining in Vivinal® would be the materials used in the manufacturing process—citric acid, sodium hydroxide, activated carbon, and cellulose. In addition, Vivinal® contains lactose and glucose and a small amount of galactose. Lactose, cellulose, galactose, and glucose are natural constituents of various foods. Cellulose is a major constituent of many edible plants. Citric acid, sodium hydroxide, and activated carbon are GRAS for use in food production and are not of toxicological concern. As shown in *Table 8*, no heavy metals—lead, cadmium, arsenic, or mercury—could be detected in Vivinal®. The Certificate of Analysis for this testing is provided in *Appendix B*

Table 8. Heavy Metals in Vivinal®

	Batch Number (ppm in Formulation*)				
	340061	339080	338111	338113	340019
Lead	<0 04*	<0 04*	<0 04*	<0 04*	<0 04*
Cadmium	<0 04*	<0 04*	<0 04*	<0 04*	<0 04*
Arsenic	<0 10*	<0 10*	<0 10*	<0 10*	<0 10*
Mercury	<0 0005*	<0 0005*	<0 0005*	<0 0005*	<0 0005*

* These values indicate that these metals were not found in the formulation The values are the limits of detection (LOD).

c) Chemical and Physical Properties

Table 9 lists the properties of Vivinal®.

Table 9. Properties of Vivinal®

Property	Vivinal®	Method of Analysis
GOS (percent of dry matter)	57–63	HPAEC-PAD
pH	3 2–3 8	ISO 10523
Viscosity (cPs at 25°C)	1000–5000	HAAKE
Appearance	clear to yellowish (flavescent) viscous liquid	IDF 99C (1997)
Solubility in water	completely soluble	IDF 129A (1988)
Density (g/ml at 20°C)	1 38	IDF 134A (1995)
Taste	slightly sweet	IDF 99C (1997)
Odor	no characteristic odor	IDF 99C (1997)

d) Stability

Vivinal® is stable during long-term storage at pH 3.6 and temperature of 30°C for a period of 14 months. Both the total amount and the composition of the GOS remain unchanged. Data regarding the stability of one lot of Vivinal® during storage are given in *Figure 3*

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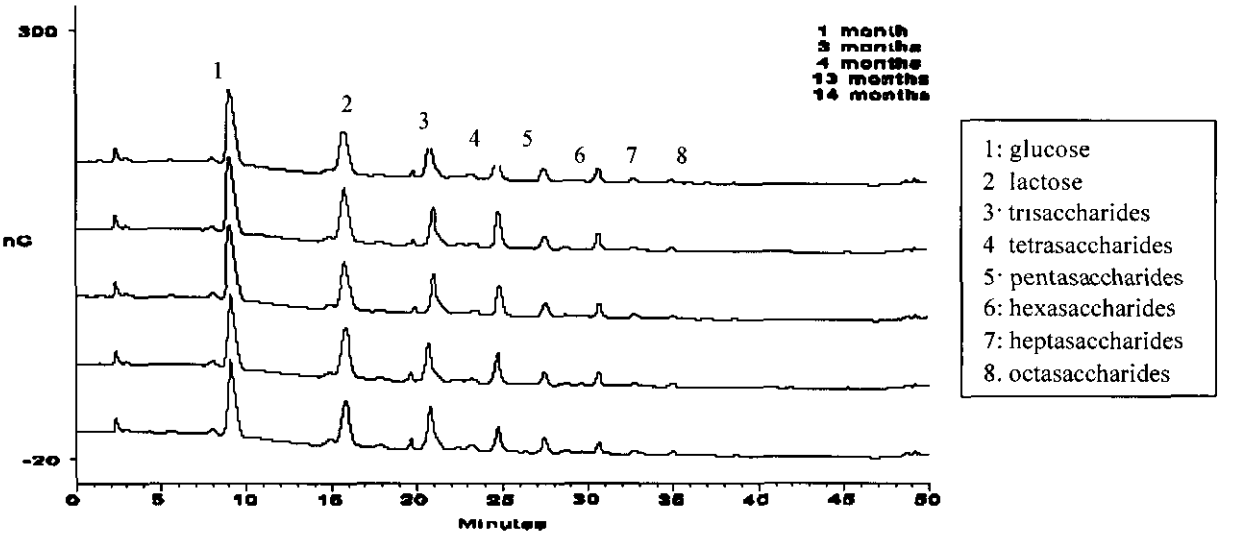

Figure 3. *Chromatogram of the Composition of Vivinal®*
(The top line represents 1 month of storage, the second line 3 months, etc.)

GOS is stable under other extreme conditions of storage or use. Experiments have shown that GOS does not break down at temperatures of up to 120°C and at pH values of 3–7. The stability of GOS also has been tested in a soft drink model. GOS does not break down after pasteurization (5 minutes at 85°C) at pH 3 and during storage for 6 months. In this experiment the GOS content was measured by means of HPAEC-PAD (AOAC method 2001.02).

e) Method of Analysis for the Substance in Food

For quantitative analysis of GOS in food products, the HPAEC-PAD method is used; this method is approved by AOAC as an official AOAC method (2001.02).

To analyze the GOS content in products high in lactose (> 20% w/w) and low in GOS (1–2% w/w), an extraction procedure is used in conjunction with AOAC method 2001.02.

000130

II. USE OF GOS

A. Historical Exposure to GOS

1. Naturally Occurring GOS

a) Human Milk

Human milk contains more than 130 different oligosaccharides (Vandenplas 2002), accounting for the third largest solid constituent in human milk after lactose and fat (Newburg 1997). The total amount of complex oligosaccharides in mature human milk is estimated to exceed 12 g/L, and oligo-saccharide levels in human colostrum can reach levels as high as 22 g/L (Newburg 1997). Vandenplas (2002) reported that the highest concentration of oligosaccharides in human milk, 20 g/L, is reached on the fourth day of life. Differences between term and preterm milk have not been observed (Kunz et al. 2000)

Based on milk samples from 50 human donors, Chaturvedi et al. (1997) identified twelve major types of oligosaccharides ranging from tri- to octasaccharides that accounted for more than 70% of total milk oligosaccharides by weight. These oligosaccharides exist in many different molecular forms—some linear and some branched, some composed of simple sugars and others containing sugar derivatives, some acidic and some neutral (Vandenplas 2002). Schmelzle et al. (2003) noted that GOS resembles the oligosaccharides in human milk with respect to the molecular weight profile and high galactose content, but differs in that human milk oligosaccharides comprise a complex mixture of different structures.

b) Bovine Milk

In bovine milk, only trace amounts of oligosaccharides are detectable, with sialyllactose being the major component (Kunz et al., 2000). Thus, most infant formulas based on bovine milk are essentially devoid of GOS unless it is added.

2. Manufactured GOS

a) Dietary Supplements

Currently, dietary supplements containing GOS manufactured by Borculo Domo are available in the United States. The GOS ingredient is sold in a mixture under the brand name Elix'or, and the products are sold as a syrup or a powder. The Elix'or product is identical to Vivinal®. When taken according to manufacturer instructions, the supplements provide 7.5–15 g of GOS per day. A New Dietary Ingredient (NDI) notification for the Elix'or products was filed with FDA in March, 1998 by EM Industries, Inc (EM Industries, Inc. 1998). The NDI notification was filed without comment by the FDA.

b) Infant Formula

Infant formulas containing GOS have been marketed in Europe since March 2000 (Savino et al. 2003). Such formulas are marketed under the names "Omneo" (manufactured by Nutricia) and "Conformil" (manufactured by Milupa). Both Nutricia and Milupa are owned by Numico. Both formulas contain 8 g oligosaccharides per L; 90% (7.2 g/L) of the oligosaccharide is GOS, and the balance (0.8 g/L) is fructooligosaccharide. The GOS used in these formulas is Vivinal®.

c) Fortified Foods

Galactooligosaccharides of the same type as Vivinal® GOS have been used as a food ingredient in Japan for several years. A list of some foods that contain GOS is presented in *Table 10.*

Table 10. Japanese Foods that Contain GOS

Product Name	Description	Manufacturer
Fiber & Oligoa	soft drink	Wakodo
Be-feel	dairy drink	Yakult
C100, Seni & Oligo	drink	Takeda
Umer	fermented dairy product	Coberco

B. Intended Use of Vivinal® and Vivinal® GOS 10

1. Addition Level of GOS, Vivinal® and Vivinal® GOS 10 to Infant Formula

MJN intends to add Vivinal® and Vivinal® GOS 10 to milk-based infant formula as prebiotic ingredients, along with polydextrose. When defining the maximum addition levels, the manufacturer specifications were taken into consideration. In addition, according to experimental trials of our infant formula production environment, we have observed loss of the notified substance during processing of the infant formulas containing these ingredients. We have considered both factors when defining the maximum addition levels.

The maximum addition levels of Vivinal® is 5.0 g/L. Considering losses during the manufacturing of our infant formula and that Vivinal® comprises approximaely 42% GOS, its maximum intended addition level is equivalent to 2 g/L of GOS. When GOS is added as Vivinal® GOS 10 the maximum addition level is 7.7 g/L (also equivalent to 2 g GOS/L).

2. Estimated Daily Intake of GOS and Vivinal®

According to tables of daily energy intake by formula-fed infants provided by Fomon (1993), the subpopulation of infants with the highest intake per kg body weight is boys age 14–27 days. The 90th percentile energy intake by this group is 141.3 kcal/kg bw/day. Among girls, the highest energy intake is found in the same age group, 14–27 days, and is nearly as high as boys: 138.9 kcal/kg bw/day. MJN's Enfamil LIPIL with Iron, along with most other standard formulas, contains 0.676 kcal/mL when ready to consume. Therefore, to obtain 141.3 kcal energy/kg, an infant boy must consume 209.0 mL/kg of formula. To reach her 90th percentile of energy consumption, 138.9 kcal/kg bw/day, an infant girl must consume 205.5 mL/kg of formula. The 90th percentile of formula intake for the two sexes combined is about 207 mL/kg bw/day.

The 90th percentile daily intake of Vivinal® added at a maximum concentration of 5.0 g/L is thus estimated to be 1 g/kg bw. The 90th percentile intake of GOS, comprising 42% of Vivinal®, is estimated to be 0.4 g/kg bw/day. The 90th percentile daily intake of Vivinal® GOS 10 added at a maximum concentration of 7.7 g/L is thus estimated to be 1.6 g/kg bw. The 90th percentile intake of GOS, comprising 27% of Vivinal® GOS 10, is estimated to be 0.4 g/kg bw/day.

As the infant grows, formula intake increases, but more slowly than weight gain, so that consumption assessed as mL formula per kg body weight is lower for infants older than 27 days. As a result, intake per kg body weight of Vivinal® and of GOS decreases as the infant grows older and larger. Estimates of the mean and 90th percentile intakes of GOS at various ages, based on Fomon's (1993) data on energy intakes, are shown in *Figure 4*.

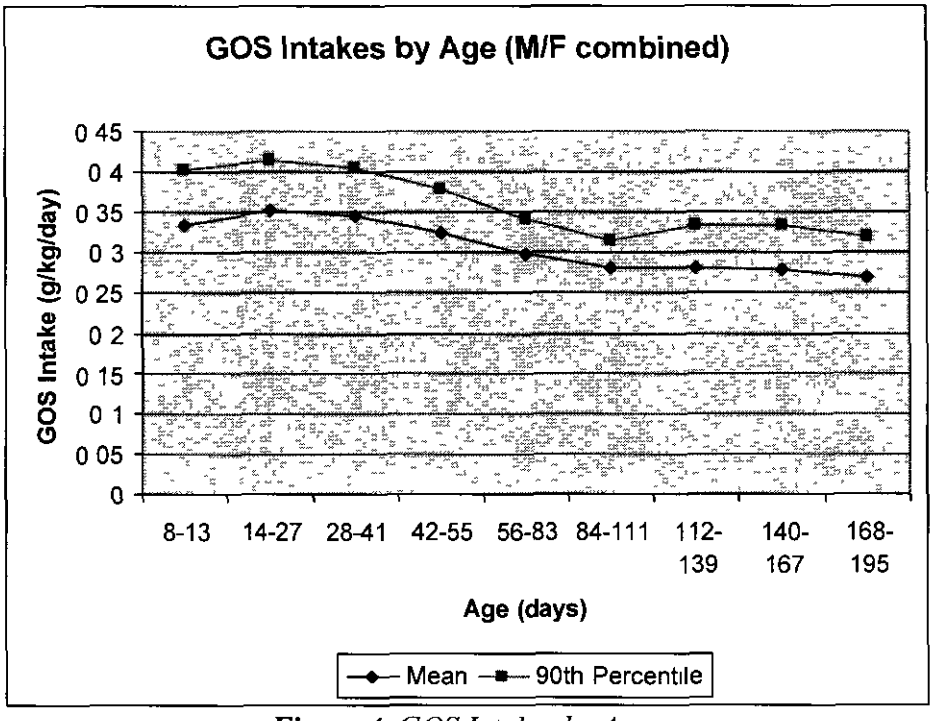

Figure 4. *GOS Intakes by Age*

Figure 4 shows that the estimated 90th percentile intake of GOS peaks at about 0.4 g/kg bw/day during the first 6 weeks of life, then declines to about 0.3 g/kg bw/day by weeks 8–12. Establishing the EDI of GOS at 0.4 g/kg bw/day, and the EDI of Vivinal® at 1 g/kg bw/day and Vivinal® GOS 10 at 1.6 g/kg bw/day, is thus a conservative basis for estimating long-term exposure.

Since bovine milk does not contain GOS, there are no other sources of GOS or of Vivinal® and Vivinal® GOS 10 in the diets of formula-fed infants, and therefore the above estimated daily intake constitutes the total daily exposure of infants to GOS at the 90th percentile.

III. SAFETY DATA

A. Safety of the Production Organism and Enzyme

1. Production Organism

In the production of Vivinal® GOS from lactose, lactose ß-galactosidase is used to promote the transgalactosylation. This enzyme is produced by *Bacillus circulans* Jordan (ATCC 4516). The *B. circulans* species was established in 1890 and is listed on the Approved List of Bacterial Names (Skerman et al. 1980). Strain ATCC 4516, a non-genetically modified organism, is sourced from the American Type Culture Collection (http://www.atcc.org/), where it was deposited by W. W. Ford (Smith et al. 1952). The ATCC website indicates that this strain is at biosafety level 1, the lowest concern level.

2. Production Enzyme: ß-Galactosidase

The ß-galactosidase used in the production of Vivinal® GOS is obtained from Daiwa Kasei K. K. of Japan, in the form of a powder trade-named Biolacta®. The enzyme ß-galactosidase is listed by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) as safe for use in the preparation of food (www.amfep.org/main.html; last updated October 2001). The production of GOS by ß-galactosidase produced by *Bacillus circulans* was characterized using NMR spectroscopy and fast atom bombardment mass spectrometry (Zervosen et al. 2001). ß-galactosidase produced by *Bacillus circulans* was further characterized by Yanahira et al. (1995), who also confirmed by *in vitro* studies using human intestinal bacteria that the GOS units were predominantly used by human intestinal bifidobacteria.

To substantiate the safety of Biolacta® ß-galactosidase, multiple tests have been performed and certifications have been provided to Borculo Domo by Daiwa Kasei K. K. The company certified that no cases of allergic reactions have occurred in the 6-year history of the product among the approximately 40 employees who routinely come into contact with it, and no reports indicating allergenicity have been received from customers (Daiwa Kasei 1994).

Based on tests of antibacterial activity of Biolacta® ß-galactosidase conducted by the Japan Food Research Laboratories (1991), the enzyme showed no antibacterial activity against any organism tested: *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 11229), *Bacillus cereus* (ATCC 2), *Bacillus circulans* (ATCC 4516), *Streptococcus pyogenes* (ATCC 12344), and *Serratia narcescens* (ATCC 14041).

The Mycotoxin Research Association (MRA) of Japan tested Biolacta® for the presence of mycotoxins (MRA 1991); tests were negative for all mycotoxins tested: aflatoxin B_2, ochratoxin A, sterigmatocystin, Trichothecene 2, and zearalenone.

Tests of mutagenicity of Biolacta® conducted at TNO provided no indications of chromosomal damage or damage to the mitotic apparatus in bone marrow cells of mice injected with Biolacta® at the limit dose of 2000 mg/kg (Verhagen 1995) or of

clastogenicity based on chromosome aberration assays with Chinese hamster ovary cells in both absence and presence of S-9 activation (de Vogel 1995).

Finally, an acute toxicity study with 10 male and 10 female mice, conducted by the Japan Food Research Laboratories (1991), found that oral administration of Biolacta® at 2000 mg/kg caused no deaths, apparent symptoms, abnormal necropsy findings, or effects on body-weight gain. Thus, the $LD_{50} > 2000$ mg/kg.

B. Absorption, Distribution, Metabolism and Excretion of GOS

More than 90% of prebiotic material is estimated to arrive in the colon intact (Van Loo et al. 1999; Aggett et al. 2003). This non-digestibility is an essential precondition for the function of GOS as a prebiotic (Miniello et al. 2004). Data from both *in vitro* and *in vivo* studies summarized in *Tables 13* through *16* at the end of this chapter provide further evidence of the indigestibility of this oligosaccharide.

1. *In Vitro* Studies

A series of *in vitro* experiments was sponsored by Borculo Domo to assess the hydrolysis of GOS by human intestinal enzymes and the acid stability of the oligosaccharide (Asp 1994). Results from these unpublished studies show that when small human intestinal biopsy homogenates with normal disaccharidase activities were incubated with GOS for 6 hours, liberation of glucose and galactose from GOS was less than 10% that from lactose. The liberation of glucose appeared to reach a plateau, while galactose liberation proceeded in a linear fashion throughout the study. When ten different intestinal biopsies were incubated with GOS at 37°C for 2 hours, the oligosaccharidase activity (as assessed by glucose and galactose liberation) was 5–10% of the lactase activity, with greater liberation of galactose than glucose. Additionally, GOS was found to be stable against acid hydrolysis at pH 1–5 during at least 2 hours at 37°C.

Other investigators have also observed that GOS is slowly digested under conditions mimicking conditions found in the small intestine. GOS showed slow liberation of galactose and glucose when exposed to human small intestine homogenates *in vitro*, and almost no liberation when the activity of intracellular ß-galactosidases was inhibited (Burvall et al. 1980). In the same study, prolonged incubations with portions of human intestine indicated that galactose was liberated slowly, and no significant amount of glucose was released even after 6 hours of incubation.

Ohtsuka et al. (1990) measured the digestion *in vitro* of GOS synthesized from lactose by *Cryptococcus laurentii* OKN-4 using artificial gastric juice, a-amylase of human saliva and of pig pancreas, and rat small intestinal mucosa homogenates. GOS was not hydrolyzed by the artificial gastric juice or a-amylase, and only a very small amount of GOS was digested by the mucosa homogenates.

Ito and colleagues (1990) report the unpublished observation that GOS was not hydrolyzed by exposure to rat intestinal mucosa homogenate at 37°C for 2 hours.

2. Animal Studies

In an unpublished *in vivo* study conducted by Borculo Domo (cited in EM Industries 1998), the GOS in Vivinal® (Elix'or) passed the small intestine almost completely intact in pigs that had been cannulated at the end of their ileum.

Ohtsuka et al. (1991) employed a radioisotope technique to assess the utilization and metabolism of GOS synthesized from lactose by *Cryptococcus laurentii* OKN-4 in conventional rats, antibiotic-treated rats, and germ-free rats. Results from the study show that GOS is metabolized by intestinal microorganisms in the gastrointestinal tract. In the same study, no GOS was found in the feces of conventional rats fed a 5% GOS diet (approximately 5.1 g/kg) for 2 weeks. Ohtsuka et al. (1990) reported that feeding rats GOS for 6 weeks did not lead to the production of GOS-hydrolyzing enzymes in the small intestine and therefore an increase in the digestion of GOS. Results from this study provide evidence that digestive enzymes do not adapt in response to long-term feeding of GOS, and thus most GOS reaches the colon intact.

Results of hydrogen breath tests are also often viewed as demonstrating the indigestibility of GOS in experimental animals. The breath hydrogen test provides a measure of unspecified microbial fermentation in the large intestine, as hydrogen produced by carbohydrate fermentation is absorbed and subsequently expired in the breath. Hydrogen is typically found in the breath of breast-fed infants (Brand-Miller et al. 1998; Laforgia et al. 1995) and is thought to result from colonic fermentation of human milk oligosaccharides (Brand-Miller et al. 1998), although organisms such as the bifidobacteria do not produce hydrogen as part of their metabolism.

In a study of germ-free rats inoculated with a human fecal flora, intake of a 4% GOS diet (approximately 2 g/kg) for 4 weeks led to a nearly 7-fold increase in hydrogen excretion as compared to rats fed a control diet, and a 2.5-fold higher methane excretion (Djouzi and Andrieux, 1997). Hydrogen production was elevated 10-fold in rats fed a 5 or 10% GOS diet (approximately 2.8 and 5.6 g/kg, respectively) for 4 weeks as compared to a control group, though methane production was not significantly modified (Kikuchi et al. 1996). Only the 5% GOS diet group showed a significant increase in hydrogen plus methane excretion. In another study, intake of a 4% GOS diet (approximately 2 g/kg) for 1 month resulted in significant increases in hydrogen and methane production in germ-free rats inoculated with human fecal flora as compared to controls, though no changes were measured in conventional rats fed the GOS diet (Meslin et al. 1993).

3. Human Studies

Results from human studies employing hydrogen breath tests also demonstrate the indigestibility of GOS and its fermentation in the colon. Following daily intake of GOS by healthy adults for 3 weeks, breath-hydrogen concentrations increased by 130% in individuals consuming 14.4 g GOS as compared to no increase in the placebo group (Alles et al.1999). Daily intake of 8.5 g GOS had no effect on breath hydrogen. Additionally, no fecal excretion of GOS was detected in an analysis of fecal samples. The increase in breath hydrogen and absence of GOS in the feces suggest complete fermentation of GOS in the human colon.

In a study of five healthy men who consumed a single dose of 30 g GOS (0.5 g/kg), breath-hydrogen excretion was elevated in the 4-hour period following ingestion (Tanaka et al., 1983). The response to GOS was reported to be similar to the response following administration of lactulose, a non-digestible disaccharide. The results therefore suggest that following poor absorption of GOS in the small intestine, the oligosaccharide passes into the colon, where it is metabolized by intestinal bacteria.

Not all investigators, however, have reported an increase in breath-hydrogen excretion following intake of GOS. Bouhnik and colleagues (1997) reported a significant drop in breath-hydrogen excretion and no change in methane excretion following daily intake of 10 g GOS for 3 weeks by eight healthy adults. Daily intake of 15 g GOS by 12 healthy men had no effect on hydrogen in expired air (van Dokkum et al. 1999).

4. Conclusions

Results of studies conducted by Borculo Domo as well as those of several studies in the published literature, discussed above, demonstrate that GOS is not digested to any appreciable extent in the upper gastrointestinal tract. Given the absence of GOS in fecal samples following administration of GOS, the oligosaccharide appears to be completely fermented by the microflora in the large intestine.

C. Changes in Microflora and Stool Characteristics in Response to GOS

The effects of GOS intake on microflora, related measures of microbial fermentation, and stool characteristics have been studied *in vitro* as well as in animals and humans. These studies are summarized in *Tables 13* through *16* at the end of this chapter.

1. *In Vitro* Studies

The studies summarized in *Table 13* provide data on the *in vitro* effects of GOS on fecal microflora and measures of microbial fermentation. A variety of study designs were employed, including studies using feces from humans, studies using feces from animals, and studies in which GOS was incubated with selected bacterial species.

In five studies, feces were collected from humans. Flickinger et al. (2000) fed an unspecified dose of GOS to humans prior to collection of feces. Incubation in an anaerobic system for 24 hours showed a time-dependent decrease in pH as well as a time-dependent increase in organic acid production. After 24 hours, the GOS sample had the lowest pH and highest concentration of organic acids of all 6 substrates tested. In other studies, GOS was added to fecal samples post-collection. Bouhnik et al. (1997) collected feces from 3 humans and incubated a fecal homogenate with 10 g GOS per day for 14 days. On day 4, the maximum (95%) degradation rate was reached and pH was significantly reduced by day 7. Total short-chain fatty acids (SCFAs) doubled by day 7, with an increased proportion of acetate and decreased proportion of propionate. Similarly, Durand et al. (1992) homogenized human fecal samples and found that maximum degradability occurred by day 3 when incubated with 20 g GOS per day; in

this study, concentrations of short-chain fatty acids, carbon dioxide, and methane also increased. Boehm et al. (2004) cultured stools from infants fed a formula containing a GOS/LOS blend (8 g/L), a control formula, or human milk, and found that the SCFA production of the stools from infants who received GOS/LOS resembled that from the stools of breast-fed infants. McBain and Macfarlane (2001) incubated feces from one individual with 10 g GOS in a three-vessel continuous culture system designed as a model for the proximal, transverse, and distal colon. In specific sections of this system, lactobacilli and bifidobacteria increased significantly while bacteroides and peptostreptococci growth were inhibited. In all vessels, activities of ß-glucosidase and ß-glucuronidase were inhibited. In a fecal slurry to which GOS was added, bacteroides and bifidobacteria increased while no changes in total bacteria, clostridia, lactobacilli, or *E. coli* were observed; production of lactate, propionate, and acetate also was observed in these samples (Rycroft et al. 2001).

