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October 18, 2007

Paulette Gaynor
GRAS Notification Program
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Galacto-oligosaccharides (GOS)

Dear Dr Gaynor:

On behalf of Friesland Foods Domo, ENVIRON International Corporation is pleased to submit this Notification of the Generally Recognized as Safe (GRAS) Determination for the use of galacto-oligosaccharides (GOS) in foods and in foods for term infants. This Notification contains 1) the GRAS Exemption Claim, 2) the Statement of Consensus of an Expert Panel; and 3) the GRAS Determination narrative document.

Sincerely,

(b) (6)

Gavin Thompson
Principal Consultant

10-19-07P02:15 RCVD

24-15175C

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GRAS Exemption Claim for Galacto-Oligosaccharides (GOS)

A. NAME AND ADDRESS OF NOTIFIER

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Contact:

Rob van Vliet
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B. COMMON OR USUAL NAME OF GRAS SUBSTANCE

The substance that is the subject of this GRAS determination is galacto-oligosaccharides (GOS) prepared with a β -galactosidase derived from *Bacillus circulans*. Other chemical names for this GOS are galactooligosaccharide, transgalactosylated oligosaccharide, transgalacto-oligosaccharide and oligogalactosyl-lactose. The common name for a product currently manufactured by Friesland Foods Domo that contains the GOS that is the subject of this GRAS determination is Vivinal®GOS (formerly known as Elix'or and "OLIGO").

C. INTENDED USE

GOS prepared with a β -galactosidase derived from *Bacillus circulans* is intended to be added to a variety of foods and also infant formulas for the routine feeding of term infants. The food categories to which GOS will be added and the maximum concentrations of GOS in foods and infant formulas are detailed in Table I-1 below.

GRAS Determination for GOS
Friesland Foods Domo

Table 1. Intended Uses of GOS

| Food Group | Food Group Category^a | Approximate serving size^b (g) | Maximum g GOS per serving^c |
|---|--|---|--|
| Bars | bars | 40 | 5.0 |
| Dairy products | yogurt | 227 | 7.5 |
| | frozen dairy desserts | 70 (½ cup) | 3.0 |
| Fruit drinks and waters/quenchers | fruit drinks (vitamin/mineral fortified) and energy drinks | 240 (240 mL) | 5.0 |
| | fitness water and thirst quenchers | 240 (240 mL) | 3.0 |
| Fruit preparations | fruit pie filling | 85 | 5.0 |
| | fruit prep | 40 (2 Tbsp) | 5.0 |
| | jelly/jam | 20 (1 Tbsp) | 5.0 |
| Infant formulas for term infants and baby foods | infant formula | 8 g per L | NA ^d |
| | infant meal replacement drinks | 250 | 3.0 |
| | baby juice | 120 (120 mL) | 3.0 |
| | baby yogurt drink | 125 (120 mL) | 3.0 |
| | baby dessert | 110 | 3.0 |
| | baby snack | 7 | 1.0 |
| Milk beverages | milk | 244 (240 ml) | 5.0 |
| | milk drinks | 250 (240 mL) | 7.5 |
| | syrup flavoring for milk | 40 (2 Tbsp) | 5.0 |
| | meal replacement drinks | 250 (240 mL) | 5.0 |
| | milk substitutes | 245 (240 mL) | 5.0 |

^a In some food group categories, not all types of foods are intended for addition of GOS (e.g., addition of GOS is limited to nonfat and low fat milk only not whole and reduced fat milk). The specific types of products intended for addition of GOS are detailed in Table III-3.

^b Serving sizes based on Reference Amounts Customarily Consumed (RACC) (21 CFR §101.12). Actual product serving sizes may differ slightly from these values.

^c GOS concentrations in foods or beverage as consumed.

^d Not applicable.

D. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of GOS (prepared with a β -galactosidase derived from *Bacillus circulans*) as an ingredient in foods and term infant formulas at the maximum levels described in Section C of this chapter is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of GOS from the intended uses specified above, as estimated by ENVIRON International Corporation (ENVIRON), 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 GOS is safe, and GRAS, under the intended conditions of use, the safety of the intake of GOS 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 GOS as an ingredient in foods and infant formulas has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

- 1) Results from *in vitro* and *in vivo* studies provide evidence that GOS is largely resistant to gastric acidity, hydrolysis by mammalian enzymes, and absorption in the gastrointestinal tract. The available evidence indicates that GOS is fermented in the lower gastrointestinal tract.
- 2) Human milk contains a complex mixture of oligosaccharides, and oligosaccharides account for the third largest solid constituent in human milk after lactose and fat. The total amount of complex oligosaccharides in mature human milk is typically estimated to range from 5 to 8 g/L, though higher levels have also been reported.
- 3) GOS are chains of galactose units, usually with a single terminal glucose molecule ((galactose(Gal))_n-glucose (Glu)). The type of β -glycosidic linkage between the monomer units is mainly 1→4 Gal, though other β -glycosidic

linkages including 1→6 Gal, 1→2 Glc, 1→3 Glc, 1→4 Glc, 1→6 Glc, 1→2 Gal and 1→3 Gal may also be present.

- 4) GOS prepared with a β -galactosidase derived from *Bacillus circulans* is a well characterized oligosaccharide. It is a mixture of di-octasaccharides composed of 1-7 galactose units linked to a glucose molecule. The product reproducibly meets compositional standards and complies with limits on contaminants appropriate for food-grade ingredients. Product specifications are set to assure that GOS is suitable for use in food.
- 5) The β -galactosidase enzyme preparation derived from *Bacillus circulans* and used in the production of GOS is well characterized and reproducibly meets compositional and activity standards and complies with limits on contaminants appropriate for food-grade ingredients. Product specifications are set to assure that the β -galactosidase preparation is suitable as a processing aid. The starting organism, *Bacillus circulans*, is appropriately maintained and tested to assure purity of the organism. *Bacillus circulans* has no pathogenic activity, is not mutagenic, is negative for all tested toxins, has low acute toxicity, and has regulatory approvals in other countries.
- 6) Mean and 90th percentile 2-day average intakes of GOS by all individuals ages 2 and older who reported consumption of at least one food potentially fortified with GOS are 8.0 and 16.8 g/day, respectively. The mean estimated intake by infants 0-5 months old is 8.1 g/day and the mean GOS intake by infants 6-11 months old is 8.4 g/day. Teenage males have the highest estimated intake of GOS; their estimated mean and 90th percentile 2-day average intakes are 9.4 and 18.9 g/day, respectively. On a g/kg-bw/day basis, infants are estimated to have the highest intakes of GOS. The estimated 90th percentile 2-day average intake of GOS by infants 0-5 months old is 1.88 g GOS/kg-bw/day, and the estimated 90th percentile intake by infants 6-11 months of age is 1.55 g GOS/kg-bw/day. The estimated 90th percentile 2-day average intake of GOS by all users age 2 and older is 0.33 g GOS/kg-bw/day.

- 7) GOS has been tested for potential toxicity in two repeat dose 90-day studies in rats. Under the conditions of the tests, No Observed Adverse Effect Levels (NOAELs) were established at the highest doses tested due to the absence of adverse events. In one published study, GOS was administered to male and female Sprague Dawley Crl:CD[®](SD)IGS BR rats at doses of 0, 1.13 or 2.25 g GOS/kg-bw/day. The NOAEL of GOS in rats in this study was 2.25 g GOS/kg-bw/day. In an unpublished 90-day repeat dose study, GOS was administered to Wistar rats at doses of approximately 0, 1.6, 3.2, or 6.1 g GOS/kg-bw/day in male rats, and 0, 1.8, 3.6, or 6.9 g GOS/kg-bw-day in female rats. The NOAEL of GOS in rats in this study was 6.9 g GOS/kg-bw/day based on the highest dose tested in females. Other repeat dose and chronic studies of GOS consumption were conducted in rats, mice, pigs and dogs. Findings from these studies and the unpublished 90-day study corroborate the safety of GOS under the conditions of use.
- 8) A total of seven published studies and two unpublished studies of the effects of adult ingestion of GOS produced by Friesland Food Domo were reviewed; the studies represent six separate clinical trials. Results from published clinical studies involving the administration of GOS produced by Friesland Foods Domo to adults daily for time periods up to 3 weeks at doses of 8.1 to 20.8 g GOS per day (provided in 2 or 3 equivalent portions) indicate that consumption of GOS at these levels of intake is generally well tolerated; results from the unpublished studies corroborate this finding. The adverse effects associated with these levels of GOS intake were typically increased flatulence or gastrointestinal discomfort, and generally mild in nature. In one study, intake of 20.8 g GOS was reported to be well tolerated over a period of 5 days; GOS was consumed in lower doses in the preceding 4 days. In another study, intake of 20 g GOS daily for 4 days followed intake of lower doses of GOS for 4 days; gastrointestinal complaints were reported during GOS consumption, though they were considered to be mild in nature.
- 9) Nine additional studies (representing 10 study populations) of the effects of GOS consumption by adults were reviewed. The GOS used in these studies was from sources other than Friesland Foods Domo. In these studies GOS was administered for time periods ranging from 6 days to 4 weeks with doses ranging

from 2.4 to 15 g GOS per day. Findings from these studies corroborate the safety of GOS consumption.

- 10) Results from a published study of term infants consuming 2.4 g GOS per L in infant formula daily for 6 months indicate that the supplemented formula was well tolerated. Results from two other studies in which infants consumed GOS-fortified formula for 3 or 18 weeks, or children consumed milk containing GOS for a period of 1 year, also indicate that the GOS-containing products were well tolerated. Additionally, results from 11 clinical studies in which term infants consumed infant formula containing a combination of GOS and fructo-oligosaccharide (FOS) at levels up to 7.2 g GOS per L to age 6 months indicate that the supplemented formulas are well tolerated by infants, produce no adverse effects such as diarrhea, reflux or increased incidence of crying, and support normal growth. Results from these studies also suggest that formulas with added GOS or GOS in combination with FOS may influence shifts in infant gut microflora that result in gut microflora more similar to the gut microflora of breast-fed infants.
- 11) Results from two clinical trials in a special population, preterm infants, indicate that formulas containing 9 g GOS per L and 1 g FOS per L were well tolerated and supported normal growth over the 2 or 4 week study periods. Findings from these studies in preterm infants corroborate safety of the GOS-supplemented formulas in term infants.

Determination of the GRAS status of GOS and the β -galactosidase enzyme preparation derived from *Bacillus circulans* used in the production of GOS, under the intended conditions of use, has been made through the deliberations of A. Wallace Hayes, Ph.D., D.A.B.T., E.R.T., F.A.T.S. (Harvard School of Public Health, Boston, MA); David J. A. Jenkins, MD, Ph.D., D.Sc. (Professor, Canada Research Chair in Nutrition and Metabolism, Department of Nutritional Sciences); and Judith K. Jones, M.D., Ph.D. (President and CEO, The Degge Group, Ltd.). 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 GOS and the potential human

GRAS Determination for GOS
Friesland Foods Domo

exposure to GOS resulting from its intended use as an ingredient in foods and term infant formulas and have concluded

*There is no evidence in the available information on GOS or the β -galactosidase enzyme preparation derived from *Bacillus circulans* and used in the production of GOS, that demonstrates or suggests reasonable grounds to suspect a hazard to the public when the β -galactosidase enzyme preparation and GOS are used at levels that might reasonably be expected from the proposed applications. GOS and the β -galactosidase enzyme preparation derived from *Bacillus circulans* and used in the production of GOS are GRAS for use in products as proposed by Friesland Foods Domo*

Therefore, GOS and the β -galactosidase enzyme preparation derived from *Bacillus circulans* and used in the production of GOS are safe, and GOS is GRAS at the proposed levels of addition to foods and infant formula. GOS and the β -galactosidase enzyme preparation derived from *Bacillus circulans* and used in the production of GOS are, 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 Gavin P Thompson, Ph.D., Principal Consultant, ENVIRON International Corporation, 4350 North Fairfax Drive, Suite 300, Arlington, Virginia 22203. Telephone: 703-516-2300, Facsimile: 703-516-2393, Email: gthompson@environcorp.com

F. SIGNATURE

This notice of a GRAS Exemption Claim for Galacto-Oligosaccharides (GOS) under its intended conditions of use is submitted by Friesland Foods Domo

(b) (6)



Rob van Vliet
Manager, Quality Assurance
Friesland Foods Domo

12 Oct. 2007

Date

THE GENERALLY RECOGNIZED AS SAFE STATUS OF GALACTO-OLIGOSACCHARIDES (GOS)

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of food and food ingredients, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized as Safe (GRAS) status of galacto-oligosaccharides (GOS) and β -galactosidase derived from *Bacillus circulans* used as a processing aid in the manufacturing of GOS. The information for GOS and β -galactosidase is summarized in the GRAS determination document, Generally Recognized As Safe Determination for the Use of Galacto-Oligosaccharides in Foods and Term Infant Formulas

GOS is intended for use as a dietary ingredient in selected foods and beverages at maximum concentrations ranging from 1 to 7.5 g GOS per serving depending upon the specific food group. The GRAS determination for the use of GOS as an ingredient in bars, dairy products, fruit drinks and waters/quenchers, fruit preparations, baby foods, and milk beverages at the maximum use levels described in the GRAS determination, is based upon scientific procedures as described under 21 CFR §170.30(b), and corroborated by a history of safe exposure, unpublished data and use of GOS in the European Union in foods. GOS is also intended for use as a dietary ingredient in infant formula for term infants at a maximum proposed concentration of 8.0 g GOS per L infant formula, is based upon scientific procedures as described under 21 CFR §170.30(b), and corroborated by a history of safe exposure, unpublished data and approval for use of GOS in the European Union in infant formula. The intake of GOS 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.

Therefore, the β -galactosidase enzyme preparation derived from *Bacillus circulans* used in the production of GOS and GOS are GRAS for the intended uses as proposed by Friesland Foods DOMO. Because the β -galactosidase enzyme preparation derived from *Bacillus circulans* used in the production of GOS and GOS are GRAS for the intended uses, they are excluded from the definition of a food additive, and thus may be marketed for these uses without the need to promulgate a specific food additive regulation under 21 CFR

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Based on independent and collective critical evaluation of the available information, the Expert Panel concludes that the β -galactosidase enzyme preparation derived from *Bacillus circulans* used in the production of GOS and GOS, produced in accordance with current Good Manufacturing Practice (cGMP), meeting the specifications referenced in the GRAS determination document, Generally Recognized As Safe Determination for the Use of Galacto-Oligosaccharides in Foods and Term Infant Formulas, is safe for the intended uses. The Expert Panel further concludes that this use is GRAS based on scientific procedures and corroborated by history of safe exposure and unpublished data. The Expert Panel believes that other experts qualified by training and/or experience to evaluate the safety of food ingredients would concur with this conclusion

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Signature: _____
Date: 3/30/07

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Scientific Advisor to the Expert Panel
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Signature: _____
Date: 3/30/07

ENVIRON

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

Prepared for:
Friesland Foods Domo
Zwolle
The Netherlands

Prepared by:
ENVIRON International Corporation
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September 6, 2007

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I. INTRODUCTION

Friesland Foods Domo engaged ENVIRON International Corporation (ENVIRON) to assemble, summarize, and critically review the available physical, chemical, and toxicological data for galacto-oligosaccharides (GOS) under the proposed conditions of use. ENVIRON compiled the dossier of supporting information, estimated the dietary intakes under the conditions of use, and prepared this safety assessment report. This dossier provides supporting documentation for the GRAS exemption claim for GOS and presents a critical review of the published literature, data, and other information regarding GOS.

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 a β -galactosidase derived from *Bacillus circulans*. Other chemical names for GOS prepared with this enzyme 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 Vivinal® GOS (formerly known as Elix'or and "OLIGO").

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 (Glu)). In Vivinal® GOS, the saccharides vary in chain length from disaccharides to octasaccharides. The type of β -glycosidic linkage between the monomer units is mainly 1→4 Gal; 55% in the trisaccharide fraction and 72% in the higher oligosaccharide fraction (Chockchaisawasdee et al. 2004). This specific subfraction of GOS is also referred to as β -1,4-galacto-oligosaccharide or 4'-galactosyllactose. 1→6 Gal linkages occur 3 to 4% of the time. Other β -glycosidic linkages including: 1→2 Glc, 1→3 Glc, 1→4 Glc, 1→6 Glc, 1→2 Gal and 1→3 Gal can also be present (Chockchaisawasdee et al. 2004). Rabiou et al. (2001) determined that GOS can be made of differing β -glycosidic linkages depending on the enzyme source. In

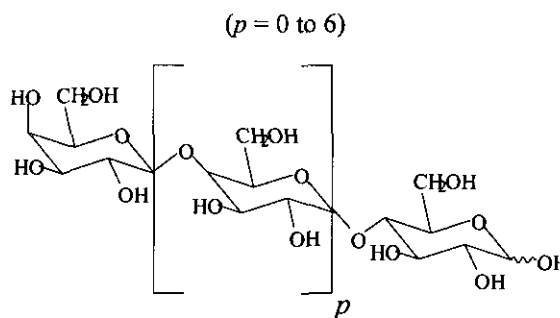
another study, GOS derived from *Bacillus circulans* and *Cryptococcus laurentii* was found to predominantly produce GOS with 1→4 Gal β-glycosidic linkages (Sako et al. 1999).

Vivinal® GOS is a product containing galacto-oligosaccharides (GOS), lactose, glucose, and a small amount of galactose. Vivinal® GOS is marketed in a syrup form but could also be applied in combination with other components in a co-spray dried form.

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 Vivinal® GOS Components by Chain Length

GOS is a mixture of di-octasaccharides composed of 1-7 galactose units linked to a glucose molecule at the reducing end. The distribution of the Vivinal® GOS components by chain length is shown in Table II-1. The major saccharide in the GOS fraction of Vivinal® is the tri-saccharide O-beta-D-galactopyranosyl-(1-4)-O-beta-D-galactopyranosyl-(1-4)-beta-D-glucose. The molecular weights of the individual oligosaccharides range between 342 (disaccharide) and 1315 (octasaccharide) Daltons. The average molecular weight (M_w) of the GOS fraction is approximately 522.28 Daltons, and the number average molecular weight (M_n) of GOS is 472.21. The average number of moles of saccharide per kg GOS is 2.12.

| Table II-1. GOS Fraction | | | | | | | |
|--|---------|------------------|--|-----------------------|--------------------------------------|-------------------------------|------------------|
| Chain Length of Saccharide (Gal) _n Glu | | p ^(a) | Empirical Formula | MW (Daltons) | Wt DM Fraction of GOS ^(b) | g Sacc ^(c) /kg GOS | Mole Sacc/kg GOS |
| Disaccharide ^(d) | n = 1 | 0 | C ₁₂ H ₂₂ O ₁₁ | 342.30 | 0.33 | 330 | 0.96 |
| Trisaccharide | n = 2 | 1 | C ₁₈ H ₃₂ O ₁₆ | 504.44 | 0.39 | 390 | 0.77 |
| Tetrasaccharide | n = 3 | 2 | C ₂₄ H ₄₂ O ₂₁ | 666.58 | 0.18 | 180 | 0.27 |
| Pentasaccharide | n = 4 | 3 | C ₃₀ H ₅₂ O ₂₆ | 828.72 | 0.07 | 70 | 0.08 |
| Hexa-, hepta-, octasaccharides | n = 5-7 | 4-6 | C ₄₂ H ₇₂ O ₃₆ ^(d) | 1153.0 ^(d) | 0.03 | 30 | 0.03 |
| | | | | Total | 1.00 | 1000 | 2.12 |
| Note (a) See Figure 1, p = (n - 1), (b) Other than lactose, (c) lactose Sacc = Saccharide, (d) Average of hexa-, hepta- & octasaccharide | | | | | | | |

B. Physical and Chemical Properties

The physical and chemical properties of Vivinal[®] GOS are identified in Table II-2. The solubility in ethanol cannot be measured, as Vivinal[®] GOS is an aqueous syrup.

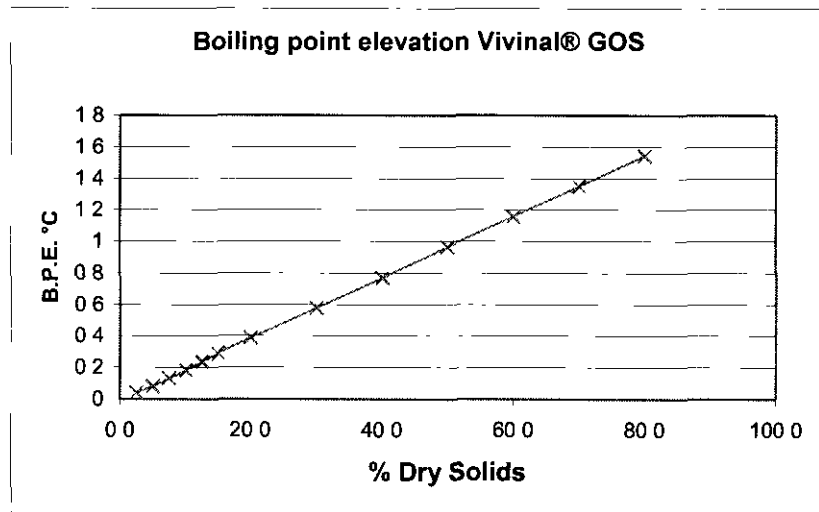
Vivinal[®] GOS is not completely soluble in vegetable oil.

| Table II-2. Properties of Vivinal [®] GOS | | |
|--|---|--------------------|
| Property | Vivinal [®] GOS | Method of Analysis |
| Purity (% GOS) | 57 (on DM) | HPAEC-PAD |
| PH | 3.2 – 3.8 | ISO 10523 |
| Appearance | clear to yellowish (flavescent) viscous liquid | IDF 99C (1997) |
| Solubility in: | | |
| Water | completely soluble | IDF 129A (1988) |
| Ethanol (g/mL) | NA | NA |
| Vegetable Oil | NA | NA |
| Density (g/ml (20°C)) | 1.38 | IDF 134A (1995) |
| Boiling Point (Range?) | NA | NA |
| Taste | slightly sweet | IDF 99C (1997) |
| Odor | No Characteristic odor | IDF 99C (1997) |
| Note: NA = not applicable, DM = dry matter Source: Friesland Foods Domo | | |

Vivinal[®] GOS is stable when boiling and will not decompose, as heat-treatment of 120°C for 30 minutes did not result in the breakdown of GOS. The boiling point increase (Figure 2) has been calculated from the freezing point depression (Figure 3) by means of the equation:

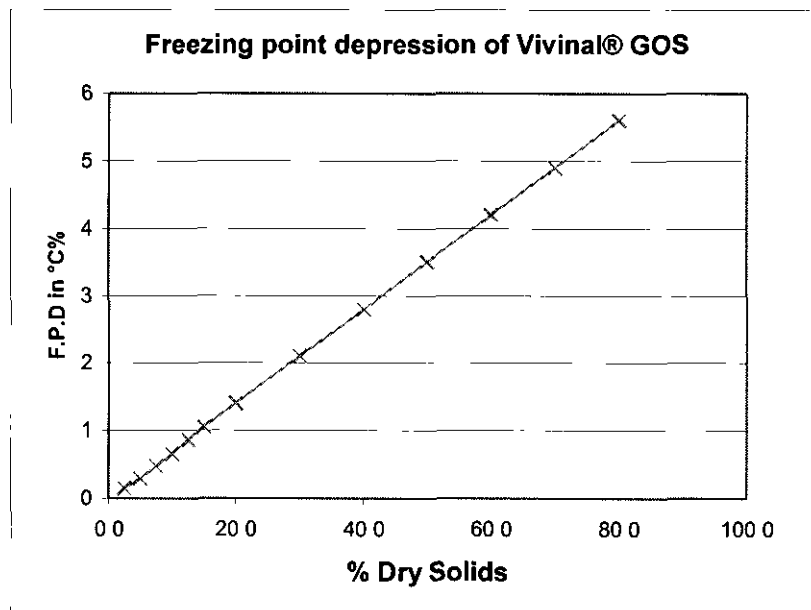
$$\Delta T_{\text{boiling point}} = \Delta T_{\text{freezing point}} \times (0.51/1.85).$$

Figure 2. Boiling Point Elevation of Vivinal® GOS



Source: Friesland Foods Domo

Figure 3. Freezing Point Depression of Vivinal® GOS



Source: Friesland Foods Domo

000020

C. Production Process

1. Starting Materials

Vivinal® 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 (Glu). Other raw materials used in the production of Vivinal® GOS are listed in Table II-3. Specifications for the materials identified in Table II-3 are listed in Appendix 1.

| Table II-3. Materials Used to Produce GOS | | | |
|--|------------|------------------|---------------------------------------|
| Material | CAS Number | Function | Food Use Approvals (21 CFR Section) |
| Lactose | 63-42-3 | substrate | 168.122 |
| β-galactosidase | 9031-11-2 | Enzyme catalyst | 184.1387 / 184.1388 |
| Water | 7732-18-5 | Solvent | NA ^a |
| Citric acid | 77-92-9 | production aid | 184.1033 |
| Sodium hydroxide | 1310-73-2 | production aid | 184.1763 |
| Activated carbon | 7440-44-0 | production aid | 173.25 |
| Cellulose | 9004-34-6 | purification aid | 172.868 / 172.872 / 172.870 / 172.874 |
| Hydrochloric acid | 7647-01-0 | purification aid | 182.1057 |
| Perlite filter aid | 93763-70-3 | purification aid | NA ^b |
| Resin 1 | NA | purification aid | 173.25 |
| Resin 2 | NA | purification aid | 173.25 |
| Resin 3 | NA | purification aid | 173.25 |
| ^a Not applicable | | | |
| ^b Perlite is qualified as a filtration aid in food processing under 7 CFR 205.605 | | | |
| Source: Friesland Foods Domo | | | |

2. Beta-Galactosidase

β-galactosidase is the enzyme used in the production of GOS. This enzyme has a long history of use in food ingredients. The bacterial species used to make the β-galactosidase preparation in the production of Vivinal® GOS is *Bacillus circulans*, strain ATCC 31382. Once the enzyme preparation is generated, Biolacta® N5 is then produced. Information on the bacterial strain used to produce β-galactosidase, the identity of the enzyme, enzymatic properties, historical use and safety data of the enzyme as well as the production process of Biolacta® N5 are located in Appendix 2. Information presented in Appendix 2 demonstrates that *Bacillus circulans* has no pathogenic activity, is not

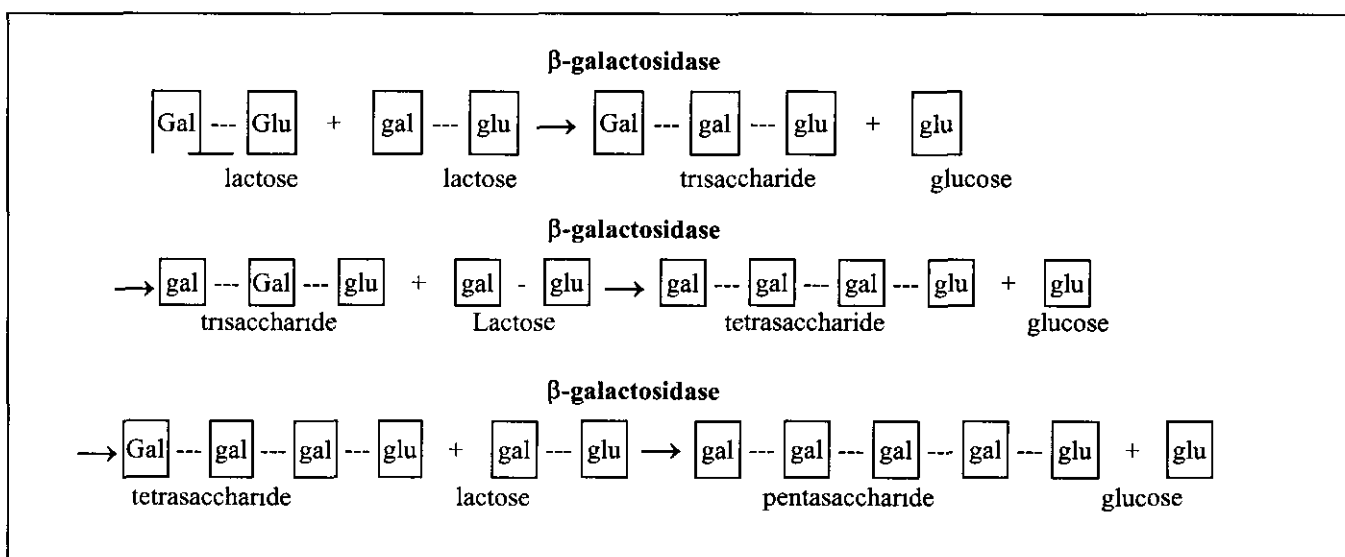
000021

mutagenic, is negative for all tested toxins, has low acute toxicity, and has regulatory approvals in other countries.

3. Vivinal® GOS Production Process

Galacto-oligosaccharides are produced through the enzymatic conversion of lactose (Matsumoto 1993). Vivinal® GOS is prepared from edible lactose in suspension, isolated from sweet whey (derived from cow's milk). The lactose is subjected to the action of a β -galactosidase. This enzyme reaction gives rise to galacto-oligosaccharides with increasing chain lengths by a series of transglycosylation reactions (Figure 4).

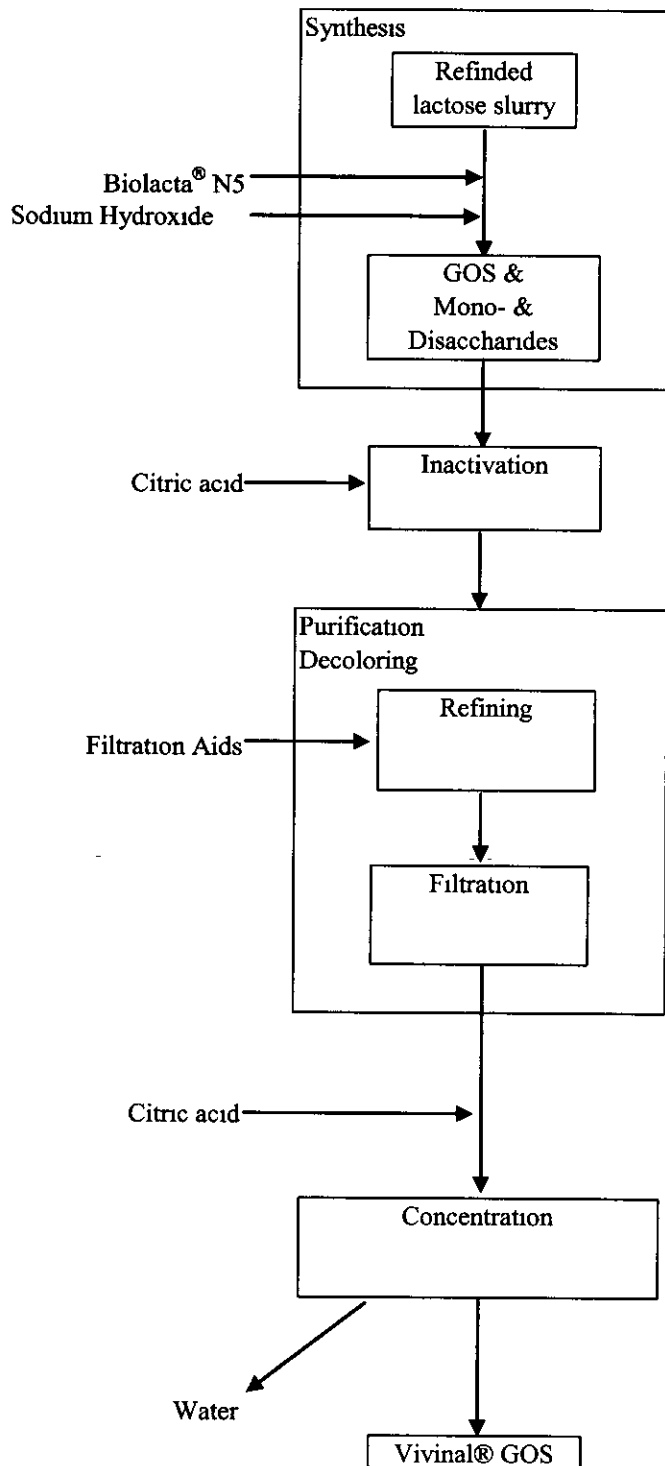
Figure 4. Formation of GOS from Lactose by β -Galactosidase



The enzyme β -galactosidase and sodium hydroxide (to adjust the pH) are added to the liquid lactose slurry and mixed. The mixture is then heated and citric acid (to lower the pH) is added in order to inactivate the enzyme and stop the reaction. Once the enzyme is inactivated, the residues and other possible impurities are removed from the product by adsorption and filtration processes. These processes remove the denatured enzyme. For these processes activated carbon, cellulose hydrochloric acid, perlite and several resins are used. These components are removed from the product by filtration. The processing aids used during the production are indicated in Table II-3. The material is then concentrated by evaporation of water to produce the product Vivinal® GOS. Figure 5 is a flow chart representation of the GOS manufacturing process.

