AM

Spherix Consulting, Inc.

August 17, 2010

RECEIVED
AUG 18 2010
Division of
Biotechnology and
GRAS Notice Review

Moraima J. Ramos Valle, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Food and Drug Administration

Dear Moraima:

Please find enclosed a hard copy of the revised version of the submission for GRN 334. We have included the appropriate changes in the revised version to address comments received from the Agency. The revised version supersedes the previously submitted GRN 334 dossier.

Kindly let us know if you have any additional questions or comments.

Best regards,

(b) (6)

Claire L, Kruger, Ph.D., D.A.B.T.
Chief Executive Officer

6430 Rockledge Drive, Suite 503
Bethesda, MD 20817
Tel.: (301) 897-2540; Fax: (301) 897-2567
http://spherix.com/healthSciences.html

RECEIVED
AUG 18 2010
Division of
Biotechnology and
GRAS Notice Review

Generally Recognized as Safe (GRAS) Determination for the Use of Galacto-Oligosaccharides (GOS) in Foods and Infant Formulas

Prepared for:

Yakult Pharmaceutical Industry Co., Ltd.
1796 Yaho, Kunitachi-shi
Tokyo 186-8650
Japan

Prepared by:

Spherix Consulting, Inc.
6430 Rockledge Drive #503
Bethesda, MD 20817
USA

February 3, 2010

TABLE OF CONTENTS

I. GRAS EXEMPTION CLAIM 1

A. NAME AND ADDRESS OF SPONSOR 1
B. COMMON OR USUAL NAME OF GRAS SUBSTANCE 1
C. INTENDED USE 1
D. BASIS FOR GRAS DETERMINATION 1
E. AVAILABILITY OF INFORMATION 6
F. SIGNATURE 6

II. DESCRIPTION OF SUBSTANCE 7

A. IDENTITY 7
1. Chemical Name and Identity 7
2. Common or Trade Names 7
3. CAS Registry Number 7
4. Composition 7
5. Chemical Structure of GOS 8
6. Distribution of Oligomate GOS Components by Chain Length 8
B. PHYSICAL AND CHEMICAL PROPERTIES 9
C. PRODUCTION PROCESS 10
1. Beta-Galactosidases 10
2. Oligomate GOS Production Process 13
3. Starting Material and Processing Aids 15
D. PRODUCT CHARACTERIZATION 16
1. Batch Analysis Results and Product Specifications 16
2. Residual Impurities 17
3. Stability of Oligomate GOS 17
E. METHOD OF ANALYSIS OF OLIGOMATE GOS IN FOODS 17

III. HISTORY OF USE 19

A. HISTORICAL EXPOSURE TO GOS 19
1. Naturally Occurring GOS 19
2. GOS Added to Foods 19
3. Dietary Supplements 20
B. EXISTING REGULATORY APPROVALS FOR THE USE OF GOS IN FOODS 20
C. INTENDED USES OF OLIGOMATE 55N/55NP GOS 21
D. ESTIMATED INTAKES OF GOS FROM THE INTENDED USES 23

IV. INTENDED EFFECT 27

V. SAFETY 28

A. SAFETY OF THE SPOROBOLOMYCES SINGULARIS YIT 10047 YEAST ORGANISM ("YC-Y") 28
1. Identification and Taxonomic Description 28
2. Non-toxigenicity of Sporobolomyces singularis YIT 10047 ("YC-Y") 29
3. Enzymatic Activity of Sporobolomyces singularis (parent strain) 31
4. Enzymatic Activity of Sporobolomyces singularis 7B6 or YIT 10047 33
5. Safety Studies on Sporobolomyces singularis YIT 10047 ("YC-Y") Yeast Concentrate 35
6. Use of Sporobolomyces singularis YIT 10047 in the Production of Oligomate 55N/55NP GOS 39
B. SAFETY OF THE KLUYVEROMYCES LACTIS PURIFIED BETA-GALACTOSIDASE ENZYME 40

C. GOS PRODUCT PROFILES 40
1. Organisms and/or Their Enzymes Utilized in the Synthesis of GOS 41
2. Saccharide Polymer Length Distribution in GOS Products 41
D. SAFETY OF GOS 44
1. Introduction 44
2. Summary of In Vitro Studies of GOS 45
3. Summary of Animal Studies of GOS 56
4. Summary of Adult Studies of GOS 91
5. Summary of Infant Studies of GOS 117

VI. REFERENCES 140

TABLES

TABLE II-1. EXAMPLE OF SUGAR COMPOSITION IN OLIGOMATE * 9
TABLE II-2. PROPERTIES OF OLIGOMATE GOS 9
TABLE II-3. BATCH ANALYSIS DATA AND SPECIFICATIONS FOR OLIGOMATE GOS 18
TABLE III-1. INTENDED USES OF GOS 22
TABLE III-2. ESTIMATED 2-DAY AVERAGE INTAKES OF GOS FROM ALL PROPOSED USES IN FOODS25
TABLE V-1. MYCOTOXINS ABSENT IN SPOROBOLOMYCES SINGULARIS YC-Y YEAST CONCENTRATE 30
TABLE V-2. SUMMARY OF IN VITRO GOS STUDIES 48
TABLE V-3. SUMMARY OF GOS STUDIES IN ANIMALS 64
TABLE V-4. SUMMARY OF STUDIES OF GOS IN ADULTS 99
TABLE V-5. SUMMARY OF STUDIES OF GOS IN INFANTS 124

FIGURES

FIGURE 1. STRUCTURE OF GOS 8
FIGURE 2. MANUFACTURING PROCESS OF YEAST CONCENTRATE-YAKULT (YC-Y) 12
FIGURE 3. FORMATION OF GOS FROM FOOD GRADE LACTOSE BY ß-GALACTOSIDASE 13
FIGURE 4. MANUFACTURING PROCESS OF OLIGOMATE GOS 14
FIGURE 5. GOS PRODUCT PROFILES 43

I. GRAS EXEMPTION CLAIM

A. NAME AND ADDRESS OF SPONSOR

Yakult Pharmaceutical Industry Co., Ltd.
1796 Yaho, Kunitachi-shi
Tokyo 186-8650
Japan

B. COMMON OR USUAL NAME OF GRAS SUBSTANCE

The substance that is the subject of this Generally Recognized As Safe (GRAS) determination is galacto-oligosaccharides (GOS) prepared with two β-galactosidases, one derived from *Sporobolomyces singularis* and the other derived from *Kluyveromyces lactis*. Other chemical names for GOS are: galacto-oligosaccharide, transgalactosylated oligosaccharide, transgalacto-oligosaccharide, and oligogalactosyl-lactose. The trade name for a product containing this GOS is Oligomate which is marketed in a syrup form (Oligomate 55N) and in a powder form (Oligomate 55NP).

C. INTENDED USE

Yakult intends to use Oligomate 55N/55NP GOS as an ingredient in term infant formulas at a level of 7.2 grams per liter and in selected foods for general food use at levels ranging from 0.3 – 11 g GOS per serving.

D. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of Oligomate 55N/55NP as an ingredient in term infant formulas at a level of 7.2 g/L and in selected foods for general food use at levels ranging from 0.3 – 11 g GOS per serving is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of Oligomate 55N/55NP from the intended uses specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that Oligomate 55N/55NP is safe, and GRAS, under the intended conditions of use, the safety of the intake of Oligomate 55N/55NP has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of Oligomate 55N/55NP as an ingredient in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1) Oligomate 55N/55NP GOS is a mixture of di- through hexasaccharides composed of 1-5 galactose units linked to a glucose molecule at the reducing end. The total GOS content of Oligomate is defined as the portion of the formulation that consists of the summed non-lactose disaccharides plus trisaccharides, tetrasaccharides, and trace amounts of higher oligosaccharides, excluding the lactose and monosaccharide content. The major saccharide in the GOS fraction of Oligomate 55N/55NP is the trisaccharide O-beta-D-galactopyranosyl-(1-4)-O-beta-D-galactopyranosyl-(1-4)-beta-D-glucose. *Sporobolomyces singularis* YIT 10047 has been used by Yakult in the production of Oligomate 55N/55NP since 2003 and is the main processing aid used to produce galacto-oligosaccharides from food-grade lactose. This organism is a chemically induced strain derived from *Sporobolomyces singularis* Phaff & do Carmo-Sousa, a ballistospore yeast organism and has been determined to be safe and GRAS for this use. The lactase (IUB No. 3.2.1.23) enzyme preparation derived from *Kluyveromyces lactis* that is used in the production of Oligomate 55N/55NP GOS for the purpose of degrading redundant lactose and preventing product crystallization has FDA GRAS (Generally Regarded As Safe) status under 21CFR §184.1388.

2) Oligomate 55N/55NP reproducibly meets compositional standards and complies with limits on contaminants appropriate for food-grade ingredients. Product specifications are set to assure that Oligomate 55N/55NP is suitable for use in food.

3) GOS has a long history of use in the food industry. In Europe, the first GOS-containing product was launched in 1997. By 1995, numerous products were commercially available in Japan, including Oligomate 50, Oligomate 55, Oligomate 55N/55NP (Yakult Pharm. Ind. Co.), CupOligo H-70 and CupOligo P (Nissin Sugar Mfg. Co.). Numerous food products contain GOS, including infant formulas, functional foods and clinical nutrition products.

4) The European Union plus the Dutch, Italian and United Kingdom governments recognize GOS as an approved ingredient in select foods in Europe. Four GOS products (GRAS Notifications #233, 236, 285, & 286) have been determined to be GRAS for use in the U.S. for addition to a variety of foods (GRAS 2007a, 2007b, GRAS 2009a, 2009b).

5) Different methods have been utilized for production of GOS products; the consequence of these process changes is a difference in the final product with respect to the profile of the oligosaccharide polymers and the position of the chemical bonds between the saccharide units. These chemical differences were evaluated for purposes of bridging studies done with different GOS products to establish safety, including physiological activity, of Oligomate 55N/55NP. GOS is generally defined as including the total content of non-lactose disaccharides plus trisaccharides, tetrasaccharides, and trace amounts of higher oligosaccharides, excluding the lactose and monosaccharide content of the formulation. GOS products consist mainly of β4'-type linkages or β6'-type linkages, depending on the specific enzymes and organisms utilized. From the perspective of chemical linkage, all β-linked GOS products are handled similarly by the gastrointestinal tract. The β-linkages between the saccharide units are resistant to digestion by α-amylase and the GOS passes undigested into the colon where it can be fermented. Oligomate 55N/55NP, and other GOS products with different degrees of oligomerization or chain lengths and either exhibit similar physiologic effects. They support the growth of various *Bifidobacterium* species in animals and humans and decrease the intestinal or fecal pH in animals or in humans as a result of the increased production of short-chain fatty acids (SCFA) from microbial fermentation. Oligomate 55N/55NP consists mainly of 4'-GOS, with some 6'-GOS. Both 4'-GOS and 6'-GOS are utilized for growth by the Bifidobacteria of the colon. Therefore, studies investigating safety of other β4'-type or β6'-type linked GOS products can be used to corroborate safety of Oligomate 55N/55NP.

6) As a consequence of lack of digestion, GOS products produce similar physical and physiological effects on the gastrointestinal system such as increases in colonic microflora fermentation, reduction of intestinal and fecal pH, changes in fecal characteristics and frequency, increased cecal tissue weight, flatulence, borborygmi, and abdominal distension. GOS products of varying chain lengths have also been shown to be fermented in the colon. As a result of fermentation, an increase in the numbers of Bifidobacteria, Lactobacilli and other beneficial bacteria in intestinal contents or feces have been measured. GOS ingestion has also been shown to decrease the numbers of specific pathogens, and decrease the adherence and invasion of host cells by pathogenic bacteria.

7) The favorable changes in the microflora profile results in increasing activity levels of bacterial-related β-galactosidases and β-glucosidases, changes in the concentrations of short-chain fatty acids (as an indirect measurement of microbial metabolic activity), and an increase in breath hydrogen concentration.

8) Systemic effects produced by GOS in some studies of animals and in human trials include positive changes in mineral balance (i.e., calcium, magnesium, and phosphorus), beneficial effects on plasma levels of cholesterol, increased production of soluble immunoglobulin A (IgA), modulation of systemic cytokine levels and immune cell responses toward a less inflammatory state, increased numbers of immune cells that could phagocytose invading pathogens, and decreased incidence of atopic disease in at-risk subjects.

9) Genotoxicity and subchronic rodent toxicology studies of Oligomate 55N/55NP document safety of intake at the proposed intake levels. Oligomate 55N is not genotoxic and the no observed adverse effect (NOAEL) level was determined to be ≥ 2000 mg Oligomate 55N syrup/kg/d (equivalent to 825 mg GOS/kg/d) in a subchronic rat study (Kobayashi et al. 2009).

10) *In vitro*, subchronic toxicology studies and other animal studies of essentially equivalent GOS products at levels up to 2250 mg GOS/kg/d corroborate safety of ingestion.

11) Clinical trials in healthy and compromised adults and preterm and term infants of GOS products, including studies using Oligomate 55N/55NP, substantiate safety. Endpoints reported from single or multiple dose adult studies of GOS (from 2.4-40.1 g GOS/d) include breath hydrogen levels, fecal frequency and characteristics, flatus/flatulence and abdominal distention occurrence, laxative threshold, dose for diarrhea onset in 50% of subjects, anaerobe, *Bifidobacterium*, and *Lactobacillus* counts, cytokine and inflammatory marker levels, fecal SCFA concentration changes, reduction in infection occurrence in compromised patients, reduction in length of hospital stay and antibiotic use rate, body weight gain, changes in serum lipid levels, modulation of fecal bile acid content, calcium absorption, and glucose tolerance and insulin response. No adverse effects other than mild gastrointestinal intolerance at levels of ingestion of Oligomate 55N at up to 0.30 g GOS/kg (approximately 18 g GOS) in a bolus dose were noted. Frequency of gastrointestinal symptoms increase in subjects at higher doses.

12) Safety of GOS ingestion has been determined in infant studies of GOS given at 0.2-0.9% w/v GOS (mainly in the form of GOS/FOS (9:1)) in formula. Parameters assessed that support the safety of ingestion of GOS at the proposed use level in infant formula, include adverse and serious adverse events, food or infant formula intake, body weight and length, dropouts according to treatment, fecal microflora measurements, antibiotic treatment, infection and hospitalization rates, stool

frequency and consistency, development of atopic disease, rates of regurgitation and/or colic, fecal SCFA content, reduction in fecal pH, calcium levels, and anthropometric measurements such as head circumference and sum of skin folds.

13) The Scientific Committee on Food (SCF), stated that it had no major concerns with the inclusion of up to 8 g oligosaccharides/L of a combination of 90% GOS and 10% FOS in both infant formulas and follow-on formulas. This corresponds to a maximum of 7.2 g GOS per liter of formula. In addition, four galacto-oligosaccharide products (GRAS Notifications #233, 236, 285, & 286) have been determined to be GRAS in the U.S. for use in a variety of foods from 1 to 7.5 g per serving and as an ingredient in term infant formula and follow-on formula at a level of 7.2 grams of galacto-oligosaccharides per liter. Publicly available GRAS Notifications present an estimated daily consumption from selected foods (excluding infant formulas) of up to approximately 17 g/person/day at the 90th percentile.

14) The intended use of GOS in infant formula is 7.2 g/L. The intended use in other selected foods resulted in an estimated daily intake (EDI) of 12.2 g GOS/person/day or 0.28 g GOS/kg body weight/day in the general population. The 90th percentile (heavy consumer) intakes of GOS in the general population from all proposed food uses was estimated to be 25.3 g GOS/person/day or 0.70 g GOS/kg body weight/day.

15) The safety of exposure to levels of GOS estimated from its intended uses is supported by a determination of a NOAEL of 2000 mg syrup/kg/day (825 mg GOS/kg/d) in a 90-day oral gavage study of Oligomate 55N, as well as lack of adverse effects observed in a battery of genotoxicity studies of the product. Corroborating animal studies of other sources of GOS support safety of ingestion. Numerous clinical studies of adults and infants consuming Oligomate 55N and other GOS formulations supports the safety and tolerance of ingestion of Oligomate 55N/55NP at the current estimate of daily GOS exposure from the proposed uses.

Determination of the GRAS status of Oligomate 55N/55NP under the intended conditions of use has been made through the deliberations of Dr. Roger Clemens, Dr. A. Wallace Hayes, and Dr. Judith Jones. 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, including the safety of Oligomate 55N/55NP and the potential human exposure to Oligomate 55N/55NP resulting from its intended use as an ingredient in foods and have concluded:

There is no evidence in the available information on Oligomate 55N/55NP *that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when* Oligomate 55N/55NP *is used at levels that might reasonably be expected from the proposed applications.* Oligomate 55N/55NP *is GRAS for use in foods as proposed by Yakult.*

Therefore, Oligomate 55N/55NP is safe and GRAS at the proposed levels of addition to foods. Oligomate 55N/55NP is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

E. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, Ph.D., D.A.B.T., CEO, Spherix, Incorporated, 6430 Rockledge Drive, Westmoreland Bldg, Suite 503, Bethesda, Maryland, 20817, Telephone: 301-897-0611; Facsimile: 301-897-2567; Email: ckruger@spherix.com.

F. SIGNATURE

Pursuant to the criteria provided in proposed 21 CFR 170.36, Yakult Pharmaceutical Industry Co., Ltd. hereby notifies the Food and Drug Administration that the use of Oligomate 55N/55NP as an ingredient in foods under the intended conditions of use is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Yakult Pharmaceutical Industry Co., Ltd. has determined that such use is generally recognized as safe (GRAS) through scientific procedures.

Signature _____ Date _____

II. DESCRIPTION OF SUBSTANCE

A. IDENTITY

1. Chemical Name and Identity

The substance that is subject of the evaluation for the GRAS determination is galacto-oligosaccharides (GOS) prepared with two β-galactosidase, one derived from *Sporobolomyces singularis* and the other derived from *Kluyveromyces lactis*. Other chemical names for GOS are: galacto-oligosaccharide, transgalactosylated oligosaccharide, transgalacto-oligosaccharide, and oligogalactosyl-lactose.

2. Common or Trade Names

The trade name for a product containing this GOS is Oligomate which is marketed in a syrup form (Oligomate 55N) and in a powder form (Oligomate 55NP).

3. CAS Registry Number

The CAS Registry Number 66455-21-8 for "oligosaccharides" (comprising carbohydrates, sugars, oligosaccharides, β-oligosaccharides and oligomeric monosaccharides) covers oligosaccharides in general. The GOS that is the subject of this GRAS determination is comprised mainly of 4'-galacto-oligosaccharides. The CAS Registry Number for this specific type of oligosaccharide is 6587-31-1.

4. Composition

GOS are chains of galactose units, usually with a single terminal glucose molecule ((galactose(Gal))$_n$-glucose(Glc)). The chain length of Oligomate 55N (syrup form) and Oligomate 55NP (powder form) varies. The total GOS content has been defined as the portion of the formulation that consists of the summed non-lactose disaccharides plus trisaccharides, tetrasaccharides,and trace amounts of higher oligosaccharides, excluding the lactose and monosaccharide content (De Slegte 2002). From this definition, and referring to Table II-1, Oligomate 55N consists of approximately 41.25% (w/w) GOS on a wet weight basis and Oligomate 55NP consists of approximately 55% (w/w) GOS on a dry weight basis. The type of β-glycosidic linkage between the monomer units is mainly 1→4 Gal (Sar *et al.*, 2004; Mwenya *et al.*, 2005). This specific subfraction of GOS is also referred to as β-1,4-galacto-oligosaccharide or 4'-galactosyllactose. 1→6 Gal linkages occur occationally (Table II-1). Other β-glycosidic linkages including: 1→2 Glc, 1→3 Glc, 1→4 Glc, 1→6 Glc can also be present (Sar *et al.*, 2004; Mwenya *et al.*, 2005). Rabiu et al. (2001) determined that GOS can be made of

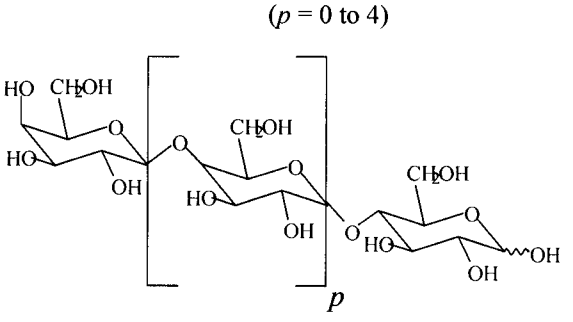
differing β-glycosidic linkages depending on the enzyme source. GOS derived from *Bacillus circulans* and *Cryptococcus laurentii* was found to predominantly produce GOS with 1→4 Gal β-glycosidic linkages (Sako et al. 1999).

Oligomate GOS is a product containing galacto-oligosaccharides (GOS), lactose, glucose, and galactose. Oligomate GOS is marketed in a syrup form (Oligomate 55N) and in a powder form (Oligomate 55NP).

5. Chemical Structure of GOS

The chemical structure of GOS is identified in Figure 1 below. As shown in the figure, the number of galactose units may vary.

Figure 1. Structure of GOS



6. Distribution of Oligomate GOS Components by Chain Length

Oligomate GOS is a mixture of di- through hexasaccharides composed of 1-5 galactose units linked to a glucose molecule at the reducing end. The distribution of the Oligomate GOS components by chain length is shown in Table II-1. Saccharide composition of Oligomate GOS was also analyzed by Japan Food Research Laboratories, which confirms the results presented in Table II-1. The major saccharide in the GOS fraction of Oligomate is the tri-saccharide O-β-D-galactopyranosyl-(1-4)-O-β-D-galactopyranosyl-(1-4)-β-D-glucose. The molecular weights of the individual oligosaccharides range between 342 (disaccharide) and 991 (hexasaccharide) Daltons. Chemical tests carried out by the manufacturer also show that the saccharide composition of Oligomate GOS produced in past seven years has not changed.

Table II-1. Example of Sugar Composition in Oligomate *

		p[(a)]	Empirical Formula	MW (Daltons)	Weight fraction (g/100g Oligomate)	GOS fraction (g/100g Oligomate)
Monosaccharides	Glc		$C_6H_{12}O_6$	180.16	22.4	-
	Gal				8.6	-
Disaccahrides	Gal β 1-4Glc (lactose)	0	$C_{12}H_{22}O_{11}$	342.30	12.7	-
	Gal β 1-3Glc				3.8	3.8
	Gal β 1-2Glc				2.4	2.4
	Gal β 1-6Glc				5.0	5.0
	Others				3.6	3.6
Trisaccharides	Gal β 1-6Gal β 1-4Glc	1	$C_{18}H_{32}O_{16}$	504.44	5.0	5.0
	Gal β 1-4Gal β 1-4Glc				18.5	18.5
	Others				10.9	10.9
Tetrasaccharides	Gal β 1-6Gal β 1-4Gal β 1-4 Glc	2	$C_{24}H_{42}O_{21}$	666.58	3.9	3.9
	Gal β 1-4Gal β 1-4Gal β 1-4 Glc				0.8	0.8
Others including trace amounts of higher oligosaccharides		>2			2.5	2.5
Total					**100.1**	**56.4**

Note:
*. Based on dry mass
(a) See Figure 1; Glc-Glucose; Gal=Galactose

B. PHYSICAL AND CHEMICAL PROPERTIES

The physical and chemical properties of Oligomate GOS are identified in Table II-2.

Table II-2. Properties of Oligomate GOS

Property	Oligomate 55N	Oligomate 55NP	Method of Analysis
Purity (% GOS on dry mass)	≥55	≥55	HPLC
pH (Brix 26.5 solution)	3.0 to 5.5	Not applicable	JSFA (2007)
Appearance	Clear to yellowish viscous liquid	White powder	Visual check
Solubility in in water	Completely soluble	Completely soluble	SMAFSR (1990)
Density g/ml at 15 °C Density g/ml at 40 °C	1.38 1.37	0.67	JSFA (2007) for syrup; Internal method for powder
Taste	Slightly sweet	Slightly sweet	Sensory test
Odor	No characteristic odor	No characteristic odor	Sensory test

Note: NA = not applicable; DM = dry matter
JSFA: Japanese Standards for Food Additive
SMAFSR: Standard Methods of Analysis in Food Safety Regulation
Source: Yakult Pharmaceutical Industry Co., Ltd

Tests with 10 Brix solution showed that Oligomate GOS is very stable against heating under both neutral and acidic conditions. Oligomate GOS is thermostable at tempertatures as high as 160°C and does not decompose. In addition, it has been demonstrated that Oligomate GOS is more stable than sucrose. Thus, there is no concern for potential decomposition of Oligomate GOS during typical food processing conditions. In addition, at the same concentrations, Oligomate GOS reduces water activity and increases osmotic pressure to the same degree as sucrose.

C. PRODUCTION PROCESS

Oligomate GOS (Oligomate 55N and Oligomate 55 NP) is produced by San-ei Sucrochemical Co., Ltd. located at 24-5 Kitahama-machi, Chita City, Aichi, Japan. This facility is certified by Japanese Quality Assurance Organization for its compliance with the requirement of ISO 9001:2000/JIS Q 9001:2000, and is licensed by the Public Health Center, Chita City, Aichi Prefecture for the production of food additives. Edible lactose is used as a starting material. Each batch of lactose received must meet the internal specifications established by Yakult. Processing aids used for the production of Oligomate GOS include two β-galactosidases, one is derived from *Sporobolomyces singularis* and the other is derived from *Kluyveromyces lactis*, activated carbon, ion exchange resins and filters. With the exception of β-galactosidases derived from *Sporobolomyces singularis*, these processing acids are widely used in the production of food and food ingredients and must meet the specifications established for them. The safety of these processing aids is discussed below in detail.

1. Beta-Galactosidases

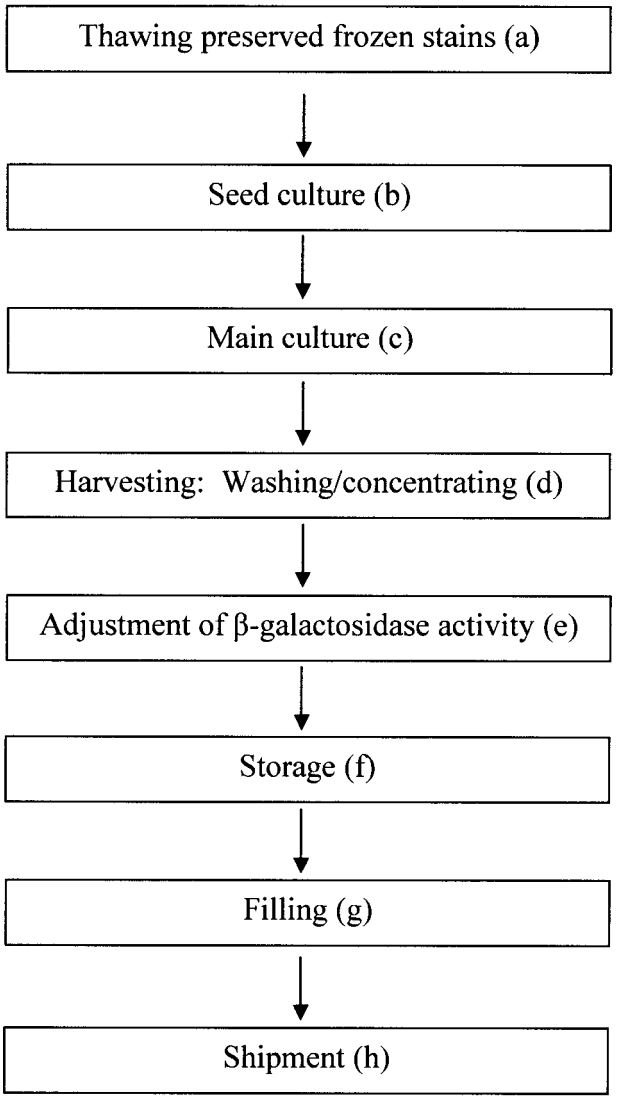
Oligomate GOS is prepared from food-grade lactose. The reaction is catalized by two β-galactosidases, one is derived from *Sporobolomyces singularis* and the other is derived from *Kluyveromyces lactis*. β-Galactosidases derived from *Sporobolomyces singularis* (Yeast Concentrate-Yakult or YC-Y) has potent transglactosylation activity, and is used to produce galacto-oligosaccharide from lactose. The enzyme derived from *Kluyveromyces lactis* (GODO-YNL) is used primarily to degrade unreacted lactose.

Sporobolomyces singularis is used as a biocatalyst in GOS production because of its potent transgalactosylation activity. The production strain, *Sporobolomyces singularis* YIT 10047, is a mutant of *Sporobolomyces singularis* ATCC 24193. This production strain, formally reported as *Sporobolomyces singularis* 7B6, has been deposited in the International Patent Organism Depository (http://unit.aist.go.jp/pod/cie/index.html) under the registration number FERM P-18817 (Ishikawa *et al.*, 2005). This mutant has 10-fold higher transgalactosylation activity, which is represented as β-galactosidase activity, when it is grown on glucose medium

compared with the parent strain, *Sporobolomyces singularis* ATCC 24193 (Ishikawa *et al.*, 2005). The safety studies conducted on *Sporobolomyces singularis* YIT 10047 includes a bacterial reverse mutation assay and a micronucleus test with peripheral blood of mice, and conclude that *Sporobolomyces singularis* YIT 10047 (reported as *Bullera singularis* YIT 10047 in the studies) is not genotoxic. In addition, mycotoxin screening test has shown that *Sporobolomyces singularis* YIT 10047 does not product mycotoxins.

The Yeast Concentrate-Yakult (YC-Y) with potent transgalactosylation activity (represented as β-galactosidase activity) is produced by culturing *Sporobolomyces singularis* YIT 10047 following standard microbial cell culture practices; media sterilization, seed culture, main culture, pH and temperature control, cell harvest and packaging. The YC-Y production flow chart is presented in Figure 2. In summary, production process starts with cryopreserved strain. Cryopreserved strain is thawed following a standard thawing protocol (Step a). The culture of *Sporobolomyces singularis* YIT 10047 is scaled up from flasks (Step b) to fermenters (Step c); both accomplished under aerobic conditions. Nutrient-rich broth containing a nitrogen (protein) source and an energy source (carbohydrate) is sterilized to obtain a pure culture of *Sporobolomyces singularis* YIT 10047. The temperature, pH and β-galactosidase activity are monitored during the main culture. When a predefined β-galactosidase activity is reached, yeast cells are then harvested by centrifugation (Step d). Recovered yeast cells are further washed with water to remove remaining medium (Step d). The β-galactosidase activity of YC-Y is then standardized by adding water and homogenization (Step e) to meet the specifications established by Yakult. The obtained YC-Y product is then packaged, released and kept refrigerated (Steps f to h).

Figure 2. Manufacturing Process of Yeast Concentrate-Yakult (YC-Y)



Safety evaluation of YC-Y is detailed in Section 5.

β-Galactosidases derived from *Kluyveromyces lactis* has a long history of use in food ingredients. *Kluyveromyces lactis* is nonpathogenic and nontoxicogenic, and the β-galactosidases (reported as lactase) derived from it is Generally Recognized AS Safe (GRAS) as a direct food substance under the regulation of 21CFR184.1388 in the U.S. This lactase is supplied by Godo Shusei Co., Ltd. The trade name of this enzyme is GODO-YNL.

2. Oligomate GOS Production Process

Galacto-oligosaccharides are produced through the enzymatic conversion of lactose (Ishikawa *et al.*, 2005). Oligomate GOS is prepared from edible lactose, isolated from sweet whey (derived from cow's milk). The lactose is subjected to the action of β-galactosidases. β - Galactosidases possess two kinds of activity: hydrolytic activity, which breaks the galactose- β 1-4 glucose bond to release glucose and galactose, and transgalactosylic activity, which converts lactose into galacto-oligosaccharides. β-Galactosidase derived from *Sporobolomyces singularis* (YC-Y) has potent transgalactosylation activity. This enzyme is used primarily to catalyze the reaction to give rise to galacto-oligosaccharides with increasing chain length by a series of transglycosylation reactions and is used to produce galacto-oligosaccharide from lactose (Figure 3). On the other hand, the enzyme derived from *Kluyveromyces lactis* (GODO-YNL) is used primarily to degrade unreacted lactose.

Figure 3. Formation of GOS from Food Grade Lactose by ß-Galactosidase

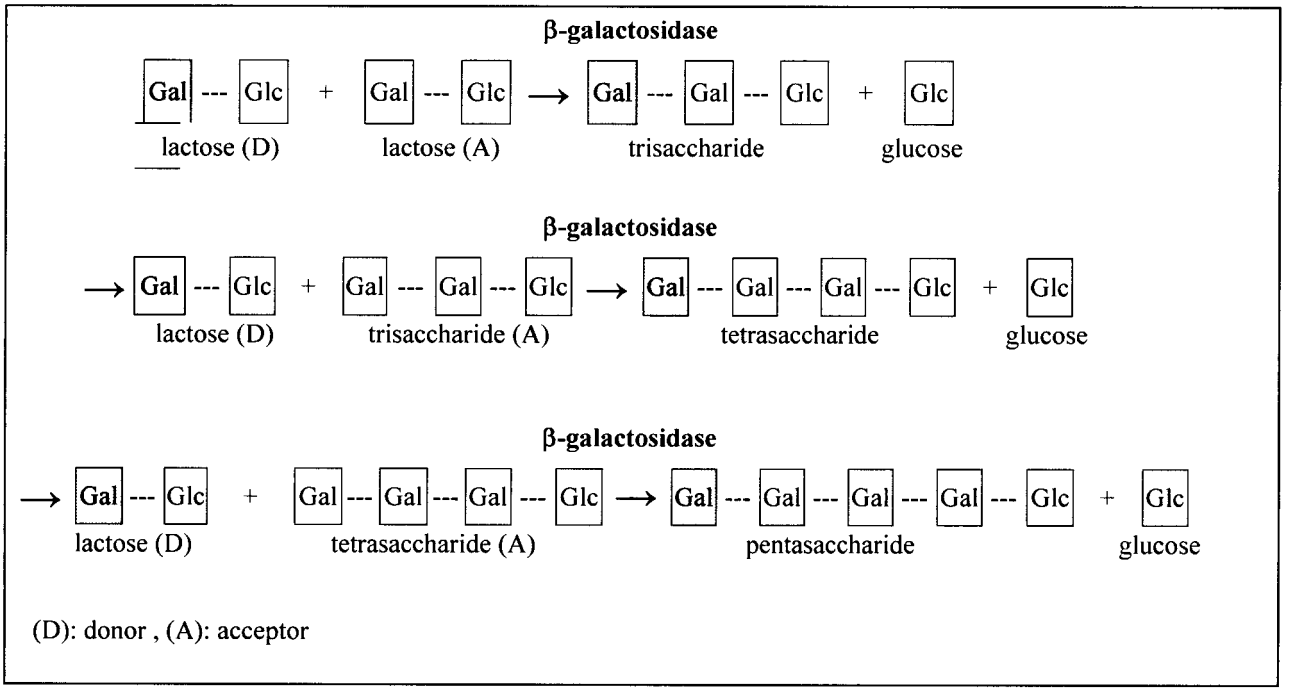
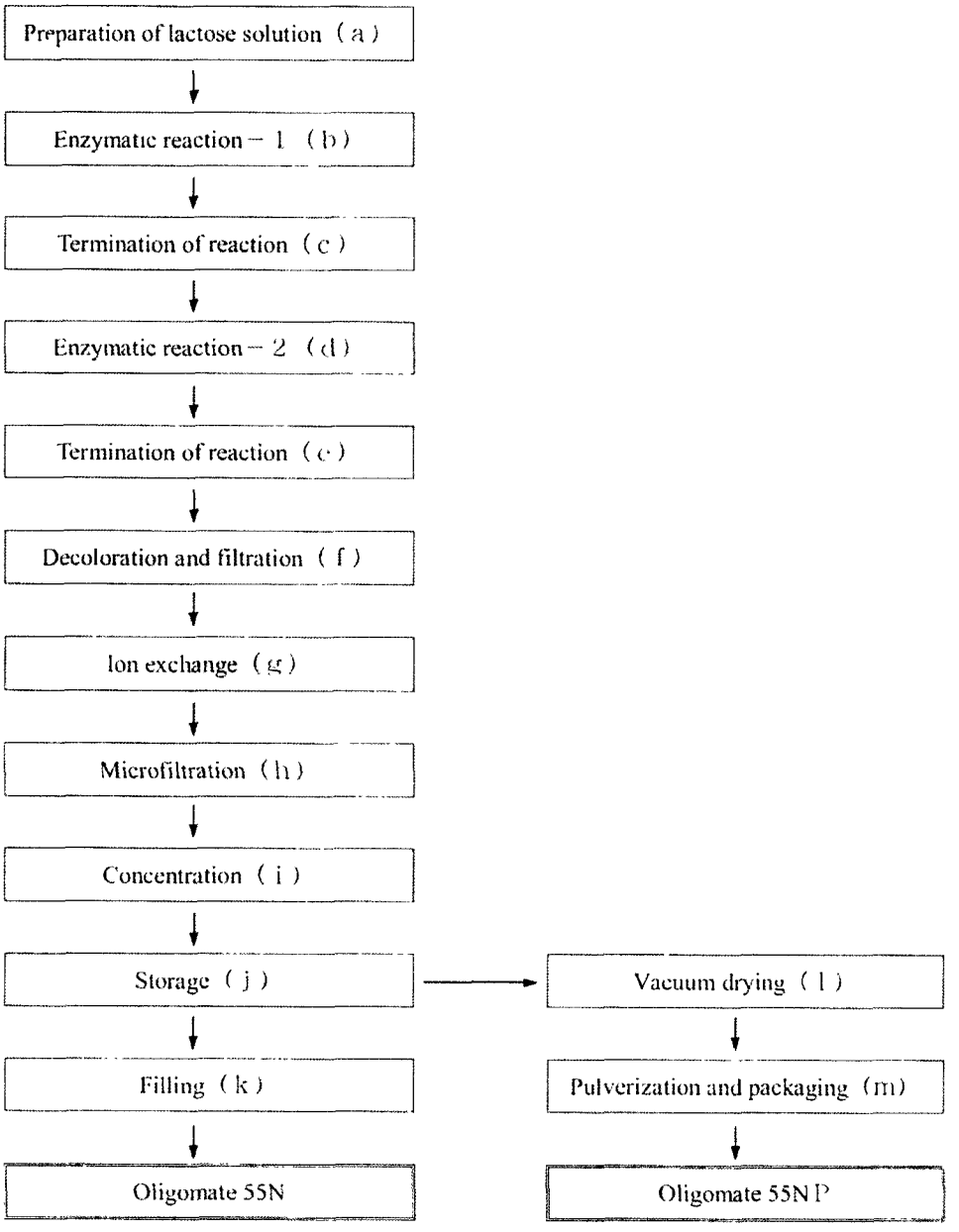


Figure 4. Manufacturing Process of Oligomate GOS



The process flow chart for the production of Oligomate GOS is presented in Figure 4. The production process is summarized below. Food grade lactose is dissolved in water that meets or exceeds public health standards by heating while mixing until complete dissolution of lactose (Step a). The lactose solution is then cooled and pH is adjusted to the optimum reaction temperature and pH ranges for YC-Y (β-galactosidase with potent transglactosylation activity).

Into the resulting lactose solution, YC-Y is added to catalyze the reaction to give rise to galacto-oligosaccharides (Step b). When a predefined saccharide composition is reached, the reaction is terminated by deactivating the YC-Y enzyme by heating (Step c). The resulting GOS mixture is cooled and the pH is adjusted to the optimum temperature and pH ranges for GODO-YNL, a β-galactosidase derived from *Kluyveromyces lactis*. GODO-YNL is then added to hydrolyze the unreacted lactose (Step d). Again, the GODO-YNL enzyme is deactivated by heating after the reaction finishes, which is indicated by the achievement of a predefined saccharide composition (Step e). Further purification steps include:

- decoloration by mixing the GOS solution with powdered activated carbon (Step f);
- filtration with ceramic membrane filter to remove activated carbon, enzymes and other proteins originated from lactose (Step f);
- demineralization with ion exchange resins (Step g);
- microfiltration to further remove microparitcles and microorganisms (Step h);
- concentration with vacuum evaporation to obtain a syrup with water content of less than 25%, followed by ultraviolet sterilization (Step i); The resulting Oligomate 55N (a syrup form GOS product) is stored in storage tanks (Step j) before it is fed to the filling line (Step k).

Alternatively, the concentrated GOS syrup (Step j) is further dried to produce Oligomate 55NP (a powder form GOS product) by heating under vacuum (Step l). The dried Oligomate GOS with water content of less than 3% is further pulverized and packaged (Step m)

For each step, the process is well controlled by controlling operation parameters such as concentration, pH, temperature and reaction time and by following redefined out of specification recovery process.

3. Starting Material and Processing Aids

Oligomate GOS is prepared from food-grade lactose. The disaccharide lactose (milk sugar) is a natural component of milk and sweet whey and is comprised of the monomers galactose (Gal) and glucose (Glc). The quality of edible lactose is well controlled. Yakult has established standards for the lactose, from which the provider generates a product specification sheet. Heavy metals are not analyzed for every batch. However, heavy metals are monitored on a quarterly basis to prevent contamination. Yakult analyzes every batch of lactose received to control the lactose quality.

In addition to the lactose starting material, all processing aids are well controlled. Activated carbon is a standard decolorizing, taste- and odor-removing, and purification agent for use in food processing. Activated carbon is approved as a food additive.

After decolorization with activated carbon, the suspension passes through cross-flow filtration with a Membralox® ceramic membrane for the removal of particles and proteins. Membralox® ceramic membrane has been widely used for clarification, concentration and filtration of various products including milk and dairy products, sugar and sweeteners, food ingredients and additives, beverages and potable water, and other foods.

Three types of ion exchange resins are used sequentially for the demineralization of the GOS solution. These resins include Purolite® A103S, Purolite® A510S, and Purolite® C150S. The functional groups for these resins are tertiary amine, Type 2 quaternary ammonium and sulphonic acid, respectively. These resins have the same polymer structure, which is macroporous polystyrene crosslinked with divinylbenzene. The sugar, sweetener and food industries have been using ion exchange resins for different types of decalcification, decolorization, demineralization, taste and odor removal, chromatography and ion exclusion. Products such as Purolite® A103S, Purolite® A510S, and Purolite® C150S have been widely used for these applications.