In three studies, animal digesta were incubated with GOS. Houdijk et al. (1997) incubated feces and ileal digesta of pigs fed a 4% GOS diet for up to 96 hours. No effects were apparent on asymptomatic gas production. Feces inoculated with GOS produced less butyrate, but no difference in total short-chain fatty acids was observed. Incubation of ileal digesta produced no difference in total short-chain fatty acids. Kikuchi-Hayakawa et al. (1997) inoculated cecal digesta of rats fed a 5% GOS diet with 10 mg GOS. By day 1, the amount of lactic acid in GOS-treated samples was higher than in control samples; no other differences in amounts of organic acids were observed. More recently, Zentek et al. (2002) collected feces from dogs fed a dose of 1 g GOS/kg bw/day for 10 days. In samples incubated for 24 hours, total short-chain fatty acid production, proportion of acetic acid, ammonia concentration, and gas volume increased.

Finally, several investigators incubated bacteria on media inoculated with a specific GOS concentration. Hopkins et al. (1998) found that growth rates significantly increased for several bifidobacteria isolates. Tanaka et al. (1983) observed growth of *Bifidobacterium* and *Lactobacillus* species within 24 hours when grown on a 1% GOS medium. Growth *of Enterobacteriaceae* and some *Streptococcus* species was also observed.

2. Animal Studies

Twenty-nine studies that examined efficacy parameters, including effects of GOS ingestion on fecal microflora, measures of microbial fermentation, and stool characteristics were identified (*Table 14*). Research was primarily conducted using the rat model (22 studies), but prebiotic effects also were studied in the mouse, rabbit, pig, calf, and dog. Several species were in both weanling and adult stages. The GOS products administered consisted of 37.9–100% GOS syrup, and were incorporated into the solid diet, into an infant formula fed to the animals, or into drinking water, or were administered via gavage. The GOS doses varied widely, and were typically higher for rats and mice on a per kilogram basis (0.8–14 g/kg bw/day) than for larger species (0.05–1.4 g/kg bw/day). Study duration ranged from 7 days to 12 months.

a. Microflora Response

Ten of the studies conducted in animals evaluated microbial response to GOS supplementation. In the majority of these studies, GOS appeared to be bifidogenic, as indicated by significant increases in fecal or cecal bifidobacteria counts (Djouzi and Andrieux 1997; Holma et al. 2002; Morishita et al. 1992; Morishita et al. 2002; Rowland and Tanaka 1993; Suzuki et al. 1999). In the study by Djouzi and Andrieux (1997), rats were fed 2 g GOS/kg bw/day for 4 weeks; the lack of effect on total anaerobes or other anaerobic species measured indicated that GOS ingestion likely increased the proportion of bifidobacteria at the expense of other bacterial species. In rats fed a GOS dose 2-fold higher (4 g/kg bw/day) for half the duration (13 days), total fecal bacteria increased, but no significant change in proportion of bifidobacteria was detected (Holma et al. 2002). No influence on growth of bifidobacteria (Houdijk et al. 1997; Kikuchi-Hayakawa et al. 1997) or total fecal bacteria (Houdijk et al. 1997; Kikuchi-Hayakawa et al. 1997) was observed in other studies. The maximum GOS dose tended to be higher in studies showing a bifidogenic effect (2–7.5 g/kg bw/day, 13–35 days) as compared to studies in which no such effect was detected (0.6–0.8 g/kg bw/day, 16–50 days), though the study durations were comparable (13–35 days versus 16–50 days).

Other commonly evaluated bacteria included lactobacilli, enterobacteria, bacteroides, and clostridia. Morishita et al. (1992) reported that feeding 4.5 g/kg bw/day GOS for 4 weeks restored lactobacilli counts that were initially reduced by cellulose ingestion. Similarly, Rowland and Tanaka (1993) reported increased lactobacilli in conjunction with decreased enterobacteria and no changes in bacteroides, staphylococci, or clostridia when feeding rats 2–3 g/kg bw/day GOS for 4 weeks. Kikuchi-Hayakawa (1997) noted no change in lactobacilli or total bacteria, but increased bacteroidaceae and decreased enterobacteriaceae in rats fed 0.8 g/kg bw/day GOS. Feeding 7.5 g/kg bw/day GOS for 7 weeks, Morishita et al. (2002) reported a decrease in putrefactive *C perfringens* in germ-free mice and a suppression of *C perfringens* growth during early bowel microflora development when GOS was fed in combination with an anaerobic bacterial inoculation. Feeding germ-free rats inoculated with bacteria characteristic of infant feces 5.3 g/kg bw/day GOS for one week, Suzuki et al. (1999) also reported a decrease in *C. perfringens*; no changes in *S. epidermidis*, *Eubacterium aerofaciens*, *Bacteroides* spp., *Staphylococcus* spp., yeasts, or *Clostridium* spp. were apparent.

Dose, duration, and outcome of GOS feeding varied widely among the studies. It is important to note that in many of the animal studies reviewed, data regarding food intake and body weight were not provided. It was therefore necessary to use default values in the calculations of daily GOS dose, and all calculated doses should be regarded as approximate. Overall, these reports suggest that GOS is bifidogenic and promotes growth of beneficial lactobacilli at a dose greater than or approximately equal to 2 g/kg bw/day. Amounts of *C perfringens* and enterobacteria may be reduced by feeding GOS at slightly higher levels.

b. Measures of Microbial Fermentation

Bacterial fermentation of non-digestible oligosaccharides (NDO) results in production of SCFAs. Absorption and utilization of these fatty acids by colonic enterocytes is thought to exert a trophic effect, as well as promote salt and water

absorption (Sako et al. 1999). Measurable increases in lower GI tract weight or size are thought to reflect these effects. Changes in pH that result from production of different fatty acids are thought to cause alterations in enzyme activity, or production of enzymes by enteric bacteria.

Measures of microbial fermentation were studied extensively in animals, with outcomes reported in nearly all studies. These included changes in pH and cecal or fecal weight or dry matter, SCFA concentrations, enzyme activity, ammonia concentration, and gas production.

The effect of GOS intake on cecal and fecal pH was measured in the majority of these studies. In nearly all studies, GOS intake was associated with a decrease in cecal pH, and results suggest that the decrease is dose-dependent (Kikuchi et al. 1996). Zentek and colleagues (2002), however, reported no effect of GOS on fecal pH in dogs consuming 1g/kg bw/day GOS for ten days, while Houdijk et al. (1997) observed increased fecal pH in weaner pigs ingesting 0.4 or 1.6% dietary GOS (0.2-0.6 g/kg bw/day) for approximately 1 month.

In nearly all studies identified, increased cecal whole weight, cecal wall weight, or cecal size was noted (Chonan et al. 1995; Chonan and Watanuki 1995; Chonan and Watanuki 1996; Chonan et al. 1996; Chonan et al. 2001; Djouzi and Andrieux 1997; Hayashi et al. 1991; Kikuchi et al. 1996; Kikuchi-Hayakawa et al. 1997; Morishita et al. 1992; Ohtsuka et al. 1990; Rowland and Tanaka et al. 1993; Sakaguchi et al. 1998; Shimura et al. 1991; Wijnands et al. 1999; Yanahira et al. 1997). Only in weanling and adult rabbits was a change in cecal weight not observed (Maertens and Peeters 1992). Daily intake of low levels of GOS (approximately 0.05–0.4 g/kg bw/day) by weanling and adult rabbits had no effect on cecal wet weight (Maertens and Peeters 1992). No effect on colon wet weight was observed in a study in which rats received 4 g/kg bw/day GOS for 13 days (Holma et al. 2002). Other researchers, however, have reported greater weight effects in the cecum than in the colon following GOS feeding in rats (Ohtsuka et al. 1990).

Reported effects on fecal weight were variable. Increased fecal weight (Chonan et al. 2001; Hayashi et al. 1991) and fecal excretion (Kikuchi et al. 1996) were reported in studies of rats and pigs. This increase was dose-dependent in rats fed GOS at 0.9–1.7 g/kg bw/day (Hayashi et al. 1991). In contrast, Houdijk et al. (1999) observed a dose-dependent decrease in feces production in pigs fed from 0.4–1.4 g GOS/kg bw/day, and Wijnands et al. (1999) reported reduced feces production in rats fed a high-fat diet containing 3.0 g GOS/kg bw/day. GOS feeding had no effect on fecal dry matter in studies of pigs and dogs ingesting low levels (0.3–1.4 g/kg bw/day) of GOS (Houdijk et al. 1997, 1998; Zentek 2002), but increased fecal dry weight was reported in a study of rats fed 4.9 or 9.4 g/kg bw/day GOS (Ohtsuka et al. 1990).

Studies measuring SCFAs (or volatile fatty acids) focused on production in either feces or cecal contents. An increase in total SCFAs was noted in studies providing from 0.8–8.6 g GOS/kg bw/day to rats (Chonan et al. 1995; Chonan and Watanuki, 1996; Chonan and Watanuki, 1995; Chonan et al. 1996; Djouzi and Andrieux 1997; Kikuchi et

al. 1996; Kikuchi-Hayakawa et al. 1997; Meslin et al. 1993; Morishita et al. 1992; Sakaguchi et al. 1998; Yanahira et al. 1997). Individual fatty acids contributing to the increased total levels were most commonly acetic, propionic, butyric, and succinic acids. In contrast, other investigators (Houdijk et al. 1997; Zentek et al. 2002; Morishita et al. 1992) reported no difference in total SCFAs when feeding 1.5 g GOS/kg bw/day or less to pigs, dogs, or rats.

Five studies measured fecal enzyme concentrations. The activity of ß-galactosidase increased in all studies where it was measured (Djouzi and Andrieux 1997; Kikuchi et al. 1996; Meslin et al. 1993; Suzuki et al. 1999). Results for other enzymes varied. Djouzi and Andrieux (1997), providing 2 g GOS/kg bw/day, reported no change in ß-glucosidase, a-glucosidase, or ß-glucuronidase. Kikuchi et al. (1996) reported that activities of ß-glucosidase and ß-glucuronidase decreased in rats fed GOS at levels of 2.8 and 5.6 g/kg bw/day, while Rowland and Tanaka (1993) observed increased ß-glucosidase activity and decreased ß-glucuronidase and nitrate reductase when feeding 2–3 g GOS/kg bw/day. Suzuki et al. (1999) described enhanced ß-glucosidase, ß-glucuronidase, and a-galactosidase activities in rats fed 5.3 g/kg bw/day GOS for 1 week.

In two studies, cecal ammonia concentration decreased with a dose of 2–5.6 g/kg bw/day GOS (Djouzi and Andrieux 1997; Kikuchi et al. 1996). In two other studies, the ammonia concentration remained unchanged when 0.1–3 g/kg bw/day GOS was provided (Maertens and Peeters 1992; Rowland and Tanaka 1993).

Gas production was evaluated in only three studies. Hydrogen excretion increased in rats fed 2.8–5.6 g GOS/kg bw/day while methane remained unchanged (Kikuchi et al. 1996); in two additional studies, both hydrogen and methane excretion increased in rats fed 2 g GOS/kg bw/day (Djouzi and Andrieux 1997; Meslin et al. 1993).

c. Stool Characteristics

Stool characteristics described in animal studies included stool consistency, water content, and transit time. Studies examining the effects of GOS intake on stool characteristics consistently reported a softening of feces in animals consuming less than 1–3 g/kg bw/day GOS (Hayashi et al. 1991; Houdijk et al. 1997; Wijnands et al. 1999, 2001). In other studies, however, GOS had no measurable effect on ratings of fecal score (Quigley et al. 1997) or stool quality (Zentek et al. 2002). Increased fecal water content was observed in a study of rats fed a 5% GOS diet (2.5 g/kg bw/day) for 21 days (Kikuchi-Hayakawa et al. 1997), though no change in cecal water content was measured in a study of germ-free rats inoculated with human feces prior to feeding 2.8 or 5.6 g/kg GOS daily for 4 weeks (Kikuchi et al. 1996). Intestinal transit time was not affected in rats fed 5.5 (Shimura et al. 1991) or 8.4 g GOS/kg bw/day (Sakaguchi et al. 1998).

d. Conclusions from Animal Studies

Data from studies of GOS intake by animals suggest that GOS is bifidogenic and promotes growth of beneficial lactobacilli at doses greater than or approximately equal to 2 g/kg bw/day. Amounts of *C. perfringens* and enterobacteria may be reduced by feeding GOS at slightly higher levels. Results also indicate that intake of GOS has effects on

measures of microbial fermentation, including reduction of cecal pH, increase in cecal weight, increased production of total SCFAs due primarily to increases in acetic, propionic, butyric, and succinic acids, and increased ß-galactosidase activity. GOS intake also tends to produce a stool-softening effect.

3. Human Studies

a. Studies of Infants

Seven studies that examined the effects of providing a prebiotic-containing formula to populations of either term or preterm infants were identified; these studies are summarized in *Table 15*. In five of the studies, the prebiotic added to the formula consisted of a blend of 90% GOS and 10% FOS. The studies were designed to assess the effects of the prebiotic formulas on fecal microflora, stool characteristics, growth, and potential side effects.

Boehm and colleagues (2002, 2003) studied the effects of feeding a formula containing 10 g/L prebiotics (90% GOS, 10% FOS) on the fecal flora and stool characteristics of preterm infants. All infants initially received pasteurized mother's milk. When the mother was no longer able to provide milk, 30 infants were randomly assigned to receive one of two formulas: a formula containing 10 g/L oligosaccharide blend (n = 15), or a similar formula containing maltodextrins in place of the added oligosaccharides (n = 15). Twelve infants who continued to receive mother's milk served as a reference group. Measurements were repeated after 7, 14, and 28 days. On each of these days, fecal microflora were investigated, and stool characteristics and possible side effects were recorded.

At the beginning of the formula feeding, there was no difference between groups in fecal bifidobacteria counts. After 4 weeks of formula feeding, infants receiving the GOS+FOS supplemented formula had significantly higher fecal bifidobacteria counts than infants fed the control formula. The bifidobacteria counts in the GOS+FOS group were in the range of those in the human milk reference group. Prebiotic versus control formula, however, had no effect on fecal counts of *Lactobacilli*, *Bacteroides*, *Clostridium* spp., *E coli*, *Enterobacter*, *Citrobacter*, *Proteus*, *Klebsiella*, or *Candida*. Infants fed formula with added oligosaccharides had significantly more stools per day than infants receiving the control formula, and a similar number of stools per day as the infants in the human milk reference group. Stool consistency was also similar in infants receiving the GOS+FOS formula and the reference group, and these infants had significantly softer stools than infants in the control group.

A study of the bifidogenic effects of formula containing a mixture of prebiotics (90% GOS, 10% FOS) was also conducted in term infants (Moro et al. 2002, 2003). In this study, 90 infants were randomized to receive one of three formulas: either 8 g/L or 4 g/L of the prebiotic mixture, or a control formula that contained maltodextrin in place of the prebiotic blend. Prior to receiving the formulas, the infants were fed mother's breast milk. On the first day of formula feeding and also after 28 days of feeding, stools were collected, fecal flora and stool pH were measured, and stool characteristics and any side effects were recorded.

On the first day of feeding, the counts of fecal bifidobacteria and lactobacilli did not differ between groups. At the end of the study, infants receiving the GOS+FOS-supplemented formulas had significantly higher fecal bifidobacteria counts than infants in the control group. Additionally, infants fed the 8 g/L prebiotic formula had significantly higher fecal bifidobacteria counts than infants fed the 4 g/L prebiotic formula (log 9.7 versus log 9.3 CFU/g). Infants fed the prebiotic formulas also had significantly higher fecal lactobacilli counts than infants in the control group, though there was no difference between the prebiotic formula groups. Oligosaccharide supplementation had no significant effect on fecal *Bacteroides*, *Clostridium*, *E. coli*, *Enterobacter*, *Citrobacter*, *Proteus*, *Klebsiella*, or *Candida*. Fecal pH decreased in a dose-dependent manner in infants fed the oligosaccharide formulas and increased in infants fed the control formula. Stool frequency increased significantly only in the group of infants fed the 8 g/L prebiotic formula. Stool consistency was significantly softer among infants fed the prebiotic formulas as compared to those in the control group.

Results from a study conducted by Knol et al. (2001), as presented in an abstract, indicate that term infants receiving a formula supplemented with GOS and FOS (dose not specified) had an increase in fecal bifidobacteria as determined by fluorescent *in situ* hybridization (FISH) with a specific 16S rRNA probe: at baseline (0–2 weeks of life), the level was log 9.2 while at 6 weeks the level was log 10. The proportion of fecal bifidobacteria as a percentage of total microorganisms also increased significantly from 31% to 59%, whereas no change was observed in the group of infants receiving a control formula. Results from this study suggest that ingestion of the prebiotic formula promotes the growth of bifidobacteria to dominate the fecal flora in term infants.

Savino et al. (2003) studied the effects of feeding a blend of Vivinal® GOS and FOS for 14 days on 214 infants with colic, 201 infants with regurgitation problems, and 232 infants with constipation. The dose used was 8 g/L; intake was 0.99 g/kg bw/day of the blend, or 0.89 g/kg bw/day GOS. Among the infants with colic, 79% showed reduced frequency, while 70% of those with regurgitation problems showed improvement. The only measure of stool characteristics was among those infants with constipation, 63% of whom increased their stool frequency.

Both nutritional efficacy and bifidogenic characteristics of a GOS+FOS blend (90% GOS, 10% FOS) were studied by Schmelzle et al. (2003). The primary endpoint was growth over the 12-week duration of the study, and the study was powered to be able to detect a weight-gain difference of 3 g/day between the 49 healthy term infants in the test group and the 53 in the control group, which received the identical formula without the GOS+FOS blend. The blend was at a concentration of 8 g/L, resulting in a daily intake of 1.08 g blend/kg, or 0.97 g GOS/kg. The prevalence of bifidobacteria among the total microorganism count increased in the oligosaccharide group relative to the control group, and the bifidobacteria count in the test group increased 1 log. The group receiving the prebiotic also exhibited softer stools than did infants in the control group.

Two studies of healthy term infants who were given only GOS, rather than a GOS+FOS blend, have also appeared in the literature. Napoli et al. (2003) studied the effect on fecal microflora of formula containing 0.7% (w/v) GOS given over 21 days to

13 healthy term infants versus a formula containing lactose as a positive control; the study also included 24 infants in a breast-milk reference group. The infants receiving GOS showed a 1 log increase in bifidobacteria count to match the range of the reference group. GOS also caused a 4-fold increase in production of lactic acid and a resulting decrease in fecal pH.

Finally, in a randomized controlled study by Ben et al. (2004), term infants received formula containing GOS at a concentration of 2.4 g/L for a period of 6 months. The study included 69 infants in the test group, 52 in the control group, and 26 in a human milk reference group. In the test group, infants showed increased counts of bifidobacteria and lactobactilli to the same level as the human milk reference group; no change was apparent in the prevalence of *E coli*. The test group, relative to the control group, also showed increased production of SCFAs and resulting decreased pH, as well as increased frequency of stools and softer stools.

Results from these seven studies in term and preterm infants indicate that addition of 2.4–10 g/L prebiotic, either 90% GOS and 10% FOS, or 100% GOS, results in increased fecal counts of bifidobacteria and the proportion of bifidobacteria in total fecal bacteria. The prevalence of bifidobacteria resembles that found in infants fed human milk. GOS may also be lactogenic, but little effect is apparent on other microorganisms. The change in fecal microflora is accompanied by increased production of SCFAs and decreased fecal pH. Additionally, stools become softer and stool frequency increases, resulting in stool characteristics similar to those of breast-fed infants.

b. Studies of Adults

Of the 13 studies identified in *Table 16*, 11 examined efficacy parameters including effects on fecal microflora, measures of microbial fermentation, and stool characteristics in populations of adults. Doses and durations of GOS administration varied widely. Doses ranged from 2.5–30 g/day, or approximately 36–500 mg/kg bw/day, and durations from a single dose to 3 weeks. In all but one study where GOS was provided to elderly subjects with constipation (Teuri and Korpela 1998), subjects were healthy men or women aged 18–75 years. When described, GOS was provided as syrup containing 45–85% galactooligosaccharide. These syrups were prepared by different manufacturers and marketed under a variety of names, including Elix'or (Alles et al. 1999; Teuri and Korpela 1998; van den Heuvel et al. 2000), Oligomate-50® (Ito et al. 1990; Ito et al. 1993), and CUP OLIGO P (Tamai et al. 1992), and were administered in a food matrix or as a supplement. In a number of studies, the source of the GOS was not provided. (Elix'or is identical to Vivinal® and is produced by the same manufacturer.)

(1) Effect on Fecal Microflora

Several studies examined the effect of GOS feeding on microflora populations as assessed in feces. In five of these studies, numbers of bifidobacteria increased with ingestion of approximately 36–143 mg GOS/kg bw/day (Bouhnik et al. 1997; Ito et al. 1990; Ito et al. 1993; Tamai et al. 1992; Tanaka et al. 1983).

Ito et al. (1990) provided subjects in a crossover study with 36, 71, or 143 mg GOS/kg bw/day for 1 week and found that fecal bifidobacteria counts and the percentage of bifidobacteria among total microorganisms increased in a dose-dependent manner. In this study, the lowest dose, corresponding to 2.5 g GOS/day, caused an increase in fecal bifidobacteria, but the effect was not significant. Ito et al. (1993) later reported that providing 36 mg GOS/kg bw/day to subjects with a low baseline bifidobacteria population (mean = log 9.46) caused a significant increase in bifidobacterial growth. Bifidobacteria levels in these two studies returned to near baseline levels when GOS administration was discontinued.

Tanaka et al. (1983) provided subjects with 43 mg/kg bw/day GOS for one week, followed by 143 mg/kg bw/day GOS for a second week. Endogenous bifidobacteria counts increased in four of the five subjects. In this study, investigators also examined the effect of feeding GOS + *Bifidobacterium breve* (*B. breve*) and *B. breve* alone on the fecal microflora. Resident and supplemented *Bifidobacterium* spp. increased in the GOS + *B. breve* group, while no change was found in the group that received *B breve* only. These results suggest that GOS promotes growth of both resident and supplemented bifidobacteria.

Both Bouhnik et al. (1997) and Tamai et al. (1992) observed increased bifidobacteria in healthy individuals after approximately 20 days of consuming GOS at 143 or 114 mg/kg bw/day.

In subjects provided with 116 mg GOS/kg bw/day for 3 weeks, a slight but non-significant increase in bifidobacteria was observed (Alander et al. 2001). GOS provided at higher levels (206–214 mg/kg bw/day) for 14–21 days did not elicit a bifidobacteria response significantly different from that of a placebo group (Alles et al. 1999) or compared to baseline levels (Teuri et al. 1998). Satokari et al. (2001) also failed to find a bifidogenic effect from administration of 8 g/day (c. 114 mg/kg bw/day) GOS for 2 weeks.

In all of the studies in which fecal bifidobacteria were measured, investigators also examined the effects of GOS intake on populations of other fecal bacteria. In general, intake of GOS produced no consistent effect on raising or lowering fecal populations of total bacteria (Tamai et al. 1992; Ito et al. 1992; Ito et al. 1993), total anaerobes (Teuri et al. 1998; Alles et al. 1999), bacteroides (Ito et al. 1990; Ito et al. 1993; Tamai et al. 1992; Tanaka et al. 1983), lactobacilli (Ito et al. 1990; Ito et al. 1993; Tamai et al, 1992; Tanaka et al. 1983), or *C perfringens* (Alander et al. 2001; Ito et al. 1993; Tamai et al. 1992). In other studies, GOS consistently had no effect on fecal populations of enterobacteria (Bouhnik et al. 1997; Ito et al. 1990; Ito et al. 1993), clostridia (Tamai et al. 1992; Alles et al. 1999), and enterococci (Ito et al. 1990; Ito et al. 1993).