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Figure 5. Vivinal® GOS Production Process



Source: Friesland Foods Domo

D. Product Characterization

1. Batch Analysis Results and Product Specifications

The composition of five batches Vivinal® GOS was determined; these data are shown in Table II-4. Product specifications are also shown in the table. The content of heavy metals was determined for another five batches (Table II-5). As can be seen, the values constitute the limits of detection, indicating that these metals were not found in the formulation. Documentation for the batch analysis results of Vivinal GOS and details of the methods of analysis are located in Appendix 3.

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GRAS Determination for GOS
Prepared for Friesland Foods Domo

Table II-4. Batch Analysis Data and Specifications for Vivinal® GOS

| | Lot Number | | | | | Mean | SD ^a | Specification | Analytical Method |
|--|------------|--------|--------|--------|--------|--------|-----------------|----------------|---|
| | 611698 | 612924 | 613006 | 614405 | 618025 | | | | |
| Chemical Composition | | | | | | | | | |
| Dry matter, Total | 74.0 | 75.1 | 74.7 | 75.3 | 75.0 | 74.82 | 0.51 | 74 – 76% | IDF 26A (1993), 2.5h 102± 2°C |
| GOS | 58.4 | 60.8 | 60.0 | 60.1 | 60.4 | 59.94 | 0.92 | min 57% DM | AOAC vol 85 (2002), method 2001.02 |
| Lactose (anh) ^b | 20.3 | 18.5 | 18.7 | 18.0 | 17.4 | 18.58 | 1.08 | max 23% DM | AOAC vol 85 (2002), method 2001.02 |
| Glucose (anh) | 20.2 | 19.7 | 20.1 | 20.7 | 21.0 | 20.34 | 0.51 | max 22% DM | AOAC vol 85 (2002), method 2001.02 |
| Galactose | 0.9 | 1.1 | 1.2 | 1.2 | 1.3 | 1.14 | 0.15 | min 0.8% DM | AOAC vol 85 (2002), method 2001.02 |
| Sulphated Ash | 0.13 | 0.16 | 0.19 | 0.2 | 0.26 | 0.188 | 0.05 | max 0.3% DM | AOAC 17ed. (2000) 930.30, sulphated <550°C till constant weight |
| Nitrogen | 0.000 | 0.006 | 0.006 | 0.009 | 0.03 | 0.0102 | 0.01 | max 0.016% DM | IDF 20B (1993), Kjeldahl |
| Nitrite | 0.08 | 0.06 | 0.06 | 0.4 | 0.0 | 0.12 | 0.16 | max 2ppm DM | IDF 97A (1984), spectrophotometric |
| pH | 3.51 | 3.3 | 3.5 | 3.4 | 3.6 | 3.46 | 0.11 | 3.2 – 3.8 | ISO 10523 (1994), potentiometric (10% w/w) |
| Viscosity (25°C) | 2059 | 2074 | 1918 | 2221 | 1677 | 1989.8 | 205.14 | 1000-5000 cPs | HAAKE |
| Microbiological Components | | | | | | | | | |
| Total Plate Count (30°C)/g | 3 | <1 | 19 | 3 | 1 | 6.5 | 8.39 | max 3000 cfu/g | IDF 100B (1991), PCMA 72h 30°C |
| Enterobacteriaceae | absent | absent | absent | absent | absent | ---- | ---- | absent in 1 g | BDI 23, VRBG 24h 30°C |
| E.coli per g | absent | absent | absent | absent | absent | ---- | ---- | absent in 5 g | IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C |
| Yeasts per g | <1 | <1 | <1 | <1 | <1 | <1 | 0 | max 50 cfu/g | IDF 94B (1990), OGYE 5 days 25°C |
| Molds per g | <1 | <1 | <1 | <1 | <1 | <1 | 0 | max 50 cfu/g | IDF 94B (1990), OGYE 5 days 25°C |
| Staph. Coag. Pos | absent | absent | absent | absent | absent | ---- | ---- | absent in 1 g | IDF 60C (1997), GCB 48h 37°C, BPA 48 h 37°C |
| Salmonellae | absent | absent | absent | absent | absent | ---- | ---- | absent in 25 g | IDF 93B (1995) |
| ^a SD = standard deviation | | | | | | | | | |
| ^b (anh) = anhydrous | | | | | | | | | |
| Source: Friesland Foods Domo, Appendix 3 | | | | | | | | | |

GRAS Determination for GOS
Prepared for Friesland Foods Domo

| Table II-5. Heavy Metals in Vivinal® GOS | | | | | | | |
|---|--------------|--------|--------|--------|--------|---------------|---|
| | Batch Number | | | | | Specification | Analytical Method |
| | 339080 | 338111 | 340061 | 338113 | 340019 | | |
| Arsenic (mg/kg) | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 | <0.4 | NEN-EN 14084 |
| Lead (mg/kg) | <0.04 | <0.04 | <0.04 | <0.04 | <0.04 | <0.2 | NEN-EN 14084 |
| Cadmium (mg/kg) | <0.04 | <0.04 | <0.04 | <0.04 | <0.04 | <0.06 | NEN-EN-ISO 11885, NPR 6425 and NEN-EN 13804 |
| Mercury (µg/kg) | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | NEN-EN 13806 and NEN-EN 13804 |
| Note: All values for the batch data analyses correspond to the limit of detection for each metal. Source: Friesland Foods Domo, Appendix 3 | | | | | | | |

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2. Residual Impurities

After the synthesis of GOS, the β -galactosidase is inactivated by the addition of acid (citric acid) and heating. Activated carbon is then added to adsorb impurities and decolorize the product. After the addition of cellulose a filtration step is performed to remove the denatured enzyme and impurities. Any impurities in Vivinal[®] GOS would be the raw materials used in the manufacturing process: lactose, water, citric acid, sodium hydroxide, activated carbon, cellulose and the monomers of the disaccharide lactose (galactose and glucose). Lactose, galactose, glucose and cellulose are natural constituents of various foods. Cellulose is a major constituent of many edible plants and is the major component of cellophane. In addition, various cellulose derivatives are approved for direct addition to foods (21CFR §172.868 ethyl cellulose; §172.872 methyl ethyl cellulose; §172.870 hydroxypropyl cellulose; §172.874 hydroxypropyl methyl cellulose). Citric acid and sodium hydroxide are GRAS (21 CFR §184.1033 and 184.1763, respectively). Activated carbon is used extensively for purification by adsorption of food and pharmaceutical products.

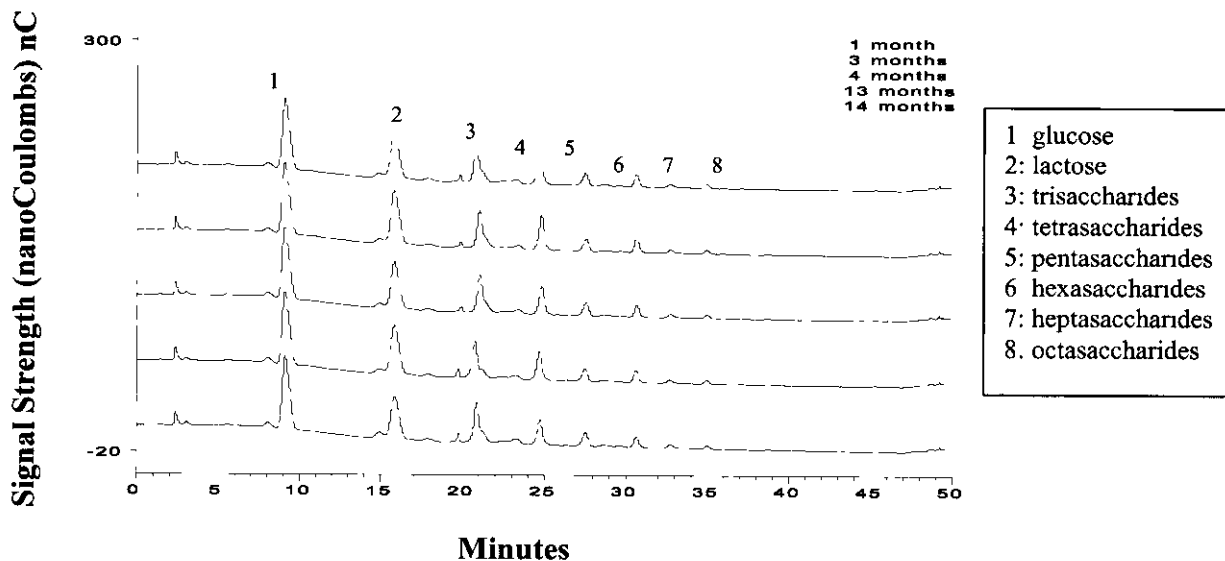
3. Stability of Vivinal[®] GOS

Vivinal[®] GOS is stable during long-term storage at pH 3.6 and 30°C for a period of 14 months. Both the total amount and the composition of the GOS remain unchanged. An example of the stability of Vivinal[®] GOS during storage is given in Figure 6. The vertical axis, nC (nanoCoulombs), represents the signal strength of the detector while the horizontal axis, minutes, represents the retention times of the various peaks.

A chromatographic analysis of five Vivinal[®] GOS batches produced between 1999 and 2002, all of which were analyzed simultaneously in 2002, is presented in Appendix 3. These results indicate that Vivinal[®] GOS is stable over an extended period of storage.

In a shelf life test performed in August 2001 (Table II-6), the contents of GOS, lactose, glucose and galactose were measured in samples of Vivinal[®] GOS after 14 to 20 weeks of storage at 18- 22°C. When multiple lot numbers are listed, the average of the samples is represented in the table. These data indicate that the GOS, lactose, glucose and galactose were stable during these 20 weeks, as their content still complies with the product specification of Vivinal[®] GOS.

Figure 6. Storage Stability of Vivinal® GOS



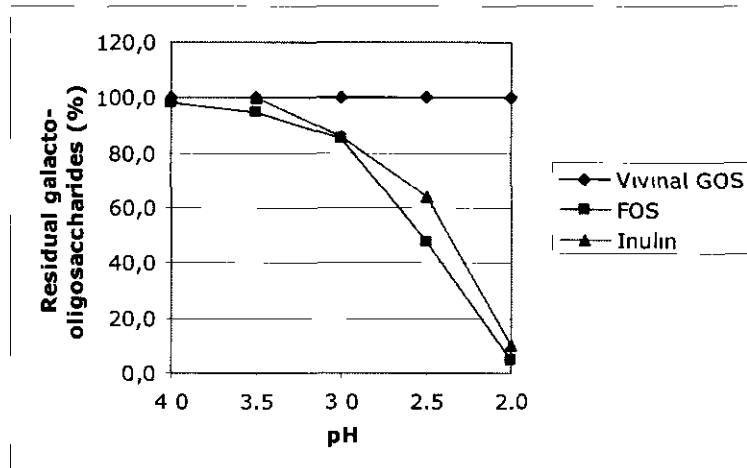
Source: Friesland Foods Domo

| Table II-6. GOS Composition During Storage | | | | |
|---|-----------------|---------|---------|-----------|
| Weeks | GOS | Lactose | Glucose | Galactose |
| | % in dry matter | | | |
| 14 | 59.8 | 21.1 | 18.2 | 0.96 |
| 16 | 59.2 | 20.5 | 19.0 | 1.33 |
| 17 | 58.6 | 21.8 | 18.7 | 0.94 |
| 18 | 59.0 | 20.7 | 19.3 | 1.01 |
| 19 | 59.5 | 19.0 | 20.0 | 1.47 |
| 20 | 58.6 | 20.4 | 19.8 | 1.22 |
| Note. 1 to 3 samples analyzed in duplicate at each time point, samples were from different lot numbers at each time point Source: Friesland Foods Domo | | | | |

Friesland Foods Domo has studied the stability of Vivinal® GOS under varying conditions of pH and temperature. Figures 7 and 8 show findings of tests conducted containing 2% GOS or 2% FOS.

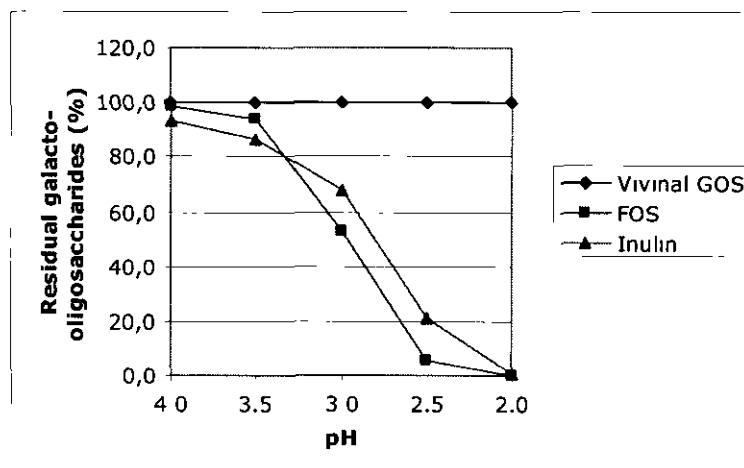
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Figure 7. Stability of Vivinal® GOS at Low pH's and 85°C for 5 Minutes



Source. Friesland Foods Domo

**Figure 8. Stability of Vivinal® GOS at Low pH's and 85°C for 5 Minutes
Followed by 7-day Storage at 30°C**



Source Friesland Foods Domo

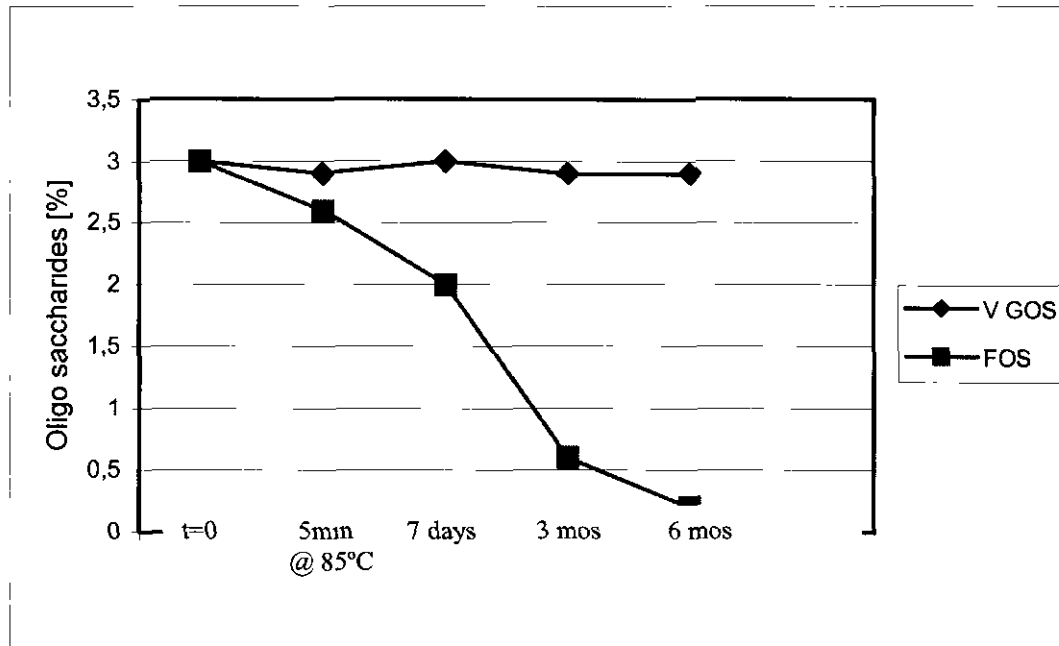
These findings show that Vivinal® GOS syrup is stable under low pH conditions and at high temperatures. Pasteurization and sterilizations at low pH will not affect Vivinal® GOS and the product will maintain its structure, appearance and content of GOS. This experiment shows that GOS does not break down under these conditions.

The stability of GOS has also been tested in a soft drink model, as illustrated in Figure 9. The model soft drink consisted of water, saccharose, oligosaccharides and citric acid in order to adjust the pH to 3.0. Galacto-oligosaccharides (GOS) or fructo-oligosaccharides (FOS) constituted 3.0% of the mixture. The amount of GOS or FOS in the model soft drink system was analyzed by use of ion exchange chromatography at five different time points including: pre-pasteurization, post-pasteurization and at storage periods of 7 days, 3 months and 6 months. GOS did not break down after pasteurization followed by a storage time of 6 months.

In food products requiring heat treatment, GOS remains stable at temperatures up to 80°, 100° and 120°C and pH values of 3-7 (Figure 10).

C00030

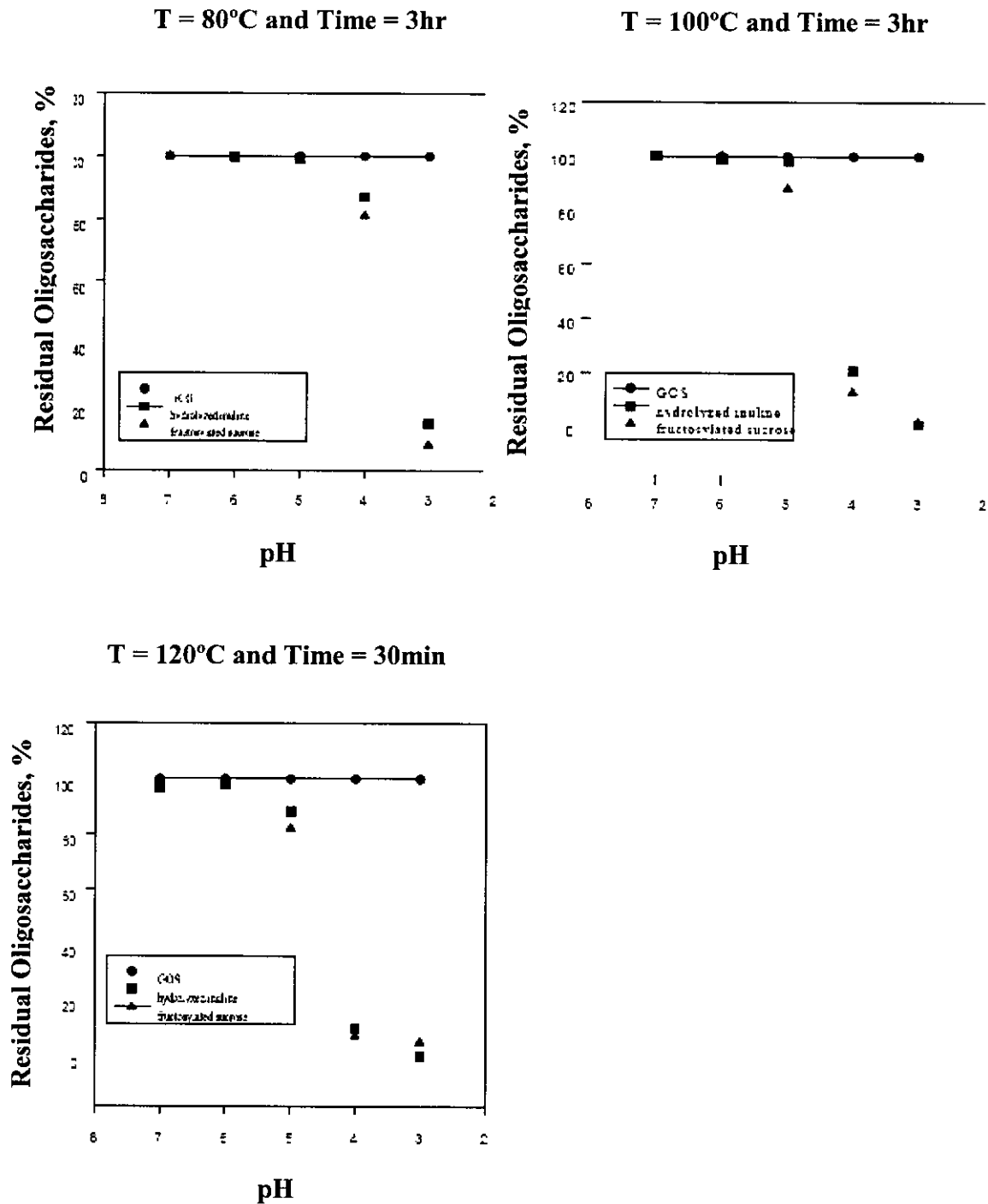
Figure 9. Stability of GOS and FOS in a Soft Drink Model



Source: Friesland Foods Domo

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Figure 10. Stability of GOS at 80 °, 100 ° and 120°C at pH values 3-7



Source: Friesland Foods Domo

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E. Method of analysis of Vivinal® GOS in foods

For a quantitative analysis of GOS in food products the HPAEC-PAD method (High Performance Anion Exchange Chromatography, coupled with Pulsed Amperometric Detection) is used. This method is approved by the AOAC as an official AOAC method (2001.02). The analysis method has been described by De Slegte (2002).

For the analysis of the GOS content in products high in lactose (>20% w/w) and low in GOS (1-2% w/w), an adapted extraction procedure for method 2001.02 has been developed (Appendix 3).

III. HISTORY OF USE

A. Historical Exposure to GOS

1. Naturally Occurring GOS

Human milk contains a complex mixture of approximately 130 different oligosaccharides (McVeagh and Miller 1997). Oligosaccharides account for the third largest solid constituent in human milk after lactose and fat (Newburg 2000). The total amount of complex oligosaccharides in mature human milk is estimated to range from 5 to 8 g/L (Kunz et al. 2000), though other investigators have reported oligosaccharide levels as high as 25.6 g/L in human colostrum and 15.4 g/L in mature milk (Coppa et al. 1991, 1997). The oligosaccharide content of human milk varies with the infant's gestation, with the duration of lactation, diurnally, and with the genetic makeup of the mother (McVeagh and Miller 1997, Erney et al. 2000). Kunz and colleagues (2000), however, reported no differences between term and preterm milk oligosaccharide levels. GOS with the structure $\text{Gal}\beta 1 \rightarrow 6\text{Gal}\beta 1 \rightarrow 4\text{Glc}$ was isolated from human milk (Yamashita and Kobata 1974). In milk samples collected from five women, the GOS content ranged from 1.9 to 3.9 mg/L.

In comparison to human milk oligosaccharides, which have a large and diverse array of molecules, the composition of bovine milk oligosaccharides is simple, though structurally the compounds are similar to those found in human milk (Gopal and Gill 2000). Bovine colostrum has been reported to contain 8.5 mg/L GOS (Saito et al. 1987). Mature bovine milk contains only trace amounts of total oligosaccharides and no GOS has been detected (Kunz et al. 2000, Saito et al. 1987). Commercial yogurt (a fermented milk product), however, has been reported to contain low levels (0.03%-0.09%) of GOS due to the enzymatic activity of β -galactosidases on lactose (Toba et al. 1982).

2. GOS Added to Foods

GOS has a history of use in the food industry. Industrial scale production of GOS started in the early 1980s. In 1995, several products were available in Japan. The first product containing GOS to be sold in Europe was launched in 1997- a Dutch product called "Umer". Umer is a fermented milk product having a consistency between that of yogurt and quark and is used as a dessert. A summary of foods containing GOS on market in 1995 is presented in Table III-1.

C00034

| Table III-1. Foods Containing GOS in Japan in 1995 | | |
|---|-----------------------|------------------------|
| Food | Product | Company |
| Lactic acid beverage | Yakult 80 Ace, BIFIL | Yakult |
| Soft Drink | Hi-Line | Yakult |
| Candy | Oligo-Throat-Candy | Novel Confectionary Co |
| | Lemon Candy | Nisshin Sugar Mfg. Co. |
| Table Sugar | Cupoligo Sweet | Nisshin Sugar Mfg. Co. |
| Ice Cream | Strawberry Palflavour | Nisshin Sugar Mfg. Co. |
| Powdered Milk | Revenge | Nisshin Sugar Mfg. Co. |
| Nutrient Food | Calorian | Nisshin Sugar Mfg. Co. |
| Note: Data provided by Friesland Foods Domo | | |

Galacto-oligosaccharides including Oligomate 50 and Oligomate 55 (Yakult Pharm. Ind. Co.), Cupoligo H-70 and Cupoligo P (Nisshin Sugar Mfg. Co.) have been used as food ingredients in Japan for a number of years. At present, numerous foods contain GOS. Applications, among others, include its use in infant nutrition, functional foods and clinical nutrition (see Table III-2). The amount of GOS in foods varies by product. Current infant formula products contain up to 8 g GOS per L of formula as consumed. Current functional foods contain up to 5.0 g GOS per 100 g of food. Dairy products contain 0.24-5.0 g GOS per 100 g food. Beverages contain 0.75 g GOS per 110 ml. Other types of food products contain 0.25 to 1.5 g GOS per 100 g food.

| Table III-2. GOS-Containing Products Currently on the Market in Europe and Asia | | |
|--|--|-------------------|
| Food Application | Product Names | Company |
| Infant Nutrition | Nutrilon, Nutrilon 2 Pepti, Omneo, Powdered Baby Milk, Pepti 2 Powdered Baby Milk, Starting Milk, Baby Milk New Packaging, Toddler Growth Formula, Hami, Stage 2 Formula | Nutricia |
| | Comformul, Aptamil HA 2, HA 1 Baby Food, Ha Pre Baby Food, Growing Up Milk, Follow-On Baby, Anti-Allergic Baby Food, Milumil 3 Junior Milk, Milumil Wachstums Milch Milk Powder, Forward Baby Milk | Milupa |
| | Flesvoedingen Bottle Feeds, Infant Milk 1, Baby formulae, Follow-On Formula, Follow-On Milk 2 for Infants, | Friso |
| | Lenilac 1&2 | Plasmon |
| | HN Heilnahrung, Folgemilch, Folgemilch 3 Baby Milk | Humana |
| | Sustagen, Enfakid DH*RA 4 Infant Powdered Milk Product, Enfalac 1 Infant Formula, Enfagrow, | Mead Johnson |
| | Enfapro 2 Follow-on-Formula Infant Milk Powder, School with 3 Prebiotics Chocolate Powder, School Vanilla Flavour | |
| Functional Foods | Blemil, Baby Milk Drinks | Ordesa |
| | Step-Up Follow-On Milk | Cow & Gate |
| | Gefilus drink, Valio Gefilus Tehojuoma - Vanilja | Valio |
| | Dairy Drink, Latte Parzialmente scremato fermentato con | Amarci e Piacersi |

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| Table III-2. GOS-Containing Products Currently on the Market in Europe and Asia | | |
|--|---|----------------|
| Food Application | Product Names | Company |
| | probiotici | |
| | Biola dairy drink, Strawberry & Blueberry Soured Milk with LGG, Symet Melk med Fersken Peach Flavoured Milk | TINE |
| | Baby'up bifidus | Yoplait |
| | Children's Instant Chocolate Drink | Milupa |
| | Barre de Croissance | Nutricia |
| Clinical Nutrition | Resource 2 0 fibre | Novartis |
| Note: List of products provided by Friesland Foods Domo; list is not exhaustive. | | |

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, Inc in 1998 for GOS (under the brand name Elix'or®) (FDA 1998). The recommended use of GOS as a dietary supplement is 7.5 to 15 g GOS, evenly spread over the course of the day. The Notification was filed without comment by FDA.

B. Existing Regulatory Approvals for the Use of GOS in Foods

The European Union as well as Dutch, Italian and UK governments recognize Vivinal® GOS as an approved ingredient in select foods in Europe (Appendix 4). 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 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

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carbohydrates may be added to infant formulas for technical reasons or if they provide a “source of fermentable substrates for the gut microflora” (SCF 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® (SCF 2003).

A closely related oligosaccharide, fructo-oligosaccharide, has been determined to be GRAS for use in a variety of foods (FDA 2000). This oligosaccharide was determined to be safe when added to a variety of foods, including baby foods, at levels of 0.1-3.6%. Inulin, another non-digestible carbohydrate, has been determined to be GRAS for use in a variety of foods (FDA 2003).

C. Intended Uses of Vivinal® GOS

Friesland Foods DOMO intends to add GOS to a variety of foods and infant formula. The intended uses and levels of GOS per product are shown in Table III-3.

Table III-3. Intended Uses of GOS

| Food Group | Food Group Category | Examples of Foods in Category | Approximate serving size^a (g) | Maximum g GOS per serving^b |
|---|--|--|---|--|
| Bars | bars | snack bars, meal replacement bars, breakfast bars | 40 | 5.0 |
| Dairy products | yogurt | yogurt, excluding frozen yogurt | 227 | 7.5 |
| | frozen dairy desserts | frozen desserts such as ice creams and frozen yogurts, frozen novelties | 70 (½ cup) | 3.0 |
| Fruit drinks and waters/quenchers | fruit drinks (vitamin/mineral fortified) and energy drinks | fruit drinks (<100% real juice) identified as a vitamin and/or mineral fortified product; energy drinks | 240 (240 mL) | 5.0 |
| | fitness water and thirst quenchers | water with added vitamins/minerals, thirst quenchers | 240 (240 mL) | 3.0 |
| Fruit preparations | fruit pie filling | fruit fillings for pies | 85 | 5.0 |
| | fruit prep | fruit fillings in bars, cookies, yogurt, cakes, etc. | 40 (2 Tbsp) | 5.0 |
| | jelly/jam | jellies, jams, fruit preserves, fruit butters | 20 (1 Tbsp) | 5.0 |
| Infant formulas for term infants and baby foods | infant formula for term infants | infant formula and follow-on formulas, includes ready-to-drink formula or formula prepared from powder or liquid concentrate | 8 g per L | NA ^c |
| | infant meal replacement drinks | meal replacement products such as Pediasure [®] | 250 | 3.0 |
| | baby juice | all types of juice identified as "baby" juices | 120 (120 mL) | 3.0 |
| | baby yogurt drink | yogurt and juice beverages identified as "baby" drinks | 125 (120 mL) | 3.0 |
| | baby dessert | fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts) | 110 | 3.0 |
| | baby snack | baby crackers, pretzels, cookies, snack items such as Gerber Graduates [®] finger foods | 7 | 1.0 |
| | | | | |
| Milk beverages | milk | all acidophilus or fortified milks; nonfat and lowfat fluid milks; includes fluid milk and unreconstituted milk powder | 244 (240 mL) | 5.0 |
| | milk drinks | flavored milks including chocolate milk, coffee drinks, cocoa, smoothies (dairy or fruit based), other fruit and dairy combinations, kefir, includes ready-to-drink and powder mixes | 250 (240 mL) | 7.5 |

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GRAS Determination for GOS
Prepared for Friesland Foods Domo

| Table III-3. Intended Uses of GOS | | | | |
|---|--------------------------|--|--|--|
| Food Group | Food Group Category | Examples of Foods in Category | Approximate serving size ^a (g) | Maximum g GOS per serving ^b |
| | syrup flavoring for milk | syrups used to flavor milk beverages | 40 (2 Tbsp) | 5.0 |
| | meal replacement drinks | meal replacement beverages or diet beverages; includes ready-to-drink beverages and powder mixes | 250 (240 mL) | 5.0 |
| | milk substitutes | soy milk | 245 (240 mL) | 5.0 |
| ^a Serving sizes based on Reference Amounts Customarily Consumed (RACC) (21 CFR §101.12) Actual product serving sizes may differ slightly from these values ^b GOS concentrations in foods or beverage as consumed ^c Not applicable. | | | | |

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D. Estimated Intakes of GOS from the Intended Uses

Estimates of potential intakes of GOS resulting from the intended uses of the oligosaccharide in select foods and infant formula 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). This NHANES data set provides nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States (NCHS 2006).

As part of the examination, trained dietary interviewers collect detailed information on all foods and beverages consumed by respondents in the previous 24 hour time period (midnight to midnight). A second dietary recall was administered by telephone 3 to 10 days after the first dietary interview, but not on the same day of the week as the first interview. A total of 9,043 respondents provided complete dietary intakes for the Day 1 recall, and 8,354 of the individuals provided a complete Day 2 recall.

The data files used to process the NHANES 2003-2004 dietary recalls include 6940 food codes, each identified by a unique number and descriptive name. The food codes represent single component foods or ingredients such as milk or vegetable oil; finished foods such as bread or margarine; and also food mixtures such as a grilled cheese sandwich. The database was reviewed, and all food codes (or portions of food codes) corresponding to one of the intended use categories of GOS were identified. Beverages containing milk as a component of a mixture, for example milk with chocolate syrup or a latte, also were identified, and the proportion of milk in each beverage (g per 100 g food code) was estimated using USDA survey files (USDA 2006). The weight per serving of each food code also was estimated using USDA survey files (USDA 2006).