After demineralization, the GOS solution is further filtered sequentially through a 1 μm filter, and then 0.6 μm and 0.3 μm zeta potential filters to remove microparticles and microorganisms. The filter is made of polypropylene, which is physically inactive and thus has no untoward effect on human health. Three raw materials are used for the production of zeta potential filters (Depth-Clear™). Cellulose and diatomaceous earth have been identified by the respective suppliers to be generally recognized as safe (GRAS) in their use as a filter aid for foods. The third material, the binding resin, is appropriate for use in food applications under FDA 21CFR176.170.

In addition, treated industry water is used in the production process. The water quality must meet the requirements of Ministerial Ordinance No. 101 of 2003 "Water Quality Standards" issued by the Ministry of Health, Labour and Welfare.

D. PRODUCT CHARACTERIZATION

1. Batch Analysis Results and Product Specifications

The composition of three batches of oligomate GOS was determined; these data are shown in Table II-3. Product specifications are also shown in the tables. The test results show that the products comply with the specifications established.

2. Residual Impurities

After the synthesis of GOS, the β-galactosidase is inactivated by heating. Activated carbon is then added to adsorb impurities and decolorize the product. A filtration step is performed to remove the denatured enzyme and impurities. Potential impurities in Oligomate GOS would be the raw materials used in the manufacturing process: lactose, water, activated carbon, and the monomers of the disaccharide lactose (galactose and glucose). Lactose, galactose and glucose are natural constituents of foods. Activated carbon is used extensively for purification of food and pharmaceutical products.

3. Stability of Oligomate GOS

The stability of Oligomate 55N and 55NP was evaluated over a period of 18 months or 2 years. Changes of sugar composition, physical and chemical properties were monitored. Results show that Oligomate GOS is highly stable. As for Oligomate 55N, a slight decrease of pH was noticed. In addition, GOS syrup (55N) became darker over time of storage. We expect that this is because of the presence of monosaccharides in GOS, since the change of pH and color over time is also seen in sugar syrups such as high fructose corn syrup.

The stability of Oligomate syrup (55N) was further evaluated over a period of 3 months. Oligomate 55N was stored at elevated temperature as high as 55 °C at pH of either 2 or 3. In addition, heat stability of Oliggomate 55N was evaluated at a pH range between 2 to 7 by heating at temperature as high as 120 °C for 10 minutes. The results showed that Oligomate 55N is highly heat stable under the pH range tested.

E. METHOD OF ANALYSIS OF OLIGOMATE GOS IN FOODS

GOS content in food products can be quantified using high performance liquid chromatography.

Table II-3. Batch Analysis Data and Specifications for Oligomate GOS

Oligomate 55N					
Parameters	**Specifications**	**Lot YPS0925E**	**Lot YPS0922F**	**Lot YPS0915E**	**Method of Analysis**
GOS	≥ 55% based on dry weight	56.4%	56.7%	57.4%	HPLC
Solid content (Bx.)	75.0 to 76.0	75.2	75.4	75.9	Refractometer at 20°C
pH	3.0 to 5.5	4.4	4.8	4.6	26.5 Bx. Solution (pH meter)
Ash	≤ 0.1%	≤ 0.1%	≤ 0.1%	≤ 0.1%	Burn at 550°C
Protein	≤ 0.1/100g	≤ 0.1/100g	≤ 0.1/100g	≤ 0.1/100g	Kjeldahl method
Color	≤ 0.3	0.06	0.03	0.06	Difference of absorbance at 420 nm and 720 nm of 26.5 Bx. solution
Turbidity	≤ 0.05	0.00	0.00	0.00	Absorbance at 720 nm of 26.5 Bx. solution
Heavy metal (as Pb)	≤ 5μg/g	≤ 1μg/g	≤ 1μg/g	≤ 1μg/g	Na_2S colormetric method
Lead	≤ 1μg/g	≤ 0.1μg/g	≤ 0.1μg/g	≤ 0.1μg/g	ICP atomic emission spectrometry
Arsenic (as As_2O_3)	≤ 1μg/g	≤ 1μg/g	≤ 1μg/g	≤ 1μg/g	Atomic absorption spectrometry
Aerobic plate count	≤ 300 /ml	≤ 300 /ml	≤ 300 /ml	≤ 300 /ml	Plate count agar
Mold and yeast	≤ 50 /ml	≤ 50 /ml	≤ 50 /ml	≤ 50 /ml	Potato dextrose agar
Coliform	Negative	Negative	Negative	Negative	BGLB broth
Oligomate 55NP					
Parameters	**Specifications**	**Lot 0809291**	**Lot 0807161**	**Lot 0806171**	**Method of Analysis ***
GOS	≥ 55% based on dry weight	56.5%	56.4%	56.8%	HPLC
Moisture content	≤ 3%	0.4%	0.4%	0.5%	Vacuum (<2kPa) at 105°C for 5 hours
Ash	≤ 0.1%	≤ 0.1%	≤ 0.1%	≤ 0.1%	Burn at 550°C
Protein	≤ 0.1/100g	≤ 0.1/100g	≤ 0.1/100g	≤ 0.1/100g	Kjeldahl method
Heavy metal (as Pb)	≤ 5μg/g	≤ 5μg/g	≤ 5μg/g	≤ 5μg/g	Na_2S colormetric method
Lead	≤ 1μg/g	≤ 1μg/g	≤ 1μg/g	≤ 1μg/g	ICP atomic emission spectrometry
Arsenic (as As_2O_3)	≤ 1μg/g	≤ 1μg/g	≤ 1μg/g	≤ 1μg/g	Atomic absorption spectrometry
Aerobic plate count	≤ 300 /g	≤ 10 /g	≤ 10 /g	≤ 10 /g	Plate count agar
Mold and yeast	≤ 50 /g	≤ 10 /g	≤ 10 /g	≤ 10 /g	Potato dextrose agar
Coliform	Negative	Negative	Negative	Negative	BGLB broth

III. HISTORY OF USE

A. HISTORICAL EXPOSURE TO GOS

1. Naturally Occurring GOS

Galacto-oligosaccharides occur naturally as a complex mixture in human breast milk and colostrum, as well as in bovine milk. Human milk galacto-oligosaccharides exhibit a complex and diverse chemical profile of over 130 different compounds (McVaegh and Miller 1997). Oligosaccharides account for a large portion of the solids content of milk, in addition to lactose and fat (Newburg 2000). The total concentration of oligosaccharides is variable and estimates range from 5-8 g/L (Kunz et al. 2000) to 15.4 g/L (Coppa et al. 1991, *ibid* 1997) in mature human milk. Human colostrum may contain higher concentrations of oligosaccharides; one estimate calculated the level at 25.6 g/L (Coppa et al. 1991, *ibid* 1997). The galacto-oligosaccharide fraction of the total oligosaccharides content was found to range from 1.9 to 3.9 mg/L in milk samples collected from five women (Yamashita and Kobata 1974). The individual oligosaccharide chemical profile of human milk is known to vary with the infant's gestational state (for preterm infants), the duration of lactation (Sumiyoshi et al. 2004) as well as the genetic makeup of the mother (McVaegh and Miller 1997, Erney et al. 2000). However, total oligosaccharide content has been reported to be similar in term and preterm milk (Kunz et al. 2000). The major galacto-oligosaccharides isolated from human milk have the structure Galβ1→6Galβ1→4Glc (6'-GL) (Yamashita and Kobata 1974, Sumiyoshi et al. 2004), Galβ1→3Galβ1→4Glc (3'-GL) (Sumiyoshi et al. 2004) and Galβ1→4Galβ1→4Glc (4'-GL) (Sumiyoshi et al. 2004) indicating that the β-type linkage between saccharide units occurs naturally.

When compared with human milk oligosaccharides, the chemical profile of bovine milk oligosaccharides is much less diverse, but structurally similar (Gopal and Gill 2000). Bovine colostrum has been reported to contain 8.5 mg/L galacto-oligosaccharides (Saito et al. 1987), but mature bovine milk contains only traces of total oligosaccharides and no GOS at all (Kunz et al. 2000, Saito et al. 1987). However, low levels of GOS (0.03-0.09%) may still occur in fermented milk products, such as commercial yogurt, due to the enzymatic activity of microbial β-galactosidases on the lactose in the milk (Toba et al. 1982).

2. GOS Added to Foods

GOS has a history of use in the food industry. Industrial scale GOS production began in the 1980s. By 1995, numerous products were commercially available in Japan. In Europe, the

first GOS-containing product was launched in 1997: a Dutch product called "Umer." Umer is a dessert having a consistency between that of yogurt and quark (fresh curd cheese), and is produced from fermented milk. GOS has been used as a food ingredient in Japan for several years, including Oligomate 50, Oligomate 55, Oligomate 55N/55NP (Yakult Pharm. Ind. Co.), CupOligo H-70 and CupOligo P (Nissin Sugar Mfg. Co.). Numerous food products contain GOS, including infant formulas, functional foods and clinical nutrition products

3. Dietary Supplements

In the U.S., Vivinal® GOS is approved as a dietary supplement ingredient. A New Dietary Ingredient (NDI) notification was submitted to the U.S. Food and Drug Administration (FDA) by EM Industries, on 3-19-98, for galacto-oligosaccharides derived from lactose (under the brand name Elix'or®) (FDA 1998). The recommended use of GOS as a dietary supplement is 7.5-15 g GOS, spread evenly throughout the course of the day. FDA NDI Report 24 for Vivinal® was filed without comment by FDA.

B. EXISTING REGULATORY APPROVALS FOR THE USE OF GOS IN FOODS

The European Union (EU) plus the Dutch, Italian and United Kingdom governments recognize Vivinal® GOS as an approved ingredient in select foods in the EU. In a document issued by the Dutch government on May 6, 1996, to Friesland Foods, the government refers to Article 2 of the Dutch Commodities Act Decree on the Permitted Use of New Foodstuffs. This article regulates ingredients classified as novel foods. The Dutch Government provided an exemption to this article for Vivinal® GOS. As such, Vivinal® GOS, in practice, is not a novel food and is qualified as a normal food ingredient in the Netherlands. Vivinal® GOS is used in other European countries as an ingredient in foods, including formulas for term infants. In the EU, a combination of 90% GOS and 10% fructo-oligosaccharides (FOS) may be added to infant formulas and follow-on formulas such that their total content does not exceed 8 g per L; this maximum concentration is equivalent to 7.2 g GOS per L infant formula.

In a review of infant formula composition, the European Commission, Scientific Committee on Food (SCF), stated that it had no major concerns with the inclusion of up to 8 g oligosaccharides/L of a combination of 90% GOS and 10% FOS in both infant formulas and follow-on formulas. According to the Committee, non-digestible carbohydrates may be added to infant formulas for technical reasons or if they provide a "source of fermentable substrates for the gut microflora" (Scientific Committee on Food 2003). The oligosaccharide addition approved by the Committee corresponds to a maximum of 7.2 g GOS per liter of formula (and 0.8 g FOS

per liter), or 16 g Vivinal® GOS per liter assuming 45% (weight basis) GOS in Vivinal® (Scientific Committee on Food 2003).

Four galacto-oligosaccharide products (GRAS Notifications #233, 236, 285, & 286) have been determined to be GRAS for use in a variety of foods (GRAS 2007a, 2007b, GRAS 2009a, 2009b). The first product is a mixture of GOS plus polydextrose for use as an ingredient in milk-based term infant formula at levels not to exceed 2 grams per liter (g/L) for galacto-oligosaccharides and 2 g/L for polydextrose. The second is a GOS product for use as an ingredient in term infant formula at a level of 5 grams (g) per liter, and in bars, yogurt, frozen dairy desserts, fruit drinks and energy drinks, fitness water and thirst quenchers, fruit pie filling, fruit preparation, jelly and jam, baby juice, baby yogurt drink, baby dessert, baby snack, milk, milk drinks, syrup flavorings for milk, meal replacement drinks, meal replacement drinks for children, and milk substitutes at levels ranging from 1 to 7.5 g per serving. The third is a GOS product for use as an ingredient in certain baby, infant, and toddler foods at levels ranging from 0.86 to 1.28 grams (g) per serving; and, in certain beverages and beverage bases, dairy product analogs, milk products, bakery products, cereal and other grain products, desserts, dessert toppings and fillings, fruit and fruit juices, snacks, soups, and soft and hard candy at a level of 1.28 g/serving. The fourth is a GOS product for use as an ingredient in term infant formula and follow-on formula at a level of 7.2 grams of galacto-oligosaccharides per liter. Publicly available GRAS Notifications present an estimated daily consumption from selected foods (excluding infant formulas) of up to approximately 17 g GOS/person/day at the 90th percentile.

C. INTENDED USES OF OLIGOMATE 55N/55NP GOS

Yakult Pharmaceutical Ind. Co., LTD (Yakult) intends to add Oligomate 55N/55NP to a wide variety of selected foods in the U.S. food supply. The product categories to which Oligomate 55N/55NP will be added and corresponding use-levels for GOS employed in the current intake analysis are summarized in Table A. Specifically, Yakult plans to bring "vinegar drinks", which is new to American consumers, to the US market. "Vinegar drinks" are popular to the Japanese consumers, and are consumed in a way that is similar to "energy drinks" in the US.

Table III-1. Intended Uses of GOS

Food Group	Proposed Food Uses	Approximate serving size [a] (g or ml)	Maximum g GOS per serving [b]
Milk and milk products	Milk, milk substitute such as soy milk	250 g (240 ml)	5.0
	Milk drink	250 g (240 ml)	9.5
	Yogurt	225 g	7.5
	Milk based meal replacement	250 g (240 ml)	5.0
	Infant formula	7.2 g per liter	NA
	White sauces, milk gravies and cheese sauces	80 g (1/4 cup or 60ml)	1
	Milk desserts, frozen like ice creams	75 g (1/2 cup)	1.5
	Pudding and custards including baby foods	108 g (1/2 cup)	1.5
	Cheese soups	245g	1.5
Soups	Egg soups; soups with legumes as major ingredient; soups with grain products as major ingredient; potato soups; deep-yellow vegetable soups; tomato soups; other vegetable soups	245g	1.5
Nut beverages	Coconut beverages	250 g (240 ml)	4
Bakery products	Bread	50 g	0.5
	Brownies	40 g	0.4
	Cakes, heavy weight	125 g	1.25
	Cakes, medium weight	80 g	0.8
	Cakes, light weight	55 g	0.55
	Coffee cakes, crumb cakes, doughnuts, Danish, sweet rolls, sweet quick type breads, muffins, toaster pastries	55 g	0.55
	Cookies	30 g	0.3
	Crackers that are usually used as snacks	30 g	0.3
	French toast, pancakes	110 g	1.1
	Pies, cobblers, fruit crisps, turnovers, other pastries	125 g	1.25
	Waffles	85 g	0.85
	Grain-based bars with or without filling or coating, e.g., breakfast bars, granola bars, rice cereal bars	40 g	0.4

Table III-1. Intended Uses of GOS

Food Group	Proposed Food Uses	Approximate serving size [a] (g or ml)	Maximum g GOS per serving [b]
Cereals	Ready-to-eat cereals	35 g	0.7
	Ready-to-eat cereals (dry) for baby food	15 g	0.6
	Ready-to-serve cereals for baby food	110 g	0.6
Fruit and vegetable juices	Fruit juices (including citrus fruit juices) and nectars	250 g (240 ml)	4
	Vegetable juices	250 g (240 ml)	4
	Fruit juices, vegetable juices and juice mixtures baby food	125 g (120 ml)	2
Sugars and sweets	Jellies, jams, preserves	20 g (1 tbsp)	5.0
Nonalcoholic beverages	Fruit drinks such as fruit juice drinks, fruit flavored drinks, sports drinks, etc	250 g (240 ml)	5.0
	Non fruit beverages including energy drinks [d]	250 g (240 ml)	11
	Beverage concentrate (powder)	250 g (240 ml) [c]	5.0

a. Serving sizes based on Reference Amounts Customarily Consumed (RACC) (21 CFR §101.12) revised as of April 1, 2009. Actual product serving sizes may differ slightly from these values.
b. GOS concentrations in foods or beverage as consumed.
c. After dissolution of powder in water.
d. Yakult plans to bring "vinegar drinks", which is new to American consumers, to the US market. "Vinegar drinks" are popular to the Japanese consumers, and are consumed in a way that is similar to "energy drinks" in the US.
NA= not applicable

D. ESTIMATED INTAKES OF GOS FROM THE INTENDED USES

Estimates of potential intake of GOS from Oligomate 55N/55NP resulting from the intended uses in select foods were calculated using food consumption data reported in the United States Department of Health and Human Service's 2003-2004 National Health and Nutrition Examination Survey (NHANES). The intake estimates are based on GOS only.

The NHANES 2003-2004 survey provides the appropriate data for evaluating food-use and food-consumption patterns in the United States. The survey contains 2 years of data on individuals selected *via* stratified multistage probability sample of civilian non-institutionalized population of the U.S. NHANES 2003-2004 survey data were collected from individuals and households *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all 4 seasons of the year. Day 1 data were collected in-person, and Day 2 data were collected by telephone in the following 3 to 10 days, on different days of the week, to achieve the

desired degree of statistical independence. The data were collected by first selecting Primary Sampling Units (PSUs), which were counties throughout the U.S. Small counties were combined to attain a minimum population size. These PSUs were segmented and households were chosen within each segment. One or more participants within a household were interviewed. Fifteen PSUs are visited each year. For the 2003-2004 NHANES, there were 12,761 persons selected; of these 9,643 were considered respondents to the Day 1 in-person examination and data collection. However, only 9,034 of the in-person respondents provided complete dietary intakes for Day 1. Furthermore, of those providing the Day 1 data, only 8,354 provided complete dietary intakes for Day 2.

In addition to collecting information on the types and quantities of foods being consumed, NHANES 2003-2004 collected socioeconomic, physiological, and demographic information from individual participants in the survey, such as sex, age, height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population. Sample weights were incorporated with NHANES 2003-2004 to compensate for the potential under-representation of intakes from specific population groups as a result of sample variability due to survey design, differential non-response rates, or other factors, such as deficiencies in the sampling frame.

Among 8,354 respondents who not only completed food intake survey on both Day 1 and Day 2, 8227 respondents also provided physiological information including age, sex and weight. Only 27 of 8227 respondents reported no intake of food products containing GOS during the 2 survey days and thus were "zero" consumers. When sample weights are considered, 99.7% of the total U.S. population was identified as consumers of GOS from the proposed food uses. Thus, only the estimated daily intakes of GOS-users (reported intake of food products containing GOS on either Day 1, or Day 2 or on both days) are reported.

The estimated total intake of GOS from all proposed food uses of GOS in the U.S. by population group is summarized in Table B. Consumption of GOS containing foods by the total U.S. population resulted in estimated mean intakes of GOS of 12.2 g/person/day or 0.28 g/kg body weight/day. The 90th percentile (heavy consumer) intakes of GOS from all proposed food-uses by the total population were estimated to be 25.3 g/person/day or 0.70 g/kg body weight/day.

On an individual population basis, the greatest mean intake of GOS on an absolute basis was observed to occur in children at 18.1 g/person/day. Infants displayed the greatest mean

intake of GOS on a body weight basis, with a value of 1.44 g/ kg body weight/day. When infants were further grouped in months, the greatest mean intakes of GOS were observed to occur in infants aged between 1 and 2 years with intakes of 19.4 g/person/day on an absolute basis and 1.73 g/kg body weight/day on a body weight basis, respectively.

When heavy consumers (90th percentile) were assessed, intakes of GOS from all proposed food-uses were determined to be greatest in male teenagers at 33.0 g/person/day on an absolute basis and in infants at 2.42 g/ kg body weight/day on a body weight basis. When infants were further grouped in months, greatest mean intake of GOS was observed to occur also in infants aged between 1 and 2 years at 2.60 g/kg body weight/day.

Table III-2. Estimated 2-Day Average Intakes of GOS from All Proposed Uses in Foods

Population	N_u/N_p [a]	Percent users [b]	2-Day Average GOS Intakes Per User			
			g GOS/day		g GOS/kg-bw/day	
			Mean	90th Percentile	Mean	90th Percentile
Infants, 0-5 months	145/145	100	6.9	11.3	1.16	1.82
Infants, 6-11 months	215/217	99.6	7.9	11.8	0.90	1.40
Infants, 12-23 months	301/301	100	19.4	27.8	1.73	2.60
All infants, 0-1 year	***661/663***	***99.9***	***14.7***	***26.8***	***1.44***	***2.42***
Children, 2-11 years	1518/1518	100	18.1	30.4	0.77	1.42
Teen females 12-19 years	989/991	99.1	12.4	24.2	0.22	0.47
Teen males, 12-19 years	993/997	99.4	17.4	33.0	0.28	0.57
Adult females, 20+ years	2118/2129	99.8	9.5	19.4	0.14	0.29
Adult males, 20+ years	1921/1929	99.7	11.7	24.8	0.14	0.30
Total population	8200/8227	99.7	12.2	25.3	0.28	0.70

[a] Number of people consuming one or more foods containing GOS on either Day 1 or Day 2 of the survey over the number of people surveyed.
[b] Percent of user when sample weights are considered.

Because GOS has physiological effects similar to fibers, its intakes thus are compared with the recommended daily intake of fiber. The Institute of Medicine (IOM) has not determined Dietary Reference Intake (DRI) of fiber for the infants aged between 0 to 12 months. For the children aged between 1 and 3 years, the DRI for fiber is 19 g per day. For children aged between 4 and 8 years and for adults, the DRI for fiber is between 21 and 38 g per day. The estimated daily intakes of GOS based on the proposed uses and use levels (Table III-2) are well below or about the same as the DRIs the IOM determined.

It is important to note that theses estimates are likely large overestimates of actual intakes of GOS from Oligomate 55N/55NP resulting from the proposed uses in the food supply. In the calculations of estimated intakes, any reported intake of a food corresponding to one of the proposed use categories (Table A) was assumed to contain added Oligomate 55N/55NP. Additionally, all foods were assumed to contain the maximum proposed concentration of GOS per serving. It is likely that consumers may in fact consume only a subset of these foods containing added Oligomate 55N/55NP, and not all products may contain the maximum proposed use levels.

IV. INTENDED EFFECT

The subject of the GRAS determination, Oligomate 55N/55NP galacto-oligosaccharides (GOS), is composed of indigestible carbohydrates that preferentially stimulate the growth and/or activity of a limited number of bacteria in the colon. The intended effect of Oligomate 55N/55NP is to provide a dietary source of these oligosaccharides.

V. SAFETY

A. SAFETY OF THE SPOROBOLOMYCES SINGULARIS YIT 10047 YEAST ORGANISM ("YC-Y")

1. Identification and Taxonomic Description

The specific organism utilized in the production of Oligomate 55N/55NP GOS is *Sporobolomyces singularis* YIT 10047, a mutant producing large amounts of β-glucosidase derived from *Sporobolomyces singularis* Phaff & do Carmo-Sousa (ATCC 24193), a ballistospore yeast organism. Ballistosporous yeasts are known to occur naturally on the surface of plant leaves and stems (Nakase et al. 1993, Nakase and Suzuki 1987, Stadelmann 1975). *S. singularis* was initially isolated from insect frass (excrement) found in the bark of the coast or western hemlock tree (*Tsuga heterophylla* (Raf.) Sargent) and was characterized by H. J. Phaff and Lidia Do Carmo-Sousa in 1962 (Phaff and Do Carmo-Sousa 1962). *S. singularis* has also been referred to by the older synonym of *Bullera singularis* (Phaff & do Carmo-Sousa) Rodrigues de Miranda (Japan Society for Culture Collections Online Database and DSMZ, The German Resource Centre for Biological Material), and some references use this Latin binomial. *S. singularis*, when cultured on corn meal agar, forms kidney- to sickle-shaped ballistospores. However, ballistospore formation does not occur on malt or potato dextrose agar. Additionally, in contrast to other species of the genus *Sporobolomyces*, which are red or salmon-pink due to their ability to synthesize carotenoids (Lodder and Kreger-van Rij 1952), *S. singularis* is non-pigmented. Due to this observation, Phaff and Do Carmo-Sousa proposed an amended diagnosis for the description of the genus *Sporobolomyces* Kluyver and van Niel:

> "Usually red or salmon-pink organisms, but some species are non-pigmented. Thallus consists of oval to elongate cells; true hyphae and pseudohyphae may be present. Vegetative reproduction mainly by budding. On part of the vegetative cells typical kidney-shaped or sickle-shaped spores (ballistospores) develop in an oblique position to the aerial sterigmata. When mature, these spores are discharged into the air by means of a peculiar mechanism: a drop of liquid is excreted at the hilum of the spore; the drop increases in size and is discharged, carrying the spore with it. These spores either germinate and form normal yeast cells or they form again aerial sterigmata at which spores develop. The metabolism is strictly oxidative." (Phaff and Do Carmo-Sousa 1962)

The parent organism *Sporobolomyces singularis* is available from the American Tissue Type and Culture Collection (ATCC; www.atcc.org) as ATCC® Number 24193™, and is reported to produce β-glycosyl-nucleoside and vitamin derivatives via formation of β-galactosyl linkages

(Suzuki et al. 1994, Suzuki et al. 1992), the key enzymatic ability that is involved in the production of Oligomate 55N/55NP GOS. The *Sporobolomyces singularis* YIT 10047 strain that is utilized as a processing aid in the manufacturing of Oligomate 55N/55NP was isolated in 2005 by Ishikawa and co-workers as *S. singularis* 7B6 for its high β-glucosidase-like specific activity (Ishikawa et al. 2005). *S. singularis* 7B6 (also referred to as *Sporobolomyces singularis* YIT10047) was generated by briefly incubating the parent strain with methyl-*N'*-nitro-*N*-nitrosoguanidine, washing the cells, and then selecting for the resulting mutants that could grow in lactose medium containing 2-deoxy-D-glucose (2-DG), an inhibitor of glucose utilization (Ishikawa et al. 2005). This process selected for yeast strains that could break down lactose to a usable energy source using its β-galactosidase or a β-galactosidase-like enzyme, since β-glucosidases should be blocked by the 2-DG. The β-galactosidase-like enzyme produced by the *S. singularis* YIT10047 strain is described in further detail in the section "*Enzymatic activities of Sporobolomyces singularis*."

2. Non-toxigenicity of *Sporobolomyces singularis* YIT 10047 ("YC-Y")

In order to assess the non-toxigenic nature of *Sporobolomyces singularis*, a search for the natural occurrence of compounds produced by the genus *Sporobolomyces*, the species *Sporobolomyces singularis*, and the related *Rhodotorula* genus (Nakase et al. 1993, Nakase 2000, Hamamoto and Nakase 2000, Fell et al. 2000) was completed using Pubmed, Toxline, the Dialog chemical and toxicology databases, plus the specialty natural products database NAPRALERT^{SM} (NAtural PRoduct ALERT, Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago; www.napralert.org). A summary of the chemical classes of secondary metabolites produced by *Sporobolomyces* and *Rhodotorula* species was compiled. All of the compound classes identified and the specific compounds identified are not limited in their occurrence to *Sporobolomyces* and *Rhodotorula*. Compound classes reported in various *Sporobolomyces* species include the following: alkaloids (spermidine, single report; no additional alkaloids reported), aromatic acids (benzoic acid), carotenoids (β-carotene), organosulfur compounds (methanethiol), proteins (glucosidase-like BglA enzyme), quinoids (coenzyme Q10), short-chain aliphatic acids (butyric acid, valeric acid), short-chain aliphatic alcohols (isoamyl alcohol), and short-chain aliphatic lactones (γ-decalactone, δ-decalactone). Additional chemical classes reported in *Rhodotorula*, but not in *Sporobolomyces*, include: amino acids (L-lysine), benzenoids (guaiacol), carbohydrates (mannan), lipids (linoleic acid), phenylpropanoids (*trans*-ferulic acid), sterols (ergosterol) and vitamins (biotin). The compound classes listed for *Rhodotorula* may or may not also be produced by *Sporobolomyces* species. However, as no compounds of toxicological concern were noted from the searches of both *Sporobolomyces* and *Rhodotorula*, this

information helps establish the non-toxigenicity of *Sporobolomyces singularis* YIT 10047 for use as a processing aid in the production of Oligomate 55N/55NP GOS.

Individual NAPRALERT^{SM} searches were carried out to determine if there were a reported occurrence of the following known mycotoxins in the *Sporobolomyces* and *Bullera* (an older name for the *Sporobolomyces* genus) genera: citrinin, ochratoxins, cytochalasins, penitrem, patulin, penicillic acid, sterigmatocystin, and gliotoxin. No hits were returned for any of these mycotoxins. However, it was unknown whether *Sporobolomyces singularis* possessed the biosynthetic potential to produce any mycotoxins under special conditions (*i.e.*, induction under stress). Therefore, a list of mycotoxins was generated based on mycotoxins produced by certain *Fusarium*, *Aspergillus*, *Penicillium*, and other fungal species; these are listed in Table V-1. The mycotoxins selected for testing were chosen to represent a wide range of biosynthetic enzymatic pathways that might be present, but are as yet uncharacterized, in *S. singularis*. For example, citrinin is a known toxin found in contaminated "red yeast rice" cultured using *Monascus* spp., the ochratoxins are produced by *Aspergillus* and *Penicillium* spp, aflatoxins are made by *Aspergillus* spp., patulin is synthesized by *Acremonium*, *Aspergillus*, *Penicillium*, and other species, sterigmatocystin is made by *Aspergillus*, *Bipolaris*, and *Monocillium* spp., and nivalenol, deoxynivalenol, the fumonisins, and zearalenone are produced by *Fusarium* spp. Multiple lots of the *S. singularis* yeast concentrate used in the production of Oligomate 55N/55NP were tested for the presence of these mycotoxins, and none was detected at the respective analytical limits.

Table V-1. Mycotoxins absent in *Sporobolomyces singularis* YC-Y yeast concentrate

Compound Name	Detected (Y/N)	Limit of Detection
Citrinin	N	0.1 ppm
Ochratoxin A	N	0.5 ppb
Aflatoxins B_1, B_2, G_1, G_2	N	0.5 ppb each
Patulin	N	0.01 ppm
Sterigmatocystin	N	20 ppb
Nivalenol, deoxynivalenol (vomitoxin)	N	0.2 ppm each
Zearalenone	N	50 ppb
Fumonisins B_1, B_2	N	0.1 ppm each

3. Enzymatic Activity of *Sporobolomyces singularis* (parent strain)

In 1962, it was reported that *Sporobolomyces singularis* could utilize glucose, lactose and cellobiose, but not galactose, for growth (Phaff and do Carmo-Sousa 1962), implying that the organism contains a β-glucosidase and/or a β-galactosidase. When *S. singularis* is grown in the presence of 1% lactose, a new reducing sugar (a trisaccharide) was formed, suggesting that *S. singularis* carries out a transgalactosylation reaction wherein the glucose is utilized for energy by the organism while the remaining galactose is transferred to excess lactose in the medium, creating a novel oligosaccharide(s) (Phaff and do Carmo-Sousa 1962). It is now recognized that GOS can be synthesized from either β-galactosidase, β-glucosidase, or organisms harboring these enzymes (Sakai et al. 2008). Later reports on *S. singularis* describe the isolation of an enzyme that is characterized as having either β-hexosidase (Blakely et al. 1969; these workers mentioned a dual β-galactosidase/β-glucosidase ability by the same enzyme) or β-galactosidase activity (Cho et al. 2003; these researchers noted that the activity is inhibited by sodium dodecylsulfate (SDS), suggesting that the active enzyme may be a dimer). Phaff and do Carmo-Sousa did not detect any lactase activity in the culture medium during their feeding experiments, indicating that any lactase present is either not soluble (sequestered intracellularly) or may be bound to the cell membrane. A more recent study has demonstrated that a β-galactosidase-like activity is concentrated in the cell debris isolated from crude cellular preparations of *S. singularis* 7B6, a mutant of the parent organism (*Sporobolomyces singularis* Phaff & Carmo-Sousa, ATCC 24193) (Ishikawa et al. 2005), corroborating the earlier observation that the enzyme activity is localized to the yeast cell wall.

Gorin and co-workers (Gorin et al. 1964a and *ibid* 1964b) reported that *Sporobolomyces singularis* could synthesize tri- and tetrasaccharides containing galactose and glucose reducing end-units as main products, suggesting the presence of either a β-glucosidase and a β-galactosidase, or one enzyme capable of utilizing both glucose and galactose/lactose as substrates. They also noted that the β-galactosidase was associated with the cell wall since culture filtrates did not harbor the activity. In their report on the preparation of reaction products from numerous substrates, the authors noted that disaccharides and trisaccharides were formed that contained β1→4 linkages. Control of the pH was found to be essential for the production of oligosaccharides from *S. singularis*, as no oligosaccharides were produced at pH ≥ 4.0. In their research on the structural elucidation of the specific oligosaccharides formed by *S. singularis*, Gorin and co-workers also identify *O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-D-glucose and *O*-D-galactopyranosyl-(1→4)-*O*--D-galactopyranosyl-(1→4)-*O*-D-galactopyranosyl-(1→4)-D-glucose (with specific rotation suggestive of β-linkages). These

compounds are two of the specific trisaccharides and tetrasaccharides found in Oligomate 55N/55NP.

Shin and Yang (Shin & Yang 1998) partially purified a β-galactosidase from *Bullera singularis* ATCC 24193 (an older name for *Sporobolomyces singularis*; note the identical ATCC number) and optimized GOS production to a maximum yield of 40% (w/w) from 300 g lactose/liter medium at 45 °C, pH 3.7, when the lactose conversion was 70%. Under these conditions, the major trisaccharide was proposed to have the structure *O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucose, the major trisaccharide of Oligomate 55N/55NP. Temperatures above 50 °C significantly inhibited the enzymatic reaction and the formation of GOS was subject to feedback inhibition generated by the production of galactose and glucose during the reaction. Trisaccharides were produced in greater amounts than disaccharides for this enzyme preparation. The weight percent yield of GOS did not increase with increasing initial concentrations of lactose, which was itself limited by the solubility of lactose at the reaction temperature of 45 °C. The β-galactosidase was tolerant of a range of pH values (3.5-6.0) and was insensitive to the presence of sodium and potassium salts. Productivity was reported as 4.8 g/L•h with a GOS yield of 40% (w/w).

Shin and co-workers (Shin et al. 1998; same group as above) also immobilized the *Bullera singularis* β-galactosidase on chitosan beads to compare the continuous production of GOS versus the standard batch reaction format. They noted that the maximum hydrolysis of the colorimetric substrate used to measure β-galactosidase activity, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), was observed at 50 °C for both production formats. Productivity was measured at 4.8 g GOS/L•h, with 54% yield for the batch production and 4.4 g GOS/L•h, with 55% yield for the continuous production. In the continuous production, the major oligosaccharides produced were a disaccharide and a trisaccharide, presumably the ones identified by Gorin and co-workers (Gorin et al. 1964a and *ibid* 1964b). The yield and composition of oligosaccharides produced during the continuous reaction and the batch reaction were nearly the same, however, and the type of oligosaccharides produced were not altered by the immobilized enzyme format. Therefore, immobilizing the yeast cells is not necessary from a GOS manufacturing perspective. However, Shin and co-workers (Shin et al. 1998) did mention that use of an enzyme having high activity and stability was a critical factor for the future optimization of GOS production by the *Bullera* (*Sporobolomyces*) *singularis* enzyme. This issue may have led other researchers to attempt to generate *S. singularis* mutants having higher β-galactosidase enzyme activity (see next section).

4. Enzymatic Activity of *Sporobolomyces singularis* 7B6 or YIT 10047

Sporobolomyces singularis YIT 10047 (Sakai et al. 2008) is the specific organism utilized in the production of Oligomate 55N/55NP GOS, and this yeast is identical to *S. singularis* 7B6 (Ishikawa et al. 2005), a mutagen-induced mutant that over-produces a β-galactosidase-like enzyme that catalyzes the formation of GOS from lactose in high yield versus the native enzyme found in the parent organism *Sporobolomyces singularis* Phaff & Carma-Sousa (ATCC 24193).

Mutagen-induced 2-deoxy-D-glucose-resistant, lactose-utilizing mutants of *Sporobolomyces singularis* ATCC 24193 have been isolated that have enhanced cellular β-galactosidase-like activity relative to the parent strain (Ishikawa et al. 2005), further supporting this organism's utility in the commercial production of GOS. Ishikawa and co-workers carried out experiments to characterize the substrate preference of the purified β-galactosidase-like enzyme from the selected mutant strain *S. singularis* 7B6. They also directly compared the substrate specificity of crude cellular β-galactosidase-like activities in the *Sporobolomyces singularis* parent strain and in selected mutants. In the case of the *Sporobolomyces singularis* 7B6 mutant, its purified β-galactosidase-like enzyme was discovered, via matching the deduced amino acid sequence to that of known enzymes, to actually be a β-glucosidase that exhibited high transgalactosidase activity. The gene coding for the enzyme was named *bglA* and contains 18 introns, 19 exons, and produces a product of 594 amino acids. The BglA protein exhibited 35% sequence identity and 50% sequence similarity to known plant β-glucosidases in the family 1 glycosyl hydrolases, excepting its unique 110-amino-acid N-terminal sequence. This additional protein sequence has 30% identity to the cell wall-bound BglB β-glucosidase of *Candida wickerhamii*, which also contains a unique 100-130 amino-acid N-terminal sequence and is of similar molecular weight as the reported BglA enzyme (Ishikawa et al. 2005). Ishikawa and co-workers found that the BglA enzyme had a molecular weight of 73.9 kDa on sodium dodecyl sulfate polyacrylamide gel (denaturing gel) and 146 kDa by gel filtration (native conditions), indicating it is likely a homodimer.

In the Ishikawa study, the crude cellular β-galactosidase-like enzymes for both the parent strain and *Sporobolomyces singularis* 7B6 were evaluated for their substrate specificity by spectrophotometrically measuring the release of colored *o*-nitrophenol or *p*-nitrophenol from the substrates *o*-nitrophenyl-β-D-galactopyranoside (for galactose) and *p*-nitrophenyl-β-D-glucopyranoside (for glucose), respectively, in the presence of the enzymes. The authors demonstrated that the cellular enzyme found in the parent yeast strain mainly utilized lactose as a substrate (2.15 units β-galactosidase-like activity per gram wet cell weight), versus glucose (0.79

U/g), but exhibited lower overall activity, when compared with the high-activity, low-specificity 7B6 mutant cellular enzyme that could use either lactose or glucose (7.99 and 7.57 U/g, respectively) as a substrate (Ishikawa et al. 2005).

Ishikawa and co-workers reported that the BglA enzyme is insensitive to catabolite repression, meaning it is not easily switched off by feedback from the increasing levels of GOS or other lactose hydrolysis products. Additionally, the cellular β-galactosidase-like activity is 10-fold higher than that observed for the parent organism. After a three-step purification procedure, the purified *Sporobolomyces singularis* 7B6 BglA enzyme was isolated in 14.1% yield, had 8.69 units β-galactosidase activity per mg protein, and was found to have a molecular weight of approximately 146 kDa (gel filtration)/73.9 kDa (SDS-PAGE), indicating it consists of two similar or identical subunits (Ishikawa et al. 2005). The 7B6 BglA enzyme had a K_m value of 5.40 mM for the substrate ONP-Gal (measuring galactose turnover) and a V_{max} = 3.07 μmol/min per mg protein. Disaccharides and trisaccharides were the main products formed from lactose by the purified BglA enzyme after 48 h at 40°C at pH 6.0. Much lower amounts of tetra-, penta-, and hexasaccharides were formed in comparison to the shorter oligomers (Ishikawa et al. 2005).

Reverse transcription-polymerase chain reaction (RT-PCR) experiments by Ishikawa and co-workers indicated that the *bglA* gene from 7B6 cells is expressed at much higher levels than expression levels observed for the parent strain, when grown in both lactose and glucose media (Ishikawa et al. 2005). This result supports the hypothesis that production of the BglA enzyme is constitutive and insensitive to catabolite repression. Ishikawa and co-workers could not detect any mutations in the *bglA* gene of the 7B6 mutant, compared with the same gene in the parent organism, suggesting that the alteration in enzyme regulation occurs at the level of a transcription-regulating factor or transcription enhancer.

Sakai and co-workers carried out additional experiments on the organism *Sporobolomyces singularis* 7B6, re-naming it *S. singularis* YIT 10047, and deposited the strain in the International Patent Organism Depositary (IPOD, Tsukuba, Japan) as *S. singularis* FERM P-18817 (Sakai et al. 2008). These researchers immobilized the *S. singularis* YIT 10047 onto a calcium chloride-stabilized sodium alginate gel for the purpose of carrying out repeated-batch synthesis of GOS from lactose. They found that the GOS yield can be increased by increasing the concentration of lactose in the yeast culture medium, but this effect is limited by the tendency of lactose to crystallize out of solution at both high concentrations and low temperatures (Sakai et al. 2008). The enzyme activity of the crude cells, when subjected to a temperature increase prior to their reaction with lactose, was stable up to temperatures of 50°C preincubation in acetate buffer, pH 5.5. Sakai and co-workers also showed that the cellular enzyme exhibited

similar temperature stability and time courses for GOS production in both the free and immobilized cell formats, indicating that immobilization of the cells does not compromise their ability to enzymatically convert lactose to GOS. This observation was also noted for the enzyme derived from the parent organism (Shin et al. 1998). Nearly 60% lactose conversion plus greater than 40% GOS production (as percent of total saccharides) could be achieved in either free culture or immobilized cell format. For a single-batch reaction, at a given ratio of immobilized cells to grams of lactose present in the medium, it was observed that lactose conversion was driven by increasing temperature over the range of 55-65°C. However, this trend did not hold for subsequent repeated-batch reactions, and the GOS yields rapidly declined for repeated reactions carried out at the higher temperatures of 65 and 60°C. However, GOS production appeared stable for repeated-batch reactions carried out with immobilized *S. singularis* YIT 10047 at 55°C, 600 g lactose/L and pH 6.0. In a separate experiment, it was demonstrated that GOS production was most stable and reproducible over repeated-batch production at pH 5.0, 55°C and 600 g lactose/L (Sakai et al. 2008). This study indicates that GOS can readily be produced in high yield from lactose by *S. singularis* YIT 10047 at 55°C, pH 5.0-6.0, with 600 g lactose/L, and that immobilizing the yeast cells is not necessary from a GOS manufacturing perspective.