Overall, the response of the microflora to GOS ingestion was highly variable across the studies summarized above, and it remains difficult to identify the most beneficial dose or duration of GOS administration in adults. Changes in bifidobacteria may have remained undetectable due to small sample sizes; five of the reports examining

microflora changes studied only 8–12 subjects. Differences in individual response or endogenous bacterial populations may cause additional variability among results. Generally, it appears that in individuals with particularly low endogenous bifidobacteria count, GOS supplementation at 36 mg/kg bw/day (2.5 g) may be sufficient to stimulate bifidobacterial growth. Although providing GOS at levels as high as 206-214 mg/kg bw/day (approximately 15 g/day) did not promote growth of bifidobacteria in two studies (Alles et al. 1999; Teuri et al. 1998), studies providing subjects with 143 mg/kg bw/day (10 g/day) showed positive bifidobacteria growth, suggesting this as a level sufficient to detect a bifidogenic effect in healthy adults (Bouhnik et al. 1997; Ito et al. 1990; Tanaka et al. 1983).

(2) Measures of Microbial Fermentation

The majority of studies of GOS intake by adults evaluated one or more changes in microbial fermentation. Measures of bacterial activity and carbohydrate fermentation included: pH, fecal weight, fecal dry matter, SCFA production, ammonia production, changes in specific enzyme activities or amounts, and breath-hydrogen excretion.

Across studies, GOS had no effect on the fecal pH of adults regardless of dose, study duration, or subject age (Bouhnik et al. 1997; Ito et al. 1993; Tamai et al. 1992; Teuri et al. 1998; Teuri and Korpela; 1998; van den Heuvel et al 1998; van Dokkum et al. 1999). Daily intake of GOS also had no effect on fecal weight g/kg bw/day (Alles et al. 1999; Bouhnik et al. 1997; Ito et al. 1990; van Dokkum et al. 1999), levels of total fecal SCFAs (Alles et al. 1999; Ito et al. 1993; van den Heuvel et al. 1998), or percentage dry matter (Alles et al. 1999; Teuri and Korpela 1998). Although van Dokkum et al. (1999) reported no effect on fecal total weight, a decrease in fecal dry weight was noted. In two of the studies in which SCFAs were measured, levels of acetic acid in GOS-fed subjects increased. GOS intake appeared to have no consistent impact on levels of other SCFAs. The concentration of fecal ammonia also was not affected by GOS feeding (Tanaka et al. 1983; Ito et al. 1993). A decrease was noted, however, in four of five subjects receiving both GOS and *B. breve* (Tanaka et al. 1983). Ito et al. (1993) reported no change in ß-glucuronidase activity after feeding 36 mg GOS/kg bw/day, though van Dokkum et al. (1999) reported increased ß-glucuronidase activity when subjects were provided 188 mg GOS /kg bw/day. GOS intake also had variable effects on breath-hydrogen excretion. A significant increase was reported by Alles et al. (1999), who provided subjects 206 mg/kg (14.4 g) GOS daily for 3 weeks and Tanaka et al. (1983), who provided subjects 500 mg/kg (30 g) GOS in a single dose. Results from other studies in which lower doses of GOS were tested indicated a decrease (Bouhnik et al. 1997) or no change in breath-hydrogen excretion (van Dokkum et al. 1999).

(3) Stool Characteristics

Many studies of the effects of GOS intake by adults assessed stool characteristics, including stool consistency, water content, frequency, and transit time.

Intake of 2.5, 5, or 10 g (36, 71, or 143 mg/kg) GOS daily for one week by healthy males resulted in softer stools as reported by Ito et al. (1990). Iiu et al. (1994) found that constipation and incidence of no bowel movement and incomplete bowel movement were reduced in young adult volunteers fed 143 mg GOS/kg bw/day for 10

days. GOS intake by elderly females with constipation, however, had no effect on stool consistency (Teuri and Korpela 1998). Results from several studies indicate that GOS intake for 3 weeks had no effect on the percentage of water in feces (Bouhnik et al. 1997; Ito et al. 1993; Tamai et al. 1992).

Two of the four studies evaluating stool frequency reported no difference with ingestion of 36–206 mg GOS/kg bw/day for 3 weeks (Alles et al. 1999; Ito et al. 1990). An increase in defecation frequency was reported when GOS was provided for 2 weeks at a dose of 214 mg/kg bw/day (Teuri et al. 1998) and in 8 of 14 elderly subjects with constipation provided with 133 mg/kg bw/day (Teuri and Korpela 1998).

One study estimated transit time and reported no effect with GOS treatment (van Dokkum et al. 1999).

(4) Conclusions of Studies in Adults

Overall, results from the majority of studies identified suggest that GOS promotes growth of bifidobacteria in adult humans. A dose as low as 36 mg/kg bw/day GOS may be bifidogenic in populations with low endogenous bifidobacterial counts. It is difficult to draw conclusions regarding the effect of GOS on other bacteria, as studies evaluating these forms are inconsistent in methods and reported outcomes. There is no evidence to suggest that adult fecal pH, stool weight, total SCFA production, fecal ammonia concentration, or fecal enzyme activity is altered by GOS ingestion at the levels tested in these studies.

Results from these studies suggest that GOS intake may have a softening effect on feces in healthy adults, and may help to relieve constipation in elderly individuals with this condition. GOS intake may also increase frequency of defecation in healthy adults, though results are somewhat equivocal.

4. Conclusions

Evidence from studies providing infants with a prebiotic formula indicate that ingestion of GOS at a concentration of up to 9 g/L is bifidogenic and may also promote growth of lactobacilli. It may also result in increased production of SCFAs and resulting reduced fecal pH. Formula containing 3.6–9 g/L GOS was well tolerated by both preterm and term infants and resulted in softer stool consistency. In healthy adults, ingestion of 143 mg GOS/kg bw/day appears to promote growth of bifidobacteria and inconsistently induce changes in other anaerobic species while improving constipation. Effects on other indicators of microbial fermentation, including fecal pH, stool weight, and total SCFA production, are not apparent. Animal and *in vitro* data support a bifidogenic effect of GOS and increased concentrations of short-chain fatty acids.

D. Safety

1. Animal Studies

a. Safety Findings from Studies of Efficacy

Few animal studies reported or specifically evaluated safety endpoints. Across studies, there were no reports of animal death as a result of GOS treatment.

With the exception of selected reports of diarrhea, there were no reports of intolerance in animals fed from 0.05–14 g/kg bw/day. In a study of pigs in which 2 GOS test groups received 0.1–1.4 g GOS/kg bw/day, 2 fructooligosaccharide (FOS) test groups received similar levels of FOS, and a control group received no non-digestible oligosaccharides (NDO), 2 pigs developed severe diarrhea and were excluded from the data analyses of efficacy (Houdijk et al. 1998). The authors did not report which diet these pigs received. Ohtsuka et al. (1990) reported that some rats consuming a 10% (w/v) GOS diet (9.4 g/kg bw/day) initially developed diarrhea but the condition resolved after 2 weeks. Discrete signs of diarrhea also were observed in two rabbits after weaning and consuming a diet of 0.05 g/kg bw/day GOS (Maertens and Peeters 1992).

Nearly all studies documented that GOS ingestion did not cause any significant changes in body weight or food intake. A small number of studies, however, did indicate that animals consuming GOS had greater body-weight gains compared to animals consuming a control diet (Kikuchi-Hayakawa et al. 1997; Quigley et al. 1997) or compared to animals consuming a lower (Ohtsuka et al. 1990) or higher (Hayashi et al. 1991) dose of GOS.

The effects of GOS on calcium, magnesium, or phosphorous absorption and retention were assessed in several studies in rats (Chonan et al. 1995; Chonan and Watanuki 1995; Chonan and Watanuki 1996; Chonan et al. 1996; Chonan et al. 2001) and a study in dogs (Zentek et al. 2002). Studies in rats consistently indicated increased absorption of these minerals with GOS intake, though GOS intake had no effect on mineral absorption in dogs.

In two studies, GOS administration reduced plasma cholesterol but did not affect serum triglycerides (Djouzi and Andrieux 1997; Kikuchi et al. 1996).

Findings from these animal studies corroborate that GOS is safe and well tolerated. Overall, at the levels of daily GOS tested in animal studies, GOS intake was associated with few adverse events and did not appreciably affect food intake or weight gain in animals. Results also indicate that intake of GOS may reduce cholesterol and promote calcium, magnesium, and phosphorous absorption in rats.

b. Toxicity Studies of Vivinal®

(1) Subchronic Feeding Study (Lina 1995)

A 90-day oral feeding study in rats (Lina 1995) was conducted to investigate the potential toxicity of transgalacto oligosaccharide syrup (Vivinal®) produced by Borculo Whey Products.

Wistar rats were randomized to 1 of 4 groups as shown in *Table 11*. The test substance was incorporated in the feed at the dose levels indicated.

Table 11. Study Design (Lina 1995)

Dose Group	Males	Females	Dose Level (% of Feed)	Dose Level (mg/kg bw/day)	
				Males	Females
1	20	20	0	0	0
2	20	20	5	3,625	4,100
3	20	20	10	7,250	8,200
4	20	20	20	14,500	16,400

General condition and behavior were not adversely affected in any of the groups and none of the rats died during the study. Ophthalmological examination at the conclusion of the study did not reveal any treatment-related changes. In the first few weeks of the study, there was a slight decrease in food intake in both sexes and transient growth retardation was observed in males at the high dose. The growth retardation did not continue, and was regarded as due to irritation or poor palatability of the test material rather than toxicity. Water consumption was not affected by the treatment.

Values obtained for red blood cell variables, clotting potential, and total and differential white blood cell counts did not show significant changes. Decreases were observed in plasma phospholipids in males of the high-dose group and in urea levels in females of the middle- and high-dose groups. Urinary density was decreased in males of the high-dose group. These changes were within the range of historical controls and were judged to be toxicologically insignificant. There were no significant changes in semi-quantitative observations in the urine or in microscopy of the urinary sediment.

At autopsy, a dose-related decrease in the pH of the cecal content was observed in both males and females of the middle- and high-dose groups. The weights of the full and empty cecum showed a dose-related increase in both sexes in all dose groups. Spleen weights were slightly increased in females of the high dose group. Gross examination at autopsy did not reveal any abnormalities attributable to the administration of the test substance, and microscopic examination revealed no treatment-related changes.

The physiological responses to the feeding of the test substance were regarded as expected and non-adverse intestinal effects of administration of high levels of non-digestible carbohydrate and not as evidence of toxicity. The study authors determined that the no observed adverse effect level (NOAEL) for Vivinal® administered in the feed for 90 days under the conditions of the study was 14,500 mg/kg bw/day for males and 16,400 mg/kg bw/day for females, the highest doses tested.

(2) Subchronic Gavage Study (Anthony et al. 2006)

A 90-day oral (gavage) study in rats (Anthony et al. 2006) was conducted according to Good Laboratory Practices to investigate the potential toxicity of Vivinal® galacto-oligosaccharides syrup (GOS).

Vivinal® was provided to Springborn Laboratories as a pale yellow liquid and was stored refrigerated at 2–8°C.[1] Reference controls, including fructose oligosaccahrides (FOS), sucrose, lactose, and dextrose were also provided as white powders. These served as controls to simulate the differing amounts of fructose in each of the two different test materials and to determine whether the high fructose content had an adverse impact on food consumption. The reference-control materials were stored at room temperature. The vehicle reference control was prepared weekly as 70% w/v[2] FOS (a cloudy pale yellow suspension of lactose and glucose mixed with reverse osmosis deionized tap water [RODI]). This was stored refrigerated, and a sufficient amount was removed each day to prepare the solution for dosing the vehicle reference control animals. The homogeneity and stability (over 10 days) of the reference control were confirmed analytically by KAR Laboratories. The concentration of each reference and vehicle control was verified analytically after dose preparation at weeks 0, 7 and 11.

Sprague Dawley Crl:CD®(SD)IGS BR rats, male and female, were housed (2–3/sex/cage) for 6 days after receipt. During the remainder of the acclimation period, and while on study, the rats were housed individually in suspended stainless steel cages. After acclimation for 13 days, animals were randomized to 1 of 4 groups as noted in *Table 12*. (Groups 2–4, not shown, were the FOS reference control and test groups for SOS.)

Table 12. Study Design (Anthony et al. 2006)

Dose Group	Males	Females	Dose Material	Dose Level[3] (mg/kg bw/day)	Dose Volume[4] (mL/kg)

[1] Documentation concerning chemical identification, purity, strength, stability, and other required data is maintained by MJN.

[2] w/v = weight to volume

[3] Dosage levels were based on a prior range-finding/tolerability study. The high-dose level was selected as it is an acceptable upper limit dosage level for longer term oral studies by regulatory agencies

[4] Dosage volumes were calculated based on density determinations performed at SLI Vivinal® = 1400 mg/mL.

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1	15	15	RODI Water	0	14 7
5	15	15	FOS Reference Control	0	7 8
6	15	15	Vivinal®	2500	3 9
7	15	15	Vivinal®	5000	7 8

On day -1, animals were weighed and examined for clinical observations and randomized to one of the above four groups. Animals were approximately 6 weeks of age, with body weights ranging from 201–254 g for males and 139–187 g for females. The test article, reference control preparation, and vehicle were administered each morning by oral gavage on study days 0–89 (first 5 animals/sex/group), days 0–90 (second 5 animals/sex/group), or days 0–91 (last 5 animals/sex/group). Individual doses were based on the most recent body weights. The reference control was utilized at the same dose volume as the Vivinal® high-dose level to mimic caloric intake and fiber content.

A wide variety of endpoints was assessed in this study, as follows:

1) Clinical observations for general health/mortality and moribundity were performed twice daily. Observations were performed once weekly prior to dosing and prior to scheduled euthanasia. Signs of overt toxicity were monitored approximately 2 hours following dosing for each group.

2) Body weights and food consumption were recorded on day -1 and weekly thereafter. A final body weight was taken prior to sacrifice on days 90, 91, or 92.

3) To perform clinical pathology, blood was collected from fasted rats on study day 90 (first 5 rats/sex/group), day 91 (second 5 rats/sex/group), or day 92 (last 5 rats/sex/group). Hematology,[5] coagulation,[6] and clinical chemistries[7] were conducted on all samples.

4) Urine samples[8] were collected overnight prior to initiation of blood collection. Feed was withheld during urine collection, but water was available.

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[5] Erythrocyte (RBC), hematocrit (Hct), hemoglobin (Hgb), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), platelet count, reticulocyte count, and total and differential leucocyte counts (including RBC morphology).

[6] Activated partial thromboplastin time (APTT), prothrombin time (PT) and fibrinogen

[7] Alanine aminotransferase (ALT), albumin, albumin/globulin (calculated), alkaline phosphatase, aspartate aminotransferase (AST), blood creatinine, blood urea nitrogen (BUN), calcium Cholesterol, electrolytes (sodium, potassium and chloride), gamma glutamyl transferase (GGT), globulin (calculated) phosphorus, total bilirubin, and total serum protein.

[8] Included qualitative analysis for bilirubin, blood, glucose, ketones, leucocytes, nitrites, protein, specific gravity, and urobilinogen Gross appearance, pH, and total volume were also recorded. Microscopy was done on the spun deposit.

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5) Ophthalmological examinations were performed on all rats prior to in-life initiation (day -2) and prior to the end of the study (day 83).

6) All animals were subjected to a complete gross necropsy examination[9] upon death or scheduled sacrifice on days 90–92.

7) Organs and tissues were obtained at scheduled sacrifice and preserved for possible histological evaluation.[10] All tissues collected at necropsy from Groups 1 and 4, and the lungs, liver, kidneys and gross lesions from the remaining groups, were processed for histological examination. Appropriate statistical analyses were conduced on the data collected on each parameter.[11]

When Vivinal® was administered to rats by gavage at 2500 or 5000 mg/kg bw/day for 90 days, there were no premature deaths attributed to treatment. Clinical signs were unremarkable, and there were no ocular findings in any animal. Analysis of clinical pathologies, including blood biochemistries, hematology,[12] urinalysis and coagulation revealed only random statistically significant effects. There were also occasional effects noted on absolute and relative organ weights. These differences were generally found only in the RODI water controls as opposed to the FOS reference controls (which were included in the study to account for the higher caloric and fiber intake in the treatment groups). There were no corroborative findings at study termination in either macroscopic or histopathologic examinations that indicated any of these effects were related to treatment. In addition, the random occasional observations in hematology and blood chemistries were within the range of intralaboratory controls, not consistent between sexes, and not dose related. As noted above, none were corroborated by macroscopic or histological findings.

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[9] Gross necropsy examinations included evaluation of external surfaces of the body, all orifices, and the cranial, thoracic, abdominal, and pelvic cavities, and their contents. Surviving rats were euthanized by CO_2 inhalation followed by exsanguinations

[10] Genital organs, adrenals, gross lesions, aorta, bone marrow smear (femur), brain, cecum, colon, duodenum, esophagus, lachrymal glands, eyes, femur, ileum, jejunum, kidneys, liver, lungs, mammary glands, lymph nodes, nasal cavity, pancreas, peripheral (mediastinal, mesenteric, and submandibular) nerve (sciatic) pituitary, prostate, rectum, skeletal muscle (thigh), skin, spinal cord (cervical, midthoracic, and lumbar), spleen, stomach, salivary gland, testes/ovaries, tongue, trachea, and bladder

[11] Body weights, body-weight gain and food consumption were analyzed by One-Way Analysis of Variance (ANOVA). If significance was detected with ANOVA ($p < 0.05$), pair-wise group comparison was conducted using the Tukey-Kramer test. Clinical pathology data and absolute and relative organ weights were analyzed for homogeneity of variance using Levene's test If $p < 0.01$, multiple group comparisons proceeded using the Kruskal-Wallis non-parametric ANOVA, followed by Dunn's test, when $p < 0.05$. The following comparisons were made between the groups

Group 1 vs. Groups 5, 6, and 7
Group 5 vs Groups 6 and 7

[12] For example, mean eosinophil value for the Vivinal® reference control (G5) was higher than the RODI water control. Mean hematocrit and hemoglobin values for 5000 mg/kg bw/day Vivinal® (G7) females were higher than the reference control for Vivinal® (G5) There were other similar changes that were within historical control ranges, not consistent between sexes, or not dose related.

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Body-weight effects were not observed in either sex in either Vivinal® group when compared to their FOS controls. Decreased food consumption was noted in both sexes of the FOS reference control group when compared to the RODI reference controls. A similar decreased food consumption was seen in both sexes of the animals dosed at 5000 mg/kg bw/day Vivinal® when compared to the RODI water controls. It can be hypothesized that the effects that were observed on food intake in the high-dose Vivinal® and FOS reference controls may be the result of higher caloric load, greater gavage volumes, and/or higher osmolarity of these test solutions in the GI tract compared to the lower Vivinal® dose group or RODI water controls. Based on these considerations, as well as the lack of toxicologically relevant effects on other parameters in the study, the no observed adverse effect level (NOAEL) for Vivinal® administered by gavage for 90 consecutive days is 5000 mg/kg bw/day.

2. Human Studies

a. Studies in Infants

Seven studies were identified that examined the effects of providing prebiotic-containing formulas to populations of both term and preterm infants. In five of the studies (Boehm et al. 2002, 2003; Knol et al. 2001; Moro et al. 2002, 2003; Savino et al. 2003; Schmelzle et al. 2003), the prebiotic added to the formula consisted of 90% GOS and 10% frutooligosaccharides (FOS). In two studies (Ben et al. 2004; Napoli et al. 2003), GOS alone was added to the formula. The durations of feeding in these studies ranged from 14 days to 6 months. The studies were designed to assess the effects of the prebiotic formulas on fecal microflora, stool characteristics, and growth as well as to evaluate potential side effects.

In the single study of preterm infants (Boehm et al. 2002), 15 infants born at =32 weeks gestational age consumed infant formula containing 9 g/L GOS and 1 g/L FOS daily for 4 weeks while 15 infants consumed a control formula; the study also included 12 breast-fed infants as a human-milk reference group. The estimated intake of the prebiotic blend was 1.74 g/kg bw/day, including 1.57 g/kg bw/day of GOS. Intake of the prebiotic formula resulted in no differences in weight or length gains of the infants as compared to infants in the control group, and the GOS+FOS-supplemented formula had no effect on incidence of crying, regurgitation, or vomiting.

Moro et al. (2002, 2003) studied intake of a formula containing either 4 or 8 g/L of a prebiotic blend (3.6 g/L GOS + 0.4 g/L FOS or 7.2 g/L GOS + 0.8 g/L FOS, respectively) or a control formula containing maltodextrin for 4 weeks. The study population was healthy term infants; 30 infants received 4 g/L prebiotic blend, 27 received 8 g/L prebiotic, and 33 infants consumed the control formula. The estimated average intake of the prebiotic blend was 0.73 or 1.57 g/kg bw/day; the estimated average intake of GOS was 0.66 or 1.41 g/kg bw/day. Intake of the prebiotic formulas resulted in no differences in weight or length gains of the infants as compared to infants in the control group, and the GOS+FOS-supplemented formulas had no effect on incidence of crying, regurgitation, or vomiting. No adverse effects were observed.

Savino et al. (2003) studied the effects of consumption for 14 days of formula containing 8 g/L of a GOS+FOS blend (90% GOS, 10% FOS) on 604 term infants: 214 with colic, 201 with regurgitation problems, and 232 with constipation. Intake of the prebiotic blend reduced the severity of these problems with no reported adverse effects and with positive judgments from both parents and pediatricians regarding the infants' GI comfort.

In another study of the prebiotic blend of 90% GOS and 10% FOS (Schmelzle et al. 2003), healthy term infants received either a formula containing 8 g/L of the blend or a control formula for 12 weeks. This study, with 49 infants in the test group and 53 in the control group, was intended to evaluate the nutritional efficiency of the test formula and was powered to detect a difference in weight gain as small as 3 g/day. The estimated daily intake of the blend was 1.08 g/kg, and the estimated daily intake of GOS was 0.97 g/kg. No adverse effects were observed, and there was no difference in tolerance between the test and control groups. There was no difference in gains in weight, length, head circumference, or skinfold thickness. Finally, measures of blood biochemistry found no differences in total protein, albumin, or urea and no clinically significant differences in prealbumin, tyrosine, threonine, isoleucine, or lysine.

Ben et al. (2004) studied the effects of feeding term infants a formula containing 2.4 g/L of GOS for 6 months. The study included 69 infants in the test group and 52 controls, along with a human milk reference group of 26 infants. No adverse side effects such as crying, vomiting, or regurgitation were observed. There was no difference between the test group and the controls in gain in weight or length.

Results from all of these studies demonstrate that the prebiotic supplemented formulas supported normal growth in healthy term and preterm infants at the tested doses, and caused no untoward side effects related to tolerance of the formulas. Schmelzle et al. (2003) further showed that 0.97 g GOS/kg bw/day had no significant impact on blood biochemistry.

b. Studies in Adults

Several studies of GOS intake by adults examined safety endpoints, including adverse events, tolerance of the supplement, gastrointestinal distress, clinical chemistries or hematology, and mineral absorption.

Overall, few adverse events were reported by subjects consuming the levels of GOS provided in these studies. Intake of GOS up to 286 mg/kg bw/day (20 g/day; GOS provided in one to three portions per day in each study) had no effect on tolerance or gastrointestinal distress (Alles 1999; Bouhnik et al. 1997; Teuri and Korpela 1998; van den Heuvel et al. 2000; van Dokkum et al. 1999). Flatulence was the most commonly reported side effect in studies assessing safety endpoints. In one study, subjects taking 36–143 mg/kg bw/day experienced a dose-dependent, but non-significant increase in flatulence with transient gas and bloating (Ito et al. 1990). Teuri et al. (1998) reported a significant increase in flatulence in subjects ingesting 214 mg/kg bw/day (provided in two portions), but incidence of other abdominal symptoms did not differ from controls. However, when pooled, overall GI symptoms (poor appetite, flatulence, loose stools, soft

stools, abdominal distention, other abdominal symptoms) appeared more frequently in the test group. Occasional "mild diarrhea" was reported, but it is not clear that true diarrhea was observed or merely increased defecation frequency, more watery stools, and other indications of altered stooling characteristics. In other studies, mild flatulence was reported by some subjects (van Dokkum et al. 1999; Alles et al. 1999). In a study by Alander et al. (2001), 1 of 30 subjects reported pain from intestinal bloating; though no other complaints were reported. Several studies specifically noted that no subjects experienced diarrhea (Bouhnik et al. 1997; Ito et al. 1990; Teuri et al. 1998).

Van den Heuvel et al. (1998; 2000) examined the effect of GOS on mineral absorption. In the 1998 study, healthy men were provided with 188 mg/kg bw/day, delivered in three portions daily for a period of 3 weeks. In these subjects, GOS feeding had no effect on calcium absorption, mean iron status, or percentage of iron absorption. In the subsequent study, calcium absorption was measured in women confirmed to be postmenopausal for =5 years. These women were provided with GOS initially at a dose of 149 mg/kg bw/day and gradually increased to 285 mg/kg bw/day over a period of 9 days. During the period of GOS intake, the women experienced a 16% increase in calcium absorption (van den Heuvel et al. 2000).