Using the list of food codes and the NHANES 2003-2004 dietary recall data files from individuals with two complete days of dietary recall, ENVIRON estimated mean and 90th percentile 2-day average intakes of GOS from the individual product categories and also all categories combined. The 2-day average intakes represent the total estimated intakes of GOS during the two days of recall divided by two (i.e., $(\text{Intake}_{\text{Day 1}} + \text{Intake}_{\text{Day 2}})/2$). Intakes were calculated for subpopulations of infants (0-5 mo M+F, 6-11 mo M+F, 12-23 mo M+F), children (2-5 y M+F, 6-11 y M and F separately), teenagers (12-18 y M and F separately), adults (19+ y M and F separately), and all individuals ages 2+ y. Survey respondents were categorized into age groups based on ages reported at the time of the examination component in NHANES. The estimates were generated using survey

sample weights to adjust for differences in representation of subpopulations; results therefore are representative of the U.S. population.

Estimates of GOS intake from all uses in foods and infant formula combined are shown in Table III-4. As shown in the table, mean and 90th percentile 2-day average intakes of GOS by all individuals ages 2 and older who reported consumption of at least one food potentially fortified with GOS are 8.0 and 16.8 g/day, respectively. The mean estimated intake by infants 0-5 months old is 8.1 g/day and the mean GOS intake by infants 6-11 months old is 8.4 g/day. Teenage males have the highest estimated intake of GOS; their estimated mean and 90th percentile 2-day average intakes are 9.4 and 18.9 g/day, respectively. On a g/kg-bw/day basis, infants are estimated to have the highest intakes of GOS. The estimated 90th percentile 2-day average intake of GOS by infants 0-5 months old is 1.88 g GOS/kg-bw/day, and the estimated 90th percentile intake by infants 6-11 months of age is 1.55 g GOS/kg-bw/day. The estimated 90th percentile 2-day average intake of GOS by all users age 2 and older is 0.33 g GOS/kg-bw/day. The estimated intakes of GOS (g/day) by population and food group category are shown in Appendix 5.

Table III-4. Estimated 2-Day Average Intakes of GOS from All Proposed Uses in Foods and Infant Formula

| Population ^a | n ^b | Percent users ^c | 2-Day Average GOS Intakes Per User | | | |
|-------------------------|----------------|----------------------------|------------------------------------|-----------------|----------------------------|-----------------|
| | | | g GOS/d | | g GOS/kg-bw/d ^d | |
| | | | Mean | 90th Percentile | Mean | 90th Percentile |
| Infants, 0-5 mo | 101 | 100.0 | 8.1 | 11.2 | 1.34 | 1.88 |
| Infants, 6-11 mo | 172 | 99.5 | 8.4 | 12.5 | 0.98 | 1.55 |
| Infants, 12-23 mo | 229 | 85.6 | 5.3 | 11.2 | 0.47 | 1.03 |
| Children, 2-5 y | 621 | 92.3 | 7.8 | 18.2 | 0.45 | 1.01 |
| Boys, 6-11 y | 346 | 94.7 | 9.1 | 18.8 | 0.30 | 0.59 |
| Girls, 6-11 y | 404 | 92.5 | 8.9 | 16.4 | 0.28 | 0.51 |
| Teen males, 12-18 y | 693 | 79.1 | 9.4 | 18.9 | 0.16 | 0.38 |
| Teen females 12-18, y | 685 | 76.7 | 8.1 | 16.7 | 0.15 | 0.30 |
| Adult males, 19+ y | 1428 | 69.2 | 8.3 | 17.7 | 0.10 | 0.21 |
| Adult females, 19+ y | 1673 | 73.1 | 7.4 | 15.3 | 0.11 | 0.22 |
| Total population, 2+ y | 5850 | 75.0 | 8.0 | 16.8 | 0.15 | 0.33 |

^a Breastfeeding infants and children were excluded from the sample population

^b Number of people consuming one or more foods containing GOS during the two 24-hour periods of dietary recall

^c Weighted percent

^d Analysis of intake in terms of g GOS/kg-bw/d was limited to people with a measured body weight

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It is important to note that all estimates of intake presented in Table III-4 are likely overestimates of actual intakes of GOS 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 III-3) was assumed to contain added GOS. 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 GOS, and not all products may contain the maximum proposed use levels of GOS.

The estimates of potential GOS intake by infants were based on the survey population of non-breastfeeding infants. The youngest infants (i.e., prior to introduction of weaning foods) in the sample population therefore are presumably consuming exclusively infant formula. Some infants, however, consume a combination of human milk and infant formula. The intakes of GOS by infants consuming a combination of human milk and formula would likely be lower than the estimates of GOS intake based on the population of exclusively formula fed infants.

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IV. INTENDED EFFECT

The subject of the GRAS determination, galacto-oligosaccharide (GOS), is a non-digestible carbohydrate that selectively stimulates the growth and/or activity of one or a limited number of bacteria in the colon. The intended effect of GOS is to provide a dietary source of this oligosaccharide.

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V. SAFETY DATA

A. Introduction

A prebiotic was first defined as “a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (Gibson and Roberfroid 1995). Since the introduction of the prebiotic concept, three specific criteria have been established for classification of a food ingredient as a prebiotic (Roberfroid 2007). These criteria are: (1) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; (2) fermentation by intestinal microflora; and (3) selective stimulation of the growth and/or activity of thus intestinal bacteria that contribute to health and well being.

Galacto-oligosaccharides have been determined to meet the prebiotic criteria (Roberfroid 2007). A review of the evidence regarding GOS’s resistance to digestion and absorption, fermentation by intestinal microflora, and stimulation of selective intestinal growth and/or activities provides an understanding of the fate of GOS in the human gastrointestinal tract and the impact of GOS on human health. Results from sub-chronic studies of GOS in rats provide information on the systemic effects and toxicity of the oligosaccharide, while results from clinical studies provide evidence of the safe and well tolerated use of GOS by adults and infants.

Friesland Foods Domo provided ENVIRON with references to studies conducted with Vivinal® GOS. Additionally, ENVIRON conducted searches for toxicological and safety information related to ingestion of all sources of galacto-oligosaccharides. The searches were conducted using the DIALOG® Toxicology and Medicine categories in November and December of 2006. The searches covered all available publication years. Search terms used were various names of the substance including, but not limited to: GOS, TOS, prebiotic, galacto-oligosaccharide and/or oligogalactose. The search was further refined using terms including, but not limited to: fermentation, digestion, safety, toxicity, microflora, tolerance and infants. An additional search of publication years 2006-2007 was conducted in early February 2007 to identify any literature that may have appeared since the previous searches. Potentially relevant articles were retrieved and reviewed and summaries of the studies are presented in Tables V-6 to V-11 and Tables V-13 and V-14. The review was limited to English-language documents.

B. Resistance of GOS to Gastric Acidity, Hydrolysis and Absorption

Results from *in vitro* and *in vivo* studies provide evidence that GOS is largely resistant to gastric acidity, hydrolysis by mammalian enzymes, and absorption in the gastrointestinal tract. More than 90% of the GOS is estimated to arrive in the colon (Van Loo et al. 1999). Resistance of GOS to gastric acidity has been shown *in vitro* at pH as low as 1.0 and a temperature of 37°C. Results from both *in vitro* and *in vivo* experiments with human intestinal or salivary enzymes or porcine digestive enzymes indicate that GOS is largely resistant to hydrolysis. Results from studies of the intestinal permeability of lactulose (a disaccharide) provide information on the potential absorption of an oligosaccharide such as GOS. The resistance of GOS to normal endogenous digestive processes in the small intestine results in material for fermentation in the large intestine, with the major products of fermentation including carbon dioxide, hydrogen, short chain fatty acids and bacterial cell mass (Cummings et al. 2001).

Incubation of 4'galactosyllactose (GOS) prepared from lactose by *Cryptococcus laurentii* with artificial gastric juice for a period of 6 h at 37°C and pH of 1.0, 1.5 or 2.0 resulted in no hydrolysis as measured by liberated glucose (Ohtsuka et al. 1990). Under these same conditions, sucrose and fructooligosaccharides demonstrated hydrolysis.

Ohtsuka et al. (1990) studied the digestibility of 4'galactosyllactose (GOS) prepared from lactose by *Cryptococcus laurentii* in a series of *in vitro* experiments. No increase in reducing sugar concentration (glucose) was observed in a 1% solution of 4'GOS exposed to alpha-amylase from human saliva or hog pancreas during a 60 minute incubation; in contrast, soluble starch (the control) resulted in a steady increase in reducing sugar during the first 30 minutes of incubation, after which the glucose concentration remained relatively stable. The investigators also studied the hydrolysis by rat small intestinal mucosa. The susceptibility of GOS to saccharidase activity in the jejunum was low, as it was approximately 1/1,300 of that for maltose, 1/270 of that for sucrose and 1/85 of that for lactose. Daily intake of GOS for a period of 6 weeks did not alter hydrolyzing activities of GOS in the duodenum or jejunum, though there was a small but significant increase in the hydrolysis of GOS in the ileum after the GOS feeding period (13.5 vs 9.8 nmol substrate hydrolyzed per mg protein per h).

Burvall et al. (1980) investigated the digestibility of oligosaccharides containing galactosyl residues in beta-(1→6) linkages. In this *in vitro* study, liberation of galactose during incubation of the tri- and tetrasaccharides with human intestinal homogenate was

observed to be less than 10% of the liberation observed during incubation of the homogenate with lactose. In the presence of p-chloromercuric benzoate (PCMB), an inhibitor of beta-galactosidase activity, liberation of galactose from the tri- and tetrasaccharides was less measured to be 2% or less of the liberation from lactose. These findings suggest that oligosaccharides are minimally digested in the small intestine, and that hydrolysis of the oligosaccharides is due primarily to the action of beta-galactosidase.

Unpublished studies on the Friesland Foods Domo GOS provide corroborative evidence of the resistance of GOS to hydrolysis and digestion (Asp 1994; see Appendix 6). In an *in vitro* experiment, a pool of human small intestinal biopsy homogenates with normal disaccharidase activities was incubated with GOS for 6 hours. At the end of the observation period, liberation of glucose and galactose from GOS was less than 10% of that from lactose. The liberation of glucose appeared to reach a plateau, while galactose liberation proceeded in a linear manner. In a separate investigation, 10 different intestinal biopsies were incubated with GOS at 37°C and at a pH of 1, 2, 3, 4 or 5. After 2 hours, the liberation of glucose from GOS was approximately 5-10% of that from lactose, more galactose was liberated than glucose, and there was no detectable lactose in the GOS preparation, indicating that the oligosaccharide is stable against acid hydrolysis.

In an *in vivo* study of the digestibility of Friesland Foods Domo's GOS, GOS was observed to pass the small intestine in pigs almost completely intact (cited in EM Industries, Inc. NDI submission, FDA 1998).

C. Fermentation of GOS and Selective Stimulation of Intestinal Bacteria

1. *In Vitro* Evidence

More than 90% of the GOS is estimated to arrive in the colon (Van Loo et al. 1999). Several *in vitro* studies (Table V-1) have been conducted to study the fermentation of GOS by intestinal microbes, and to investigate if GOS stimulates selective growth or activity of intestinal bacteria associated with human health. Results from these studies suggest that growth of bifidobacteria is stimulated by GOS. The available *in vitro* data also indicate that GOS is fermented in the colon as measured by increased production of short-chain fatty acids.

Based on pure culture studies, Tanaka and colleagues (1983) reported that all nine strains of bifidobacteria incubated in a 1% GOS medium expressed active growth within 24 hours, as did two strains of bacteroides and four strains each of lactobacilli and enterobacteria. Within two to three days, growth of four additional strains of lactobacilli, two strains of streptococci and another strain of enterobacteria were observed, while no growth of fusobacteria, eubacteria, clostridia, *Propionibacterium acnes*, and *Staphylococcus aureus* was observed.

In another *in vitro* study, all seven test strains of bifidobacteria were able to utilize two different GOS preparations as measured by maximum specific growth rates (Hopkins et al. 1998). Vernazza et al. (2006) also reported that GOS was well utilized by the five test strains of bifidobacteria. Vulevic et al. 2004 measured bacterial populations and short-chain fatty acid production in a batch culture fermentation vessel containing a human fecal slurry and GOS. The investigators reported that GOS was almost exclusively selective towards bifidobacteria while growth of bacteroides was inhibited. Production of all short-chain fatty acids peaked at approximately 10 hours; lactic and acetic acid were the predominant short-chain fatty acids. Rycroft and colleagues (2001) reported increased counts of bifidobacteria in a static batch culture during fermentation of GOS, while no effects on total bacterial counts, clostridia, bacteroides, lactobacilli, streptococci or *E. coli* were observed. Lactate and acetate levels also increased during the fermentation of GOS.

McBain and MacFarlane (2001) studied the metabolism of GOS in a three-stage continuous culture model of the colon. Increases of bifidobacteria and lactobacilli (\log_{10} cfu/mL of 0.2 and 0.4, respectively) were observed in the proximal region, while populations of *Bacteroides* spp. decreased in the proximal and distal regions (\log_{10} cfu/mL of -0.3 to -0.4). Fermentation of GOS primarily occurred in the proximal region as indicated by acid production.

Table V-1. Summary of *in vitro* Studies with GOS

| Reference | Objective | Study Design | Effect |
|----------------------------|---|--|--|
| Hopkins et al 1998 | To determine the abilities of seven bifidobacterial isolates to utilize 15 different carbohydrate sources (including GOS and Oligomate 55). | Batch culture with bacterial growth determined using spectrophotometry Source: GOS (85% pure) & Oligomate (55% pure); Yakult, Japan | -Both GOS preparations were generally good substrates for all test strains. -Higher cell yields and specific growth rates were attained during culture of bifidobacteria on oligosaccharides compared to their monomeric constituent. |
| McBain and MacFarlane 2001 | To investigate the metabolism of inulin and GOS with respect to bacterial growth, bifidobacterial stimulatory properties and anti-mutagenicity potential in a 3-stage continuous culture model of the colon | Three-stage continuous culture model of the colon, which reproduces the physicochemical characteristics of the proximal (V1) and distal (V2, V3) colons. Samples were taken from the colon at regular time intervals over a period of 30 h. The model contained fecal slurry from 1 healthy male. Source: Yakult Pharmaceutical, Japan (85% purity) | -Within 3 h of adding GOS, fermentation was markedly stimulated in the proximal region and to a lesser extent in the distal region as indicated by production of fermentation acids. -Increases in bifidobacteria ($0.2 \log_{10}$ cfu/mL) and lactobacilli ($0.4 \log_{10}$ cfu/mL) were seen in the proximal region. -Counts of <i>Bacteroides</i> spp decreased in proximal and distal regions (0.3 to $0.4 \log_{10}$ cfu/mL). -Compared to inulin, GOS strongly suppressed β -glucosidase and β -glucuronidase as well as arylsulphatase. |
| Rycroft et al. 2001 | To compare the fermentation properties of commercially available prebiotic oligosaccharides, including GOS, by fecal bacteria using a batch culture method. | Static batch culture evaluated by fluorescent <i>in-situ</i> hybridization. Samples were removed for analysis at baseline and after 5 h fermentation to determine immediate effects of the oligosaccharides, and after 24 h to observe overall effects on bacterial composition. Gas and SCFA production also were determined. Source: Oligomate 55 (50% purity); Yakult, Tokyo, Japan. | -Bifidobacteria counts were higher than baseline values after 5 and 24 h of GOS fermentation. No significant differences in total bacterial count, clostridia, bacteroides, lactobacilli, streptococci, or <i>E. coli</i> were observed. -Lactate and acetate concentrations increased throughout the 24 h of fermentation; propionate levels were comparable at 5 and 24 h, and higher than levels at baseline. No change in the concentration of butyrate was seen. -GOS resulted in a relatively low gas production as compared to the other substrates. |
| Sharp et al 2001 | To determine the <i>in vitro</i> effect of GOS and other short-chain carbohydrates on human intestinal | Batch culture fermentation system with fecal sample for 24 h. Bacterial counts estimated via plate counts and analysis of small subunit rRNA | -No significant differences in individual species or total populations bifidobacteria via plate counts. -SSU rRNA targeted oligonucleotide probes revealed |

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Table V-1. Summary of *in vitro* Studies with GOS

| Reference | Objective | Study Design | Effect |
|----------------------|--|---|--|
| | bifidobacteria and <i>E. coli</i> | abundance. Source: Yakult, Japan | overall increase in bifidobacteria and higher numbers of <i>B. adolescentis</i> and <i>B. angulatum</i> - Growth of <i>E. coli</i> was reduced. |
| Tanaka et al. 1983 | To determine if GOS (TOS) is exclusively utilized by bifidobacteria <i>in vitro</i> | Bacterial species were incubated in 1% GOS medium at 37°C for 5 days. The bacterial species included 9 species/strains of bifidobacteria and 55 cultures of other genera. Source: Yakult, Japan | -All 9 bifidobacteria strains tested expressed active growth within 24 h -None of the cultures of other genera showed growth except two strains of <i>Bacteroides</i> and four strains each of <i>Lactobacillus</i> and <i>Enterobacteriaceae</i> Within a day, no growth was seen of <i>Fusobacterium</i> , <i>Eubacterium</i> , <i>Clostridium</i> , <i>Propionibacterium acnes</i> , <i>Streptococcus</i> , or <i>Staphylococcus aureus</i> |
| Vernazza et al. 2006 | To assess suitability of bifidobacteria inclusion in synbiotic products on the basis of carbohydrate preference and acid and bile tolerance | Five strains of bifidobacteria were analyzed for their carbohydrate preference from 12 substrates based on maximum growth rates. Source: Oligomate 55P Yakult (55% purity), Tokyo, Japan | -Maximal fermentation growth rates demonstrated that GOS was well utilized by all bifidobacteria species. -GOS was non-significantly better fermented than glucose in all but one case. |
| Vulevic et al. 2004 | To quantitatively evaluate a variety of prebiotics including TOS using the MPE (measure of prebiotic effect) equation using healthy human fecal samples. | Batch culture fermentation vessels containing a fecal slurry from one human. Samples were extracted from each vessel to determine bacterial populations via FISH, analysis of SCFA, and total carbohydrate measurements. Source: Elix'or Borculo Domo, Netherlands | -GOS was almost exclusively selective towards bifidobacteria, growth of <i>Bacteroides</i> was inhibited. -Production of all SCFAs peaked at approximately 10 h; lactic and acetic acids were the predominant acids |

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2. *In Vivo* Evidence

Studies have been conducted in rats, mice, pigs and dogs to assess the effects of GOS ingestion on fermentation by intestinal microbes and effects on growth or activity of intestinal bacteria. These studies are summarized in Table V-2. Results from these studies suggest that GOS ingestion is associated with increased numbers of fecal bifidobacteria in rats and mice, and the ingested GOS is fermented as indicated by increased short-chain fatty acid production, decreased cecal pH, and increased cecal weight. Increases in breath hydrogen also have been reported in some, though not all, studies in animals. Results from these studies also indicate that GOS is fermented and produces increased amounts of gases that are expired in the breath.

Rowland and Tanaka (1993) studied the effects of GOS on cecal bacteria and their metabolic activities in germ-free rats inoculated with human fecal flora. GOS was administered in drinking water at a dose of 5% (w/w) in the diet, which was equivalent to approximately 3 g GOS/kg-bw/d. At the end of the 4-week feeding period, cecal concentrations of total anaerobes, bifidobacteria and lactobacilli were significantly higher than levels in control animals, and cecal concentrations of enterobacteria were significantly lower. Cecal pH decreased in animals consuming GOS. GOS consumption was also associated with increased beta-glucosidase activity and decreased activities of beta-glucuronidase and nitrate reductase.

Djouzi and Andrieux (1997) also examined the effects of GOS on intestinal bacterial metabolism in germ-free rats inoculated with human fecal flora. In this study, rats consumed approximately 2.4 g GOS/kg-bw/day for a period of 4 weeks. Fecal counts of bifidobacteria were significantly higher in animals consuming the GOS diet as compared to the control diet. GOS had no effects on fecal counts of total anaerobes, bacteroides, clostridia, enterococci, or enterobacteria. Beta-galactosidase activity was increased in rats in the GOS group as compared to the control group, and hydrogen and methane excretion were 7-fold and 2.5-fold higher than levels from control animals, respectively. Cecal weight increased and cecal pH decreased in animals fed GOS and short-chain fatty acid concentrations increased.

The effects of GOS on cecal short-chain fatty acid production were studied in a series of experiments by Kikuchi-Hayakawa et al. (1997). In one experiment, male Wistar rats consumed 5% GOS in the diet (approximately 1.4 g GOS/kg-bw/day) for a period of 16 days. At the end of the feeding period, GOS consumption was associated with increased

fecal *Bacteroidadeae* and decreased fecal *Enterobacteriaceae*, though GOS had no effects on total bacteria, bifidobacteria or lactobacilli. Cecal concentrations of acetic, propionic and butyric acids were significantly higher in animals fed GOS as compared to animals fed a control diet. The isobutyric levels were lower in animal fed GOS. In the second experiment, rats were fed 5% GOS in the diet for up to 21 days and cecal contents were assessed at 1, 2, 7 or 21 days. The concentrations of lactic and acetic acids in the cecal contents were not affected by the diet or time of adaptation. The concentrations of propionic, butyric and total acids in the cecal contents were higher in the rats fed the GOS diet than in those fed the control diet, but were not affected by the interaction of the time of adaptation.

In a study of rats with induced colitis, increased counts of fecal bifidobacteria and total bacteria were seen during ingestion of approximately 4 g GOS/kg-bw/day (Holma et al. 2002). No consistent effects of GOS were seen on fecal bifidobacteria counts in 3-week old rats fed a formula containing 1.2, 5.0 or 10% GOS (Perez-Conesa et al. 2006a).

Morishita et al. (2002) fed a diet containing 5% GOS to 5 male germ-free Balb/c mice while 5 additional mice were fed a control diet. Fecal populations of *B. breve* and *B. vulgatus* increased while *C. perfringens* decreased after supplementation with a GOS diet for a period of 9 weeks.

Increases in breath hydrogen also have been reported in some, though not all, studies in animals. In a study of germ-free rats inoculated with human fecal flora, hydrogen excretion of animals consuming a 4% GOS diet for a period of four weeks was nearly 7-fold higher than excretion by rats fed a control diet, and GOS-fed rats also were found to have a 2.5-fold higher methane excretion (Djouzi and Andrieux 1997). Breath hydrogen production was increased 10-fold in rats fed a 5% or 10% GOS diet for 4 weeks as compared to rats fed a control diet, though methane production was not different between the diet groups (Kikuchi et al. 1996). In another study, germ-free rats inoculated with human fecal flora and fed a diet containing 4% GOS for one month had greater hydrogen and methane production as compared to rats fed a control diet, though no differences in gas production were observed in conventional rats (Meslin et al. 1993).

Ohtsuka et al. (1991) used a radioisotope technique to measure the excretion and distribution of [U-¹⁴C]4'-GOS in rats. In the 24-hours following administration of a single dose of [U-¹⁴C]4'-GOS to conventional rats, 48.8% of the radioactivity was recovered as ¹⁴CO₂, and the radioactivity of contents of the gastrointestinal tract, urine and feces

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accounted for another 8.7, 4.8 and 4.1% of the administered dose, respectively. An additional 12.8% of the radioactivity was recovered in the carcass, 1.4% in the liver and 0.6% in serum. In total, the investigators accounted for 81.2% of the administered radioactivity within 24 hours after administration of the [U-¹⁴C]4'-GOS. Excretion of ¹⁴CO₂ was delayed and total excretion in the 24 hours following dosing was reduced in rats treated with antibiotics. Excretion was further reduced in germ-free rats (who lack an established microbiota required to allow fermentation to occur in the gut). A total of 90.7% of the administered radioactivity was recovered in germ-free rats in the 24-hours following dosing. In these rats, the majority of GOS (58.3% of total radioactivity) was recovered in the intestine and its contents, 17.7% of activity was recovered as ¹⁴CO₂, 7.7% was found in the carcass, and smaller amounts were found in urine, feces, liver and serum. Results from this study indicate that ingested GOS is fermented in the colon by intestinal microorganisms. Ohtsuka et al. (1991) fed conventional rats a diet containing 5% GOS for a period of two weeks and used HPLC (High Performance Liquid Chromatography) to measure GOS in feces collected at the end of the feeding period. No GOS was detected in the feces, which indicates that GOS was fermented in the colon.

Other researchers have studied effects that GOS may have on other characteristics of the colon. Meslin et al. (1993) investigated the effect GOS had on mucin distribution in the gastrointestinal tract. The investigators concluded that GOS administered to three different strains of rats (germ-free, conventional and heteroxenic) at 2.4 g/kg-bw/d increased β-D-galactosidase and other glycolytic activity while also producing an intrinsic effect on mucus cell distribution in the colon.

Smiricky-Tjardes et al. (2003) reported that supplementation of 3% of the diet with GOS resulted in significant increases in bifidobacteria and *Lactobacilli* in pigs. A subsequent study conducted by Mountzouris et al. (2006) found that dietary addition of 1% GOS to a swine diet did not alter total cell populations, bifidobacteria or *E. coli*. The investigators offered explanations for a lack of observation of prebiotic effect including the possibility of 'dilution effects' due to the presence of resistance starch in the milled corn component of the diet as well as the fact that pigs naturally harbor a high number of lactobacilli and bifidobacteria which may mean their response in prebiotic supplementation might not be dramatic.

D. Systemic Effects and Safety of GOS in Animals

Results from one published subchronic toxicity study in rats demonstrated that under the conditions of the test, No Observed Adverse Effect Level (NOAEL) was estimated at the highest dose tested due to the absence of adverse events (Anthony et al 2006). Anthony

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et al (2006) reported a NOAEL of 2.25 g GOS/kg-bw/day. Another 90-day subchronic toxicity study in rats was conducted using Vivinal GOS (Lina 1995, see Appendix 7); a NOAEL of approximately 6.9 g GOS/kg-bw/day in rats was established in this study. The highest dose resulted in physiological changes such as increased cecal weight that are consistent with consumption of high levels of non-digestible carbohydrates. Results from this unpublished study therefore provide additional evidence to support the safety of GOS at the tested levels. Both of the subchronic toxicity studies are summarized in Table V-3.

Other repeat-dose and chronic studies of GOS consumption were conducted in rats, mice, pigs and dogs. These studies were designed to evaluate various efficacy endpoints; however, many also had measures of safety. GOS was administered for time periods ranging from 7 days to 12 months with dosages up to 17 g/kg-bw/day (dosages based on studies in which GOS doses on a body weight basis were provided or could be calculated using feed intake data and a default body weight). These studies corroborate the conclusions reported in the sub-chronic studies concerning the safety of ingestion of GOS.

1. Subchronic Toxicity of GOS in Sprague-Dawley Rats

The toxicity of GOS (Vivinal syrup) was investigated in male and female Sprague-Dawley Crl:CD (SD)IGS BR rats given 2.5 or 5 g GOS syrup/kg-bw/day by oral gavage daily for 90 days (Anthony et al. 2006). The GOS syrup contained approximately 45% GOS, 15% lactose and 14% glucose. The amount of GOS in the diet was 1.125 and 2.25 g/kg-bw/day. A reference control containing fructooligosaccharides (FOS), lactose and glucose (70% FOS, 15% each lactose and glucose) was included to match the digestible sugars in the test material. The reference control diet was prepared weekly and tested for homogeneity and stability over a 10-day period. Animals were approximately 6 weeks of age with initial mean body weights of 229.3 g for males and 160.7 g for females. The test material and the reference and vehicle controls were administered each morning by oral gavage to groups of 15 rats/sex/control or treated groups for 90 consecutive days. Groups of 5 animals/sex/control or treated groups dosed on study days 0 to 89, 0 to 90, and 0 to 91. These results were combined and reported as Group 1 (vehicle control), Group 2 (reference control), Groups 3 and 4 (1.125 or 2.25 g GOS/kg-bw/day, respectively).

Animals were checked twice daily for general health and morbidity with detailed clinical observations made once weekly prior to dosing and prior to scheduled euthanasia.

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Animals were monitored for 2 hours following dosing for signs of overt toxicity. Individual body weights and food consumption were recorded on the first day of treatment and weekly thereafter with a final body weight taken on day 90, 91, or 92 for the first, second, and last groups of rats, respectively. Feed efficiency values were calculated. Blood was collected following the last day of treatment (90, 91, or 92 days) from the orbital plexus of slightly anesthetized rats that were fasted overnight. Hematological and clinical chemistry parameters were evaluated. Urinalysis was conducted on urine collected overnight prior to blood collection. Ophthalmologic examinations were performed prior to the dosing and at the end of the study. All animals were subjected to a complete gross necropsy examination upon death or scheduled sacrifice on days 90, 91, or 92. Organ weights were obtained at scheduled sacrifice and selected tissues and organs were preserved for histological examinations.

Body weights, body weight gain, and food consumption were analyzed by ANOVA and, if significant, by pair-wise comparison using the Tukey-Kramer test. Clinical pathology data and absolute and relative organ weights were analyzed for homogeneity of variance using Levene's test. If significant ($p < 0.01$), multiple group comparisons were conducted using the Kruskal-Wallis, non-parametric ANOVA, followed by the Dunn's test (when $p < 0.05$). The vehicle control was compared to the results from the reference control and the two treated groups, and the reference control was compared to the results in the two treated groups.

All animals survived until scheduled sacrifice with the exception of 2 deaths due to gavage error (confirmed at necropsy) in females in the 2.25 g GOS/kg-bw/day treated group. Clinical signs, such as hair loss, ocular discharge, dark material around the eyes and noses were observed sporadically in controls and treated groups and were not considered treatment-related. No statistically significant or toxicologically relevant differences in mean body weights in either sex or dose when compared to either the vehicle or reference control were noted. Food consumption was significantly decreased in treated groups compared to controls at some time points (decreases noted in groups consuming 1.125 and 2.25 g GOS/kg-bw/day were 10% to 14% during days 49 to 70 and 14% to 19% during days 0 to 7, 14 to 21, and 33 to 89; decreases also noted in only in 2.25 g GOS/kg-bw/day group were 7% to 11% during days 14 to 21, and days 28 to 29). No significant differences in food consumption were recorded when the treated groups were compared to the reference control.

Feed efficiency was significantly increased in males taking 5.0 g GOS/kg-bw/day on days 56 to 63 and in males taking either 1.125 or 2.25 g GOS/kg-bw/day on days 84 to 89

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when compared to the vehicle controls. No differences in food efficiency were noted in any of the treated groups when compared to the FOS reference control.

Occasional statistically significant changes were noted in hematological parameters (e.g., mean hematocrit and hemoglobin values in the 2.25 g GOS/kg-bw/day females were higher compared to the FOS control). These observations were minor, inconsistent, not related to dose and within the intralaboratory historical control. Consequently, they were not considered to be of toxicological significance. All other hematological and coagulation parameters including: erythrocytes, hemoglobin, mean corpuscular volume, MCH, MCHC, platelets, nucleated RBCs, reticulocytes, prothrombin time, activated prothrombin time, fibrinogen, leukocytes, segmented neutrophils, lymphocytes, monocytes, basophils and eosinophils were comparable to both the vehicle and reference controls. Similarly, occasional statistically significant changes were seen in clinical chemistry parameters (mean ALT value was lower in the 2.25 g GOS/kg-bw/day males compared to the FOS control). These differences were also minor, inconsistent, and not dose-related within the intralaboratory historical control and consequently considered to be of no toxicological relevance. The clinical chemistry parameters evaluated were AST, ALT, alkaline phosphatase, total bilirubin, total protein, albumin, globulin A/G ratio, phosphorus, urea nitrogen, creatinine, glucose, sodium, potassium, chloride, calcium, cholesterol, and serum GGT. Sporadic differences in urine specific gravity were noted but these findings were again inconsistent, not dose-related and therefore not considered to be toxicologically relevant. No treatment-related abnormalities were found upon ophthalmological examinations.

When necropsied, no remarkable abnormalities were observed in any animals. Fresh organ weights were obtained at scheduled sacrifice for adrenals, brain, heart, kidneys, spleen and pituitary in males and females in the prostate and testes in males and the uterus in females. Absolute and relative organ weights were not significantly different from either vehicle or reference controls with three exceptions. Mean relative spleen weight in males in 2.25 g GOS/kg-bw/day group was higher than that found in the vehicle controls. Mean absolute ovary weight in females in the 2.25 g GOS/kg-bw/day group and mean relative liver weight in females in the 1.125 g GOS/kg-bw/day group was found to be lower than the FOS controls. These findings were not considered to be of toxicological significance because they were inconsistent and not dose-related. All organs and other tissues were examined microscopically (Note: the published report stated in the Methods section that the lungs, liver and kidneys from the reference group and 1.125 g GOS/kg-bw/day group were not examined; however, histological data for these organs were reported in Table 4). All histopathological findings were considered

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normal for this age and strain of rat. The investigators concluded that this study demonstrated that administration of GOS up to 2.25 g/kg-bw/day by gavage for 90 days did not cause signs of treatment-related toxicity. A NOAEL was estimated at 2.25 g GOS/kg-bw/day, the highest dose tested.