5. Safety Studies on *Sporobolomyces singularis* YIT 10047 ("YC-Y") Yeast Concentrate

Repeated-dose 90-day oral toxicity study (021B201)

Bullera singularis YIT 10047 (also known as *Sporobolomyces singularis*) cell concentrate was suspended in saline and dosed for 90 days orally via gastric tube in rats in order to test the safety of the yeast concentrate (Kaneko, Study Number 021B201). The undiluted yeast concentrate concentration was standardized to contain at least 2500 mU/mL of β-galactosidase activity using o-nitrophenyl-β-D-galactopyranoside as a substrate. Four-week old Crj:CD (SD) IGS rats were acclimatized for two weeks. After the quarantine period, forty males (189.9-220.0 g body weight) and forty females (130.9-155.3 g body weight) showing normal and healthy development were selected for the study. Rats were divided into four groups of 10 males and 10 females each and received either 10 mL of undiluted yeast extract per kg body weight, 1 mL yeast extract/kg bw or 0.1 mL yeast extract/kg bw, made up in 10 mL total volume per dose per kg body weight. A saline (Japanese Pharmacopoeia Saline Solution, Fuso Pharmaceutical Industries, Ltd., Osaka, Japan) control group was also included in the study. Body weights were recorded weekly, individual feed consumption was determined during a three-day period each week, and the general condition of the rats was observed daily. Tests carried out include the following: gross and fundus examinations at days -8 and 87, urinalysis on days 86-87,

hematology at the study's end, serum biochemistry, plus gross examination and organ weights on all animals, and histopathology on the high-dose and saline groups.

There were no inter-group differences in body weight gain and feed consumption. Compared with the saline control group, the high-dose females had a statistically significant decrease in renal sodium excretion. However, no changes in plasma sodium or electrolytes or kidney-related serum biochemistry or pathological changes accompanied this observation in the high-dose group, suggesting that the decreased renal sodium excretion was toxicologically insignificant. Regarding the urinalysis results, no other differences were noted between treatment groups and the control group. Mean corpuscular hemoglobin concentrations were statistically significantly higher in the high-dose females versus saline control females. However, this result was judged to be incidental because no statistically significant changes were observed in hemoglobin concentration or red blood cell count. Platelet counts were statistically significantly higher in the middle- and high-dose females versus control females, but histopathologic examination of bone marrow in the high-dose and control groups revealed no differences between these groups. When compared with historical data on the CD(SD)IGS rats, the saline control group had relatively low platelet counts, suggesting that the elevated platelet counts in the middle- and high-dose females were incidental. There was a statistically significant increase in relative cardiac weight for the high-dose female group versus controls that was not associated with a change in either the absolute organ weight or the mean group body weight. Statistically significantly lower relative spleen weight was reported for the middle-dose male group versus control males. This change in relative splenic weight was not associated with a change in either the absolute organ weight or the mean group body weight. There were no other treatment-related changes in the high-dose group compared with the control group as regards gross observations and histopathologic examinations.

When administered orally for 90 days in rats, the *Sporobolomyces singularis* YIT 10047 extract did not induce any relevant changes versus saline controls. The no observed adverse effect level (NOAEL) in rats for this study was considered to be greater than 10 mL yeast extract (standardized to $\geq$ 2500 mU/mL of β-galactosidase activity) per kg body weight.

Bacterial reverse mutation assays (021D701)

Yeast extract from *Sporobolomyces singularis* (described as *Bullera singularis* YIT 10047) was tested for its ability to induce mutations in the Ames *Salmonella typhimurium* reverse mutation assay conducted in the presence and absence of metabolic activation (rat liver S9 mix plus cofactors) (Yasutake, Study Number 021D701). *S. typhimurium* strains TA 1535,

TA1537, TA98 and TA100 and *Escherichia coli* WP2*uvrA* were used. The yeast concentrate tested had a standardized β-galactosidase activity of ≥ 2500 mU/mL as measured by release of *o*-nitrophenol from *o*-nitrophenyl-β-D-galactopyranoside. When tested in an initial dose-finding assay at 1.2, 3.7, 11.1, 33.3 and 100 µl yeast concentrate/plate, the *S. singularis* extract did not increase the number of revertants, measured as number of colonies per plate, by greater than two-fold over the number of revertants observed for the solvent control (distilled water), for any of the five bacterial strains tested and no dose- response effects were observed. At the highest concentration of 100 µl yeast concentrate per plate, in the absence of metabolic activation the number of colonies was slightly less than the number of colonies that was observed for the solvent control for *S. typhimurium* strains TA98, TA100, and TA1535. In plates containing S9 mix (+ metabolic activation), only the *S. typhimurium* strain TA98 exhibited reduced numbers of colonies, versus the negative control, when treated with high concentrations of the yeast concentrate. These results indicated that, at the highest concentration, the *Sporobolomyces singularis* yeast concentrate may exhibit some growth inhibition of some of the test strains and that 100 µl/plate was the maximum dose appropriate for use in the definitive test to avoid excessive cytotoxicity.

The definitive test used a separate lot of the standardized *Sporobolomyces singularis* YIT 10047 yeast extract at test concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 µl per plate and yielded similar results as the first test. No bacterial strains had more than a two-fold increase in revertants relative to the negative control group. No dose-response effects were observed, except for a reduction in the number of colonies at the 100 µl dose for *Salmonella typhimurium* strains TA98, TA 1535, and TA1537 in the absence of S9 mix, again suggesting possible growth inhibition of some of the test strains at this dose.

In both tests, a positive result was defined as a substance reproducibly eliciting more than twice the number of revertant colonies observed for the negative control, and exhibiting a dose-response increase. All positive control substances (2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, sodium azide, 9-aminoacridine, 2-aminoanthracene, benzo[a]pyrene) were required to produce significant increases in mutant frequencies >2-fold for tests to be valid.

Reticulocyte micronucleus formation and bone marrow cytotoxicity (021D101)

Yeast extract from *Sporobolomyces singularis* (*Bullera singularis* YIT 10047) was tested for its ability to produce chromosome damage by determination of incidence of micronuclei in reticulocytes and for cytotoxicity by suppression of bone marrow proliferation in mice after a single oral administration of the test article (Ohyama, Study Number 021D101). Eight-week old

male Crj:CD-1 (ICR) mice were received and acclimated for 8 d, after which thirty mice were randomized into five groups of six mice each. Treatment and solvent control groups were fasted for 18 h prior to treatment. A fifth group was not fasted and was administered the positive control test article (mitomycin C) via intraperitioneal injection. Test article was administered via oral gavage to three groups at 20, 6.7 or 2.2 mL yeast concentrate/kg body weight (standardized to ≥ 2500 units/mL of β-galactosidase activity). The negative control group received saline at 20 mL/kg via gavage. The volume administered was 20 mL/kg across all dosed groups. The positive control article was 1 mg mitomycin C in a dose volume of 10 mL/kg, given as a single intraperitoneal injection.

Peripheral blood was collected from the tail vein at 48 and 72 h post-administration of the test and control articles, and specimens were prepared for fluorescent microscopic determination of numbers of micronucleated reticulocytes in the blood of the dosed and control animals. Two thousand cells were observed per animal. Body weight was measured on the day of administration and on days 2, 3, and 4 afterwards. Animals were observed immediately after administration, followed by 1, 2, 4, and 6 h post-treatment and then once a day thereafter until the end of the study.

No changes in clinical signs were observed across all groups and the mice receiving yeast concentrate exhibited good body weight increase over the course of the study. A few animals in both the test article and negative control groups exhibited sporadic body weight decreases of 0.1-0.8 g, but these were not persistent and thought to be incidental. Body weights of all test article and negative control groups were similar throughout the study. Body weight was significantly but transiently increased at day 1 post-administration in the positive control group versus the test article and negative control groups.

Mean micronucleus (MN) frequencies in reticulocytes were 0.10% (2.2 mL of yeast concentrate/kg body weight), 0.13% (6.7 mL yeast concentrate/kg bw), and 0.16% (20 mL yeast concentrate/kg bw) at 48 h post-administration of the *S. singularis* yeast concentrate. MN frequencies were 0.15%, 0.16%, and 0.15%, respectively, at 72 h post-administration of test article. These values were not significantly different from the saline control whose MN frequencies were 0.13% and 0.16% at 48 and 72 h, respectively. The incidences of MN in the positive control group were 2.71% and 0.65% at 48 and 72 h post-administration of mitomycin C. The results indicated that yeast extract from *S. singularis* did not produce chromosome damage in mice under the conditions of this test.

There was no indication that yeast extract from *S.* singularis was cytotoxic to bone marrow by determination of reticulocyte versus total erythrocytes ratios. Mean ratios of reticulocytes:total erythrocytes for respective assessments at 48 and 72 hr post-administration were 3.28% and 3.65% for the negative control group; 2.47% and 3.45% at 2.2 mL of yeast concentrate/kg body weight; 2.77% and 4.20% at 6.7 mL of yeast concentrate/kg bw; and 3.03% and 3.84% at 20 mL of yeast concentrate/kg bw. Mean values in the positive control group were 1.34% and 1.03% at 48 and 72 h post-administration. The negative control and test article group results were not statistically different from one another.

Stability of the test article

Because of its use as a processing aid in the production of galacto-oligosaccharides from lactose, the test material was standardized based on commercial product specifications of β-galactosidase activity. Activity and stability tests were performed to ensure that the different lots of test material met similar specifications. β-galactosidase activity of the yeast extract was measured over time using *o*-nitrophenyl-β-D-galactopyranoside as the colorimetric product-generating substrate. For the two lots of yeast extract used in the mutagenicity tests, enzymatic stability was studied over approximately a one-month period and a loss of 3.4% and 3.5% of activity was observed for each lot, respectively. On the day of mutagenicity testing, the β-galactosidase activity of each dose of test article was determined and compared to the theoretical activity calculated from the dilution scheme. For each of the two lots, the actual enzyme activity of the test article was within 10% of the expected enzyme activity, confirming that test article samples were prepared correctly. Additionally, the suspension homogeneity and stability of 10-fold and 100-fold yeast extract dilutions over 4 h at room temperature and 7 d under refrigeration were tested and the yeast extract was found to be homogenous and within acceptable limits (-6.4% and 4.7%, respectively; criteria was ≤ 10% loss) for activity loss under these storage conditions. For the reticulocyte micronucleus and bone marrow cytotoxicity tests, the specific lot of test article declined only 3.8% in activity over approximately one month, which is in good agreement with results obtained from the two lots (Lots 020806 and 020924) used in mutagenicity testing. The *Sporobolomyces singularis* YIT 10047 ("YC-Y") yeast extract exhibited stable β-galactosidase activity over the course of testing in various genotoxicity assays, and enzymatic activity levels were reproducible from lot-to-lot.

6. Use of *Sporobolomyces singularis* YIT 10047 in the Production of Oligomate 55N/55NP GOS

Sporobolomyces singularis YIT 10047 has been used by Yakult in the production of Oligomate 55N/55NP since 2003 and is the main processing aid used to produce galacto-

oligosaccharides from food-grade lactose. This strain is prevented from undergoing spontaneous mutation or any other change across multiple lots by the use of individual stock vials of the strain which are stored frozen at -80 °C. Frozen vials are thawed for one-time use in each production lot and continuous passages of the organism are not kept in culture in order to prevent mutations or other changes in the strain and to maintain its quality. During production of Oligomate 55N/55NP GOS, multiple levels of processing controls are in place to prevent carry-over of the organism or any of its unwanted metabolites into the final product. These include, but may not be limited to, the use of a heat inactivation step after the initial enzymatic GOS production by *S. singularis* YIT 10047, multiple downstream filtration steps that remove particulate matter down to the cellular level, the concentration of the final product to a syrup form that has a water activity level sufficiently low to prevent microbial contamination and growth, plus an antimicrobial treatment step prior to final packaging of the GOS syrup or its conversion to powder form. Additionally, the use of multiple ion-exchange resins during the production process is expected to prevent the carry-over of any unwanted yeast metabolites into the final product.

B. SAFETY OF THE KLUYVEROMYCES LACTIS PURIFIED BETA-GALACTOSIDASE ENZYME

The lactase (IUB No. 3.2.1.23) enzyme preparation derived from *Kluyveromyces lactis* that is used in the production of Oligomate 55N/55NP GOS for the purpose of degrading redundant lactose which caused product crystallization has FDA GRAS (Generally Regarded As Safe) status under 21CFR §184.1388. It is listed under FDA's Partial List of Enzyme Preparations that are Used in Foods found at www.fda.gov/Food/FoodIngredientsPackaging/ucm084292.htm (accessed 12-09-09). The specific *K. lactis* lactase used is called GODO-YNL, a food-grade enzyme preparation containing lactase and glycerin that is manufactured by Godo Shusei Co., Ltd., 250, Nakahara, Kamihongo, Matsudo, Chiba, 271-0064 Japan, Phone: 81-47-362-1260, Fax: 81-47-362-1263.

C. GOS PRODUCT PROFILES

Over the past twenty years, different methods have been utilized for production of GOS products; the consequence of these process changes is a difference in the final product with respect to the profile of the oligosaccharide polymers and the position of the chemical bonds between the saccharide units. These chemical differences could have potential implications for the physiological activity of the final product, and are important to understand for purposes of bridging studies done with different products to establish safety, including physiological activity, of Oligomate 55N/55NP. Figure 5 details the oligosaccharide profile composition of various

GOS products reported in the scientific literature. The figure contains data as reported by the study authors, and was not normalized to oligosaccharide content based on wet or dry weight of GOS syrups versus powders. It provides a perspective on the oligosaccharide profiles and chemical linkages comprising the various GOS products, for the purpose of understanding how Oligomate 55N/55NP is chemically related to other GOS products.

1. Organisms and/or Their Enzymes Utilized in the Synthesis of GOS

Studies retrieved from the public literature for GOS products have reported production using various organisms, including: *Bacillus circulans* (Chonan et al. 1995, *ibid* 1996, *ibid* 2001), *Cryptococcus laurentii* (Sako et al. 1999), *Streptococcus thermophilus* (Ito et al. 1993), *Aspergillus oryzae* (Tanaka et al. 1983; Chonan & Watanuki 1995; Bouhnik et al. 1997), *Bifidobacterium bifidum* (Tzortzis et al. 2005), *Sporobolomyces singularis* YIT 10047/*Kluyveromyces lactis* (current product; Matsumoto et al. 2004), plus various combinations of these organisms (Kikuchi et al. 1996; Chonan & Watanuki 1996; Kikuchi-Hayakawa et al. 1997). When cultured in the presence of lactose as the starting material, the resulting GOS consists mainly of 4'-type linkages or 6'-type linkages, depending on the specific enzymes and organisms utilized. The β-linkage, compared to an α-linkage, confers gastrointestinal stability to the GOS products (Kanai & Matsumoto 2009) because the α-amylase produced by humans is capable of only breaking down α1→4 glycosidic bonds. Therefore, GOS passes undigested through the gastrointestinal tract until it reaches the colon, where bacterial β-glycosidases can act on it. 4'-GOS is the predominant linkage produced from lactose by *Bacillus circulans* or *Cryptococcus laurentii*, while *Aspergillus oryzae* and/or *Streptococcus thermophilus* (Sako et al. 1999) will produce mainly 6'-GOS. Oligomate 55N/55NP, produced by *Sporobolomyces singularis* YIT 10047 and *Kluyveromyces lactis* β-galactosidase, is a mixture of 4'-GOS and 6'-GOS. *S. singularis* has been reported to form mainly 4'-GOS (Gorin et al. 1964). From the perspective of chemical linkage, β-linked GOS products are handled similarly by the gastrointestinal tract. The β-linkages between the saccharide units are resistant to digestion by α-amylase and the GOS passes undigested into the colon where it can be fermented.

2. Saccharide Polymer Length Distribution in GOS Products

GOS products described in the literature can also be categorized with regard to their degree of oligomerization: 1) those with average chain length of one to four saccharides (monosaccharides to tetrasaccharides), 2) those with average chain length of two to five saccharides (disaccharides to pentasaccharides), and 3) those average chain length of three to five saccharides (trisaccharides to pentasaccharides). All three categories contain at least some trisaccharide content (Figure 5). Products falling within each of the three categories have been

reported to support the growth of Bifidobacteria in animals and humans. Trisaccharide GOS alone has been reported to support the growth of Bifidobacteria (Matsumoto et al. 2004). Linear oligosaccharides are broken down more efficiently by bacteria than branched oligosaccharides and are preferentially utilized; in addition Bifidobacteria are reported to utilize shorter chain oligosaccharides preferentially, while bacteroides primarily utilize GOS having longer chain lengths (Cummings et al. 2001). Oligomate 55N/55NP GOS falls within category 1, with its average saccharide chain length of 1 to 4 (monosaccharides to tetrasaccharides), and its GOS profile is comprised of nearly one third trisaccharides.

Oligomate 55N syrup contains 41.25% GOS content on a wet weight basis and is a mixture of 4'- and 6'-GOS, mainly in the form of monosaccharides (31% w/w), disaccharides (27.5%, including lactose), trisaccharides (34.4%), and a small amount of tetrasaccharides (7.2%) (25% moisture). Oligomate 55NP is the dried powder form of Oligomate 55N containing 53.55% GOS content on a dry weight basis (3% moisture). The powder has an identical oligosaccharide chemical profile as the syrup except for the moisture content. As illustrated in Figure 5, the oligosaccharide profile of Oligomate 55N/55NP is most similar to that of Oligomate 55 (Mwenya et al. 2005), Oligomate 55P (Kawakami et al. 2005), and Oligomate 50 (Ito et al. 1993), as well as Elix'or (Holma et al. 2002), Vivinal (Gonzalez et al. 2008), *Bifidobacterium bifidum*-produced GOS (Tzortzis et al. 2005), and Bi^{2}muno (3'-/4'-/6'-GOS: Vulevic et al. 2008).

Oligomate 55N/55NP, and other GOS products that fall within all three oligosaccharide profile categories have similar physiologic effects. They support the growth of *Bifidobacterium* species in animals and humans and decrease the intestinal or fecal pH in animals or in humans as a result of the increased production of short chain fatty acids (SCFA) from microbial fermentation. Both 4'-GOS and 6'-GOS are utilized for growth by the Bifidobacteria (Sako et al 1999). Many of the GOS formulations reported in the literature to exhibit bifidogenic activity in animals or humans are reported to contain 6'-GOS. *Bifidobacterium bifidum* (Tzortis et al. 2005) has been reported to produce 6'-GOS, suggesting that (at least some) Bifidobacteria may preferentially synthesize and/or utilize 6'-galacto-oligosaccharides as an energy source. Rabiu and co-workers indicate that Oligomate 55, which is chemically very similar to Oligomate 55N/55NP, exhibited a similar bacterial growth profile as that of BS-4, an oligosaccharide derived from *Bifidobacterium angulatum* (Rabiu et al. 2001). Oligomate 55N/55NP contains both 4'- and 6'-GOS and has been shown to significantly increase *Bifidobacterium* counts and the overall % Bifidobacteria in the total fecal microflora population in humans at doses of 2.5 and 5.0 g GOS/day, with a greater effect observed at the 5.0 g/d level (Matsumoto et al. 2004). GOS also inhibits attachment of certain strains of *Escherichia coli* and *Salmonella* spp. to HT29

cells *in vitro* (Tzortzis et al. 2005). Oligomate 55N/55NP is expected to produce similar physiologic effects and have a similar safety profile to other GOS products due to equivalent gastrointestinal handling and fate (refer to Section V.D. Safety of GOS).

Figure 5. GOS Product Profiles

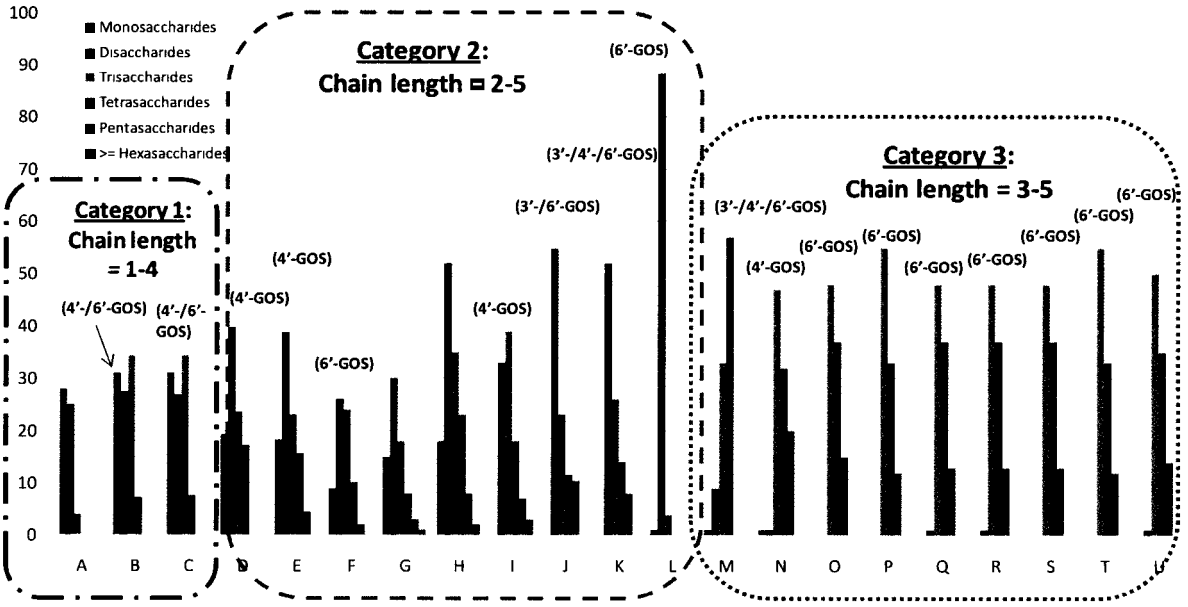


Figure 5. Graphical representation of the oligosaccharide content of various galacto-oligosaccharide (GOS) products. A: Valio product (organism not reported), Holma 2002; B: Yakult's Oligomate 55N/55NP (*Sporobolomyces singularis/Kluyveromyces lactis*), Matsumoto 2004; C: Yakult's Oligomate 55N (organism not reported), Kimura 2004; D: Yakult's Oligomate 55P (organism not reported), Kawakami 2005; E: Yakult's Oligomate 55 (organism not reported), Mwenya 2005; F: Yakult's Oligomate 50 (organism not reported), Ito 1993; G: Elix'or (organism not reported), Holma 2002; H: Elix'or (organism not reported), Alles 1999; I: Vivinal (*Bacillus circulans*), Gonzalez 2008, GRN 236; J: *Bifidobacterium bifidum* GOS, Tzortzis 2005; K: Bi^{2}muno (organism not reported), Vulevic 2008; L: *Streptococcus thermophilus* GOS, Ito 1993; M: Unspecified GOS (organism not reported), Djouzi & Andrieux 1997; N: Yakult *Bacillus circulans* GOS, Chonan 1995, *ibid* 1996, *ibid* 2001; O: *Aspergillus oryzae* GOS, Bouhnik 1997; P: Yakult *Aspergillus oryzae* GOS, Chonan & Watanuki 1995, Tanaka 1983; Q: Yakult product (organism not reported), Sakaguchi 1998, Djouzi & Andrieux 1997; R: Yakult *Aspergillus oryzae/Streptococcus thermophilus* GOS, Kikuchi-Hayakawa 1997; S: *Aspergillus oryzae/Streptococcus thermophilus* GOS, Kikuchi 1996; T: *Aspergillus oryzae/Streptococcus thermophilus* GOS, Chonan & Watanuki 1996; U: Yakult product (organism not reported), Rowland & Tanaka 1993.

D. SAFETY OF GOS

1. Introduction

GOS products that contain only β-linkages, including Oligomate 55N/55NP, are not digested by endogenous enzymes. As a consequence of this lack of digestion, various GOS products produce similar physical and physiological effects on the gastrointestinal system such as increases in colonic microflora fermentation, reduction of intestinal and fecal pH, changes in fecal characteristics and frequency, increased cecal tissue weight, flatulence, borborygmi, and abdominal distension. GOS products of varying chain lengths have also been shown to be fermented in the colon. As a result of fermentiaton, an increase in the numbers of Bifidobacteria, Lactobacilli and other beneficial bacteria in intestinal contents or feces have been measured. The increase in microflora results in increasing activity levels of bacterial-related β-galactosidases and β-glucosidases, changes in the concentrations of short-chain fatty acids (as an indirect measurement of microbial metabolic activity), and an increase in breath hydrogen concentration. GOS ingestion has also produced decreases in the numbers of specific pathogens, and decreases in adherence and invasion of host cells by pathogenic bacteria. In addition, systemic effects produced by GOS in some studies of animals and in human trials include positive changes in mineral balance (*i.e.*, calcium, magnesium, and phosphorus), beneficial effects on plasma levels of cholesterol, increased production of soluble immunoglobulin A (IgA), modulation of systemic cytokine levels and immune cell responses toward a less inflammatory state, increased numbers of immune cells that could phagocytose invading pathogens, and decreased incidence of atopic disease in at-risk subjects.

Genotoxicity and subchronic rodent toxicology studies of Oligomate 55N/55NP document safety of intake at the proposed intake levels. Oligomate 55N is not genotoxic and the no observed adverse effect (NOAEL) level was determined to be ≥ 2000 mg Oligomate 55N syrup/kg/d (equivalent to 825 mg GOS/kg/d) in a subchronic rat study (Kobayashi et al. 2009). *In vitro* experiments, plus subchronic toxicology studies and other animal studies of essentially equivalent GOS products at levels up to 2250 mg GOS/kg/d (Anthony et al. 2006) or 10% GOS in the diet (Pérez-Conesa et al. 2007, *ibid.* 2006a, *ibid.* 2006b, Sakaguchi et al. 1998, Kikuchi et al. 1996, Hayashi et al. 1991, Ohtsuka et al. 1990) corroborate safety of ingestion.

Clinical trials, including studies using Oligomate 55N/55NP, in healthy and compromised adults and preterm and term infants substantiate safety of GOS products. Endpoints reported from single or multiple dose adult studies of GOS (from 2.4-40.1 g GOS/d) include breath hydrogen levels, fecal frequency and characteristics, flatus/flatulence and abdominal distention

occurrence, laxative threshold, dose for diarrhea onset in 50% of subjects, total anaerobes, *Bifidobacterium*, and *Lactobacillus* counts, cytokine and inflammatory marker levels, fecal SCFA concentration changes, reduction in infection occurrence in compromised patients, reduction in length of hospital stay and antibiotic use rate, body weight gain, changes in serum lipid levels, modulation of fecal bile acid content, calcium absorption, and glucose tolerance and insulin response. Safety parameters assessed in infant studies of GOS given at 0.1-0.8% w/v GOS (mainly in the form of GOS/FOS (9:1)) in formula or in doses up to 3 g GOS/d and 4.5 g GOS/FOS (9:1)/d include adverse and serious adverse events, food or infant formula intake, body weight and length, dropouts according to treatment, fecal microflora measurements, antibiotic treatment, infection and hospitalization rates, stool frequency and consistency, development of atopic disease, rates of regurgitation and/or colic, fecal SCFA content, reduction in fecal pH, calcium levels, and anthropometric measurements such as head circumference and sum of skin folds.

2. Summary of *In Vitro* Studies of GOS

In vitro studies, excluding genotoxicity studies of Oligomate 55N that are summarized in Section V3., are presented here and in Table V-2. Oligomate 55N 4'-/6'-GOS is an indigestible complex carbohydrate that resists digestion by human salivary enzymes, porcine pancreatic α-amylase, and artificial gastric juice (Chonan et al. 2004), due to its nonhydrolyzable β-linkages connecting the saccharide units. GOS is not absorbed in its native form to any appreciable extent and survives the human gastrointestinal tract until it reaches the colon, where it is acted upon by the local populations of gut flora and broken down for energy and growth. Incubating GOS with cultured fecal bacterial slurries *in vitro* results in an increased gas production (Hernot et al. 2009, Rycroft et al. 2001, 6'-GOS: Bouhnik et al. 1997, Kikuchi and Sakata 1992), and a decrease in the culture pH (Hernot et al. 2009, Rycroft et al. 2001). Because GOS is not absorbed in its native form, it is not distributed throughout the body. Once in the colon, however, it stimulates the growth of beneficial bacteria and is acted upon by the local gut flora population which rapidly catabolizes the GOS.

Oligomate 55N GOS was found to support growth of the following *Bifidobacterium* species in batch culture format: *B. adolescentis*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. infantis*, and *B. longum* (Matsumoto et al. 2004). Other GOS products that exhibit Bifidogenic activity *in vitro* include: Oligomate 55P (Vernazza et al. 2006), Oligomate 55 (Hopkins et al. 1998, Rycroft et al. 2001, Rabiu et al. 2001), oligolactose (McBain and Macfarlane 2001), *Aspergillus oryzae*-produced 6'-GOS (Tanaka et al. 1983), Vivinal (Hernot et al. 2009, Depeint et al. 2008), *Bifidobacterium* spp.-derived GOS (Rabiu et al. 2001) and other, uncharacterized GOS

formulations (3'-/4'-GOS: Ryu et al. 2007). Certain of these GOS formulations also support the growth of *Lactobacillus* spp. *in vitro: Bifidobacterium* spp.-derived GOS (Rabiu et al. 2001), Vivinal (Depeint et al. 2008, Hernot et al. 2009), *Aspergillus oryzae*-produced 6'-GOS (Tanaka et al. 1983), oligolactose (McBain and Macfarlane 2001), and Oligomate 55 (Makras et al. 2005, Rabiu et al. 2001). However, this activity is reported less frequently for GOS and generally tends to be weaker versus the ability of GOS to support the growth of *Bifidobacterium* spp.

When Oligomate 55N/55NP is separated into its disaccharide, trisaccharide, and tetrasaccharide components and given to various strains of bacteria, the longer chain length oligosaccharides support growth of fewer species of bacteria compared to the shorter oligosaccharides (Matsumoto et al. 2004). In this study, the disaccharide fraction was utilized by fifteen bacterial strains consisting of six *Bifidobacterium* species, seven *Lactobacillus* species, *Bacteroides vulgates*, *Clostridium perfringens*, *Enterococcus faecium*, *Streptococcus salivarius*, and *Escherichia coli*. This growth pattern was said to be similar to that of lactose, implying a broad spectrum of bacterial utilization of the disaccharides present in Oligomate 55N/55NP. The trisaccharide fraction supported growth of six *Bifidobacterium* species, three *Lactobacillus* species, *Bacteroides vulgates*, *Clostridium clostridiiforme*, *Bacteroides fragilis*, *Eubacterium rectale*, and *Clostridium perfringens*. The tetrasaccharide fraction was utilized by five *Bifidobacterium* species, *Bacteroides vulgates*, *Lactobacillus reuteri*, *Eubacterium rectale*, and *Clostridium perfringens*. Oligomate 55N/55NP is Bifidogenic, in that it supports the growth of numerous *Bifidobacterium* species, however, it is not completely selective for this group of bacteria.

A consequence of stimulating the growth of Bifidobacteria and other beneficial gut bacteria is the production of short-chain fatty acids, which are absorbed locally in the colon/cecum/rumen and serve as an energy source for colon cells (Macfarlane et al. 2008). When cultured with fecal bacteria, the products Vivinal (Hernot et al. 2009), Oligomate 55 (Rycroft et al. 2001), and an unnamed *Aspergillus oryzae*-produced 6'-GOS (Bouhnik et al. 1997) were catabolized into the short-chain fatty acids acetate, propionate, butyrate and lactate. 6'-GOS also increases the production of adenosine triphosphate (ATP), a cellular energy source, in culture (Bouhnik et al. 1997).

If present in high enough amounts at the appropriate time of exposure, GOS may also block local cellular binding sites in the GI tract from binding and invasion by pathogenic bacteria. When purified to 100% GOS content, Oligomate 55 was reported to inhibit adherence of enteropathogenic *Escherichia coli* to HEp-2 and Caco-2 cells *in vitro* and to inhibit bacterial autoaggregation (Shoaf et al. 2006). However, the autoaggregation effect was weak, as only

56.4% inhibition occurred at 16 mg GOS/mL. Additionally, the antiadherence effect was only effective when GOS was preincubated with the cells prior to the addition of the *E. coli*. Searle and co-workers found that 2 mM of Bimuno® GOS could reduce the invasion of mucus-secreting colon cells by *Salmonella* Typhimurium, and the effect was observed regardless of the order of addition of the GOS and bacteria (Searle et al. 2009). This suggests that there are differing inhibitory effects of GOS on specific strains of pathogenic bacteria.

Table V-2. Summary of *in vitro* GOS Studies

Reference	Objective/Test Article	Study Design	Effect
Oligomate 55N (current formulation for GRAS)			
Matsumoto et al. 2004	To investigate the prebiotic effect of GOS on 22 bacterial genera *in vitro*. Source: Oligomate 55N (4'- and 6'-GOS) *Formulation B, Category 1 (Figure 5)*	Batch culture of individual tester strains.	• Oligomate 55N syrup supported growth of all *Bifidum* strains tested: *B. adolescentis*, *B. Bifidum*, *B. Breve*, *B. catenulatum*, *B. infantis*, *B. longum*.
Chonan et al. 2004	To study the resistance of GOS to digestive enzymes. Source: Oligomate 55N (4'- and 6'-GOS)	*In vitro* incubation of purified saccharide fractions from GOS with various digestive enzymes.	• Neither the GOS syrup nor any of the purified saccharide fractions were digested by human salivary or porcine pancreatic α-amylase or artificial gastric juice. • Lactose and the disaccharide fraction were degraded by rat intestinal mucosal enzymes.
Oligomate 55P (older formulation)			
Vernazza et al. 2006	To compare carbohydrate substrate preferences of five strains of human-specific *Bifidobacterium*. Source: Oligomate 55P	Comparison of maximum growth rates of *Bifidobacterium* strains on each of twelve carbohydrate substrates added to broth media, as measured by optical density (spectrophotometry).	• Oligomate 55P supported good growth of the five *Bifidobacterium* strains tested: *B. adolescentis* DSM20083, *B. infantis* DSM 20088, *B. lactis* Bb12, *B. longum* 46, and *B. longum* DSM 20219.
Oligomate 55 (older formulation)			
Hopkins et al. 1998	To determine the abilities of seven bifidobacterial isolates to utilize 15 different carbohydrate sources (two GOS formulations, including Oligomate 55). Source: GOS (85% pure) & Oligomate 55 (55% pure).	Batch culture with bacterial growth determined by optical density (spectrophotometry).	• The GOS and Oligomate 55 preparations supported growth of all *Bifidobacterium* species tested. • Specific growth rates and total cell yields varied, when compared with the mono- and disaccharide constituents. • *Bifidobacterium infantis* demonstrated some selectivity for GOS and Oligomate 55 in comparison with galactose, glucose and lactose.

Table V-2. Summary of *in vitro* GOS Studies

Reference	Objective/Test Article	Study Design	Effect
Rycroft et al. 2001	To compare the fermentation properties of various commercial prebiotic oligosaccharide formulations in human fecal cultures. (various GOS formulations, including Oligomate 55) Source: Oligomate 55 (50% pure).	Batch culture with fluorescent *in situ* hybridization (FISH)-based measurement of bacterial genera composition and growth, short-chain fatty acid and lactate measurement via HPLC, and gas evolution (total volume) over time for each prebiotic substrate.	• Oligomate 55 produced statistically significant increases in lactate, acetate and propionate production and gas volume emission from fecal slurries after 24h incubation. • Oligomate 55 caused a statistically significant and selective increase in *Bifidobacteria* spp. at both 5 and 24h post-inoculation, but did not cause an increase in the total bacterial population count.
Rabiu et al. 2001	To test the ability of various oligosaccharides to support growth of the various human-specific *Bifidobacterium spp.* plus mixed populations of gut bacteria in comparison with Oligomate 55 as a control. Source: Oligomate 55	Synthesis of oligosaccharides by purified enzymes from specific *Bifidobacterium* spp. and batch culture experiments to measure the growth rate (optical density) of selected gut bacteria on various oligosaccharide substrates compared with Oligomate 55. Agar plate experiments to follow growth of selected bacterial genera.	• In pure culture experiments, Oligomate 55 supported the growth of seven *Bifidobacterium* species plus *Lactobacillus acidophilus* ANR-1. • In mixed gut bacteria culture experiments, Oligomate 55 supported growth of Lactobacilli and Bacteroides.
Hopkins and Macfarlane 2003	To determine the bifidogenicity of three nondigestible oligosaccharide (NDO) preparations in the context of normal and antibiotic-treated human fecal flora. Also, to measure *Clostridium difficile* colonization in the presence and absence of the NDOs and clindamycin. Oligomate 55 was used as the representative GOS test article. Source: Oligomate 55	Batch fecal cultures were inoculated with a toxigenic strain of *Clostridium difficile*, in the presence or absence of NDO and/or antibiotic. *C. difficile* and *Bifidobacterium* spp. growth on agar plates was followed over 48 h. Specific rRNA for *C. difficile*, Bifidobacteria and bacteroides-prevotella-porphyromonas group bacteria were measured, as well as short-chain fatty acid production.	• In the absence of clindamycin, Oligomate 55: --Caused a non-significant decrease in counts of *Clostridium difficile.* --Did not support growth of the total Bifidobacteria population, but increased rRNA levels of this group of bacteria, suggesting possible metabolic support. --Slightly increased the production of acetate and butyrate versus controls. --Caused a transient increase in lactate and succinate levels. • In the presence of clindamycin, Oligomate 55: --Did not prevent the increase in *Clostridium difficile* growth. --Did not rescue the inhibited Bifidobacteria population.

Table V-2. Summary of *in vitro* GOS Studies

Reference	Objective/Test Article	Study Design	Effect
Makras et al. 2005	To assess the capacity of ten strains of *Lactobacillus* to degrade inulin-type fructans (FOS) and a transgalacto-oligosaccharide (TOS) mixture (Oligomate 55). Source: Oligomate 55	Anaerobic incubation of test strains of *Lactobacillus* in the presence of FOS or Oligomate 55 on agar plates impregnated with bromocresol purple as a color indicator.	• Oligomate 55 as a sole energy source supported the growth of nine *Lactobacillus* strains in agar plate assays.
Shoaf et al. 2006	To examine the ability of various prebiotic oligosaccharides to inhibit the adherence of enteropathogenic *Escherichia coli* (EPEC) to HEp-2 and Caco-2 cells. Source: Oligomate 55 purified to 100% GOS by size exclusion chromatography.	Co-culture of enteropathogenic *Escherichia coli* and HEp-2 or Caco-2 mammalian cells in the presence or absence of various prebiotic oligosaccharides to measure percent adherence inhibition. Direct cell counts to measure number of bacterial microcolonies/cell and number of bacteria/microcolony via microscopy. Also, the ability of the oligosaccharides to inhibit the autoaggregation of the *E. coli* strain was investigated.	• Purified Oligomate 55 GOS inhibited adherence of enteropathogenic *E. coli* to both HEp-2 and Caco-2 cells. A weak dose-response effect was observed for Oligomate GOS on HEp-2 cells and gave 56.4% maximal inhibition of EPEC autoaggregation at 16 mg/mL. • The purified Oligomate GOS was only effective when preincubated with the mammalian cells prior to the addition of the *E. coli*. Experiments to measure the ability of GOS to compete off pre-adhered *E. coli* gave negative results.
Other formulations			
Hernot et al. 2009	To compare the *in vitro* microbial metabolism of various fructans, galacto-oligosaccharides, and polydextrose. Source: Vivinal GOS; Friesland Foods Domo, Zwolle, The Netherlands	Tube fermentation of pooled human fecal samples, inoculated with test substrates.	Vivinal GOS: • Caused increased gas production, including hydrogen and methane. • Lowered pH of fecal culture. • Increased production of acetate, propionate, and butyrate. • Supported increased growth of bificobacteria and lactobacilli, but *Escherichia coli* growth remained the same. • In general, effects were comparable to the pectin standard.

Table V-2. Summary of *in vitro* GOS Studies

Reference	Objective/Test Article	Study Design	Effect
Searle et al. 2009	To investigate the ability of GOS to prevent the adherence and invasion of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* to tissue culture cells. Source: Bimuno®	Plate-based cell culture experiments to measure bacterial adhesion to and invasion of human colonic cells (HT-29-19A, non-mucus-secreting; HT-29-16E, mucus-secreting) in the presence or absence of 2 or 20 mM GOS.	• 2 mM GOS reduced invasion of mucus-secreting colon cells by *S.* Typhimurium. The effect was similar whether cells were pre-treated with GOS or received GOS + bacteria together. • 20 mM GOS increased adherence of both *Salmonella* strains to both types of colonic cells. Invasion of one strain was inhibited.
Depeint et al. 2008	To assess the prebiotic potential of a novel GOS. Source: Vivinal GOS: 57% galacto-oligosaccharides, 23% lactose, 22% glucose, 0.8% galactose (*Bacillus circulans*-produced) and "B-GOS" = Novel GOS produced by *Bifidobacterium bifidum*: 48% galacto-oligosaccharides, 22% lactose, 18% glucose, 12% galactose	Human fecal flora batch culture experiments to measure growth of individual *Bifidobacterium* and *Lactobacillus* species in the presence of two different GOS preparations.	• Vivinal GOS supported the growth of: -- *Bifidobacterium animalis* (lactis) -- *Lactobacillus reuteri* -- *Lactobacillus acidophilus* • B-GOS supported the growth of: -- *Bifidobacterium bifidum* -- *Bifidobacterium infantis* -- *Bifidobacterium longum* -- *Bifidobacterium animalis* (lactis)
González et al. 2008	To analyze transcriptional patterns of *Bifidobacterium longum* grown in human milk, formula milk and GOS-supplemented medium. Source: GOS (33% disaccharides, 39% trisaccharides, 18% tetrasaccharides, 7% pentasaccharides, 3% hexa-/hepta-/octasaccharides); Friesland Foods, The Netherlands *Formulation I, Category 2 (Figure 5)*	RNA was isolated from log-phase active cultures of *Bifidobacterium longum* and reverse-transcribed, then hybridized to microarrays and analyzed using glucose medium as the baseline comparison.	• GOS (vs. glucose medium) induced two-fold or greater transcription of genes coding for: -- β-Galactosidases -- Possible lactaldehyde reductases -- Probable glycosyltransferase -- Solute binding proteins and permease of ABC transporter system for sugars -- Galactoside symporter (*lacS*) -- *pflA* pyruvate formate-lyase 1 activating enzyme -- Sugar transport system permease protein -- Formate acetyltransferases It was judged that there was "substantial overlap" in the genes induced in *B. longum* by GOS and breast milk.