One study (van Dokkum et al. 1995) included assessments of blood lipid concentrations during intake of 188 mg/kg bw/day GOS by healthy men; no adverse effects were observed.

c. Conclusions of Human Studies

Daily intake in infant formula of 8 g/L prebiotics (90% GOS, 10% FOS) by healthy term infants and 10 g/L prebiotics by preterm infants appears to be safe and well tolerated as demonstrated by normal growth and lack of adverse effects on crying, regurgitation, or vomiting. Results from studies of GOS intake by adults suggest that daily intake of up to 286 mg/kg bw/day GOS, provided in two portions, may be well tolerated in this population. Overall, increased laxation, occasional flatulence, and a greater incidence of unformed watery stools were seen at higher levels of intake of GOS and prebiotic blends containing GOS. While increased laxation may result in some change in water balance, it is likely to remain within the normal range and was therefore judged not an issue of concern.

Table 13. *In vitro* Studies of Galactooligosaccharide (GOS)

Reference	Objective	Study Design	Results[a]
Boehm et al 2004	Examine fermentation of feces with added GOS/FOS or human milk oligosaccharide fraction v control	Fecal samples were collected from healthy formula or breast fed infants A 0 1% solution of either GOS/FOS or human milk oligosaccharide fraction was added, control samples were left untreated After 24 hours incubation, SCFAs were measured Source Not specified	-SCFA production resulting from GOS/FOS was similar to that from human milk oligosaccharide fraction -SCFA patterns were similar for the GOS/FOS groups and the human milk oligosaccharide group with acetate dominating
Bouhnik et al 1997	Examine consequences of prolonged administration of GOS on metabolism of bacteria found in human feces	Fecal samples were collected from 3 humans and homogenized Two aliquots were fermented in a semi-continuous culture system, one was inoculated with 10 g GOS/d for 14 d Samples were tested on days 1, 2, 7, and 14 2 treatments control, GOS Source Yakult Institute, Japan	-Maximum degradation rate (95%) reached on day 4, no subsequent changes -pH decreased in 2 d, decreased further by 7 d, remained at this level through day 14 -Total SCFAs doubled between 1 and 7 d, increased proportion of acetate, decreased proportion of propionate -Lactate detected on d 7 -Total gas production similar at all time points, though initial rate of production increased -Increased ATP concentrations
Durand et al 1992 *Abstract*	Study effects of GOS on fermentation metabolism and balance of human fecal microflora	Fecal samples were collected from 3 methane-producing humans and homogenized Duplicate aliquots were fermented in a continuous culture system After a 7 d adaptation period, one sample was inoculated with 20g GOS/d, delivered in 2 portions, for 15 d Measurements were taken at 3, 5–6, and 15 d 2 treatments control, GOS Source Yakult Institute, Japan	-Maximum degradability (95%) at 3 d After d 3, fermentation stabilized -Increased total SCFAs, increased acetate, propionate, and butyrate -Increased CO_2 and CH_4 production

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Table 13. *In vitro* Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Results[a]
Flickinger et al 2000	Examine fermentation characteristics of feces from humans fed 6 different carbohydrate sources	Randomized, complete block design Fecal samples were collected from 3 adults (avg = 30 y) after feeding with each of six substrates Each sample was incubated in an anaerobic system for 24 h Dose was not specified Samples were collected at 0, 1 5, 3, 6, 11, 18, and 24 h 7 treatments control, GOS, maltodextrin-like oligosaccharide, FOS, gum arabic, guar gum, and hydrolyzed guar gum Source Bioecolians, Solabia, Pantin Cedex, France	-Time-dependent decrease in pH, lowest pH of all substrates at 24 h -Time-dependent increase in organic acid production (acetate, propionate, and butyrate), highest concentration of organic acids after 24 h
Hopkins et al 1998	Study bifidobacteria growth rates and bacterial cell mass production in response to 15 different carbohydrate sources	*Bifidobacteria* were incubated in an anaerobic system for 48 h The medium contained one of 15 different carbohydrate sources Bacterial growth was measured at the end of the incubation period 15 treatments 15 carbohydrates, including GOS (85%) and Oligomate 55® Source Yakult Institute, Japan	-Significant growth rates observed for 7 bifidobacterial isolates on both GOS and Oligomate-55® -Highest culture optical density of *B adolescentis* and best growth of *B bifidum* with GOS and galactose, growth of *B longum*, *B pseudolongum*, *B catenulatum*, and *B infantis* observed
Houdijk et al 1997 *Conference Proceedings*	Study effects of bacterial fermentation kinetics using feces and ileal digesta from pigs fed GOS	Feces and ileal digesta of pigs fed 4% GOS diet for 13–18 d were collected Samples were incubated in an anaerobic system for up to 96 h 3 treatments control, FOS, GOS Source Oligostroop®, Borculo Whey Products, The Netherlands	-Incubation of feces from pigs fed GOS and inoculated with GOS resulted in lower % butyrate, no apparent difference in total VFAs, % acetate, or % propionate -Incubation of ileal digesta from pigs fed GOS and inoculated with GOS resulted in no differences in total VFAs or % acetate, butyrate, or propionate -Higher VFAs from ileal digesta compared to feces -No effect on asymptomatic gas production

000158

Table 13. *In vitro* Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Results[a]
Kikuchi-Hayakawa et al 1997 *Abstract*	Examine effects of GOS feeding on fermentation in rat cecal contents	Cecal digesta of rats fed 5% GOS for 1, 2, 7, and 21 d were collected Samples were incubated and inoculated with 10 mg GOS Samples were taken at 1, 2, 4, 8, or 24 h of incubation 2 treatments control, GOS Source Yakult Institute, Japan	-Higher lactic acid at 1 d and acetic acid at 2, 7 and 21 d, propionic acid at 2 d, and butyric acid at 1 and 21 d -Higher amount of lactic acid at 1 d, no differences in amounts of other organic acids
McBain and Macfarlane, 2001	Study bacterial growth, bifidogenicity, and anti-mutagenicity of GOS and inulin using a human colon model	Human feces from 1 healthy donor were collected The sample was incubated with 10 g GOS in a 3-stage continuous culture system modeled after the human colon Samples were taken at regular intervals over 30 h 3 treatments control, inulin, GOS Source Yakult Pharmaceutical, Japan	-Increased *Lactobacillus* in proximal colon vessel, increased *Bifidobacterium* in distal colon vessels -*Bacteroides* and *Peptostreptococcus* growth inhibited in proximal colon vessel, most bacterial populations changes occurred in the proximal colon vessel -Inhibition of ß-glucosidase and ß-glucuronidase activity in all vessels -Stimulated azoreductase in all vessels and nitroreductase in the distal colon vessels -Vigorous gas formation observed within minutes of adding GOS
Ohtsuka et al 1990	Measure the digestion of GOS prepared from *Cryptococcus laurentii* in several different reaction mixtures	GOS was added to reaction mixtures containing human salivary a-amylase, hog pancreatic a-amylase, artificial gastric juice with NaCl, pepsin, and HCl, or rat small intestinal mucosa homogenates Source GOS produced using ß-galactosidase from *Cryptococcus laurentii*	-GOS was not well digested and no increase in reducing sugar was noted in any of the reaction mixtures

Table 13. *In vitro* Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Results[a]
Rycroft et al 2001	Compare the fermentation of various prebiotics by fecal bacteria in fecal slurry made from healthy humans	Substrate (0 01 g/ml) was added to 10% fecal slurry made from fresh feces of 1 healthy individual Samples were incubated at 37°C and collected and analyzed at 0, 5, and 24 h Each substrate was evaluated in triplicate using feces from 3 different donors 7 treatments lactulose, fructooligosaccharide, inulin, xylooligosaccharide, galacto-oligosaccharide, soybean oligosaccharide, isomaltooligosaccharide Source LACT, Sigma, Poole, UK	-Increased *Bacteroides* at 5 h, increased *Bifidobacterium* at 5 and 24 h -No changes in the total count, *Clostridium*, *Lactobacillus*, *Streptococcus*, or *E coli* -Increased lactate, acetate, and propionate production, no change in butyrate -Low gas production relative to other prebiotics
Sharp et al 2001	Examine effect of GOS and other NDO on population of *Bifidobacterium* and inhibition of enterobacteria	Fresh fecal samples were homogenized and filtered 4 triplicate samples control, or 10 g/L added GOS, FOS, or fructose Bacterial enumeration at 3, 6, 12, 24, and 48 hours Source Yakult Institute, Japan	-Increased numbers of *Bifidobacteria* by both GOS and FOS, approximately at 6 hr , FOS superior at 12 & 24 hr , GOS superior at 48 hr -Reduced *E coli*
Tanaka et al 1983	Study effects of GOS on bacterial growth	Bacterial species were incubated in 1% GOS medium for 24 h Bacterial growth was measured at the end of the incubation period Treatment GOS Source Not specified	-Growth observed within 24 hours of *Bifidobacterium* (*B bifidum*, *B infantis*, *B lactentis*, *B liberorum*, *B breve*, *B longum*, *B adolescentis*), *Lactobacillus* (*L plantarum*, *L casei*, *L fermentum*, *L acidophilus*, and *L salivarius*) -Growth observed in *Enterobacteriaceae* spp and some *Streptococcus* -No growth observed in *Fusobacterium*, *Eubacterium*, or *Propionibacterium* acnes

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Table 13. *In vitro* Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Results[a]
Zentek et al 2002	Investigate effects of GOS on products of microbial metabolism using feces of dogs fed GOS	Feces of dogs fed 1 0 g GOS/kg/d for 10 d were collected Each sample was incubated for 24 h Samples were analyzed at the end of the incubation period 5 treatments control, GOS, mannanoligosaccharide, lactose, lactulose Source Borculo Whey Products, Borculo, The Netherlands	-Increased VFA production, increased proportion of acetic acid -Decreased proportion of propionic and n-butyric acids -Increased ammonia -Increased gas volume

[a] Results limited to endpoints reflecting effects of GOS on microflora and measures of microbial fermentation

Table 14. Animal Studies of Galactooligosaccharide (GOS)

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Anthony et al *2006*	Evaluate the potential toxicity of Vivinal® GOS	90-day oral gavage study, 2 dose levels of GOS, FOS reference control, water reference control	Sprague Dawley rats, 30 (15M, 15F) per group	2500 mg/kg Vivinal® GOS (1500 mg/kg GOS) 5000 mg/kg Vivinal® GOS (3000 mg/kg GOS)	90 d	-Not tested	-No test article-related mortality was observed -No body weight or food consumption effects were observed -No toxicologically meaningful differences were observed in hematology, coagulation, clinical chemistry, or urinalysis -No test article-related ocular or gross internal effects were observed -No toxicologically meaningful differences were observed in relative or absolute organ weights or in microscopic analysis -5000 mg/kg Vivinal® GOS (3000 mg/kg GOS), the highest dose tested, was considered the NOAEL for M and F

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Appel et al 1997	Study effects of GOS and dietary fat on pancreatic carcinogen-esis in rats	Longitudinal feeding study 4 treatments in rats injected with azaserine at 2 and 3 wks of age low- or high-fat with 2 GOS levels	156 M Wistar rats, 39 per group	3 1, 10 4% (low-fat) 3 6, 10 8% (high-fat) (0 9, 3 1 g/kg (low-fat), 0 9, 2 7 g/kg (high-fat)) Source Elix'or, Borculo Whey Products, The Netherlands	12 mo	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -High GOS doses resulted in higher absolute and relative cecum content weights	-No effect on number or size of atypical acinar cell nodules, tumor incidence or tumor multiplicity -No difference in food intake or body weight between GOS groups
Chonan et al 1995	Examine effect of GOS on VFA production, bone loss and serum cholesterol in ovari-ectomized rats	Feeding study in ovari-ectomized and normal rats 2 treatments per group control, GOS	36 F Wistar rats, 9 per group	5% (2 9 g/kg) Source Research conducted by Yakult Central Institute, Japan	30 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -Lower cecal pH -Higher whole cecal, cecal wall, and cecal content weights -Increased total VFA production and increased acetic, propionic, butyric, and succinic acids, no change in lactic acid -No effect on fecal bile salts	-Increased Ca absorption during study, no difference on last day of study -No difference in body weight within each group, in ovariectomized rats, GOS rats consumed less food -Decreased serum total cholesterol in ovariectomized rats -Overall increased dry weight, ash weight, and Ca content of bone

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Chonan & Watanuki 1995	Study effects of GOS on Ca absorption in rats	Parallel feeding study 5 treatments control, 2 lactose levels, 2 GOS levels	30 M Wistar rats, 6 per group	5, 10% (4 3 or 8 6 g/kg) Source Research conducted by Yakult Central Institute, Japan	10 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -Lower cecal pH -Higher whole cecal, cecal wall, and cecal digesta weights -Increased total VFAs in cecal lumen, increased acetic and butyric acids, no change in propionic, succinic, and lactic acids	-Increased Ca absorption and retention (%) -No difference in final body weights and food intake
Chonan & Watanuki 1996	Study effects of GOS on Ca absorption and bone mineralization in rats adapted to high and low Ca diets	Parallel feeding study with 5 d pre-study adaptation to normal or low dietary Ca levels 4 treatments normal Ca (control, GOS), low Ca (control, GOS)	32 M Wistar rats, 8 per group	5% (4 5 g/kg) Source Research conducted by Yakult Central Institute, Japan	30 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -Lower cecal pH -Higher whole cecal, cecal tissue, and cecal digesta weights -Higher total VFA cecum content, increased acetic, butyric, propionic, and succinic acids, no effect on lactic acid	-Increased net Ca absorption during study, no difference in last days of study -Increased bone mineralization with normal Ca diet, no effect with low Ca diet -No differences in body weight or food intake

000164

Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Chonan et al 1996	Study effects of GOS on Ca and Mg utilization in rats fed excessive P and Ca to induce Mg deficiency	Feeding study 4 treatments standard diet, 3 induced Mg deficiency diets (control, GOS, or Mg)	24 M Wistar rats, 6 per group	5% (1 9 g/kg) Source Research conducted by Yakult Central Institute, Japan	28 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -Lower cecal pH -Increased whole cecal and cecal tissue weights -Increased cecal VFAs, increased acetic, propionic, butyric, and succinic acid	-Increased Mg absorption in rats with induced Mg deficiency (absorption comparable to rats fed standard diet) -Lower kidney weight and kidney accumulation of P and Ca, and heart accumulation of Ca in induced Mg deficiency rats -No differences in body-weight gain or food intake
Chonan et al 2001	Study effects of GOS-induced microflora on Ca and Mg absorption in rats with and without suppression of microbial growth by neomycin	Feeding study 4 treatments control, neomycin, GOS, neomycin+ GOS	20 M Fischer 344 rats, 5 per group	5% (1 4 g/kg) Source Research conducted by Yakult Central Institute, Japan	7 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -Lower cecal pH -Higher whole cecal and tissue weight -Increased fecal weight	-Increased Ca and Mg absorption ratios (%) with neomycin-free diet, no effect with neomycin diet -No difference in body weight gain or food intake

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Djouzi & Andrieux 1997	Study effects of FOS, GOS, and gluco-oligosac-charides on human microflora obtained from a CH_4 producer using a gnobiotic rat model	Feeding study in germ-free rats inoculated with human feces 4 treatments control, GOS, FOS, TOS	24 M germ-free Fischer 344 rats, 6 per group	4% (2 0 g/kg) Source Yakult Institute, Japan	4 wk	Stool Characteristics -No data reported Microflora Response -Increased fecal *Bifidobacterium* -No change in total anaerobes, *Bacteroides*, *Clostridium*, *Enterococcus*, and enterobacteria Measures of Microbial Fermentation -Decreased cecal pH -Increased cecal weight -Increased total SCFAs, increased acetate, butyrate, iso-acids, and ethanol, no change in propionate and lactate -Increased ß-galactosidase, no change in ß-glucosidase, a-glucosidase, ß-glucuronidase, N-a acetylgalactosaminidase, or a-L-fucosidase -Increased H_2 and CH_4 excretion -Decreased cecal ammonia	-Decreased plasma cholesterol in treatment groups, no change in plasma triglycerides

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Hayashi et al 1991	Study the effects of GOS on Na and Mg metabolism in rats	Feeding study with three periods of 3-d balance studies 3 treatments control, 2 GOS levels	18 M Wistar rats, 6 per group	1 0, 1 9% (0 9 or 1 7 g/kg) Source Nishin Seito Kaisha, Japan	62 d	Stool Characteristics -Softening of feces was observed Microflora Response -No data reported Measures of Microbial Fermentation -Increased relative and absolute cecum weight -Greater fecal weight, greatest fecal weight in 1 9% GOS group -Dose-dependent increase in fecal wet weight	-No difference in Na or K retention (µmol/3 d, %) except for Na in second balance period (1 9% diet < 1% diet) -Decreased absolute and relative liver and stomach weights, and increased relative large intestine weight in 1 9% diet group -No difference in food intake or weight gain except for weight gain between GOS groups (1 9% diet <1% diet)
Holma et al 2002	Study effects of whey and lactose-derived GOS on intestinal microflora and inflammation in rats	Feeding study in rats with induced colitis on day 10 4 treatments control, whey-derived GOS, lactose-derived GOS, dexa-methasone, healthy rats served as control	42 M rats of HY WIST stock, 7–9 per group	4 0 g/kg (lactose-derived), 3 9 g/kg (whey-derived) Sources whey, Valio Ltd , Finland or lactose, Elix'or, Borculo Domo, The Netherlands	13 d	Stool Characteristics -No data reported Microflora Response -Increased total fecal bacteria and fecal *Bifidobacterum*, no change in proportion of *Bifidobacterium*, growth dependent on medium used to enumerate bacteria Measures of Microbial Fermentation -No change in colon wet weight.	-No colitis-ameliorating effects

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Houdijk et al 1999	Study the effects of GOS on nutrient digestion in growing pigs	Feeding study 5 treatments control, 2 GOS levels, 2 FOS levels	25 growing pigs, 5 per group	0 4, 0 8% (0 1, 0 2 g/kg) Source Borculo Whey Products, The Netherlands	42–47 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -No effect on feces production -No effect on fecal dry matter content	-No difference in fecal digestibility of nutrients, increased ileal digestibility of crude fiber with high GOS diet -No effect on food intake -4 pigs excluded from analysis due to diarrhea
	Study the effects of GOS on micronutrient digestion in growing pigs	Feeding study 4 treatments control, 2 GOS levels, 2 FOS levels	20 weanling pigs, 4 per group	1, 4% (0 4, 1 4 g/kg) Source Borculo Whey Products, The Netherlands	33–37 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -NDOs caused a dose-dependent decrease in feces production -No effect on fecal dry matter	-4 pigs excluded from analysis because of diarrhea -No change in fecal digestion of Ca, Mg, Fe, Cu, Zn, or PO_4 -Increased N excretion -Decreased ileal digestion of nonstarch carbohydrate and increased digestion of hemicellulose and cellulose
Houdijk et al 1998	Study of GOS and FOS on growth parameters and fecal characteristics of piglets	Feeding study 5 treatments control, 2 GOS levels, 2 FOS levels	50 M piglets, 10 per group	0 4, 0 8% (0 3, 0 6 g/kg) Source Oligostroop®, Borculo Whey Products, The Netherlands	6 wk	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -No difference in fecal pH, though fecal pH in all animals increased with time -No change in fecal dry matter	-During wk 1–3, lower mean daily weight gain and daily dry matter intake in pigs fed FOS or GOS, no effect over 6 wk

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Houdijk et al 1997 *Conference Proceedings*	Study effects of GOS and FOS on nutrient digestibility and microbial ecology of weaner pigs	Longitudinal feeding study 5 treatments control, 2 GOS levels, 2 FOS levels	20 weaner pigs, 4 per group	0 4, 1 6% (0 2, 0 6 g/kg) Source Oligostroop®, Borculo Whey Products, The Netherlands	33–37 d	Stool Characteristics -Softening of feces was observed Microflora Response -No difference in total fecal or ileal digesta anaerobes, *Bifidobacterium*, or fecal total aerobes -Decreased ileal digesta total aerobes Measures of Microbial Fermentation -Dose-dependent increase in fecal pH, decreased ileal digesta pH -No difference in total fecal or ileal VFAs (% acetate, propionate, and butyrate)	-N-balance not affected -No effect on digestibility of Ca, P, Mg, or Cu, increased Zn digestibility

Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Kikuchi et al 1996	Study effect of GOS on glycolytic activities, fermentation metabolites and bacterial steroid transformation in rats	Controlled feeding study, rats inoculated with human feces prior to feeding 3 treatments control, 2 GOS levels	12 M germ-free Fischer 344 rats, 4 per group	5, 10% (2 8, 5 6 g/kg) Source Yakult Institute, Japan	4 wk	Stool Characteristics -No difference in cecal water content Microflora Response -No data reported Measures of Microbial Fermentation -Dose-dependent decrease in cecal pH -Greater fecal excretion -Increased cecal weight -Increase in total SCFAs, increase in acetic and propionic acids, no change in butyric acid, decrease in isobutyric, valeric, isovaleric, and caproic acids -No difference in total bile acid excretion -Decreased cecal ammonia -No change in N-acetyl-β-galactosaminidase activity, increase in β-galactosidase activity, dose-dependent decrease in β-glucosidase and decrease in β-glucuronidase -Increased H_2 production, no change in CH_4	-Decreased plasma cholesterol -No change in plasma triglyceride and glucose concentrations -No difference in digestibility of dry matter -No effect on food intake or body weight

000170

Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Kikuchi-Hayakawa et al 1997 *Abstract*	Study the effect of GOS on organic acid production in rats	Feeding study with 10 d diet adaptation 2 treatments control, GOS	12 M Wistar rats, 6 per group	5% (0 8 g/kg) Source Research conducted by Yakult Institute, Japan	16 d	Stool Characteristics -No data reported Microflora Response -No change in total bacteria, *Lactobacillus*, and *Bifidobacterium* -Increased number of *Bacteroides* -Decreased number of *Enterobacteriaceae* Measures of Microbial Fermentation -Increased cecal organic acids	-No difference in food intake -Greater body weight gain
		Feeding study 8 groups, 2 treatments (animals taken from each group for different measures) control, GOS	48 M Wistar rats, 6 per group	5% (2 5 g/kg) Source Research conducted by Yakult Institute, Japan	21 d	Stool Characteristics -Increased fecal water content Microflora Response -No data reported Measures of Microbial Fermentation -Decreased cecal pH -Increased cecum and cecal tissue weight with time -Higher total cecal organic acid concentration, increase in propionic and butyric acids, no change in lactic or acetic acids	-No data reported

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Maertens & Peeters 1992 *Report*	Study effect of GOS provided before and after weaning on cecal parameters and cecal biochemistry of rabbits	Feeding study, rabbits received treatment 10 d before weaning, half were then inoculated with *E coli* 2 treatments each for control and inoculated rabbits control, GOS	48 weanling rabbits, 12 per group	0 1% (0 05 g/kg) Source Eurolysine, France	10 d before weaning, and 7–10 d after weaning	Stool Characteristics -No data reported Microflora Response -Increased *E coli* number in inoculated groups, no effect in uninoculated animals Measures of Microbial Fermentation -Higher mean cecal pH in infected groups. -No difference in cecal weight -No effect on cecal acetic and propionic acids in uninoculated group, increased butyric acid -Lower cecal acetic acid in GOS + *E coli* group	-No effect on diarrhea score -No depression or diarrhea was observed in any of the animals before weaning -After weaning, two animals showed discrete signs of diarrhea -No apparent differences in weight gain or feed intake in uninoculated animals, severe anorexia and growth depression in *E coli* group, no change in uninoculated group
	Study effect of GOS provided before and after weaning on cecal parameters and cecal biochemistry of rabbits	Feeding study 6 treatments control, 5 GOS levels	270 rabbits, 45 per group	0 1, 0 2, 0 3, 0 4, 0 5% (0 1, 0 2, 0 2, 0 3, 0 4 g/kg) Eurolysine, France	10 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -No effect on cecal weight -Decreased cecal pH -Increased acetic and butyric acids, no effect on propionic acid -No effect on cecal ammonia	-No effect on weight gain or food intake