2. Subchronic Toxicity of GOS in Wistar Rats

GOS syrup was administered to groups of male and female Wistar outbred (CrI:(WI(WU)BR) rats (20 rats/sex/group) in the diet at levels of 0%, 5%, 10% or 20% daily for 90 days in a sub-chronic toxicity study of GOS syrup (Lina 1995, see Appendix 7). This study was conducted by TNO Nutrition and Food Research Institute; results from the study have not been published. In this study, GOS doses in male rats corresponded to approximately 1.6, 3.2, or 6.1 g GOS/kg-bw/day, and doses in female rats were approximately 1.8, 3.6 or 6.9 g GOS/kg-bw/day. The highest dose of pure GOS given was estimated to be 6.9 g/kg-bw/day. The GOS syrup contained 72.9% (wt%) sugar (other constituents were not listed). The syrup was diluted with water to a final water content of 35% to improve distribution in the diet. The general health of young animals, approximately 3 weeks of age, was evaluated and rats were acclimated for a week after arrival prior to being randomly assigned to four groups proportionately by weight class. Animals were housed five to a cage, separated by sex, for the entire experiment. A modified diet containing pregelatinized potato starch and water, which replaced 20% of the wheat and maize cereals, was used. Batches of the test and control diets were prepared four times during the experiment, tested for homogeneity, and frozen. Weekly, portions of the diet were thawed, kept refrigerated and dispensed daily. Food and water were provided *ad libitum*. Body weights were measured at the start of the experiment and weekly until the end of the experiment. The amount food consumed was measured per cage weekly throughout the experiment, while water intake was measured per cage in weeks 1, 6 and 12.

The general condition and behavior of the animals were evaluated twice daily (morning and afternoons) on weekdays and once in the mornings on weekends or holidays. Hematology, clinical chemistry, urinalyses and determination of cecal pH were conducted in the same 10 rats/sex/group (2 to 3 rats per cage were randomly selected). Toward the end of the treatment period (days 86 and 87), rats were fasted from water and food (for 24 and 16 hours, respectively), placed in individual metabolism cages and urine collected during the last 16 hours of fasting. Urine volume and density was measured to evaluate the concentrating ability of the kidneys. Urinalysis was carried out in samples pooled per cage. Also after overnight fasting, blood glucose was determined from blood

samples collected from the tail vein. Other clinical chemistry and hematology parameters were evaluated in blood samples collected at necropsy from the abdominal aorta. Organs were weighed and selected tissues were preserved for microscopic examination.

Body weight data, food and water intake, food conversion efficiency, hematological and clinical chemistry values, and organ weights were analyzed using ANOVA followed by Dunnett's test to determine whether group means were significantly different. Percentages of white blood cell counts, semi-quantitative urinary observations and cecal pH were analyzed by the Kruskal-Wallis test followed by pairwise comparisons using the Mann-Whitney U-test when means were significantly different.

All animals survived until scheduled sacrifice. General conditions and behavior were not adversely affected by the test substance in any group. Ophthalmoscopic changes were those common in rats of this age and strain. Transient decreases in mean body weight, body weight gain, and food intake were noted in males in the high-dose group during the first few weeks of the study only. This was considered to be due to poor acceptability rather than less efficient utilization of the diet, as food consumption efficiency was unaffected.

A number of other treatment-related changes seen in the high-dose group were considered to be physiological responses to the ingestion of a substance with dietary fiber properties and were not considered toxicologically significant. A decrease in cecal pH in both sexes was attributed to increased microbial activity resulting in the formation of acidic degradation products. Similarly, decreased plasma urea concentrations in females in the mid- and high-dose groups were caused by increased use of ammonia derived from blood urea by intestinal microorganisms. Decreased levels of phospholipids in males in the high-dose group were also noted but considered by the investigators to be non-specific physiological changes seen with other oligosaccharides, such as oligofructose or inulin. Also observed in the males high-dose group was a decrease in urinary density that was attributed to an increase in fecal excretion and decrease in urinary excretion of sodium and potassium.

No changes in hematological parameter (hemoglobin, packed cell volume, red blood cell count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), prothrombin time, thrombocyte count, total white blood cell count, or differential white cell count) were seen. No other changes in any of the clinical chemistry or urinary parameters measured were seen. The clinical chemistry parameters measured included alkaline phosphates activity (ALP), aspartate

aminotransferase activity (ASAT), alanine aminotransferase activity (ALAT), gamma glutamyl transferase activity (GGT), total protein, albumin, ratio albumin to globulin, urea, creatinine, total bilirubin, electrolytes, cholesterol, triglycerides, and phospholipids. Urinary parameters evaluated included appearance, glucose, occult blood, ketones, protein, bilirubin, urobilinogen, and microscopy of the sediment.

With two exceptions, no significant changes in absolute or relative organ weights were noted in any treated animals. The organs evaluated included the adrenals, brain, cecum, kidneys, liver, spleen, testes, and thymus. A dose-related increase in the weight of the full and empty cecum (cecal enlargement) was seen in all dose groups of both sexes. The investigators concluded this was a result of an adaptation to an increased load of osmotically active substances due to increased microbial fermentation of the undigested GOS. The lack of any histopathological changes in the cecum wall supported the observation. An increase in absolute and relative spleen weight occurred in the high-dose group females. This was considered to be of doubtful toxicological significance because there was no change in red blood cell counts or treatment-related histopathological changes in the spleen.

No treatment-related abnormalities were found during the gross necropsy examination nor were treatment-related microscopic changes noted in any of the organs or tissues examined. All changes seen were considered to be common findings for rats of this strain and age. The investigators concluded that dietary levels up to 20% GOS syrup were tolerated without signs of toxicity. The NOAEL for this study was estimated to be 6.9 g GOS/kg-bw/day, the highest dose tested.

3. Corroborative Repeat-Dose Studies of GOS Administration

Data including final body weights, body weight gains, food intake, or food consumption efficiency from repeat-dose studies of GOS administration provide corroborative information regarding the toxicity of GOS. Studies of the effects of GOS on mineral absorption, lipid levels, and growth performance in animals also are summarized in Table V-2. The findings are briefly reviewed below.

Kikuchi-Hayakawa et al. (1997) reported an increase in body weight gain but no change in food intake in male rats fed a diet containing 5% GOS (estimated to be 1.36 g/kg-bw/day) for 10 days in a study designed to investigate the effects of GOS on organic acid production. An increase in body weight gain was also reported by Ohtsuka et al. (1990) in male rats given 10% GOS in the diet for 39 days but not in rats fed a diet containing

5% GOS. Food consumption was increased at both doses in the Ohtsuka et al. (1990) study but food conversion efficiency was unaffected. Diarrhea was noted in the high-dose group rats during the first two weeks of the study, which apparently had no effect on body weight gain. Relative organ weights (stomach, small intestine, liver, heart, kidney and spleen) were unchanged compared to controls in either dose group in the Ohtsuka et al. (1990) study. The investigators determined body weight gain in both studies to be due to an increase in cecal weight. Chonan et al. (1995) reported an increase in food intake in ovariectomized rats fed a diet containing GOS (estimated to be 3 g/kg-bw/day) for 30 days. Food intake was reported to be normal in sham-operated rats fed a diet containing the same amount of GOS. Body weights were not affected in any groups.

In other studies, GOS has demonstrated no effect on final body weights, body weight gains, food intake, or food consumption efficiency when administered to male rats in the diet at GOS concentrations up to 19% (estimated to be 17 g/kg-bw/day) for up to 365 days (Appel et al. 1997; Chonan and Watanuki 1995; Chonan and Watanuki 1996; Chonan et al. 1996; Chonan et al. 2001; Djouze and Andrieux 1997; Hayashi et al. 1991; Holma et al. 2002; Kawakami et al. 2005; Kikuchi et al 1996; Rowland and Tananka 1993; Sakaguchi et al. 1998; Yanahira et al. 1997).

Effects on liver and serum or plasma lipids were evaluated in male rats fed diets containing GOS at concentrations of approximately 4% to 10%. Plasma cholesterol concentrations were significantly reduced in rats fed diets containing 5% or 10% GOS for 28 days; serum triglyceride and glucose concentrations were unchanged (Kikuchi et al. 1996). Plasma cholesterol concentrations were lower in rats fed a diet containing approximately 4% GOS for 28 days; plasma triacylglycerol concentrations were not modified (Djouzi and Andrieux 1997). Serum lipid concentrations (cholesterol, HDL-cholesterol, phospholipids and triglyceride) and liver lipid concentrations (cholesterol, triglyceride, and phospholipids) were not significantly different from controls in male rats fed diets containing 5% or 10% GOS for 39 days (Ohtsuka et al. 1990). Vos et al. (2006) showed results of an investigation to determine the impact of the dietary addition of a GOS+FOS mixture on the immune response of 53 female mice administered an influenza vaccination. GOS was given at levels equivalent to 0.9, 2.25, 4.5 and 9% of the diet. The investigators found that GOS enhanced the DTH (delayed-type hypersensitivity) response in a dose-dependent manner, while supplementation of an FOS/inulin mixture did not. GOS was also found to increase the proportion of bifidobacteria and lactobacilli in a dose-dependent manner without any differences in animal weight or feed intake during the study among groups.

The effect of GOS on absorption of essential minerals has also been investigated. Using the same test group in their bifidobacteria study, Perez-Conesa et al. (2006b) determined that administration of 12, 50 and 100 g GOS/kg-bw to rats for 30 days increases apparent calcium absorption. The high-dose group also demonstrated an increase in bioavailability of magnesium and phosphorus. The investigators noted that fecal moisture content was not affected by GOS supplementation at the three doses tested. In male rats made magnesium-deficient by feeding a diet high in phosphorus and calcium (control diet), administration of 3 g GOS/kg-bw/day in the diet for 28 days resulted in an increase in magnesium absorption compared to the control diet but was equal to that seen with the standard diet. Increases in kidney weight and accumulation of phosphorus and/or calcium in the kidney and heart seen in rats on the control diet were reversed in rats fed GOS and returned to levels seen in rats fed the standard diet (Chonan et al. 1996). The apparent calcium absorption ratio was increased in rats fed GOS in the diet for up to 30 days at concentrations ranging from 2.3 to 8.7 g GOS/kg-bw/day (Chonan and Watanuki 1995; Chonan and Watanuki 1996; Chonan et al. 1995; Chonan et al. 2001; Yanahira et al. 1997). Calcium ratios were reported to return to normal by day 30 (Chonan et al. 1995; Chonan and Watanuki 1996). In rats fed GOS in the diet at concentrations of 3.0 or 4.5 g GOS/kg-bw/day for 30 days, calcium content of femur and tibia bones was increased (Chonan et al. 1995; Chonan and Watanuki 1996). The apparent absorption ratio of magnesium was also increased in male rats fed up to approximately 4 g GOS/kg-bw/day in the diet for one to two weeks compared to a standard diet (Chonan et al. 2001; Yanahira et al. 1997). No effects on the apparent absorption or retention of sodium or potassium were seen in rats fed a diet at concentrations of 9.5% or 19% (estimated to be 8 and 17 g/kg-bw/d, respectively) for 62 days except for a decrease in the apparent absorption of potassium in the high-dose group on days 45-47. Decreased liver and stomach weights (absolute and relative to body weight) and increased relative large intestine weights were reported in the high-dose group. No effects related to liver, stomach, or large intestine weights were seen in low-dose groups (Hayashi et al. 1991).

Houdijk et al. (1998) studied the effects of FOS and GOS on the growth performance and fecal characteristics of young growing pigs by feeding male piglets diets containing 0.54% or 1.08% FOS and GOS for 6 weeks. Dry matter intake and body weight gain was decreased in the treated pigs when compared to controls in week one through three of the study; however, the mean growth performance was not affected by treatment in week one through six of the study. The investigators note that this suggests an adaptation period for non-digestible carbohydrates in pigs. There was no effect on fecal pH and FOS and GOS were not detected in the feces. The absence of GOS in the feces suggested to the

investigators that an alteration in pig microbiota may have occurred. In order to study the effects of FOS and GOS on nutrient digestion in both weanling and growing pigs, Houdijk et al. (1999) fed weanling pigs diets containing 1% or 4 % FOS or GOS and growing pigs diets containing 0.4% or 0.8% FOS or GOS for up to 27 days (weanling) or 47 days (growing). Results in the weanling group indicated there was an increase in nitrogen excretion, but no change in the ileal digestion of calcium, phosphorous, magnesium, iron, copper, or zinc in the treated group. There was also a decrease in the ileal digestion of nonstarch carbohydrates and an increase in the digestion of hemicellulose and cellulose in the treated groups. In the growing group of pigs there was increased ileal digestion of crude fiber in the 0.8% GOS group with no effects seen in the ileal digestion of other nutrients. Smiricky-Tjardes and colleagues (2003) found GOS digestibility in the ilea of pigs to be 100% for the test animals. The investigators also noted that addition of GOS to the diet resulted in a 5.5% unit decrease in digestibility of all nutrients investigated. The investigators attributed the decrease in digestion to the relatively high amount of OS in the standard diet.

The effect of oligosaccharides, lactose, and lactulose on fecal quality, nutrient and mineral digestibilities, and microflora metabolism was studied in dogs (Zentek et al. 2002). Mannan oligosaccharide, GOS, lactose, or lactulose was added to the diet of adult female beagle dogs at a dose of 1 g/kg-bw/day for 10 days. Results indicated no changes in the absorption of calcium, phosphate, magnesium or potassium compared to controls. Also, there were no changes in stool quality, no changes in unbound water, no effects on fecal pH or dry matter, and no effect on fecal ammonia. The investigators noted that higher intakes might have produced pronounced changes, although preliminary testing demonstrated a very narrow window of tolerance for dogs, with 2 g/kg-bw/d inducing diarrhea.

The effects of GOS on colorectal or pancreatic cancer have been investigated. In rats fed GOS at concentrations of 0.87, 0.88, 2.71, or 3.13 g/kg-bw/day for 12 months, no changes in multiplicity or incidence of chemically (azaserine)-induced atypical acinar cell nodules in the pancreas were reported, and GOS had no effects on final body weight, weight gain, or food consumption (Appel et al. 1997). In studies conducted to evaluate the effects of GOS on chemically-induced (1,2-dimethylhydrazine or azoxymethane) colorectal cancer, rats were given GOS by diet at low concentrations (estimated to be 0.68 and 2.04 g/kg-bw/day) and high concentrations (estimated to be 2.75 and 6 g/kg-bw/day) for 9 to 10 months (Wijnands et al. 1999; Wijnands et al. 2001). Decreases in tumor incidence, size and multiplicity were seen in high GOS groups when compared to a

corresponding group given either cellulose or low GOS, and no safety concerns with the administration of GOS were observed.

4. Summary of Animal Studies

Sub-chronic safety studies in rats have estimated No Observed Adverse Effect Levels (NOAELs) of 2.25 g GOS/kg-bw/day (Anthony et al. 2006) and 6.9 g GOS/kg-bw/day (Lina 1995, see Appendix 7) over 90-day periods. Repeat dose studies in rats, mice, pigs and dogs have shown no adverse effects of GOS on weight gain, growth or mineral absorption. A chronic rat study has demonstrated no effect on final body weight, body weight gain, or food consumption at estimated intakes up to 3.13 g/kg-bw/day for 12 months (Appel et al. 1997).

Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration * |
|--------------------|---|--|---|---|--------------------|---|
| Rats | | | | | | |
| Appel et al. 1997 | Study the effects of GOS on dietary fat-promoted pancreatic carcinogenesis induced by azaserine. Feeding study | 156 M Wistar rats, 39 per group | 4 treatments: low-fat low GOS (8.3% of diet), low-fat High GOS (27.4%), High-fat low GOS (9.54%), High-fat high GOS (28.63%). GOS delivered in diet in syrup form. | 3.1, 10.4% (low-fat), 3.6, 10.8% (high-fat) 0.88, 3.13 g/kg-bw/d (low-fat); 0.87, 2.71 g/kg-bw/d (high-fat) Source: Borculo Whey Products, Borculo, The Netherlands | 12 mo | -High GOS caused an increase in absolute and relative cecum content weight. -Increase in cell proliferation -No effect on food consumption or body weight -No effect on multiplicity or incidence of atypical acinar cell nodules. -For the High-fat diets, neither low-dose GOS nor high-dose GOS kept the high levels of fat from increasing carcinoma development. |
| Chonan et al. 1995 | Determine the effects of GOS on calcium absorption and bone loss prevention in ovariectomized (OVX) rats. Feeding study. | 36 F Wistar rats, 9 per group | 4 treatments: Sham-operated control, Sham-operated GOS, OVX control, OVX GOS | 3.0 g/kg-bw/d Source: 99% Prepared from lactose by researchers | 30 d | -Increased whole cecal, cecal wall, and cecal contents weight in GOS rats. -Decreased cecal pH -GOS increased total VFA, acetic, propionic, butyric, succinic and lactic acids -No effect on body weight. -Decreased food intake in OVX rats fed GOS -Increased Ca absorption on days 8-10 and 18-20 in GOS groups with no effect seen on days 28-30. - GOS groups demonstrated overall increase in calcium content of femur and tibia bones compared to control. |
| Chonan et al. 1996 | Study the effects of GOS on the use of magnesium and the degree of | 24 M Wistar rats (4 weeks old), 6 per group. | 4 treatments: standard diet, control, GOS (5 g/100 g | 0.75 g/d 3 g/kg-bw/d (Weight not | 28 d | -Increased whole cecal weight and cecal tissue weights. -Decreased cecal pH. -Increased cecal SCFA |

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Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration* |
|--------------------------|---|--------------------------------|--|---|--------------------|---|
| | calcification of the kidney and heart in magnesium deficient rats. Feeding study | | diet), Mg | provided in study Therefore, default rat weight of 250 g assumed.) Source: prepared from GOS by researchers | | -No effect on body weight gain or food intake. -Increased magnesium absorption when compared to rats fed standard diet. -Decreased kidney wt and accumulation of P and CA. -Decreased heart accumulation of Ca. |
| Chonan et al 2001 | Study the effects of GOS on calcium and magnesium absorption in rats Feeding study | 20 M F344 rats, 5 per group. | 4 treatments: control, neomycin sulfate, 4'-GOS, neomycin sulfate + 4'-GOS | 5% 2.3 g/kg-bw/d (Weight not given therefore, 250 g bw assumed) Source: Prepared from lactose by researchers | 7 d | -Increased whole cecal weight. -Increased cecal tissue weight -Decreased cecal pH. -No change in fecal weight -Increased Ca and Mg absorption in GOS group, no effect in GOS + Neomycin group. -No effect on body weight gain or food intake |
| Chonan and Watanuki 1995 | Study effects of TOS on calcium absorption in rats. Parallel feeding study | 30 M Wistar rats, 6 per group. | 5 treatments control, 2 lactose levels, 2 6'-GOS levels | 5, 10% (4.3, 8.7 g/kg-bw/d) Source: Prepared from lactose by researchers | 10 d | -Decreased cecal pH. -Increased whole cecal, cecal wall, and cecal digest weights. -Increased total VFA -No change in final body weight, weight gain, or food intake. -Increased percentages of calcium absorption and retention. |
| Chonan and Watanuki 1996 | Study effects of 6'-GOS on calcium absorption and | 32 M Wistar rats, 8 per group | 4 treatments: normal calcium | 5% (4.5 g/kg-bw/d) | 30 d | -Increased whole cecal, cecal tissue, and cecal digesta weights. -Decreased cecal pH. |

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Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration* |
|-------------------------|--|---|--|--|--------------------|---|
| | bone mineralization in rats adapted to different levels of dietary calcium Parallel feeding study | | control, normal calcium 6'-GOS, low calcium control, low calcium 6'-GOS. | Source: Prepared from lactose by researchers. | | Increased total VFA; Increased acetic, propionic, butyric, succinic, and lactic acid -No effect on body weight or food intake -Increased femur and tibia calcium content in normal calcium diet GOS animals. No effect in low calcium diet animals -Increased calcium absorption in normal calcium diet GOS animals during study with no effect seen on days 28-30 No effects seen in GOS animals fed the low calcium diet |
| Djouzi and Andreux 1997 | Study effects of GOS, FOS, and gluco-oligosaccharides on intestinal bacterial metabolism. Feeding study | 24 M germ-free Fischer rats (2.5 months old), 6 per group. (inoculated with human feces 2 weeks prior to feeding period) | 4 treatments: control, FOS, GOS, gluco-OS | 4 % GOS in diet. Body weight and food intake not given. 2.4 g/kg-bw/d (Weight and food intake not given therefore, 250 g bw and 15 g/d intake assumed). Source: Yakult Institute, Tokyo, Japan. | 4 wk | -Increased fecal bifidobacteria -Increased β -galactosidase, α -galactosidase, β -glucosidase and β -glucuronidase. -Increased CH ₄ and H ₂ excretion -Increased cecal weight -Decreased cecal pH and ammonia -Increased total SCFA, increased acetate and propionate. -Decreased plasma cholesterol. -Small decrease (non-significant) in plasma triacylglycerol concentrations. |
| Hayashi et al 1991 | Study the effects of GOS on bioavailability of Na and K in rats | 18 M Wistar rats, 6 per group | 3 treatments: control, 2 4'-GOS levels. | 9 5, 19% (8, 17 g/kg-bw/d) | 62 d | -Increase in absolute and relative cecum weights. -Increased wet fecal weights in both dose groups. -Decreased urine volume and increased softening of feces reported. |

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Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration * |
|---------------------|--|---|--|---|---|---|
| | | | | Source Nishin Seito Kaisha, Tokoyo, Japan. | | -No effect on weight gain or food intake in GOS groups when compared to controls. -Decreased weight gain in high GOS group when compared to low GOS group -Decreased liver and stomach weights (absolute and relative) in high-dose group. Increased relative large intestine weight in high-dose group. No effects related to liver, stomach, or large intestine seen in low- dose group -No effect on Na or K absorption or retention in high- or low-dose groups (umol/3 d) except for decrease in potassium absorption in high-dose group on days 45-47. |
| Holma et al. 2002 | Study effects of GOS on the development of inflammation and the growth of bifidobacteria. Feeding study | 42 M rats (HY WIST), 7-9 per group | 5 treatments: healthy control, colitis control, whey-derived GOS, lactose-derived GOS, dexamethasone | 4.0 g/kg-bw/d (whey-derived) Source. Valio Ltd, Helsinki, Finland 3.9 g/kg-bw/d (lactose-derived) Source Elix'or, Borculo Whey Products, The Netherlands | 13 d (induced colitis on day 10) | -No change in colon wet weight. -Increased fecal bifidobacteria and total bacteria. -No effect on percent bifidobacteria. -No effect on body weight -No effect on colonic inflammation. |
| Kawakami et al 2005 | Study the effects of GOS on unconjugated phenols in blood | 18 M Wistar rats (Charles River Japan, Inc), 6weeks | 3 treatments: control feed, feed with 2.5% tyrosine, feed | 0.66 g/d 2.6 g/kg-bw/d (Weight not | 14 d | -Decrease in cecal pH -increase in cecal tissue and content weight. -Decrease (non-significant) in cecal phenol levels. -Increase (non-significant) in phenol (serum, |

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Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration * |
|---------------------|--|--|---|--|--------------------|---|
| | | old, 6 per group | with 2.5% tyrosine and 5%GOS (GOS composed of 59 1% galacto-oligosaccharides) | provided in study. Therefore, default rat weight of 250 g assumed) Source: Oligomate 55P, Yakult Yakuhin Kogyo, Tokyo, Japan | | urine) and p-cresol (fecal, cecal, serum, urine) levels. -Authors suggest that GOS had no effect on weight gain. -Small difference in energy intake |
| Kikuchi et al. 1996 | Study effects of GOS on bacterial glycolytic activity, fermentation and bacterial steroid transformation in rats. Feeding study | 12 M germ-free Fischer 344 rats (8 weeks old), 4 per group (Inoculated by oral intubation with 2ml of a 10 ⁻² dilution of a human fecal suspension from three healthy methane-producers) | 3 treatments control, 2 GOS levels | 5, 10% (2 8, 5 6 g/kg-bw/d) Source Yakult Central Institute for Microbiological Research, Tokyo, Japan | 4 wk | -Increased fecal excretion. -Increased H ₂ production. -Dose-dependent increase in β-galactosidase, dose-dependent decrease in β-glucosidase, decrease in β-glucuronidase in high-dose group. -Increased cecal weight. -Dose-dependent decrease in cecal pH, decrease in cecal ammonia. -Increased total SCFA, -No change in food intake, body weight or digestibility of dry matter. -Decreased plasma cholesterol concentrations. |
| Kikuchi- | Study effects of | 12 M Wistar | 2 treatments: | 5% | 16 d | -No effect on total bacteria, <i>Lactobacilli</i> , or |

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Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration* |
|----------------------|---|--|--|--|--------------------|--|
| Hayakawa et al. 1997 | GOS on organic acid production in rats | rats (CLEA Japan), 7 weeks old, 6 per group | control, GOS | (0.34 g/d) 1.4 g/kg-bw/d (Weight not provided in study. Therefore, default rat weight of 250 g assumed.) | | bifidobacteria. -Increase in number of <i>Bacteroidaceae</i> -Decrease in number of <i>Enterobacteriaceae</i> . -Increase in acetic, propionic, and butyric acids. -Decrease in isobutyric acid. -Increased body weight gain. -No change in food intake. |
| | Feeding studies | 48 M Wistar rats, 7 weeks old, 6 per group. | 2 treatments control, GOS (animals taken from each group for different measures) | 5% | 21 d | -Increased cecum and cecal tissue weight with time. -Decrease in cecal pH. -Organic acids-increased propionic and butyric acids, no effect on lactic or acetic acid |
| Meslin et al 1993 | Study effects of GOS on mucin distribution, glycolytic activities & bacteria metabolism in GF, CV & HE rats. Feeding study | 12 M F344 rats (germ-free, conventional, and heteroxenic), 3 months old, 2 per group | 6 treatments control and GOS for each of 3 rat models (GF, CV and HE) | 4% of diet 2.4 g/kg-bw/d (Weight and food intake not given therefore, 250 g bw and 15 g/d intake assumed) Source: Not Specified | 1 mo | -TOS increased β -D-galactosidase and other glycolytic activities. -TOS demonstrated an intrinsic effect on mucus cell distribution in the colon; although the presence of microflora abolished this effect in CV and HE rats. |
| Ohtsuka et al. | Evaluate the effects | 30 M | 5 treatments | 5%, 10% | 39 d | -Increased fecal dry weight. |

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Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration* |
|---------------------------|---|--|---|---|--------------------|--|
| 1990 | of GOS on body weights, organ weights, serum lipids, and liver lipids in rats. Feeding study | Sprague-Dawley rats, 6 per group | control, lactose, lactulose, 2 GOS levels. | (4.9, 9.4 g/kg-bw/d) Source: prepared from lactose by researchers | | -Increased relative cecum and colon weights. -Increased cecal contents weight -Increased weight gain in both low-dose group (non-significant) and high-dose group (significant). -Increased food intake. -Diarrhea observed in high-dose group during first 2 weeks. -No effect on relative organ weights -Increases (non-significant) in serum lipids in the high- dose group |
| Ohtuska et al. 1991 | Study the excretion and metabolism of GOS in rats using a radioisotope technique | 16 M Sprague-Dawley rats, CV and GF, 4 per group | 4 treatments. CV rats with tagged-lactose or tagged-GOS diet, GF rats with tagged-lactose or tagged-GOS diet Note Several CV rats were given penicillin and chloramphenicol for 1 week before use. | 5% (approx. 3.8 g/kg-bw/d) Assuming body weight of 195 g and food intake of 15 g/d Source: prepared from labeled lactose by researchers | 2 wk | - ¹⁴ CO ₂ excretion slower for treated CV rats. -Antibiotics caused a 2 hour delay in ¹⁴ CO ₂ excretion in treated CV rats. - ¹⁴ CO ₂ excretion for treated GF rats one third the excretion of treated CV rats over a 24 h period. -Radioactives in serum, liver and carcass lower in treated group than lactose group -Radioactives in the feces and urine higher in treated group than lactose group. -No GOS detected in feces |
| Perez-Conesa et al. 2006a | Study the effects of different follow-up | 48 M Sprague- | 8 treatments: control, | 1 2, 5.0, 10% | 30 d | -In 1 st period, only bifidobacteria+GOS @ 10% showed higher bifidobacteria count compared to |

Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration* |
|------------------------------|--|---|---|--|--|--|
| | formulas bifidobacteria and/or GOS on fecal bifidobacteria in rats. Feeding study | Dawley rats, 3 weeks old, 6 per group | bifidobacteria, 3 GOS, 3 GOS+bifidoba cteria | (Unable to calculate GOS intake due to absence of formula intake in study) Source: Oligomate 55P, Yakult Pharmaceutical Ind. Co., Tokyo, Japan | Divided into 3 periods 1 (d8-10), 2 (d 18-20) and 3 (d 28-30) | control -In 2 nd period, higher counts found in all bifidobacteria+GOS groups -In 3 rd period, only GOS @ 1.2% had higher bifidobacteria count than control |
| Perez-Conesa et al. 2006b | Study alterations in bioavailability of calcium, magnesium and phosphorus in rats fed GOS follow-up infant formulas Feeding study | 18 M Sprague- Dawley, 3 weeks old, 6 per group | 3 treatments: 3 GOS | 12, 50, 100 g/kg formula Source: Oligomate 55P; Yakult, Japan. | 30 d | -Apparent absorption of Ca increased for at all doses. -Apparent absorption of Mg and P increased in high-dose group -Test substance did not increase fecal volume |
| Rowland and Tanaka 1993 | Study effects of GOS on cecal bacteria metabolic activities in rats. Feeding study | 8M/9F germ-free Lister Hooded rats inoculated with human feces, 5-6 per group. | 3 treatments: control, GOS, GOS+B. breve | 5% of daily diet 3 g/kg-bw/d (Weight & food intake assumed: 250 g bw and 15 g/d). Source: Yakult, Japan | 4 wk | -Increase in total anaerobe count, bifidobacteria, <i>Lactobacilli</i> . -Decreased <i>Enterobacteria</i> -Decreased cecal pH. -Increased β -glucosidase activity; decreased β - glucuronidase and nitrate reductase activity. -No effects on body weight gain |

Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration * |
|----------------------|--|---|--|--|--------------------|---|
| Sakaguchi et al 1998 | Study effects of sucrose, GOS and FOS on the gastrointestinal tract in growing rats Feeding study | 35 M Wistar rats, 7 per group | 5 treatments: basal diet, restricted basal diet, sucrose, GOS, FOS | 10% 6 g/kg-bw/d (Weight and food intake not given therefore, 250 g bw and 15 g/d intake assumed) Source: Yakult Honsha Co. Ltd., Tokyo, Japan. | 50 d | -Increase in cecal tissue and content weights. -Increased total cecal organic acids; Increased succinic, lactic, formic, acetic, butyric acid, decreased propionic, isobutyric, and isovaleric acid. -No effect on food intake or weight gain. |
| Wijnands et al 1999 | Study effects of GOS on colorectal cancer in rats induced by 1,2-dimethylhydrazine. Feeding study | 468 M pathogen-free Wistar rats, 8 weeks old, 39 per group. | 12 treatments: low, medium, or high-fat diets each with high or low levels of cellulose or GOS | 3.4, 10% 2.04, 6 g/kg-bw/d (Weight and food intake not given therefore, 250 g bw and 15 g/d intake assumed). Source Borculo Domo Ingredients, The Netherlands | 9 mo | -Softer feces in high-dose groups. -Dark feces -Decrease in cecal pH in low (non-significant) and high (significant) GOS groups. -Marginal differences in final body weights and energy intake -No occurrences of diarrhea. -Decreased tumor incidence (non-significant) in high GOS groups. -Decrease in tumor size and multiplicity in high GOS groups -Enlarged cecum in high GOS group |
| Wijnands et al. 2001 | Study the effects of GOS on the development of aberrant crypt foci and colorectal tumors in rats | 204 M Fischer 344 rats, 3 weeks old, 102 per group. | 4 treatments . Low GOS, High GOS, L/HGOS (LGOS diet first 7 weeks, | 0.34, 1.26 g/d (0.68, 2.75 g/kg-bw/d) | 10 mo | -Marginal increase in final body weight in LGOS group compared to HGOS group. -Increase (non-significant) in food consumption and energy intake in LGOS group compared to HGOS group. -Decrease in tumor incidence in HGOS group |