Table V-2. Summary of *in vitro* GOS Studies

Reference	Objective/Test Article	Study Design	Effect
Ryu et al. 2007	To determine the hygroscopicity, Bifidogenicity, and anticariogenicity of various oligosaccharides. Source: GOS (β-D-Gal*p*-(1→4)-α-D-Gal*p*, β-D-Gal*p*-(1→3)-α-D-Gal*p*, β-D-Gal*p*-(1→4)-α-D-Glc*p*, and β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*); Samyang Genex Co., Seoul, Korea	Anaerobic culture of various *Bifidobacterium* spp., *Escherichia coli*, *Clostridium botulinum*, and *Staphylococcus aureus* in the presence of various oligosaccharides.	• GOS supported the growth of *Bifidobacterium longum* BORI, *B. longum* ATCC 15707, *B. bifidum* ATCC 15700, but not *B. breve* ATCC 29521, *Escherichia coli*, *Clostridium botulinum*, or *Staphylococcus aureus.*
Tzortzis et al. 2005	To assess the prebiotic potential of GOS in a 3-stage continuous culture system. Source: GOS produced by the activity of galactosyltransferases from *Bifidobacterium bifidum* NCIMB 41171 on lactose (powder consisting of (w/w): 45% lactose, 9.9% disaccharides[Gal (β 1-3)-Glc; Gal(β 1-3)-Gal; Gal(β 1-6)-Gal; Gal(α 1-6)-Gal], 23.1% trisaccharides [Gal (β 1-6)-Gal(β 1-4)-Glc; Gal(β 1-3)-Gal (β 1-4)-Glc], 11.55% tetrasaccharides [Gal (β 1-6)-Gal(β 1-6)-Gal(β 1-4)-Glc], and 10.45% pentasaccharides [Gal(β 1-6)-Gal(β 1-6)-Gal(β 1-6)-Gal(β1-4)-Glc] *Formulation J, Category 2 (Figure 5)*	Fecal flora from healthy volunteers was used to inoculate a 3-stage continuous fermentor, representing the proximal, transverse, and distal colon. Bacterial growth was measured in the presence or absence of 1% (w/v) GOS. Cell culture-based cytotoxicity and bacterial adhesion assays were also carried out.	• *Bifidobacterium* spp. were only slightly increased, as measured by fluorescence *in situ* hybridization (FISH) in Vessels 1 and 2, intended to represent the proximal and transverse colon. There was no change in *Lactobacillus* spp. • GOS was not cytotoxic to HT29 cells at concentrations up to 10 mM (5.5 mM, correcting for %purity) for 48 h. • GOS, as a mixture, or as individual chromatographically purified oligomer fractions (final concentrations not stated), did not affect (dat not shown) the adhesion of *Escherichia coli* O157:H7 (VT-) to HT 29 cells. • GOS mixture (final test concentration not stated) "strongly inhibited" (data not shown) attachment of *E. coli* (EPEC) O26 NCTC 08620 and *Salmonella enteric* serotype Typhimurium to HT29 cells. This activity was attributed mainly to the disaccharide fraction of GOS by the authors (data not shown).

Table V-2. Summary of *in vitro* GOS Studies

Reference	Objective/Test Article	Study Design	Effect
McBain and Macfarlane 2001	To investigate the effects of inulin and oligolactose on human-specific bifidobacterial and lactobacillus populations and on genotoxic enzyme activity in an *in vitro* colon model. Source: Yakult oligolactose (85% pure; contains small amounts of free glucose, galactose and lactose)	A three-stage continuous *in vitro* culture system with nonmethanogenic fecal slurry was utilized to measure the rate of alkali consumption in the presence of oligosaccharides. Also, the prevalence of specific bacterial populations was examined, and the effect of oligosaccharides on various enzyme activities was studied *in vitro*.	• Oligolactose was weakly Bifidogenic, supported growth of Lactobacilli in the fecal slurry, and caused increased utilization of NaOH. • After 30h, anaerobes, facultative species, and total lactobacilli were increased in the vessel (V_1 of a three-vessel series) mimicking the proximal colon. • Oligolactose decreased the activity of beta-glucosidase, beta-glucuronidase, and arylsulfatase *in vitro*. • Oligolactose increased the activity of azoreductase *in vitro*.
Rabiu et al. 2001	To use extracted β-galactosidases from various *Bifidobacterium* strains to synthesize galacto-oligosaccharides and study their ability to support growth of selected gut bacteria. Source: Custom-synthesized GOS preparations, each made from purified β-galactosidases isolated from various *Bifidobacterium* species.	Pure bacterial and mixed fecal bacteria batch culture fermentation in the presence of various GOS preparations synthesized by the purified β-galactosidases.	• No control (usually: lactose) was included for comparison. • Oligosaccharides synthesized by β-galactosidases from *Bifidobacterium bifidum*, *B. infantis*, *B. pseudolongum*, *B. adolescentis*, and *B. angulatum* generally supported the growth of nearly all *Bifidobacterium* spp. tested, plus *Lactobacillus acidophilus* ANR-1. • The bacterial growth profile for BS-4 oligosaccharide derived from *B. angulatum* was very similar as that observed for Oligomate-55 (Yakult).
Bouhnik et al. 1997	To assess the effects of prolonged administration of GOS on the fermentative activity of colonic flora *in vitro*. Source: Yakult 6'-GOS (*Aspergillus oryzae*-produced GOS: 48% trisaccharides, 37% tetrasaccharides, 15% penta-hexasaccharides) *Formulation O, Category 3 (Figure 5)*	Two semi-continuous fermentors inoculated with combined stool specimens from three healthy volunteers and followed in the presence or absence of 10 g GOS/d × 14 d. *Note: control data not shown for comparison.*	• GOS increased production of ATP in culture; onset and rapidity of production increased after 1 d adaptive period. • GOS rapidly decreased fermentor pH; the rapidity of the pH drop increased over time, indicating culture adaptation to GOS. • GOS increased total short-chain fatty acid production in the fermentor. • Gas volume production and its onset increased over time.

Table V-2. Summary of *in vitro* GOS Studies

Reference	Objective/Test Article	Study Design	Effect
Kikuchi and Sakata 1992	To study the kinetics of gas release from the breakdown of transgalactosylated oligosaccharide (TOS) in rat cecal culture. Source: transgalactosylated oligosaccharides (TOS; purity not stated)	Mini-scale batch culture model system based on a culture apparatus inoculated with rat cecum was utilized. Gas volume production was followed over time.	• TOS exhibited similar kinetics of initial rate of gas release (ml/hr) compared with glucose. • The half maximal concentration (K_s*; mg/mL) and maximum velocity of gas release (V_{max}'*; mL/hr) did not differ statistically from values observed for glucose.
Tanaka et al. 1983	To study the utilization of GOS for growth by various intestinal microflora strains. Source: Yakult 6'-GOS (*Aspergillus oryzae*-produced: 55% trisaccharides, 33% tetrasaccharides, 12% penta-/hexasaccharides), *Bifidobacterium breve* 4006, or combination of both. *Formulation P, Category 3 (Figure 5)*	Individual strain culture and growth measurement with or without 1% GOS.	• 1% GOS in culture medium supported growth of all *Bifidobacterium* spp. tested; GOS supported growth of some, but not all, *Lactobacillus* spp., and selected Enterobacteriaceae.

Table V-2. Summary of *in vitro* GOS Studies

Reference	Objective/Test Article	Study Design	Effect
Burvall et al. 1980	To study the digestibility of GOS *in vitro* by human intestinal enzymes. Source: GOS (Maxilact®) fractionated as follows: Fraction A: β-D-Gal-(1→6)-β-D-Gal-(1→6)- β-D-Gal-(1→4)-D-Glc; β-D-Gal-(1→6)- β-D-Gal-(1→6)—D-Gal; β-D-Gal-(1→6)-β-D-Gal-(1→6)—D-Glc Fraction B: β-D-Gal-(1→6)- β-D-Gal-(1→4)-D-Glc Fraction C: β-D-Gal-(1→6)-D-Gal; β-D-Gal-(1→6)-D-Glc Fraction D: ~80% lactose; β-D-Gal-(1→6)-D-Gal; β-D-Gal-(1→6)-D-Glc; another disaccharide (uncharacterized)	Crude intestinal homogenates were incubated with oligosaccharide substrate fractions or lactose and libaration of monosaccharides was measured.	• Versus lactose, only Fraction D was appreciably hydrolyzed by the intestinal homogenate. Glucose and galactose were liberated.

3. Summary of Animal Studies of GOS

Refer to Table V-3 for tabular summaries of the studies detailed below. Unless otherwise noted in this section, "GOS" refers to β-linked GOS.

Absorption/distribution/metabolism/excretion (ADME)

The complete extent to which GOS is fermented *in vivo* in the presence, but not absence, of an intact gut flora (4'-GOS: Ohtsuka et al. 1991) suggests that, until it reaches the colon, the galacto-oligosaccharide β-linkages are mainly unhydrolyzed and remain stable even in the acidic environment of the stomach. Oligomate 55N/55NP GOS is a mixture of β4'-GOS and β6'-GOS, and each of these forms of GOS has been demonstrated to be indigestible by artificial gastric juice and α-amylase from human saliva (β4'-GOS; Ohtsuka et al. 1990) and human small-intestinal enzymes (β6'-GOS; Burvall et al. 1980). Therefore, Oligomate 55N/55NP will be undigestible by these enzymes and this GOS formulation will survive in the gut until it reaches the colon. In rats with intact gut flora, radiolabeled GOS is mainly metabolized to expired CO_2, with less than 5% being excreted in urine or feces and 8.5% remaining in the intestines; however, germ-free rats retained most of the labeled GOS within their intestines (58.31%), indicating that GOS is indigestible until it reaches the appropriate gut flora in the colon (4'-GOS: Ohtsuka et al. 1991).

Microbiota effects

GOS survives the gastrointestinal tract until it reaches the colon, where it is metabolized by gut bacteria and utilized for growth and energy. Bacteria break down GOS with their β-glycosidases, hydrolyzing the oligosaccharides into monomeric and shorter polymer units. Germ-free rats did not demonstrate increased activity of β-glycosidases when they were fed GOS (Meslin et al. 1993). In pigs consuming 1% GOS in the diet, β-galactosidase activity was strongly induced in the cecum, ascending colon and rectum (Mountzouris et al. 2006). Rats on a 4, 5 or 10% GOS diet had a marked increase in β-galactosidase, α-glucosidase, and/or β-glucosidase activity (3'-/4'-/6'-GOS: Djouzi and Andrieux 1997, 6'-GOS: Kikuchi et al. 1996). Rats consuming a 5 or 10% 4'-GOS diet exhibited increased duodenal maltase and sucrase and ileal lactase activities (Ohtsuka et al. 1990).

GOS can support the growth of many strains of Bifidobacteria and some strains of Lactobacilli in the gastrointestinal tract, and this, in turn, supports the health of the host organism by a number of mechanisms. Increasing the local intestinal population of Bifidobacteria and/or

Lactobacilli may help block binding sites that would otherwise be available to pathogenic microbes for use in binding to and invading the intestinal mucosal cells. Biasing the gut flora toward a higher percent content of Bifidobacteria may also protect against developing allergies later in life and improve the immune response to certain vaccines (Macfarlane et al. 2008, Veereman-Wauters 2005). Bifidobacteria and Lactobacilli are also considered to be beneficial because of their ability to produce short-chain fatty acid metabolites which have positive effects on host intestinal cells (Macfarlane et al. 2008, Sako et al. 1999). There have been numerous studies on the ability of GOS to stimulate growth of Bifidobacteria and Lactobacilli in animals; however, sample handling is of critical importance to note, as frozen or aged stool will exhibit significantly reduced bacterial counts.

In rats, 0.66% (Pérez-Conesa et al. 2006a, Pérez-Conesa et al. 2006b), 4% (6'-GOS: Djouzi and Andrieux 1997), and 5% (6'-GOS: Rowland and Tanaka 1993) GOS in the diet significantly increased Bifidobacteria in the fecal flora. In the Rowland and Tanaka study, Lactobacilli counts were also increased, but in general this is not the case for GOS as Bifidobacteria are more strongly stimulated by this substrate. The Rowland and Tanaka study used gnotobiotic rats that were colonized with human microflora, and this may account for some of the variation observed versus the other rat studies. In a surgical rat model of severe acute pancreatitis, 1 g GOS/d prevented loss of the Bifidobacteria population, as measured by reverse transcription-polymerase chain reaction (RT-PCR) analysis of 16S rRNA, versus control-fed rats (Zhong et al. 2009). Another study examined the effects of orally dosed GOS (administered via drinking water followed by gastric gavage) in rats with induced colitis and found that GOS at 3 or 3.3% in water for 10 d, followed by GOS at 4 g/kg/d for 72 h, statistically significantly increased fecal Bifidobacteria, versus the colitis control (Holma et al. 2002).

A gnotobiotic mouse study found that 5% GOS in the diet stimulated growth of *Escherichia* coli, *Lactobacillus salivarius*, and *Bifidobacterium breve* in the feces, versus control diet (Morishita et al. 2002).

One study in pigs did not note any additional growth of Bifidobacteria or Lactobacilli in the cecum, ascending colon or rectum of animals fed a 1% GOS diet for up to 51 d (Mountzouris et al. 2006), but digesta samples were frozen prior to analysis. A second pig study found that 0.9% GOS in the diet slightly increased *Lactobacillus* populations in the feces and 2.2% 3'-/6'-GOS increased both *Bifidobacterium* and *Lactobacillus* populations in the proximal and distal colon and feces (Tzortis et al. 2005). A third pig study noted that 3.5% GOS increased both Bifidobacteria and Lactobacilli in the feces but not in ilium contents (Smiricky-Tjardes et al. 2003).

In addition to supporting growth of beneficial gut bacteria, GOS has also been shown to inhibit the proliferation and/or colonization ability of specific pathogenic bacteria in animals. A study in gnotobiotic mice consuming 5% dietary GOS found that growth of *Clostridium perfringens* was inhibited in the GOS group, versus control, and that GOS had less effect when *C. perfringens* was introduced first to the (initially) germfree mice (Morishita et al. 2002). Conventional mice that received a single dose of 2.5 g GOS/kg 30 min prior to exposure to *Salmonella enterica* serovar Typhimurium SL1344nal^r exhibited lower numbers of bacteria colonizing the spleen, liver, ileum, cecum and colon (Searle et al. 2009).

Production of short-chain fatty acids (SCFAs)

Short-chain fatty acid (SCFA) production is one of the consequences of GOS-stimulated gut bacterial growth. However, measuring concentrations of short-chain fatty acids can be technically challenging, as these are volatile compounds which may or may not be detectable in fecal and intestinal contents samples depending on the method of sample collection, the sample preparation method and time to analysis. Additionally, the majority of SCFA generated by gut flora is absorbed by the local colon cells and therefore may not be detectable in the stool (Macfarlane et al. 2008). Therefore, SCFA measurement has limited utility as a general indicator of the overall ability of a substance to stimulate the growth of gut flora. However, when detected, increased SCFA levels in the GI tract or feces are a positive indication of enhanced bacterial fermentation.

Conventional, but not germ-free, rats exhibit increased levels of SCFAs when fed GOS (Meslin et al. 1993), confirming the role of intact gut flora in the production of SCFAs. However, the profiles of SCFAs produced (*i.e.*, acetate, propionate, butyrate, valerate, etc.) tend to vary across gut flora species and host species. Due to these considerations, an increase in SCFA production can reflect an increase in gut bacterial growth or metabolic activity, but an absence of induction of SCFAs in a study cannot be interpreted to mean there was a lack of stimulation of gut flora. Many studies have reported on the ability of GOS to stimulate the production of SCFA in animals. Rumen contents of cows fed 1.2% 4'-GOS in the diet contained higher levels of acetate versus the control diet (Mwenya et al. 2005). Rats fed 4, 5 or 10% dietary GOS had overall increased levels of SCFAs in their cecal or fecal contents versus the control diet group (3'-/4'-/6'-GOS: Djouzi and Andrieux 1997, 6'-GOS: Kikuchi-Hayakawa et al. 1997, 4'-GOS: Chonan et al. 1996, 6'-GOS: Chonan and Watanuki 1996, 6'-GOS: Kikuchi et al. 1996, 4'-GOS: Chonan et al. 1995, 6'-GOS: Sakaguchi et al. 1998). Pigs receiving 0.9 or 2.2% 3'-/6'-GOS in their diet produced SCFAs in their proximal colon (Tzortis et al. 2005). A separate study found that pigs fed 1 or 2% GOS had increased SCFA production in the cecum (Houdijk et al.

2002b). Some studies have produced negative results (Mwenya et al. 2004), possibly due to an insufficient amount of GOS dosed per animal body weight or to the technical reasons mentioned above.

Increased concentrations of SCFAs and enhanced ion absorption due to decreased pH can alter the osmotic balance of the GI tract and enhance the fluid volume of enterocytes. An oral dose of 2000 mg Oligomate 55N/kg/day (825 mg GOS/kg/d) in rats increased cecum weight over 90 d (Kobayashi et al. 2009). In general, feeding GOS at levels from 2.96-20% in the diet of rats will result in an increased cecum weight (4'-/6'-GOS: Kobayashi et al. 2009, 4'-GOS: Kawakami et al. 2005, 4'-GOS: Chonan et al. 2001, 6'-GOS: Djouzi and Andrieux 1997, 6'-GOS: Kikuchi-Hayakawa et al. 1997, 6'-GOS: Chonan and Watanuki 1996, 6'-GOS: Kikuchi et al. 1996, 4'-GOS: Chonan et al. 1995, 6'-GOS: Chonan and Watanuki 1995, 4'-GOS: Hayashi et al. 1991, 4'-GOS: Ohtsuka et al. 1990). Feeding a diet containing 2% GOS has a similar effect in pigs (Houdijk et al. 2002b).

Increased digestion and protein content in pig colon from feeding 1 or 2% dietary GOS has also been reported (Houdijk et al. 2002b).

Gastrointestinal tract (GI) effects

Evidence from studies of GOS intake indicate that it produces an increase in fecal wet weight in rats (Hayashi et al. 1991, Ohtsuka et al. 1990) and an increase in cecal contents weight in rats (4'-GOS: Kawakami et al. 2005, 6'-GOS: Kikuchi-Hayakawa et al. 1997, 6'-GOS: Chonan and Watanuki 1996) and in pigs (Houdijk et al. 2002b).

pH effects

As would be expected based on generation of short-chain fatty acids during microbial fermentation, levels of 2.96-20% dietary GOS reduce cecal pH in rats (4'-GOS: Kawakami et al. 2005, 4'-GOS: Chonan et al. 2001, 3'-/4'-/6'-GOS: Djouzi and Andrieux 1997, 6'-GOS: Kikuchi-Hayakawa et al. 1997, 4'-GOS: Chonan et al. 1996, 4'-GOS: Chonan et al. 1995, 6'-GOS: Chonan and Watanuki 1995, 6'-GOS: Rowland and Tanaka 1993) and ruminal pH in cows (4'-GOS: Mwenya et al. 2005).

Mineral balance

Beneficial GOS-mediated alterations in mineral balance have been reported in rats, including increased absorption and retention of iron (4'-GOS: Pérez-Conesa et al. 2007), magnesium, calcium (4'-GOS: Chonan et al. 2001) and phosphorus (Pérez-Conesa et al. 2006b),

the restoration of normal magnesium levels in the context of excess dietary calcium and phosphorus (4'-GOS: Chonan et al. 1996), and calcium retention in the femur and tibia (6'-GOS: Chonan and Watanuki 1996). Additionally, 6'-GOS preserved calcium content of the tibia, but not the femur, in ovariectomized rats after 30 d exposure (Chonan et al. 1995). Acidification of the GI tract from fermentation by the gut flora is the likely mechanism for increased mineral absorption following GOS intake. Increased calcium, magnesium, and iron absorption has also been observed during dietary intake of fructo-oligosaccharides (FOS) (Zafar et al. 2004, Morohashi et al. 1998, Wang et al. 2009). One study demonstrated a decrease in potassium absorption and urinary excretion accompanied by an increase in fecal potassium and sodium content (4'-GOS: Hayashi et al. 1991) at levels of GOS in the diet of 10 and 20%. At these high levels of GOS addition to the diet, the observed shift in ion excretion from the urine to the feces is likely due to bulk absorption effects in the colon and thus is not a safety concern for GOS at the proposed levels of intake.

Other effects

A few studies have reported the ability of GOS to alter plasma and fecal cholesterol levels and to change the balance of saturated versus unsaturated fatty acids in the body content of rats. 10% GOS in the diet caused increased content of cholesterol and its metabolites in the feces of rats (6'-GOS: Kikuchi et al. 1996). However, 5% dietary GOS decreased lithocholic acid (bile acid) and coprostosterol (sterol) content of feces. Another rat study demonstrated that 4% dietary 3'-/4'-/6'-GOS was sufficient to produce lower plasma cholesterol levels versus controls (Djouzi and Andrieux 1997). In a different study, 5% dietary 4'-GOS reduced serum cholesterol in ovariectomized rats versus those on control diet or sham-operated rats (Chonan et al. 1995). Rats fed a diet containing 10% 6'-GOS for 50 d had reduced body fat content of linoleic and oleic acids and increased content of myristic acid compared with the control-fed group (Sakaguchi et al. 1998). The authors hypothesize that the unsaturated:saturated fatty acid ratio was reduced due to the extremely high levels of acetic acid (a SCFA) that was available for *de novo* cholesterol and triacylglycerol synthesis in the GOS-fed rats. Corroborating this conclusion, 10% dietary FOS elicited the same shift in body content of unsaturated:saturated fatty acids due to an overabundance of butyrate (a SCFA) which also serves as a precursor molecule in the cholesterol biosynthesis pathway (Zambell et al. 2003). The food intake of these rat studies (Kikuchi et al. 1996; Chonan et al. 1995; Sakaguchi et al. 1998) were in the range of 50 to 90 g/kg bw/day with an average food intake of 70 g/kg bw/day. These studies used diet containing 4 to 10% GOS, giving a GOS intake by rat of 2.8 to 7 g GOS/kg bw/day, which is an order of magnitude higher than the estimated daily intake (EDI) of GOS by humans based on the proposed uses and use

levels (Table III-2). Therefore, adverse effects on lipid parameters are not a safety concern for GOS at the proposed levels of intake.

One study demonstrated that GOS could modulate mucins in the colon of germ-free, but not conventional or heteroxenic (human flora-inoculated) rats (Meslin et al. 1993). Mucus-containing cells were decreased in the proximal colon and increased in the distal colon.

A study investigating the effects of GOS in rats with surgically-induced severe acute pancreatitis found that supplementation of 1 g GOS/d restored soluble immunoglobulin A (IgA) levels in intestinal mucus and partially reduced the apoptosis index in small intestinal epithelium versus enteral-fed-only rats (Zhong et al. 2009).

In a murine influenza vaccination model, 2.5% GOS/FOS (9:1) in the diet was found to produce a maximal ear swelling response in a delayed-type hypersensitization (DTH) experiment (Vos et al. 2006), vs. 0.5, 1.25, and 5% GOS/FOS. GOS/FOS produced more swelling compared with FOS/inulin, short chain fructo-oligosaccharides (sc-FOS), inulin or the control, suggesting that the GOS portion of the GOS/FOS mixture was responsible for the DTH response. Carrier-mediated effects were ruled out.

Safety endpoints from animal studies

In a ninety-day subchronic oral gavage safety study of Oligomate 55N, male and female Crl:CD(SD) rats were dosed with 0, 500, 1000 and 2000 mg 4'-/6'-GOS syrup/kg/d (0, 206.3, 412.5, and 825 mg GOS/kg/d) (Kobayashi et al. 2009). The no observed adverse effect (NOAEL) level was determined to be ≥ 2000 mg syrup/kg/d [calculated GOS dose based on 41.25% w/w content: 825 mg GOS/kg/d]. The only clinical change observed was reddish tears and the frequency of its occurrence was similar across the treatment and control groups. There were no GOS-related changes in body weight, feed or water intake, urinalysis, hematology, clinical chemistry or ophthalmological tests in any test group. Cecum weight was higher in males in the 2000 mg syrup/kg/d (825 mg GOS/kg/d) group versus the control group, but no concerning histopathology results were observed. Bacterial reverse mutation and chromosomal aberration assays were also carried out on Oligomate 55N in the Kobayashi study, and no significant mutagenesis or chromosomal aberrations were observed for any GOS concentrations tested *in vitro*.

A ninety-day oral gavage safety study was carried out on Vivinal® GOS syrup, which has a chemical profile similar to that of Oligomate 55N (see Figure 5) (Anthony et al. 2006). In this study, 2.5 and 5.0 g syrup/kg body weight/day [calculated GOS doses based on 45% w/w

content: 1.125 and 2.25 g GOS/kg/d] was administered to Sprague Dawley Crl:CD®(SD)IGS BR rats and there were no significant differences in body weight for either GOS group versus either the carrier or FOS reference controls. No gross organ abnormalities or test article-related histopathological changes were observed. At 5 g syrup/kg/d (2.25 g GOS/kg/d), there was a small decrease in feed intake by both males and females at various times over the course of the study, but the effect was not significant when compared with the FOS reference group. At 5 g syrup/kg/d (2.25 g GOS/kg/d), males had a higher mean spleen weight relative to final body weight, compared with the carrier control group. At 2.5 g syrup/bw/d (1.125 g GOS/kg/d), females had decreased feed intake for a short period during the study. There were no toxicologically significant effects noted for Vivinal GOS syrup.

GOS has been reported to affect feed intake and body weight in other studies, although in some cases GOS was tested in combination with FOS. Vos and co-workers fed Vivinal GOS/Raftiline HP long-chain FOS (9:1) at 0.5, 1.25, 2.5, and 5% GOS/FOS in the diet of C56BL/6JolaHsd mice (gender unstated) for 45 or 51 days and did not observe any differences in weight change or feed intake over time versus the control group (Vos et al. 2006).

GOS effects on body weight and feed intake in rats are mixed. The need for precise caloric balancing in feeding studies may be responsible for these results. Pérez-Conesa and co-workers fed 3 wk old male Sprague-Dawley rats 0.66, 2.75 or 5.5% 4'-GOS (Oligomate 55P) in the diet for 30 d and found that only rats in the 0.66% GOS group had lower final body weight and weight gain versus control (4'-GOS: Pérez-Conesa et al. 2007). Sakaguchi and co-workers fed male Wistar rats (mean body mass = 70 g) 10% 6'-GOS for 50 d and observed no difference in feed intake, feed efficiency and weight gain versus the control group (Sakaguchi et al. 1998). Chonan and Watanuki fed 4 wk old male Wistar rats 5% dietary 6'-GOS for 30 d and observed no effect on body weight, feed intake or feed efficiency versus control (Chonan and Watanuki 1996). Kikuchi and co-workers fed 10 wk old male Fisher 344 gnotobiotic (germ-free rats inoculated with human fecal flora) 5 or 10% 6'-GOS in the diet for 4 wk and found no changes in feed intake or body weight compared with control (Kikuchi et al. 1996). Chonan and co-workers studied the effects of GOS in ovariectomized Wistar rats, feeding 5% 6'-GOS in the diet for 30 d (Chonan et al. 1995). They noted a slight decrease in feed intake in the ovariectomized (OVX) group consuming 5% 6'-GOS. There was no effect on body weight or feed intake across all other GOS-fed groups (sham-operated or OVX). An earlier study by Chonan and Watanuki in 4 wk old male Wistar rats demonstrated that 5 and 10% dietary 6'-GOS had no effect on body weight, feed intake or feed efficiency versus control (Chonan and Watanuki 1995). Hayashi and co-workers fed male Wistar rats 10 or 20% 4'-GOS in the diet for 62 d and found that rats fed the 20% 4'-GOS diet had reduced body weight gain versus the 10% 4'-GOS and control groups;

however, final body weight was not significantly affected and feed intake was similar across all groups (Hayashi et al. 1991). Ohtsuka and co-workers noted a net body weight increase in male SD rats fed 10% 4'-GOS for 6 wk versus the control group (Ohtsuka et al. 1990). The feed conversion ratio was decreased in the 10% GOS group vs. control, and feed intake was increased in both the 5 and 10% GOS groups vs. control.

Houdijk and co-workers fed 57 d old castrated pigs a diet containing Oligostroop® syrup at 1 or 2% GOS (w/w) for 44 d (mean) and found that body weight and feed intake was lower in the 2% GOS group versus control (Houdijk et al. 2002b). Body weight gain per day was also decreased in the GOS groups versus the control group. In another study, Houdijk and researchers fed 57 d old castrated pigs a diet containing Oligostroop® syrup at 0.4 or 0.8% GOS (less than was used in the 2002b study) for 42-47 d and found that feed intake was similar across all treatment groups versus control (Houdijk et al. 1999). An older study by the same group (Houdijk et al. 1998) demonstrated that Oligostroop® syrup at 1 or 2% GOS in pigs (38 d old castrated piglets, fed for 6 wk) had an initial lower mean daily weight gain and daily dry matter intake versus control-fed pigs. This effect disappeared over time, suggesting that the pigs adapted to the dietary GOS. Together, these three studies suggest that there may be a threshold in pigs for GOS consumption above which feed intake and/or body weight begin to be affected.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Oligomate 55N/55NP (current formulation for GRAS)			
Kobayashi et al. 2009	To investigate the oral subchronic safety and the genotoxicity of Oligomate 55N syrup in rats. (75% solids with 55% of solids as galacto-oligosaccharides) To investigate gene mutation. Peripheral blood micronuclei testing for bone marrow suppression in mice was also carried out. Source: Oligomate 55N syrup (75% solids; 55% galacto-oligosaccharides based on dry weight)	90-day oral gavage safety study in Crl:CD(SD) rats, plus bacterial reverse mutation in the Ames assay and in *Escherichia coli* WP2*uvrA*, chromosomal aberration testing in Chinese hamster lung fibroblasts, and micronucleus test assays in mouse peripheral blood reticulocytes. Ninety day safety study in rats: Four groups of rats: 10 male, 10 female in each. Doses: 0, 500, 1000 and 2000 mg syrup/kg body weight/d [0, 206.3, 412.5, and 825 mg GOS/kg/d] Bacterial reverse mutation *in vitro* assays: 312.5, 625, 1250, 2500 and 5000 µg syrup/plate (128.9, 257.8, 515.6, 1031, 2063 µg GOS/plate). Chromosomal aberration *in vitro* tests: 1250, 2500 and 5000 µg syrup/mL (515.6, 1031, 2063 µg GOS/mL).	• The no observed adverse effect (NOAEL) for Oligomate 55N was ≥ 2000 mg syrup/kg/d (825 mg GOS/kg/d) for both male and female rats. • Clinical changes observed: reddish tears, similar frequency of occurrence in test and control groups. • No GOS-related changes in body weight, feed/water intake, urinalysis, hematology, clinical chemistry or ophthalmological tests in male or female rats in any test group. • Cecum weight (absolute and relative) was higher in males in the 2000 mg syrup/kg 825 mg GOS/kg/d) group compared with the control group. No concerning histopathology results were observed. • No significant mutagenesis or chromosomal aberrations were observed for any GOS concentrations tested *in vitro*.
Oligomate 55P (older formulation)			

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Pérez-Conesa et al. 2007	To investigate the effect of 4'-GOS alone or in combination with probiotics on iron bioavailability in weanling rats. Source: Oligomate 55P powder (55% 4'-galacto-oligosaccharides based on dry weight)	Fifty-four 3 wk old weanling male Sprague-Dawley rats were divided into eight groups of six rats each and fed one of eight infant formula-based diets for 30 d. Mineral balancing for iron was performed on days 8-10, 19-20, and 28-30. Diets were: control, probiotic (Bifidobacteria), prebiotic (1.2, 5, 10% Oligomate 55P; 0.66, 2.75, 5.5% GOS), synbiotic (Bifidobacteria plus 1.2, 5, or 10% 4'-GOS). Species: Rats Dose of GOS: 0.66, 2.75, 5.5% GOS in diet Exposure period: 30 d	• 0.66% GOS group: -- Lower final body weights and weight gains observed in this group versus control. Effect not observed for 2.75 and 5.5% GOS groups, but was even more pronounced in the 2.75% GOS + Bifidobacteria (synbiotic) group. • 5.5% GOS group: -- Had increased apparent iron absorption and retention, plus decreased fecal excretion at all mineral balancing time points examined. Urinary iron excretion was unaffected by any level of GOS.
Pérez-Conesa et al. 2006a	To evaluate the effect of various follow-up infant formulas containing pre- and/or probiotics on rat fecal bifidobacteria counts. (Oligomate 55P in infant formula with or without *Bifidobacterium bifidum* and *B. longum*) Source: Oligomate 55P powder (55% galacto-oligosaccharides based on dry weight)	Fifty-four three-week old male Sprague-Dawley rats were fed infant formula plus Oligomate 55P at 1.2, 5.0, and 10.0% (w/w) (0.66, 2.75, 5.5% GOS) with or without concomitant addition of *Bifidobacterium bifidum* and *B. longum* (synbiotic treatment), or a control formula containing no pre- or probiotics, for 30 d. Feces were collected and cultured on agar plates for total aerobe, anaerobe and bifidobaceria counts. Species: Rats Dose of GOS: 0.66, 2.75, 5.5% GOS in diet Exposure period: 30 d	• 0.66% GOS group: --Transient initial decrease in both aerobes and anaerobes in rat fecal flora. -- Bifidobacteria increased significantly by 28-30 d. Higher percent GOS in the formula did not cause additional Bifidobacteria growth. • 5.5% GOS group: --Later decrease (28-30 d) in anaerobe count in rat fecal flora compared with non-supplemented formula.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Pérez-Conesa et al. 2006b	To evaluate the effect of probiotics and prebiotics added singly or in combination to infant formulas on the absorption and bioavailability of Ca, Mg and P in rats. Source: Oligomate 55P powder (55% galacto-oligosaccharides based on dry weight)	This publication reports on different endpoints measured during the Pérez-Conesa et al. 2006a study. Mineral absorption ratios were calculated based on intake minus fecal excretion and expressed as a percentage of mineral intake for Ca, Mg and P. Body weights and feed intakes were recorded. Species: Rats Dose of GOS: 0.66, 2.75, 5.5% GOS in diet Exposure period: 30 d	• 0.66% GOS group: --Transient initial decrease in both aerobes and anaerobes in rat fecal flora. -- Bifidobacteria were increased significantly by 28-30d. -- By 30d, rats had significantly lower final body weight and weight gain compared with other GOS groups and the control group. • 5.5% GOS group: -- Later decrease (present at 28-30 d) in anaerobe count in fecal flora compared with rats on non-supplemented formula. • GOS did not affect water intake, urine excretion or feces wet weight, but feces moisture content declined in the 5.5% GOS group versus control. [This extraphysiological effect may be due to consumption of an excess amount of fiber.] • Apparent percent absorption and retention of Ca, Mg and P increased in all GOS groups, while fecal excretion in mg/day decreased. • Initial increased urinary excretion of Ca was observed in GOS groups and declined over time. • Urinary effects for Mg and P were more variable and bidirectional.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Kawakami et al. 2005	To study the ability of GOS to prevent an increase in phenol and *p*-cresol in rats fed a tyrosine-supplemented diet. Source: Oligomate 55P powder (59.1% galacto-oligosaccharides [18.2% disaccharides, 23.6% trisaccharides, 17.3% tetra-/hexasaccharides], 19.3% monosaccharides, 21.6% lactose) *Formulation D, Category 2 (Figure 5)*	Eighteen 6-wk old male Wistar rats were fed a basal diet for 7 d, and then assigned into three groups of 6 rats/group. Six rats were maintained on basal diet throughout the study. Twelve were fed a 2.5% tyrosine-supplemented diet for 9 d. The latter group was then assigned into two groups of 6 rats/each: diet containing 2.96% GOS plus 2.5% tyrosine or 2.5% tyrosine for 14 d. Species: Rats Dose of GOS: 2.96% GOS + 2.5% tyrosine in diet Exposure period: 14 d	• 2.96% GOS plus 2.5% tyrosine: -- Offset the tyrosine-induced increase in fecal, cecal, serum and urine phenol and *p*-cresol levels. Levels of phenol and *p*-cresol in all excreta from treatment groups were not statistically different from measurements taken in the basal diet group. -- Cecal tissue and cecal content weight was significantly increased compared with rats fed the basal diet. -- Cecal pH was significantly decreased compared with rats fed the basal diet.
Oligomate 55 (older formulation)			
Mwenya et al. 2005	To evaluate the effects of supplementing dairy cows with yeast culture and/or GOS on ruminal fermentation and nitrogen levels. Source: Oligomate 55 (18.3% monosaccharides, 38.8% disaccharides, 23.1% trisaccharides, 15.7% tetrasaccharides, 4.51% penta-/hexasaccharides, 3.7% water) *Formulation E, Category 2 (Figure 5)*	Four nonlactating, ruminally cannulated Holstein cows were fed a basal diet or one containing 10g/d yeast extract, 1.2% GOS, or both, for 27 d. Species: Cows Dose of GOS: 1.2% GOS in diet Exposure period: 27 d	• 1.2% GOS: -- GOS-supplemented dairy cows had decreased uric acid and allantoin excretion in urine, versus control diet group. -- Microbial nitrogen was decreased, compared with the control diet. -- Ruminal pH was decreased versus control. -- Acetate production was increased, while propionate was decreased. -- Protozoal counts were higher in the GOS group compared with those in the control.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Sar et al. 2004	To determine the effect of a combination of nitrate with beta-1,4-galacto-oligosaccharides on rumen methanogenesis, nitrate intoxication and metabolic rate in sheep. Source: Oligomate 55 (60% GOS w/w)	Feed study with measurement of respiratory gas exchange and metabolic rate plus simultaneous collection of rumen fluid and blood from four rumen-fistulated wethers. Treatments were: physiological saline (control), nitrate (1.3 g $NaNO_3/kg^{0.75}$ BW, from a 30% w/v solution), nitrate plus GOS (20 g Oligomate 55/day in two equal portions), and nitrate plus nisin (3 $mg/kg^{0.75}$BW/day). One-week washout between each 8 d treatment. Species: Sheep Dose of GOS: nitrate + 20 g Oligomate 55/d (11.94 g GOS/d) in diet Exposure period: 8 d	• Concurrent administration of nitrate plus GOS: -- Decreased rumen and plasma nitrite concentration versus nitrate alone. -- Decreased methane production and metabolic rate versus control. --Partially offset the nitrate-induced methemoglobinemia. -- GOS was degraded in the rumen within 2-3 hr in the presence of nitrate.