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Meslin et al 1993	Study effects of GOS on mucin distribution, bacterial metabolism, and glycolytic activities in conventional, germ-free, and heteroxenic (HE) rats	Feeding study in 3 rat models (conventional, germ-free, and heteroxenic inoculated with human feces) 6 treatments control, GOS for each rat model	12 M rats, 2 per group	4% (2 g/kg) Source Yakult Institute, Japan	1 mo	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -Decreased pH in conventional and HE rats -Increased SCFAs in conventional and HE rats -Increased β-galactosidase activity in conventional and HE rats -Decreased N-acetyl-β-D-galactosaminidase and N-acetyl-β-D-glucosaminidase in conventional rats -Increased α-L-fucosidase in HE rats -Increased H_2 and CH_4 production in HE rats, no change in conventional rats	-No data reported
Morishita et al 2002	Study the effects of GOS on intestinal microflora of germ-free mice	Feeding study Mice were inoculated with bacterial strains during the feeding period 2 treatments control, GOS	10 M germ-free BALB/c mice, 5 per group	5% (7 5 g/kg) Source Snow Brand Milk Co , Japan	7 wk	Stool Characteristics -No data reported Microflora Response -*B breve* increased and *C perfringens* decreased -*C perfringens* was suppressed by GOS in combination with anaerobic bacteria during early normal development of the bowel microflora Measures of Microbial Fermentation -No data reported	-No data reported

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Morishita et al 1992 *Abstract and tables only* *Article in Japanese*	Study effects of FOS and GOS on intestinal microflora and cecal SCFAs in rats	Feeding study 4 treatments control, 2 GOS levels, FOS	M Wistar rats, n or groups not stated	3, 5% (1 5, 2 5 g/kg) Source Not specified	4 wk (3%), 7 wk (5%)	Stool Characteristics -No data reported Microflora Response -Increased *Bifidobacterium* Measures of Microbial Fermentation -Decreased cecal pH -Increased total SCFA concentration in 5% GOS group, increase in acetic and propionic acids, decrease in iso-butyric, butyric, and iso-valeric acids, no change in valeric acid	-No data reported
	Study effects of GOS and FOS on intestinal microflora and cecal SCFAs in mice	Feeding study 2 treatments control, GOS	BALB/c mice, n or groups not stated	3% (4 5 g/kg) Source Not specified	4 wk	Stool Characteristics -No data reported Microflora Response -Restored *Bifidobacteria* and *Lactobacilli* counts reduced by dietary cellulose Measures of Microbial Fermentation -Increased total cecal SCFA concentration (increased acetic acid and butyric acids, no change in propionic, iso-butyric, iso-valeric, or valeric acids)	-No data reported
	Study effects of GOS provided in drinking water on cecal SCFAs	Feeding study 3 treatments control, 2 GOS levels	ICR mice, n or groups not stated	2, 4% (drinking water) (7, 14 g/kg) Source Not specified	3 wk	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -Increased total SCFAs at 4% GOS, increased acetic, propionic, and butyric acids, no change in iso-butyric or valeric acids	-No data reported

000174

Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Ohtsuka et al 1991	Study of excretion and metabolism of GOS product in rats	Feeding study 2 treatments GOS, lactose	8 M Sprague Dawley rats (some rats treated with antibiotics), 8 M germ-free Wistar rats, 4 per group	5% (5 1 g/kg (conventional), 12 0 g/kg (germ-free)) Source Prepared by researchers	2 wk	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -In germ-free rats, no difference in total respiratory CO_2 -No GOS detected in feces	-No difference in body weight
Ohtsuka et al 1990	Study influence of GOS, lactulose, and lactose on weight gain, organ weight, serum lipids, and liver lipids of rats	Feeding study 5 treatments control, lactose, lactulose, 2 GOS levels	30 M Sprague Dawley rats, 6 per group	5, 10% (4 9, 9 4 g/kg) Source Prepared by researchers	39 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -Higher relative cecal and colonic weights. -Higher fecal dry weight -Higher cecal content weights -Higher cecal acetic acid concentration	-Diarrhea developed in 10% group initially, resolved after 2 wks -No changes in organ weights (stomach, small intestine, liver, heart, kidney, spleen) were observed -No effects on serum or liver lipid levels -Higher food intake and weight gain in 10% group
Quigley et al 1997	Study the effect of addition of GOS product to milk replacer on growth and health of bull calves	Feeding study 3 treatments control, GOS, antibiotics	94 Holstein bull calves, 30–32 per group	1% GOS product in dry matter (0 1 g/kg), delivered in 2 portions Source Snow Brand Milk Products, Ltd , Japan	26 d	Stool Characteristics -No difference in fecal score Microflora Response -No data reported Measures of Microbial Fermentation -No data reported	-Increased daily body weight gain -No difference in milk replacer intake

000175

Table 16. Adult Human Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Alles et al 1999	Examine effects of 2 levels of GOS on intestinal microflora composition and activities, and bile acids in fecal water of healthy men and women	Blinded parallel design with a run-in diet and two consecutive experimental periods 3 treatments control, 2 GOS levels	22 M, 18 F, 13–14 per group criteria 18–75 y	8 5 or 14 4 g (121 or 206 g/kg), delivered in 3 portions Source Elix'or, Borculo Whey Products, The Netherlands	3 wk	Stool Characteristics -No effect on stool frequency -No effect on percentage of fecal dry matter Microflora Response -No effect on microflora composition as compared to control group in total aerobes, total anaerobes, *Lactobacillus*, *Bifidobacterium*, *Clostridium*, or *E coli* Measures of Microbial Fermentation -No effect on fecal pH -No difference in fecal weight -Total SCFAs and bile acids in fecal water not different among groups -No GOS detected in feces -Fecal nitrogen density increased in high GOS group only -Breath H_2 higher	-Some reported flatulence in both low and high GOS groups -No other adverse effects were observed

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Table 16. Adult Human Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Bouhnik et al 1997	Study GOS tolerance and impact on fecal *Bifidobacterium* and fermentation activity of colonic flora in healthy men and women	Feeding study 1 treatment GOS	4 M, 4 F, 24–32 y	10 g (143 mg/kg), delivered in 2 portions Source Yakult Institute, Japan	21 d	Stool Characteristics -No change in fecal water content (%) Microflora Response -Fecal *Bifidobacterium* concentrations increased 1 log during GOS feeding -Fecal enterobacteria not affected Measures of Microbial Fermentation -No effect on fecal pH -No effect on stool weight -Decreased breath H_2 excretion, methane was unchanged	-Subjects did not experience any symptoms following ingestion of GOS based on a grading scale for rectal gasses, bloating, barbarygmi, and abdominal pains -No subjects experienced diarrhea -No change in stool frequency or consistency
liu et al 1994 *Abstract and tables only* *Article in Chinese*	Study effects of oligosaccharides (FOS, IMO, GOS) and polydextrose on defecation and putrefactive metabolites in young volunteers	Parallel study design 5 treatments control, FOS, IMO, GOS, polydextrose	50 M/F, age not specified 10 per group	10 g (143 mg/kg) Source Not specified	18 d	Stool Characteristics -Incidence of no bowel movement and incomplete bowel movement were reduced -Constipation was reduced in all patients Microflora Response -No data reported Measures of Microbial Fermentation -Decreased fecal p-cresol concentration	-No data reported

000177

Table 16. Adult Human Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Ito et al 1990	Study effects of GOS on fecal microflora, stool weight, and abdominal sensation in healthy men	Single-blind cross-over, with ≥7-d washout between treatments 4 treatments control (no GOS), and 3 GOS levels	12 M, 26–48 y	2 5, 5 0, or 10 0 g (36, 71, 143 mg/kg) Source Oligomate-50®, Yakult Institute, Japan	7 d	Stool Characteristics -Softer stools -No differences observed in stool frequency at any dose Microflora Response -Fecal *Bifidobacterium* and % of *Bifidobacterium* increased in a dose-dependent manner -*Lactobacillus* increased -No changes in total bacteria, *Bacteroides*, *Enterobacteriaceae*, or *Enterococcus* Measures of Microbial Fermentation -No differences observed in stool weight at any dose	-No subjects reported diarrhea -Frequency of fullness, wind, and abdominal pain increased with increasing dose This increase was not significant except for fullness, which was higher at the 10 g/d dose compared to controls -Gas and bloating were transient

0001178

Table 16. Adult Human Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Ito et al 1993	Study effects of GOS intake on fecal microflora and their metabolism in healthy men	Feeding study 1 treatment GOS	12 M, 35–55 y, mean=49 y, selected for low indigenous *Bifidobacterium* counts (mean=9 46 $\log_{10}$)	2 5 g (36 mg/kg) Source Oligomate-50® Yakult Institute, Japan	3 wk	Stool Characteristics -No change in stool water content (%) Microflora Response -Increased fecal *Bifidobacterium* -No changes in total bacteria, *Bacteroidaceae*, *C perfringens*, *Enterobacteriaceae*, *Enterococci*, *Lactobacillus*, *Bacillus*, *Staphylococcus*, and *Candida* spp Measures of Microbial Fermentation -No change in fecal pH -No change in stool weight -No change in ß-glucuronidase, ammonia, or p-cresol -Fecal nitroreductase significantly decreased -No change in acetic, propionic, butyric, isobutyric, valeric, or total organic acids -Decreased isovaleric acid	-No data reported

000179

Table 16. Adult Human Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Tamai et al 1992 *Abstract and tables only* *Article in Japanese*	Study the proliferation of fecal *Bifidobacterium*, fecal microflora, fecal moisture, and pH in healthy men	A 10-d pre-study preceded the feeding period and was followed by a 15-d washout 1 treatment GOS	11 M, 25–60 y	8 g (114 mg/kg) Source CUP OLIGO P (COP), Manufacturer not specified	20 d	Stool Characteristics -No effect on fecal water (%) Microflora Response -Increased *Bifidobacterium*, percentage of *Bacteriodaceae*, and total bacteria -No changes in fecal *C perfringens*, *Clostridium* (others), *Lactobacillus*, *Bacteroidaceae*, *Eubacterium*, or yeast -No change in percentages of *Peptostreptococcus*, or *Eubacterium* Measures of Microbial Fermentation -No effect on fecal pH	-No data reported

000180

Table 16. Adult Human Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Tanaka et al 1983	Study effects of GOS administered with and without *Bifidobacterium breve* (*B breve*) on ecology or metabolism of the fecal flora in healthy men	Feeding study 3 treatments GOS, *B breve*, GOS+ *B breve*	16 M, 25–35 y 5–6 per group	3 g in first wk, 10 g in second wk (43 and 143 mg/kg) Source Yakult Institute, Japan	2 wk (GOS only)	Stool Characteristics -No data reported Microflora Response -Promoted growth of resident *Bifidobacterium* (GOS only group) and an increase in administered *Bifidobacteria* (GOS+*B breve* group) -Decreased *Bacteroidaceae* (GOS with or without *B breve*) -Decreased *Enterobacteriaceae* and total bacterial counts in GOS+*B breve* group -Increased *Lactobacillus* in GOS only group Measures of Microbial Fermentation -No changes in fecal ammonia with GOS only, decrease in 4 of 5 subjects in GOS+*B breve* group	-No data reported

0001181

Table 16. Adult Human Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Teuri et al 1998	Study effects of GOS intake on GI symptoms, fecal frequency, and fecal microflora in healthy adults	Feeding study with 2-wk pre-clinical period 1 treatment GOS	3 M, 9 F, 25–55 y mean=38 y	15 g (214 mg/kg), delivered in 2 portions Source Not specified	2 wk	Stool Characteristics -Increased frequency of defecation Microflora Response -Higher number of total fecal anaerobic bacteria -No changes in fecal *Bifidobacterium* Measures of Microbial Fermentation -No effect on fecal pH	-Flatulence increased -No effect on appetite, loose stools, diarrhea, hard stools, abdominal distention, or other abdominal symptoms when evaluated separately -When pooled, overall GI symptoms appeared more frequently in the test period
Teuri and Korpela 1998	Study effect of GOS intake on constipation in elderly people	Controlled, double-blind, two-period crossover Study began with 1-wk run-in, and ended with a 1-wk follow-up No washout between treatments 2 treatments control, GOS	14 F, 69–87 y mean age =79 6 y, mean wt= 67 7 kg, subjects suffered from constipation	9 g (133 mg/kg), delivered in 2 portions Source Elix'or, Borculo Whey Products, The Netherlands	2 wk	Stool Characteristics -Higher defecation frequency/wk in 8 of 14 subjects -In 2 subjects, defecation was higher in the control period -Consistency of feces and ease of defecation not affected by diet -Fecal dry weight not affected Microflora Response -No data reported Measures of Microbial Fermentation -No effect on fecal pH	-Flatulence, abdominal pain, abdominal distention, and total symptoms not affected

000182

Table 16. Adult Human Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
van den Heuvel et al 2000	Study effects of GOS on Ca absorption in postmenopausal women	Randomized, crossover, double-blind study, feedings separated by a 19-d washout 2 treatments control, GOS	12 F, 55–65 y mean=62 y, mean wt= 73 1 kg	20 g, dose increased from mean of 10 42 to 15 64 to 20 84 g during study (285 mg/kg), delivered in 2 portions Source Elix'or, Borculo Domo Ingredients, The Netherlands	9 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -No data reported	-No adverse side effects GI complaints or change in stools reported after intake of 20 g GOS -Ca absorption increased by 16% -No change in urinary Ca excretion
van den Heuvel et al 1998	Study effects of inulin, FOS, and GOS on intestinal iron and Ca absorption in healthy men	Randomized, crossover, double-blind study 4 treatments control, inulin, FOS, GOS	12 M, 20–30 y mean wt= 79 8 kg	15 g (188 m g/kg), delivered in 3 portions Source Not specified	21 d	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -No effect on fecal pH -Increased concentration of acetic acid, no effect on most other SCFAs	-No effect on mean iron status (hemoglobin, ferritin, transferrin saturation, and total-iron-binding capacity) and percentage absorption -No effect on Ca absorption

000183

Table 16. Adult Human Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
van Dokkum et al 1999	Study effects of inulin, FOS, and GOS on parameters of large-bowel function, blood lipid concentrations, and glucose absorption in healthy men	Randomized, crossover, double-blind study 4 treatments control, inulin, FOS, GOS	12 M mean=23 y, mean wt= 79 8 kg	15 g (188 mg/kg), delivered in 3 portions Source Not specified	21 d	Stool Characteristics -Decreased fecal dry weight -No effect on transit time Microflora Response -No data reported Measures of Microbial Fermentation -No effect on fecal pH -No effect of diet on fecal total weight -Increase in acetic acid, no effect on lactic, propionic, butyric, valeric, iso-valeric, or iso-butyric acids -No effect on bile acid concentrations or fecal neutral steroids -Decrease in ß-glucuronidase activity -No effect on H_2 expiration	-Well tolerated, most subjects reported only mild cases of flatulence -No effect on blood lipids -Body weight remained stable

[a] Number of subjects who completed the study

[b] Approximate dose was calculated using a standard body weight of 70 kg (Derelanko & Hollinger 1995), unless otherwise noted All doses were delivered in one portion unless otherwise noted

[c] Duration of each GOS (and any other treatment) feeding period, excluding pre-study and washout periods

[d] Results limited to endpoints reflecting effects of GOS

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VI. SAFETY ASSESSMENT/GRAS DETERMINATION

A. Introduction

This chapter presents an assessment that demonstrates that Vivinal®, in both syrup form (Vivinal®) and powder form after blending with whey protein concentrate and drying (Vivinal ® GOS 10), is safe, and is also GRAS under the Federal Food, Drug, and Cosmetic Act (FFDCA) for direct addition to milk-based infant formula at levels up to 5.0 g/L (Vivinal®) and 7.7 g/L (Vivinal® GOS 10), providing 2 g/L of GOS, as one component (along with polydextrose) of a prebiotic blend. This safety assessment and GRAS determination entail two steps. In step one, the safety of Vivinal® and Vivinal® GOS 10 under their intended conditions of use is demonstrated. In the second step, Vivinal® and Vivinal® GOS 10 are determined to be GRAS by demonstrating that its safety under its intended conditions of use is generally recognized among qualified scientific experts.

The regulatory framework for establishing whether a substance is GRAS in accordance with Section 201(s) of the FFDCA is set forth under 21 CFR 170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under 21 CFR 170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under 21 CFR 170.30(c). This GRAS determination employs scientific procedures established under 21 CFR 170.30(b).

In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination consists of two components: 1) the data and information relied upon to establish the scientific element of safety must be generally available; and 2) there must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific procedures GRAS determination are applied below in an analysis of whether Vivinal®and Vivinal® GOS 10 are safe and GRAS for the uses and at the use levels intended.

B. Safety of Vivinal® and Vivinal® GOS 10

A scientific procedures GRAS determination requires first that information about the material establish that the intended use of the material is safe. The FDA has defined "safe" or "safety" for food additives under 21 CFR 170.3(i) as "a reasonable certainty in the minds of competent scientists that the substance is not harmful under its intended conditions of use." This same regulation specifies that three factors must be considered in determining safety. These three factors are:

1) The probable consumption of the substance and of any substance formed in or on food because of its use (i.e., the EDI);

2) The cumulative effect of the substance in the diet, taking into account any chemically or pharmacologically related substance or substances in such diet; and

3) Safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food and food ingredients, are generally recognized as appropriate.

An EDI for the material is derived based on the probable human consumption of the material, taking into account any existing sources of consumption of the material. Finally, the EDI for a substance is compared against a level of consumption that has been shown to be reasonably certain to be without harm. As long as the EDI is less than or approximates this level, the substance can be considered safe for its intended use (FDA 1993).

1. EDI of GOS, Vivinal® and Vivinal® GOS 10

As indicated above, 21 CFR 170.3(i) requires that, in evaluating the safety of the proposed use of a new food additive, the probable consumption (i.e., the EDI) of the substance and of any substance formed in or on food because of its use be considered. Also to be considered is the cumulative effect of the substance in the diet, taking into account any chemically or pharmacologically related substance or substances in such diet. Thus, because a scientific procedures GRAS determination requires the same quantity and quality of evidence as is required to obtain approval of the substance as a new food additive, a scientific procedures GRAS determination must also consider the probable consumption and cumulative effect of the substance in the diet. The EDI derivation described below provides a conservative estimate of the intake of GOS, Vivinal® and Vivinal® GOS 10 under the intended conditions of use.

The derivation of the estimated exposure to Vivinal®, Vivinal® GOS 10 and GOS expected to result from the proposed use, which calls for the addition to milk-based infant formula of up to 5.0 g/L of Vivinal® or 7.7 g/L of Vivinal® GOS 10 to provide 2 g/L of GOS. Based on data regarding infants' energy needs, the 90th percentile of formula intake was determined for the period with the highest level of consumption per kg body weight. This in turn allows determination of the EDI for GOS, Vivinal® and Vivinal® GOS 10, representing the 90th percentiles of intake during the infants' peak period of formula consumption. The EDI for GOS is 0.4 g/kg bw/day, the EDI for Vivinal® is 1 g/kg bw/day and the EDI forVivinal® GOS 10 is 1.6 g/kg bw/day.

These EDIs are based on formula consumption per kg body weight between the ages of 14 and 27 days, when it is highest. According to Fomon (1993), infants' energy intake, and thus their formula consumption, is lower both prior to this period and following it. This was shown in Figure 3 in Chapter III. Thus, the EDIs are conservative estimates of long-term exposure.

Additionally, the EDIs assume that the infant receives all of his or her energy from infant formula to which Vivinal® or Vivinal® GOS 10 has been added. Parents may feed their infants a variety of formulas such that this is not the case; further, as the infant grows older and other foods are introduced, formula ceases to provide 100% of energy intake.

2. Safe Level of Consumption for Vivinal® GOS

The effects of consumption of GOS, and specifically Vivinal®, have been investigated in numerous studies in animals, human adults, and human infants, as discussed in Chapter V. In all except two studies in animals, the primary goals of the studies were to investigate the effects of GOS on colonic microflora, measures of fermentation, stool frequency, or stool characteristics; the studies in infants also usually included measures of weight gain in infants fed formula with GOS versus those fed a control formula.

Experimental animals in efficacy studies were given a wide range of doses of GOS, depending on the size of the animals tested. The doses were generally as follows (*Table 17*):

Table 17. Doses of GOS Administered to Different Species of Test Animals

Species	Dose (g/kg bw/day)
Mouse	4.5–14
Rat	2–12
Rabbit	0 05–0 4
Dog	1
Pig	0 6–1.4
Bovine	0 1

In the nearly 30 studies of the prebiotic effects of GOS in animals (including rats, mice, rabbits, dogs, pigs and piglets, and calves), it was clearly established that GOS has a bifidogenic and lactogenic effect on the colonic microflora as well as beneficial effects on production of SCFAs. Most, but not all, studies that addressed nutrient intake found increased absorption and retention of calcium and magnesium; effects on other minerals were not clear, but in no case was interference with nutrient absorption observed. GOS did not affect feed consumption or weight gain, and only isolated adverse effects (most often transient diarrhea) were reported. As was noted above, it is not clear that the laxation characteristics reported as "transient diarrhea" constituted true diarrhea rather than merely looser, less well formed, or more watery stools.

Two subchronic studies of the potential oral toxicity of Vivinal® found no toxicity at any dose tested. An oral feeding study in Wistar rats provided doses as high as 14.5 g/kg bw/day to males and 16.4 g/kg bw/day to females without any signs of overt

toxicity; the no observed adverse effect level (NOAEL) for Vivinal® in this study was the highest dose tested in both sexes, 14.5 g/kg bw/day.

The subchronic gavage study of potential toxicological effects of consumption of up to 5.0 g/kg bw/day of Vivinal® by male and female Sprague Dawley rats also found no adverse effects at any dose level; the NOAEL in this study was 5.0 g/kg bw/day of Vivinal®, the highest dose tested.

The doses of GOS in studies of adults ranged from 2.5–20.0 g/day; in seven studies the dose was 8–10 g/day, in four studies it was 14–15 g/day, and in two studies it was 20 g/day. The estimated intakes of GOS per kg body weight were mostly in the range of 140–200 mg/kg bw/day. While these studies were designed primarily to evaluate the prebiotic effects of GOS, many of them reported data related to safety. No adverse effects were found regarding nutrient absorption, blood lipids, stool characteristics, or body weight. There were no reports of diarrhea. While many studies found no effects of GOS intake on GI discomfort, overall there were significantly more reports of GI discomfort, most often flatulence or transient bloating, in the GOS test groups than among controls. Not all studies have shown these effects to be dose-related; thus, the relationship between dose of GOS and GI effects is not fully understood.

In studies of the effects of consumption of GOS by infants, concentrations of GOS in the formula ranged from 2.4–10 g/L—this last in a study of preterm infants. Average intakes of the blend of prebiotics in these studies were from 0.5-2.0 g/kg bw/day, resulting in intakes of GOS of 0.4–1.6 g/kg bw/day. The durations of these studies were from 14 days to 6 months. As with studies in adults, studies in infants were directed primarily toward evaluating GOS' prebiotic effects rather than its safety, although two of them included weight gain as a primary endpoint and one study was specifically powered to detect any difference in weight gain between the GOS and control groups. The studies generally found dose-dependent bifidogenic effects of GOS and increases in stool frequency and softness. No adverse effects of any type were observed with any of the doses of GOS tested. No measures of GI discomfort such as crying, regurgitation, or study drop-out showed any difference between test and control groups. No effect on growth—weight, length, head circumference, or skinfold thickness—were found in the four studies that reported measures of growth. In the single study that included blood biochemistries, no adverse effects were found due to consumption of 1.0 g GOS/kg bw/day for 12 weeks.

Based on the review presented in Chapter V, the following conclusions were reached regarding a safe level of intake of GOS and Vivinal®:

- The totality of the evidence found in the published scientific literature demonstrates that consumption of up to 10–15 g GOS per kg body weight by experimental animals causes no adverse effects on microbial populations, nutrient absorption and retention, weight gain, or food consumption.

- The totality of the evidence found in the published scientific literature demonstrates that consumption of up to 15–20 g GOS per day by human

adults causes no long-term adverse effects and no short-term adverse effects other than some transient GI discomfort.

- The totality of the evidence found in the published scientific literature has shown no adverse effects on microbial populations, nutrient absorption, blood biochemistry, or growth from consumption of 1–1.6 g GOS/kg bw/day by human infants.
- Although consumption of GOS may have a small effect on water balance, the totality of the evidence demonstrates that this effect is not of sufficient magnitude to pose a safety concern.

3. Establishing the Safety of Vivinal® and Vivinal® GOS 10

As discussed above, the EDIs for Vivinal® or Vivinal® GOS 10 in the intended use as prebiotic ingredients to be added to milk-based infant formula are 1g/kg bw/day and 1.6 g/kg bw/day, respectively. Those EDIs for the ingredients are equivalent to an EDI of 0.4 g/kg bw/day for GOS. The EDI for GOS is well within the exposures that have been found to be safe in the extensive published literature regarding consumption of GOS by animals, human adults, and human infants. Furthermore, the EDI for Vivinal® is about 15% and 40% of the NOAELs found in the two published subchronic toxicology studies of Vivinal®, which corroborate the safety of the material that was already apparent in the published literature. Additionally, Vivinal® and Vivinal® GOS 10 are food-grade products that contains no impurities of toxicological concern. Thus, the proposed uses and use levels of Vivinal® and Vivinal® GOS 10 can be considered safe.