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Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration* |
|-----------------------|--|--|--|---|--------------------|---|
| | induced by azoxymethane. Crossover study | | HGOS thereafter) H/LGOS (HGOS diet first 7 weeks, LGOS thereafter) | Source Borculo Domo Ingredients, The Netherlands | | when compared to LGOS group during promotion phase. -GOS did not have deleterious effects on rat subjects |
| Yanahira et al 1997 | Evaluate the effects of lactitol-oligosaccharide, lactose, lactitol, and GOS on calcium and magnesium absorption in rats. Feeding study | 30 M Sprague-Dawley rats, 6 per group | 5 treatments control, lactose, lactitol, lactitol-oligosaccharide, GOS | 5% (3.9 g/kg-bw/d) Source prepared from GOS by researchers | 2 wk | -Increased cecal weight and cecal content weight. -Decreased cecal pH -Increases in total SCFA, total VFA, acetic, propionic, and butyric acid -Decreased (non-significant) food intake, final body weight, and weight gain. -Increased Ca and Mg absorption (%) |
| Mice | | | | | | |
| Morishita et al. 2002 | Determine the effects of GOS on intestinal microflora in mice. Feeding study | 10 M germ-free Balb/c mice, 6 weeks old, 5 per group. | 2 treatments: control, GOS | 5% Source: Snow Brand Milk Co., Tokyo, Japan. | 9 wk | -Increase in <i>B. breve</i> , <i>L. salivarius</i> , and <i>E. coli</i> . -Decrease in <i>C. perfringens</i> , and <i>E. fecalis</i> . -No change in <i>S. epidermidis</i> and <i>B. vulgatus</i> -Decrease in <i>C. perfringens</i> by a combination of GOS and anaerobic bacteria during early normal development of the bowel microflora |
| Vos et al. 2006 | To determine whether dietary intervention of a GOS+FOS affect DTH immune response to an | 53 F C56BL/6Jol aHds mice, 10 per test group, 3 in sham group; | <u>Intervention phase</u> 6 treatments 4 dosage levels of GOS+FOS, control diet, | 0.9%, 2.25%, 4.5%, 9% Source Vivinal | 31 d | -GOS+FOS enhanced DTH in dose-dependent manner -GOS+FOS increased the proportion of fecal bifidobacteria and lactobacilli in a dose-dependent manner. -In a comparative experiment, FOS/inulin did not |

000072

Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration * |
|---------------------|---|---|---|--|--------------------|---|
| | influenza vaccination | 6-10wk old | sham diet | GOS, Borculo Domo, Zwolle, The Netherlands | | enhance DTH responses. -No difference in animal weight or feed intake was observed during the study |
| Piglets/Pigs | | | | | | |
| Houdijk et al. 1998 | Study effects of FOS and GOS on growth performance and fecal characteristics of young growing pigs Feeding study | 50 M castrated piglets, 10 per group, 57 days old | 5 treatments. control, 2 GOS levels, 2 FOS levels | 0.54, 1.08% of diet Source: Oligostroop® (i.e. Vivinal GOS), Borculo Whey Products, The Netherlands | 6 wk | -No effect on fecal pH -No effect on fecal dry matter. -Fecal pH increased in all animals with time -Decreased daily weight gain and daily dry matter intake in OS groups during week 1-3, no effect over 6 week period. |
| Houdijk et al. 1999 | Study effects of GOS and FOS on nutrient digestion in growing pigs. | 25 growing pigs, 5 per group | 5 treatments: control, 2 GOS levels, 2 FOS levels | 0.4, 0.8% of diet Source: Oligostroop®, Borculo Whey Products, The Netherlands. | 42-47 d | -No effect on feces production. -No change in fecal dry matter content. -Increased ileal digestion of crude fiber (%) in high GOS diet, No effects seen in ileal digestion of other nutrients. -No change in food intake. -3 pigs excluded from analysis due to diarrhea |
| | Study effects of GOS and FOS on nutrient digestion in weanling pigs. | 20 weanling pigs, 4 per group. | 5 treatments control, 2 GOS levels, 2 FOS levels | 1, 4% of diet | 23-27 d | -Dose-dependent decrease in fecal production in OS group. -No change in fecal dry matter content. -No change in ileal digestion of Ca, P, Mg, Fe, Fe, CU, or Zn in OS group -Increased N excretion -Decreased ileal digestion of nonstarch carbohydrate and increased digestion of hemicellulose and cellulose in OS group. |

000073

Table V-2. Summary of Repeat Dose Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration* |
|------------------------------|--|--|---|---|--------------------|--|
| Houdijk et al. 2002a | Study the effects of TOS on microbial characteristics of feces in pigs. | 20 castrated pigs, 4 per group | 5 groups: control, 2 GOS levels, 2 FOS levels | 0.45, 1.8% of diet. Source: Oligostroop®, Borculo Whey Products, The Netherlands | 27 d | -No effects on ileal pH, VFA, or bacterial counts. -No effect on fecal production, dry matter content, VFA, or bacterial counts. -Increased fecal pH in high-dose group |
| Houdijk et al. 2002b | Study the effect of TOS on gut contents and portal plasma. | 25 castrated pigs, 5 per group | 5 groups: Control, 2 GOS levels, 2 FOS levels | 0.45, 0.9% of diet Source: Oligostroop®, Borculo Whey Products, The Netherlands | 44 d | -Colonic VFAs count similar across diets. -High-dose TOS group had more cecal VFAs than controls |
| Mountzouris et al. 2006 | Study the effects of TOS on microflora in growing pigs. | 12 castrated growing pigs [Duroc x (Large White x Landrace)], 4 per group. | 3 treatments: control, FOS, 6'-GOS | 10 g/kg diet; 1% of diet. Source: Vivinal GOS 10, Borculo Domo Ingredients, The Netherlands. | 32 d | -No effect on microflora in pigs including <i>Bacteroides</i> , <i>Eubacterium</i> , <i>Clostridium</i> , <i>E. coli</i> , <i>Bifidobacterium</i> , and <i>Lactobacillus</i> -No effect on total VFA, acetic, propionic, or butyric acid. -Increased β -galactosidase activity in cecum, ascending colon, and rectum. No effect on α -glucosidase, β -glucosidase, or β -glucuronidase. |
| Smiricky-Tjardes et al. 2003 | To evaluate GOS impact on swine nutrient digestibility, ileal and fecal bacterial populations. | 12 T-cannulated pigs (crossbred; PIC 326 sire line x C22 | 3 treatments: TOS, SS and control | TOS group: 3.5% (bw = 25 kg) TOS: 3.5 g/kg-bw/d | 42 d | - TOS resulted in 5.5% unit decrease in digestibility of all nutrients investigated including DM, OM and N -SCFA concentrations were increased -Supplementation with TOS resulted in significant increases in bifidobacteria and |

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| Table V-2. Summary of Repeat Dose Animal Studies with GOS | | | | | | |
|---|--|-------------------------------------|---|---|--------------------|---|
| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration * |
| | | dams; PIC, Franklin, KY) | | Source (TOS): Borculo Domo Ingredients, Borculo, The Netherlands | | lactobacilli. -Ileal GOS digestibility was 100% for pigs consuming the TOS. |
| Dogs | | | | | | |
| Zentek et al. 2002 | Study effects of OS, lactose, and lactulose on fecal quality, nutrient & mineral digestibilities and microflora metabolism in dogs. Crossover study | 4 adult F beagles, 1 per treatment. | 4 treatments: mannan-oligosaccharide, GOS, lactose, lactulose | 1 g/kg-bw/d Source: Borculo Whey Products, Borculo, The Netherlands. | 10 d | -No negative effects on stool quality -No change in unbound water (%). -No effect on fecal pH or dry matter. No effect on fecal ammonia. -No difference in fecal SCFA content -No influence on absorption of Ca, PO ₄ , Mg, Na, or K. |

* Results statistically significant unless noted otherwise.

Table V-3. Summary of Subchronic Toxicity Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration* |
|--------------------|--|--|--|---|--------------------|---|
| Anthony et al 2006 | Investigate the safety of GOS syrup in a sub-chronic 90-day oral (gavage) toxicity study | Rats, 4 groups (15M/15F per group) 6 weeks of age. Sprague Dawley Crl:CD(SD) IGS BR | 4 treatments control (deionized water), FOS reference control, GOS | 1 125 or 2.25 g/kg-bw/d syrup (2.5 or 5.0) g/kg-bw/day administered once daily. Source: Vivinal® GOS syrup (45%) | 89-91 d | -Decrease in mean food consumption for females at 2500 and 5000 mg/kg-bw/day and males at 5000 mg/kg/day. Decreased food consumption noted in FOS control groups as well. -No differences in body weights. -Authors indicated an increase in relative spleen weight in the 5000 mg/kg males. Mean absolute and relative spleen weights in female reference controls were increased. These changes were not considered to be adverse. -No changes seen in hematology, clinical chemistry, ophthalmology, gross necropsy, or histopathology. -The authors determined a NOAEL of at least 2.25 g/kg-bw/d GOS, or 5 g/kg-bw/day Vivinal GOS syrup, based on the lack of toxicological effects in the study. |

Table V-3. Summary of Subchronic Toxicity Animal Studies with GOS

| Reference | Study Objective & Design | Animal Species and Number | Test Substance | GOS Dose | Treatment Duration | Results of GOS Administration* |
|----------------------------|---|--|--|--|--------------------|---|
| Lina 1995 [unpublished] | Investigate the safety of GOS syrup in a sub-chronic oral toxicity study. Feeding study. | 80 M/ 80 F Wistar rats, 3 weeks old, albino, 20M and 20F per group. | 4 treatments. Control, low level, medium level, and high level GOS diet. 0% (control), 5%, 10% and 20% in feed | syrup doses: 3.7, 7.6, 14.5 g/kg-bw/d in Males, 4.2, 8.6, 16.4 g/kg- bw/d in Females. Highest dose 6.9 g/kg-bw/d Source: Borculo Whey Products (42%)—based on 58.2% GOS DM and 72.9% sugar DM. | 13 wk | -Transient growth retardation observed in males of the top-dose group. -No effect on food conversion efficiency. -Dose-related increase in the weight of the full and empty cecum, decreased plasma urea concentration and decreased cecal pH. -Decreased urinary density in males of the top-dose group. -Increased relative weight of the spleen in females of the top dose group not accompanied by changes in red blood cells or by histopathology -No adverse effects were observed; consequently authors established an NOAEL of at least 6.9 g/kg-bw/d—the highest dose tested. |

* Results statistically significant unless noted otherwise.

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E. Human Studies of GOS and Effects on GI Function and Microflora

1. Lack of Permeability of GOS through the Intestinal Epithelium

Permeability refers to the ability of the intestinal epithelium or any membrane to allow molecules to pass through it by non-mediated (passive) diffusion (Travis and Menzies 1992). The gut of a newborn infant, especially a pre-term infant, is permeable to macromolecules, such as intact sugars and proteins (Weaver et al. 1984). This period of permeability is followed by a period of considerably reduced movement of macromolecules, which has been termed “gut closure,” and is considered to represent intestinal maturation in the infant (Axelsson et al. 1989). Increased intestinal permeability in the infant can have beneficial effects, such as the ability to uptake larger nutritional molecules, as well as the development of systemic tolerance. However, this increased permeability can also have disadvantageous effects, such as increased uptake of infectious agents leading to the development of infection, inflammation, and systemic hypersensitivity (Insoft et al. 1996, as cited in van Elburg et al. 2003). An evaluation of the permeability of the infant gut is important to demonstrate that it would have limited permeability to sugars such as GOS. Information is presented in this section to show that adults and infants overall would handle GOS similarly with respect to gut permeability and that there would be no safety issues in this regard.

Intestinal permeability is assessed noninvasively *in vivo* by measuring urinary excretion of orally administered nondigestible test substances, typically a disaccharide and a monosaccharide. Lactulose is the most widely used disaccharide probe for intestinal permeability studies while L-rhamnose and mannitol are commonly used monosaccharides (Bjarnason et al. 1995). Mannitol theoretically enters the cell through the hydrophilic portion of the cell membrane, whereas lactulose passes through the tight junctions and extrusion zones of the intervillous spaces. Consequently, the loss of mucosal integrity should cause increased lactulose absorption, while the loss of these absorptive areas decreases the absorption of mannitol (Fleming et al. 1990).

Results from intestinal permeability studies in individuals free from disease conditions that may compromise gastrointestinal health provide important information on the extent to which the infant gut may be permeable to sugars in healthy term infants. Studies of intestinal permeability in healthy and compromised term infants, children, and adults are summarized in Tables V-4 and V-5. The urinary L/M ratios are summarized by age for healthy infants and older individuals in Tables V-6 and V-7, respectively, and the ratios

from single load intestinal permeability tests are shown as a function of age in Figures 11 and 12.

Results from the single load intestinal permeability studies in healthy infants show some variations in L/M urinary ratios across infant populations of the same age (Table V-6 and Figure 11). In general, however, L/M urinary ratios are highest in the first days of life (approximately 0.15), and then rapidly decrease (van Elburg et al. 2003; Catassi et al. 1995; Martinez-Augustin et al. 1995; Goto et al. 1999; Colome et al. 2007). At approximately 3 to 18 months of life, measured L/M urinary ratios were in the range of 0.05 or lower (Goto et al. 1999; Dupont and Goutail-Flaud 1990; Isolauri et al. 1989). Colome et al. (2007) studied intestinal permeability in infants with a mean age of 72.5 ± 30.52 days using a single oral load method and reported urinary L/M ratios in the range of 0.268 ± 0.149 to 0.341 ± 0.250 . Results from the study indicate that a prebiotic supplemented formula has no effect on the L/M urinary ratio as compared to other types of infant formula. Several investigators have evaluated intestinal permeability in infants under steady-state conditions of lactulose and mannitol input and urinary output (Weaver et al. 1984, 1988; Catassi et al. 1995). Under these test conditions, urinary L/M ratios were typically higher as compared to ratios collected using the single load test (Table V-6).

The urinary L/M ratio in healthy children is generally comparable to the ratio in adults (Table V-7 and Figure 12), with values typically in the range of 0.02 to 0.04 (Barboza et al. 1999; van Elburg et al. 1995; Hamilton et al. 1987; Marsilo et al. 1998; Celli et al. 1995; Miki et al. 1996; Elia et al. 1987; Fleming et al. 1990; Cox et al. 1999; Johnston et al. 2000; Saltzman et al. 1995). These findings suggest that intestinal permeability continues to decrease in the early years of life and then remains relatively stable throughout adulthood.

The routine use of intestinal permeability tests in individuals with conditions compromising small bowel integrity provides further evidence regarding the safety of ingestion of nondigestible carbohydrates by populations known to exhibit increased permeability to these test markers. Overall, results from permeability studies in infants, children, and adults show that the extent of absorption from the small intestine of carbohydrate markers, such as lactulose, is extremely low. The amount of lactulose, a good marker for nondigestible carbohydrate, which is absorbed from the GI tract by infants and children in these studies is very small. Approximately 1.7% or less of the ingested dose of lactulose was excreted by infants in the first month, and excretion of

lactulose was reportedly 1.3 times higher in infants fed exclusively human milk as compared to infants fed milk-based formula (Dmitriev et al. 1997). In older children, lactulose absorption was typically found to be below 1% of the ingested dose as assessed via urinary excretion, and was comparable to the very low levels of absorption observed in healthy adults (van Elburg et al. 1995; Miki et al. 1996; Noone et al. 1986).

In summary, the absorbed dose of nondigestible carbohydrate is very low and elimination of the dose is rapid and nearly complete via the urine. Intestinal permeability appears to rapidly decrease during the first months of life and is relatively stable after childhood. Based on this information, it is reasonable to conclude that nondigestible carbohydrates would not present safety concerns for infants with regard to intestinal permeability.

Table V-4. Studies of Gut Permeability Using Lactulose in Pediatric Populations

| Reference | Subject Description | Study Design | Intestinal Permeability / Urinary Excretion Results |
|---------------------|--|--|--|
| Barau & Dupont 1994 | 3 infants diagnosed with cow's milk allergy (CMA), 3- 9 mo of life. 17 healthy infants (controls) | Initial intestinal permeability test: After a minimum 6-hour fast and voiding of overnight urine, subjects were given a 1.001 mmol/L aqueous marker solution containing mannitol and lactulose at a dosage of 0.1 g/kg bw for each sugar. Urine was then collected for 5 hours. The second, a provocation test, was conducted in a similar manner but the marker solution was mixed with either 100 mL breast milk or formula. | Control infants: mean P/F value was 0.85; positive provocation test defined as P/F > 1.10. 9 mo F, breast fed for the fasting test, the L/M ratio was 5.57%. Upon elimination of mother's intake of dairy products, fasting L/M ratio was 2.36%. Provocation test with breast milk L/M ratio was 2.95% (P/F = 1.25). 4 mo M, breast fed: fasting test L/M ratio was 3.31%, provocation test with cow's milk and then breast milk increased so that P/F = 10.69. Removal of dairy products from mother's diet resulted in provocation test of infant male as normal. 3 mo F: normal fasting test (3.67%), elevated prevocational tests with milk substitutes, including soy proteins. Tests were normal after elimination of all milk- and soy-based formulas. This study indicates that with infants with active CMA, ingestion of breast milk, cow's milk or soy proteins during provocation test results in increased gut permeability to lactulose as indicated by an increased L/M ratio. Elimination of dairy products from the diet of lactating mothers can eliminate the intestinal adverse reactions associated with CMA. |
| Barboza et al. 1999 | Group 1: 15 children (age < 5 y) defined as having 3+ liquid stools in the last 24 h. Group 2: 15 children with no episode of diarrhea in the last 2 wk The groups were matched for age within \pm 3 mo. | After a 3 hour fast, children ingested a solution (2 ml/kg) containing mannitol (50 mg/ml) and lactulose (200 mg/ml). Urine was collected for 5 hours. | Analysis of the intestinal permeability of healthy children using the L/M test showed a significant decrease in percent lactulose excretion in urine compared to children with diarrhea (0.3029 ± 0.2846 vs. 0.1183 ± 0.0855). A significant reduction in the L/M ratio was also observed when controls were compared to children with diarrhea (0.1404 ± 0.1206 vs. 0.0394 ± 0.0235). |

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Table V-4. Studies of Gut Permeability Using Lactulose in Pediatric Populations

| Reference | Subject Description | Study Design | Intestinal Permeability / Urinary Excretion Results |
|---------------------|---|--|--|
| Catassi et al. 1995 | 72 full term, healthy infants fed exclusively human milk or a formula | On days 1 and 7 of life, a dual-marker, steady-state L/M test was performed. Infants received regular feeds at 3- to 4-h intervals. The marker solution contained 200 mg lactulose and 40 mg mannitol per ml water, and was given at the end of each milk feed during a 24-h period (6 marker doses). On day 1, 0.5 ml of marker solution was given at each feed, and on day 7, 1 ml was given per feed. After 24 h, a random urine sample of at least 2 ml was obtained. At 1 mo of age, a single oral load test was performed. After an overnight fast, 3 g lactulose and 600 mg mannitol were given in an isotonic aqueous solution. All urines passed in the subsequent 5 h was collected. | In infants fed human milk, the L/M ratios on d 1, 7 and 30 were 1.27 ± 0.74 , 0.22 ± 0.25 , and 0.24 ± 0.26 . In infants fed formula, the L/M ratios on d 1, 7 and 30 were 1.29 ± 0.73 , 0.47 ± 0.41 , and 0.21 ± 0.17 . The L/M ratios differed by feeding group only on d 7. In the 47 infants who completed the 1-month study, the single load urinary L/M ratio (calculated as the ratio of percentage of urinary recovery of each sugar) was 0.09 ± 0.08 . |
| Celli et al. 1995 | 28 control children, ages 2-15 (mean 9 years) without evidence of gastrointestinal or systemic disease. 28 patients with active gluten-sensitive enteropathy (GSE), ages 3-16 years (mean 10 years). | The subjects followed a diet free of mannitol, lactulose, mannose, and fructose for 24 hours before the test. After an overnight fast, the subjects voided a pretest urine sample and then ingested (0.55 ml/kg) a solution containing 18.2 g mannitol and 18.2 g lactulose in 100 ml water. Urine was collected for a total of 5 h. | Control subjects showed a mean lactulose recovery of $0.28\% \pm 0.04\%$. In contrast, the GSE patients showed a mean recovery of $0.73\% \pm 0.5\%$. The mean L/M recovery ratio value was 0.022 ± 0.007 in control subjects and 0.084 ± 0.054 in GSE patients. The differences between mean lactulose and mannitol urinary recovery and mean L/M in GSE children compared with controls were statistically significant. |
| Colome et al. 2007 | 57 healthy infants, average age 72.5 ± 30.52 d. Infants grouped by feeding type: human milk, formula with prebiotics, formula with nucleotides, formula with long-chain polyunsaturated fatty acids (LC-PUFA), or formula with nucleotides and LC-PUFA. | The test solution contained 250 mg lactulose and 100 mg mannitol per 5 ml water. The test solution was put into infants' mouths with a syringe. Fasting before the test was not mandatory. Urine was collected by parents in a plastic bag for 6 h. | L/M, L/C and M/C ratios were not different between infants in fed human milk or infant formula. The L/M ratios in infants fed formula supplemented with prebiotics (fructo-oligosaccharide and galacto-oligosaccharide) and infants fed other formulas were 0.331 ± 0.222 and 0.299 ± 0.183 , respectively. The L/C and M/C ratios were higher in infants fed the prebiotic formulas vs the other formulas (L/C: 4.921 ± 3.957 vs 2.442 ± 1.731 , $p=0.024$; M/C: 18.019 ± 15.187 vs 9.374 ± 5.982 , $p=0.039$). |

Table V-4. Studies of Gut Permeability Using Lactulose in Pediatric Populations

| Reference | Subject Description | Study Design | Intestinal Permeability / Urinary Excretion Results |
|---------------------------------|--|---|---|
| Dmitriev et al. 1997 [abstract] | 96 infants (27 to 40 weeks gestation), 5 to 75 d of age. Infants had no evidence of gastrointestinal disease. | Urinary excretion of lactulose was measured within 12 hours after oral administration of a test solution providing 0.7 g/kg lactulose | During the first month of life, urinary excretion of lactulose decreased to less than 1.7% of the test dose, and excretion was not different between infants born over 33 weeks' gestation and those born under 33 weeks' gestation. Excretion of lactulose by infants exclusively fed breast milk was 13 times more than infants receiving milk-based infant formula. |
| Dupont & Goutail-Flaud 1990 | 29 infants, 3 d-20 mo of life, hospitalized with various illnesses or surgeries; all but one experienced diarrhea during permeability studies. 24 infants, as controls, for whom GI, skin or cardiovascular disease had been ruled out. | Subjects were given an aqueous solution of 10% mannitol and 65% lactulose at a dosage of 0.1 g/kg bw for each sugar. Urine was then collected for 12 h. | 7 subjects with small bowel resection: mannitol recovery was $4.3 \pm 1.9\%$ vs $18.1 \pm 7.2\%$ in 24 age-matched healthy infants. 9 subjects with necrotizing enterocolitis (avg age approx 4 mo) had a mean L/M ratio of $13.9 \pm 8.0\%$ vs $3.1 \pm 1.2\%$ in 22 age-matched healthy infants. 4 of 7 patients with abdominal parietal wall defect had an L/M ratio of $>7.2\%$. 6 patients with varying illnesses had L/M ratios during diarrhea of $25.3 \pm 19.4\%$ versus $5.0 \pm 3.7\%$ in absence of diarrhea. The study indicated that episodes of diarrhea in compromised infants were associated with increases in the L/M ratio |

Table V-4. Studies of Gut Permeability Using Lactulose in Pediatric Populations

| Reference | Subject Description | Study Design | Intestinal Permeability / Urinary Excretion Results |
|---------------------|---|---|---|
| Ford et al. 1985 | 39 children with diarrheal illness were divided into three groups Acute gastroenteritis: 14 children, ages 2-15 mo (mean 7.3 mo) Resolving gastroenteritis: 11 children, ages 3-17 mo (mean 8.5 mo) Chronic diarrhea 14 children, ages 4-19 mo (mean 12.9 mo). 15 children from the acute and resolving gastroenteritis groups whose parents agreed to a second permeability study, to be performed when the child had fully recovered. 27 children (2 mo to 15 y of age) undergoing duodenal biopsy were also studied in 28 permeability studies. | After an overnight fast of at least 6 hours, an isotonic load containing 3.5 g lactulose, 5 g lactose, 0.5 g rhamnose and 0.5 g D-xylose was given orally. Urine was collected over the next 5 hours. | The L/R ratio in the acute gastroenteritis group was 0.43 ± 0.31 (normal < 0.07). The chronic diarrhea group had an L/R ratio of 0.12, and the ratio in the recovered group was 0.045 ± 0.018 . A strong positive correlation was found between an increasing L/R ratio and the percentage of the oral lactulose dose excreted in the urine. There was also a weaker negative correlation between L/R ratio and the percentage excretion of rhamnose. These findings indicate that an elevation of the L/R ratio was predominantly caused by increased lactulose absorption, although frequently, there was a concomitant fall in rhamnose absorption. When histological appearance was considered, there was a strong association between the degree of mucosal abnormality and an elevated L/R excretion ratio. This was not so for the L/X ratio. |
| Goto et al. 1999 | 158 nonacutely ill Guatemalan infants (no diarrhea for at least 1 wk, age birth to 11 mo, mean age 4.7 ± 3.4 mo) provided adequate collections for a total of 200 acceptable permeability studies | Sugar solutions of 400 mg lactulose and 100 mg mannitol in 3 ml water were used. Children were given the sugar solution (3 ml/kg) by a dropper at least 2 hours after the previous meal. A urine collection bag was placed shortly after the infants consumed the test dose and all urine was collected for the next 5 hours. | Using a definition of abnormal intestinal permeability (L/M recovery ratio ≥ 0.07), the overall prevalence of apparent intestinal dysfunction in the study subjects was 29.5%. The rate of altered permeability varied according to age, feeding practices, and nutritional status (weight-for-age). Older infants, those who stopped breast feeding and those with poor nutritional status had elevated L/M ratios. Median L/M ratios in infants ages 0-2, 3-5, and 6-11 mo were 0.041, 0.049, and 0.054, respectively. The median L/M ratios (based on % L and M recovery) in breastfed infants, non-breast-fed infants, and infants fed mixed diets were 0.041, 0.060 and 0.054, respectively. The geometric mean of L/M ratios in breast-fed infants 0-5 and 6-11 mo were 0.046 and 0.058, and the ratios in non-breastfed infants 0-5 and 6-11 mo were 0.094 |

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Table V-4. Studies of Gut Permeability Using Lactulose in Pediatric Populations

| Reference | Subject Description | Study Design | Intestinal Permeability / Urinary Excretion Results |
|----------------------|--|---|--|
| | | | and 0.056, respectively. |
| Hamilton et al. 1987 | 37 children with clinical suspicion of celiac disease (presenting with diarrhea, failure to thrive or short stature); mean age was 6.5 y (range 3 mo – 15 y). 33 children (mean age 5.5 y, range 7 mo – 14 y) admitted for minor surgery served as the control population | The solution for the permeability test contained 2 g mannitol, 5 g lactulose and 9 ml glycerol, made up to 100 ml with water. Fasting subjects provided a baseline urine sample and ingested 2 ml solution/kg up to a maximum of 100 ml. Urine was collected for the following 5 hours. | In the control group, mean urinary recovery of mannitol was $16.2 \pm 7.4\%$, mean lactulose recovery was $0.76 \pm 0.49\%$ and the median L/M recovery ratio was 0.036 (range 0.005 – 0.09). In children with a normal jejunal biopsy and no intestinal disease demonstrable to explain their symptoms, mean lactulose recovery was 0.42% and the median L/M ratio was 0.03 (range 0.008 – 0.54). In the patients with celiac disease, mean lactulose excretion was 0.77%, which does not differ significantly from the other groups tested. The mean mannitol recovery (2.6%), however, was significantly lower than controls; and the L/M ratio was abnormally high (0.24 - 5.47). In children diagnosed with cows' milk intolerance, lactulose recovery was 0.71% (not different from all other groups), however, mannitol recovery (2.68%) was significantly lower. Thus, intestinal permeability was abnormal in children with celiac disease and cows' milk intolerance, however, it was manifested by a reduction in absorption of mannitol. The lactulose recovery was not affected. |

Table V-4. Studies of Gut Permeability Using Lactulose in Pediatric Populations

| Reference | Subject Description | Study Design | Intestinal Permeability / Urinary Excretion Results |
|-------------------------------|--|---|---|
| Isolauro et al. 1989 | Studies were conducted in 41 children aged 3-25 mo with acute gastroenteritis (73% rotavirus). Group A: 9 children had been fasted and fluid replacement was inadequate. Group B: 17 children had received oral fluids continuously during diarrhea, but had not been given food. Group C: 12 children were given fluids and uninterrupted feedings. Two control groups: one group of 19 children, aged 7-26 mo (avg age 17.2 mo) hospitalized because of other acute febrile infections, with no GI symptom and a second control group of 9 children aged 2-23 mo (avg age 8.0 mo) with no signs of acute infections. | The intestinal permeability tests in children with acute gastroenteritis and febrile controls were performed during their first 12 hours in hospital. After 6 h initial fluid therapy, a 100 ml solution containing 4 g lactulose and 0.8 g mannitol was given by mouth. In the afebrile control group, the permeability test was done after an overnight fast of at least 6 h. All urine was collected over 5 h. | The L/M excretion ratios were significantly higher in the patients with acute gastroenteritis than in the controls, mainly due to decreased urinary recovery of mannitol. The mean L/R ratio in each control group was approximately 0.02. The gastroenteritis patients who had received uninterrupted feeding in addition to adequate fluid replacement before hospitalization had a normal urinary L/M ratio, with a mean of 0.04 whereas in fasted children with inadequate or adequate fluid replacement, the respective mean ratios were 0.24 and 0.14. The fasting associated rise was caused by increased lactulose excretion. The study indicated that fasting maintains the increased intestinal permeability associated with acute gastroenteritis. |
| Marsilio et al. 1998 | 30 healthy children, ages 0.4-13 years, mean 7.4 y (controls), 10 patients with Crohn's ileocolitis (ages 13.2-21 y, mean 14.7 y) and 10 patients with celiac disease (ages 1.3-17.9 y, mean 5.8 y) in the moderate or severe activity phases of their diseases. | Patients drank a solution containing 5 or 10 g of lactulose and 2 or 5 g of mannitol in 50-100 ml of deionized water, according to the age. Urine was collected during the next 6 hours and total volume of urine was measured. | The excretion ratio L/M in the urine of healthy subjects was 0.024 ± 0.006 . The control subjects showed a mean lactulose recovery of 0.33% (range 0.07-0.522%) and a mean mannitol recovery of 14.12% (range 3.92-29%). Lactulose excretion was $2.25 \pm 2.10\%$ in patients with active Crohn's disease. The mean L/M recovery ratio in these patients was 0.200 ± 0.082 . In patients with active celiac disease, the L/M ratio was 0.072 ± 0.025 ; the urinary recoveries of lactulose and mannitol were 0.53% and 8.00%, respectively. |
| Martinez-Augustin et al. 1995 | 27 healthy infants (6 term, 21 preterm). | At 7 days of life, a test dose of lactulose and mannitol (300 and 60 mg, respectively) was administered dissolved in the liquid diet. | The urinary L/M excretion ratios were not statistically significantly different among term infants fed human milk, preterm infants fed human milk (n=4) and preterm infants fed |