Other GOS formulations			
Searle et al. 2009	To study the effect of oral administration of GOS on bacterial host immunity in mice using various models. Source: Bimuno® GOS mixture	*Study #1*: Murine ligated ileal gut loop model to study the prevention of infection by administering 10^9 CFU *Salmonella enterica* serovar Typhimurium SL1344nal^r transmurally with or without 2 mM Bimuno. Four 8 wk old female SLC:ICR mice were inoculated with bacteria with or without GOS and ileal gut loops plus target organs were harvested 30 min later. *Study #2*: Two groups of 15 female BALB/c (Charles River) mice were given 10^7 CFU *S.* Typhimurium alone or 2.5 g Bimuno/kg followed by *S.* Typhimurium 30 min later by oral gavage. Feces and liver, spleen, ileum, caecum, colon and heart blood were sampled at intervals up to 96 h post-inoculation *Study #3*: Four groups of 30 female BALB/c mice were dosed by oral gavage with sterile PBS, 2.5 g Bimuno/kg, 10^7 CFU *S.* Typhimurium (SL1344nal^r) or 2.5 g GOS/kg prior to bacterial challenge 30 min later. Necropsies were carried out at 4, 8, 24, 72, 96 and 120 h post-inoculation. Species: Mice Dose of GOS: Single dose 2.5 g Bimuno/kg bw Exposure period: N/A	*Study #1:* • Ileal gut loops treated with GOS exhibited no pathology and normal tissue architecture via histopathological examination. • Gut loops inoculated with two strains of *S.* Typhimurium + GOS retained their epithelial structure and mucosa and had fewer signs of bacterial invasion, versus bacteria-treated tissue. *Study #2:* • No clinical signs of toxicity were reported in the GOS + bacteria group. • GOS pre-feeding (30 min) could not protect against *Salmonella enterica* Typhimurium infection-induced pyogranuloma. Additionally, in all groups, nearly all tissue segments from liver, spleen, ileum, caecum and colon contained lymphoid hyperplasia, submucosal oedema, portal inflammation, autolysis or other histological change. *Study #3:* • All groups receiving GOS remained clinically normal throughout the study, and no gross lesions were observed at necropsy. • Background mucosal autolysis was present in some intestinal samples, and lymphoid hyperplasia, submucosal oedema, portal inflammation, red pulp splenic hyperplasia and lifting/sloughing mucosal epithelium was commonly noted across all groups. • GOS pre-feeding (30 min) resulted in lower numbers of *S.* Typhimurium SL1344nal^r colonizing the spleen, liver, ileum, caecum and colon.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Zhong et al. 2009	To investigate the protective effect of GOS on intestinal barrier function in rats with severe acute pancreatitis (SAP). Source: GOS, 90% purity, Xi'an Deshipu Bio-industry Company, China. (Impurities not described.)	Sixty adult male Sprague-Dawley rats were divided into six groups of 10 rats/group: --Sham operation with 4 d sacrifice --Sham operation with 7 d sacrifice --SAP-induced, with enteral nutrition (EN), 4 d sacrifice --SAP-induced, with EN, 7 d sacrifice --SAP-induced, with EN + 0.9 g GOS/d, 4 d sacrifice --SAP-induced with EN + 0.9 g GOS/d, 7 d sacrifice Species: Rats Dose of GOS: 0.9 g GOS/d Exposure period: 7 d (maximal)	• GOS supplementation prevented loss of Bifidobacteria population in SAP rats versus enteral-fed-only rats (as measured by RT-PCR of 16S rRNA genes). Partial prevention of loss of Lactobacilli was observed. • GOS supplementation restored soluble IgA concentration in intestinal mucus and partially reduced the apoptosis index in small intestinal epithelium versus enteral-fed-only rats. • GOS supplementation slightly increased levels of occludin mRNA relative to enteral-fed-only rats, but not to levels present in sham operated rats.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Anthony et al. 2006	To conduct a 90 d oral gavage safety study on Vivinal® GOS syrup in rats and establish its NOAEL. Source: Vivinal® GOS syrup (*Bacillus circulans*-derived; 45% GOS; 15% lactose, 14% glucose, 1% galactose), Borculo Domo	120 six-wk old male and female Sprague Dawley Crl:CD®(SD)IGS BR rats were divided evenly among four groups and fed the following via oral gavage daily for 90 d: --Reverse osmosis deionized (RODI) water control --FOS reference control --2.5 g syrup /kg/d [1.125 g GOS/kg/d] --5.0 g syrup /kg/d [2.25 g GOS/kg/d] Species: Rats Dose of GOS: 5.0 g syrup/kg/d via gavage [2.25 g GOS/kg/d] Exposure period: 90 d	• No statistically significant differences in body weight for either GOS group, compared with either RODI or FOS controls. • No internal gross abnormalities were observed in GOS groups. • No test article-related histopathological changes were observed. • 5 g syrup/kg/d [2.25 g GOS/kg/d]: -- Decreased feed intake (7-11%) in males during days 14-21 and 28-89 and in females (14-19%) during days 0-7, 14-21, and 35-89, versus RODI control. Effect not significant vs. FOS reference group. -- Males had increased feed efficiency during two isolated weeks, versus RODI control. Effect not significant vs. FOS reference group. -- Males had higher mean spleen weight relative to final body weight, vs. RODI group. -- Females had lower mean absolute ovary weights, vs. FOS group. -- Males had lower specific gravity of urine vs. RODI. -- Females had increased hemoglobin and hematocrit values, versus the FOS control, on days 90-92. -- Males had decreased ALT levels, versus FOS control, on days 90-92. -- The latter four findings were deemed not toxicologically significant by the authors. •2.5 g syrup/kg/d [1.125 g GOS/kg/d]: -- Decreased feed intake (10-14%) in females during days 49-70. -- Females had lower mean liver weights relative to final body weight, vs. FOS group. -- Specific gravity of urine was higher in females vs. FOS group. -- The latter two effects were determined to be non-toxicologically significant by the authors.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Mountzouris et al. 2006	To profile the composition and metabolic activity of colonic microflora in growing pigs fed various oligosaccharides. Source: Vivinal GOS 10, Borculo Domo Ingredients, The Netherlands	Twelve castrated 35 d old [Duroc × (Large White × Landrace)] pigs were randomized to three isocaloric/isonitrogenous diets: control, 10 g/kg FOS or 10 g/kg GOS. A 7 d adaptation period was followed by the experimental diet for 30-32 d, when digesta were harvested. Species: Pigs Dose of GOS: 1% GOS in diet Exposure period: 51 d (maximal)	• GOS did not induce additional growth of Bifidobacteria or Lactobacilli in the caecum, ascending colon or rectum, vs. control diet. • GOS diet slightly increased acetic acid production in caecum and rectum relative to control (not statistically significant). • GOS strongly induced β-galactosidase activity in the caecum, ascending colon and rectum.
Vos et al. 2006	To analyze the immunomodulatory properties of prebiotic oligosaccharides in a murine influenza vaccination model. Source: 50% (w/w) of test article as GOS/FOS (9:1 ratio): Vivinal GOS (Borculo Domo, Zwolle, The Netherlands; degree of polymerization 3-8) plus Raftiline HP long-chain FOS (Orafti, Wijchen, The Netherlands; average degree of polymerization > 23) and 19% maltodextrin, 16% lactose, 14% glucose and 1% galactose.	GOS/FOS (9:1) was given in the diet at 0.5, 1.25, 2.5, or 5% (w/w) to 10 C56BL/6JolaHsd mice (gender unspecified) and various immune responses were followed, including delayed-type hypersensitivity (DTH) to an influenza vaccine (injection, booster 21 d later, DTH test 9 d afterwards), splenocyte proliferation, vaccine-specific antibody production. Fecal bifidobacteria and lactobacilli were measured in the same experiment. Control diet was given for 5 d prior to the study. Supplementation was given for 20 or 14 d prior to the first vaccination and lasted until 31 d after the first vaccination (study's end). GOS/FOS was also compared against FOS/inulin, SC-FOS and inulin to measure its comparative ability to induce DTH and alter gut microbiota (mechanism of action study). Species: Mice Dose of GOS: 5% GOS/FOS (9:1) in diet Exposure period: 51 d (maximal)	• No significant differences in weight or feed intake were observed for GOS/FOS-fed groups, versus control diet. • 2.5% GOS/FOS in the diet produced the maximal ear swelling response in the DTH experiment. • GOS/FOS caused significantly more ear swelling in the DTH model vs. FOS/inulin, sc-FOS, inulin, or the control (all equivalent), suggesting that the GOS portion was responsible for the DTH response. The DTH response was not a carrier-mediated effect. • GOS/FOS had a dose-dependent effect on Bifidobacteria and Lactobacilli (~10-fold weaker; qRT-PCR), decreased fecal pH, increased lactate levels, and increased fecal levels of acetic, propionic, and butyric acids. • GOS/FOS produced comparable increase in Bifidobacteria and Lactobacilli as FOS/inulin.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Tzortis et al. 2005	To assess the prebiotic potential of a GOS preparation in a parallel continuous randomized pig trial. Source: GOS produced by the activity of galactosyltransferases from *Bifidobacterium bifidum* NCIMB 41171 on lactose (powder consisting of (w/w): 45% lactose, 9.9% disaccharides[Gal (β 1-3)-Glc; Gal(β 1-3)-Gal; Gal(β 1-6)-Gal; Gal(α 1-6)-Gal], 23.1% trisaccharides [Gal (β 1-6)-Gal(β 1-4)-Glc; Gal(β 1-3)-Gal (β 1-4)-Glc], 11.55% tetrasaccharides [Gal (β 1-6)-Gal(β 1-6)-Gal(β 1-4)-Glc], and 10.45% pentasaccharides [Gal(β 1-6)-Gal(β 1-6)-Gal(β 1-6)-Gal(β1-4)-Glc]	Forty weaned male 28 d old pigs were acclimated to the unit and control diet for 7 d. Then pigs were fed either the control diet or diet containing 1.6% (w/w) GOS mixture (0.9% GOS), 4% GOS mixture (2.2% GOS), or 1.6% inulin for 33-35 d, after which the colonic contents were sampled. Species: Pigs Dose of GOS: 0.9 or 2.2% GOS in diet Exposure period: 33-35 d	• 2.2% dietary GOS increased *Bifidobacterium* and *Lactobacillus* spp. in the proximal and distal colon contents and in feces, and decreased the pH of the proximal, but not distal, colon contents. • 0.9% GOS mildly increased *Lactobacillus* populations in the feces. • Only the proximal colon was affected by GOS as regards the production of short chain fatty acids: -- 0.9% GOS increased lactic and propionic acid content. -- 2.2% GOS increased lactic and acetic acid content.
Mwenya et al. 2004	To evaluate the effect of yeast culture, lactic acid bacteria and beta-1-4 galacto-oligosaccharides on rumen methanogenesis, energy and nitrogen utilization in sheep. Source: Unspecified Yakult GOS product	Digestion study followed by a respiratory study in four rumen-fistulated wethers. Diets were fed for 10 d followed by a 9 d collection period. Treatments were: basal diet (control), basal diet plus 20 g GOS, basal diet plus 4 g yeast concentrate, basal diet plus 1 g lactic acid bacteria/kg feed. Species: Sheep Dose of GOS: 20 g GOS/d in diet Exposure period: 10 d	• GOS-fed sheep (vs. controls): -- Had increased ruminal oxidation-reduction potential. -- Had reduced ruminal ammonium nitrogen levels. -- Had lower acetate:proprionate ratios. -- Had reduced methane and heat production. -- Had higher energy intake. -- Exhibited lower hemicellulose digestibility. -- Volatile fatty acid production did not change.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Smiricky-Tjardes et al. 2003	To evaluate the effect dietary oligosaccharide addition on swine nutrient digestibility and intestinal microbiota and fermentation. Source: GOS as 58.3% galacto-oligosaccharides (uncharacterized), 20% lactose, and 18% glucose (Borculo Domo Ingredients, Borculo, The Netherlands).	Twelve crossred pigs (30 ± 2 kg; PIC 326 sire line × C22 dams) were T-cannulated and then fed an oligosaccharide-free control diet for 21 d. On d 22 ileal digesta samples were taken for *in vitro* fermentation experiments. Pigs were fed one of three isonitrogenous diets: control diet, diet + 17% soy soluble, or diet + 3.5% GOS (w/w), for 6 wk. On d 64, another *in vitro* experiment was carried out on ileal effluent. Species: Pigs Dose of GOS: 3.5% GOS in diet Exposure period: 6 wk	• GOS diet increased levels of bifidobacteria and lactobacilli in the feces, but not the ileum. • GOS did not increase levels of short-chain fatty acids in the ileum. • *In vitro* experiments could not be evaluated because results from a control group were not included for comparison.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Holma et al. 2002	To investigate the effects of GOS on inflammation and growth of bifidobacteria in a rat model of colitis. Source: GOS-1: prepared from cow milk whey; 12% GOS concentrate containing 8% disaccharides, 4% trisaccharides, 9% lactose, 17% glucose, 11% galactose, 6% protein, 0.7% fat, 0.2% ash (Valio Ltd, Helsinki, Finland). Production organism unknown. GOS-2: Elix'or® prepared enzymatically from lactose; 45% GOS concentrate containing 15% disaccharides, 18% trisaccharides, 8% tetrasaccharides, 3% pentasaccharides, 1% hexa-/hepta-/octasaccharides, 15% lactose, 14% glucose, 1% galactose, 0.03% protein, 0.2% ash (Borculo Whey Products, Borculo, Zwolle, The Netherlands). Production organism unknown.	Forty-two adult male rats of outbred HY:WIST stock were divided into five groups of 7-9 rats/group consisting of healthy controls, induced-colitis controls, plus induced-colitis rats receiving either 4 g GOS-1/kg/d, 4 g GOS-2/kg/d, or 2 mg subcutaneous dexamethasone/kg/d as a positive treatment control. GOS supplementation was given in drinking water (3.3% GOS as GOS-1, 3% GOS as GOS-2) for 10 d prior to colitis induction and then given via gastric gavage after the colitis induction. Animals were followed for 72 h. Species: Rats Dose of GOS: 3 or 3.3% (w/v) GOS in drinking water for 10 d, then 4 g/kg/d GOS via gastric gavage for 72 h Exposure period: 10 d + 72 h	• Neither GOS preparation could prevent colitis-associated loss in body weight or reduce colon wet weight, a crude marker of inflammation. • A colitis-associated increase in myeloperoxidase activity was not inhibited by GOS, and colonic damage scores (visual evaluation) for GOS-treated rats were not significantly different from the colitis control. • Fluid intake was somewhat reduced in the GOS groups in comparison with healthy control rats. • Both GOS preparations caused an increase in fecal Bifidobacteria , although GOS-2 appeared to more strongly stimulate growth.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Houdijk et al. 2002a	To study the effects of dietary oligosaccharides on the microbial characteristics of ileal chime and feces in weaner pigs. Source: Oligostroop® (45% transgalacto-oligosaccharides (TOS), uncharacterized; Borculo Whey Products, Borculo, The Netherlands)	Twenty 38 d old castrated pigs [(Great Yorkshire × Landrace) ♂ × (Great Yorkshire) ♀] (10.4 kg avg) were ranked on bw, divided into five weight classes, then randomized to diet (4 pigs/diet): control diet, diet containing 10 or 40 g/kg TOS, or diet containing 10 or 40 g/kg GOS. Pigs were allowed to adapt to diet for 13 d after which feces (d 13-17), and then ileal chyme (d 33, 34, 36, 37; collected by means of a T-cannula) were collected over time and assayed for bacteria and their metabolites. Species: Pigs Dose of GOS: 1 or 4% GOS in diet Exposure period: 37 d	• GOS did not cause any changes in short chain fatty acid content, chyme production, ileal pH, or bacterial populations in ileal chyme. • GOS at 40 g/kg feed caused an increase in fecal pH and an increase in fecal levels of *iso*-butyric acid.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Houdijk et al. 2002b	To determine the effect of dietary oligosaccharides on intestinal and portal plasma fermentation characteristics in growing pigs. Source: Oligostroop® (uncharacterized transgalacto-oligosaccharides; Borculo Whey Products, Borculo, The Netherlands)	Five groups of five 57 d old castrated pigs [(Great Yorkshire × Landrace) ♀ × (Great Yorkshire) ♂] were fed either a control diet or diet containing 7.5 or 15 g FOS/kg feed or 10 or 20 g GOS/kg feed for 44 d (42 d-46 d), after which pigs were slaughtered under anaesthesia and gut contents removed. Species: Pigs Dose of GOS: 1 or 2% GOS in diet Exposure period: 42-46 d	• Body weight and feed intake was lower in groups fed 2% GOS versus control diet. • Body weight gain per day was lower in both GOS groups versus control diet. • Fecal and ileal dry matter digestion were similar across all diet groups. • Fresh weight of caecal contents was greater in the 2 % GOS group versus control diet, and dry matter concentration for caecal contents was higher for 1% GOS vs. control. • GOS did not affect gut pH at any portion of the intestines measured, at either dietary dose level. • Mmol of volatile fatty acids measured in the total caecum section were higher in the 2% GOS group versus control. • Crude protein concentration in colon contents was higher in 1% GOS-fed pigs vs. control diet. • Crude protein pool in the total caecum section was higher for the 2% GOS-fed pigs vs. control. • Volatile fatty acid concentrations were unchanged in the portal plasma of pigs on either GOS diet, vs. control diet.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Morishita et al. 2002	To examine the effect of GOS in combination with *Bifidobacterium* and *Bacteroides* on the population of in the intestine of gnotobiotic mice. Source: GOS (unspecified; Snow Brand Milk Co., Tokyo, Japan)	Six wk old male germfree BALB/c mice were orally administered a mixture of *Escherichia coli* 128, *Enterococcus faecalis* 132, *Staphylococcus epidermidis* 24, and *Lactobacillus salivarius* ATCC11741, followed by *Clostridium perfringens*, *Bacteroides vulgates* YM-18, and *Bifidobacterium breve* YMM-1 sequentially over time while they were being fed either a control diet or a diet containing 5% GOS. Populations of the various bacteria were measured in the feces at 2 wk sampling intervals through wk 9. Species: Mice Dose of GOS: 5% GOS in diet Exposure period: 9 wk	• GOS stimulated growth of *Escherichia coli*, *Lactobacillus salivarius*, and *Bifidobacterium breve* versus control diet. • GOS inhibited growth of *Enterococcus faecalis* (weakly), *Staphylococcus epidermidis* (only transiently), and *Clostridium perfringens* (significantly), versus control diet. • GOS had less effect on *C. perfringens* populations when *C. perfringens* was introduced first to the germfree mice.
Zentek et al. 2002	To evaluate the intestinal effects of feeding various oligosaccharides to dogs. Source: GOS as Lactifit (uncharacterized; Borculo Whey Products, Borculo, The Netherlands)	Four adult female beagles (11.7 ± 2.2 kg) were fed a basal diet during two control periods. A 4 × 4 Latin square study design was utilized and four experimental diets were given: mannanoligosaccharides, GOS, lactose, and lactulose (1 g/kg bw/d per oligosaccharide treatment). Species: Dogs Dose of GOS: 1 g GOS/kg bw/d via diet Exposure period: 4 supplementation periods ×10 d/period	• GOS had no negative effect on stool quality. • GOS did not affect fecal fatty acid levels (data not shown), renal nitrogen, urea, or indican excretion. • GOS did not affect other measures of microbial metabolism, including changes in pH, ammonia levels, or gas volume in fecal cultures after 24 h exposure.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Chonan et al. 2001	To determine whether the action of intestinal bacteria is necessary for influencing the absorption of Ca and Mg in rats by feeding GOS with or without neomycin. Source: Unspecified Yakult *Bacillus circulans*-produced GOS product, consisting of: 47% trisaccharides, 32% tetrasaccharides, 20% penta-/hexasaccharides. *Formulation N, Category 3 (Figure 5)*	Twenty 6-wk old male F344 rats were fed a control diet (AIN-76) or diet formulated with 5% (w/w) GOS, 0.67% (w/w) neomycin sulfate or both, for 4+7 d (control diet followed by treatment diet). Five animals were utilized per treatment group. Deionized water was provided. Species: Rats Dose of GOS: 5% GOS in diet Exposure period: 7 d	• 5% GOS-fed group: -- Had decreased Ca and Mg excretion compared with control group. -- Had increased Ca and Mg absorption compared with control group. -- Had increased whole cecal and cecal tissue weight compared with control group. -- Had decreased cecal pH compared with control group. • 5% GOS plus neomycin group: -- Had Ca, Mg excretion values intermediate between control and GOS-only groups. -- Had Ca, Mg absorption values similar to control group, suggesting that presence of intact gut flora is necessary for GOS-associated Ca and Mg uptake.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Houdijk et al. 1999	To investigate the effects of feeding FOS and GOS on nutrient digestion in growing and weanling pigs. Source: Oligostroop® GOS-rich syrup (Borculo Whey Products, Borculo, The Netherlands)	*Study #1:* Twenty-five 57 d old growing castrated [Great Yorkshire × Landrace sires × Great Yorkshire dams] pigs were fed a control diet or diet containing 6.8 or 13.5 g FOS/kg feed or 4.0 or 8.0 g GOS/kg feed (5 pigs/diet). Feces were collected from d 28-32 and small intestinal digesta were harvested on d 42-47 (end of study). Species: Pigs Dose of GOS: 0.4 or 0.8% GOS in diet Exposure period: 42-47 d *Study #2:* Twenty 38 d old weanling pigs (gender unspecified) were fed a control diet from d 10 until weaning, followed by either control diet or diet containing 10 or 40 g FOS/kg feed or 10 or 40 g GOS/kg feed, at four pigs/diet. Feces and urine were collected on d 13-17 and ileal digesta were sampled via a postvalve T-cecum cannula on d 33-37. Nitrogen and mineral balances were carried out. Species: Pigs Dose of GOS: 0.4 or 0.8% GOS in diet Exposure period: 33-37 d	*Study #1:* • Feed intake and feces production were similar across all treatment groups, vs. the control diet. • All measured digestive parameters were unchanged on GOS diets vs. the control diet. *Study #2:* • Feed intake and feces production were similar across all treatment groups, vs. the control diet. • Certain parameters for apparent ileal digestion were increased in the high-GOS-fed, but not low-GOS-fed group, vs. control: dry matter, organic matter, hemicelluloses, cellulose, nonstarch neutral-detergent soluble carbohydrates. • Nitrogen, calcium, phosphorus, magnesium, iron, copper and zinc balance were unchanged in GOS-fed groups vs. control diet group.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Houdijk et al. 1998	To study the effects of dietary FOS and GOS on growth and fecal characteristics of young growing pigs. Source: Oligostroop® GOS-rich syrup produced from whey (75% dry matter by weight; 40% GOS, unclear if this refers to a dry or wet weight basis; Borculo Whey Products, Borculo, The Netherlands)	Fifty-six 38 d old castrated male piglets [(Great Yorkshire × Landrace) ♂ × (Great Yorkshire)] received control diet for 19 d and were then ranked by body weight to exclude six pigs having the lowest body weight. The remaining 50 piglets were divided into weight classes and randomized to one of five diets (10 pigs/diet) for six weeks: control diet, diet containing 7.5 or 15 g FOS/kg feed or diet containing 10 or 20 g GOS syrup/kg feed. [It is likely that the GOS dose is the same as in Houdijk et al. 1999: 4 or 8 g GOS/kg feed or 0.4% and 0.8% in the diet.] Species: Pigs Dose of GOS: 1 or 2% GOS syrup in diet Exposure period: 6 wk	• GOS syrup-fed pigs had lower mean daily weight gain and daily dry matter intake vs. control pigs; the effect was most pronounced during the first week on diet and disappeared over time. • GOS syrup-fed pigs had no significant weight differences from control-fed pigs by the end of the 6 wk study. • GOS syrup did not affect fecal pH. • Dry matter fecal contents were slightly lower in GOS syrup-fed pigs vs. control-fed pigs by 6 wk.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Sakaguchi et al. 1998	To compare the energetic characteristics of various indigestible oligosaccharides with that of sucrose in rats. Source: Unspecified Yakult 6'-GOS product, consisting of: 1% disaccharides, 48% trisaccharides, 37% tetrasaccharides, 13% penta-/hexasaccharides, <5% moisture. Production organism unknown. *Formulation Q, Category 3 (Figure 5)*	Thirty-five male Wistar rats having a mean body mass of 70 g were divided into five groups of 7 rats/group and fed either a basal diet (no oligosaccharides), a basal-restricted diet, or a diet consisting of 10% (w/w) sucrose, 10% FOS or 10% GOS for 50 d. Species: Rats Dose of GOS: 10% GOS in diet Exposure period: 50 d	• 10% GOS-fed group: -- Had no difference in feed intake, weight gain or feed efficiency versus control group. -- Had overall increased levels of short-chain acids and fatty acids versus control group. -- Had reduced body fat content of linoleic and oleic acids compared with the control group. -- Had increased body fat content of myristic acid compared with the control group. -- The overall unsaturated:saturated fatty acid body content was reduced in rats fed GOS, versus control. However, the same effect was observed in rats fed 10% fructooligosaccharides (FOS). This result is a physiological effect due to an overwhelmingly large amount of SCFA that is produced by fermentation in the gut to produce acetate and butyrate. The increased levels of available acetic (GOS) and/or butyric (FOS) acids result in an altered state of *de novo* cholesterol and triacylglycerol synthesis that proceeds at an unusually high rate.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Djouzi and Andrieux 1997	To study the effects of various oligosaccharides on intestinal bacterial metabolism in gnotobiotic rats (inoculated with human fecal flora). Source: GOS (unnamed product; BioEurope, Toulouse, France), 1% monosaccharides, 9% disaccharides, 33% tri- to hexasaccharides, 57% tetra- to heptasaccharides. Unspecified Yakult "TOS" product having 1-3'-, 1-4'-, 1-6'-oligosaccharides, and consisting of: 1% mono-/disaccharides, 48% trisaccharides, 37% tetrasaccharides, 13% penta-/hexasaccharides. Production organism unknown. *Formulation Q, Category 3 (Figure 5)*	Twenty-four 2.5-mo old germ-free male Fischer 344 rats were fed a sterilized control diet for 1 wk, then inoculated with human fecal flora. Two weeks post-inoculation animals were divided into four groups of 6 rats each. Groups were fed a sterilized control diet, or diet containing 4% GOS, FOS or TOS for 4 wk adaptation, followed by a 4 d respiratory study. Species: Rats Dose of GOS: 4% GOS in diet Exposure period: 4 wk + 4 d	• 4% GOS-fed rats had lower plasma cholesterol levels vs. the control group. • No changes in intestinal microflora were observed for GOS-fed rats vs. controls. • The following enzymes were increased in GOS-fed rats vs. controls: -- β-galactosidase -- α-glucosidase -- β-glucosidase • β-glucuronidase was decreased in GOS-fed rats vs. controls. • GOS-fed rats produced less hydrogen and more methane, vs. control-fed rats. • GOS-fed rats had significantly lower cecal pH values and higher levels of acetate and propionate vs. controls. Cecal weight was also significantly increased vs. controls. • 4% TOS-fed group: -- Had lower plasma cholesterol versus the control group. -- Had significantly higher intestinal populations of *Bifidobacterium* microbes versus the control group. -- Had significantly higher beta-galactosidase activity versus control group. -- Produced significantly higher amounts of methane and hydrogen compared with controls. -- Exhibited higher cecal weights versus controls. -- Had lower cecal pH versus controls. -- Increased production of acetate, butyrate, iso-acids and ethanol versus controls. --Excreted less ammonia versus controls.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Kikuchi-Hayakawa et al. 1997	To study the physiological and metabolic effects of GOS supplementation in rats. Source: Unspecified Yakult *Aspergillus oryzae/Streptococcus thermophilus*-produced GOS product = 48% trisaccharides, 37% tetrasaccharides, 13% penta-/hexasaccharides. *Formulation R, Category 3 (Figure 5)*	*Experiment #1*: Twelve 7 wk old male Wistar rats were fed a control diet for 10 d and then were randomized into two groups of six rats each. Groups were fed the control diet or a 5% GOS diet for 16 d. Fecal samples were taken at 15 d and cultured for total bacteria. The cecum was harvested. *Experiment #2*: Forty-eight 7 wk old male Wistar rats were fed a control diet for 10 d and then randomized into eight groups of six rats each. Groups were fed either control diet or 5% GOS diet for 1, 2, 7 or 21 d. Cecal contents were batch-cultured and pH of contents was measured. Species: Rats Dose of GOS: 5% GOS in diet Exposure period: 21 d (longest)	• (Expt #1): 5% GOS-fed group: -- Cecal contents contained higher acetic, propionic, and butyric, and less isobutyric, acids versus controls. • (Expt #2): 5% GOS-fed group: -- At all time points (1, 2, 7, 21 d), GOS-fed rats had greater weight of cecum contents, cecal tissue, and percent fecal water versus controls. The difference was greatest at 21 d. -- Production of propionic and butyric acids were increased in GOS-fed groups versus controls; production of acetic acid was less consistent. Differences between GOS-fed and non-GOS-fed groups disappeared when bacterial batch cultures were fed additional GOS *in vitro*. -- Cecal pH was slightly decreased in all GOS-fed groups versus controls.
Chonan et al. 1996	To study the effect of GOS on magnesium (Mg) absorption and storage and calcium/phosphorus toxicity in Mg-deficient male Wistar rats. Source: Unspecified Yakult *Bacillus circulans*-produced GOS product = <1% monosaccharides, <1% disaccharides, 47% trisaccharides, 32% tetrasaccharides, and 20% penta- and hexasaccharides. *Formulation N, Category 3 (Figure 5)*	Researchers induced Mg deficiency by over-feeding calcium (Ca) and phosphorus (P) to male Wistar rats and then attempted to mitigate the resulting toxicity by feeding 5% GOS. Twenty-four 4 wk old rats were adapted to a standard diet for 7d, then randomized to new diet for 28d. The "control" diet was purposefully overloaded with Ca and P. Feed consumption and body weight were recorded, blood samples were taken, and a the cecum was harvested. Species: Rats Dose of GOS: 5% GOS in diet Exposure period: 28 d	• Excess P, Ca plus 5% GOS-fed group: -- Had normalized absorption and distribution of Mg: reduced fecal excretion, increased absorption and apparent absorption ratio, increased femur and serum Mg. • Exhibited reduced toxicity compared with the control diet containing excess Ca and P: reduced kidney weight, reduced phosphorus and calcium content of the kidney, and reduced calcium content of the heart. • Had increased whole cecal weight, cecal tissue weight, decreased cecal pH, and increased production of short chain fatty acids compared with both the standard and P, Ca-overloaded diets.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Chonan and Watanuki 1996	To study the effect of GOS on calcium (Ca) absorption and bone mineralization in rats adapted to various levels of dietary Ca. Source: Unspecified Yakult *Aspergillus oryzae/Streptococcus thermophilus*-produced 6'-GOS containing 55% trisaccharides, 33% tetrasaccharides, and 12% penta- and hexasaccharides. *Formulation T, Category 3 (Figure 5)*	Four-week old male Wistar rats were fed one of four experimental diets containing high or low Ca (0.5g Ca/100g diet; 0.05g Ca/100g diet) with or without GOS (5g GOS/100g diet) for 30d. Feed intake and body weight were recorded, fecal samples were collected, and the cecum and right femur and tibia were harvested at the end of the study. Species: Rats Dose of GOS: 5% GOS in diet Exposure period: 30 d	• General observations for 5% GOS groups: -- No effect on body weight, feed intake or feed efficiency. -- Increased whole cecal, cecal tissue and cecal digesta weight. -- Increased production of acetic, propionic, butyric, succinic and lactic acids, regardless of Ca status. • Normal Ca diet with 5% GOS, vs. control: -- Early but transient decrease in the fecal excretion of Ca. -- Increased Ca content of femur and tibia. -- Increased Ca content of both liquid and solid phases of the cecum, while simultaneously increasing the %Ca partitioning into the liquid phase. • Low Ca diet with 5% GOS, vs. control: -- No effect of GOS on fecal excretion of Ca. -- No effect on Ca content of femur or tibia.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Kikuchi et al. 1996	To study the effect of TOS on bacterial glycolytic activity, fermentation and bacterial steroid transformation in gnotobiotic rats. Source: Unspecified Yakult *Aspergillus oryzae/Streptococcus thermophilus*-produced "TOS" product containing 48% trisaccharides, 37% tetrasaccharides, and 13% penta- and hexasaccharides. *Formulation S, Category 3 (Figure 5)*	Twelve 8 wk old germ-free Male Fischer 344 rats were inoculated with human fecal flora and acclimated to a control diet for 2 wk. Then rats were assigned to one of three groups for 4 wk: control diet, 5% TOS, or 10% TOS. Feed intake and body weight were recorded, feces were collected, and blood lipids, gas production, bacterial enzyme activity and metabolite production was measured. Species: Rats Dose of GOS: 5 or 10% GOS in diet Exposure period: 4 wk	• General observations for TOS groups: -- Both TOS groups (5 and 10%) increased hydrogen production. -- No changes in feed intake, body weight, or fecal excretion, compared with control. -- Increased activity of beta-galactosidase and a decrease in beta-glucosidase enzyme activity. -- Increased caecal weight. -- Decreased caecal pH. -- Increased production of short chain fatty acids. -- Decreased ammonia production. -- Decreased content of lithocholic acid (bile acid) in feces. --Decreased content of coprosterol (steroid) in feces. • 10% TOS group: -- Decreased beta-glucuronidase activity. -- Increased lactic and succinic acid production. -- Decreased valeric and caproic acid production. -- Increased beta-muricholic acid in feces. -- Increased content of cholesterol and its metabolites in feces.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Chonan et al. 1995	To study the effect of dietary GOS (5g/100g) on calcium absorption in ovariectomized (OVX) Wistar rats. Source: Unspecified Yakult *Bacillus circulans*-produced 6'-GOS product containing <1% monosaccharides, <1% disaccharides, 47% trisaccharides, 32% tetrasaccharides, and 20% penta- and hexasaccharides. *Formulation N, Category 3 (Figure 5)*	Thirty-six 4 wk old female Wistar rats were divided into two groups; eighteen were ovariectomized while the rest were sham-operated. Rats were fed either AIN-76 diet (control) or control diet containing 5g GOS/100g for 30d. Body weight and feed intake was recorded and fecal and blood samples were taken. The cecum was harvested. Species: Rats Dose of GOS: 5% GOS in diet Exposure period: 30 d	• 5% GOS: -- Had no effect on body weight or feed intake for sham-operated rats; slight decrease in feed intake in OVX group. -- Increased whole cecal weight, cecal wall weight and cecal contents in both sham-operated and OVX rats, compared with the control diet. -- Reduced serum cholesterol in the OVX group, compared with control diet. This effect was not observed in sham-operated rats. -- Caused an initial, short-lived decrease in fecal Ca excretion that disappeared by the end of the study. -- Preserved calcium content of the tibia, but not femur, in OVX rats after 30 d. Sham-operated rats had increased calcium content of the femur but not tibia. -- Increased production of short chain volatile fatty acids in the cecum, and reduced the cecal pH in both OVX and sham-operated mice, versus control diet.
Chonan and Watanuki 1995	To study the effect of GOS (5 or 10g/100g) in the diet of rats on calcium balance and calcium absorption in the cecum. Source: Unspecified Yakult *Aspergillus oryzae/Streptococcus thermophilus*-produced GOS product containing 55% trisaccharides, 33% tetrasaccharides, and 12% penta- and hexasaccharides. *Formulation P, Category 3 (Figure 5)*	Four-week old male Wistar rats were fed a diet containing GOS (5 or 10g/100g) for 10d, followed by assessment of Ca absorption. Calcium absorption was also studied in an *in situ* ligated cecum model. Body weight and feed intake was measured and fecal and urine samples were collected. The cecum was harvested. Species: Rats Dose of GOS: 5 or 10% GOS in diet Exposure period: 10 d	• 5% GOS and 10% GOS groups: -- Had no effect on body weight, feed intake or feed efficiency. -- Had significantly increased whole cecal, cecal wall and cecal digesta weights. -- Had decreased cecal pH. -- Had increased Ca absorption and retention. • *In situ* ligated cecum model: -- GOS caused a mild elevation in serum calcium after 4 h incubation. -- GOS caused increased production of acetic and butyric acids by 2 h post-injection. -- GOS caused an increase in Ca in the liquid phase of the cecal lumen that was sustained for 4 h post-injection.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Meslin et al. 1993	To study the effects of trans-galactosylated oligosaccharides (GOS) on both mucin distribution in the gastrointestinal mucosa and bacterial metabolism. Source: Unspecified Yakult GOS product	Three-month old germ-free (GF), conventional (CV) and heteroxenic (HE; human fecal flora-inoculated) male inbred F344 rats (four rats/group) were fed a control diet or one containing 40 g trans-galactosylated oligosaccharide (GOS)/kg for one month. Metabolic and histological endpoints were measured. Species: Rats Dose of GOS: 40 g GOS/kg feed (4% GOS) Exposure period: 1 mo	• GOS: -- Had greatest ability to modulate mucins in the colon of germ-free rats. Presence of any flora abolished the effect. Mucus-containing cells were decreased in the proximal colon and increased in the distal colon. -- Increased activity of beta-D-galactosidase in both CV and HE, but not germ-free, rats. -- Significantly increased production of both hydrogen and methane gas in HE rats. -- Mildly increased caecal pH in CV and HE rats. -- Increased short chain fatty acid production in CV rats.
Rowland and Tanaka 1993	To study the effect of a diet containing 5% TOS with or without *Bifidobacterium breve* (synbiotic diet) on the caecal bacteria in rats colonized with human microflora. Source: Unspecified Yakult GOS product containing 50% trisaccharides, 35% tetrasaccharides, 14% penta- and hexasaccharides and 1% lactose. Production organism unknown. *Formulation U, Category 3 (Figure 5)*	Male and female gnotobiotic Lister Hooded rats were studied in three groups: control diet, diet containing 5% TOS (w/w), and diet containing 5% TOS plus *Bifidobacterium breve* at 10^9/mL in drinking water. After four weeks, caecal samples were tested for pH, protein concentration, bacterial numbers, nitrate reductase, beta-glucosidase, beta-glucuronidase and ability to convert 2-amino-3-methyl-7H-imidazo[4,5-*f*]quinoline (IQ) to its reactive genotoxic metabolite 7-hydroxy-2-amino-3,6-dihydro-3-methyl-7H-imidazo[4,5-*f*]quinoline-7-one (7-OHIQ). Species: Rats Dose of GOS: 5% GOS in diet Exposure period: 4 wk	• 5% TOS-fed rats, vs. controls: -- Had decreased caecal pH and decreased Enterobacteria counts. -- Had increased total anaerobe, Bifidobacteria, and Lactobacilli counts. -- Increased beta-glucosidase activity. -- Decreased beta-glucuronidase and nitrate reductase activities. -- Had somewhat decreased conversion of IQ into 7-OHIQ in the caecum contents.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Hayashi et al. 1991	To investigate the effect of different levels of GOS on the bioavailability of sodium and potassium in rats. Source: GOS as "4'-GL" = *O*-β-D-galactopyranosyl-(1-4)-*O*-β-D-galactopyranosyl-(1-4)-D-glucopyranose: > 95% galacto-oligosaccharides, 0.5% glucose, 0.5% galactose, 1% lactose (Nishin Seito Kaisha, Tokyo, Japan)	Eighteen male Wistar rats (65-75 g; age not stated) were randomized to three groups (6 rats/group): control diet, 10% (w/w) 4'-GOS trimer (4'-GL) GOS diet, or 20% GOS diet. Three 3 d metabolic balance studies were conducted during 62 d of feeding, on d 30-32, 45-47 and 60-62. Species: Rats Dose of GOS: 10 or 20% GOS in diet Exposure period: 62 d	• Rats fed a 20% GOS diet had lowered body weight gain, vs. 10% GOS or control. Final body weight was slightly decreased in 20% GOS-fed rats vs. control (not statistically significant). • Feed intake was similar across all groups during all three balance periods. • Liver and stomach weights were significantly decreased (absolute wt and as % bw) in 20% GOS group vs. control. • Large intestine and cecum weights were significantly increased as % bw for 20% GOS vs. control. Cecum weight was also increased for 10% GOS vs. control (absolute and % bw). • Fecal wet weight was significantly greater in both GOS groups vs. control during all balance periods. • Urinary volume was decreased in the 20% GOS group during the first two balance periods and in the 10% GOS group during the middle balance period. • Fecal sodium excretion was substantially increased in the 10 and 20% GOS groups, vs. control. • Apparent sodium absorption was somewhat decreased in GOS-fed rats during two balancing periods. • Fecal potassium excretion was significantly increased in the 20% GOS group during all three balancing periods, and was increased in only one balancing period for the 10% GOS group. • Urinary potassium excretion was reduced in the 20% GOS group during all three balancing periods, and in the 10% GOS group for one period. • Apparent potassium absorption was decreased in the 20% GOS group across the three balancing periods. • 20% GOS caused significant decreases in potassium content of large intestine and sodium in the cecum, vs. control.

Table V-3. Summary of GOS Studies in Animals

Reference	Objective/Test Article	Study Design	Effect
Ohtsuka et al. 1991	To study the metabolism and distribution of orally administered ^{14}C-labelled GOS in rats. Source: 14C-labelled GOS produced from [*U*-^{14}C] lactose fermented by *Cryptococcus laurentii* OKN-4 and fractionated to produce [*U*-^{14}C] *O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose ["4'-GL"].	Conventional SD male rats (~230 g; age not provided) were fed either control diet or diet containing 5% 4'-GOS trimer (4'-GL) for 2 wk and then dosed with radiolabeled 4'-GL or lactose. Several conventional male rats were given penicillin G and chloramphenicol (50 units/mL and 50 µg/mL in drinking water, respectively) for a week prior to dosing with radiolabeled 4'-GL or lactose and were treated similarly as conventional rats. Germ-free Wistar male rats (~80g) were also used in radioligand studies. Experiments were carried out under conventional conditions. Species: Rats Dose of GOS: 5% GOS in diet Exposure period: 2 wk	• When given 0.5 mL of [U-14C]4;-GL (37 kBq/0.16 mg 4'-GL) in saline via a gastric tube, the relative ranking for expired total $^{14}CO_2$ (within 24 h) was as follows: conventional > antibiotic-treated >> germ-free rats. [Note that the dose of the radioligands may not have been adjusted for the decreased mass of the germ-free rats. It was also unclear as to whether germ-free rats were acclimated to the 4'-GL diet prior to the study.] • Conventional rats metabolized and excreted nearly half of the labeled 4'-GLvia expired CO_2. Less than 5% was excreted in the urine or feces, 8.5% remained in the intestine, 1.43% in liver, 0.62% in serum and 12.83% in carcass. Researchers accounted for 81.20% of the total dose of labeled 4'-GL given. • Germ-free rats exhibited less fecal excretion and significantly higher intestinal retention (58.31%) of labeled 4'-GL versus the conventional rats.
Ohtsuka et al. 1990	To study the effect of chronic ingestion of GOS on body weight gain, organ weight, serum lipids and liver lipids in the rat. Source: "4'-GL" GOS produced from lactose fermented by *Cryptococcus laurentii* OKN-4 and fractionated to produce [*U*-^{14}C] *O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose ("4'-GL").	Male SD rats (3 wk) were fed either control diet or diet containing 5% GOS, 10% GOS, 10% lactose, or 10% lactulose for 6 wk (6 rats/group). Body weight, feed intake, serum and liver lipids were measured, fecal samples were taken and body organ weights were measured at the end of the study. Species: Rats Dose of GOS: 5 or 10% GOS in diet Exposure period: 6 wk	• Net body weight gain was increased in the 10% GOS group vs. control. Feed conversion ratio was decreased vs. control. • Feed intake and fecal weight was increased in both 5 and 10% GOS groups vs. control. • Feeding 5 and 10% GOS caused a significant increase in cecum and colon wet weight as a percent of body weight, vs control. Cecal contents and feces weights were similarly increased. • GOS had no effect on serum or liver lipid levels. • GOS was stable to human and porcine α-amylase, and artificial gastric juice at pH 1.0-2.0. • Feeding GOS decreased duodenal maltose and sucrose saccharidase activities, and increased ileal GOS and lactose saccharidase activities.

4. Summary of Adult Studies of GOS

Refer to Table V-4 for summaries of the studies detailed in this section.

Studies of GOS indicate that it is indigestible and survives the gastrointestinal tract until it reaches the colon, where it is fermented by Bifidobacteria, among other bacterial species, with the resultant formation of gas and/or short-chain fatty acids. Physiologically, GOS causes changes in fluid balance in the colon and GOS intake can result in softer stools, increased defecation frequency, occasional diarrhea, and changes in the absorption of minerals. In certain contexts, decreases in counts of pathogenic organisms are observed, which is expected due to competition by the increasing population of Bifidobacteria in the gut. Refer to Table V-4 for summaries of these studies.

Studies of Oligomate 55N/55NP

Four pivotal published clinical studies directly support the safety of Oligomate 55N GOS syrup in humans. When fed an appropriate carbohydrate substrate, the anaerobes living in the large intestine can generate hydrogen gas which is then absorbed into the blood circulation and transported to the lungs for excretion via the breath. Chonan and co-workers demonstrated the undigestability of Oligomate 55N by measuring an increase in hydrogen gas levels in the breath of subjects consuming GOS (Chonan et al. 2004). Sixteen healthy adults (8 women, 8 men; ages unstated) who did not experience uncomfortable subjective abdominal symptoms such as diarrhea after dairy product intake were divided into two groups and given either one dose of 34.7 g Oligomate 55N syrup (15 g as GOS) in 100 mL of water or a placebo beverage containing identical amounts of lactose, glucose, and galactose but no GOS. After one week washout, the subjects were crossed over to the other treatment. On the day of GOS syrup consumption, breath samples were collected before intake of the beverage and then every 30 min thereafter for a total of 8 h. Baseline values were subtracted from all measurements and the hydrogen concentrations were summed to calculate the total breath hydrogen excretion. A questionnaire was also filled out by the subjects to record their defecation and abdominal symptoms within 24 h of beverage intake. Subjects were instructed to avoid intake of large amounts of milk, fermented milk and oligosaccharide-containing foods prior to the test. Breath hydrogen concentrations were significantly higher ($P < 0.05$; $n = 16$) in the GOS group (303 ± 229 Δ ppm/8 h), versus placebo (5 ± 7 Δ ppm/8 h), beginning at 1.5 h after syrup intake, peaking at 3-4 h, and then gradually tapered off toward the end of the sampling period (after ~ 6h). Subjective abdominal symptoms were reported by five subjects consuming GOS, but not by any placebo-administered subjects. Reported symptoms included gurgling sounds ($n = 5$; statistically significant at $P < 0.05$),

flatulence (n = 1), and increased flatus (n = 4). There was no significant difference in either the frequency of defecation or stool characteristics after intake of the test beverage; mushy and watery stools were not reported. Oligomate 55N (37.4 g syrup containing 15 g GOS) causes an increase in breath hydrogen within 1.5-2 h and creates flatus within 24 h, indicating its indigestibility until it reaches the colon.