C. General Recognition of the Safety of Vivinal® and Vivinal® GOS 10

In September 2001, the EU's Scientific Committee on Food (SCF) reviewed the suitability and safety of a blend comprising 90% GOS (from Vivinal®) and 10% oligofructose (from chicory roots) for use at an addition level of 8 g/L in infant formula and follow-on formula (SCF 2001a). At that time, the SCF found that the prebiotic blend indeed had a bifidogenic effect, although it noted that the evidence of a relationship between a bifidobacteria dominated flora and appropriate outcomes of infant health and well-being is not conclusive. The Committee noted the effect of the prebiotic blend on stool frequency and consistency, and expressed some concern regarding a potential for dehydration in young infants, although this was not a major concern for older infants. Thus, the SCF found the potential for adverse effects of the addition of 8 g/L of the prebiotic blend to be low for older infants and approved its use in follow-on formula while recommending the submission of additional information regarding the safety of the blend for young infants.

Three months later, in December 2001, based on additional data from clinical studies that had not been previously available, the SCF determined that it no longer had concerns about the addition of 8 g/L of the prebiotic blend to infant formula intended for consumption by neonates (SCF 2001b). It did, however, indicate a desire to continue to

receive evidence regarding the suitability and safety of the addition of prebiotics to infant formula, in particular with respect to infant nutrient absorption, water balance, and growth.

In its comprehensive review of infant formula composition in 2003 (SCF 2003), the Committee reaffirmed its previous statement that it has no major concerns with the inclusion of up to 8 g/L of a combination of 90% GOS and 10% FOS in both infant formulas and follow-on formulas.

The prebiotic addition approved by the Committee represents 7.2 g/L of GOS (and 0.8 g/L of FOS), 17 g/L of Vivinal® or 27 g/L of Vivinal® GOS 10.

Much of the data cited in the present review, especially the clinical trials of GOS or GOS+FOS in infants, have become available since the SCF rendered its initial judgment in 2001 that the addition to infant formula of 8 g/L of a prebiotic blend containing 90% GOS is suitable and safe for young infants as well as older infants. The totality of these data, both animal and human, is consistent with the SCF's December 2001 judgment.

The proposed use and use levels of Vivinal® and Vivinal® GOS 10 have been determined to be safe through scientific procedures set forth under 21 CFR 170.30(b). Furthermore, because this safety assessment satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of Vivinal® for direct addition to milk-based infant formula at up to 10 g/L on a dry basis (equivalent to 14 g/L of Vivinal ® or 22 g/L of Vivinal® GOS 10) , providing 6 g GOS/L (three times the intended addition level), was made through the deliberations of an Expert Panel comprising Dennis M. Bier, M.D, Michael P. Doyle, Ph.D., George C. Fahey, Ph.D., Glenn R. Gibson, Ph.D., Berthold V. Koletzko, M.D., Robert A. Rastall, Ph.D., and John A. Thomas, Ph.D. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, and have concluded:

> *Ingestion of Vivinal® from the proposed use results in intakes of Vivinal® and of GOS by infants that remain within safe limits established by published animal and human studies Vivinal® has been sufficiently characterized to ensure that it is a food-grade product. No evidence exists in the available information on GOS or on Vivinal® that demonstrates, or suggests reasonable grounds to suspect, a hazard to infants when Vivinal® is added as a prebiotic ingredient to milk-based infant formula at a level of up to 10 g/L*

It is their opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same scientific conclusion. The Expert Panel thus determined that Vivinal® is safe and GRAS for addition to infant formula at up to 10 g/L, with or without combination with other prebiotic ingredients.

Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Rowland & Tanaka 1993	Study effect of GOS on metabolic activities of cecal bacteria in rats colonized with human fecal microflora	Feeding study in rats inoculated with human feces 3 treatments control, GOS, GOS + *B breve*	8 M, 9 F germ-free Lister-Hooded rats inoculated with human feces, 5–6 per group	5% (2 0–3 0 g/kg) Source Yakult Institute, Japan	4 wk	Stool Characteristics -No data reported Microflora Response -Increased total anaerobe count, *Lactobacillus*, and *Bifidobacterium* in both GOS groups, decreased *Enterobacteria* in both groups -Decreased enterobacteria and decreased *Streptococcus* in GOS+ *B breve* group -No change in *Bacteroides*, *Staphylococcus*, yeasts, or *Clostridium* Measures of Microbial Fermentation -Lower cecal pH -No difference in cecal ammonia -Higher cecal content weight in GOS+ *B breve* group -Increased β-glucosidase activity, decreased β-glucuronidase and nitrate reductase activity	-No difference in terminal body weight or body-weight gain

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Sakaguchi et al 1998	Study energy contribution and effects of GOS and FOS on the gastro-intestinal tract	Feeding study 5 treatments basal diet, restricted basal diet, sucrose, FOS, GOS	35 M Wistar rats, 7 per group	10% (8 4 g/kg) Source Yakult Honsha Co , Ltd , Japan	50 d	Stool Characteristics -No effect on transit time Microflora Response -No data reported Measures of Microbial Fermentation -Increased cecal tissue and content weights -Increased total cecal organic acids, increased succinic, lactic, formic, acetic, and butyric acids, decreased propionic, iso-butyric, and iso-valeric acids	-Decreased linoleic and oleic acids and ratio of unsaturated fatty acids to saturated fatty acids, increased myristic acid in body fat -Decreased crude protein and crude fat digestibility, increased crude ash digestibility -No difference in food intake or weight gain
Shimura et al 1991 *Abstract and tables only* *Article in Japanese*	Study effects of GOS, FOS, and lactose on Ca, P, and Fe utilization in weanling rats	Feeding study 4 treatments control, GOS, FOS, lactose	31 M weanling Wistar rats, 7–8 per group	15 4% (5 5 g/kg) Source Not specified	30 or 180 d	Stool Characteristics -No difference in transit time Microflora Response -No data reported Measures of Microbial Fermentation -Decreased cecal pH after 30 d and increased cecal pH after 180 d -Higher cecal content weight	-Increased Mg retention at 180 d, no Mg absorption -No change in P or Ca retention -Decreased absorption of Fe after 30 d -No differences in food intake or weight gain at 180 d, decreased food intake at 30 d

Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Suzuki et al 1999 *Abstract and tables only* *Article in Japanese*	Study effects of GOS given to mice on formation of intestinal microflora typically found in the infant gut	Controlled feeding study Mice inoculated with bacterial species in infant feces 2 treatments control, GOS	Germ-free mice, n or groups not stated	3 5% (5 3 g/kg) Source Not specified	7 d	Stool Characteristics -No data reported Microflora Response -Increase in *E coli*, *B breve*, and total bacteria, decreased *C perfringens* and *En faecalis* -No change in *S epidermidis* and *Eub aerofaciens* Measures of Microbial Fermentation -Lower cecal and fecal pH -Enhanced fecal α-galactosidase, β-galactosidase, β-glucosidase, and β-glucuronidase activities	-No data reported
Wijnands et al 1999	Study the effects of GOS and cellulose on colorectal cancer in rat models	Feeding study Rats were inoculated with 1,2-dimethyl-hydrazine to induce colorectal tumors 12 treatments low-, medium-, or high-fat diets, each with 2 cellulose levels or 2 GOS levels	468 M Wistar rats, 39 per group	3 4 (low), 10 0% (high) (1, 3 0 g/kg) Source Borculo Domo Ingredients, The Netherlands	9 mo	Stool Characteristics -Softer feces consistency in high GOS groups -Dark feces Microflora Response -No data reported Measures of Microbial Fermentation -Decreased cecal pH in high GOS groups. -Lowest feces production in high-fat, high GOS group -Enlarged cecum in high GOS group	-No diarrhea occurred -Decreased tumor incidence, multiplicity, and size with GOS feeding -Marginal differences in energy intake and body weights among groups

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Wijnands et al 2001	Study effect of GOS and dietary fat on colorectal cancer and aberrant crypt focci formation in rats	Crossover study Rats treated with azoxy-methane to induce colorectal tumors 4 treatments high- or low-fat diets, each with 2 GOS levels	204 M Fischer rats, 102 per group	0 3, 1 3 g/d (0 7, 2 7 g/kg) Source Borculo Domo Ingredients, The Netherlands	7 wk, up to a total of 10 mo	Stool Characteristics -Softer feces consistency Microflora Response -No data reported Measures of Microbial Fermentation -No data reported	-No difference in food consumption or final body weights -Decreased incidence of tumors in rats fed high GOS diet
Yanahira et al 1997	Study effects of GOS, lacitololigo-saccharide, lactose, and lactitol on Ca and Mg absorption in rats	Feeding study 5 treatments control, lactose, lacitol, lacitol-oligosac-charide, GOS	56 M Sprague Dawley rats, 6 per group	5% (3 9 g/kg) Source Not specified	2 wk	Stool Characteristics -No data reported Microflora Response -No data reported Measures of Microbial Fermentation -Decreased cecal pH -Higher cecum weight and cecal contents weight -Elevated total VFA concentration, increased acetic, propionic, and butyric acids	-Increased absorption ratios of Ca and Mg -No difference in final body weight or food intake

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Table 14. Animal Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Species[a]	Daily GOS Dose in diet and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Zentek et al 2002	Study effects of indigestible carbohydrates on fecal quality, nutrient and mineral digestibilities, and products of intestinal microflora metabolism in dogs	Crossover study 4 treatments mannanoligo-saccharide, GOS, lactose, lactulose	4 adult F dogs, 1 per treatment	1 g/kg Source Borculo Whey Products, Borculo, The Netherlands	10 d	Stool Characteristics -No negative effect on stool quality -No change in unbound water (%) Microflora Response -No data reported Measures of Microbial Fermentation -No difference in fecal pH -No change in fecal dry matter -No difference in fecal VFA content -No change in fecal ammonia	-No change in absorption of Ca, PO_4, Mg, Na, or K^+

[a] Number of animals completing the study.

[b] Approximate dose calculated using standard weight and food intake data for the species studied unless reported in paper Doses in units of g/kg correspond to intake per kilogram body weight, doses in units of percent correspond to proportion of diet

[c] Duration of each GOS (and any other treatment) feeding period, excluding pre-study and washout periods.

[d] Results limited to endpoints reflecting effects of GOS Short chain fatty acids (SCFAs) and volatile fatty acids (VFAs) are the same.

Table 15. Infant Studies of Galactooligosaccharide (GOS)

Reference	Objective	Study Design	Subjects	Daily GOS Dose and Source	Duration of Feeding	Efficacy Results	Safety Results
Ben et al 2004	Study the effects of GOS on microbial populations, fermentation endpoints, and infant growth	Randomized controlled (negative control) with a human milk reference group	271 term infants 69 in test group, 52 controls, 26 human milk reference group, 124 mixed human milk and test formula	2 4 g/L GOS Intake Not reported Source Not reported	6 mo	Stool Characteristics -Increased stool frequency and decreased hardness Microflora Response -Increased *Bifidobacterium* and *Lactobacillus* to same level as human milk reference group, no change in *E coli* Measures of Microbial Fermentation -Increased SCFA, decreased pH	Side Effects -No side effects (crying, vomiting, regurgitation) Growth -No effect on weight or length gain
Boehm et al 2002	Measure stool characteristics, fecal flora, growth, and side effects in preterm infants consuming formula with GOS+FOS (90% GOS, 10% FOS)	Randomized parallel study 2 treatments Standard preterm formula, formula with GOS+FOS blend, plus human milk reference group	30 M/F preterm infants ≤32 wks 15 per group, 12 in reference group	10 g/L GOS+FOS Intake Est 1 87 g blend/kg/d = 1 68 g GOS/kg/d Source Vivinal GOS, Borculo Domo Ingredients	28 d	Stool Characteristics -Higher stool frequency to range of reference group -Softer stools to range of reference group Microflora Response -Fecal *Bifidobacterium* counts higher in the GOS+FOS fed infants to the range of the reference group -No effect on *Bacteroides*, *Lactobacillus*, *Clostridium* species, *E coli*, enterobacter, and others Measures of Microbial Fermentation -No data reported	-No adverse effects were observed Side Effects -No side effects (crying, regurgitation, vomiting) Growth -No effect on weight or length gain

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Table 15. Infant Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects	Daily GOS Dose and Source	Duration of Feeding	Efficacy Results	Safety Results
Knol et al 2001 *Abstract*	Study effects of a prebiotic formula on microflora in term infants	Randomized, prospective, double-blind 2 treatments standard formula, GOS+FOS formula	30 M/F term infants 0–14 d postpartum 14 in test group, 16 in control group	Not specified Source Research conducted by Numico	4–6 wks	Stool Characteristics -No data reported Microflora Response -Fecal *Bifidobactena* increased 1 log and % of *Bifidobacteria* in total fecal bacteria was higher Measures of Microbial Fermentation -No data reported	-No data reported
Moro et al 2002	Determine if a formula containing GOS+FOS (90% GOS, 10% FOS) has a dose-dependent bifidogenic effect in term infants	Randomized, controlled, double-blind, crossover study 3 treatments positive control (malto-dextrin) formula, 8g/L GOS+FOS formula, 4g/L GOS+FOS formula	90 M/F term infants 27 received 8g/L, 30 received 4g/l, 33 received control formula	4 or 8 g/L GOS+FOS Intake Est 0 73 g or 1 57 blend/ kg/d= 0 66 or 1 41 g GOS/kg/d Source Vivinal GOS, Borculo Domo Ingredients	28 d	Stool Characteristics -Stool frequency increased in the 8 g/L group -Stools softer in both GOS+FOS groups Microflora Response -Dose-dependent increase in fecal *Bifidobacterium* -Fecal *Bifidobacterium* counts higher in the 8g/L group as compared to the 4g/L group -Increased *Lactobacilli* -No effect on number of infants with *Bacteroides*, *Clostridium* species, *E coli*, *Enterobacter*, and other bacteria Measures of Microbial Fermentation -Dose-dependent decrease in fecal pH	-No adverse effects were observed Side Effects -No side effects (crying, regurgitation, vomiting) Growth -No effect on weight or length gain

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Table 15. Infant Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects	Daily GOS Dose and Source	Duration of Feeding	Efficacy Results	Safety Results
Napoli et al 2003 *Abstract*	Study the effects of GOS on fecal microflora in formula-fed infants	Randomized controlled (positive control= lactose) with a human milk reference group	26 healthy full-term infants 13 per group, 24 in reference group	0 7% GOS (approx 7 g/L GOS) Intake Not reported Source Not reported	21 d	Microflora Response -Increased *Bifidobacterium* 1 log to range of reference group Measures of Microbial Fermentation -Increased lactate 4-fold (NS), decreased fecal pH	-Not reported
Savino et al 2003	Study effects of a formula containing FOS & GOS (90% GOS, 10% FOS) on infants with colic, regurgitation problems, and/or constipation	Observational prospective study	604 M/F term infants ≤90 days 214 with colic, 201 with regurgitation problems, 232 with constipation	8 g/L GOS+FOS Intake Est 0 99 g blend/kg/d = 0 89 g GOS/kg/d Source Vivinal GOS, Borculo Domo Ingredients	14 d	Stool Characteristics -63% increased the number of stools Other -79% showed reduced frequency of colic -70% showed reduced regurgitation problems	-No adverse effects were reported -Both parents and pediatricians gave positive judgments of the effect on GI comfort

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Table 15. Infant Studies of Galactooligosaccharide (GOS), cont.

Reference	Objective	Study Design	Subjects	Daily GOS Dose and Source	Duration of Feeding	Efficacy Results	Safety Results
Schmelzle et al 2003	Evaluate the nutritional efficacy and bifidogenic characteristics of formula with a GOS/FOS blend	Randomized double-blind controlled (negative control) study, powered to detect a weight-gain difference of 3 g/d	102 healthy term infants 49 in test group, 53 in control group, completed the study	8 g blend/L, 90% GOS & 10% FOS, Est 1 08 g blend/kg/d= 0 97 g GOS/kg/d Source Numico	12 wk	Stool Characteristics -Softer stools Microflora Response -Increased *Bifidobacterium* 1 log, increased percentage of bacteria were *Bifidobacterium* Measures of Microbial Fermentation -None reported	-No adverse effects were observed Growth -No difference in gains in weight, length, head circumference, skinfold thickness Blood biochemistry -No significant differences in total protein, albumin, or urea, no clinically significant differences in prealbumin, tyrosine, threonine, isoleucine, or lysine Tolerance -No difference in tolerance was noted Study drop-outs were not different between test and control groups

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Table 16. Adult Human Studies of Galactooligosaccharide (GOS)

Reference	Objective	Study Design	Subjects[a]	Daily GOS Dose and Source[b]	Duration of Feeding[c]	Efficacy Results[d]	Safety Results[d]
Alander et al 2001	Examine effects of GOS and *Bifidobacterium lactis* (*B lactis*) B12 on fecal microflora in healthy men and women	Randomized, blinded, parallel design with 2-week pre-feeding and 2-week washout 3 treatments GOS, *B lactis*, GOS+ *B lactis*	3 M, 27 F, 10 per group 22–47 y, mean=32 y	8 1 g (116 mg/kg), delivered in 2 portions Source Borculo Domo Ingredients, The Netherlands	2 wk	Stool Characteristics -No data reported Microflora Response -GOS + *B lactis* treatment produced increase in fecal *Bifidobacterium* -GOS alone caused decrease in *C perfringens* -No changes detected in lactic acid bacteria or coliforms Measures of Microbial Fermentation -No data reported	-One subject in GOS group reported pain from intestinal bloating -No other adverse effects were observed

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Relying on the opinion of the Expert Panel, along with more recent information (such as Ziegler et al. (2007) that was not available to the Expert Panel, MJ concludes that Vivinal® and Vivinal® GOS 10 are safe and GRAS for their intended use as dietary ingredients to be added to infant formula as sources of GOS, along with polydextrose, at levels not to exceed 5.0 g/L and 7.7 g/L, respectively.

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Appendix A

Product Specification Sheets

DOMO®

VIVINAL®
GOS

Product Specification Sheet

Description : galacto-oligosaccharide syrup.

Typical analysis : dry matter 75% of which galacto-oligosaccharides 59%, lactose 21%, glucose 19% and galactose 1%

Sensorial : clear syrup, slightly sweet taste.

Product specification :

	Specification	Method of analysis
Chemical/ physical:		
Dry matter	74-76%	IDF 26A (1993), 2½ h 102±2°C
Galacto-oligosaccharides	min. 57% on DM	AOAC vol 85 (2002), method 2001.02
Nitrogen	max. 0.016% on DM	IDF 20B (1993), Kjeldahl
Sulphated ash	max. 0.3% on DM	AOAC 17ed (2000) 930.30, sulphated ≤550°C till constant weight
Lactose anhydrous	max. 23% on DM	AOAC vol 85 (2002), method 2001.02
Glucose anhydrous	max. 22% on DM	AOAC vol 85 (2002), method 2001.02
Galactose	min. 0.8% on DM	AOAC vol 85 (2002), method 2001.02
Viscosity	1000-5000 cPs	HAAKE
Nitrite	max. 2 ppm on DM	IDF 97A (1984), spectrophotometric
pH	3.2 - 3.8	ISO 10523 (1994), potentiometric (no dilution)
Microbiological:		
Total plate count 30°C	max. 3000 cfu/g	IDF 100B (1991), PCMA 72h 30°C
Enterobacteriaceae	absent in 1 g	BDI 23, VRBG 24h 30°C
E. coli	absent in 5 g	IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C
Yeasts	max. 50 cfu/g	IDF 94B (1990), OGYE 5 days 25°C
Moulds	max. 50 cfu/g	IDF 94B (1990), OGYE 5 days 25°C
Staphylococci coagulase-positive	absent in 1 g	IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C
Salmonellae	absent in 25 g	IDF 93B (1995)

Packaging : container, 650 or 1200 kg.

Storage : keep in clean, dry and dark conditions, keep away from strongly odorous materials.

Shelf life : 18 months after production date.

August 6th, 2007 (version 13)

Friesland Foods Domo
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The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements are intended only as a source of information. No warranties, expressed or implied, are made. On the basis of this information it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product. The information contained herein should not be construed as permission for violation of patent rights.

DOMO®

Product Specification Sheet

VIVINAL®
GOS 10

Description : galacto-oligosaccharide rich whey product

Typical analysis : galacto-oligosaccharides 28.5%, lactose 36.0%, glucose 9.5%, galactose 0.5%, protein 17 5%, minerals 3.5%, fat 1.5%, moisture 3 0%.

Sensorial : creamy coloured, milky powder

Product specification:

	Specification	Method of analysis
Chemical / physical:		
Protein (N x 6.38)	15 – 20 %	IDF 20B (1993), Kjeldahl
Ash	max. 5 0 %	AOAC 17ed. (2000) 930.30
Total moisture	max. 4.5 %	IDF 23A (1988), Karl Fischer
Fat	max. 2.0 %	IDF 9C (1987), Röse Gottlieb
Galacto-oligosaccharides	min 27.0 %	AOAC 2001-02, HPAEC-PAD
Scorched particles	disc A	ADPI bulletin 916 (1990)
pH	6 0 – 7 0	ISO 10523 (1994), 10% w/w
Insolubility index	max. 0.5 ml	IDF 129A (1988)
Taste and flavour	Bland, sweet, no off-flavour	IDF 99C (1997)
Nitrate	max. 50 ppm	IDF 97A (1994)
Nitrite	max 1 ppm	IDF 97A (1994)
Microbiological:		
Total plate count 30°C	max. 1000 cfu/g	IDF 100B (1991)
Enterobacteriaceae	absent in 10 x 10 g	BDI 23 VRBG 24h 30°C
Yeasts	max. 10 cfu/g	IDF 94B (1990)
Moulds	max. 10 cfu/g	IDF 94B (1990)
Staphylococcus aureus	absent in 1 g	IDF 60C (1997)
E. coli	absent in 10 g	IDF 170A-1 (1999)
Bacillus cereus	max. 100 cfu/g	ISO 7932 (1993)
Salmonella	absent in 1500 g	IDF 93B (1995)
E sakazakii	absent in 300 g	ISO/DTS 22964
Suph. red. clostridia	max. 10 cfu/g	BDI 29
Clostridium perfringens*	absent in 1 g	ISO 7937 (1997)

* only to be done in case 2 values for SRC > 10 cfu/g

Packaging : multiple layered paper bag with polyethylene inner liner, 25 kg nett.

Storage: keep in clean, cool (average 10-20°C), dry (average 60% RH; advice max. 65% RH) and dark conditions, keep away from strongly odorous materials.

Retest: this product is very stable when stored under the correct conditions, however, for storage periods exceeding 12 months after production date we recommend re-sampling.

July 13th, 2007 (version 3)

Friesland Foods Domo
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The information contained herein is, to the best of our knowledge, correct. The data outlined and the statements are intended only as a source of information. No warranties expressed or implied, are made. On the basis of the information it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product. The information contained herein should not be construed as permission for violation of patent rights.