Table V-4. Studies of Gut Permeability Using Lactulose in Pediatric Populations

| Reference | Subject Description | Study Design | Intestinal Permeability / Urinary Excretion Results |
|------------------------|--|---|---|
| | | Breast-fed infants received the test dose in water. Total urine was collected for the following 5 hours | formula (n=17), the L/M ratios were 0.18 ± 0.19 ; 0.20 ± 0.16 ; 0.32 ± 0.31 , respectively |
| Miki et al 1996 | 14 healthy children and adolescents (median age 9.2 y, range from 5.3 to 16 y). | After an overnight fast, a pretest urine sample was collected. The subjects then drank the test solution, containing 5 g of lactulose, 1 g of L-rhamnose, and 1 g mannitol in 100 ml of water. Urine was collected for a total of 5 h | The range and mean of percentage of urinary excretion of lactulose in the 14 healthy subjects studied was $0.29 \pm 0.14\%$ (range of 0.11 – 0.67%), and the mean urinary excretion of mannitol was $14.0 \pm 3.4\%$. The mean excretion ratios of L/R and L/M were 0.047 ± 0.018 and 0.021 ± 0.010 , respectively. |
| Noone et al 1986 | 15 infants aged 6 wk to 2 y, and 3 adults with acute gastroenteritis in whom rotavirus was the sole pathogen isolated, were investigated. 10 healthy infants (mean 7.9 mo) and 10 healthy adults (mean 22 y) were used as controls. 7 infants and 5 adults with varying levels of intestinal lactase had both an oral lactose/lactulose test and jejunal biopsy. | Infants and adults with acute gastroenteritis were investigated during their acute illness and again after clinical recovery 4 wk later. After withdrawal of food for 4 hours and fluids for 2 h, the infants were given the test solution containing 10 g lactose, 3.5 g lactulose and 0.5 g L-rhamnose dissolved in water to a volume of 150 ml. Adults received double this dose in 300 ml after an overnight fast. Urine collection was done for 5 h (adults) or 5 h plus the next void (infants) | Healthy adult subjects excreted a mean of 0.05 ± 0.02 , 0.21 ± 0.05 and $10.1 \pm 2.8\%$ of ingested doses of lactose, lactulose and L-rhamnose, respectively. Healthy infants excreted 0.06 ± 0.03 , 0.20 ± 0.10 and $7.5 \pm 2.5\%$ of ingested doses of lactose, lactulose and L-rhamnose, respectively. The mean lactose/lactulose excretion ratios were 0.023 and 0.029 for healthy adults and infants, respectively. Adults and infants with acute gastroenteritis showed an increased urinary lactulose excretion of 0.64% compared with 0.215% for healthy controls (mean percentage of dose). The L/R % excretion ratio was abnormal in all patients, being 0.46 during the acute illness, and fell to within control range (0.008-0.052) after recovery. |
| van Elburg et al 1995 | 30 healthy children (median age 5 y, range of 0-16 y) and 40 healthy adults (median age 31 y) were tested to determine reference values | After an overnight fast of 8 h in patients older than 2 y, and 4 h in younger patients, the patient drank 2 ml/kg (maximum 100 ml) of test solution. Test solution was 2 g mannitol, 40 g sucrose and 10 g lactulose 50% in 100 ml solution with water. Urine was collected during the next 5 h. | The reference values for percent of ingested lactulose dose absorbed are not different between children and adults (mean 0.62% (range 0-1.71%) and 0.45% (range 0-1.03%), respectively with mean L/M ratios (based on % ingested dose) of 0.034 and 0.027, respectively. |
| van Elburg et al. 2003 | 116 preterm infants (26-36 wk gestation, median age 31 wk, 2 d) and 16 term infants (>37 weeks gestation) delivered by elective | The test solution containing 250 mg lactulose and 100 mg mannitol per 5 ml water was administered by nasogastric tube (2 ml/kg). Urine was collected for 6 hours. Infants were | In term infants, L/M ratios were 0.170 when measured within the first 2 d of life, and 0.123 when measured 3-6 d later. |

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Table V-4. Studies of Gut Permeability Using Lactulose in Pediatric Populations

| Reference | Subject Description | Study Design | Intestinal Permeability / Urinary Excretion Results |
|--------------------|---|--|--|
| | caesarian section completed the first test A second test was performed in 102 of the preterm and 9 of the term infants. | tested within 2 d of birth. A second test was performed 3-6 d after the first test | |
| Weaver et al. 1984 | 33 infants (gestational ages ranging from 27 to 41 wk) were studied from birth to age 7 d. | Infants received 2 to 4 hourly oral feeds containing 5.8 mmol/l (200 mg/l) lactulose and 2.2 mmol/l (40 mg/100 ml) mannitol. All preterm and some term infants received SMA Gold Cap concentrated liquid feed (Wyeth Laboratories), and the rest were given Milumil (Milupa) After 24 hours, when a steady state of marker input and output had been reached, a random urine sample was collected daily for 7 d from preterm infants, and until discharge from term infants. | Infants born at >37 wk gestation (mean 40 wk) showed no difference in L/M ratios during the first 4 d after starting oral feeds (median 0.56-0.59). |
| Weaver et al. 1987 | 19 full-term infants; 10 infants (40 ± 1.2 wk gestation) were breast-fed and 9 infants (40 ± 1.3 wk gestation) were fed a cows' milk formula. | The breast-fed infants received an aqueous solution containing 200 mg/ml lactulose and 40 mg/ml mannitol by syringe during each breast feeding The formula-fed infants received ready-to-feed Milupa, to which 200 mg lactulose and 40 mg mannitol per 100 ml feed had been added. Infants received feeds at 4 to 6 hourly intervals After 24 hours, when a steady state of marker input and output was reached, a daily random urine sample was obtained from each infant | In the breast-fed infants, the urinary L/M and lactulose/creatinine ratios fell as measured on d 2 through 6 of life, whereas in the cows' milk formula group there was no significant change in ratios over time. The parallel falls in the group of breast-fed infants indicate a sequential decline in intestinal lactulose uptake. |

Table V-4. Studies of Gut Permeability Using Lactulose in Pediatric Populations

| Reference | Subject Description | Study Design | Intestinal Permeability / Urinary Excretion Results |
|--------------------|---|--|--|
| Weaver et al. 1988 | 38 healthy term English infants (25 male, 13 female), assessed at 6, 12 and 18 wk of life. 39 healthy term Gambian infants (23 male, 16 female) assessed at 6, 12 and 18 wk of life. 8 English infants were breast-fed, 30 were fed cow's milk formula (CMF), weaning was at the discretion of the mothers. All Gambian infants were breast-fed and received first weaning diet at 3 mo. | Subjects were divided into 7 groups by postnatal age, feeding regimen, and nationality. CMF infants received lactulose and mannitol to their feed resulting in 200 mg and 40 mg, respectively, per 100 mL feed Breast-fed infants received a solution containing 200 mg lactulose and 40 mg mannitol per mL by syringe during feeding. Random daily urine samples were collected from each infant. | CMF-fed infants had higher median L/M ratio than either English or Gambian breast-fed infants at 6 wk of life or all 12-wk-old infants. At 12 wks, there was no significant difference in L/M ratios between English infants (82% receiving weaning foods) and unweaned Gambian infants. At 18 wk, weaned English and Gambian infants had slightly higher median L/M ratios than unweaned 12-wk old infants of the same nationality These are much lower than the L/M ratios of English and Gambian infants with gastroenteritis and undernutrition |

Table V-5. Studies of Gut Permeability Using Lactulose in Adult Populations

| Reference | Subject Description | Study Design | Urinary excretion |
|-----------------------|---|---|--|
| Blomquist et al. 1997 | 65 control subjects and 70 patients with a disease or treatment affecting the small intestine and/or cecum and ascending colon were investigated. | After an overnight fast, subjects voided a pre-test urine sample and ingested a test solution (52 ml aqueous solution containing 30 μCi ^{51}Cr -EDTA, 0.5 μCi $1\text{-}^{14}\text{C}$ -mannitol, 1 g D-mannitol, 2 g glycerol, and 10 g lactulose). Urine was collected for 6 hours. | In the control group, excretion of lactulose was weakly correlated with urinary volume. In the patient group, excretion of large pore markers (lactulose and ^{51}Cr -EDTA) tended to be higher and that of small pore markers (mannitol) was lower than in the control group. Excretion of both types of markers tends to decrease with age, the large pore/small pore marker ratio remaining unchanged. The 0-6 h urinary excretion of lactulose in control subjects was approximately 0.25%. |
| Cox et al. 1999 | 24 newly diagnosed celiacs (mean age 34 y) and 10 control subjects (mean age 34 y). | Test subjects and patients ingested 10 g lactulose and 2.5 g mannitol in water. In 10 of the celiac patients and all the controls, urine was collected for 6 hours; blood was taken before and every 30 minutes for 2 hours following ingestion of the test solution. | At 1 hour after ingestion, the mean mannitol level in normals was significantly higher than in untreated celiacs. The 1 hour mean serum lactulose level in normals (0.125 $\mu\text{mol/liter}$) was significantly lower than in untreated celiacs (0.56 $\mu\text{mol/liter}$). The median lactulose/mannitol excretion ratio in untreated celiacs was 0.42 compared with 0.023 in normals. Urinary lactulose recovery in the control subjects was 0.2%. |
| Elia et al. 1987 | 35 oral permeability tests and 7 intravenous tests were carried out in lean and obese subjects who had no history of GI disease. Mean age of lean subjects was 28 y. 3 oral permeability tests were conducted in 3 subjects who had an ileostomy as a result of surgery for ulcerative colitis. | Intravenous injection of 0.1 g lactulose was followed by blood collected at intervals up to 8 hours after injection. Urine was collected at intervals up to 48 hours. For the oral permeability test, each subject drank a solution containing 10 g lactulose, 1.5 g lactose, 5 g mannitol, 0.5 μCi ^{14}C -mannitol and about 30 μCi ^{51}Cr -EDTA in 50 ml water; urine was collected at intervals up to 48 hours after dosing. | The excretion of lactulose (% of dose) after oral administration over the time periods 0-2 hours, 2-4 hours and 4-6 hours was 0.069 ± 0.006 , 0.088 ± 0.008 and 0.091 ± 0.016 , respectively. Over 24 hours, excretion (% of dose) over 0-6 hours, 6-12 hours and 12-24 hours was 0.26 ± 0.022 , 0.101 ± 0.016 and <0.1 , respectively. The L/M ratio over the first 6 h of urine collection was 0.021 ± 0.003 . The corresponding values for lactulose excretion by the ileostomy patients were 0.30, 0.14, and 0.05%, respectively. After intravenous administration, the amount of lactulose recovered in urine of healthy adults after 48 hours was $99.7 \pm 0.6\%$. |
| Fleming et al. 1990 | 18 healthy adults with no history of gastrointestinal disease, mean age 30 y. | Subjects ingested test solution containing 10 g lactulose and 5 g mannitol after an overnight fast and urine was collected for the next 6 hours. | The mean % excretion of lactulose was $0.33 \pm 0.04\%$ SEM of the administered dose. The mean L/M ratio was 0.027 ± 0.003 . |
| Johnston et al. 2000 | 60 subjects with positive serology for enteropathy. | The serological screening tests have been proposed as a means of identifying asymptomatic patients or | There were no significant differences between the lactulose excretion or L/M excretion ratio in control subjects who repeated |

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Table V-5. Studies of Gut Permeability Using Lactulose in Adult Populations

| Reference | Subject Description | Study Design | Urinary excretion |
|----------------------|--|---|--|
| | or celiac disease and 21 healthy controls (mean age 34.0 y) | those with unrecognized celiac disease. Subjects with positive serology were asked to complete the intestinal permeability test and jejunal biopsy After an overnight fast, subjects consumed a solution containing 5 g lactulose, 2 g mannitol and 22.3 g glucose. All urine was collected for the following 5 hours. Ten of the healthy controls performed the test on two occasions to assess inter-biological variation; one subject performed the test on 10 different occasions to assess intra-biological variation. | the test at two different time points (0.28 vs. 0.38% lactulose excretion and 0.016 vs 0.018). Intra-biological variation in a single control subject was found to be 57%, all results being within the normal range ($L/M \leq 0.024$) Mean % lactulose excretion (0.85 vs. 0.26) and mean L/M (0.105 vs 0.013) were significantly higher in the untreated symptomatic celiac group compared with healthy controls. |
| Karaeren et al. 2002 | 37 adults (18 to 68 years; mean age 37.3 y) with no history of gastrointestinal disease. | Subjects were fasted overnight and subsequently ingested a solution containing 10 g lactulose Urine was collected for 6 hours. | The mean urinary lactulose concentration was 0.58 ± 0.39 mmol/L. |
| Maxton et al. 1986 | 6 healthy adults received lactulose intravenously, 10 healthy adults (27-53 y) received lactulose orally | Intravenous administration of 1.46 mmol lactulose over 2 minutes was followed by urine collection over 24 hours After an overnight fast, a test solution containing lactulose (13.7 mmol) was ingested, urine was collected over a 24 hour period. | Urinary recovery of lactulose after intravenous administration reached 75% by 5 hours and exceeded 90% at 24 hours. Intestinal permeation of ingested lactulose was $0.415 \pm 0.050\%$ during the first 5 hour period |
| Saltzman et al. 1995 | 56 healthy male and female subjects, ages 20-39 y (n = 20), 40-59 y (n = 9); ≥ 60 y (n = 17) | After an 8 h fast, subjects ingested 10 g of lactulose and 5 g of mannitol. Urine was collected for 6 h. | With increasing age, there was a progressive decrease in the percentages of lactulose and mannitol excreted, however, the L/M ratio did not change The percentages of lactulose and mannitol excreted were directly proportional to creatinine clearance values, which indicated that the age-related decline in lactulose and mannitol excreted was caused by a decline in renal function In addition, small-intestine permeability did not increase with increasing age. At age 50 y, the percent lactulose excretion was approximately 0.18%, and the L/M ratio was approximately 0.28. |

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Table V-6. Measures of Intestinal Permeability in Healthy Term Infants

| Reference | Study population | Type of feeding | Reported Age | Calculated Age (mo) | L/M ^a | L/M units ^b | Assessment |
|-------------------------------|---------------------------|---------------------------------|----------------------------|---------------------|------------------|------------------------|--------------|
| van Elburg et al. 2003 | term infants | not specified | within first 2 days | 0.03 | 0.17 | median | single load |
| van Elburg et al. 2003 | term infants | not specified | 3-6 days after first test | 0.20 | 0.123 | median | single load |
| Martinez-Augustin et al. 1995 | term infants | human milk | 7 d | 0.23 | 0.18 | mean | single load |
| Catassi et al. 1995 | term infants | formula | 30 d | 0.99 | 0.09 | mean | single load |
| Goto et al. 1999 | infants | human milk | 0-5 mo | 2.00 | 0.046 | GM | single load |
| Goto et al. 1999 | infants | formula | 0-5 mo | 2.00 | 0.094 | GM | single load |
| Colome et al. 2007 | term infants | human milk | 18-120 d; mean age 65.54 d | 2.16 | 0.313 | mean | single load |
| Colome et al. 2007 | term infants | formula - prebiotics | 18-120 d; mean age 74.41 d | 2.45 | 0.331 | mean | single load |
| Colome et al. 2007 | term infants | formula - nucleotides | 18-120 d; mean age 74.41 d | 2.45 | 0.341 | mean | single load |
| Colome et al. 2007 | term infants | formula - LC-PUFA ^c | 18-120 d; mean age 74.41 d | 2.45 | 0.296 | mean | single load |
| Colome et al. 2007 | term infants | formula - LC-PUFA & nucleotides | 18-120 d; mean age 74.41 d | 2.45 | 0.268 | mean | single load |
| Dupont and Goutail-Flaud 1990 | healthy infants | not specified | <13 mo | 4.00 | 0.031 | mean | single load |
| Goto et al. 1999 | infants | human milk | 6-11 mo | 8.00 | 0.058 | GM | single load |
| Goto et al. 1999 | infants | formula | 6-11 mo | 8.00 | 0.056 | GM | single load |
| Isolauri et al. 1989 | control infants, afebrile | not specified | 2-23 mo; mean age 8.0 mo | 8.00 | 0.02 | GM | single load |
| Isolauri et al. 1989 | control infants, febrile | not specified | 7-26 mo; mean age 17.2 mo | 17.20 | 0.02 | GM | single load |
| Weaver et al. 1984 | term infants | formula | 1 d | 0.03 | 0.56 | median | steady state |
| Catassi et al. 1995 | term infants | human milk | 1 d | 0.03 | 1.27 | mean | steady state |
| Catassi et al. 1995 | term infants | formula | 1 d | 0.03 | 1.29 | mean | steady state |
| Weaver et al. 1984 | term infants | formula | 4 d | 0.13 | 0.59 | median | steady state |
| Catassi et al. 1995 | term infants | human milk | 7 d | 0.23 | 0.22 | mean | steady state |
| Catassi et al. 1995 | term infants | formula | 7 d | 0.23 | 0.47 | mean | steady state |
| Catassi et al. 1995 | term infants | human milk | 30 d | 0.99 | 0.24 | mean | steady state |
| Catassi et al. 1995 | term infants | formula | 30 d | 0.99 | 0.21 | mean | steady state |
| Weaver et al. 1988 | infants (English) | formula | 6 wk | 1.40 | 0.30 | median | steady state |
| Weaver et al. 1988 | infants (English) | human milk | 6 wk | 1.40 | 0.20 | median | steady state |

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Table V-6. Measures of Intestinal Permeability in Healthy Term Infants

| Reference | Study population | Type of feeding | Reported Age | Calculated Age (mo) | L/M ^a | L/M units ^b | Assessment |
|--------------------|-------------------|-----------------|--------------|---------------------|------------------|------------------------|--------------|
| Weaver et al. 1988 | infants (Gambian) | human milk | 6 wk | 1.40 | 0.22 | median | steady state |
| Weaver et al. 1988 | infants (English) | formula | 12 wk | 2.79 | 0.21 | median | steady state |
| Weaver et al. 1988 | infants (Gambian) | human milk | 12 wk | 2.79 | 0.17 | median | steady state |
| Weaver et al. 1988 | infants (English) | weaned | 18 wk | 4.19 | 0.32 | median | steady state |
| Weaver et al. 1988 | infants (Gambian) | weaned | 18 wk | 4.19 | 0.27 | median | steady state |

^a L/M = the ratio of lactulose/mannitol concentrations in urine.

^b GM = geometric mean

^c LC-PUFA = long-chain polyunsaturated fatty acids

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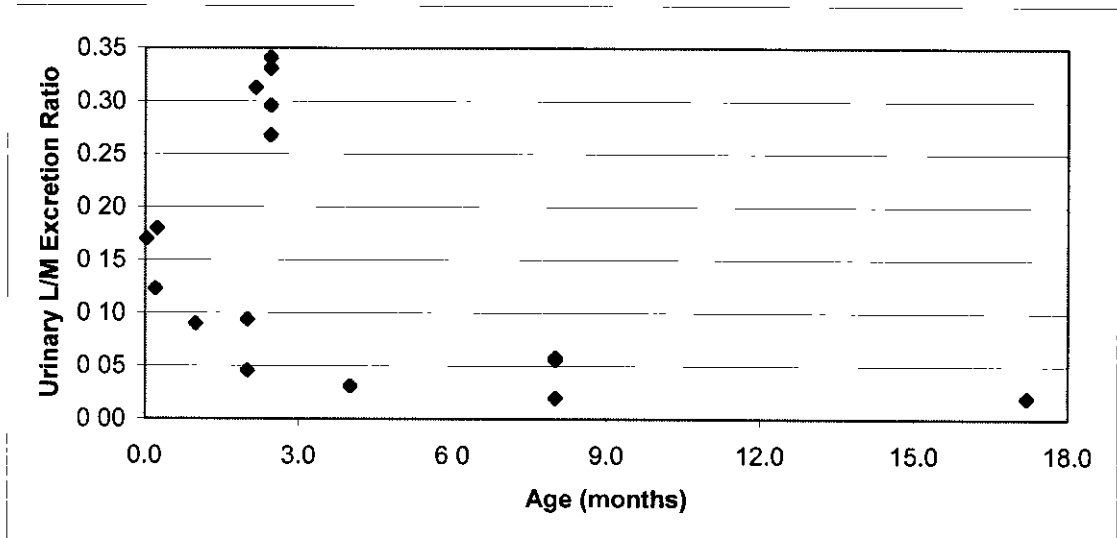
Table V-7. Measures of Intestinal Permeability in Healthy Children and Adults

| Reference | Study population | Age | Age (y) | L/M | L/M units | % L Excretion |
|------------------------|--------------------------|---------------------------------|---------|--------|-----------|---------------|
| Barboza et al. 1999 | children (controls) | <5 y; assume maximum of 4 y | 4 | 0.0394 | mean | 0.11 |
| van Elburg et al. 1995 | healthy children | 0-16 y; median 5 y | 5 | 0.034 | mean | 0.62 |
| Hamilton et al. 1987 | healthy children | 7 months - 14 y, mean 5.5 y | 5.5 | 0.036 | median | 0.76 |
| Marsilo et al. 1998 | healthy children | 0.4-13 y; mean 7.4 y | 7.4 | 0.024 | mean | 0.33 |
| Celli et al. 1995 | healthy children | 2-15 y; mean 9 y | 9 | 0.022 | mean | 0.28 |
| Miki et al. 1996 | healthy children | 5.3-16 y, median 9.2 y | 9.2 | 0.021 | mean | 0.29 |
| Elia et al. 1987 | healthy and obese adults | mean age of healthy adults 28 y | 28 | 0.021 | mean | 0.26 |
| Fleming et al. 1990 | healthy adults | mean 30 y | 30 | 0.027 | mean | 0.33 |
| van Elburg et al. 1995 | healthy adults | 21-59 y; median 31 y | 31 | 0.027 | mean | 0.45 |
| Cox et al. 1999 | healthy adults | 25-42 y; mean 34 y | 34 | 0.023 | median | 0.20 |
| Johnston et al. 2000 | healthy adults | mean 34 y | 34 | 0.013 | mean | 0.26 |
| Saltzman et al. 1995 | healthy adults | 20+ y | 50 | 0.028 | mean | 0.16 |

^a L/M = the ratio of % lactulose recovery to % mannitol recovery in urine, L/M ratios assessed via a single load test

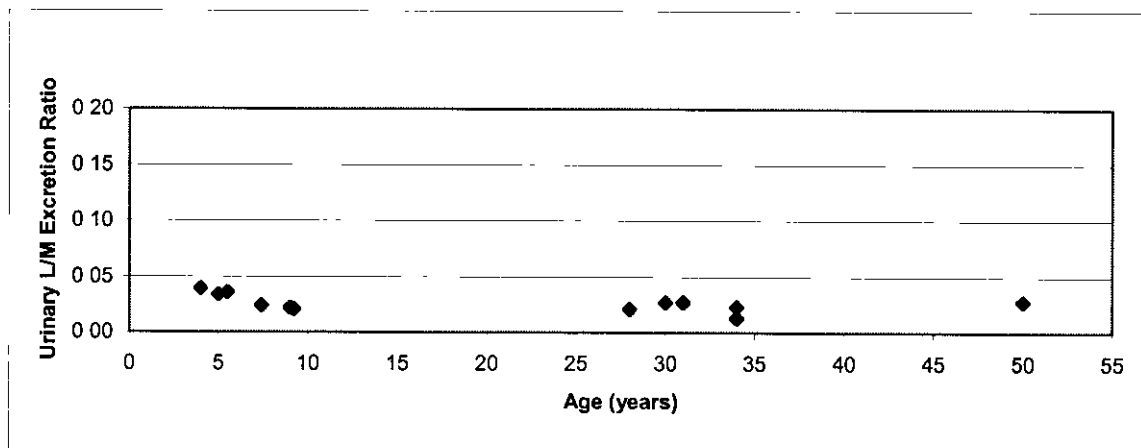
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Figure 11. Lactulose/Mannitol Ratios in Healthy Term Infants



Data source: Table V-6, results from single load intestinal permeability tests

Figure 12. Lactulose/Mannitol Ratios in Healthy Children and Adults



Data source: Table V-7; results from single load intestinal permeability tests

2. Normal Development and Function of Intestinal Microflora

a) Development of Microflora in the Infant

At birth, the intestinal tract of a human infant is sterile. The development of infant microflora occurs in several stages: initial acquisition of microflora during birth and the first week of life, development of microflora during feeding, and changes during weaning. Various factors influence this development, including method of delivery (vaginal or Cesarean section), birth environment (home or hospital), hygienic measures in place at the time of birth, developmental stage at birth (preterm/term), type of infant feeding (breast fed versus formula fed), and the use of antimicrobials (Heavey and Rowland 1999 as cited in Rodricks et al. 2007; Orrhage and Nord 1999; Penders et al. 2006). In addition, following birth, the mother delivers additional microbial strains to the infant during suckling, kissing, and caressing (Mackie et al. 1999).

Because of these various factors, the 'normal' microflora of infants is variable. During vaginal birth, an infant is colonized by bacteria from its mother (vaginal and feces microflora) and, to a lesser extent, from the environment (Boehm et al. 2005; Dai and Walker 1999 as cited in Rodricks et al. 2007; Hammerman et al. 2004 as cited in Rodricks et al. 2007; Mackie et al. 1999). Infants delivered via Cesarean-section (c-section) often have microflora dominated by environmental/hospital isolates. Consequently, the gastrointestinal microflora of vaginally-delivered infants is quite different from infants delivered via Cesarean section (Penders et al. 2006). Infants born through C-section have lower numbers of bifidobacteria and *Bacteroides*, whereas they are more often colonized with *C. difficile* compared to vaginally-born infants (Penders et al. 2006).

Over the first weeks of life, facultative anaerobic bacteria begin to predominate as the oxygen in the gut is utilized and depleted (Heavey and Rowland 1999 as cited in Rodricks et al. 2007; Orrhage and Nord 1999; Conway 1997; Wold and Adlerberth 2000 as cited in Rodricks et al. 2007; Fanaro et al. 2003, Penders et al. 2006). Anaerobic bacteria including various bifidobacteria species and *Bacteroides* spp. are found as early as day 2 in the breast-fed infant's microflora, though peak counts are reached closer to day 7 (consistent with the depletion of oxygen). Other anaerobic bacteria that appear during this change include *Clostridium* spp., *Eubacterium* spp., and *Lactobacillus* spp. (Stark and Lee 1982; Lundequist et al. 1985; Balmer and Wharton 1989 as cited in Rodricks et al. 2007, Harmsen et al. 2000, Penders et al. 2006). Streptococci, Enterobacteria (including *Escherichia coli*), staphylococci, and Enterococci have been

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identified as colonizing the infant gut one to two days after birth (Conway 1997, Lundquist et al. 1985; Balmer and Wharton 1989 as cited in Rodricks et al. 2007, Stark and Lee 1982; Balmer et al. 1989).

An important determinate of gut microflora in infants is feeding; differences have been found between the microflora of breast-fed infants and bottle-fed infants. The earliest studies of the infant microflora showed that breast-fed infants had a microflora dominated by bifidobacteria, which easily out-compete other genera and the presence of which are thought to depend on the occurrence of certain glycoproteins in human breast milk (Cummings et al. 2004 as cited in Rodricks et al. 2007). In contrast, formula-fed infants have a more complex flora which resembles the adult gut in that bacteroides, clostridia, bifidobacteria, lactobacilli, gram positive cocci, coliforms, and other groups are all represented in fairly equal proportions (Cummings et al. 2004 as cited in Rodricks et al. 2007). Formula-fed infants have also been found to have higher fecal levels of potentially harmful bacterial metabolic by-products (Edwards and Parrett 2002).

A prospective cohort study of 1032 infants at 1 month of age used quantitative real-time polymerase chain reaction assays for the enumeration of bifidobacteria, *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis* group, lactobacilli, and total bacterial counts in fecal samples (Penders et al. 2006). Most infants ($n = 700$) were breastfed exclusively up to the first 1 month of life, whereas 232 infants were formula fed exclusively and 98 infants received a combination of breastfeeding and formula feeding. Exclusively formula-fed infants were more often colonized with *E coli*, *C. difficile*, *B fragilis* group, and lactobacilli than were their exclusive breastfed counterparts (Table V-8). The counts of *E coli*, *C. difficile*, *B fragilis* group, and lactobacilli were also significantly higher for formula-fed infants compared with breastfed infants.

In this population, 4 brands of formula were frequently used. Brand B contained locust bean gum and brand D was enriched with oligosaccharides, whereas the others were not. Infants fed exclusively with 1 of these formulas were compared. As shown in Table V-8, infants fed the oligosaccharide-enriched formula (brand D) harbored greater numbers of bifidobacteria in their stools. After adjustment for the other determinants under study, counts of bifidobacteria (coefficient: 0.60; $P = .04$) and also counts of lactobacilli (coefficient: 0.75; $P = .02$) tended to be higher for infants fed formula D, compared with reference formula A.

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**Table V-8. Median Counts and Prevalence of Colonization with Selected Gut Bacteria
in Feces of Infants 1 Month of Age ($n = 1032$)**

| Characteristics | n ^a | Bifidobacteria | | <i>E. coli</i> | | <i>C. difficile</i> | | <i>B. fragilis</i> Group | | Lactobacilli | | Total Counts |
|--|----------------|-----------------------------------|------------------|-----------------------------------|-----------------|-----------------------------------|-----------------|-----------------------------------|-----------------|-----------------------------------|-----------------|---------------------------------------|
| | | Counts, Median, log10 CFU/g Feces | Prevalence % | Counts, Median, log10 CFU/g Feces | Prevalence % | Counts, Median, log10 CFU/g Feces | Prevalence % | Counts, Median, log10 CFU/g Feces | Prevalence % | Counts, Median, log10 CFU/g Feces | Prevalence % | Median, log ₁₀ CFU/g Feces |
| Type of infant feeding | | | | | | | | | | | | |
| Exclusively breastfed ^b | 700 | 10.67 | 99 | 9.06 | 85 | 4.53 | 21 | 8.99 | 79 | 8.54 | 29 | 10.98 |
| Exclusively formula fed | 232 | 10.69 | 97 | 9.84 | 94 ^c | 7.43 | 33 ^c | 9.76 | 88 ^c | 8.93 | 41 ^c | 11.43 ^c |
| Combination | 98 | 10.78 | 99 | 9.76 | 93 ^c | 5.58 | 35 ^c | 9.53 | 83 ^d | 8.71 | 34 | 11.36 ^c |
| Type of infant formula | | | | | | | | | | | | |
| Brand A ^b | 47 | 10.51 | 96 | 9.83 | 91 | 7.68 | 40 | 9.84 | 89 | 8.84 | 34 | 11.45 |
| Brand B (with locust bean gum) | 19 | 10.80 | 95 | 9.81 | 89 | 6.56 | 21 | 9.80 | 95 | 8.40 | 47 | 11.43 |
| Brand C | 39 | 10.81 | 95 | 9.82 | 93 | 7.28 | 25 | 9.70 | 87 | 8.68 | 38 | 11.28 |
| Brand D (with oligosaccharides) | 20 | 11.19 | 100 ^d | 9.63 | 95 | 6.23 | 30 | 10.11 | 75 | 9.29 | 55 | 11.65 |
| ^a The study included 1032 infants, totals may not add up to 1032 because of missing values. Counts were calculated from positive samples only. | | | | | | | | | | | | |
| ^b Reference category. | | | | | | | | | | | | |
| ^c <i>P</i> < 0.001, as determined with the Mann-Whitney rank-sum test, calculated from all samples (the statistical significance refers to an overall difference incorporating both counts and prevalence). | | | | | | | | | | | | |
| ^d <i>P</i> < 0.01, as determined with the Mann-Whitney rank-sum test, calculated from all samples (the statistical significance refers to an overall difference incorporating both counts and prevalence). | | | | | | | | | | | | |

b) Adult Microflora

The colonic microflora of infants can be described as “adult-like” after the age of two years, although populations of facultative anaerobes are often observed to be greater than those of healthy adults (Hopkins et al. 2002). Different analytical methodologies were utilized to compare the bacterial composition of feces obtained from children (16 months to 7 years), young adults (21 to 34 years), elderly subjects (67-88 years) and patients with *C. difficile* associated diarrhea (68-73 years) (CDAC) (Hopkins et al. 2002). Bacteria were determined by viable count, 16S rRNA, and community cellular fatty acids (CFA) methodologies. The results of the study suggest that gut microflora is not completely adult-like until much later in life than originally thought and probably continues to change throughout the life of an individual.

While total anaerobe counts were similar in all four subject groups, bacterial compositions at the genus level varied markedly (Hopkins et al. 2002). Populations of bacteroides-group organisms were significantly lower in CDAC patients compared to the other populations, while numbers of bifidobacteria were reduced in geriatric patients, irrespective of *C. difficile* infection. Viable counts of predominant bacterial species isolated from the feces of adults and children showed some variation such as higher bifidobacterial and clostridial populations in children. However, the principal microbiological difference between adults and children was the occurrence of higher numbers of enterobacteria, which were 100-fold higher in the children’s feces (determined by viable counts and 16S rRNA measurements). This finding suggests that while the overall microflora composition is similar in children and adults, facultative anaerobes often remain at elevated levels in the large intestine of infants. It is probable that as the microflora matures, competition from other bacterial species increases causing a reduction in facultative anaerobic populations. The rRNA analysis showed that the proportions of enterobacterial, bifidobacterial and bacteroides-group organisms were elevated in the children, thus demonstrating that the intestinal flora of this group was bacteriologically less complex than that of the adults.

The microbiota of CDAC patients was markedly different from all other samples with greatly reduced species diversity of bifidobacteria, prevotella and bacteroides with a concomitant increase in clostridia and lactobacilli (Hopkins et al. 2002). Although a less sensitive diagnostic tool, marked alterations in bacterial populations detected in the CDAD patients were revealed by a greatly altered CFA profile obtained from these stools. The CFA analysis correlated with the viable bacterial counts and 16S rRNA measurements. The altered composition of metabolically active bacteria such as

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bacteroides and eubacteria probably leads to profound changes in the biochemical capacity of the gut microbiota with advancing age (Hopkins et al. 2002). Especially evident with aging was a reduction in the putatively protective bifidobacteria.

Once the microflora composition has become established, the major bacterial groups in the feces of adults remain relatively constant over time. It is noted, however, that elderly people have fewer bifidobacteria and higher populations of enterobacteria compared to younger adults. The frequency of isolation of *C. difficile* is also greater in the elderly. A number of physiological changes occur in the body with advancing age. These include decreased acid secretion by the gastric mucosa and greater permeability of mucosal membranes in the gut. Thus, it is likely that certain bacterial strains take advantage of new ecological niches, thereby inducing a shift in the composition of the gut microflora (Hopkins et al. 2002).