Deguchi and co-workers (Deguchi et al. 2003) investigated the effects of Oligomate 55N on bowel habits in healthy adults who had a tendency to be constipated. Subjects were recruited via a survey and considered elegible according to the following criteria: women defecating ≤ 4 times a week, who consumed three meals per day for at least 4 days/week, had infrequent consumption of yogurt or fermented milk drinks, did not regularly use cathartics and other drugs, and who had poor stool consistency; men who defecated ≤ 5 times/week and defecated on ≤ 4 days/week. Sixty-two women (17-29 yr) and ten men (19-45 yr) were enrolled. After a one-week pre-intake period, subjects were assigned to either a 5.0 g GOS (12.1 g Oligomate 55N syrup) section or a 2.5 g GOS (6.1 g Oligomate 55N syrup) section. Each section was further divided into subgroups to receive either GOS or placebo. There were a total of four groups per gender that received either placebo, 2.5 g GOS/beverage, or 5.0 g GOS/beverage daily for two weeks. There was an interim wash-out period of two weeks, after which subjects were crossed over and given the alternate treatment for a further two weeks. Subjects completed a 24-h recall questionnaire to report their defecation frequency, stool color, flatus frequency and abdominal symptoms such as distention and borborygmi. They also were instructed to measure the amount of stool and record its color and consistency, and document use of any medicines and to make note of any particular physical conditions. Subjects were directed to avoid excessive intake of fermented soybeans and milk products. For data analysis, the first week of the non-intake intake period was considered to be a wash-out period so only data from the second week of non-intake were used to stratify subjects into subgroups based on their defecation frequencies. Ten subjects were excluded from the study on the basis of 1) antibiotic use (n = 3), 2) daily defecation frequency (not constipated; n = 3), 3) not completing the initial questionnaire (n = 3), and 4) not consuming the test article (n = 1). Fifty-two women and ten men were included in the final analysis. No clinically significant abnormalities were reported. In the total study population there was a higher defecation frequency during the first week of the 5.0 g GOS intake period compared with the second week of the non-intake period. However, the defecation frequency during the first week of the 5.0 g GOS intake period and the first week of the placebo intake period did not differ significantly. In subjects having baseline defecation frequency of 3-5 times/week, consumption of 5.0 g GOS/day (12.1 g Oligomate 55N syrup/d) significantly increased their defecation frequency and number of days of defecation/week versus placebo

treatment. In severely constipated subjects having a baseline defecation frequency of 2 times/week or less GOS did not improve the defecation frequency versus placebo; nor did defecation frequency increase in persons having baseline frequency of 6 times/week or more. Consuming 2.5 g GOS/day (6.1 g Oligomate 55N syrup/d) did not have a significant effect on defecation frequency versus placebo for the total study population. Regarding improvement in stool consistency (softening), there was no difference between intake of 5.0 g GOS versus placebo for all subjects. However, in six subjects having poor baseline stool consistency (mostly scybala), there was a significant increase in the number of banana-like and semi-solid stool during consumption of 5.0 g GOS, verus control beverage. Frequency of flatus significantly increased during the 5.0 g GOS intake period versus placebo intake, suggesting that GOS remains undigested until it reaches the colonic microflora. Oligomate 55N increases defecation frequency at the 5.0 g GOS/d (12.1 g Oligomate 55N syrup) level in individuals with a defecation frequency of 3-5 times/week.

Kimura and co-workers (Kimura et al. 2004) determined the no effect level (NOEL) and the 50% effective dose (ED_{50}) of Oligomate 55N for the onset of diarrhea in men and women. Twenty-four adult men and women who were free of gastrointestinal disease, did not have a propensity for diarrhea and who had not experienced diarrhea and abdominal pain after intake of 200 mL milk/day were recruited. During the six-week study period, consisting of a one-week preliminary period, followed by a five-week intake period, the subjects consumed one GOS syrup-containing beverage per week, in order of increasing dose, over the course of the study. Oligomate 55N syrup was administered at 11.7, 23.3, 46.7, 70.0, and 93.3 g (5.1, 10.1, 20.1, 30.3, and 40.1 g GOS, respectively) in a beverage. Subjects completed a questionnaire every day to document their defecation frequency, stool consistency and diarrhea incidence, physical conditions and any unusual life events, in addition to noting any adverse effects and their frequency. Diarrhea was defined as "watery stool occurring from the intake of the test beverage until 6:00 on the following morning." The NOEL and ED_{50} for diarrhea onset was determined from a plot of the cumulative incidence (%) of diarrhea versus the minimum intake of GOS syrup per body weight required to induce diarrhea (g/kg) in subjects who experienced it. The x-intercept was calculated to be the NOEL and the dose that caused a cumulative incidence of diarrhea of 50% was considered the ED_{50}. The NOEL was estimated to be 0.68 g syrup/kg (0.30 g GOS/kg) and the ED_{50} was calculated at 1.39 g syrup/kg (0.60 g GOS/kg). Men and those having a high initial defecation frequency ($\geq$ 7/wk) were noted to be more sensitive to diarrhea caused by GOS intake. Other side effects of GOS intake included borborygmus, flatus, flatulence, abdominal discomfort and pain, nausea, and diarrhea. The first three symptoms occurred at or above the threshold of 0.7 g syrup/kg (0.29 g GOS/kg). The authors did not find

an association between age and the threshold dose of GOS which caused diarrhea, nor was there an association between gender and the occurrence of diarrhea at increasing doses. Among the 16 subjects experiencing diarrhea, the dose at which the first episode emerged was significantly higher in women than in men. In most subjects, the diarrhea resolved within 5-10 hr and disappeared by the end of the day of intake. There was no association between diarrhea duration and GOS dose. The NOEL for onset of diarrhea by Oligomate 55N is 0.68 g syrup/kg (0.30 g GOS/kg) and the ED_{50} to cause diarrhea is 1.39 g syrup/kg (0.60 g GOS/kg).

In the Kimura study, investigators also examined the subchronic toxicity of consumption of 15 g GOS/day (36.4 g Oligomate 55N syrup/d), versus placebo, in twenty healthy adults for 2 weeks. According to the authors, 15 g GOS is three times the effective dose for improvement of bowel movement. The subchronic study consisted of a one-week observational period, a two-week intake period, and a one-week post-intake observational period. Daily questionnaires covered the same questions as in the first study, and the incidence of mushy and watery stools was tracked. One subject was excluded due to taking antibiotics during the intake and post-intake period. Another subject in the GOS group withdrew from the study due to having a cold and being in poor physical condition during the pre-intake period; this person experienced flatulence, abdominal pain on day 1 of intake and had mushy stool and chills 20 h later. The symptoms resolved within 24 h and the subject was included only in the analysis of mushy and watery stools. Incidence of mushy and watery stools and stool consistency did not differ between the placebo and GOS groups during the intake period. There was no difference between placebo and GOS for flatus, gurgling sounds, and flatulence incidence during the intake period, although flatus increased with GOS intake. Besides the one subject who discontinued the study, there was no difference in the frequency of non-abdominal symptoms between the placebo and GOS groups.

Matsumoto and co-workers studied the effects of Oligomate 55N GOS syrup on human intestinal microflora (Matsumoto et al. 2004). Total bacteria count and *Bifidobacterium* count in the feces was measured in forty volunteers (mean age 37 ± 9 yr; range: 25-59 yr). Twenty-two subjects (mean age = 39 ±10 yr) having low Bifidobacteria counts of $10^{9.4 \pm 0.3}$ CFU/g feces and low total percent Bifidobacteria (mean = 13 ± 7%) were randomized into two groups of 11 each and fed 2.5 or 5.0 g GOS (6.1 or 12.1 g Oligomate 55N syrup, respectively) in a 110 mL beverage daily for 2 wk after a 2 wk pre-intake period. The study was 6 wk total, including a 2 wk post-intake period. Subjects were instructed to avoid excessive intake of fermented milk products, fermented soybeans, foods containing dietary fiber and oligosaccharides, and excessive alcohol use. Fecal samples were collected at the end of the 2 wk pre-intake period, during week 1 and 2 of the intake period, and at the end of the 2 wk post-intake period to measure the levels of

fecal flora, organic acids, putrefactive metabolies, bile acids, β-glucuronidase activity, water content and pH. A questionnaire was used to document intake of the test beverage, foods, medications and daily changes in physical conditions. The *Bifidobacterium* count increased significantly in the GOS groups at one and two weeks of feeding, versus the pre-intake period, and the magnitude of the increase was greater in the 5.0 g GOS (12.1 g Oligomate 55N syrup) group versus the 2.5 g (6.1 g Oligomate 55N syrup) group. Measured levels of fecal short-chain fatty acids actually decreased somewhat after consumption of 5.0 g GOS/d (12.1 g Oligomate 55N syrup/d). However, it has been noted that the majority of SCFAs generated in the colon by gut flora are absorbed by the local environment, making it challenging to detect changes in SCFAs in feces (Macfarlane et al. 2008). A significant decrease in fecal levels of succinate was observed in the 5.0 g GOS (12.1 g Oligomate 55N syrup) group at one week of feeding. Water content of feces decreased in the 5.0 g GOS (12.1 g Oligomate 55N syrup) group at two weeks of feeding, but the effect was transient and disappeared by the post-intake period. Levels of putrefactive metabolites did not change significantly in either GOS group, versus the pre-intake period, nor did β-glucuronidase levels or fecal pH. Total bile acids were significantly decreased at one week of feeding in the 5.0 g GOS (12.1 g Oligomate 55N syrup) group, versus the pre-intake period. These results demonstrate the fermentability of Oligomate 55N GOS syrup by Bifidobacteria which is an indirect measure of its indigestibility in humans. Oligomate 55N/55NP is Bifidogenic in humans at levels of 2.5 and 5.0 g GOS/d (6.1 and 12.1 g Oligomate 55N syrup).

Studies of other GOS products

Numerous additional studies of GOS corroborate its safe consumption in adults. Ito and co-workers (Ito et al. 1990, 6'-GOS: *ibid* 1993a) found that Oligomate 50 at 2.5, 5.0 and 10.0 g GOS/d increased fecal Bifidobacterial counts in adults. Oligomate 50 at 5.0 and 10.0 g GOS/d also caused increased feelings of fullness and gas (Ito et al. 1990), indicating its indigestibility until it reaches the colon. A different Yakult 6'-GOS formulated at 15 g GOS/d in iced tea was demonstrated to increase fecal counts of Bifidobacteria and Lactobacilli, decrease fecal pH, and decrease *Candida* counts (Ito et al. 1993b). Bi^{2}muno 3'-/4'-/6'-GOS caused an increase in Bifidobacteria and decreased fecal populations of *Bacteroides* spp., *Clostridium histolyticum* group, *Escherichia coli* and *Desulfovibrio* spp., versus placebo, when given at 2.64 g GOS/d in elderly subjects (Vulevic et al. 2008). Elderly subjects consuming Bi^{2}muno also had favorable changes in the activity and numbers of their phagocytosing leukocytes, including natural killer cells, and neutrophils, and exhibited cytokine levels that were skewed toward a less inflammatory state. Elix'or elicited an increase in breath hydrogen levels when dosed at 14.4 g GOS/d in healthy adults (Alles et al. 1999). These particular GOS formulations are chemically

similar to Oligomate 55N/55NP as described in Figure 5. However, these GOS products are not only chemically similar to Oligomate 55N/55NP; they exhibit similar physiological effects as well, as Oligomate 55N also stimulates the growth of Bifidobacteria (Matsumoto et al. 2004), increases abdominal gas (Chonan et al. 2004, Deguchi et al. 2003, Kimura et al. 2004) and increases the level of breath hydrogen (Chonan et al. 2004). Taken together, these studies demonstrate the chemical and physiological equivalence of Oligomate 55N/55NP with the abovementioned GOS formulations, and demonstrate their safety and tolerability even in susceptible adult populations such as the elderly.

Additional studies of other GOS formulations support its safe use in both healthy and health-compromised or sensitive adult populations. Many of these studies did not characterize their GOS test substances as completely as investigators did in the studies described above, but the large body of evidence resulting from use of these GOS formulations in adults provides further support for their safety and tolerability in the general population.

In healthy adults, GOS can stimulate the growth of Bifidobacteria at doses of 2 g GOS/d (as a synbiotic in combination with *Lactobacillus rhamnosus* GG; Kekkonen et al. 2007), 2.4 g GOS/d (Gopal et al. 2003), 2.5 g GOS/d (Bouhnik et al. 2004), 3.36 g GOS/d (Depeint et al. 2008), and 10 g 6'-GOS/d (Bouhnik et al. 1997), depending on the formulation. A synbiotic containing 2 g GOS/d plus *Lactobacillus rhamnosus* GG caused more flatulence in subjects versus those consuming the probiotic organism alone (Kekkonen et al. 2007). Bouhnik and co-workers also reported that their 6'-GOS, when administered at 5 g 6'-GOS twice a day (10 g/d total), decreased hydrogen gas production which is in contrast to the majority of studies reporting on this endpoint (Bouhnik et al. 1997). In a single-dose experiment to measure the utilization of GOS by gut bacteria, Tanaka and co-workers observed a rapid increase in breath hydrogen production in five subjects receiving 0.5 g 6'-GOS/kg body weight (Tanaka et al. 1983). During a study of a GOS syrup being dosed at 8.1 g/d (3.65 g GOS/d), one subject in the GOS-only treatment group dropped out due to pain from intestinal bloating (Alander et al. 2001). Subjects consuming 10 g GOS/d for 18 days had improved constipation and reduced fecal *p*-cresol levels (a putrefactive metabolite) (Liu, et al. 1994; abstract only). Increased flatulence was noted in other studies testing the effect of various GOS formulations on bowel function at a dose of 15 g GOS/d, as divided into two (GOS syrup; Teuri et al. 1998) or three (van Dokkum et al. 1999) doses. Teuri and co-workers also noted an increase in defecation frequency during the GOS treatment period, versus during the run-in period (Teuri et al. 1998). The van Dokkum study reported increased fecal acetic acid production and decreased fecal dry weight, and no change in glucose tolerance, insulin response and serum lipids (van Dokkum et al. 1999). Another study of 15 g GOS/d in healthy males demonstrated increased fecal acetic acid levels, versus an

unsupplemented diet (van den Heuvel et al. 1998). These studies corroborate the finding that GOS is indigestible until it reaches the colon where it stimulates the proliferation of beneficial Bifidobacteria in the intestinal tract. During fermentation in the colon, the GOS is catabolized by the bacteria into hydrogen gas and short-chain fatty acids. These effects cause the physiological changes described above.

An interesting characteristic of GOS is its notable safety profile in health-compromised adult populations. In adult patients with severe systemic inflammatory response syndrome (SIRS) placed on synbiotic therapy (10 g GOS plus 3 g/d probiotic; Oligomate HP plus *Bifidobacterium breve* and *Lactobacillus casei*), fecal *Bifidobacterium* counts were significantly increased in the treatment group, versus untreated patients and healthy controls (Shimizu et al. 2009). Additionally, fecal acetic and propionic acids were significantly increased in the synbiotic treatment group, and the incidence of enteritis, pneumonia and bacteremia were reduced versus untreated controls. Biliary cancer patients having high-risk hepatectomy were given either a synbiotic formulation or placebo for two weeks post-operatively and assessed for various physiological effects (Kanazawa et al. 2005). Those on treatment received 12 g Oligomate 55/d plus 3 g/d of a probiotic mix via a feeding catheter. At the end of the study, *Bifidobacterium* and *Lactobacillus* counts were increased in the treatment group, versus control, and the following endpoints were also changed: decreased C-reactive protein (CRP) levels (a measure of systemic inflammation), decreased white blood cell counts, decreased fecal Enterobacteriaceae and *Candida* counts, increased fecal acetic, propionic and butyric acid concentrations, and significant reductions in infectious complications, postoperative hospital stay length and antibiotic use rates. In another study of biliary cancer patients undergoing high-risk hepatobiliary resection, 15 g Oligomate 55 GOS plus 3 g/d mixed probiotic was administered via feeding catheter either post-operatively (group A) or both pre- and post-operatively (group B), for two weeks per period (Sugawara et al. 2006). *Bifidobacterium* total counts were higher in group B versus group A, and group B exhibited lower interleukin-6 (IL-6; endogenous pyrogen) levels, white blood cell counts, and CRP levels versus group A. These subjects also had increased fecal acetic and butyric acid concentrations, lower incidence of postoperative infectious complications, shorter hospital stay, and less antibiotic use, versus the group treated post-operatively only. Adult irritable bowel syndrome (IBS) patients who consumed 3.5 or 7.0 g GOS/d exhibited increased fecal *Bifidobacterium* spp. counts (Silk et al. 2009). GOS at 3.5 g/d improved stool consistency, flatulence and bloating, and also improved the Likert scale (sum of several scored items, also called the summative scale which is a psychometric scale) and subjective global assessment (SGA) scores. 7.0 g GOS/d improved bloating and SGA scores.

A study in children and young adults, aged 2 y 6 mo to 24 y 8 mo, having short bowel and refractory enterocolitis administered 1 g GOS plus 1 g probiotic mixture three times a day for over one year and noted increased body weight gain in 6/7 patients, improved percent parental/enteral nutrition in 3 patients, dramatically reduced the fecal aerobes/anaerobes ratio, increased production of fecal short chain fatty acids, increased *Bifidobacterium* and *Lactobacillus* counts, and decreased *Candida* counts (Kanamori et al. 2004). Although no control group was included in this particular study, the fact that positive health outcomes were observed in such a vulnerable and at-risk population speaks to the safety of the GOS portion of the synbiotic therapy.

GOS products have also been tested in sensitive adult populations. Short-chain GOS/long-chain FOS (9:1) was given to pregnant women from gestation week 25 through delivery at 1.51 g GOS/FOS three times per day (total of 4.5 g GOS/FOS per day) in milk or water in order to test its effects on maternal and neonatal gut flora (Shadid et al. 2007). The GOS/FOS supplementation was well-tolerated and increased fecal Bifidobacteria counts in the women, but not in the neonates. GOS/FOS had no effect on bowel habits, stool frequency or consistency, or stool or vaginal pH. The neonates were breastfed in all the groups, including the placebo group, so any effect of GOS/FOS on fecal flora may have been masked. Van den Heuvel and co-workers administered escalating doses of Elix'or syrup, at up to 20 g GOS/d (44 g syrup/d), in two divided doses, to postmenopausal women for nine days and measured radiolabeled calcium absorption and excretion on day 8 (oral and intravenous administration of ^{44}Ca and ^{48}Ca, respectively) (van den Heuvel et al. 2000). Mean calcium absorption in postmenopausal women was increased 3.3% (statistically significant) in the GOS treatment period versus the reference period, whereas calcium excretion was unchanged. Thus, GOS may exert a physiological effect on calcium balance via increased absorption in the intestinal tract. Defecation frequency was increased in elderly women consuming 9 g GOS/d as Elix'or GOS syrup in yogurt which also contained *Lactobacillus acidophilus*, although it was not mentioned whether the cultures were still live at the time of consumption (Teuri and Korpela 1998). Adverse symptoms were similar during the run-in and GOS treatment periods, and included flatulence and abdominal distention. These demonstrations of the safety of GOS in pregnant women, postmenopausal women and elderly women further support its use in the general population.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Oligomate 55N/55NP (current product for GRAS)						
Chonan et al. 2004	To measure the digestibility of GOS by measuring hydrogen gas production.	8 F, 8 M (age ranges not provided)	Yakult Oligomate 55N GOS syrup (as described in Kimura et al. 2004)	15.0 g GOS, 3.3 g lactose, 6.1 g glucose, 2.2 g galactose	Single dose	• Breath hydrogen concentration was significantly increased 1.5-8 h after GOS intake. • GOS caused borborygmi, flatulence and flatus within 24 h after intake.
Deguchi et al. 2003	To investigate the effects of GOS on bowel habits.	52 F (17-29 yr), 10 (19-45 yr) M with constipation tendency	Yakult Oligomate 55N GOS syrup (as described in Kimura et al. 2004)	31 F, 5 M: 2.5 g or 5.0 GOS/d (cross-over study) vs. sucrose-matched controls	1 wk survey 2 wk intake 2 wk non-intake 2 wk intake (cross-over)	• 5.0 g GOS/d increased defecation and flatus frequency, # days with defecation, and improved stool consistency.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Kimura et al. 2004	*Study #1*: Estimation of NOEL, ED_{50} for GOS-caused diarrhea. *Study #2*: Safety of subchronic daily intake of GOS.	*Study #1*: 12 women, 12 men; aged 20-58 yr, 39.0-96.0 kg. *Study #2*: 10 women, 10 men; age and weight unstated.	Yakult Oligomate 55N GOS syrup: 22.9% (w/w) glucose, 8.2% galactose, 12.5% lactose, 14.4% transgalactosylated disaccharides, 34.4% trisaccharides, 7.6% tetrasaccharides and larger oligosaccharides; in the form of a canned beverage.	*Study #1*: Escalating dose structure: 11.7, 23.3, 46.7, 70.0, 93.3 g syrup/190 mL beverage (5.1, 10.1, 20.1, 30.3, and 40.1 g GOS/190 mL beverage, respectively), administered once per week; one dose per week. *Study #2*: 15 g GOS/100 mL (36.4 g syrup/100 mL) beverage daily.	*Study #1*: 6 wk: 1 wk survey period, followed by 5 wk intake period; one dose per week in escalating dose regimen, versus placebo beverage. *Study #2*: 2 wk study; one dose daily for entire study period, versus placebo beverage.	• *Study #1*: -- The estimated laxative threshold was 0.68 g syrup/kg (0.30 g/kg as GOS) for the entire population. Men: 0.65 g syrup/kg (0.27 g GOS/kg); women: 0.90 g syrup/kg (0.37 g GOS/kg). -- ED_{50} for diarrhea onset was 1.39 g syrup/kg (0.60 g/kg as GOS) for the entire population. Men: 1.13 g syrup/kg (0.47 g GOS/kg); women: 1.50 g syrup/kg (0.62 g GOS/kg). -- Men in general and subjects with a higher defecation frequency (≥ 7 times/wk) were more sensitive to the induction of GOS-associated diarrhea. -- Reported abdominal symptoms included borborygmus, flatus, flatulence, abdominal discomfort and pain, nausea, and diarrhea. • *Study #2*: -- No differences in incidence of loose stools or diarrhea were observed between treatment and control groups. -- GOS-consuming subjects experienced increased flatus versus control.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Matsumoto et al. 2004	To measure the effect of a GOS beverage on intestinal flora.	22 volunteers with "low" total and relative *Bifidobacterium* counts were enrolled (gender unspecified; mean age 39 ± 10 y). 11: 2.5 g GOS/d; 11: 5.0 g GOS/d.	Yakult Oligomate 55N GOS syrup (as described above in Kimura et al. 2004)	11 subjects: 2.5 g GOS/d (6.1 g syrup/d) 11 subjects: 5.0 g GOS/d (12.1 g syrup/d)	2 wk pre-intake 2 wk intake 2 wk post-intake	• Both GOS doses increased total anaerobe and *Bifidobacterium* counts. • No major effects on fecal organic acids for GOS groups. -- Decrease in succinate for 5.0 GOS (12.1 g syrup) group. • Transient decreases in fecal water content and total bile acids in 5.0 g GOS (12.1 g syrup) group.
Oligomate 55 (older formulation)						
Sugawara et al. 2006	To investigate the effect of synbiotics on intestinal, immunological and general outcomes in biliary cancer patients undergoing high-risk hepatobiliary resection.	Adults: 101 patients enrolled; 81 completed study: *Group A*: 23 M/17 F *Group B*: 23 M/18 F	Yakult Oligomate 55 (unspecified formulation)	15 g Oligomate 55/d, plus 3 g/d Yakult BL Seichōyaku (*Lactobacillus casei* strain Shirota, *Bifidobacterium breve* strain Yakult), administered via feeding catheter.	*Group A*: 2 wk post-operative *Group B*: 2 wk pre-operative and 2 wk post-operative	• Group B exhibited lower IL-6 (endogenous pyrogen), white blood cell (WBC) counts, and C-reactive protein (CRP) levels, versus Group A. • *Bifidobacterium* counts were higher in Group B vs. Group A at 1 d before hepatectomy. • Group B had increased acetic and butyric acid fecal concentrations vs. Group A after hepatectomy. • Group B had significantly lower incidence of infectious complications, postoperative hospital stay, and cumulative length of antibiotic therapy, vs. Group A.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Kanazawa et al. 2005	To investigate the effect of synbiotics on intestinal integrity and microflora and surgical outcome in patients undergoing high-risk hepatectomy.	Adults: 54 biliary cancer patients enrolled; 44 completed study: 21 (15 M/6 F) synbiotics, 23 (14 M/9 F) controls	Yakult Oligomate 55 (unspecified formulation)	12 g GOS/d, plus 3 g/d Yakult BL Seichōyaku (*Lactobacillus casei* strain Shirota, *Bifidobacterium breve* strain Yakult), administered via feeding catheter.	2 wk post-operative	• Significant reductions in C-reactive protein (CRP) levels and white blood cell counts at post-operative day10 were observed in GOS group, vs. control. • *Bifidobacterium* and *Lactobacillus* counts were increased in GOS group, vs. control; effect was sustained for 2 wk post-intervention. Enterobacteriaceae and *Candida* counts were similarly decreased. • Fecal acetic, propionic, and butyric acid concentrations were increased in GOS group. • Striking reductions in infectious complication occurrences, postoperative hospital stay lengths, and antibiotic use rates were noted in GOS group vs. control.
Oligomate 50 (older formulation)						

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Kanamori et al. 2004	To treat short bowel patients with refractory enterocolitis using synbiotic therapy.	Children and young adults: 7 patients having short bowel and refractory enterocolitis: 5 M/2 F (2-24 yr); no control group	Yakult Oligomate GOS (content unspecified) plus Yakult Honsya Biolactis® Powder (*Bifidobacterium breve Yakult:B. breve* and *Lactobacillus casei Shirota:L. casei*)	[(1 g GOS + 1 g Biolactis) × 3]/d	> 1 yr (15-55 mo)	• Body weight gain (kg/yr) increased in 6/7 patients. • Increased prealbumin levels in 6/7 patients. • Improved percent parental/enteral nutrition in 3 patients. • Increased Bifidobacterium and Lactobacillus counts; decreased *Candida*. • Dramatically reduced fecal aerobes/anaerobes ratio. • Increased short chain fatty acid production in feces.
Ito et al. 1993a	To investigate the effects of GOS on fecal microflora and their metabolism.	28 healthy M recruited (35-67 yr; mean 50 yr): retained 12 M (35-55 yr; mean 49 yr) with low bifidobacteria counts; no control group.	Yakult Oligomate 50 (Syrup containing 52% (w/w) GOS: 38% monosaccharides, 10% lactose, 16% disaccharides, 24% trisaccharides, 10% tetrasaccharides, 2% penta-/hexasaccharides)	2.5 g/d (6.7 g Oligomate 50 syrup) in apple juice	3 wk	• GOS significantly increased fecal Bifidobacteria counts after 1 wk and persisted through 3 wk, compared with pre-feeding counts. • GOS reduced levels of nitroreductase at 1-3 wk and indole at 3 wk, vs. the pre-feeding period. • Isovaleric acid was reduced at 2-3 wk in GOS group, vs. the pre-feeding period.
Ito et al. 1990	To investigate the effects of GOS on fecal microflora.	12 healthy M (26-48 yr)	Yakult Oligomate 50 (content described above in Ito et al. 1993)	0, 2.5, 5.0, or 10.0 g GOS	7 d/treatment; Latin square cross-over study with 1 wk washout between each treatment	• GOS increased fecal Bifidobacteria and Lactobacilli counts, and increased the relative ratio of Bifidobacteria to total bacteria • GOS at 5.0 and 10.0 g/d increased feelings of fullness and wind. 10.0 g/d caused a slight increase in abdominal pain.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Oligomate HP (Older formulation)						
Shimizu et al. 2009	To evaluate the effects of synbiotic therapy on deteriorated gut flora and environment.	Adults: Patients with severe systemic inflammatory response syndrome (SIRS): 14 M/15 F (55 ± 21 yr) synbiotic therapy, 20 M/6 F (55 ± 16 yr) previously untreated patients, and 14 healthy controls.	Yakult Oligomate HP (unspecified GOS formulation) plus Yakult BL Seichoyaku (*Bifidobacterium breve* and *Lactobacillus casei*)	10 g GOS/d plus 3 g probiotic/d	Study lasted 40 d; unclear whether subjects were dosed daily.	• *Bifidobacterium* counts (selective media plate culture) were significantly increased in fecal flora, versus untreated and normal controls. • Acetic and propionic acids were significantly increased in GOS group, versus untreated controls. • Incidence of enteritis, pneumonia and bacteremia were reduced in GOS group, versus untreated controls.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Other GOS formulations						
Silk et al. 2009	To investigate the ability of GOS to alter colonic microflora and symptoms of IBS patients. Single center, parallel crossover, controlled study	Adult IBS patients: 18-80 yr: 60 randomized to study, 44 completed study. IBS-diarrhea dominant: 9F/14M (mean ages 44, 47 yr, respectively) IBS-constipation dominant: 8F/1M (42, 61 yr) IBS-diarrhea/constipation alternating: 11F/1M (56, 61yr)	*Bifidobacterium bifidum* NCIMB 41171-produced GOS (from lactose): Mixture of 48% (w/w) galacto-oligosaccharides with degree of polymerization 2-5, 22% lactose, 18% glucose, 12% galactose. Patients received either (study period #1/study period #2): 1) Placebo/3.5 g GOS (n = 16) 2) Placebo/7.0 g GOS (n = 14) 3) Placebo/Placebo (n = 14)	3.5 or 7.0 g GOS/d, unspecified as to whether this is the crude mixture or oligosaccharides delivered.	2 wk baseline period 28d study period #1 14 d washout 28 d study period #2	• Both 3.5 and 7.0 g GOS/d increased fecal *Bifidobacterium* spp. • Fecal *Clostridium perfringens-hystolyticum* subgroup and *Bacteroides-Prevotella* spp. were decreased in the 7.0 g GOS group, whereas fecal *Eubacerium rectale/Clostridium coccoides* was increased in the 3.5 g GOS group. • 3.5 g GOS/d improved stool consistency, flatulence, bloating, Likert scale, and subjective global assessment (SCA) scores. • 7.0 g GOS/d improved bloating and SGA scores.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Depeint et al. 2008	To assess the prebiotic potential of a novel GOS. Randomized, blinded, placebo-controlled studies. *Study #1:* Evaluation of GOS and its matrix for prebiotic effects. *Study #2:* Evaluation of the dose-response of GOS for prebiotic effects.	Healthy subjects: *Study #1:* 13M/16F (mean age = 32.5 yr) *Study #2:* 12M/18F (mean age = 36.3 yr)	*Study #1:* "V-GOS" = Vivinal GOS: 57% galacto-oligosaccharides, 23% lactose, 22% glucose, 0.8% galactose (*Bacillus circulans*-produced) *Study #2:* "B-GOS" = Novel GOS produced by *Bifidobacterium bifidum*: 48% galacto-oligosaccharides, 22% lactose, 18% glucose, 12% galactose	*Study #1:* 15 g fat-filled milk powder (FFMP; 35% lactose, 28% vegetable fat, 26% protein, 5.5% minerals, 2% sucrose, 0.25% lecithin, 3% moisture) or 15 g FFMP + 7 g V-GOS (4.0 g GOS) *Study #2:* 15 g FFMP + 7 g sucrose 15 g FFMP + 7 g B-GOS (3.36 g GOS) 15 g FFMP + 3.6 g B-GOS (1.73 g GOS)	*Study #1:* 7d run-in 7d treatment 7d washout 7d treatment *Study #2:* 7d run-in 7d treatment #1 7d washout 7d treatment #2 7d washout 7d treatment #3	*Study #1:* • 7 g V-GOS (4.0 g GOS) + FFMP caused an increase in the *Bifidobacterium* and a decrease in the *Bacteroides-Prevotella* populations. *Study #2:* • 3.6 g B-GOS (1.74 g GOS) + 3.4 g sucrose + FFMP increased *Bifidobacterium* and *Clostridium perfringens-hystolyticum* subgroup counts. • 7 g B-GOS (3.36 g GOS) + FFMP increased *Bifidobacterium* counts. • There was a dose-response between B-GOS dose and amount of increase in *Bifidobacterium* counts vs. matrix (FFMP)-treatment alone.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Vulevic et al. 2008	To measure the ability of GOS to modulate various immune parameters and fecal flora composition in the elderly. Double-blind, placebo-controlled, crossover study	Elderly: Forty-four subjects: 16 M/28 F (mean age = 69.3 ± 4.0 yr)	Bi^{2}muno: 48% (w/w) total galacto-oligosaccharides, present as: 52% dimers, 26% trimers, 14% tetramers, 8% pentamers (mainly β1→6 linkages [51%], plus β1→3 [26%] and β1→4 [23%])	5.5 g Bi^{2}muno/d (2.64 g GOS) or 5.5 g/d maltodextrin (placebo)	10 wk treatment 4 wk washout 10 wk treatment (cross-over)	• By 10 wk, GOS increased fecal populations of *Bifidobacterium* spp., *Lactobacillus-Enterococcus* spp., and *Clostridium coccoides-Eubacterium rectale*, vs. placebo. • By 10 wk, GOS decreased fecal populations of *Bacteroides* spp., *Clostridium histolyticum* group, *Escherichia coli*, and *Desulfovibrio* spp., vs. placebo. • GOS had no effect on serum LDL or total cholesterol levels. • GOS treatment significantly increased activity of natural killer (NK) cells in peripheral blood. • GOS increased IL-10 levels [anti-inflammatory cytokine] and decreased IL-1β, TNF-α, and IL-6 levels [inflammatory cytokines] in peripheral blood mononuclear cells vs. placebo. • GOS significantly increased the numberof phagocytosing cells in response to *Escherichia coli* and the phagocytic capacity per cell.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Kekkonen et al. 2007	To investigate the ability of GOS + probiotic ("synbiotic") vs. probiotic alone to affect the intestinal microbiota of healthy subjects. Randomized, double-blind, cross-over study	Forty-four healthy adults (mean age = 43.3 yr): 9 M/32 F	*Lactobacillus rhamnosus* GG alone or combined with GOS (uncharacterized; Valio Ltd, Helsinki)	2 g/d in 65 mL milk-based fruit juice	4 wk run-in 3 wk treatment 4 wk washout 3 wk treatment	• Probiotic + GOS stimulated growth of fecal Bifidobacteria more than treatment with probiotic alone. • Incidence of flatulence, abdominal pain, abdominal distension and heartburn were similar across treatment groups. • Subjects consuming probiotic + GOS experienced more flatulence than those taking probiotic alone.
Shadid et al. 2007	To test how supplementation of GOS and long-chain FOS in the last trimester of pregnancy affects maternal and neonatal gut microbiota, and whether fetal immunological parameters are modulated. Randomized, double-blind, placebo-controlled pilot study	Pregnant women: Forty-eight subjects (18-45 yr) were randomized to treatment: GOS/lcFOS, *n* = 17; placebo, *n* = 16 finished the study	Short-chain GOS/long-chain FOS (9:1): 45.45% short-chain GOS, 4.91% long-chain FOS, 14.39% glucose, 15.15% lactose, 19.4% maltodextrin, 0.76% galactose	[(3 g GOS/lcFOS preparation) × 3]/d, dissolved in 200 mL water or milk. Actual GOS/lcFOS content = (1.51 g GOS/lcFOS)*3/d .	Wk 25 gestation to delivery	• GOS/lcFOS supplementation was well tolerated and increased Bifidobacteria, but not lactobacilli, in maternal stool samples. Maternal fecal flora changes were not mirrored in the neonates. • GOS/lcFOS had no effect on bowel habits, stool frequency/consistency, or stool or vaginal pH. • In neonates, neither total bacterial counts nor percent bifidobacteria and lactobacilli showed any significant differences between treatment groups at any time point, but bifidobacteria and lactobacilli increased over time. However, all neonates in the study were breastfed.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Bouhnik et al. 2004	To determine the bifidogenic potential of various nondigestible carbohydrates (NDC) in the diet. Double-blind, randomized, placebo-controlled	Two hundred healthy adults: *Study #1:* Sixty-four subjects of 8 subjects/group (8 groups) consumed 10 g/d of one of seven NDCs or placebo *Study #2:* 136 subjects of 32/group (4 groups) consumed various NDCs determined to be bifidogenic in Study #1; groups were divided into subgroups of 8 subjects/subgroup and assigned to a dose of 2.5, 5.0, 7.5 or 10 g per NDC. 8 subjects were assigned to placebo as a separate group.	GOS as Cup-oligo P (uncharacterized; Niossin Sugar MFG, Tokyo, Japan)	*Study #1:* 10 g GOS/d, divided into two 5 g doses *Study #2:* 2.5, 5.0, 7.5, or 10 g GOS/d, divided into two doses	*Study #1:* 7 d run-in 7 d treatment *Study #2:* 7 d run-in 7 d treatment	*Study #1:* • GOS supplementation significantly increased fecal bifidobacteria counts vs. placebo. • GOS caused a non-significant increase in excess flatus vs. placebo. *Study #2:* • No dose-response was observed for GOS in relation to increasing numbers of either Bifidobacteria or total anaerobes, vs. placebo. Minimal effective dose is 2.5 g GOS/d.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Tannock et al. 2004	To study the impact of consuming FOS or GOS-supplemented biscuits on the fecal microflora. Double-blind, cross-over study	Fifteen healthy subjects (7 M/8 F, ages not stated), divided into 3 groups and fed GOS- or FOS-supplemented or control biscuits in sequential order (cross-over).	GOS as Oligovite (uncharacterized; Fonterra)	2.5 g GOS/d	3 wk treatments with 2 wk washouts in-between and at study's end	• Populations of lactobacilli, lactose-fermenting enterobacteria, total enterobacteria, and enterococci did not differ throughout the study (data not shown), as measured by selective culture. • Alterations to bacterial DNA-DGGE (PCR-denaturing gradient gel electrophoresis) profiles as a result of supplementing the diet were not observed. • Analysis of RNA-DGGE results by the authors noted differences between treatment groups that may be attributed to increased metabolic activity of various bacteria. • Identical strains of fecal Bifidobacteria had drastically differing interindividual abilities to utilize various Oligovite components (disaccharides, trisaccharides, tetrasaccharides/larger oligomers), as measured by selective culture of feces from GOS-fed subjects.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Gopal et al. 2003	To study the effects of consumption of milk-derived oligosaccharides on the composition of gastrointestinal flora. Randomized, double-blind, placebo-controlled study	Thirty healthy subjects (20-60 yr; 18 M/12 F) were divided into three groups (10/group): Control, Probiotic-supplemented, and GOS-supplemented	GOS as BP*Oligo* (8% galacto-oligosaccharides (w/w) containing mainly monosaccharides, disaccharides and trisaccharides) in 250 mL reconstituted milk	2.4 g GOS/d	2 wk run-in 4 wk treatment 2 wk washout	• One dropout due to upset stomach (control group). • Microflora from the GOS-treated group contained increased amounts of Bifidobacteria at 14, 21, and 28 d and Lactobacilli at 28 d, versus that of the control group. • Subjects with lower initial counts of Lactobacilli showed a more significant increase after dietary intervention in comparison with those having higher initial Lactobacilli counts.
Malinen et al. 2002	To measure changes in Bifidobacteria populations during and after a feeding trial of GOS syrup or probiotic.	Thirty healthy subjects (3 M/27 F; mean age 32 yr) were divided into three groups: GOS (n = 8, 2 dropouts), GOS + probiotic (n = 10), or probiotic (n = 10).	GOS syrup added to yogurt. GOS not characterized; source not stated.	8.1 g GOS syrup/d	2 wk run-in 2 wk treatment 2 wk washout	• No changes in distribution of various *Bifidobacterium* species were observed in the GOS-only group after treatment. Measurements were carried out using a new PCR-ELISA method.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Alander et al. 2001	To study the effect of GOS supplementation on fecal microflora and on the survival of *Bifidobacterium lactis* Bb-12 in the gastrointestinal tract. Randomized, non-patient-blinded study	Thirty subjects (22-47 yr; 3 M/27 F) were randomized to one of three groups: GOS (n = 10), probiotic (n = 10), or GOS + probiotic (n = 10)	GOS as GOS syrup (containing 75% (w/v) solids, present as 60% (w/w) GOS, 20% (w/w) lactose, 19% (w/w) glucose, and 1% (w/w) galactose). GOS component uncharacterized; Borculo Domo Ingredients, The Netherlands.	8.1 g GOS syrup/d (3.65 g GOS/d)	2 wk pre-feeding (non-supplemented, diet acclimation) 2 wk treatment 2 wk washout	• One subject in the GOS group dropped out due to pain from intestinal bloating. • There was a slight, non-significant, increase in fecal Bifidobacteria in the GOS group in the post-feeding perios vs. the pre-feeding period. • In the GOS group there was a slight, but significant, decrease in the number of fecal *Clostridium perfringens* post-feeding vs. pre-feeding.
van den Heuvel et al. 2000	To investigate the effect of GOS on calcium absorption in postmenopausal women. Double-blind, randomized, cross-over study	Postmenopausal women: Twelve healthy subjects (mean age = 55-65 yr) that passed a prechallenge with ~10 g GOS to assess tolerance. Subjects received both a placebo treatment and the GOS treatment.	GOS syrup as Elix'or in yogurt (Borculo Domo Ingredients, The Netherlands). GOS syrup contained 75% solids (w/v) which are composed of 60% GOS (w/w), 20% lactose (w/w), 19% glucose (w/w), and 1% galactose (w/w).	Maximal 20 g GOS/d (44 g Elix'or syrup/d) (5 d of every 9 d treatment period), divided into two doses per day	9 d treatment, including 5 d dose escalation for diet acclimation (^{44}Ca and ^{48}Ca administered orally and intravenously, respectively, on d 8 of each treatment period) 19 d washout 9 d treatment	• Calcium absorption was increased in the GOS treatment period vs. the reference period, whereas calcium excretion was unchanged.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Alles et al. 1999	To evaluate the effect of two doses of GOS on the composition and metabolic activity of intestinal microflora. Placebo-controlled, patient-blinded study	Forty-one subjects (22 M/19 F) were divided into three groups: Placebo (*n* = 13; 7 M/6 F; mean age = 37.8 yr), Low-GOS (*n* = 13; 7 M/6 F; mean age = 36.5 yr), or High-GOS (*n* = 14; 8 M/6 F; mean age = 42.9 yr)	GOS syrup as Elix'or (Borculo Domo Ingredients, The Netherlands) in fruit juice. GOS syrup contained 75% solids (w/v) which are composed of 62% GOS (w/w) [32% disaccharides, 35% trisaccharides, 23% tetrasaccharides, 8% pentasaccharides, 2% hexasaccharides and higher], 20% lactose (w/w), and 18% monosaccharides (mainly glucose).	Divided into three doses per day: Low-dose GOS: 8.5 g GOS/d High-dose GOS: 14.4 g GOS/d	3 wk run-in 3 wk treatment	• One female subject withdrew due to personal reasons; data are excluded from final analyses. • Breath hydrogen increased in the high-GOS group vs. low-GOS and control groups. • Nitrogen density in fecal matter was increased in the high-GOS group vs. control. • No changes in fecal concentrations of short-chain fatty acids were observed across groups during the intervention period. • No changes in bile acid, ammonia, indole or skatole concentrations were observed across groups during the intervention. • There was no treatment effect on composition of intestinal microflora; however, bifidobacterial counts increased by 4%, independently of diet.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
van Dokkum et al. 1999	To study the effect of GOS on large bowel function, blood lipid concentrations and glucose absorption. Latin square, randomized, double-blind, controlled study	Twelve healthy male subjects (mean age = 23 yr) assigned to: Inulin, FOS, GOS, or Control group	GOS (85% (w/w) GOS, 3.3% (w/w) glucose/fructose/sucrose, 0.3% (w/w) galactose, 10.7% (w/w) lactose, and 1% (w/w) ash). GOS source unidentified.	15 g GOS/d, divided into three doses	4 × 3 wk treatment periods --Subjects consumed dinner in a metabolic ward for 14 d, then lived in the ward the final 7 d during treatment periods	• Increased flatulence was reported in the inulin, FOS and GOS groups. • 15 g of inulin, FOS, or GOS was generally well-tolerated. • Percent fecal dry weight was decreased in the GOS group relative to control. • No change in fecal bile acid or neutral steroid content was observed for the GOS group, vs. control. • Acetic acid concentration was increased in the GOS group, vs. control. • Hydrogen production was only slightly increased at 240 min post-ingestion for the GOS group, vs. control. • Glucose tolerance, insulin response and serum lipids were not affected by GOS.
Teuri et al. 1998	To study the effect of GOS on fecal frequency and gastrointestinal symptoms.	Twelve healthy subjects (3 M/9 F; mean age = 38 yr, 25-55 yr) were all fed GOS-containing yogurt.	GOS syrup (uncharacterized; Valio Ltd., Helsinki, Finland) in yogurt.	15 g GOS/d, divided into two doses	1 wk run-in 2 wk treatment	• Defecation and flatulence frequency increased during the GOS treatment period. • There was a non-significant trend toward increased incidence of loose stools and abdominal distension during GOS treatment. • A consistent effect on fecal Bifidobacteria populations was not observed.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Teuri and Korpela 1998	To investigate whether GOS can relieve constipation in elderly people. Double-blind, two-period, cross-over study	Elderly: Fourteen female subjects (mean age = 79.6 yr) were randomized into blocks of four	GOS syrup as Elix'or (Borculo WP, The Netherlands) in yogurt, which also contained *Lactobacillus acidofilus*. GOS syrup contained 45% GOS, 25% water, 15% lactose, 14% glucose and 1% galactose.	9 g GOS/d, divided into two doses	1 wk run-in 2 × 2 wk treatments 1 wk follow-up	• Defecation frequency per week was higher during the GOS period than during the control period. • Flatulence and abdominal distention were similar during control and GOS treatment periods. • pH and dry weight of feces did not differ between groups. • GOS increased the ease of defecation, but the effect was not statistically significant.
van den Heuvel et al. 1998	To measure the effect of GOS on intestinal absorption of iron and calcium in men. Randomized, controlled, double-blind, cross-over study	Twelve healthy, nonanemic male subjects (20-30 yr) received four treatments on top of a constant basal diet: Unsupplemented (control), Inulin, FOS, or GOS.	GOS (85.7% GOS as disaccharides-hexasaccharides, 10.7% lactose, 3.3% glucose/fructose/sucrose, 0.3% galactose, 1% ash); source not stated.	15 g GOS/d	21 d per treatment (cross-over) --Iron absorption measured over last 7 d of treatment --Calcium absorption measured on d 21 of each treatment	• No significant differences in iron status or absorption were found among treatments. • Acetic acid was significantly increased in feces during GOS supplementation, but no change in fecal pH was observed (data not shown). • No effect of GOS on calcium absorption was found.
Bouhnik et al. 1997	To assess tolerance of GOS and effects of prolonged administration on colonic microflora.	8 healthy subjects: 4 M/4 F (20-32 yr); no control group	Yakult TOS (*Aspergillus oryzae*-produced, powdered, GOS: 48% trisaccharides, 37% tetrasaccharides, 15% pent-/hexasaccharides	(5 g) × 2/ d	21 d	• No change in stool weight, fecal water or pH. • Bifidobacteria counts increased. • Hydrogen gas production decreased.