Biolacta N5 **SG505263**

1. GENERAL INFORMATION

Name product	BIOLACTA N5
General discription	Enzyme for the production of galacto-oligosacchande, standardized by lactose
Legal declaration	Raw materials have to comply with Dutch and E C regulations for food products
Remark	Ingredients must not be derived from genetically modified raw materials. Also free of foreign material and allergens that are not listed on the ingredients declaration. Enzyme is derived from Bacillus circulans (ATCC code 31382). This micro-organism should be non GMO
Resource number	Number 505263 to be mentioned on each unit
Production and expiry date	To be mentioned on each unit
Shelf life	At least 6 months
Storage conditions	< 5°C
Packaging	1,5 kg PE bags in carton drum
Documents	Packing list + certificate of analysis

2. PRODUCT SPECIFICATION

Properties	Specification	Method of analysis
Chemical		
Lactose hydrolizing capacity	> 5,000 LU/gram	Daiwa method
Arsenic	< 3 ppm	JP XII (*)
Heavy metals (as Pb)	< 40 ppm	JP XII (*)
Physical		
Appearance	Slightly tan powder	
Microbiology		
Aerobic total plate count	< 10000/gram	JP XII (*)
Coliforms	< 30/gram	AOAC (**)
E coli	Abs. in 25 gram	FDA (***)
Salmonella	Abs in 25 gram	ISO 6579, 2nd revised ed. 1992

(*) Japanese pharmacopoeia, 12 edition
(**) Association of Official Analytical Chemists
(***) FDA Bacteriological Analytical Manual for Foods, 5th ed. 1978

3. CERTIFICATE OF ANALYSIS

- Production and expiry date
- Lactose Hydrolizing capacity
- ONPG hydrolyzing capacity
- Arsenic
- Heavy metals (as Pb)
- Aerobic plate count
- Coliforms
- E coli
- Salmonella

Citroenzuur **SG010118**

1. GENERAL INFORMATION

Name product	Citric acid monohydrate
General discription	Organic acidifier, food grade
Legal declaration	Raw materials have to comply with Dutch and E C. regulations for food grade products.
Remark	Ingredient must not be derived from genetically modified raw materials Also free of foreign material and allergens that are not listed on the ingredient declaration
Resource Number	RN 010118 to be mentioned on each unit
Documents	Cert. of conformance
Production and expiry date	To be mentioned on each unit
Guaranteed shelflife	At least 12 months
Packaging	Bags 25 kg (multiwall + p e liner)
Pallet type	Industrial (100x120), dry and clean
Bags/pallet	40 (8 x 5)
Additional	Shrinkwrapped/ cardboard layer on pallet
Storage conditions	Original unopened package below 25°C

2. PRODUCT SPECIFICATION

Properties	Specification	Method of analysis
Chemical		
Moisture	7 7 - 8.8 %	USP 921
pH	1 5 - 2.5	0 5 % Solution FNZ 14.13
Heavy metals	Max 10 ppm	USP 231
Mesh size	Min 95 % trough 0 841 mm	Alpine
	Max 10 % trough 0 149 mm	Alpine
Reaction on citrate	Positive	USP 191
Ash	Max 0 02 %	FNZ-Method
Bulk dentisy	Approx 0 85 kg/litre	IDF 134
Physical		
Appearance	Colourless crystals or white granular to fine powder	Sensory evalution method
Flavour	Acidic	Sensory evalution method
Aroma	Normal	Visual observation method

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Natronloog 33% (SG60220004010) **SG504877**

1. GENERAL INFORMATION

Name product	Sodium Hydroxide 33%
General discription	Sodium hydroxide solution in water
Legal declaration	Raw materials have to comply with E C legislation for food additives (96/77/EC) and the Food Chemicals Code
Remark	Ingredient must be free of foreign material and allergens that are not listed on the ingredient declaration
Resource Number	SAP nr. 504877 to be mentioned on delivery note
Documents	Delivery note, Cert of analysis per batch, cleaning certificate (unless former load was the same product)
Production and expiry date	To be mentioned on certificate of analysis
Guaranteed shelflife	Indefinite, under correct storage conditions
Packaging	Bulk product, delivered by road tanker
Storage conditions	Temp > 20°C, in airtight tank
Security full truck loads	Hoses carriers and outlet valves must be end capped and security tagged Hatches or hatch covers must be security tagged. All security tag numbers must be recorded and available for inspection before unloading All security tags must be intact on arrival

2. PRODUCT SPECIFICATION

Properties	Specification	Method of analysis
Chemical		
Iron	Max 10 mg/kg *	SAM 104 04
NAOH	32 -34 %	SAM 284.01
Chloride	Max 0 02 % *	SAM 269.03
CO_3 (as Na_2CO_3)	Max 0 1 %	SAM 183 04
Arsenicum	Max 1 mg/kg	
Lead	Max 0.15 mg/kg	
Mercury	Max 0 3 mg/kg	
Physical		
Appearance	Liquid	Sensory evaluation method
Colour	Clear	Visual observation method
Aroma	Odourless	Sensory evaluation method
Microbiological		
Salmonella	Absent in 25 ml	IDF 93B (1995)
Staphylococcus aureus	< 100.000 cfu/ml	IDF 60C (1997)

3. CERTIFICATE OF ANALYSIS

- Delivery number
- Production date
- NaOH (% w/w)

Note: * = based on a 100% concentration

Actieve kool **SG504952**

1. GENERAL INFORMATION

Name product	Norit PN 2
General discription	Neutralized steam activated carbon with a high adsorptive capacity, dedicated for removal of small colour bodies and undesired taste and odour compounds.
Legal declaration	Raw materials have to comply with Dutch and E C. regulations for food products.
Remark	Ingredient must not be derived from genetically modified raw materials. Also free of foreign material and allergens that are not listed on the ingredient declaration
Resource Number	Number 504952 to be mentioned on each unit
Production and expiry date	To be mentioned on each unit
Shelf life	At least 1 year
Storage conditions	No special requirements
Packaging	Paper bags 20 kg
Documents	Packing list + certificate of analysis
Change control	Norit informs FFD on any process change which influences the specification of the final product.

2. PRODUCT SPECIFICATION

Properties	Specification	
Chemical		
Molasse number	Max. 390	NSTM (*)
Moisture (W/W %) as packed	Max.10	NSTM (*)
PH	6 0 - 7.2	NSTM (*)
Physical		
Appearance	Typical	

(*) Norit standard test methods

3. CERTIFICATE OF ANALYSIS

- Production and expiry date
- Molasse number
- Moisture
- PH

Alpha-Cel HKB200 **SG504969**

1. GENERAL INFORMATION

Name product	Alpha-Cel HKB200 ™
General discription	100% purified powdered cellulose filter aid
Legal declaration	Material has to comply with requirements in The Food Chemical Codex, 4th Edition, pages 96-97. Furthermore material must comply with relevant regulations for food grade products
Remark	Ingredient must not be derived from genetically modified raw materials. Also free of foreign material and allergens that are not listed on the ingredient declaration
Resource number	Number 504969 to be mentioned on each unit
Production and expiry date	To be mentioned on each unit
Shelf life	At least 1 year
Storage conditions	No special requirements
Packaging	paper bags 23 kg
Documents	Packing list + certificate of analysis
Change control	Significant changes in raw materials, processing etc. need to be approved by Friesland Foods Domo prior to implementation

2. PRODUCT SPECIFICATION

Properties	Specification	Method of analysis
Chemical		
pH	5,0 – 7,5	10% solution
Ash (total)	< 0,5%	
Heavy metals, as lead	< 0,01 ppm	
Physical		
Appearance	Fine white powder	
Average particle length	100 µm	
Loose bulk Density	170 – 190 g/l	
Packed bulk Volume	140 – 200 ml/50 gram	
Typical Screen analysis		
On > 40 mesh (420 µm)	0%	
Thru < 100 mesh (150 µm)	>95%	
Thru < 200 mesh (75 µm)	>80%	

3. CERTIFICATE OF ANALYSIS

- Lotnumber
- Screen analysis
- Packed bulk density
- Moisture
- Color

Results microbiol quarterly analysis company water.

Sample:	Date analysis	Total plate count/1 ml. 22°C. (0)	Total plate count/1 ml. 37°C. (0)	Coliforms/100 ml. 37°C. Yellow col.	Coliforms/100 ml. 37°C. Red col.	Coliforms/100 ml. 44°C. Yellow col.	Coliforms/100 ml. 44°C. Red col.	Faecal str 100 ml	Spores src. 100 ml.
Source 1	05-03-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 3	05-03-03	<1	<1	<1	1	<1	1	NEG	<1
Source 4	05-03-03	3	<1	<1	<1	<1	<1	NEG	<1
Source 6	05-03-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 6	05-03-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 7	05-03-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 8	05-03-03	<1	<1	<1	<1	<1	<1	NEG	1
Clean water tank 1	20-03-03	<1	<1	<1	<1	<1	<1	NEG	<1
Clean water tank 2	20-03-03	<1	<1	<1	<1	<1	<1	NEG	<1
Standards		<=100/1 ml.	<=10/1 ml.	<=1/100 ml.	n.v.t.	<=1/100 ml.	n.v.t.	Neg./100 ml.	<=1/100 ml.

BRRP - MB044 2004/0312
Kwartaalonderzoek bedrijfswater
c:\exc\bitrap\documenten\microbiologisch mb lijsten

Appendix II

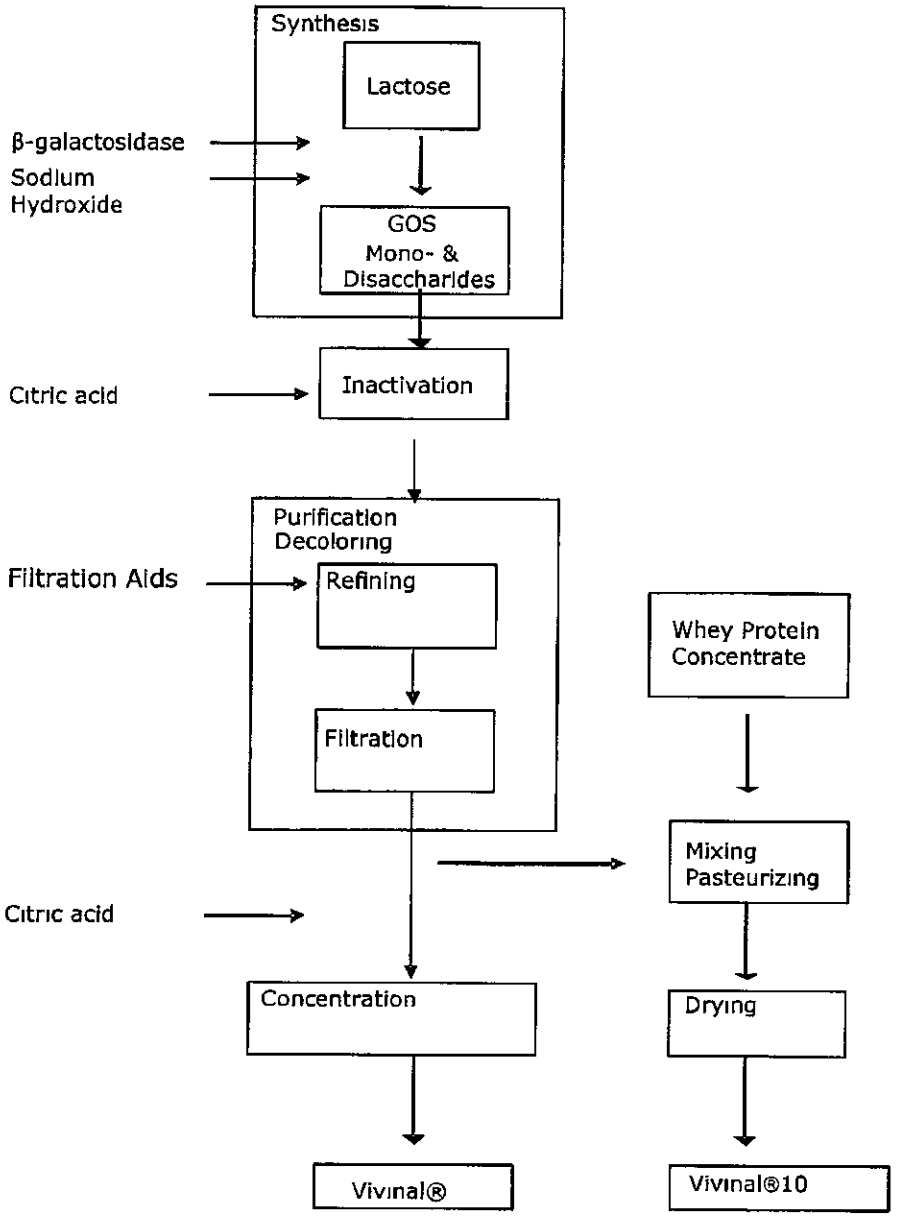
Results microbiol quarterly analysis company water.

Sample	Date analysis	Total plate count/1 ml. 22°C. (0)	Total plate count/1 ml. 37°C. (0)	Coliforms/100 ml. 37°C. Yellow col.	Coliforms/100 ml. 37°C. Red col.	Coliforms/100 ml. 44°C. Yellow col.	Coliforms/100 ml. 44°C. Red col.	Fecal str. 100 ml	Sporen str. 100 ml
Source 1	08-05-03	1	<1	<1	<1	<1	<1	NEG	<1
Source 3 (not available in May)									
Source 4	08-05-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 5	08-05-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 6	08-05-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 7	08-05-03	1	4	<1	<1	<1	<1	NEG	<1
Source 8 (not available in May)									
Clean water tank 1	16-04-03	19	1	<1	<1	<1	<1	NEG	<1
Clean water tank 2	16-04-03	1	1	<1	<1	<1	<1	NEG	<1
Standards		<=100/1 ml.	<=10/ml.	<=1/100 ml.	n.v.t.	<=1/100 ml.	n.v.t.	Neg./100 ml	<=1/100ml

BRRP - M8014 2505031X
Wijzigingen voorbehouden
Overnemen van de gegevens in verslag alleen mit tijdens

Results microbiol quarterly analysis company water.

Sample:	Date analysis	Total plate count/1 ml.		Coliforms/100 ml. 37°C.		Coliforms/100 ml. 44°C.		Fecal str.	Spores src.
		22°C (a)	37°C. (b)	Yellow col.	Red col.	Yellow col.	Red col.	100 ml	100 ml.
Source 1	18-07-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 3									
Source 4	18-07-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 5	18-07-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 6	18-07-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 7	18-07-03	<1	<1	<1	<1	<1	<1	NEG	<1
Source 8	18-07-03	<1	<1	<1	<1	<1	<1	NEG	<1
Clean water tank 1	17-07-03	<1	<1	<1	<1	<1	<1	NEG	<1
Clean water tank 2	17-07-03	<1	<1	<1	<1	<1	<1	NEG	<1
Standards		<=100/1 ml.	<=10/ml.	<=1/100 ml.	n.v.t.	<=1/100 ml.	n.v.t.	Neg./100 ml.	<=1/100ml



Process Diagram of the Production of Vivinal® and Vivinal® GOS 10

DOMO®

HIPROTAL® 635

Product Specification Sheet

Description : ultrafiltrated whey protein powder

Typical analysis : lactose 51%, protein 35%, minerals 6%, fat 2.5%, moisture 3.5%, organic milk salts 2%.

Sensorial : creamy coloured, milky powder

Product specification :

	Specification	Method of analysis
Chemical / physical:		
Protein	min. 35%	IDF 20B (1993), Kjeldahl (N x 6.38)
Ash	max. 7.0%	AOAC 17ed. (2000) 930 30, ≤550°C till constant weight
Total moisture	max. 4 5%	IDF 23A (1988), Karl Fischer
Nitrite	max. 5 ppm	IDF 97A (1984), spectrophotometric
Scorched particles	max. disc A	ADPI bulletin 916 (1990), 25g nonfat dry matter/ ∅ 28mm
pH	6.0 – 7 0	ISO 10523 (1994), potentiometric (10% w/w)
Bacteriological:		
Total plate count 30°C	max. 25000 cfu/g	IDF 100B (1991), PCMA 72h 30°C
Enterobacteriaceae	absent in 0.1 g	BDI 23, VRBG 24h 30°C
Yeasts	max 50 cfu/g	IDF 94B (1990), OGYE 5 days 25°C
Moulds	max. 50 cfu/g	IDF 94B (1990), OGYE 5 days 25°C
Bacillus cereus	max. 100 cfu/g	ISO 7932 (1993), MYP 24h 30°C
Staphylococci coagulase-positive	absent in 1 g	IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C
E. coli	absent in 1 g	IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C
Salmonella	absent in 50 g	IDF 93B (1995)

Packaging : a multiple layered paper bag with a polyethylene inner liner, 25 kg nett, also available in big bags.

Storage : keep in clean, cool (average 10-20°C), dry (average 60% RH; advice max. 65% RH) and dark conditions, keep away from strongly odorous materials.

Retest : this product is very stable when stored under the correct conditions, however, for storage periods exceeding 24 months after production date we recommend re-sampling.

September 2nd, 2005 (version 11)

Friesland Foods Domo
Hanzeplein 25, 8017 JD Zwolle, P.O. Box 449 8000 AK Zwolle, The Netherlands,
Telephone. +31 38 46 77 444, Fax: +31 38 46 77 555, www.domo.nl, e-mail: info.domo@frieslandfoods.com

The information contained herein is, to the best of our knowledge correct. The data quoted and the statements are intended only as a source of information. No warranties expressed or implied are made. On the basis of this information it is suggested that you evaluate the product on a laboratory scale prior to use in a finished product. The information contained herein should not be construed as permission for violation of patent rights.

Pages 000224 - 000226 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

DAIWA KASEI K. K.
7-12, UEHONMACHI 5-CHOME, TENNOJI-KU, OSAKA, 543-0001, JAPAN
PHONE : +81-6-6768-5001
FAX : +81-6-6763-4767

October 30, 2003

To Borculo Domo Ingredients

Our comments regarding the safety of BIOLACTA N5

Production strain:

1. Production strain "*Bacillus circulans*" is classified into the group of microorganisms that are accepted as harmless contaminants present in food by JECFA and AMFEP.
2. The bio-safety degree of *Bacillus circulans* is classified into level 1 in accordance with the guideline of Japanese Society for Bacteriology. Microorganisms of level 1 are defined as those that are not placed on the list of pathogenic ones. The list of microorganisms that belong under level 1 and may cause opportunistic infection is published, but *Bacillus circulans* is out of the list.

Fermentation medium:

We use the fermentation medium of food grade in manufacturing lactase concentrate.

Safety data:

We have the following data regarding the safety of BIOLACTA N5.

(1) Ames test
(2) Chromosomal aberration test
(3) Acute toxicity study with a single oral dose given
(4) Mycotoxins test
(5) Antibiotic activity

DAIWA KASEI K.K.

Hirotsugu Watanabe
General Manager,
Quality Assurance Division

DAIWA KASEI K. K.
7-12, UEHONMACHI 5-CHOME, TENNOJI-KU, OSAKA, 543-0001, JAPAN
PHONE: +81-6-6768-5001
FAX: +81-6-6763-4767

October 30, 2003

To Borculo Domo Ingredients

NATURAL CERTIFICATE

We hereby certify that GMO status of the origin, medium and diluent used in manufacturing the following product is as follows:

Product :	BIOLACTA N5	
Origin :	*Bacillus circulans*	(non-GMO)
Medium :	Defatted soybean meal	(not-specified)
	Lactose	not applicable
Diluent :	Lactose	not applicable

Sincerely yours,

DAIWA KASEI K.K.

H. Watanabe
General Manager,
Quality Assurance Division

DAIWA KASEI K. K.
7-12, UEHONMACHI 5-CHOME, TENNOJI-KU, OSAKA, 543-0001, JAPAN
PHONE: +81-6-6768-5001
F A X: +81-6-6763-4767

October 30, 2003

To Borculo Domo Ingredients

CERTIFICATE

We hereby certify that BIOLACTA N5 can be used for food processing on the basis of the reasons described below in Japan.

1. Our company's Shiga plant has obtained approval of manufacturing the enzymes products as food additives from the government of Shiga prefecture in Japan.

2. β-Galactosidase(Lactase) is registered in the list of existing food additives, based on Article 2 Paragraph 1 of the Supplementary Provisions to Law No. 120, 1996, "the Law Concerning Amendments to the Food Sanitation Law and Nutrition Improvement Law" in Japan. It is permitted to use the enzymes in the list by Japanese law.

Sincerely yours,

DAIWA KASEI K.K.

H. Watanabe
General Manager,
Quality Assurance Division

— analytico®

Borculo Domo Ingredients
T.a.v. mevr. M Leusink
Postbus 449
8000 AK ZWOLLE

Analysecertificaat

Certificaatnummer : 2003075851
Rapportagedatum : 04-12-2003
Monster(s) ontvangen : 30-10-2003
Datum monstername :
Monsternemer : KLANT
Klantnummer : 2236
Klantnaam : Borculo Domo Ingredients
Contactpersoon : mevr. M Leusink
Uw projectnummer :
Uw projectnaam :
Projectcoördinator : projectcoordinatie

Nr.	Monsteromschrijving	Analytico-nr.
1	Vivinal GOS lot.no. 340051	1446227
2	Vivinal GOS lot.no. 339080	1446236
3	Vivinal GOS lot.no. 338111	1446237
4	Vivinal GOS lot.no. 338113	1446238
5	Vivinal GOS lot.no. 340019	1446239

Analyse	Eenheid	1	2	3	4	5
Arsenic (AAS oven)	mg/kg	<0.10 [3]	<0.10 [5]	<0.10 [6]	<0.10 [7]	<0.10 [8]
Cadmium (ICP-AES-USN)	mg/kg	<0.04 [9]	<0.04 [7]	<0.04 [8]	<0.04 [9]	<0.04 [10]
Kwik (koude damp)	µg/kg	<0.5	<0.5	<0.5	<0.5	<0.5
Lood (AAS-oven)	mg/kg	<0.04 [11]	<0.04 [12]	<0.04 [13]	<0.04 [14]	<0.04 [15]

Legenda
C: door RvA geaccrediteerde verrichting
B: uitgevoerd door Analytico Milieu (L010) te Breda
D: uitgevoerd door Analytico Milieu (L010) te Barneveld
E: uitgevoerd door een extern laboratorium

Zie de bijlage voor opmerkingen bij dit analysecertificaat.

* Indicatieve waarde.
De analyseresultaten hebben alleen betrekking op het monster. De toegepaste onderzoeksmethoden en bijbehorende meetonzekerheden staan vermeld in ons overzicht "Specificatie Analysemethoden", 2003/1.
De analysemonsters worden tot 3 weken na de datum ontvangst bewaard.

Bijlage: B

Accoord
Pr.coördr.
CW

Analytico Food B.V.
Leeuwerikstraatweg 129
8441 PK Heerenveen
P.O. Box 766
8440 AT Heerenveen NL
Tel. +31 (0)513 47 22 99
Fax +31 (0)513 47 22 60
E-mail info@analytico.com
Site www.analytico.com
ABN AMRO 54 43 75 329
VAT/BTW No. NL 0078.36.233.B08
KvK No. 01070347

Analytico Food B.V. is certified by the COXI (BS/92/46). All offers and agreements are subject to our General Conditions directly available upon request.

— analytico®

Bijlage (B) met opmerkingen behorende bij analysecertificaat 2003075851

Opmerking1)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking2)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking3)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking4)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking5)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking6)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking7)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking8)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking9)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking10)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking11)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking12)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking13)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking14)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opmerking15)
De bepalingsgrens is verhoogd in verband met matrixeffecten.

Opinies en interpretaties van de resultaten in dit rapport vallen buiten de scope van de accreditatie.

Analytico Food B.V.

Leeuwerderstraatweg 139
8441 PK Heerenveen
P.O. Box 744
8440 AT Heerenveen NL

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E-mail info@analytico.com
Site www.analytico.com

KVK 08080 54 05 75 328
VAT/BTW No. NL 0078.36 535.B01
EVE No. 01070347

Analytico Food B.V. is certified by the COKZ (EC/93/46). All offers and agreements are subject to our General Conditions directly available upon request.

Original

BZE62258

Certificate

CERTIFICATE

Centraal Orgaan voor
Kwaliteitsaangelegenheden
in de Zuivel

Netherlands controlling authority for milk and milk products under government supervision
PO Box 250, 3830 AG Leusden, The Netherlands
Tel. (+31 33 4965696 fax (+31)33 4965677, E-mail: certificaten@cokz.nl

We, the Netherlands controlling authority for milk and milk products (COKZ), hereby certify

- that the undermentioned establishments of Friesland Foods Domo / the Netherlands have been inspected by our institute on basis of the hygienic requirements laid down in Regulation (EC) No 853/2004 for specific hygiene rules for food of animal origin and have been approved: the establishments are registered under approval numbers as indicated below;

Processing establishments of Friesland Foods Domo located in the Netherlands

Addres	Location	EC-approval number
Boterdiep WZ 44-46, P.O. Box 3, 9780 AA	Bedum	NL Z0163 EEG
De Perk 30, P.O. Box 8, 9410 AA	Beilen	NL Z0075 EEG
Needseweg 23, P.O. Box 46, 7270 AA	Borculo	NL Z0199 EEG
it Heech, 9035 AE	Dronrijp	NL Z0148 EEG
Spoardijk 21, P.O. Box 8710 AA	Workum	NL Z0113 EEG

----------TEXT END----------

Leusden, 19-12-06
On behalf of the director of the
NETHERLANDS CONTROLLING AUTHORITY
FOR MILK AND MILK PRODUCTS,

the proxy

E.E.M.Zwiers-Bömer

Netherlands Control Authority For Milk And Milk Products C N

Any alteration or addition to the above text is not allowed without permission of COKZ and makes this certificate invalid. Such an alteration or addition is considered a offence of forgery and is punishable by e.g. imprisonment of up to six years in accordance with Section 225 of the Penal Code.