The metabolic characteristics (nine enzyme activities and 11 metabolites) of the fecal microflora were studied in children (3-15 years), adults (30-46 years) and elderly (69-89 years) subjects (Andrieux et al. 2002). The results showed large inter-individual differences in the three groups for bacterial enzyme activities. No significant differences between groups was observed for the major short-chain fatty acids (acetate, propionate, butyrate), however, the fecal concentrations of less abundant SCFA (caproate, iso-butyrate and iso-valerate) did differ among groups. Valerate, iso-butyrate and iso-valerate were significantly higher in elderly persons than in adults; they were also higher in elderly persons than in children. Ammonia concentration was significantly higher in elderly persons than in adults. The D/L lactate ratio was significantly higher in elderly persons compared to the other groups. In conclusion, data showed significant differences between elderly persons and younger adults and children, but the major metabolic characteristics of the fecal microflora were not greatly altered by the aging process.

c) Functionality of Gut Microflora

After birth the gastrointestinal tract exists in symbiosis with a large number and variety of bacteria that contribute to the health of the individual. Gut microorganisms contribute to diverse mammalian processes. Along its entirety, the microflora of the gut act as an effective barrier against opportunistic and pathogenic microorganisms. Other advantageous effects include modulation of the immune system, development of intestinal microvilli, production of short chain fatty acids (SCFA) upon which the colonic mucosa is dependent for energy, fermentation of non-digestible dietary fiber and anaerobic metabolism of peptides and proteins resulting in the recovery of metabolic energy for the host and removal of carcinogens and toxins (Rodricks et al. 2007).

The intestinal mucosa provides a natural physical cellular barrier, limiting potentially harmful microorganisms present in the intestinal lumen from colonizing enterocytes, as described earlier. The gastrointestinal tract of the preterm neonate is physiologically immature in its development at the time of birth, rendering it more susceptible to bacterial translocation than that of the adult. Many species of pathogenic bacteria are able to alter the permeability of the intestine and invade deep tissue; these include, among others, *Salmonella* spp., *Listeria monocytogenes*, *Yersinia* spp., and *Shigella* spp. Infection of the intestinal epithelium often leads to diarrhea; when this fails to resolve the infection, bacteria may move into deeper tissue and eventually cause systemic infections and the related symptoms (Pucciarelli et al. 1997 as cited in Rodricks et al. 2007).

Prebiotics such as GOS function to increase levels of anaerobic lactic acid bacteria (*Lactobacillus acidophilus*, bifidobacteria), which have a protective role against the translocation of other bacteria. This is mediated by the production via fermentation of SCFAs that produce an acidic environment unfavorable for many pathogens, as well as production of antimicrobial bacteriocins. Bacteriocins are proteins or protein complexes with bactericidal activities directed against species that are closely related to the producer bacterium (Hammerman et al. 2004 & Dai and Walker 1999, both as cited in Rodricks et al. 2007). Several studies have investigated the enteric flora of infants with NEC and found a decline in the concentration of anaerobic species and increased colonization with gram-negative bacteria (Hammerman et al. 2004 as cited in Rodricks et al. 2007).

The microflora of the intestine can have significant effects on the GALT. Studies examining the absence of a normal microflora demonstrated increased antigen transport across the gut mucosa (Isolauri et al. 2001). Additionally, intestinal colonization by non-pathogenic bacteria is an important antigenic stimulus for the maturation of the GALT; as the gut microflora is established, the capacity of the GALT to produce IgA secreting cells increases. The stimulatory effect of the microflora on the secretory IgA system and on B cell function in general is well established (Gaskins 1997 as cited in Rodricks et al. 2007).

3. Studies in Humans

Many studies have been conducted in humans, including term and preterm infants, to assess the tolerance and impact of Vivinal® GOS on gut microflora and related microbial activities. The data from these studies provide evidence of the safety of use of Vivinal® GOS. The safety of other GOS products has also been studied, and results provide corroborative data to support the safety of Vivinal® GOS. Table V-15 explains the abbreviations used in the GOS tables.

a) Studies in Adults

A total of 16 published studies of GOS and two unpublished studies (conducted on Friesland Food Domo's GOS) were identified and reviewed (Tables V-9 and V-10); the studies represent investigations of GOS ingestion in 16 study populations. In these studies GOS was administered for time periods ranging from 6 days to 4 weeks with a dosage range of 2.4 to 20.8 g GOS per day. GOS prepared by Friesland Foods Domo was consumed daily over periods of 8 to 21 days, with doses ranging from 8.1 to 20.8 g per day.

Tolerance of GOS was investigated in the clinical studies, as well as effects of GOS consumption on fecal microflora, stool characteristics, and fecal enzyme activities. Results from these studies indicate that ingestion of up to 15 g GOS per day over a period of 3 weeks is well tolerated by most adults. The adverse effects associated with GOS intake at this level were increased flatulence or gastrointestinal discomfort and generally mild in nature. In one study, intake of 20.8 g GOS was reported to be well tolerated over a period of 5 days; GOS was consumed in lower doses in the preceding 4 days. In another study, intake of 20 g GOS daily for 4 days followed intake of lower doses of GOS for 4 days; gastrointestinal complaints were reported during GOS consumption, though they were considered to be mild in nature.

(1) Adult Studies with GOS Produced by Friesland Foods Domo

All adult studies involving the use of GOS produced by Friesland Foods Domo are summarized in Table V-9. A total of seven published studies and two unpublished studies were reviewed; the studies represent six separate clinical trials. van Dokkum and colleagues (1999) present findings from a Latin square, randomized, double-blind study in which 12 healthy young men consumed GOS, FOS, inulin or no oligosaccharide (the control period) for a period of 3 weeks each. In each feeding period, the men consumed a total of 15 g GOS (or other oligosaccharide) daily in three equal portions dissolved in

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orange juice and consumed with meals. At the end of each feeding period, various parameters of large bowel function, blood lipid concentrations and glucose absorption were assessed. Consumption of 15 g GOS per day was reportedly well tolerated. The percentage weight of fecal dry matter increased during GOS consumption, while GOS ingestion had no effects on fecal total or dry weight, intestinal transit time, fecal pH, concentrations of fecal bile acids and neutral steroids, fecal enzyme activities, blood lipid concentrations, glucose tolerance, or breath hydrogen production. The concentration of acetic acid in fecal samples was increased during GOS consumption, but no effects on other short-chain fatty acids were observed. Additionally, daily consumption of 15 g GOS had no effects on iron or calcium absorption in this study population (van den Heuvel et al. 1998).

An additional investigation of the tolerance of GOS was incorporated into this larger study, though results have not been published (van Dokkum 1995, see Appendix 8). Results from the unpublished report provide additional details on the tolerance assessment of GOS. Tolerance to GOS was assessed via structured daily questionnaires regarding defecation patterns and observations about stomach or intestinal complaints. Subjects generally reported feeling “good” or “excellent” in the daily questionnaires. Four of the 12 men reported increased flatulence during the GOS period, while few incidences of other gastrointestinal disturbances were reported in the GOS period, and some disturbances were noted during the control period. During the GOS period, several men indicated that feces were softer and of larger volume. These details support the conclusion of van Dokkum et al. (1999) that ingestion of 15 g GOS per day, delivered in 3 equal doses, was well tolerated.

In another human clinical study of GOS produced by Friesland Foods Domo (Alles et al. 1999), 39 health men and women were assigned to consume a diet containing 0, 8.5 or 14.4 g GOS per day for 3 weeks after a 3 week run-in period. The GOS was consumed in three equal portions in fruit juice at meals. GOS had no effects on daily dietary intakes, stool frequency or weight, fecal pH, fecal short-chain fatty acid concentrations, concentrations of bile acids in fecal water, or fecal concentrations of ammonia, indoles and skatoles. No changes in fecal bacteria counts were observed. Some participants in both the 8.5 and 14.4 g GOS groups reported flatulence. Breath hydrogen concentrations also increased among men consuming 14.4 g GOS daily for a period of 3 weeks, though intake of 8.5 g GOS had no effect on breath hydrogen concentrations, and no GOS was detected in feces.

Alander and colleagues (2001) investigated the effects of consumption of GOS alone or in combination with *Bifidobacterium Lactis* Bb-12 on fecal microflora. The GOS used in this study was supplied by Borculo Domo Ingredients, currently Friesland Foods Domo. Ten subjects in the GOS group consumed 8.1 g GOS per day, divided in two equal doses in yogurt, for a period of two week. A decrease in fecal *C. perfringens* was seen in the subjects who consumed GOS alone, but no effects on fecal bifidobacteria were observed. One participant in the GOS group was unable to complete the study due to pain from intestinal bloating. The study was single-blinded and subjects were aware of the feeding group to which they were assigned.

van den Heuvel and colleagues (2000) studied the effects of GOS (produced by Friesland Foods Domo) on calcium absorption in postmenopausal women. In this double-blind, randomized, crossover study, 12 postmenopausal women consumed a yogurt drink twice daily that contained either GOS or a placebo (sucrose). Each study period was 9 days. GOS was administered in increasing dosages to allow for adaptation to the oligosaccharide. The total intake of GOS was 10.4 g on days 1 and 2, 15.6 g on days 3 and 4, and 20.8 g on days 5 to 9. No adverse effects were seen after consumption of 20.8 g GOS per day as compared to the control treatment. Calcium absorption increased 16% during the GOS treatment, resulting in significantly higher calcium absorption as compared to the control period.

The effect of GOS produced by Friesland Foods Domo on constipation in the elderly women was investigated by Teun and Korpela (1998). In this double-blind, cross-over study, subjects consumed a GOS-containing yogurt or a control yogurt twice daily for a period of two weeks each. The total daily dose of GOS was 9 g and was consumed in two equal portions. Ingestion of GOS had no effects on fecal pH, percent dry weight of feces, or fecal characteristics or gastrointestinal symptoms. Frequency of defecation increased during the GOS treatment in 8 of the 14 study participants.

Another study of the effects of GOS and FOS on breath hydrogen excretion and gastrointestinal well being was conducted by Borculo Domo Ingredients (Alles and Schoterman 1999, see Appendix 8); this study was not published. In this balanced, single-blind cross-over study, the effects of GOS and FOS were studied before and after daily intake of 2 portions of yogurt containing one of the oligosaccharides. Adaptation to the oligosaccharides was ensured by gradually increasing the dose: the total daily dose on days 1-2 was 10 g, on days 3-4 was 15 g, and on days 5-8 was 20 g. Participants maintained diaries of gastrointestinal complaints throughout the study, and completed questionnaires at the end of the run-in and supplement periods. On the last day of the

supplemental periods, end-expiratory breath samples were taken at 30-minute intervals over a period of 10 hours. Complaints of belching, flatulence, bloating and abdominal pains/cramps were greater during both the GOS and FOS periods as compared to the baseline period, though there were no differences in complaints between the GOS and FOS groups. Study participants reported less frequent defecation during the GOS period as compared to normal defecation patterns, while GOS ingestion had no effects on stool consistency or quantity or occurrence of diarrhea or constipation. Breath hydrogen excretion in both the GOS and FOS groups followed a similar curve, though breath hydrogen excretion was 34% higher in the FOS group as compared to the GOS group. Overall the gastrointestinal complaints were considered to be mild. Results from this study provide corroborative evidence of the tolerance of daily intakes of up to approximately 20 g GOS as reported by van den Heuvel and colleagues (2000).

(2) Adult Studies with Other Sources of GOS

Human clinical studies of GOS from sources other than Friesland Foods Domo are summarized in Table V-10. Results from these nine published studies representing 10 study populations provide additional information on the tolerance of the oligosaccharide and effects of GOS ingestion on fecal microflora composition and stool characteristics.

Bouhnik et al. (1997) reported that 8 healthy adults consuming 10 g GOS per day (in two equal doses) experienced no adverse symptoms during the 3-week study period. The investigators also reported a significant drop in breath hydrogen excretion and no change in methane excretion. Teuri and colleagues (1998) also assessed gastrointestinal symptoms during consumption of 15 g GOS per day for a period of 2 weeks. Subjects consumed the GOS in two equal doses per day in yogurt. The occurrence of flatulence increased significantly during GOS consumption, as did the sum of all gastrointestinal symptoms, though no diarrhea was reported and no changes in appetite, stool consistency, abdominal distention or other symptoms were observed.

Alterations in gut microflora, stool patterns and mineral absorption have been studied in adults of varying age groups and at varying dosages of GOS. In a study of 4 healthy men and 4 healthy women, fecal bifidobacteria counts increased during a 3-week period of daily ingestion of 10 g GOS (Bouhnik et al. 1997). In a later study, fecal bifidobacteria counts were observed to increase in 8 adults consuming 10 g GOS per day for a week (Bouhnik et al. 2004). GOS dosages of 2.5, 5.0, 7.5 and 10 g per day were not found to have a dose-dependent effect on fecal bifidobacteria (Bouhnik et al. 2004). In the study

by Tanaka et al. (1983), 5 healthy young men consumed 3 g GOS per day for one week followed by another week of GOS supplementation at 10 g per day. The investigators reported a dose-dependent increase in fecal bifidobacteria counts. Increases in breath hydrogen concentrations were observed following the intake of a single dose of 30 g GOS (Tanaka et al. 1983). In another study, daily intake of 2.5 g GOS in a beverage resulted in an increase in fecal bifidobacteria over the course of the 3-week study period (Ito et al 1993a), and an increase in fecal bifidobacteria and lactobacilli in 12 men consuming 15 g GOS (disaccharides) for 6 days (Ito et al. 1993b). This investigator also reported a dose-dependent increase in the mean number and percentage of fecal bifidobacteria in subjects consuming 2.5, 5.0 or 10.0 g GOS per day for one week (Ito et al. 1990). Gopal et al. (2003) also reported increased fecal microflora in subjects consuming 2.4 g GOS per day in milk for 4 weeks; the GOS consumed in this study was produced *in situ* in milk enzymatically treated with β -galactosidase.

In other studies in adults, GOS was reported to have no significant impact on the gut microflora. Tannock et al. (2004) concluded that ingestion of 2.5 g GOS per day in biscuits over a 3-week period did not affect fecal microflora. Teuri et al. (1998) administered 15 g GOS per day to healthy volunteers in a yogurt mixture. Although no increase in the amount of bifidobacteria was found in the 12 healthy study participants after a 2-week feeding period, the investigators did note an increase in anaerobic bacteria from fecal samples growing on MRS medium.

Studies investigating the impact of GOS supplementation on fecal characteristics and stool patterns generally have reported few effects. GOS was not found to have effects on fecal pH (Bouhnik et al. 1992, 2004; Teuri et al. 1998), fecal weight (Bouhnik et al. 1992, Ito et al. 1990), or frequency of defecation (Ito et al. 1990), though Teuri et al. (1998) reported an increase in stool frequency in adults consuming 15 g GOS per day. In one study, subjects consuming GOS reported softer feces (Ito et al. 1990). In a 4-week study of 14 elderly females suffering from constipation, however, 9 g GOS per day was not found to decrease the hardness of stools or increase ease of defecation (Teuri and Korpela 1998).

A summary of the gastrointestinal effects reported in each study of adults by total daily intake of GOS is presented in Table V-11. Direct comparisons across the studies are difficult to make because of the different methods of tolerance assessment and reporting used in the studies. The data do not suggest, however, a clear dose-response effect of total daily GOS intake on gastrointestinal symptoms in adults. Intakes of GOS in the range of 20 or 21 g per day, the highest doses tested, were reported to have no impact on

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gastrointestinal effects as compared to a control treatment in one study (van den Heuvel et al. 2000), though a similar dose was reported to increase gastrointestinal symptoms above baseline levels in another study (Alles and Schoterman 1999).

In conclusion, adult clinical studies of GOS indicate that doses as high as 15 or 20.8 g per day are generally well tolerated. Overall, the effects of GOS on gastrointestinal tolerance appear to be generally mild in nature over the range of doses assessed in adults. Some side effects were reported at these doses, but they were generally mild in nature. The ability of GOS to alter the balance of the colonic bacteria towards a potentially healthier microflora, most notably by increasing bifidobacteria levels, has been shown in some but not all studies. Moreover, GOS may positively impact fecal characteristics and stool patterns in some individuals, though results from clinical studies do not consistently support these findings.

Table V-9. Summary of Adult Studies with GOS from Friesland Foods Domo

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion* |
|---|--|---|--|---|--------------------|---|
| Alander et al 2001; Malinen et al. 2002 | Study effects of GOS and a probiotic on fecal microflora in healthy adults; determine changes in distributions of bifidobacterial species using PCR-ELISA Randomized, single-blinded, parallel study. | 3 M/ 27 F, 10 per group, 22-47 y, mean age 32 y | 3 treatments: Yogurt containing GOS syrup, <i>B. lactis</i> Bb-12 (0.5 g) or GOS+ <i>B. lactis</i> | 8.1 g; delivered in 2 daily doses. 0.14 g/kg-bw/day [†] | 2 wk | -Decrease in <i>C. perfringens</i> , no change in total fecal bifidobacteria in GOS group -One subject experienced pain from intestinal bloating during feeding period. (96.7% of participants well tolerated). -No change in distribution of bifidobacterial species in GOS group. |
| Alles et al 1999 | Evaluate effects of GOS on the composition and activity of intestinal microflora in healthy adults. Parallel designed study | 22 M/ 18 F, 13-14 per group, mean age 39 y | 3 treatments: placebo diet, 2 GOS levels | 8.5 or 14.4 g; delivered in 3 daily doses. 0.20 g/kg-bw/day | 3 wk | -No effect on intestinal microflora including bifidobacteria, <i>Lactobacilli</i> , <i>Clostridia</i> , or <i>E. coli</i> -No change in stool frequency, % fecal fecal dry matter, or fecal weight. -Increase in nitrogen density (low-dose group non-significant). -No effect on fecal SCFA, fecal water bile acids, or fecal pH. -Some flatulence reported in low- and high-dose groups. -No fecal excretion of GOS. |

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Table V-9. Summary of Adult Studies with GOS from Friesland Foods Domo

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion* |
|--|---|---|--|--|--------------------|--|
| Alles and Schoterman 1999 [unpublished] | Compare effects of GOS and FOS on breath hydrogen excretion and gastrointestinal well being in adults Balanced, single-blind crossover study | 10 M/ 6 F, mean=36.6 y | 2 treatments GOS or FOS in yogurt | 10 g on d 1-2 15 g on d 3-4 20 g on d 5-8, delivered in 2 daily doses. 0.33 g/kg-bw/day [†] | 8 d | -Complaints of belching, flatulence, bloating and abdominal pains/cramps were greater during GOS and FOS periods than baseline period; no differences between GOS and FOS. -Less frequent defecation during GOS period compared to normal, no effects on stool consistency, quantity, diarrhea or constipation. -Mean peak breath hydrogen excretion at 4½ h for both GOS and FOS, breath hydrogen excretion 34% higher with FOS vs. GOS |
| Teuri and Korpela 1998 | Investigate the effects of GOS on constipation in the elderly. Double-blind, two-period cross-over study | 14 F, 7 per group, 69-87 y, mean=79.6 y | 2 treatments: control yogurt, yogurt+GOS | 9 g/d; delivered in 2 daily doses 0.13 g/kg-bw/day | 2 wk | -No change in fecal pH or % dry weight -No effect on fecal hardness or ease of defecation. -Increased frequency of defecation in 8/14 people vs a decrease in 2/14 and no change in 4/14 -No change in fluid intake -Weight remained unchanged. |

Table V-9. Summary of Adult Studies with GOS from Friesland Foods Domo

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion * |
|--|---|---------------------|---|---|--------------------|--|
| van den Heuvel et al 1998; van Dokkum et al. 1999, van Dokkum 1995 [unpublished] | <p>Study the tolerance of GOS in healthy men.</p> <p>Study the effects of inulin, FOS and GOS on large-bowel function, blood lipid concentrations, glucose absorption, and intestinal absorption of iron and calcium in healthy men.</p> <p>Randomized, double-blind, crossover study; questionnaire administered for information on tolerance.</p> | 12 M, mean age 23 y | 4 treatments: control, inulin, FOS, GOS | <p>15 g/d; delivered in 3 daily doses</p> <p>0.19 g/kg-bw/day</p> | 3 wk | <ul style="list-style-type: none"> -Half of participants experienced improvement in defecation pattern -Increase in flatulence observed by 4 participants -Well tolerated. -No study drop outs or serious adverse events reported. -Decrease in % fecal dry weight. -Increase in acetic acid. -No change in propionic, butyric, valeric, or iso-valeric acid. -No change in H₂ expiration. -No effect on fecal bile acid concentrations or fecal neutral steroids -Decrease in β-glucuronidase activity. -No effects on body weight. -Well tolerated -Increase in flatulence. -No change in blood lipid concentration or glucose absorption. -No change in fecal pH. -Increase in fecal acetic acid. -No significant impact on iron status or calcium absorption. |

Table V-9. Summary of Adult Studies with GOS from Friesland Foods Domo

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion* |
|----------------------------|--|------------------------------|--------------------------------------|--|--------------------|---|
| van den Heuvel et al. 2000 | Study the effects of GOS on calcium absorption in postmenopausal women. Unbalanced, double-blind, randomized, crossover study | 12 F, 55-65 y, mean age 62 y | 2 treatments: control, GOS in yogurt | 10.4 g on d 1-2, 15.6 g on d 3-4, 20.8 g on d 5-9; delivered in 2 daily doses. 0.28 g/kg-bw/day | 9 d | -No adverse side effects (gastrointestinal complaints or change in stools) when compared to control -Increase in Ca absorption (16%) |

* Results statistically significant unless noted otherwise.

† Default weight of 60 kg used in calculating g/kg-bw/d intake; maximum intakes shown for all studies with multiple dose levels

Table V-10. Summary of Adult Studies with GOS from a Source Other than Friesland Foods Domo

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion* |
|---------------------|--|--|--|---|--------------------|---|
| Bouhnik et al. 2004 | To determine the bifidogenic effect of different nondigestible carbohydrates in the diets of healthy adults. Double-blind, randomized, placebo-controlled (Phase I) | 81 M/ 119 F, mean=30 y Phase I: 64 participants; 8 groups of 8 subjects | 8 treatments: placebo, sc-FOS, soybean OS, GOS, resistant starch, lactulose, long-chain inulin, isomalto-OS. | 10 g/d 0.13 g/kg-bw/day [†] Source: Cupoligo P: Niossin Sugar MFG, Tokyo. | 1 wk | -Increase in fecal bifidobacteria -No change in fecal total anaerobes, Lactobacillus, Bacteroides, enterobacteria, or pH - Increases in excess flatus, bloating, borborygm and abdominal pain in all groups including placebo group -No effect on number of stools -No diarrhea reported |
| | | Phase II: 136 participants; 8 subjects per treatment/dose | 5 treatments placebo, FOS, GOS, soybean OS, resistant starch | 0, 2.5, 5 0, 7.5, 10 0 g/d 0.17 g/kg-bw/day [†] | 1 wk | -No significant dose-response. -Increases in excess flatus, bloating and abdominal pain for all treatment groups. -No diarrhea reported. |
| Bouhnik et al 1997 | Assess tolerance and effects of GOS on fecal bifidobacteria and fermentative activity of colonic flora in healthy men and women. | 4 M/ 4 F, 20-32 y | 1 treatment: GOS in diet | 10 g/d; delivered in 2 daily doses 0.17 g/kg-bw/day [†] Source: Yakult Institute, Tokyo, Japan | 3 wk | -No effect on fecal weight, % fecal water, or fecal pH. -Decrease in breath H ₂ excretion; no change in methane excretion. -Increase in fecal bifidobacteria concentrations; no effect on fecal enterobacteria. -Participants did not experience any symptoms as a result of treatment. |

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Table V-10. Summary of Adult Studies with GOS from a Source Other than Friesland Foods Domo

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion * |
|-------------------|---|--|--|---|--------------------|---|
| Gopal et al. 2003 | Study the effects on microbiota composition due to the consumption of a milk containing GOS or <i>B. lactis</i> Randomized, double blind, placebo-controlled study | 18 M/ 12 F in 3 groups; 10 each group; 20-60 y | 3 treatments: Low fat base milk powder, enzymatically treated base milk, <i>B. lactis</i> fortified base milk | 2.4 g/d 0.04 g/kg-bw/day [†] Source: <i>in situ</i> production from β -galactosidase | 4 wk | -Increase in fecal bifidobacteria throughout study, increase in fecal lactobacilli at week 4. -No effects on fecal total anaerobes, enterobacteria, clostridia, bacteroides, or streptococci. -Subject compliance reported as excellent. -No adverse symptoms reported by participants; milk was well accepted. |
| Ito et al. 1990 | Study effects of GOS on intestinal microflora and stool weight in healthy men. Single-blind crossover study | 12 M, 26-48 y | 4 treatments: sugar mixture in 115 ml apple juice drunk after lunch daily. | 2.5, 5.0, or 10.0 g/d 0.17 g/kg-bw/day [†] Source: Oligomate-50 [®] (52% GOS) | 1 wk | -Decrease in stool hardness. -No change in stool frequency or stool weight. -Dose-dependent increase in fecal bifidobacteria (mean number and %). -Increase in <i>Lactobacilli</i> ; no changes in numbers of <i>Bacteroides</i> , <i>Enterobacteriaceae</i> or <i>Enterococci</i> . -No reports of diarrhea. -Dose-dependent increase in sensation of fullness. -Non-significant dose dependent increase in wind and abdominal pain. |

Table V-10. Summary of Adult Studies with GOS from a Source Other than Friesland Foods Domo

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion* |
|------------------|--|-----------------------------|--|---|--------------------|--|
| Ito et al. 1993a | Study effects of GOS on microflora of people with comparatively low numbers of indigenous fecal bifidobacteria | 12 M | 1 treatment. Juice containing GOS given daily before lunch | 2.5 g/d 0.04 g/kg-bw/day [†] Source: Oligomate-50 [®] (52% GOS) | 3 wk | -Fecal bifidobacteria increased during feeding period, no effect on other microflora. -Decrease in fecal nitroreductase and indole. -No effect on stool weight, fecal pH, beta-glucuronidase, ammonia, p-cresol -Decrease in fecal valeric acid, no change in other SCFAs or total SCFAs. |
| Ito et al. 1993b | Determine effects of GOS intake on fecal microflora and their metabolism in healthy volunteers. | 12 M, 33-55y, mean age 49 y | 1 treatment iced tea containing GOS | 15 g/d 0.25 g/kg-bw/day [†] Source: Oligomate-50 [®] | 6 d | -Increase in fecal bifidobacteria and Lactobacilli, decrease in fecal bacteroidaceae and <i>Candida</i> spp; no change in fecal <i>C. perfringens</i> , enterobacteriaceae, lactobacilli, bacilli, or staphylococci. -No change in stool weight or percent water content. -Decrease in fecal pH, ammonia, p-cresol, and indole. -Decrease in fecal concentrations of isovaleric, valeric, propionic, and isobutyric acids; no change in succinic, lactic, formic, butyric, or total organic acids |

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Table V-10. Summary of Adult Studies with GOS from a Source Other than Friesland Foods Domo

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion* |
|---------------------|--|--------------------------------|---|--|--------------------|--|
| Tanaka et al. 1983 | Study effects of GOS on fecal flora of healthy men. | 16 M, 25-35 y, 5-6 per group | GOS delivered in diet with or without <i>B. breve</i> . | 3 g/d wk 1, 10 g/d wk 2 0.17 g/kg-bw/day [†] Source not specified | 2 wk | -Dose-dependent increase in bifidobacteria -Dose-dependent decrease in <i>Bacteroidaceae</i> -Increase in breath hydrogen excretion. |
| Tannock et al. 2004 | Study effects of GOS on fecal microflora in healthy men and women Double-blind, crossover study | 7 M/ 8 F divided into 3 groups | Biscuits containing GOS, FOS or no OS | 2.5 g/d 0.04 g/kg-bw/day [†] Source: Oligovite (Fonterra) | 3 wk | -No effect on fecal microflora |
| Teuri et al. 1998 | Study effects of GOS ingestion on GI symptoms and fecal frequency in healthy adults | 3 M/ 9 F, 25-55 y, mean=38 y | 1 treatment: 200mL of yogurt containing 38 g/L GOS | 15 g/d; delivered in 2 daily doses 0.25 g/kg-bw/day [†] Source: Maxilact® | 2 wk | -Increase in stool frequency -No effect on fecal bifidobacteria; increase in fecal total anaerobes. -No change in fecal pH. -Increase in flatulence. -Increase all GI symptoms when summed and compared to control period. -No change in appetite, loose stools, diarrhea, hard stools, abdominal distention or other abdominal symptoms. |

*Results statistically significant unless noted otherwise

[†] Default weight of 60 kg used in calculating g/kg-bw/d intake; maximum intakes shown for all studies with multiple dose levels.

Table V-11. Summary of Gastrointestinal Effects of GOS in Adults by Total Daily GOS Intake

| Reference ^a | ≤ 5 g/d | 7.5 to <10 g/d | 10 or 10.4 g/d | 14.4, 15 or 15.6 g/d | 20 or 20.8 g/d |
|--|--|---|--|--|-------------------------|
| Gopal et al. 2003 | No G I. effects | - | - | - | - |
| Bouhnik et al. 2004 | Mild G I symptoms | Mild G.I. symptoms | Mild G.I. symptoms | - | - |
| Ito et al. 1990 ^b | Mild increase in flatulence/abdominal pain | Mild increase in flatulence/abdominal pain | Mild increase in flatulence/abdominal pain | - | - |
| Alander et al. 2001; Malinen et al. 2002 | - | Mild pain from intestinal bloating (3% of subjects) | - | - | - |
| Bouhnik et al. 2004 | - | No G.I. effects | - | - | - |
| Teuri and Korpela 1998 | - | No G I. effects | - | - | - |
| Alles et al. 1999 | - | Some flatulence | - | Some flatulence | - |
| Bouhnik et al. 1997 | - | - | No G I effects | - | - |
| van den Heuvel et al. 1998; van Dokkum et al. 1995, 1999 | - | - | - | Increased flatulence (33% of subjects) | - |
| Teuri et al. 1998 | - | - | - | Increased flatulence and total G.I. symptoms | - |
| Alles and Schoterman 1999 | - | - | Increased G I symptoms | Increased G I symptoms | Increased G.I. symptoms |
| van den Heuvel et al. 2000 | - | - | No G.I. effects | No G.I effects | No G.I effects |

^a Includes only studies in which effects of GOS intake on gastrointestinal tolerance were reported.

^b Results from study non-significant and dose-dependent

b) Studies in Infants

(1) Term Infants

A total of 16 published studies describing administration of GOS to full term infants in infant formula were identified and reviewed. These 16 studies represent eleven distinct clinical trials.

In two of the published trials, infants were fed a formula containing 2.4 g GOS per L for 6 months (Ben et al. 2004) or a follow-on formula containing 5.0 g GOS per L for 18 weeks (Sawatzki et al. 2005). In addition to the published studies, an abstract describing a clinical trial in which infants consumed formula supplemented with approximately 7 g GOS per L was identified and reviewed (Napoli et al. 2003). The effects of milk supplemented with a combination of GOS and *Bifidobacterium lactis* on growth of young children also was assessed (Sarkar et al. 2004). Summaries of all studies in which infants or children consumed formula or milk containing only the oligosaccharide GOS are presented in Table V-12.

In nine of the published studies involving infant formula, the oligosaccharide-supplemented formulas consisted of a combination of 90% GOS and 10% fructo-oligosaccharide (FOS). The GOS and FOS (GOS+FOS) supplemented formulas were administered for time periods ranging from 2 weeks to 6 months, at dosages of 3.6 -7.2 g GOS per L. These studies are summarized in Table V-13. Scholtens et al. (2006) fed infants weaning foods with added GOS and FOS. Fanaro et al. (2005) used a test formula consisting of an 80% GOS+FOS combination with 20% acidic oligosaccharides.

Friesland Foods Domo has indicated that the source of GOS used in all of the infant clinical studies shown in Tables V-12 and V-13 is Friesland Foods Domo.

In the study by Ben et al. (2004), 271 infants 7-days old or younger were enrolled in a 6-month trial. Treatment groups included a standard formula group, a breast-fed group, a GOS-formula group (2.4 g GOS per L) and a mixed breast-fed and GOS-formula group. 52 infants were enrolled in the standard formula group; 69 in the GOS-formula group; 26 in the breast-fed group; and 124 in the mixed feed group. GOS was reported to have no effect on the incidence of crying, regurgitation or vomiting over the 6-month period. Higher levels of fecal bifidobacteria and lactobacilli were found in the GOS group compared to the negative controls at both 3 and 6 months. No significant differences were found among the GOS group and the positive controls. No effect on *E. coli* was

found. The GOS group had softer stool consistency as well as increased stool frequency. Fecal acetate levels were also found to be higher in the GOS-fed infants, and fecal pH was lower.