Table V-4. Summary of Studies of GOS in Adults

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Liu et al. 1994	To study the effects of oligosaccharides on defecation and putrefactive metabolite production. *(Note: Article in Chinese; English translation not available. Information derived from abstract.)*	50 subjects: 5 groups of 10 subjects/group	Yakult GOS (unknown composition) and other oligosaccharide preparations (tested singly)	10 g/d	14 d run-in 18 d oligosaccharide supplementation	• GOS reduced fecal *p*-cresol content. • Constipation was improved.
Ito et al. 1993b	To investigate the effects of GOS intake on fecal microflora and their metabolism.	12 M healthy subjects (27-48 yr); no control group	Yakult GOS (*Streptococcus thermophilus*-produced: 79.3% disaccharides, 3.9% tri-/tetra-/penta-/hexasaccharides, 6.7% others, 9.1% lactose, 1.0% galactose + glucose)	15 g/d in 115 mL iced tea	6 d	• Bifidobacteria and Lactobacilli counts plus the ratio of Bifidobacteria:total bacteria increased. *Candida* spp. declined. • Fecal pH and ammonia, *p*-cresol and indole content were decreased. • Fecal propionic, isobutyric, isovaleric and valeric acid content decreased.
Tanaka et al. 1983	To study the effects of GOS on normal fecal flora.	Healthy subjects: 16 M (25-35 yr): 5 GOS-only, 6 *Bifidobacterium breve* only, 5 combination	Yakult GOS (*Aspergillus oryzae*-produced: 55% trisaccharides, 33% tetrasaccharides, 12% penta-/hexasaccharides), *Bifidobacterium breve* 4006, or combination of both	3 or 10 g/d Plus: single dose of 0.5 g GOS/kg body weight for hydrogen breath study	Escalating dose study: 1 wk run-in 1 wk 3 g/d 1 wk 10 g/d	• Rapid increase in breath hydrogen gas production in 5 subjects given 0.5 g GOS/kg body weight.

5. Summary of Infant Studies of GOS

Refer to Table V-5 for summaries of the studies detailed in this section.

Term infants

In term infants, the reported effects of GOS intake (mainly as GOS/FOS, 9:1; FOS = fructo-oligosaccharides) include increased gas, increased stool frequency and stool softness, and decreased colic episodes and regurgitation, among other measured endpoints. The majority of studies of GOS in infants were conducted using a 9:1 mixture of GOS/FOS that was added to infant formula at various concentrations. Although the use of a mixture of oligosaccharides confounds the interpretation of certain results, such as Bifidogenicity, as being due to GOS alone, a lack of adverse effects from consumption of the mixture supports the safety of the GOS portion. Refer to Table V-5 for summaries of the studies detailed below.

Four studies were retrieved that either used GOS alone at 0.24-0.7% (w/v) in formula or GOS in combination with polydextrose and/or lactulose in infant formula at 0.134-0.268% GOS (w/v). Nakamura and co-workers added polydextrose, GOS (as Vivinal), and lactulose anhydride (LOS) to Enfamil LIPIL with iron to investigate the effect of these substances on infant tolerance and fecal bacteria populations (Nakamura et al. 2009). Healthy term infants that were 13-92 days old received either formula (n = 105) or were breast-fed in a parallel group (n = 30). One infant in a treatment group did not consume the formula provided and was excluded from the final analysis. Another infant in the breast-fed group did not meet the gestational age requirement and was not included in intestinal bacterial composition, stool characteristics and tolerance analyses. The formula-fed infants were divided into four groups: 1) control (n = 25), 2) control formula plus 4 g/L of polydextrose (PDX):GOS (1:1) [2 g GOS/L; or, 0.2% GOS, w/v] (n = 27), 3) control formula plus 4 g/L of PDX:GOS:lactulose anhydride (LOS) (3:2:1) [1.34 g GOS/L; or, 0.134% GOS, w/v] (n = 27), and 4) control formula plus 8 g/L of PDX:GOS:LOS (3:2:1) [2.68 g GOS/L; or, 0.268% GOS, w/v] (n = 25). The study consisted of a 7 d run-in period followed by a 28 d feeding period. There were no differences in discontinuation between feeding groups; nor were there significant differences in the numbers of infants experiencing constipation or diarrhea during the run-in or feeding periods among feeding groups. Additionally, there were no clinically significant adverse events in any feeding group. Stool frequency did not change across the groups, nor did the amount of fussiness. Stool consistency increased in the supplemented formula groups versus control. Although this study was not designed to measure growth, groups 2 (0.2% GOS) and 4 (0.268% GOS) had significantly increased weight-for-age Z-scores versus control, at day 28. The increase was of the same magnitude as was noted for the breast-fed group versus control. However, the weight-for-age Z-score for group 4 (0.268% GOS) also differed

from that of the control group at the time of enrollment. Length- and head circumference-for-age Z-scores of groups 2-4 were not significantly different from the control group on day 28.

Ziegler and co-workers studied the effects of polydextrose (PDX), GOS, and lactulose (LOS), on stool consistency, weight gain and growth over 120 d in healthy term infants (Ziegler et al. 2007). Healthy infants born at > 37 wk gestational age, with birth weight ≥ 2500 g, and that had been solely formula-fed for at least 24 h prior to randomization, were enrolled in the study and assigned to one of three study formula groups. Infants received either 1) control formula (Enfamil LIPIL with iron), 2) control formula supplemented with 4 g of polydextrose (PDX) and GOS per L formula (50:50 ratio; 2 g PDX/2 g GOS per L; or, 0.2% GOS, w/v), or 3) control formula supplemented with 8 g of PDX, GOS and lactulose (LOS) per L formula (50:33:17 ratio; 4 g PDX/2.64 g GOS/1.36 g LOS per L; or, 0.264% GOS, w/v). Anthropometric measurements such as body weight, body length and head circumference were taken at visits conducted at 14, 30, 60, 90, and 120 d of age. Additionally, a 24-hr dietary recall and tolerance recall was completed by parents during visits. Of the 226 infants enrolled, 164 completed the study: 58 in the control group (group 1), 58 in group 2 (0.2% GOS), and 48 in group 3 (0.264% GOS). There were no statistically significant differences among the study groups for weight, length or head circumference at any time point. Supplemented formula groups had significantly looser stools than the control group, and supplemented formula groups exhibited a statistical difference from the control formula group in the incidence of diarrhea, eczema and irritability as adverse events. Intolerance to study formula was the most frequent reason for discontinuing the study. Specific reasons for discontinuance included gas (n = 21), fussiness (n = 16), vomiting (n = 12), and diarrhea (n = 7), with most incidents occurring in group 3. The intolerance symptoms reported may or may not be due, in part, to the GOS content of the formula, as up to two additional substances were supplemented at the same time. However, the GOS levels used in the Ziegler study, 0.2 and 0.264%, are small in comparison with other infant studies that reported good tolerance of GOS. In particular, GOS supplemented as GOS:FOS (9:1) supported normal growth in both term infants (12 wk) and term infants at high risk of allergic disorders (6 mo), respectively, when given at 0.72% w/v GOS in infant formula (Schmelzle et al. 2003; Moro et al. 2006).

Ben and co-workers investigated the effects of GOS on intestinal microbial populations and fermentation characteristics (Ben et al. 2004). Two hundred seventy-one term infants were assigned to either 1) Frisolac H formula (n = 52), 2) formula with 0.24 g GOS/100 mL (0.24% GOS, w/v) (n = 69), 3) breast milk (n = 26), or 4) breast milk plus formula with 0.24 g GOS/100 mL (0.24% GOS, w/v) (n = 124) for 6 mo. At 3 and 6 mo, infants on the GOS-supplemented formula had significantly higher fecal counts of *Bifidobacterium* and *Lactobacilli* spp. versus the control formula. Fecal short-chain fatty acid levels were significantly increased and pH values

were significantly decreased in GOS-fed infants versus control-fed infants. Incidences of crying, regurgitation and vomiting were similar across all groups and there were no differences in weight or length gains observed in groups 2-4.

Napoli and co-workers studied the ability of GOS-supplemented formula to stimulate growth of fecal Bifidobacteria in term infants (Napoli et al. 2003; abstract only). Term infants received either formula supplemented with 0.7% GOS [presumed %w/v] (n = 13) or formula supplemented with lactose as control (n = 13) for 21 d. Twenty-four breast-fed infants were followed as a reference group. GOS supplementation increased fecal Bifidobacteria to similar levels as those observed in the breast-fed group (statistically significant). Fecal pH at 21 d was significantly lower in GOS-fed infants versus control.

The above four studies demonstrate that GOS is indigestible until it reaches the colon in healthy term infants, where it is fermented by beneficial Bifidobacteria, causing a decrease in fecal pH. With or without combination with polydextrose and lactulose anhydride in infant formula, GOS was tolerated in this population. Two studies supported healthy growth and weight gain over six months at 0.24% GOS, w/v (GOS alone; Ben et al. 2004) and over four months at 0.2 and 0.264% GOS, w/v (GOS/PDX/LOS combination; Ziegler et al. 2007). The symptoms reported in the Ziegler 2007 study may or may not be due, in part, to the GOS content of the formula, as up to two additional substances were supplemented at the same time. However, the GOS levels used in the Ziegler study, 0.2 and 0.264% w/v, are small in comparison with other infant studies that report good tolerance of GOS. In particular, GOS supplemented as GOS/FOS (9:1) supported normal growth in both term infants over a 12 wk time period and term infants at high risk of allergic disorders over the course of 6 mo, when given at 0.72% w/v GOS in infant formula (Schmelzle et al. 2003; Moro et al. 2006).

Numerous studies have investigated the fermentative and physiological effects of supplementing infant formula with GOS/FOS (9:1), at concentrations varying from 0.36-0.72% GOS (w/v), versus administering control formula alone. Various GOS/FOS (9:1) combination formulations have been shown to increase Bifidobacteria counts in at least nine studies in term infants at concentrations of 0.4% (w/v) (0.36% GOS) (Moro et al. 2002; Rigo et al. 2001) for 28 d or 2 mo, 0.6% (0.54% GOS) (Bakker-Zierikzee et al. 2006) for 32 wk, and 0.8% (0.72% GOS) (Moro et al. 2006; Knol et al. 2005; Moro et al. 2005; Schmelzle et al. 2003; Moro et al. 2002) for 28 d up to 6 mo in formula and at up to 4.5 g GOS/FOS per day (Scholtens et al. 2006) for 6 wk versus control. Fecal pH was reduced in term infants consuming formula supplemented with GOS/FOS (9:1) at 0.6% (w/v) (0.54% GOS) (Bakker-Zierikzee et al. 2005) 16 wk and 0.8% (0.72% GOS) (Knol et al. 2005; Moro et al. 2002) for 28 d or 6 wk versus control. Stool frequency increased significantly at 0.4% (w/v) (0.36% GOS) (Chouraqui et al. 2008) for up to 4

mo and 0.8% (0.72% GOS) (Savino et al. 2003) GOS/FOS (9:1) for 14 d, and stool softness was increased at 0.4% (0.36% GOS) (Moro et al. 2002) for 28 d and 0.8% (0.72% GOS) for 28 d or 12 wk in formula (Schmelzle et al. 2003; Moro et al. 2002) versus control. Fecal short-chain fatty acids (bacterial metabolites) were increased at 0.8% GOS/FOS (9:1) (0.72% GOS) in formula, versus unsupplemented formula, in one infant study for 6 wk (Knol et al. 2005). Fewer colic episodes (Savino et al. 2006; Savino et al. 2003) at 14 d and regurgitation (Savino et al. 2003) at 14 d were reported in infants consuming GOS/FOS (9:1) at 0.8% (w/v) (0.72% GOS) versus control formula.

A few studies recorded data on safety and growth parameters in term infants fed GOS/FOS (9:1) supplemented formulas. Schmelzle and co-workers reported an increase in the following parameters in 76 infants consuming 0.8% GOS/FOS (9:1) (0.72% GOS, w/v) in formula, versus 74 consuming control formula, for 12 weeks: gas, body weight in females (transient; observed at 6 wk but not at 12 wk), head circumference in females, sum of skinfolds in males; these effects were accompanied by a decrease in formula intake and slightly higher drop out rate for GOS/FOS infants due to flatulence and stool quality (Schmelzle et al. 2003). In a smaller, uncontrolled study of fifteen infants fed formula containing 0.4% GOS/FOS (9:1) (0.36% GOS, w/v) from birth to two months of age, researchers observed similar growth rates compared with historical data on infants fed conventional formula or breast milk (Rigo et al. 2001). Lean body and fat mass compositions were similar between the experimental group and historical data for conventional formula-fed and breast-fed infants. In one of the larger controlled term infant studies carried out (284 infants enrolled and randomized, 227 completed and analyzed, 142 with follow-up data), Chouraqui and co-workers report that there was no significant difference in the frequency of serious adverse events or adverse events among groups consuming 1) control formula (n = 53), 2) formula plus *Bifidobacterium longum* BL999 and *Lactobacillus rhamnosus* LPR (n = 70), 3) formula plus *Bifidobacterium longum* BL999 and *Lactobacillus rhamnosus* LPR and 0.4% w/v GOS/FOS (9:1) (0.36% GOS, w/v) (n = 70), or 4) formula plus *Bifidobacterium longum* BL999 and *Lactobacillus paracasei* ST11 and 0.4% GOS/FOS (9:1) (0.36% GOS, w/v) (n = 74) for 4 mo. There were no differences in dropout rates among groups. Serious adverse events reported for the GOS/FOS groups included diarrhea (n = 1), vomiting (n = 2), surgery (n = 1), pyelonephritis (n = 1), bronchiolitis (n = 4) and inguinal hernia (n = 2). GOS/FOS (0.36% GOS) groups had no differences from the control group regarding weight gain, length, head circumference, or BMI parameters for either gender. This study included a 12 mo follow-up observation period, during which the frequency of antibiotic treatment or hospitalization rates did not vary among groups.

Positive outcomes in studies of GOS/FOS in term infants at high risk of allergic disorders are indicative of a good safety profile for GOS. Moro and co-workers investigated the long-term

effect of GOS/FOS on the incidence of atopic dermatitis in infants at high risk of atopy (Moro et al. 2006). In their six-month study, one hundred two infants were assigned to the prebiotic group (0.8% w/v GOS/FOS (9:1) (0.72% GOS, w/v) in hypoallergenic hydrolyzed cow milk whey protein-based formula) and one hundred four infants were assigned to placebo. Study drop outs were primarily due to the reestablishment of breast feeding by the infants' mothers. Stool frequency was increased and stool consistency was improved at 3 and 6 mo in the GOS/FOS group versus control. Ten infants in the GOS/FOS group developed atopic dermatitis, versus 24 in the placebo group. The degree of severity of the dermatitis was not related to the diet according to the authors. Episodes of regurgitation and crying were significantly reduced in the GOS/FOS group at 3 and 6 mo versus control. Rinne and co-workers assessed the effect of GOS/FOS on quantitative and qualitative differences in infant gut microbiota (Rinne et al. 2005). Thirty-two infants having one close relative with atopic eczema, allergic rhinitis or asthma were assigned to 1) control formula (n = 8), 2) formula + GOS/FOS (9:1) (n = 8), 3) breastmilk (n = 8), or 4) breastmilk + probiotic (the probiotic was started in the mother 2 wk prior to delivery; n = 8) for 12 mo. Unfortunately, the dose of GOS/FOS used in the formula was not stated. At the study's completion, the weight and length of the infants were comparable among all the feeding groups, suggesting the GOS/FOS-supplemented formula supported normal growth. Atopic eczema was more frequent in the unsupplemented formula group versus the others (statistically significant).

One term infant case report adds to the evidence base supporting the safety of GOS when used in synbiotic combination with probiotic mixtures. Tatekawa and co-workers treated an 8 mo old female having enteritis who underwent an extended myectomy-myotomy of the proximal jejunum for near-total intestinal aganglionosis with an unspecified dose of Yakult Oligomate HP, carrot powder containing *Lactobacillus coagulans*, plus *Bifidobacterium breve* Yakult [BBG-1] and *Lactobacillus casei* Shirota (Biolactis Powder) for an indefinite period of therapy (Tatekawa et al. 2007). Upon administering the synbiotic therapy, the authors found that stool flora shifted from methicillin-resistant *Staphylococcus aureus* to nonpathogenic bacteria, including *Lactobacillus* spp. At the time of the published report, the infant had likely been on the synbiotic therapy for at least 16 mo there was no incidence of enteritis central line infection and the infant was exhibiting good weight gain.

Preterm infants

In preterm infants, GOS/FOS has been shown to favorably modify physiological parameters such as stool viscosity, fecal frequency, fecal pH and gastrointestinal transit time. Mihatsch and co-workers assigned twenty preterm infants to either control formula (maltodextrin-supplemented) or GOS/FOS-supplemented formula (1% w/v plus maltodextrin)

for 14 d (Mihatsch et al. 2006). This particular GOS/FOS formulation was unknown as to its ratio of GOS:FOS. The GOS/FOS-supplemented group had significantly reduced stool viscosity, reduced intestinal transit time and reduced stool pH versus the placebo group. A study of 30 preterm infants investigated the influence of dietary oligosaccharides on calcium metabolism and fecal flora and stool characteristics (Lidestri et al. 2003; Boehm et al. 2002). Infants were assigned to either a control formula (n = 15) or formula containing 1% (w/v) GOS/FOS (9:1) (0.9% GOS, w/v) (n = 15) for 28 d. GOS/FOS had no influence on serum concentrations of calcium and phosphorus or alkaline phosphatase activity versus control; however, urine calcium tended to be higher in this group versus control (p = 0.055, nearly statistically significant). There was no difference between the groups for urine phosphorus levels. Weight and length gains during the study period were similar across the two groups. At 28 d, the numbers of fecal Bifidobacteria were significantly increased in the GOS/FOS group versus control. The number of stools and stool consistency for GOS/FOS-fed infants was intermediate between that of the control formula group and that observed in a breast-fed comparison group. One preterm infant case report was found for a 9 mo old female having laryngotrachoesophageal cleft (type IV) who was given 1 g Oligomate GOS plus 1 g Biolactis (*Bifidobacterium breve* Yakult plus *Lactobacillus casei* Shirota; 10^9) three times a day for 11 mo (Kanamori et al. 2002). Bowel movement was restored immediately after administration of the synbiotic therapy, fecal short-chain fatty acid content was increased by 1 mo post-therapy and body weight gain and serum choline esterase increased over time. The infant had satisfactory weight gain according to the study authors (data presented indicate an increase of growth rate post-intervention). These results support the safety of GOS in sensitive populations such as preterm infants and are suggestive of safe use in the general population.

GOS in infant formulas

Oligomate GOS has a long history of use in infant formulas in Japan. Its introduction into infant formulas can be traced back to the early 1980's (Mitsuhashi et al, 1982. Abstract only). GOS supplementation of infant formula was undertaken in an attempt to more closely mimic the health benefits of breast milk, which contains large amounts of galacto-oligosaccharides. Early researchers noticed that GOS-fed infants exhibited similar stool characteristics as those infants which were breast-fed. For example, Mitsuhashi et al (1982) found that the fecal characteristics of infants fed with 2% galactosyl lactose-supplemented (w/v) infant formula are similar to those of breast milk fed infants, which produce stools that are soft in consistency, sour or sweet in odor and deep yellow in color, suggesting that feeding infants GOS favorably affects their gut flora and the colonic fermentation characteristics. In contrast, the feces of infants fed with regular infant formula are slightly hard, have a more putrescent odor and are yellowish green or light yellow in color. Studies indicate that 0.24% (w/v) or 0.7% (w/v) GOS in infant formula for 6 mo

or 21 d, respectively, can statistically significantly increase fecal counts of Bifidobacteria and decrease fecal pH in term infants (Ben et al. 2004 and Napoli et al. 2003) versus control infants. Additionally, in the longer term infant study, incidences of crying, regurgitation and vomiting were similar across all groups and no differences in weight or length gains were observed among groups, indicating that GOS was well tolerated by and safe in term infants.

Other studies of GOS in term and preterm infants have mainly used a 9:1 combination of GOS:FOS (GOS ranging from 0.36-0.9% GOS, w/v), limiting the ability to link any reported Bifidogenic effects to the GOS component alone. However, in the GOS:FOS (9:1) studies in term infants receiving from 0.36-0.72% GOS (w/v), the weight of the evidence supports good tolerance and a lack of adverse events due to the GOS:FOS supplementation, plus favorable reductions in fecal pH and increases in stool frequency and/or stool softness versus the control formula group (Bakker-Zierikzee et al. 2005; Knol et al. 2005; Moro et al. 2002; Chouraqi et al. 2008; Savino et al. 2003; Schmelzle et al. 2003). One research group consistently reports fewer colic episodes and reduced regurgitation in term infants consuming GOS:FOS versus control formula (Savino et al. 2003 and 2006). Growth rates, body mass indicators, adverse event and serious adverse event rates are similar in GOS:FOS-supplemented term infants versus those consuming control formula (Rigo et al. 2001; Chouraqui et al. 2008; Schmelzle et al. 2003). Drop-outs in GOS:FOS treatment groups are generally due to increased flatulence and stool quality (parental reports). GOS:FOS at 0.8% (w/v) (0.72% GOS, w/v) for 6 mo in high allergic risk term infants was well-tolerated, did not contribute to the development of atopic dermatitis, and significantly reduced episodes of regurgitation and crying versus control formula (Moro et al. 2006). Preterm infants consuming 1% (w/v) GOS:FOS (9:1) (0.9% GOS, w/v) for 28 d exhibited similar weight and length gains versus the control group (Lidestri et al. 2003; Boehm et al. 2002). Number of stools and stool consistency for the GOS:FOS-fed preterm infants was intermediate between that of the control formula group and that observed in a breast-fed comparison group. These results indicate that GOS alone was tolerated and safe at 0.24-0.7% GOS (w/v), while the GOS:FOS combination is well tolerated by and safe in term infants at 0.36-0.72% GOS (w/v) in formula and in preterm infants at 0.9% GOS (w/v) in formula.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Term Infants						
Nakamura et al. 2009	To investigate the effect of supplementing infant formula with various prebiotic combinations on tolerance and fecal bacteria populations. Multi center, double blind, controlled, parallel-group feeding study	Healthy term infants 13-92 d old: 105 to receive formula; 30 as parallel-breast fed group.	4 formula groups: 1) Enfamil LIPIL with iron (control; *n* = 25), 2) control plus 4 g/L polydextrose (PDX):GOS (1:1) (*n* = 27), 3) control plus 4 g/L PDX:GOS:lactulose anhydride (LOS) (3:2:1) (*n* = 27), 4) control plus 8 g/L PDX:GOS:LOS (3:2:1) (*n* = 25).	Vivinal, GOS concentration unspecified (Friesland Foods Domo, Zwolle, The Netherlands) Individual GOS concentrations in formulas was unstated but must be ≤ 2 g/L (group 2), 1.3 g/L (group 3), and 2.6 g/L (group 4).	7 d run-in 28 d feeding period	• No differences in study discontinuation among feeding groups. • Mean formula intakes similar during run-in period and feeding trial. • Group 2 had a statistically significant difference in weight from the control group at d 28 that was similar to that observed for the breast-fed group vs. control; this difference was present at enrollment but was not statistically significant. • By d 28 all groups, including control, had increased amounts of fecal *Enterobacteriaceae* that approached levels observed in the breast-fed group. • No significant changes in *Clostridium*, *Lactobacillus*, or *Bifidobacterium* populations were observed across groups by d 28 (qPCR, FISH).

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Chouraqui et al. 2008	To evaluate the safety and tolerance of infant formulas containing probiotics and synbiotics (prebiotics + probiotics). Multi-center, prospective, controlled, double-blind, randomized study	Healthy, full-term infants ≤ 14 d old: 284 enrolled and randomized, 227 completed and analyzed after treatment, 142 with follow-up data	Unspecified mixture of 90% GOS/10% short-chain FOS (scFOS) (manufacturer unknown) Groups received: 1) Control formula (unsupplemented) (*n* = 53) 2) Formula with *Bifidobacterium longum* BL999 + *Lactobacillus rhamnosus* LPR (*n* = 70) 3) Formula with *B. longum* BL999 + *L. rhamnosus* LPR + GOS/scFOS (*n* = 70) 4) Formula with *B. longum* BL999 + *L. paracasei* ST11 + GOS/scFOS (*n* = 74)	0.4 g of GOS/scFOS mixture/100 mL formula	4 mo treatment period 12 mo follow-up observation period	• GOS/scFOS groups had no differences from control for weight gain, length, head circumference or BMI parameters, for either sex. • Study dropouts were due to change of formula (*n* = 21), adverse events (*n* = 5) and loss in follow-up (*n* = 31). • No differences in dropout rates between groups. • Frequency of antibiotic treatment or hospitalization during the observational period did not vary among groups. • Stool frequency was significantly higher in group 3 vs control, and a trend was noted for group 4. • Liquid stool occurrence was more frequent in group 4 vs. control. • No significant differences in frequency of serious adverse events or adverse events between groups.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Tatekawa et al. 2007	Case study of synbiotic therapy in a pediatric patient who underwent an extended myectomy-myotomy of the proximal jejunum for near-total intestinal aganglionosis.	Infant case report: 8 mo. F with enteritis	Synbiotic therapy: Carrot powder, *Lactobacillus coagulans*, *Bifidobacterium breve* Yakult [BBG-1], *Lactobacillus casei* Shirota [Biolactis Powder], plus GOS [Oligomate HP; unspecified formulation]	Unspecified	Not directly stated (indefinite therapy); likely ≥ 16 mo.	• Stool flora exchanged from methicillin-resistant *Staphylococcus aureus* to nonpathogenic bacteria, including *Lactobacillus spp.*, after synbiotic therapy. • No central line infection.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Ziegler et al. 2007	To investigate the effect of supplementing infant formula with various prebiotic combinations on tolerance and fecal bacteria populations. Multi center, double blind, controlled, randomized, parallel-group, prospective feeding study	Healthy term infants > 37 wk gestational age and ≥ 2500 g birth weight: 226 enrolled, 164 completed the study, 62 drop-outs	3 formula groups: 1) Enfamil LIPIL with iron (control; *n* = 58), 2) control plus 4 g/L polydextrose (PDX):GOS (1:1) (*n* = 58), 3) control plus 8 g/L PDX:GOS:LOS (lactulose) (50:33:17) (*n* = 48).	GOS concentration and source unspecified Individual GOS final concentrations in formulas were 0 g GOS/L (group 1), 2 g GOS/L (group 2), and 2.64 g GOS/L (group 3).	24 h pre-feeding with formula alone; 120 d on study formulas thereafter	• No statistically significant differences between treatment and control groups for mean weight growth rate at any time point. • No statistically significant differences between treatment and control groups for mean length growth rates (data not shown). • Treatment groups had significantly looser stools than the control group. • No statistically significant differences among the groups regarding dropout rate before visit 5 (120 d). • Intolerance cited as most frequent reason for study discontinuation; specific symptoms included gas, fussiness, vomiting and diarrhea. • Statistically significant differences in diarrhea, eczema and irritability incidence was observed in supplemented groups versus control. • No differences among groups was detected for serious adverse events. Those which occurred were deemed to be unrelated to the study product.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Moro et al. 2006	To investigate the effect of GOS/long-chain FOS on the incidence of atopic dermatitis in infants at high risk of atopy. Prospective, double-blind, randomized, placebo-controlled study	Term infants at high risk for atopy: 102 prebiotic group (50 provided stool samples) 104 placebo group (44 provided stool samples)	Hypoallergenic hydrolyzed cow's milk whey protein-based formula containing short-chain GOS/long-chain FOS (9:1). GOS/FOS uncharacterized; source not stated	0.8 g GOS/FOS(9:1)/100 mL	6 mo	• 10 infants in the GOS/FOS group developed atopic dermatitis, compared to 24 in the control group (differences between means were statistically significant); degree of severity not related to diet. • GOS/FOS caused a significant increase in fecal bifidobacteria, but not lactobacilli, vs. control. • Drop outs were mainly due to continuation/reestablishment of breast feeding. • Stool frequency was increased and stool consistency was improved at 3 and 6 mo in the GOS/FOS group vs. control. • Episodes of regurgitation and crying (including in connection with feeding) were significantly reduced in the GOS/FOS group at 3 and 6 mo, compared with control.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Savino et al. 2006	To evaluate the efficacy of GOS/FOS-supplemented formula to reduce crying episodes (colic) in infants. Prospective, single-blind, randomized study	One hundred ninety-nine healthy, term formula-fed infants (< 4 mo), having colic received either control formula plus simethicone (6 mg/kg 2×/d) (103) or new formula containing GOS/FOS (96)	Formula containing GOS/FOS (9:1). GOS/FOS uncharacterized; source not stated *Note: The control formula also contained GOS/FOS at 0.4 g/100 mL, and had differing base protein content from the test formula.*	0.8 g GOS/FOS (9:1)/100 mL	14 d	• GOS-fed infants had significantly fewer colic episodes vs. control after 1 wk, and fewer crying episodes after 2 wk. • No mention of adverse effects.
Scholtens et al. 2006	To test the effect of GOS/FOS-supplemented weaning foods on the composition of intestinal microbiota in infants. Double-blind, randomized study	Thirty-five healthy term infants (4-6 mo) randomized to weaning foods supplemented with either GOS/FOS (9:1) (n = 11; mean age = 17.9 wk) or maltodextrin (n = 9; mean age = 17.1 wk)	80 mL jars of fruits and vegetables plus 15 g sachets of rice flour were supplemented with 1.5 g GOS/FOS (9:1) per serving. GOS was represented as Vivinal GOS (Borculo Domo Ingredients, Zwolle, The Netherlands)	Up to 4.5 g GOS/FOS/d (target dose)	6 wk	• Study design allowed use of weaning products not used in the study. • Bifidobacteria/g feces was non-significantly increased for GOS/FOS group vs. control. • Percent of fecal Bifidobacteria in the bacterial population significantly increased in the GOS/FOS group vs. control. • No statistically significant difference in total short-chain fatty acid production in feces in GOS/FOS group vs. control.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Bakker-Zierikzee et al. 2006	To study the effects of standard, prebiotic and probiotic infant formulas on the fecal secretory immunoglobulin A (SIgA) levels in infants. Randomized, double-blind study	Fifty-seven infants of normal birth weight, formula-fed from birth, were randomized to: Formula + GOS/FOS (*n* = 19; 12 M/7 F) Formula + *Bifidobacterium animalis* (*n* = 19; 10 M/9 F) or Control formula (*n* = 19; 5 M; 14 F). 63 breast-fed infants (33 M/30 F) were included as a separate control group.	GOS/FOS (9:1), GOS as Vivinal (Borculo Domo Ingredients, Zwolle, The Netherlands)	0.6 g of GOS/FOS (9:1)/100 mL formula	Birth – 32 wk	• Infants in the GOS/FOS formula group exhibited statistically significantly increased fecal secretory IgA levels vs. control formula at 16 wk only. However, a trend was observed for higher SIgA levels through wk 28 for the GOS/FOS group vs. control. • There were no differences in defecation rate or stool consistency between the formula groups.
Bakker-Zierikzee et al 2005	To compare infant formulas supplemented with either a prebiotic or probiotic for their effects on infant intestinal flora. Randomized, double-blind study				16 wk	• Number of drop outs not significantly different between groups. • At wk 4, % Bifidobacteria in total bacterial population was increased in the GOS/FOS group, vs. control. This effect disappeared by wk 16. • GOS/FOS supplementation significantly decreased fecal pH, compared with control, but not as much as the breastfeeding group. • There were no significant differences in fecal levels of lactate or short-chain fatty acids between groups. • By 16 wk, butyrate and (isobutyrate + isovalerate + valerate) fecal levels were decreased in the GOS/FOS group, vs. control.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Knol et al. 2005	To study the intestinal microflora and its metabolic activity in infants supplemented with GOS/FOS (9:1). Randomized, double-blind, placebo-controlled study	Formula-fed infants (mean age = 7.7 wk; 53 completed study) fed either: Control formula (*n* = 19; 10 M/9 F) or Formula + GOS/FOS (*n* = 15; 8 M/7 F) A breast-fed group was included for comparison (*n* = 19; 12 M/7 F)	GOS/FOS (9:1) in infant formula. GOS not characterized; source not stated.	0.8 g GOS/FOS (9:1)/100 mL in formula	6 wk	• Percent bifidobacteria in fecal flora were significantly increased and fecal pH was significantly decreased in the GOS/FOS group, vs. control. • Percent of acetate in fecal short chain fatty acids was significantly increased and propionate was significantly decreased in the GOS/FOS group, vs. control.
Moro et al. 2005	To determine whether a prebiotic mixture of GOS/long-chain FOS can be detected in stools of formula-fed infants. Randomized study	Thirty-two term infants were assigned to either: Control formula (mean age = 6.2 d; *n* = 16; 9 M/7 F) or Formula plus GOS/FOS (mean age = 6.9 d; *n* = 16; 8 M/8 F)	GOS/FOS (9:1) in infant formula. GOS not characterized; source not stated.	0.8 g GOS/FOS (9:1)/dL (0.8 g/100 mL) in formula	28 d	• At 28 d, the GOS/FOS group had significantly increased numbers of fecal bifidobacteria vs. placebo group. • GOS and FOS were detected via high-performance anion-exchange chromatography in feces of GOS/FOS-supplemented infants.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Rinne et al. 2005	To assess quantitative and qualitative differences in gut microbiota in infants.	Allergy high-risk infants: Thirty-two infants (gestation age = 36-42 wk) having one close relative with atopic eczema, allergic rhinitis or asthma: Formula (*n* = 8), Formula + GOS/FOS (9:1) (*n* = 8), Breastmilk (*n* = 8), or Breastmilk + probiotic (*n* = 8; probiotic started in mother 2 wk prior to delivery)	GOS/FOS (9:1) in infant formula. GOS uncharacterized (Omneo, Nutricia, Cuijk, The Netherlands).	Dose not stated	12 mo	• At 12 mo, weight and length of infants were comparable between all feeding groups. • Atopic eczema was more common in the unsupplemented formula group vs. other groups. • Amount of Bifidobacteria and Bacteroides in feces of the GOS/FOS-fed group were similar to levels found in the breastmilk-fed group, and were increased relative to the formula-fed group. • GOS/FOS and breastmilk groups had higher incidence of *Bifidobacterium bifidum* versus the other groups.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Ben et al. 2004	To investigate effects of GOS in formula milk on term infants' intestinal microbial populations and fermentation characteristics. Randomized, controlled study	Two hundred seventy-one term infants were assigned to either: Formula (*n* = 52), Formula + GOS (*n* = 69), Breast milk (*n* = 26), or Breast milk + Formula + GOS (*n* = 124)	GOS in Frisolac H infant formula (uncharacterized; Friesland, The Netherlands)	0.24 g GOS/dL (0.24 g/100 mL)	6 mo	• At both 3 and 6 mo, infants on the GOS-supplemented formula had significantly higher counts of fecal *Bifidobacteria* and *Lactobacilli*, vs. control formula. • At 3 and 6 mo, fecal short-chain fatty acid levels and pH in the GOS-fed group were significantly increased and decreased, respectively, compared with the control group.
Napoli, et al. 2003 (Abstract; full article unavailable)	To investigate the bifidogenicity of GOS-supplemented formula on fecal microflora in healthy term infants. Randomized study	Twenty-six formula-fed, healthy term infants were randomized to receive control formula (*n* = 13) or formula + GOS (*n* = 13). A comparison group of 24 breast-fed infants was included.	GOS in infant formula. GOS not characterized; source not stated.	0.7% GOS in formula	21 d treatment	• GOS supplementation increased fecal bifidobacteria counts to similar levels as seen in the breast-fed group. • Fecal pH was significantly lower in GOS-fed infants vs. controls. • Fecal lactate levels were marginally increased in GOS-fed, vs. control, infants.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Savino et al. 2003	To test the ability of FOS plus GOS to reduce colic and feeding problems in infants. Uncontrolled study	Six hundred four formula-fed healthy term infants (≤ 3 mo; mean age = 1.35 mo) having colic (*n* = 214), regurgitation (*n* = 210) and/or constipation (*n* = 232) were enrolled.	GOS/FOS in infant formula. GOS not characterized; source not stated.	0.8 g GOS/FOS per 100 mL formula	14 d	• In infants presenting with colic, 79% had a statistically significant reduction in frequency of colic at 14 d. • In infants presenting with regurgitation, 70.15% demonstrated statistically significant improvement, versus control, at 14 d. • In infants presenting with constipation, 63.4% had a statistically significant increase in number of stools/day by 14 d.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Schmelzle et al. 2003	To evaluate the nutritional efficacy and bifidogenicity of an infant formula containing GOS/FOS (9:1). Double-blind, randomized, controlled study	One hundred fifty-four healthy, term infants (gestational age = 37-42 wk; mean age per group = 7 d), formula-fed by 14 d were assigned to control formula (*n* = 78) or formula + GOS/FOS (*n* = 76).	GOS/FOS in infant formula (Omneo/Comformil, Numico, Zoetermeer, The Netherlands). GOS not characterized.	0.8 g GOS/FOS per 100 mL formula	12 wk	• Slightly higher drop out rate for GOS/FOS group due to flatulence and stool quality, and "possibly related to feeding." • At 6 wk, girls in the GOS/FOS group had significantly higher weight compared with the control group. This effect was no longer statistically significant by 12 wk. • At 6 wk, fecal Bifidobacteria were increased in the GOS/FOS group vs. control. • At 6 wk, serum prealbumin levels were slightly, but significantly, lower in a subset of the GOS/FOS group (*n* = 12) vs. control (*n* = 23). •At 6 wk, serum levels of threonine were significantly higher for the GOS/FOS group, vs. either breast-fed or control groups. Tyrosine was lower vs. control and isoleucine and lysine were higher, vs. control. • At 12 wk, head circumference of girls in the GOS/FOS group was significantly larger vs. control. • At 12 wk, sum of skinfolds (mm/d) was significantly higher in boys in the GOS/FOS group, vs. control. • At both 6 and 12 wk, infants in the GOS/FOS group had significantly lower formula intake (mL/kg/d) vs. controls. • Stool consistency of the GOS/FOS group was significantly softer/more watery vs. control group at both 6 and 10 wk.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Moro et al. 2002	To determine the bifidogenicity of GOS/FOS (9:1) in term infants. Randomized, placebo-controlled study	Ninety term infants (*n* = 90) were assigned to receive either control formula (*n* = 33; 17 M/18 F; mean age = 6.3 d), low-GOS/FOS (9:1) formula (*n* = 30; 17 M/13 F; mean age = 6.8 d), or high-GOS/FOS (9:1) formula (*n* = 27; 12 M/15 F; mean age = 7.2 d)	GOS/FOS (9:1) in infant formula. GOS not characterized; source not stated.	0.4 g GOS/FOS (9:1) per dL formula (0.4 g/100 mL) or 0.8 g GOS/FOS (9:1) per dL formula (0.8 g/100 mL)	28 d	• Weight and length gains during study period were similar across groups. • Incidence of regurgitation, vomiting, and crying were not affected by diet (data not shown). No incidents of diarrhea were reported during the study. • The number of Bifidobacteria and Lactobacilli in stools was higher in treatment groups vs. control. • Fecal pH decreased significantly in the 0.8 g GOS/FOS group, but not for the 0.4 g GOS/FOS group, vs. control. • Stool consistency was significantly affected by GOS/FOS supplementation, in a dose-dependent manner. Stool frequency was slightly increased in the high-GOS/FOS group, vs. the other groups.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Rigo et al. 2001	To study the effect of GOS/FOS on the growth, weight gain, mineral content and intestinal flora development in term infants. Non-controlled trial (no control group for comparison)	Fifteen healthy term infants (4 M/11 F) completed the study. All were fed the test formula containing GOS/FOS.	GOS/FOS in formula (1/2-dose of GOS/FOS in Omneo/Conformil formula; GOS component uncharacterized)	0.4 g GOS/FOS/100 mL formula	Birth-2 mo	• Sixteen infants were initially enrolled; one dropped out due to gastroesophagial reflux. Of the remaining 15, one large-for-gestational-age infant was excluded from the final analysis. • Across the entire study, the growth rate of GOS/FOS-fed infants was similar to that of historical data on infants fed conventional formula or breast-fed infants. Early weight gain was lower in GOS/FOS fed infants vs. historical data on breast-fed or conventional formula-fed infants. • Lean body mass and fat mass compositions were similar between GOS/FOS-fed infants and historical data for conventional formula-fed and breast-fed infants. Bone mineral content was slightly lower in the GOS/FOS group vs. the other two. • Volumetric bone mineral density was significantly reduced during the first 2 mo of life regardless of diet (GOS/FOS vs. conventional formula vs. breast-fed), due to the high growth velocity in the presence of a relatively low mineral supply/retention at this stage of life. • Intake was lower for GOS/FOS formula vs. conventional formula (statistical significance not stated). • Bifidobacteria in fecal flora increased between d 3 and d 25.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Pre-Term Infants						
Mihatsch et al. 2006	To investigate whether feeding GOS/FOS would improve feeding tolerance in preterm infants on full enteral formula feeding. Randomized, placebo-controlled, double-blind study	Twenty healthy preterm infants, non-breastfed (mean gestational age = 27 wk; mean postnatal age = 42 d; mean weight = 1570 g), 10 infants/feeding group	GOS/FOS 0.9 g (uncharacterized; ratio of GOS:FOS unstated; Milupa[R] GmbH, 61381 Friedrichsdorf Germany) + 0.9 g maltodextrin	1 g GosFos/dL formula (1 g/100 mL)	14 d	• Weight loss, constipation, diarrhea, abdominal discomfort and flatulence were not reported. • GOS/FOS reduced stool viscosity, increased transit time and reduced stool pH vs. placebo.
Lidestri et al. 2003 (Abbreviated report)	To evaluate the influence of dietary oligosaccharides on calcium metabolism in preterm infants.	Thirty preterm (≤ 32 wk gestational age) infants assigned to control formula (*n* = 15; 8 M/7 F; mean age = 8.5 d) or formula + GOS/FOS (9:1) (*n* = 15; 7 M/8 F; mean age = 8.3 d). A breast-fed group was included for reference (*n* = 12; 7 M/5 F; mean age = 7.9 d).	GOS/FOS (9:1) in infant formula. GOS not characterized; source not stated.	1.0 g GOS/FOS (9:1) per 100 mL formula	28 d treatment	• GOS/FOS had no influence on serum concentrations of calcium and phosphorus or on the activity of alkaline phosphatase, vs. control. • Calcium in urine tended to be higher (nearly statistically significant: $p = 0.055$) in the GOS/FOS group, vs. control. No difference in phosphorus levels in urine between groups.
Boehm et al. 2002 *Same study as Lidestri et al. 2003	To investigate the effect of GOS on preterm infant fecal flora and stool characteristics. Placebo-controlled, randomized study					• Weight and length gains during study period were similar across all groups. • By 28 d, fecal Bifidobacteria were significantly increased in the GOS/FOS group, vs. control. • Number of stools and stool consistency for GOS/FOS-fed infants was intermediate between that of the control-fed and breast-fed infants.