Appendix B

Results of Analyses of Vivinal® GOS

5.Aug. 2004 17:40 BDI Zwolle Nr.0607 P. 2

BORCULO DOMO Ingredients

CERTIFICATE OF ANALYSIS

Product : Vivinal® GOS
Product code : 502551
Batchnumber : 500219
Date of production : 09-06-2004
Retest date : 09-12-2005
Contact person : Sales Support BDI Zwolle

Description : galacto-oligosaccharide syrup

Typical analysis : dry matter 75 % of which galacto-oligosaccharides 50% lactose 21 %, glucose 10 % and galactose 1%

Chemical/physical:	Specification	Results	Method of analysis
Dry matter	74 - 76 %	76 %	IDF 26A (1990), 25h 102±2°C
Galacto-oligosaccharides	min. 57 % on DM	51.5 %	AOAC vol 85 (2002), method 2001.02
Nitrogen	max. 0.016 % on DM	0.006 %	IDF 20B (1993), Kjeldahl
Sulphated ash	max. 0.3 % on DM	0.22 %	AOAC 17ed.(2000) 930.30, sulphated ±550°C till constant weight
Lactose anhydrous	max. 22 % on DM	20.3 %	AOAC vol 85 (2002), method 2001.02
Glucose anhydrous	max. 22 % on DM	17.4 %	AOAC vol 85 (2002), method 2001.02
Galactose	max. 0.8 % on DM	1.0 %	AOAC vol 85 (2002), method 2001.02
pH	3.2 - 3.8	3.47	ISO 10523 (1994), potentiometric (60% w/w)
Viscosity (25°C)	1000 - 5000 cPs	2303	HAAKE
Nitrite	max. 2 ppm on DM	0.03	IDF 37A (1984), spectrofotometric

Microbiological:	Specification	Results	Method of analysis
Total plate count 30°C	max. 3000 cfu/g	<10 /g	IDF 100B (1991), PCMA 72h 30°C
Enterobacteriaceae	absent in 1 g	absent	ISO 7402 (1993), VRBG 24h 30°C
E.coli	absent in 5 g	absent	IDF 170A-1 (1996), LSTB 48h 37°C, BCB 48h 44°C
Yeasts	max. 50 cfu/g	4 /g	IDF 94B (1990), OGYE 5 days 25°C
Moulds	max. 50 cfu/g	< 10 /g	IDF 94B (1990), OGYE 5 days 25°C
Staphylococci coag.pos	absent in 1 g	absent	IDF 60C (1987), GCB 48h 37°C, BPA 48h 37°C
Salmonella	absent in 25 g	absent	IDF 93B (1995)

Borculo, 05-08-2004

Manager QC/QA

5.Aug. 2004 17:41 BDI Zwolle Nr.0607 P. 3

BORCULO DO. Ingredients

CERTIFICATE OF ANALYSIS

Product	Vivinal® GOS
Product code	502854
Batchnumber	: 600233
Date of production	: 11-08-2004
Retest date	: 11-12-2006
Contact person	: Sales Support BDI Zwolle

Description : galacto-oligosaccharide syrup

Typical analysis : dry matter 75 % of which galacto-oligosaccharides 55% lactose 21 %, glucose 10 % and galactose 1%

Chemical/physical:	Specification	Results	Method of analysis
Dry matter	74 – 76 %	75.8 %	IDF 26A (1993), 24 h 102±2°C
Galacto-oligosaccharides	min. 57 % on DM	58.2 %	AOAC vol 85 (2002), method 2001.02
Nitrogen	max. 0.016 % on DM	0.005 %	IDF 20B (1993), Kjeldahl
Sulphated ash	max. 0.5 % on DM	0.21 %	AOAC 17ed.(2000) 930.30, sulphated <550°C til constant weight
Lactose anhydrous	max. 23 % on DM	22.6 %	AOAC vol 85 (2002), method 2001.02
Glucose anhydrous	max. 22 % on DM	20.4 %	AOAC vol 85 (2002), method 2001.02
Galactose	min. 0.8 % on DM	1.1 %	AOAC vol 85 (2002), method 2001.02
pH	3.2 – 3.8	3.27	ISO 10523 (1994), potentiometric (10% w/w)
Viscosity (25°C)	1800 – 5000 cPs	2156	HAAKE
Nitrite	max. 2 ppm on DM	0.01	IDF 97A (1996), spectrofotometric

Microbiological:

Total plate count 30°C	max. 3000 cfu/g	34 / g	IDF 100B (1991), PCA/A 72h 30°C
Enterobacteriaceae	absent in 1 g	absent	ISO 2882 (1993), VRBG 24h 38°C
E. coli	absent in 5 g	absent	IDF 170A-1 (1998), LSTB 48h 37°C, ECB 48h 44°C
Yeasts	max. 50 cfu/g	< 1 / g	IDF 94B (1990), OGYE 5 days 25°C
Moulds	max. 50 cfu/g	< 1 / g	IDF 94B (1990), OGYE 5 days 25°C
Staphylococci coagpos	absent in 1 g	absent	IDF 60C (1989), BCB 48h 37°C, BPA 48h 37°C
Salmonellae	absent in 25 g	absent	IDF 93B (1995)

Borculo, 06-08-2004

Manager QC/QA

5-Aug-2004 17:41 BDI Zwolle Nr.0607 P. 5

CERTIFICATE OF ANALYSIS

Product	: Vivinal® GOS
Product code	: 302851
Batchnumber	: 000184
Date of production	: 07-08-2004
Retest date	: 07-12-2005
Contact person	: Sales Support GOS Zwolle
Description	: galacto-oligosaccharide syrup
Typical analysis	: dry matter 75 % of which galacto-oligosaccharides 50% lactose 21 %, glucose 19 % and galactose 1%

Chemical analysis	Specification	Results	Method of analysis
Dry matter	74 - 76 %	75.5 %	IDF 26A (1993), 2h 102°C
Galacto-oligosaccharides	min. 57 % on DM	59.9 %	AOAC vol 85(2002), method 2001.02
Nitrogen	max. 0.010 % on DM	0.005%	IDF 20B (1993), Kjeldahl
Sulphated ash	max. 0.3 % on DM	0.22 %	AOAC 17ed (2000) 930.30, sulphated 550°C/8 constant weight
Lactose anhydrous	max. 23 % on DM	19.4 %	AOAC vol 85 (2002), method 2001.02
Glucose anhydrous	max. 22 % on DM	17.2 %	AOAC vol 85 (2002), method 2001.02
Galactose	max. 0.5 % on DM	1.0 %	AOAC vol 85 (2002), method 2001.02
pH	3.2 - 5.5	5.37	ISO 10523 (1994), potentiometric (10% w/w)
Viscosity (25°C)	1000 - 5000 cPs	2118	MAKE
Nisin	max. 2 ppm on DM	0.02	IDF 97A (1984), spectrophotometric

Microbiological				
Total plate count 30°C	max. 3000 cfu/g	2400 /g	IDF 100B (1991), PCMA 72h 30°C
Enterobacteriaceae	absent in 1 g	absent	ISO 7402 (1993), VRBG 24h 30°C
E.coli	absent in 5 g	absent	IDF 170A-1 (1992), LSTB 48h 37°C, ECB 48h 44°C
Yeasts	max. 50 cfu/g	< 10 /g	IDF 94B (1990), OGHE 5 days 25°C
Moulds	max. 50 cfu/g	< 10 /g	IDF 94B (1990), OGHE 5 days 25°C
Staphylococci coagpos	absent in 1 g	absent	IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C
Salmonellae	absent in 25 g	absent	IDF 93B (1996)

Borculo, 05-08-2004

Manager QC/QA

5-Aug. 2004 17:41 BDI Zwolle Nr.0607 P. 4

BORCULO DOMO ingredients

CERTIFICATE OF ANALYSIS

Product : Vivinal® GOS
Product code : 302651
Batchnumber : 630324
Date of production : 15-06-2004
Retest date : 15-12-2005
Contact person : Sales Support BDI Zwolle

Description : galacto-oligosaccharide syrup

Typical analysis : dry matter 75 % of which galacto-oligosaccharides 58% lactose 21 %, glucose 18 % and galactose 1%

Chemical/physical:	Specification	Results	Method of analysis
Dry matter	74 - 78 %	75.2 %	IDF 25A (1993), 24 h 102±2°C
Galacto-oligosaccharides	min. 57 % on DM	58.2 %	AOAC vol 85 (2002), method 2001.02
Nitrogen	max. 0.016 % on DM	0.002 %	IDF 20B (1993), Kjeldahl
Sulphated ash	max. 0.3 % on DM	0.21 %	AOAC 17ed (2000) 930.30, sulphated ≤550°C of constant weight
Lactose anhydrous	max. 28 % on DM	21.7 %	AOAC vol 85 (2002), method 2001.02
Glucose anhydrous	max. 22 % on DM	18.1 %	AOAC vol 85 (2002), method 2001.02
Galactose	min. 0.8 % on DM	1.0 %	AOAC vol 85 (2002), method 2001.02
pH	3.2 - 3.8	3.92	ISO 10523 (1994), potentiometric (10% w/w)
Viscosity (25°C)	1000 - 3000 cPs	2145	HAAKE
Nitrite	max. 2 ppm on DM	0.01	IDF 57A (1984), spectrofotometric

Microbiological:				
Total plate count 30°C	max. 3000 cfu/g		10 / g	IDF 100B (1991), PCMA 72h 30°C
Enterobacteriaceae	absent in	1 g	absent	ISO 7402 (1993), VRBG 24h 30°C
E. coli	absent in	5 g	absent	IDF 170A-1 (1990), LSTB 48h 37°C, ECB 48h 44°C
Yeasts	max. 50	cfu/g	< 1 / g	IDF 94B (1996), OGYE 5 days 25°C
Moulds	max. 50	cfu/g	< 1 / g	IDF 94B (1996), OGYE 5 days 25°C
Staphylococci coag.pos	absent in	1 g	absent	IDF 60C (1987), SCB 48h 37°C, BPA 48h 37°C
Salmonellae	absent in	25 g	absent	IDF 93B (1995)

Borculo, 05-08-2004

Manager QC/QA

Sample Number 44031

GENERAL RESULTS LISTING

List of all results entered or expected for sample 30-09-2003-DEV--NA-001

Analysis ID	Component Name	Type	Result
G1881/1	Solids (GOS)	N	83.24 % w/w

100°C 4hrs

Bristol-Myers Squibb
2400 West Lloyd Expressway
47721 Evansville
U.S.A.

CERTIFICATE OF ANALYSIS

Product : Vivinal® GOS
Product code : 259
Order no : VE044731
Customer's ref. no. : PO41500884
Destination : Evansville, IN
Quantity : 515 kg
Batch number : 340077
Date of production : 30-09-2003
Retest date : 30-03-2005
Contact person : Sales Support BDI Zwolle

Description : galacto-oligosaccharide syrup

Typical analysis : dry matter 75 % of which digestible mono- and di-saccharides 41%, galacto-oligosaccharides 59%

Chemical/ physical:	Specification	Results	Method of analysis
Dry matter	74 - 76 %	77 %	IDF 26A (1993), 2h 102±2°C 0.7 kg/ca.
Galacto-oligosaccharides	min. 57 % on DM	62 %	AOAC vol 85 (2002), method 2001.02
Nitrogen	max. 0.015 % on DM	0.002 %	IDF 20B (1993), Kjeldahl
Sulphated ash	max. 0.3 % on DM	0.24 %	AOAC 17th (2000) 930.30 weighted ≤550°C till constant weight
Lactose anhydrous	max. 23 % on DM	20 %	AOAC vol 85 (2002), method 2001.02
Glucose anhydrous	max. 22 % on DM	17.4 %	AOAC vol 85 (2002), method 2001.02
Galactose	min. 0.8 % on DM	1.0 %	AOAC vol 85 (2002), method 2001.02
pH	3.2 - 5.5	3.9	ISO 10523 (1994), potentiometric (10% w/w)
Viscosity (25°C)	1000 - 5000 cPs	3865	
Nitrite	max. 2 ppm	0.01	DM IDF 87A (1994), spectrofotometric

Microbiological:	Specification	Results	Method of analysis
Total plate count 30°C	max. 3000 cfu/g	< 10 / g	IDF 100B (1991), PCMA 72h 30°C
Enterobacteriaceae	absent in 1 g	absent	BDI 25, VRBG 24h 30°C
E. coli	absent in 5 g	absent	IDF 170A-1 (1996), LSTB 48h 37°C, ECB 48h 44°C
Yeasts	max. 50 cfu/g	< 1 / g	IDF 94B (1990), OGYE 5 days 25°C
Moulds	max. 50 cfu/g	< 1 / g	IDF 94B (1990), OGYE 5 days 25°C
Staphylococci coagulase	absent in 1 g	absent	IDF 60C (1987), GCB 48h 37°C, BPA 48h 37°C
Salmonellae	absent in 25 g	absent	IDF 93E (1995)

Borculo, 28-01-2004

Manager QA/QC

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Appendix C

Conclusion of Expert Panel

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF VIVINAL® GALACTOOLIGOSACCHARIDES AS A PREBIOTIC INGREDIENT IN INFANT FORMULA

Prepared for:
Mead Johnson & Co.
Evansville IN

November 2004

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF VIVINAL® GALACTOOLIGOSACCHARIDES AS A PREBIOTIC INGREDIENT IN INFANT FORMULA

We, the members of the Expert Panel, have individually and collectively critically evaluated the publicly available information on galactooligosaccharides (GOS) sold as Vivinal® GOS and Vivinal® GOS-10 summarized in a monograph prepared by JHEIMBACH LLC, as well as other material deemed appropriate or necessary. Our evaluation included review of the starting materials and methods of manufacture of Vivinal®; *in vitro*, animal, and human studies of the prebiotic effect and safety of Vivinal® and other GOS; the history of safe use in Europe of infant formula containing GOS; and the safety of consumption of GOS by infants at the level anticipated by the intended uses of Vivinal®. Our summary and conclusion resulting from this critical evaluation are presented below.

- The substance that is the subject of this generally recognized as safe (GRAS) determination is Vivinal®, a prebiotic ingredient comprising approximately 60% GOS along with approximately 20% lactose, 19% glucose, and 1% galactose on a dry-weight basis. Vivinal® products are available in two forms: partially dried by evaporation and sold as a syrup designated Vivinal® GOS, and mixed 1:1 with food-grade whey protein concentrate, dried, and sold as a powder designated Vivinal® GOS-10.

- GOSs are chains of galactose units with glucose at the reducing end; the number of galactose units may vary from one to eight. The GOS in Vivinal® is primarily the trisaccharide, accounting for about 39% by weight of the GOS, and the disaccharide, accounting for about 33%. The tetrasaccharide constitutes about 18% of the GOS, and the remainder is penta- through octasaccharides.

- Vivinal® GOS is formed by a transgalactosylation reaction in which lactose slurry is subjected to enzymatic hydrolysis by β-galactosidase derived from *Bacillus circulans* Jordan, ATCC 4516; this reaction produces primarily β1-4 linkages.

- During processing, heat and low pH are employed to end the transgalactosylation reaction and denature the enzyme, after which the denatured enzyme, all processing aids, and any impurities are removed using activated carbon and cellulose.

- Multiple lots of Vivinal® have been analyzed to demonstrate that the product consistently meets the physical, chemical, and microbiological specifications that have been established to ensure food-grade material. Product exposed to temperatures up to 120°C and to pasteurization, and product stored for 6 months, has been shown to be stable.

- MJN intends to use Vivinal® as a prebiotic ingredient to be added to milk-based infant formula. The maximum intended addition level of GOS is 6 g/L. This addition may be as one component of a blend of GRAS prebiotic ingredients; the total addition level of any such blend will not exceed 8 g/L of prebiotics. Since Vivinal® comprises 60% GOS, the maximum addition level of Vivinal® is 10 g/L.

- The estimated mean daily intake of Vivinal® from this use is 1 8 g/kg; the estimated 90th and 97.5th percentiles are 2.1 g/kg and 2.3 g/kg, respectively. The estimated daily intake of GOS is 60% of these amounts: 1.1 g/kg at the mean, 1.2 g/kg and 1.4 g/kg at the 90th and 97.5th percentiles. Since there are no other sources of Vivinal® or GOS in the diets of formula-fed infants, these figures represent the estimated total daily intake of these substances.

- Two subchronic animal studies of the potential oral toxicity of Vivinal® found no toxicity at any dose tested. An oral feeding study in Wistar rats provided doses as high as 14.5 g/kg/day to males and 16.4 g/kg/day to females without any signs of overt toxicity; the no observed adverse effect level (NOAEL) for Vivinal® was the highest dose tested in both sexes, 14.5 g/kg/day. A gavage study in Sprague Dawley rats providing doses up to 5.0 g/kg/day also found no adverse effects at any dose level; the NOAEL was set at 5.0 g/kg/day of Vivinal®, the highest dose tested.

- The published scientific literature through August 2004 was searched and reviewed. Based on the totality of the evidence found in the published scientific literature, the Panel found that:

    - Consumption of up to 10–15 g GOS per kg body weight by experimental animals causes no adverse effects on microfloral populations, nutrient absorption and retention, weight gain, or food consumption.

    - Consumption of up to 15–20 g GOS per day by human adults causes no long-term adverse effects and no short-term adverse effects other than some transient GI discomfort.

    - Although consumption of GOS may have a small effect on water balance, the totality of the evidence demonstrates that this effect is not of sufficient magnitude to pose a safety concern.

    - No adverse effects on microfloral populations, nutrient absorption, blood biochemistry, or growth have been shown from consumption of 1–1.6 g GOS/kg/day by human infants.

Conclusion

We, the undersigned members of the Expert Panel, have individually and collectively critically evaluated the materials summarized above and conclude that:

> Ingestion of Vivinal® GOS from the proposed uses results in intakes of GOS and other components that remain within safe limits established by published animal and human studies as well as its commercial use in Europe. Vivinal® GOS and Vivinal® GOS-10 have been sufficiently characterized to ensure that they are food-grade products. Therefore, Vivinal® GOS meeting the specifications described in the GRAS monograph, is safe when added as a prebiotic ingredient to milk-based infant formulas at up to 10 g/L.

It is also the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion. Therefore, Vivinal® GOS is safe, and is GRAS via scientific procedures, when added to milk-based infant formulas at up to 10 g/L as a prebiotic ingredient.

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CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF POLYDEXTROSE AS A PREBIOTIC INGREDIENT IN INFANT FORMULA

Prepared for:
Mead Johnson & Co.
Evansville IN

August 2005

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF POLYDEXTROSE AS A PREBIOTIC INGREDIENT IN INFANT FORMULA

We, the members of the Expert Panel, have individually and collectively critically evaluated the publicly available information on polydextrose summarized in a monograph prepared by the Burdock Group, as well as other material deemed appropriate or necessary. Our evaluation included review of the starting materials and methods of manufacture of polydextrose; *in vitro*, animal, and human studies of the prebiotic effect and safety of polydextrose; and the safety of consumption of polydextrose by infants in a blend of prebiotic ingredients added to infant formula at the level anticipated by Mead Johnson's intended use. Our summary and conclusion resulting from this critical evaluation are presented below.

- The substance that is the subject of this generally recognized as safe (GRAS) determination is polydextrose, an odorless white to light cream amorphous powder that is a polymer of randomly bonded glucose units with sorbitol end groups and citric acid attached to the polymer by mono- and diester bonds.

- The molecular weight of polydextrose ranges between 250 and 18,000 Dalton with an average of about 1,500 Dalton. The average degree of polymerization (DP) is about 12; approximately 90% of polydextrose polymers have a DP $\leq$30; while 30% have a DP $\leq$4.

- Polydextrose is prepared by the vacuum-melt condensation method, in which powdered glucose or a glucose-containing material such as hydrolyzed starch is heated under vacuum at 150 to 160°C in the presence of a polyol such as sorbitol and low levels of a polycarboxylic acid such as citric acid. The product may be purified using ion exchange, membrane filtration, or carbon treatment. Food-grade polydextrose meeting JECFA and Food Chemicals Codex specifications is available in powdered form or as an aqueous solution.

- Multiple lots of Danisco Sweeteners' polydextrose products have been analyzed to demonstrate that they consistently meet the physical, chemical, and microbiological specifications that have been established to ensure food-grade material. The powdered form of polydextrose has a shelf life of up to 24 months. The shelf life of aqueous solutions is up to 4 months at ambient temperature or up to 6 months if refrigerated. Polydextrose is also stable when subjected to an acidic environment and high temperature.

- Because of the random glucose-glucose and diester bonds, polydextrose is more resistant to enzyme or acid hydrolysis than other glucose polymers such as in soluble starch and reaches the colon largely intact, were it is fermentable by the microflora.

- MJN intends to add polydextrose to milk-based infant formula at a level not to exceed 4 g/L This addition may be as one component of a blend of GRAS prebiotic ingredients; the total addition level of any such blend will not exceed 8 g/L of prebiotics.

- The estimated mean daily intake of polydextrose from this use is 0 7 g/kg bw; the estimated 90th and 97.5th percentiles are 0 8 g/kg bw and 0.9 g/kg bw, respectively. Since there are no other sources of polydextrose in the diets of formula-fed infants, this represents the estimated total daily intake of polydextrose by this population.

- The Food and Drug Administration (FDA) approved polydextrose in 1981 as a direct food additive. Polydextrose is used to replace sugars and as a partial replacement for fat in desserts, confections, chewing gum, baked goods, instant puddings, jams, toppings and frostings, cereal bars, bakery fillings, fruit spreads, salad dressings, cakes and frozen dairy desserts. In 2000, amendments provided for the use of polydextrose as a bulking agent, texturizer, or both in table spreads and as a bulking agent or texturizer in fruit and water ices. Polydextrose is also used as an excipient and as a binder in pharmaceutical formulations. Polydextrose is approved for food use in the European Union and in Japan, China, and other countries in Asia and Latin America.

- In 1981, JECFA evaluated the use of polydextrose in food and established an acceptable daily intake (ADI) of 70 mg/kg bw/day. In 1987, JECFA re-evaluated polydextrose and revised the ADI to "Not Specified," a designation used for food ingredients that have been determined to be of very low toxicity, and for which the total dietary intake resulting from its use does not represent a health hazard to humans.

- The published scientific literature through June 2004 was searched and reviewed. Based on the totality of the evidence found in the published scientific literature, the Expert Panel found that:

    - The absorption, distribution, metabolism, and excretion of polydextrose have been extensively studied in animals and humans. Only a minor amount of polydextrose is digested or absorbed; it is not stored within tissues but is rapidly excreted.

    - Investigations in experimental animals of the effects of polydextrose on the gastrointestinal tract indicates that these effects are generally beneficial, including increased calcium absorption and neonatal maturation

    - The safety of polydextrose has been studied *in vitro* and *in vivo* in mice, rats, dogs, and monkeys. Research has included acute, subchronic, chronic, multi-generational developmental and reproductive, carcinogenicity, and genetic toxicity studies Polydextrose does not exhibit any type of toxicity or any gastrointestinal effect other than loose stools at chronic intakes as high as 14 g/kg bw/day. Chronic ingestion of 7 g/kg bw/day of polydextrose is without any observable adverse effect in experimental animals or in humans.

    - Based on numerous clinical studies in children and adults, polydextrose is not toxic in adults at levels of consumption as high as 50 g/day. No adverse effects on microfloral populations, nutrient absorption, or blood biochemistry have been shown

from consumption of up to 1 g/kg/day by human children or adults. Diarrhea is not seen in children ingesting up to 1 g/kg bw/day.

In addition to the published information cited above, the Expert Panel reviewed the results of a recently completed and not yet published study of polydextrose in human infants. These results, which corroborate the demonstration of safety provided by the published literature, were obtained in a randomized, double-blind, placebo controlled trial in which infants consumed one of three formulas between day 14 and day 120 of life. The control formula was Enfamil® LIPIL® with Iron; the test formulas were the control formula supplemented with 4 g/L of a 50:50 blend of polydextrose and galactooligosaccharides or the control formula supplemented with 8 g/L of a 50:33:17 blend of polydextrose, galactooligosaccharides, and lactulose. While there was some evidence that 8 g/L may represent a tolerance limit for some infants, neither test formula resulted in any adverse effect on growth or any observed safety-related events.

Conclusion

We, the undersigned members of the Expert Panel, have individually and collectively critically evaluated the materials summarized above and conclude that:

> Ingestion of polydextrose from the proposed uses results in a level of intake that remains within safe limits established by extensive published animal and human studies. Polydextrose has been sufficiently characterized to ensure that it is a food-grade product. Therefore, polydextrose meeting the specifications described in the Food Chemicals Codex is safe when added to milk-based infant formulas at up to 4 g/L as a component of a blend of prebiotic ingredients.

It is also the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion. Therefore, polydextrose is safe, and is GRAS via scientific procedures, when added to milk-based infant formulas at up to 4 g/L.

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