Table V-5. Summary of Studies of GOS in Infants

Reference	Study Objective & Design	Population	Test Substance	GOS Dose	Treatment Duration	Results of GOS Ingestion
Kanamori et al. 2002	To demonstrate the effects of synbiotic therapy in critically ill patients.	Infant case study: Preterm (34 wk) 9 mo. F with laryngotracho-esophageal cleft (type IV)	Synbiotic combination used in Kanamori et al. 2004	[(1 g GOS + 1 g Biolactis) × 3]/d	11 mo	• Body weight gain and serum choline esterase was increased. • Bowel movement was restored within 1 d of treatment. • Total short-chain fatty acid fecal content was increased by 1 mo.

VI. REFERENCES

Alander M, Mättö J, Kneifel W, Johansson M, Kögler B, Crittenden R, Mattila-Sandholm T, Saarela M. Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence of Bifidobacterium lactis Bb-12 in the gastrointestinal tract. International Dairy Journal 2001 11: 817-825.

Alles MS, Hartemink R, Meyboom S, Harryvan JL, Van Laere KMJ, Nagengast FM, Hautvast JGAJ. Effect of transgalacto-oligosaccharides on the composition of the human intestinal microflora and on putative risk markers for colon cancer. Am J Clin Nutr 1999 69: 980-981.

Anthony JC, Merriman TN, Heimbach JT. 90-Day oral (gavage) study in rats with galacto-oligosaccharides syrup. Food and Chemical Toxicology 2006 44: 819-826.

Bakker-Zierikzee AM, Alles MS, Knol J, Kok FJ, Tolboom JJM, Bindels JG. Effects of infant formula containing a mixture of galacto- and fructooligosaccharides or viable Bifidobacterium animalis on the intestinal microflora during the first 4 months of life. British Journal of Nutrition 2005 94: 783-790.

Bakker-Zierikzee AM, van Tol EAF, Kroes H, Alles MS, Kok FJ, Bindels JG. Faecal SIgA secretion in infants fed on pre- or probiotic infant formula. Pediatr Allergy Immunol 2006 17: 134-140.

Ben X-M, Zhou X-Y, Zhao W-H, Yu W-L, Wei P, Zhang W-L, Wu S-M, Van Beusekom CM, Schaafsma A. Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants. Chinese Medical Journal 2004 117: 927-931.

Blakely JA and Mackenzie SL. Purification and properties of a β-hexosidase from Sporobolomyces singularis. Can J Biochem 1969 47: 1021-1025.

Boehm G, Lidestri M, Casetta P, Jelinek J, Negretti F, Stahl B, Marini A. Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. Arch Dis Child Fetal Neonatal Ed 2002 86: F178-F181.

Bouhnik Y, Flourié B, D'Agay-Abensour L, Pochart P, Gramet G, Durand M, Rambaud J-C. Administration of transgalacto-oligosaccharides increases fecal Bifidobacteria and modifies colonic fermentation metabolism in healthy humans. J Nutr 1997 127: 444-448.

Bouhnik Y, Raskine L, Simoneau G, Vicaut E, Neut C, Flourié B, Brouns F, Bornet FR. The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. Am J Clin Nutr 2004 80: 1658-1664.

Burvall A, Asp N-G, Dahlqvist A. Oligosaccharide formation during hydrolysis of lactose with Saccharomyces lactis lactase (Maxilact®)—Part 3: Digestibility by human intestinal enzymes in vitro. Food Chemistry 1980 5: 189-194.

Cho YJ, Shin HJ, Bucke C. Purification and biochemical properties of a galacto-oligosaccharide producing β-galactosidase from Bullera singularis. Biotechnol Lett 2003 25: 2107-2111.

Chonan O and Watanuki M. Effect of galacto-oligosaccharides on calcium absorption in rats. Journal of Nutritional Science and Vitaminology 1995 41: 95-104.

Chonan O and Watanuki M. The effect of 6'-galacto-oligosaccharides on bone mineralization of rats adapted to different levels of dietary calcium. Internat J Vit Nutr Res 1996 66: 244-249.

Chonan O, Matsumoto K, Watanuki M. Effect of galacto-oligosaccharides on calcium absorption and preventing bone loss in ovariectomized rats. Biosci Biotech Biochem 1995 59: 236-239.

Chonan O, Takahashi R, Watanuki M. Role of activity of gastrointestinal microflora in absorption of calcium and magnesium in rats fed β1-4 linked galacto-oligosaccharides. Biosci Biotechnol Biochem 2001 65: 1872-1875.

Chonan O, Takahashi R, Yasui H, Watanuki M. Effects of β1→4 linked galacto-oligosaccharides on use of magnesium and calcification of the kidney and heart in rats fed excess dietary phosphorus and calcium. Biosci Biotechnol Biochem 1996 60: 1735-1737.

Chonan O, Shibahara-Sone H, Takahashi R, Ikeda M, Kikuchi-Hayakawa H, Ishikawa F, Kimura K, Matsumoto K. Undigestibility of galacto-oligosaccharides. Journal of the Japanese Society for Food Science and Technology (Nippon Shokuhin Kagaku Kogaku Kaishi) 2004 51(1): 28-33.

Chouraqui JP, Grathwohl D, Labaune JM, Hascoet JM, de Montgolfier I, Leclaire M, Giarre M, Steenhout P. Assessment of the safety, tolerance, and protective effect against diarrhea of infant formulas containing mixtures of probiotics or probiotics and prebiotics in a randomized controlled trial. Am J Clin Nutr 2008 87: 1365-1373.

Coppa GV, Gabrielli O, Pierani P, Zampini L, Rottoli A,Carlucci A, Giorgi PL. Quantitative and qualitative studies of carbohydrates of human colostrums and mature milk. Rev Ital Pediatr 1991 17: 303-307.

Coppa GV, Pierani P, Zampini L, Gabrielli O, Carlucci A, Catassi C, Giorgi PL. Lactose, oligosaccharide and monosaccharide content of milk from mothers delivering preterm newborns over the first month of lactation. Minerva Pediatr 1997 19: 171-175.

Cummings JH, Macfarlane GT, Englyst HN. Prebiotic digestion and fermentation. Am J Clin Nutr 2001 73(Suppl): 415S-420S.

De Slegte J. Determination of trans-galactooligosaccharides in selected food products by ion-exchange chromatography: collaborative study. J AOAC Int 2002 85: 417-423.

Deguchi Y, Makino K, Iwadate E, Kimura M, Matsumoto K, Yamaoka Y, Komatsu H, Matsumoto K, Iino H. Influence of galacto-oligosaccharides on bowel habit in healthy young volunteers with constipation tendency. J Jpn Council Adv Food Ingred Res 2003 6: 55-66.

Depeint F, Tzortzis G, Vulevic J, I'anson K, Gibson GR. Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of Bifidobacterium bifidum NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study. Am J Clin Nutr. 2008 87(3): 785-91.

Djouzi Z and Andrieux C. Compared effects of three oligosaccharides on metabolisim of intestinal microflora in rats inoculated with a human faecal flora. British Journal of Nutrition 1997 78: 313-324.

DSMZ – The German Resource Centre for Biological Material online database. Accessed 12-04-09. www.dsmz.de/microorganisms/html/yeast.genus/sporobolomyces.html

Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman-Leyer KM, O'Ryan ML, Ruiz-Palacios G, Hilty MD, Pickering LK, Prieto PA. Variability of human milk neutral oligosaccharides in a diverse population. J Pediatr Gastroenterol Nutr 2000 30: 181-192.

FDA Report 24. Galacto-oligosaccharides (derived from lactose) (ingredient of Elix'or™). EM Industries, Inc. 3/19/98. http://www.fda.gov/Food/DietarySupplements/ucm109764.htm#whatnew (Accessed 12-20-09).

Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A. Biodiversity and systematic of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. International Journal of Systematic and Evolutionary Microbiology 2000 50: 1351-1371.

González R, Klaassens ES, Malinen E, de Vos WM, Vaughan EE. Differential transcriptional response of Bifidobacterium longum to human milk, formula milk, and galactooligosaccharide. Appl Environ Microbiol. 2008 74(15): 4686-94.

Gopal PK and Gill HS. 2000. Oligosaccharides and glycoconjugates in bovine milk and colostrum. Br J Nutr. 84:S69-S64.

Gopal PK, Prasada J, Gill HS. 2003. Effects of the consumption of Bifidobacterium lactis HN019 (DR10TM) and galacto-oligosaccharides on the microflora of the gastrointestinal tract in human subjects. Nutr Res. 23:1313-1328.Gorin PAJ, Spencer JFT, Phaff HJ. The structures of galactosyl-lactose and galactobiosyl-lactose produced from lactose by Sporobolomyces singularis. Can J Chem 1964a 42: 1341-1344.

Gorin PAJ, Spencer JFT, Phaff HJ. The structures of galactosyl-lactose and galactobiosyl-lactose production from lactose by Sporobolomyces singularis. Can J Chem 1964a 42: 1341-1344.

Gorin PAJ, Spencer JFT, Phaff HJ. The synthesis of beta-galacto- and beta-gluco-pyranosyl disaccharides by Sporobolomyces singularis. Can J Chem 1964b 42: 2307-2317.

GRAS Notice #233. Combination of galacto-oligosaccharides and polydextrose. Filed 8-05-07. http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000233.pdf (Accessed 12-30-09).

GRAS Notice #236. Galacto-oligosaccharides. Filed 11-07-07. http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000236.pdf (Accessed 12-29-09).

GRAS Notice #285. Galacto-oligosaccharides. Filed 3-12-09. http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000285.pdf (Accessed 12-30-09).

GRAS Notice #286. Galacto-oligosaccharides. Filed 3-12-09. http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn000286.pdf (Accessed 12-30-09).

Hamamoto M and Nakase T. Phylogenetic analysis of the ballistoconidium-forming yeast genus Sporobolomyces based on 18S rDNA sequences. International Journal of Systematic and Evolutionary Microbiology 2000 50: 1373-1380.

Hayashi M, Taki S, Ueda F, Shimazaki Y,Ito O, Kamata S, Kakiichi N, Shitara A. 4'-Galactooligosaccharide affects sodium and potassium metabolism in rats. J Nutr 1991 121: 1606-1612.

Hernot DC, Boileau TW, Bauer LL, Middelbos IS, Murphy MR, Swanson KS, Fahey GC Jr. In vitro fermentation profiles, gas production rates, and microbiota modulation as affected by certain fructans, galactooligosaccharides, and polydextrose. J Agric Food Chem. 2009 57(4): 1354-61.

Holma R, Juvonen P, Asmawi MZ, Vapaatalo H, Korpela R. Galacto-oligosaccharides stimulate the growth of Bifidobacteria but fail to attenuate inflammation in experimental colitis in rats. Scand J Gastroenterol 2002 9: 1042-1047.

Hopkins MJ, Cummings JH, Macfarlane GT. Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources. Journal of Applied Microbiology 1998 85: 381-386.

Hopkins MJ, Macfarlane GT. Nondigestible oligosaccharides enhance bacterial colonization resistance against Clostridium difficile in vitro. Applied and Environmental Microbiology 2003 69(4): 1920-1927.

Houdijk JGM, Bosch MW, Tamminga S, Verstegen MWA, Berenpas EB, Knoop H. Apparent ileal and total-tract nutrient digestion by pigs as affected by dietary nondigestible oligosaccharides. J Anim Sci 1999 77: 148-158.

Houdijk JGM, Bosch MW, Verstegen MWA, Berenpas HJ. Effects of dietary oligosaccharides on the growth performance and faecal characteristics of young growing pigs. Animal Feed Science Technology 1998 71: 35-48.

Houdijk JGM, Hartemink R, Verstegen MWA, Bosch MW. Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chyme and faeces in weaner pigs. Arch Anim Nutr 2002a 56: 297-307.

Houdijk JGM, Verstegen MWA, Bosch MW, van Laere KJM. Dietary fructooligosaccharides and transgalactooligosaccharides can affect fermentation characteristics in gut contents and portal plasma of growing pigs. Livestock Production Science 2002b 73: 175-184.

Ishikawa E, Sakai T, Ikemura H, Matsumoto K, Abe H. Identification, cloning, and characterization of a Sporobolomyces singularis β-galactosidase-like enzyme involved in galacto-oligosaccharide production. Journal of Bioscience and Bioengineering 2005 99: 331-339.

Ito M, Deguchi Y, Matsumoto K, Kimura M, Onodera N, Yajima T. Influence of galactooligosaccharides on the human fecal microflora. J Nutr Sci Vitaminol 1993a 39: 635-640.

Ito M, Deguchi Y, Miyamori A, Matsumoto K, Kikuchi H, Matsumoto K, Kobayashi Y, Yajima T, Kan T. Effects of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. Microbial Ecology in Health and Disease 1990 3: 285-292.

Ito M, Kimura M, Deguchi Y, Miyamori-Watabe A, Yajima T, Kan T. Effects of transgalactosylated disaccharides on the human intestinal microflora and their metabolism. J Nutr Sci Vitaminol 1993b 39: 279-288.

Japan Society for Culture Collections online database. Accessed 12-04-09. www.nbrc.nite.go.jp/jscc/idb/strains?Snt=0&Sn=Sporobolomyces+singularis

Kanai H and Matsumoto K. Review. Functionality and utility of galacto-oligosaccharides. A Technical Journal on Food Chemistry & Chemicals: Food Chemicals 2009 8: 57-66.

Kanamori Y, Hashizume K, Sugiyama M, Morotomi M, Yuki N, Tanaka R. A novel synbiotic therapy dramatically improved the intestinal function of a pediatric patient with

laryngotracheo-esophageal cleft (LTEC) in the intensive care unit. Clinical Nutrition 2002 21(6): 527-530.

Kanamori Y, Sugiyama M, Hashizume K, Yuki N, Morotomi M, Tanaka R. Experience of long-term synbiotic therapy in seven short bowel patients with refractory enterocolitis. Journal of Pediatric Surgery 2004 39(11): 1686-1692.

Kanazawa H, Nagino M, Kamiya S, Komatsu S, Mayumi T, Takagi K, Asahara T, Nomoto K, Tanaka R, Nimura Y. Synbiotics reduce postoperative infectious complications: A randomized controlled trial in biliary cancer patients undergoing hepatectomy. Langenbeck's Archives of Surgery 2005 390(2): 104-113.

Kaneko K. Repeated-dose 90-day oral toxicity study of *Bullera singularis* YIT 10047 suspension in rats. Safety Research Center, Yakult Central Institute for Microbiological Research, Yakult Honsha Co., Ltd., 1796, Yaho, Kunitachi-shi, Tokyo, Japan. December 27, 2002.

Kawakami K, Makino I, Asahara T, Kato I, Onoue M. Dietary galacto-oligosaccharides mixture can suppress serum phenol and p-cresol levels in rats fed tyrosine diet. J Nutr Sci Vitaminol 2005 51: 182-186.

Kekkonen RA, Ahlroos T, Suomalainen T, Tynkkynen S, Poussa T, Nevala R, Korpela R. A combination of galacto-oligosaccharides and Lactobacillus GG increases bifidobacteria to a greater extent than Lactobacillus GG on its own. Milchwissenschaft 2007 62(3): 326-330.

Kikuchi H, Andrieux C, Riottot M, Bensaada M, Popot F, Beaumatin P, Szylit O. Effect of two levels of transgalactosylated oligosaccharide intake on rats associated with human fecal microflora on bacterial glycolytic activity, end-products of fermentation and bacterial steroid transformation. Journal of Applied Bacteriology 1996 80: 439-446.

Kikuchi H, Sakata T. Qualitative and quantitative estimation of soluble indigestible polysaccharides as substrate for hindgut fermentation by mini-scale batch culture. J Nutr Sci Vitaminol (Tokyo) 1992 38(3): 287-296.

Kikuchi-Hayakawa H, Kimura M, Watanuki M. Adaptation of rate of organic acid production of hindgut bacteria to chronic intake of galactooligosaccharide in the rat. J Nutr Sci Vitaminol 1997 43: 357-368.

Kimura M, Ikeda M, Shibata H, Ikeda S, Matsumoto K. Evaluating the laxative threshold and safety of galactooligosaccharide syrup. Japanese Journal of Food Chemistry 2004 11: 67-74.

Knol J, Scholtens P, Kafka C, Steenbakkers J, Gross S, Helm K, Klarczyk M, Schöpfer H, Böckler H-M, Wells J. Colon microflora in infants fed formula with galacto- and fructo-oligosaccharides: more like breast-fed infants. Journal of Pediatric Gastroenterology and Nutrition 2005 40: 36-42.

Kobayashi T, Yasutake N, Uchida K, Ohyama W, Kaneko K, Onoue M. Safety of a novel galacto-oligosaccharide: genotoxicity and repeated oral dose studies. Human and Experimental Toxicology 2009 28: 619-630.

Kunz C, Rudoff S, Baier W, Klein N, Stobel S. Oligosaccharides in human milk: Structural, functional, and metabolic aspects. Ann Rev Nutr 2000 20: 699-722.

Lidestri M, Agosti M, Marini A, Boehm G. Oligosaccharides might stimulate calcium absorption in formula-fed preterm infants. Acta Paediatr Suppl 2003 441: 91-92.

Liu S, Ling Y, Tsai CE. Biotechnically synthesized oligosaccharides and polydextrose reduce constipation and putrefactive metabolites in the human. Journal of the Chinese Nutrition Society 1994 19(3): 221-232.

Lodder J and Kreger-van Rij NJW. The yeasts. North Holland Publ Co, Amsterdam: 1971.

Macfarlane GT, Steed H, Macfarlane S. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol 2008 104: 305-344.

Makras L, Van Acker G, De Vuyst L. Lactobacillus paracasei subsp. paracasei 8700:2 degrades inulin-type fructans exhibiting different degrees of polymerization. Applied and Environmental Microbiology 2005 71: 6531-6537.

Malinen E, Mätto J, Salmitie M, Alander M, Saarela M, Palva A. II: Analysis of Bifidobacterium populations in human faecal samples from a consumption trial with Bifidobacterium lactis Bb-12 and a galacto-oligosaccharide preparation. System Appl Microbiol 2002 25: 249-258.

Matsumoto K, Takada T, Yuki N, Kawakami K, Sakai T, Nomoto K, Kimura K, Matsumoto K, Iino H. Effects of transgalactosylated oligosaccharides mixture (N-GOS) on human intestinal microflora. Journal of Intestinal Microbiology 2004 18: 25-35.

McBain AJ and Macfarlane GT. Modulation of genotoxic enzyme activities by nondigestible oligosaccharide metabolism in in-vitro human gut bacterial ecosystems. J Med Microbiol 2001 50: 833-842.

McVaegh P and Miller JB. Review article: Human milk oligosaccharides: Only the breast. J Paediatr Child Health 1997 33: 281-286.

Meslin JC, Andrieux C, Sakata T, Beaumatin P, Bensaada M, Popot F, Szylit O, Durand M. Effects of galacto-oligosaccharide and bacterial status on mucin distribution in mucosa and on large intestine fermentation in rats. British Journal of Nutrition 1993 69: 903-912.

Mihatsch WA, Hoegel J, Pohlandt F. Prebiotic oligosaccharides reduce stool viscosity and accelerate gastrointestinal transport in preterm infants. Acta Paediatrica 2006 95: 843-848.

Mitsuhashi S, Yoshihama M, Yahiro M, Nishikawa I, Deya E, Ahiko K, Mitsuoka T. Effect of oligosaccharides on intestinal flora and fecal characteristics of neonates. 1982. Proceedings of III. IPCR Symposium on Intestinal Flora, Tokyo (abstract only).

Morishita Y, Oowada T, Ozaki A, Mizutani T. Galactooligosaccharide in combination with Bifidobacterium and Bacteroides affects the population of Clostridium perfringens in the intestine of gnotobiotic mice. 2002 22: 1333-1341.

Moro G, Arslanoglu S, Stahl B, Jelinek J, Wahn U, Boehm G. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Arch Dis Child 2006 91: 814-819.

Moro G, Minoli I, Mosca M, Fanaro S, Jelinek J, Stahl B, Boehm G. Dosage-related Bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. Journal of Pediatric Gastroenterology and Nutrition 2002 34: 291-295.

Moro GE, Stahl B, Fanaro S, Jelinek J, Boehm G, Coppa GV. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. Acta Paediatrica 2005 94: 27-30.

Morohashi T, Sano T, Ohta A, Yamada S. True calcium absorption in the intestine is enhanced by fructooligosaccharide feeding in rats. J Nutr 1998 128: 1815-1818.

Mountzouris KC, Balaskas C, Fava F, Tuohy KM, Gibson GR, Fegeros K. Profiling of composition and metabolic activities of the colonic microflora of growing pigs fed diets supplemented with prebiotic oligosaccharides. Anaerobe 2006 12: 178-185.

Mwenya B, Santoso B, Sar C, Gamo Y, Kobayashi T, Arai I, Takahashi J. Effects of including β1-4 galacto-oligosaccharides, lactic acid bacteria or yeast culture on methanogenesis as well as energy and nitrogen metabolism in sheep. Animal Feed Science and Technology 2004 115(3-4): 313-326.

Mwenya B, Santoso B, Sar C, Pen B, Morikawa R, Takaura K, Umetsu K, Kimura K, Takahashi J. Effects of yeast culture and galacto-oligosaccharides on ruminal fermentation in Holstein cows. Journal of Dairy Science 2005 88: 1404-1412.

Nakamura N, Gaskins HR, Collier CT, Nava GM, Rai D, Petschow B, Russell WM, Harris C, Mackie RI, Wampler JL, Walker DC. Molecular ecological analysis of fecal bacterial populations from term infants fed formula supplemented with selected blends of prebiotics. Applied and Environmental Microbiology 2009 75: 1121-1128.

Nakase T and Suzuki M. Sporobolomyces inositophilus, a new species of ballistosporous yeast isolated from a dead leaf of Sasa sp. in Japan. Antonie van Leeuwenhoek 1987 53: 245-251.

Nakase T, Takematsu A, Hamamoto M, Takashima M. The expanding realm of ballistosporous yeasts. Antonie Van Leeuwenhoek. 1993 Feb;63(2):191-200.

Nakase T. Expanding world of ballistosporous yeasts: Distribution in the phyllosphere, systematic and phylogeny. J Gen Appl Microbiol 2000 46: 189-216.

Napoli J, Brand-Miller JC, Conway P. Bifidogenic effects of feeding infant formula containing galacto-oligosaccharides in healthy formula-fed infants. Proc Nutr Soc Aust 2003, Vol. 27. Asia Pac J Clin Nutr 2003 12(Suppl): S30 (abstract).

NAPRALERT^{SM} Database. Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago. Professor Norman Farnsworth, curator. www.napralert.org/

Newburg DS. Oligosaccharides in humanmilk and bacterial colonization. J Pediatr Gastroenterol Nutr 2000 30: S8-S17.

Ohtsuka K, Tsuji K, Nakagawa Y, Ueda H, Ozawa O, Uchida T, Ichikawa T. Utilization and metabolism of [U-14C]4'galactosyllactose(O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-D-glucopyranose) in rats. J Nutr Sci Vitaminol 1991 37: 173-184.

Ohtsuka K, Tsuji K, Nakagawa Y, Ueda H, Ozawa O, Uchida T, Ichikawa T. Availability of 4'galactosyllactose (O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranosyl-(1→4)-D-glucopyranose) in rat. J Nutr Sci Vitaminol 1990 36: 265-276.

Ohyama W. Micronucleus test with peripheral blood of mice on Bullera singularis YIT 10047 suspension. Safety Research Center, Yakult Central Institute for Microbiological Research, Yakult Honsha Co., Ltd., 1796, Yaho, Kunitachi-shi, 186-8650, Japan. October 28, 2002.

Pérez-Conesa D, López G, Abellán P, Ros G. Bioavailability of calcium, magnesium and phosphorus in rats fed probiotic, prebiotic and synbiotic powder follow-up infant formulas and their effect on physiological and nutritional parameters. Journal of the Science of Food and Agriculture 2006a 86: 2327-2336.

Pérez-Conesa D, López G, Ros G, Abellán P, Hartemink R. Fecal microbiota changes with the consumption of follow-up formulas containing Bifidobacterium spp. and/or galactooligosaccharides by rats and a follow-up infant formula containing Bifidobacterium spp. by human infants. Journal of Food Science 2006b 71: M7-M13.

Perez-Conesa D. Effect of probiotic, prebiotic and synbiotic follow-up infant formulas on iron bioavailability in rats. Food Science and Technology International 2007 13(1): 69-77.

Phaff HJ and Do Carma-Sousa L. Four new species of yeast isolated from insect frass in bark of Tsuga heterophylla (Raf.) Sargent. Antonie Van Leeuwenhoek, J Microbiol Serol 1962 28: 193-207.

Rabiu RA, Jay AJ, Gibson GR, Rastall RA. Synthesis and fermentation properties of novel galactooligosaccharides by β-galactosidases from Bifidobacterium species. Applied and Environmental Microbiology 2001 67: 2526-2530.

Rigo J, Pieltain C, Studzinski F. Growth, weight gain composition and mineral accretion in term infants fed a new experimental formula containing hydrolysed protein, β-palmitate and prebiotics. Pediátrika 2001 21: 387-396.

Rinne MM, Gueimonde M, Kalliomäki M, Hoppu U, Salminen SJ, Isolauri E. Similar bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and breastfeeding on infant gut microbiota. FEMS Immunology and Medical Microbiology 2005 43: 59-65.

Rowland IR and Tanaka R. The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human fecal microflora. Journal of Applied Bacteriology 1993 74: 667-674.

Rycroft CE, Jones MR, Gibson GR, Rastall RA. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. Journal of Applied Microbiology 2001 91: 878-887.

Ryu SI, Kim BG, Park MS, Lee YB, Lee SB. Evaluation of enhanced hygroscopicity, bifidogenicity, and anticariogenicity of enzymatically synthesized beta-galactosyl-trehalose oligosaccharides. J Agric Food Chem. 2007 55(10): 4184-8.

Saito T, Itoh T, Adachi S. 1987. Chemical structure of three trisaccharides isolated in free form from bovine colostrum. Carbohydr Res. 165:43-51.

Sakaguchi E, Sakoda C, Toramaru Y. Caecal fermentation and energy accumulation in the rat fed on indigestible oligosaccharides. British Journal of Nutrition 1998 80: 469-476.

Sakai T, Tsuji H, Shibata S, Hayakawa K, Matsumoto K. Repeated-batch production of galactooligosaccharides from lactose at high concentration by using alginate-immobilized cells of Sporobolomyces singularis YIT 10047. J Gen Appl Microbiol 2008 54: 285-293.

Sako T, Keisuke M, Tanaka R. Recent progress on research and applications of non-digestible galacto-oligosaccharides. International Dairy Journal 1999 9: 69-80.

Sar C, Santoso B, Mwenya B, Gamo Y, Kobayashi T, Morikawa, R, Kimura K, Mizukoshi H, Tkahashi J. Manipulation of rumen methanogenesis by the combination of nitrate with beta1-4 galacto-oligosaccharides or nisin in sheep. Animal Feed Science and Technology 2004 115(1-2): 129-142.

Savino F, Cresi F, Maccario S, Cavallo F, Dalmasso P, Fanaro S, Oggero R, Vigi V, Silvestro L. "Minor" feeding problems during the first months of life: effect of a partially hydrolysed milk formula containing fructo- and galactooligosaccharides. Acta Paeditr Suppl 2003 441: 86-90.

Savino F, Palumeri E, Castagno E, Cresi F, Dalmasso P, Cavallo F, Oggero R. Reduction of crying episodes owing to infantile colic: a randomized controlled study on the efficacy of a new infant formula. European Journal of Clinical Nutrition 2006 60: 1304-1310.

Schmelzle H, Wirth S, Skopnik H, Radke M, Knol J, Böckler H-M, Brönstrup A, Wells J, Fusch C. 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 oligosaccharides. Journal of Pediatric Gastroenterology and Nutrition 2003 36: 343-351.

Scholtens PAMJ, Alles MS, Bindels JG, van der Linde EGM, Tolboom JJM, Knol J. Bifidogenic effects of solid weaning foods with added prebiotic oligosaccharides: a randomized controlled clinical trial. 2006 42: 553-559.

Scientific Committee on Food (SCF). European Commission: Health and Consumer Protection Directorate-General. 2003. Report of the Scientific Committee on Food on the revision of essential requirements of infant formulae and follow-on formulae: Adopted April 4, 2003. Available at: http://ec.europa.eu/food/fs/sc/scf/out199_en.pdf (Accessed 12-30-09).

Searle LE, Best A, Nunez A, Salguero FJ, Johnson L, Weyer U, Dugdale AH, Cooley WA, Carter B, Jones G, Tzortzis G, Woodward MJ, La Ragione RM. A mixture containing galactooligosaccharide, produced by the enzymic activity of Bifidobacterium bifidum, reduces Salmonella enterica serovar Typhimurium infection in mice. J Med Microbiol. 2009 58(Pt 1): 37-48.

Shadid R, Haarman M, Knol J, Their W, Beermann C, Rjosk-Dendorfer D, Schendel DJ, Koletzko BV, Krauss-Etschmann S. Effects of galactooligosaccharide and long-chain fructooligosaccharide supplementation during pregnancy on maternal and neonatal microbiota and immunity—a randomized, double-blind, placebo-controlled study. Am J Clin Nutr 2007 86: 1426-1437.

Shimizu K, Ogura H, Goto M, Asahara T, Nomoto K, Morotomi M, Matsushima A, Tasaki O, Fujita K, Hosotsubo H, Kuwagata Y, Tanaka H, Shimazu T, Sugimoto H. Synbiotics

decrease the incidence of septic complications in patients with severe SIRS: A preliminary report. Digestive Diseases and Sciences 2009 54(5): 1071-1078.

Shin HJ and Yang JW. Enzymatic production of galactooligosaccharide by Bullera singularis beta-galactosidase. J Microbiol Biotechnol 1998 8: 484-489.

Shin HJ, Park JM, Yang JW. Continuous production of galacto-oligosaccharides from lactose by Bullera singularis beta-galactosidase immobilized in chitosan beads. Proc Biochem 1998 33: 787-792.

Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic Escherichia coli to tissue culture cells. Infection and Immunity 2006 74: 6920-6928.

Silk DBA, Davis A, Vulevic J, Tzortzis G, Gibson GR. Clinical trial: the effects of a trans-galactooligosaccharide prebiotic on faecal microbiota and symptoms in irritable bowel syndrome. Aliment Pharmacol Ther 2009 29: 508-518.

Smiricky-Tjardes MR, Grieshop CM, Flickinger EA, Bauer LL, Fahey, Jr. GC. Dietary galactooligosaccharides affect ileal and total-tract nutrient digestibility, ileal and fecal bacterial concentrations, and ileal fermentative characteristics of growing pigs. J Anim Sci 2003 81: 2535-2545.

Stadelmann F. A new species of the genus Bullera Derx. Antonie van Leeuwenhoek 1975 41: 575-582.

Sugawara G, Nagino M, Nishio H, Ebata T, Takagi K, Asahara T, Nomoto K, Nimura Y. Perioperative symbiotic treatment to prevent postoperative infectious complications in biliary cancer surgery: A randomized controlled trial. Annals of Surgery 2006 244(5): 706-714.

Sumiyoshi W, Urashima T, Nakamura T, Arai I, Nagasawa T, Saito T, Tsumura N, Wang B, Brand-Miller J, Watanabe Y, Kimura K. Galactosyllactoses in the milk of Japanese women: changes in concentration during the course of lactation. J Appl Glycosci 2004 51: 341-344.

Suzuki Y, Uchida K. Formation of beta-galactosyl compounds of arabinosylcytosine in growing culture of Sporobolomyces singularis. Biosci Biotechnol Biochem 1994 58: 639-643.

Suzuki Y, Uchida K. Formation of beta-galactosyl compounds of pyridoxine in growing culture of Sporobolomyces singularis. Biochim Biophys Acta 1992 1116: 67-71.

Tanaka R, Takayama H, Morotomi M, Kuroshima T, Ueyama S, Matsumoto K, Kuroda A, Mutai M. Effects of administration of TOS and Bifidobacterium breve 4006 on the human fecal flora. Bifidobacteria Microflora 1983 2: 17-24.

Tannock GW, Munro K, Bibiloni R, Simon MA, Hargreaves P, Gopal P, Harmsen H, Welling G. Impact of consumption of oligosaccharide-containing biscuits on the fecal microbiota of humans. 2004 70: 2129-2136.

Tatekawa Y, Muraji T, Takamizawa S. A case report of a patient with near total intestinal aganglionosis followed by the role of extended myectomy and synbiotics therapy. Journal of Pediatric Surgery 2007 42(4): e9-e12.

Teuri U, Korpela R, Saxelin M, Montonen L, Salminen S. Increased fecal frequency and gastrointestinal symptoms following ingestion of galacto-oligosaccharide-containing yogurt. J Nutr Sci Vitaminol 1998 44: 465-471.

Teuri U, Korpela R. Galacto-oligosaccharides relieve constipation in elderly people. Ann Nutr Metab 1998 42: 319-327.

Toba T, Watanabe A, Adachi S. 1982. Allolactose and 6-O-β-D-Galactopyranosyl-D-Galactose in commercial yogurt. J Dairy Sci. 65:702-706.

Tzortzis G, Goulas AK, Gee JM, Gibson GR. A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. J Nutr. 2005 135(7): 1726-31.

van den Heuvel EGHM, Schaafsma G, Muys T, van Dokkum W. Nondigestible oligosaccharides do not interfere with calcium and nonheme-iron absorption in young, healthy men. Am J Clin Nutr 1998 67: 445-451.

van den Heuvel EGHM, Schoterman MHC, Muijs T. Transgalactooligosaccharides stimulate calcium absorption in postmenopausal women. J Nutr 2000 130: 2938-2942.

van Dokkum V, Wezendonk B, Srikumar TS, van den Heuvel EGHM. Effect of nondigestible oligosaccharides on large-bowel functions, blood lipid concentrations and glucose

absorption in young healthy male subjects. European Journal of Clinical Nutrition 1999 53: 1-7.

Veereman-Wauters G. Application of prebiotics in infant foods. Br J Nutr 2005 93: S57-S60.

Vernazza CL, Gibson GR, Rastall RA. Carbohydrate preference, acid tolerance and bile tolerance in five strains of Bifidobacterium. Journal of Applied Microbiology 2006 100: 846-853.

Vos AP, Haarman M, Buco A, Govers M, Knol J, Garssen J, Stahl B, Boehm G, M'Rabet L. A specific prebiotic oligosaccharide mixture stimulates delayed-type hypersensitivity in a murine influenza vaccination model. International Immunopharmacology 2006 6: 1277-1286.

Vulevic J, Drakoularakou A, Yaqoob P, Tzortzis G, Gibson GR. Modulation of the fecal microflora profile and immune function by a novel trans-galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. Am J Clin Nutr 2008 88: 1438-1446.

Wang Y, Zeng T, Wang S-E, Wang W, Wang Q, Yu H-X. Fructo-oligosaccharides enhance the mineral absorption and counteract the adverse effects of phytic acid in mice. Nutrition In Press, Corrected Proof, Available online 8 August 2009. doi:10.1016/j.nut.2009.04.014.

Yamashita K and Kobata A. Oligosaccharides of human milk v. isolation and characterization of a new trisaccharide, 6'-galactosyllactose. Arch Biochem Biophys 1974 161: 164-170.

Yasutake N. Bacterial Reverse Mutation Assay on Bullera singularis YIT 10047. Safety Research Center, Yakult Central Institute for Microbiological Research, Yakult Honsha Co., Ltd., 1796, Yaho, Kunitachi-shi, Tokyo. October 28, 2002.

Zafar TA, Weaver CM, Zhao Y, Martin BR, Wastney ME. Nondigestible oligosaccharides increase calcium absorption and suppress bone resorption in ovariectomized rats. J Nutr 2004 134: 399-402.

Ziegler E, Vanderhoof JA, Petschow B, Mitmessner SH, Stolz SI, Harris CL, Berseth CL. Term infants fed formula supplemented with selected blends of probiotics grow normally and have soft stools similar to those reported for breast-fed infants. *J Ped Gastroenterol Nutr* 2007 44: 359-364.

Zambell KL, Fitch MD, Fleming SE. Acetate and butyrate are the major substrates for de novo lipogenesis in in rat colonic epithelial cells. J Nutr 2003 133: 3509-3515.

Zentek J, Marquart B, Pietrzak T. Intestinal effects of mannanoligosaccharides, transgalactooligosaccharides, lactose and lactulose in dogs. Journal of Nutrition 2002 132: 1682S-1684S.

Zhong Y, Cai D, Cai W, Geng S, Chen L, Han T. Protective effect of galactooligosaccharide-supplemented enteral nutrition on intestinal barrier function in rats with severe acute pancreatitis. Clin Nutr. 2009 28: 575-580.