Sawatzki et al. (2005) studied the effects of consumption of a follow-up formula containing 5 g GOS per L versus a standard formula in a randomized, double-blind, multicenter study. Infants were 4 months and older at study randomization, and 60 infants were randomized to each formula. The minimum amount of formula consumed was 230 mL. Concurrent use of weaning foods was permitted as part of the participants' diet during the 18-week study. There were no differences among study groups in weight or length at completion of the 18-week study. Infants consuming the GOS-supplemented formula had significantly greater increases in fecal bifidobacteria as compared to infants consuming the control formula, though the formulas had no effect on fecal bacteroides, *lactobacillus* or enterobacteriaceae. No differences in urinary osmolarity were seen between the GOS and control groups.

Napoli et al. (2003) presented findings of a clinical study of 26 term infants in an abstract. In this trial, 13 infants consumed a formula containing 0.7% GOS (approximately 7 g GOS per L) and another 13 infants consumed a control formula for a period of 3 weeks. A group of 24 breast-fed infants were followed as a reference group. No adverse events due to the supplementation of infant formula with GOS were reported. Fecal bifidobacteria significantly increased in the group of infants consuming the GOS-supplemented formula, and levels were reportedly in the range of levels in the breast-fed reference group. Fecal pH was lower in the GOS-supplemented group as compared to the control group.

The effects of milk supplemented with a combination of GOS and *Bifidobacterium lactis* on growth was assessed by Sarkar and colleagues (2004). During this year-long study of children ages 1-3 years, no adverse events were reported, and growth parameters were better in children consuming the supplemented milk as compared to the control milk.

Knol and colleagues (2005a) assessed the effects of infant formula containing 7.2 g GOS per L (and 0.8 g FOS per L) on fecal microflora over a period of 6 weeks. Infants were 7-8 weeks of age at study enrollment. Twenty-four infants were randomized to the GOS-supplemented formula group, and 23 infants were randomized to a standard control formula group. A total of 15 and 19 infants in the GOS and control groups completed the study, respectively. There was no difference in dropouts between the two groups. Symptoms of flatulence and possetting were reported to be mild in all study groups during

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the study. The percent of fecal bifidobacteria was higher in the GOS-supplemented group as compared to the control group. Infants in the GOS-group were found to have lower fecal pH, and increased proportion of fecal acetate and a decreased proportion of propionate. Haarman and Knol (2005, 2006) subsequently identified and quantified the species of bifidobacteria and *lactobacillus* present in fecal samples of 10 infants randomly selected from infants consuming the GOS-supplemented formula, the standard formula and a breast-fed reference group. The investigators found *B. infantis*, *B. breve* and *B. longum* to be the predominant bifidobacteria in both the GOS+FOS and breast-fed groups while *L. acidophilus*, *L. paracasei* and *L. casei* were the predominant lactobacilli in the two respective groups. The standard formula group tended to result in an adult-like microbiota, with lower levels of *B. breve* and *L. paracasei* and relatively higher levels of *B. catenulatum*, *B. adolescentis* and *L. debueckii*. At the end of the 6-week study, the investigators also noted that bifidobacteria and lactobacilli accounted for 80% of fecal microflora in the breast-fed and GOS+FOS groups while lactobacilli and bifidobacteria accounted for 50% of fecal microflora in infants fed the standard formula

Moro et al. (2002) studied the effects of consumption of a formula supplemented with GOS and FOS on infant microflora. Ninety term infants between 6.3 to 7.2 days old were randomly assigned to one of three formula groups: standard formula (n=33), formula supplemented with 3.6 g GOS per L (and 0.4 g FOS per L) (n=30), or formula with 7.2 g GOS per L (and 0.8 g FOS per L) (n=33). Infants consumed the assigned formula for 4 weeks. A dose-dependent increase in fecal bifidobacteria was observed in the GOS groups, with baseline levels of 8.5 CFU/g and 7.7 CFU /g, respectively, and levels of 9.3 CFU /g and 9.7 CFU /g, respectively by the end of the study period. Fecal lactobacilli increased in the GOS groups as compared to the control group, but there was not a difference between the two GOS groups. A dose-dependent decrease in stool consistency was found, while stool frequency increased and fecal pH decreased only in infants consuming formula supplemented with 7.2 g GOS per L. Weight gain and length increment were similar among groups, and the diets did not influence the incidence of crying, regurgitation or vomiting, and no infant had diarrhea during the study period. Fecal samples from infants in the high-dose group (7.2 g GOS per L) and the control groups were examined for the presence of GOS and FOS (Moro et al. 2005). The oligosaccharides were detected in stool samples from all infants consuming the GOS-supplemented formula and none of the infants in the control group. The amount of GOS detected in the feces was not quantified. The investigators noted that human milk oligosaccharides also can be detected in feces of infants consuming human milk.

Schmelzle and colleagues (2003) studied the effects of a formula containing partially hydrolyzed protein, a high level of beta-palmitic acid and nondigestible oligosaccharides (GOS+FOS) versus a standard formula in a population of healthy, term infants. Infants consumed the assigned formula from or before age 2 weeks until the age of 12 weeks. There were no differences between groups in the number of dropouts or reasons for dropping out. Infants consuming the new formula with added GOS were found to have increased fecal bifidobacteria compared to baseline levels, a higher percentage of fecal bifidobacteria as compared to infants in the control group, and a higher proportion of softer stools. Formula intake and energy intake per kg bodyweight were lower in infants consuming the supplemented formula versus the control formula, though there were no differences between groups in weight gain.

In another study involving a formula containing hydrolyzed protein, β -palmitate and GOS+FOS, 14 infants consumed the test formula from birth to 2 months of age (Rigo et al. 2001). Infants consuming breast milk or standard formulas in previous studies were used as reference controls. Growth and anthropomorphic development of the infants followed a standard healthy infant growth curved throughout the study. Infants consuming the new formula had a higher percentage of endogenous bifidobacteria compared to baseline levels when measured at 25, 45 and 68 days of age. One dropout was reported in the study due to gastro-oesophageal reflux.

Bakker-Zierikzee et al. (2005) studied the effects of a GOS+FOS-supplemented infant formula versus a probiotic formula containing *Bifidobacterium animalis* on the microflora of healthy infants over the first 16 weeks of life. At three days post-partum, 19 infants were enrolled in each of the three formula groups (5.4 g GOS per L, 6×10^{10} CFU *B. animalis* per L, or control). Sixty-three breast-fed infants were followed as a reference group. Infants consuming the GOS+FOS supplemented formula were found to have a lower fecal pH, higher percentages of fecal acetate and lower percentages of propionate, butyrate and isobutyrate, isovalerate and valerate compared with infants consuming the control formula. No differences among groups were found in the percentage of fecal bifidobacteria. During the intervention, fecal SIgA levels were higher in infants consuming the GOS+FOS supplemented formula as compared to the control formula (Bakker-Zierikzee et al. 2006). All formulas were reportedly well accepted and tolerated.

Savino et al. (2003, 2006) conducted two studies on older infants experiencing minor gastrointestinal problems including colic. The first study was an observational study in

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which 604 infants with an average age of 1.35 months consumed a partially hydrolyzed formula containing GOS+FOS (7.2 g GOS per L), palmitic acid in the β position and low levels of lactose for a period of 2 weeks (Savino et al. 2003). At the end of the feeding period, infants experienced fewer episodes of colic, fewer regurgitation problems, and less constipation. Reductions in the incidence of crying in infants with colic also were observed in a randomized, controlled trial of infants consuming either a formula with partially hydrolyzed whey proteins, GOS+FOS (7.2 g GOS per L) and a high beta-palmitic acid level or a standard formula and simethicone (Savino et al. 2006).

Rinne et al. (2005) conducted a trial that evaluated microflora of infants at 6 months of age with a family history of atopic eczema, allergic rhinitis or asthma. Inclusion criteria required infants to have been on their respective formulas since at least 2 months of age. Of the 32 infants in the study, 8 consumed the GOS+FOS formula and the remaining infants consumed a standard formula, breast-milk or a probiotic-supplemented formula. Infants consuming the GOS+FOS-supplemented formula had increased fecal bifidobacteria after 4 months, and a lower occurrence of atopic eczema compared to the standard formula group at 10 months. Weight and length of infants were comparable among all formula groups.

Moro and colleagues (2006) also tested GOS+FOS formula on infants at high risk of developing atopy. Inclusion criteria required infants to have started formula feeding within the first two weeks of life. Study visits were scheduled for each infant at 3 and 6 months of age. Of the 206 infants completing the study, 102 were in the GOS+FOS group with the remaining in a maltodextrin placebo group. In a subset of 98 of the infants, 50 coming from the GOS+FOS group, fecal samples were analyzed. Infants consuming the GOS+FOS formula were found to have increased fecal bifidobacteria levels at 3 and 6 months of age as well as an increase in stool frequency and softer stool consistency. Infants in the treatment group also had a significantly lower incidence of dermatitis. Reasons for dropouts were similar between groups, with reports of regurgitation and crying significantly lower in the GOS+FOS group.

One infant study was identified that involved the administration of GOS+FOS as a solid weaning food (Scholtens et al. 2006). This randomized, double-blind, placebo-controlled trial involved 35 fully formula fed infants with an average age of 16 weeks. Two treatments were administered: standard baby food with maltodextrin as the placebo and baby food with a GOS+FOS formulation. Nineteen infants were in the GOS-supplemented group, 11 of whom were included in the per-protocol analysis (which specified compliance of 60% consumption during the final two weeks of the study).

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Over the course of the 6-week study period, infants consumed a total of 4.05 g GOS per day in three equivalent doses. Infants consuming the GOS-supplemented food had a significant increase in fecal bifidobacteria (43% at week 0 vs. 57% at week 6) above baseline levels, indicating the ability of supplemental GOS+FOS to alter fecal microflora in the weaning period.

In a 6-week study involving 46 infants, the effects of consumption of milk containing 5.4 g per L GOS in combination with FOS and AOS (acidic oligosaccharides) were compared to milk containing exclusively AOS and a control formula (Fanaro et al. 2005). There was a significant increase in fecal bifidobacteria in the GOS+FOS-supplemented formula group (n = 15) as compared to both the AOS and control groups. Softer stools were observed compared to the control group. The investigators reported no difference in growth, crying, vomiting or regurgitation patterns among the groups.

In summary, results from studies of healthy, term infants consuming formula supplemented with up to 7.2 g GOS per L indicate that GOS is well tolerated by infants and produces no adverse effects. The GOS-supplemented formulas support normal growth. Additionally, results from these studies suggest that formulas with added GOS or GOS in combination with FOS may influence shifts in infant gut microflora that result in gut microflora more similar to the gut microflora of breast-fed infants.

(2) Preterm Infants

Two clinical trials involving the administration of infant formulas containing added GOS (90% GOS and 10% FOS) to preterm infants were identified in the published literature. The studies are summarized in Table V-14.

In the trial conducted by Boehm and colleagues (2002), preterm infants who could tolerate a formula volume of 80 mL/kg-bw/d (approximately 8 days postpartum) were randomly assigned to consume a standard preterm formula or a formula supplemented with 10 GOS+FOS per L formula (9 g GOS per L). Fifteen infants consumed each formula for a period of 28 days. A group of 12 breast-fed infants was followed as a reference group. At the end of 28 days, fecal bifidobacteria was increased in the GOS+FOS group as compared to the control group (10.0 ± 2.05 vs. $7.9 \pm .83$ log 10 CFU/g wet stools) and levels in infants consuming the supplemented formula were reported to be in the upper range of the reference group of infants, which was 10.7 log 10 CFU/g wet stools (Boehm et al. 2003). No other differences in fecal bacteria were observed. The consistency of stools from infants consuming the supplemented formula

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was softer than the consistency of infants consuming the control formula, and comparable to the stool consistency of breast-fed infants. At study completion, the hardest stools were observed in the control group, which had a significantly higher score of 3.55 ± 0.8 compared to the supplemented and reference groups which were 2.74 ± 0.7 and 2.33 ± 0.6 , respectively (Boehm et al. 2003). Infants consuming the supplemented formula also had a higher stool frequency as compared to infants in the control group, but comparable to the frequency in breast-fed infants. Weight and length gain and incidence of crying, regurgitation, and vomiting were reported to be similar among the formula groups. The GOS+FOS-supplemented formula had no effect on plasma concentrations of calcium and phosphorus, plasma activity of alkaline phosphatase, or urinary phosphate concentrations, though urinary calcium concentrations in infants fed the supplemented formula tended to be higher than those in the group fed the standard formula (1.63 ± 1.15 mmol/L vs 0.93 ± 0.72 mmol/L; $p=0.055$) (Lidestri et al. 2003). In a subset of the infants, the investigators examined the sum of selected pathogens in fecal samples (Knol et al. 2005b) and found that the sum of pathogens and pathogens as a percentage of total bacteria were lower in fecal samples from infants fed the supplemented formula as compared to the control formula.

In another clinical trial, 20 preterm infants were randomly assigned to consume a formula containing 10 g GOS+FOS per L (9 g GOS per L) or a standard formula (Mihatsch et al. 2006). The study formulas were prepared once a day by combining a standard formula with sachets containing the oligosaccharide mixture or a placebo (maltodextrin). Infants consumed the assigned formulas for a period of two weeks. Infants consuming the GOS+FOS-supplemented formula had decreased stool viscosity, change in fecal transit time, and fecal pH. During the trial the groups did not differ in clinical characteristics, formula intake or weight gain, and adverse events such as weight loss, constipation, diarrhea, abdominal discomfort or flatulence were not reported.

Results from these two clinical trials suggest that the addition of an oligosaccharide mixture consisting primarily of GOS at a level of 10 g GOS+FOS per L (9 g GOS per L) is well tolerated by preterm infants and is not associated with adverse effects over the two and four week periods of the studies.

c) Summary of Human Studies

Consumption of GOS by adults at dosages of 2.4 to 20.8 g/day for 6 days to 4 weeks has been demonstrated to be safe and overall well tolerated. Flatulence or gastrointestinal discomfort was reported by some study participants, though the effects were generally

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mild in nature. Studies of GOS consumption by term infants as young as a few days post partum have also shown GOS-supplemented infant formulas to be well tolerated at intake levels of 2.4 to 7.2 g GOS per L formula for time periods between 2 weeks to 6 months. Studies of preterm infants consuming GOS-supplemented formulas have also revealed no safety concerns with intakes of 9 g GOS per L formula for 14 or 28 days. No adverse effects on the incidence of crying, regurgitation or vomiting, or adverse effects on weight gain or loss were observed during the periods of GOS consumption.

Table V-12. Summary of Term Infant Studies with Only GOS

| Reference | Study Design & Objective | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion* |
|---|---|--|---|-------------------------|--------------------|---|
| Ben et al. 2004 | Study effects of infant formula with GOS on intestinal microflora populations and fermentation characteristics in term infants | 271 term infants: 52 standard formula, 69 formula+GOS, 26 HM, 124 mixed feed (GOS and HM); ≤ 7d postpartum | 4 treatments: standard formula, HM, Formula supplemented with 2.4 g/L GOS, HM (+) GOS group | 2.4 g/L | 6 mo | Growth: -Weight gain and length increments similar among groups Tolerance: -Increase in stool frequency and softer stool consistency -No effect on incidence of crying, regurgitation, vomiting. Dropouts: -None reported. Other Endpoints: -Increased fecal bifidobacteria and <i>Lactobacilli</i> ; no effect on <i>E. coli</i> -Decrease in fecal pH, increase in fecal SCFA (acetic) |
| Napoli et al. 2003 Only Abstract Available | Study the bifidogenic effects on GOS in formula-fed infants | 26 M/F term infants, 13 per group; 24 breast-fed infants followed as reference group | 2 treatments: GOS formula (0.7%), Lactose formula (control) | Approx 7 g/L (0.7% GOS) | 3 wk | Growth: -Data not measured/reported in abstract. Tolerance: -Data not reported in abstract. Dropouts: -None reported in abstract. Other Endpoints: -Increase in fecal bifidobacteria, into range of breast-fed reference group -Decreased fecal pH. -Non-significant increase in fecal lactate levels. |
| Sarkar et al. 2004 | To study the effect of fortification of milk with <i>Bifidobacterium lactis</i> and GOS on anemia, growth and development in children living in Delhi | 624 children, 1-3y | 2 treatments: control milk, GOS + <i>B. lactis</i> milk | 2.4 g/d | 1 y | Growth: -Higher weight for height and weight for age at 6 mo; higher weight velocity and z-scores for weight for age and weight for length at 1 y. -No effects on developmental scores. Tolerance: |

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Table V-12. Summary of Term Infant Studies with Only GOS

| Reference | Study Design & Objective | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS Ingestion* |
|----------------------|--|--|---|---|--------------------|--|
| | Double blind, randomized trial | | | Source: New Zealand Milk Limited | | -Not reported. Dropouts: -None reported. Other Endpoints: -Reduced incidence of anemia |
| Sawatzki et al. 2005 | Study the effects of GOS on infant microflora and development Double-blind, randomized, multicenter | 120 M/F term 15 infants per group, two groups in each of the 4 centers. ≥ 4 mos old. | 2 treatments: GOS follow-up formula, Standard follow-up formula Concurrent use of weaning foods was permitted. | 5 g/L (minimum of 230 mL/day of formula) | 18 wk | Growth: -At baseline infants in GOS group weighed less and were shorter; during the feeding period there were no differences between groups in height and weight. Tolerance: -Stool frequency and consistency were recorded daily; episodes of diaper rash, diarrhea and fever were also recorded. However, these data are not presented in the results of the study. Dropouts: -None reported. Other Endpoints: -Greater increase in fecal bifidobacteria; no effects on fecal bacteroides, lactobacilli, enterobacteriaceae -No effects on urinary osmolarity. |

* Results statistically significant unless noted otherwise.

Table V-13. Summary of Term Infant Studies with 90% GOS+10% FOS

| Reference | Study Design & Objective | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS+FOS Ingestion* |
|------------------------------------|---|--|---|----------|--------------------|---|
| Bakker-Zierikzee et al. 2005, 2006 | Study the effects of standard, prebiotic, and probiotic infant formulas on fecal microflora over 16 wk, and effects on fecal SIgA levels over 32 wk Randomized, double-blinded | 120 M/F term infants, 19 per group, except HM group (63), 3 d postpartum | 4 treatments: standard formula, HM, formula with <i>Bifidobacterium animalis</i> , formula with GOS+FOS | 5.4 g/L | 16 wk | Growth: -Data not measured/reported. Tolerance: -No effect on fecal consistency or frequency. Dropouts: -5 infants dropped out; not different from control group. Reasons include colic, suspicion of cow's milk allergy, constipation, and practical problems. Other Endpoints: -No change in % fecal bifidobacteria. -Decreased fecal pH. -No change in total fecal SCFA; increased % fecal acetate, decreased % fecal propionate, butyrate, isobutyrate, isovalerate, valerate. -No change in fecal lactate. -Increase in median fecal SIgA concentration at week 16. |
| Fanaro, et al. 2005 | Study tolerance and effects of formula with AO or GOS+FOS/AO on intestinal flora and stool characteristics in term infants. | 46 term infants: 15 standard formula, 15 formula+GOS+FOS/AO, 16 formula + AO | 3 treatments. Standard formula, formula with GOS+FOS + AO, formula with AO | 5.4 g/L | 6 wk | Growth: - No difference in growth patterns. Tolerance: -Softer stool consistency compared to control group -No difference in crying, vomiting or regurgitation patterns. Dropouts: -None reported. Other Endpoints: -Increased fecal bifidobacteria compared to control and AO groups. -Decreased pH compared to control and AO groups. |

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Table V-13. Summary of Term Infant Studies with 90% GOS+10% FOS

| Reference | Study Design & Objective | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS+FOS Ingestion* |
|--|--|--|--|----------------|--------------------|--|
| Knol et al. 2005a; Haarman and Knol 2005, 2006 | Determine effects of a formula containing GOS+FOS on microflora in term infants exclusively formula-fed since birth, and to identify and quantify fecal <i>bifidobacterium</i> and <i>Lactobacillus</i> species using real-time PCR assays. Randomized, double blind, placebo controlled intervention study | 53 M/F term infants, 15-19 per group, mean age 7.7 wk at start of study. 10 infants selected from each group for PCR fecal analysis. | 3 treatments: control formula, HM (studied in parallel), 8 g/L GOS+FOS | 7.2 g/L | 6 wk | Growth: -Data not measured/reported Tolerance: -Increase in median stool frequency -Mild flatulence in all groups Dropouts: -Eight dropouts; 3 from test group, 3 from control group and 2 from reference group Reasons included rotavirus-infection (n=1), medical problems of regurgitation (n=1) and formula-related complaints (n=4; 2 from test and 2 from control). Unknown reasons for drop outs in reference group Other Endpoints: -Increased % fecal bifidobacteria. - <i>B. infantis</i> , <i>B. breve</i> and <i>B. longum</i> remained predominant species in GOS and HM groups. -Decreased % <i>B. adolescentis</i> from baseline, decreased % <i>B. catenulatum</i> from control. -Increased % fecal lactobacilli -Increased <i>L. acidophilus</i> , <i>L. paracasei</i> and <i>L. casei</i> and decreased <i>L. delbrueckii</i> -At study completion fecal bifidobacteria and lactobacilli accounted for 80% of fecal bacteria. -Fecal bacteria more closely resembled HM-fed infants -Increased fecal acetate and decreased butyrate and propionate -Decreased fecal pH. -Increased fecal lactate and % D-lactate |
| Moro et al. 2002; 2005 | Evaluate effects of a formula supplemented | 90 M/F term infants, 27-33 per group, 6.3- | 3 treatments: standard formula, | 3.6 or 7.2 g/L | 4 wk | Growth: -No effect on growth. |

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Table V-13. Summary of Term Infant Studies with 90% GOS+10% FOS

| Reference | Study Design & Objective | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS+FOS Ingestion* |
|------------------|---|---|---|--------------|--------------------|---|
| | with GOS+FOS on bifidobacteria in term infants, determine if GOS and FOS are present in feces from a subset of infants fed the high-dose of GOS+FOS Randomized, placebo-controlled | 7.2 d postpartum; 16 infants selected from high-dose group for fecal analysis of GOS+FOS | formula with 4 g GOS+FOS/L, formula with 8 g GOS+FOS/L | | | Tolerance: -Dose-dependent decrease in stool hardness, and increase in stool frequency in high-dose group. -No effect on incidence of crying, regurgitation, or vomiting. -No diarrhea occurred during study. Dropouts: -None reported Other Endpoints: -Dose-dependent increase in fecal bifidobacteria, and increase in fecal <i>lactobacilli</i> (not dose-dependent). -Dose-dependent decrease in fecal pH. -GOS and FOS detected in feces. |
| Moro et al. 2006 | To investigate the effect of GOS+FOS formula on the incidence of atopic dermatitis in high risk infants Prospective, double-blind, randomized, placebo controlled. | 259 infants at risk for atopy enrolled, 206 completed study; 102 treatment group; 104 placebo (control) | 2 treatments formula with GOS +FOS, maltodextrine (control) | 7.2 g/L | <6 mo | Growth: -Data not measured/reported. Tolerance: -Increase in stool frequency and softer stool consistency in treatment group. -Lower reports of regurgitation and crying in treatment group; incidence of vomiting same between groups. Dropouts: -Reasons for dropout non-significant between groups Other Endpoints: -Increase in bifidobacteria count in treatment group -Decrease in incidence of dermatitis in treatment group compared to control group. -Severity of dermatitis unaffected by diet |
| Rinne et al. | Assess the quantitative | 32 term infants, 8 per | 4 treatments | Not reported | 4+ mo | Growth: |

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Table V-13. Summary of Term Infant Studies with 90% GOS+10% FOS

| Reference | Study Design & Objective | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS+FOS Ingestion* |
|-------------------|--|--|--|----------|--------------------|---|
| 2005 | and qualitative differences of microflora in infants following GOS+FOS intake of formula. | group, >8 wk Infants had at least one close relative afflicted with atopic eczema, allergic rhinitis or asthma. | standard formula, formula with GOS+FOS, HM, HM+ <i>Bacillus lactis</i> | | | -No differences in length or weight gain Tolerance: -Clinical characteristics comparable among groups at 6 and 12 mo of age Dropouts: -None reported. Other Endpoints: -Increase in fecal bifidobacteria after 4 mo; no effects on fecal <i>Clostridia</i> , <i>Lactobacilli/enterococci</i> or <i>Bacteroides</i> . -Lower occurrence of atopic eczema in breastfed and pre/pro-biotic groups at 10 months |
| Rigo et al. 2002 | Study the effect of a hydrolyzed protein, β -palmitic acid formula with GOS+FOS on healthy term infant growth, mineral accretion and intestinal flora development. | 14 term infants; infants entered study at birth. Breast-fed and Standard Formula infants used as reference controls | 1 treatment. New formula with GOS+FOS | 3.6 g/L | 2 mo | Growth: -Growth parameters for study infants followed standard infant growth curve Tolerance: -Investigators reported feeding tolerance as being excellent. -Adequate volume formula intake observed throughout study, although milk intakes were lower than those previously observed in infants consuming a standard formula. Dropouts: -One drop-out due to gastro-oesophageal reflux. Other Endpoints: -Increase in the percentage of endogenous bifidobacteria observed through 68 days of age (when final measurement was taken). |
| Savino et al 2003 | Study effect of a partially hydrolyzed formula with GOS+FOS, palmitic acid & low levels of lactose on | 932 infants enrolled, 604 completed study ≤ 3 mo, mean age of | 1 treatment: Study formula | 7.2 g/L | 2 wk | Growth: -Data not reported/measured. Tolerance: -Fewer regurgitation problems |

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Table V-13. Summary of Term Infant Studies with 90% GOS+10% FOS

| Reference | Study Design & Objective | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS+FOS Ingestion* |
|-----------------------|---|---|--|----------|--------------------|---|
| | formula-fed infants with G I. problems such as colic, regurgitation or constipation. Observational study. | 1 35 mo at start | | | | -Increase in daily number of stools -Fewer daily colic episodes Dropouts: -None reported. Other Endpoints: -91% positive subjective rating from parents. -95% positive subjective rating from pediatricians |
| Savino et al. 2006 | Study effects of a formula containing GOS+FOS in infants with colic Prospective randomized controlled study | 199 M/F term infants, 96 GOS+FOS, 103 control, <16 weeks | 2 treatments: Standard formula, GOS+FOS and 41% palmitic acid | 7.2 g/L | 2 wk | Growth: -Data not measured/reported. Tolerance: -Reduction in crying episodes after 7 and 14 days Dropouts: -None reported; exclusion from analysis occurred with infants from both groups due to not meeting inclusion criteria and loss to follow-up Other Endpoints: -Data not measured/reported. |
| Schmelzle et al. 2003 | Study the nutritional efficacy and bifidogenic characteristics of a formula containing partially hydrolyzed whey protein, high beta-palmitic acid content, starch and GOS+FOS vs a standard formula. Randomized, double-blind study. | 102 M/F term infants; 53 in SF group, 49 in NF group, <2 wk post-partum | Two treatments: Standard formula, GOS+FOS formula | 7.2 g/L | 10 wk | Growth: -At 6 wk, girls had larger weight gains and at 12 wk girls had larger head circumference gains -Boys had larger total skinfold thickness gains -Weight and length gains were similar at 12 wk and groups were in-line with published growth curves Tolerance: -Softer stools. -Formulas were well tolerated. Dropouts: -None reported. |

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Table V-13. Summary of Term Infant Studies with 90% GOS+10% FOS

| Reference | Study Design & Objective | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS+FOS Ingestion* |
|----------------------|--|---|--|--|--------------------|--|
| | | | | | | Other Endpoints: -Decreased energy intake from formula. -Increased fecal bifidobacteria compared to baseline, and higher % bifidobacteria vs control group at 6 wk. -No effects on serum total protein, albumin or urea; no clinically significant differences in prealbumin and amino acid values. |
| Scholtens et al 2006 | Study effects of solid foods with GOS+FOS on microflora in term infants Randomized, double blind, placebo-controlled intervention trial | <u>Intent-to-treat:</u> 35 M/F term 19 GOS+FOS, 16 control; 16wk <u>Per protocol analysis</u> 20 infants, 11 GOS+FOS, 9 control; 16 wk (data presented in paper) | 2 treatments: standard baby food (weaning food with maltodextrin as placebo), baby food with GOS+FOS | 4.05 g/d divided into 3 equal servings | 6 wk | Growth: -Data not measured/reported. Tolerance: -Non-significant decrease in stool consistency and increase in frequency compared to control for baseline measurements of 12 infants from per-protocol group. Dropouts: -11 infants withdrawn from final evaluation due to non-compliance. -1 infant withdrawn due to age exceeding study protocol. Other Endpoints: -Increase in % fecal bifidobacteria; no effect on fecal bifidobacteria count (no effect in intent-to-treat analysis). -No effect on change in fecal pH, total SCFA, or % acetate, propionate, or sum of valerate, isovalerate and isobutyrate; decrease in % butyrate. |

* Results statistically significant unless noted otherwise.

Table V-14. Summary of Preterm Infant Clinical Studies with 90% GOS+10% FOS

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS+FOS ingestion* |
|--|---|--|--|----------|--------------------|--|
| Boehm et al. 2002 and 2003; Lidestri et al 2003; Knol et al. 2005b | Investigate effects of preterm formula containing GOS+FOS on fecal flora and stool characteristics of infants born at ≤ 32 wk gestational age. | 30 preterm infants; 15 OS group, 15 control, 12 HM (reference group) study entry age: 7.9-8 3 d | 2 treatments: control; preterm formula supplemented with GOS+FOS | 9 g/L | 28 d | Growth: -No effect on weight or length gains. Tolerance: -Softer stool consistency and increased stool frequency -No effect on incidence of crying, regurgitation, vomiting Dropouts: -None reported. Other Endpoints: -Increase in fecal bifidobacteria; no effects on lactobacilli, <i>E. Coli</i> , <i>Clostridium</i> spp. <i>Bacteroides</i> , <i>Enterobacter</i> , <i>Citrobacter</i> , <i>Proteus</i> , <i>Klebsiella</i> and <i>Candida</i> -No effects on plasma concentrations of calcium and phosphorus or plasma activity of alkaline phosphatase; trend toward higher calcium concentrations in urine ($p=0.055$). -Decrease in fecal counts and % of total selected pathogens (sum of <i>Staphylococcus aureus</i> , <i>S. epidermidis</i> , <i>S. haemolyticus</i> , <i>Pseudomonas aeruginosa</i> , <i>Enterobacter</i> , <i>Klebsiella</i> , <i>Proteus</i> , <i>Streptococcus group B</i> , <i>Clostridium difficile</i> , <i>Bacillus subtilis</i> , <i>Acinetobacter</i>). |

Table V-14. Summary of Preterm Infant Clinical Studies with 90% GOS+10% FOS

| Reference | Study Objective & Design | Population | Test Substance | GOS Dose | Treatment Duration | Results of GOS+FOS ingestion* |
|----------------------|--|--|--|----------|--------------------|--|
| Mihatsch et al. 2006 | Evaluate feeding tolerance in preterm infants given formula containing GOS+FOS Double-blind, placebo-controlled trial | 20 preterm infants, 10 per group, 27 (24-31) weeks gestation, 42 (11-84) days postpartum | 2 treatments: standard preterm formula, preterm formula with GOS+FOS | 9 g/L | 14 d | Growth: -No effects on daily formula intake or daily weight gains. Tolerance: -Decrease in stool viscosity, decreased change in gastrointestinal transit time (decrease of 6 d vs increase of 9 d in control group)/ Dropouts: -No adverse effects observed. Other Endpoints: -Lower fecal pH |

*Results statistically significant unless noted otherwise

| Table V-15. Abbreviations used in GOS Tables | |
|---|---|
| Abbreviation | Meaning |
| AO | Acidic Oligosaccharides |
| CV | Conventional (animal model) |
| DM | Dry Matter |
| DTH | Delayed-Type hypersensitivity |
| FOS | Fructooligosaccharides |
| GF | Germ Free (animal model) |
| GOS | Galactooligosaccharides |
| HE | Heteroxenic (animal model) |
| HM | Human milk (Breast-fed) |
| OM | Organic Matter |
| OS | Oligosaccharides |
| OVX | Ovariectomized |
| SCFA | Short-Chain Fatty Acids |
| SIgA | Secretory Immunoglobulin A |
| SSU | Small Subunit |
| TOS | Transgalacto-oligosaccharide (i.e. GOS) |
| VFA | Volatile Fatty Acid |

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Memorandum of Telephone Conversation

Date: November 2, 2007

Between: Paulette Gaynor, Ph.D. HFS 255
Moraima Ramos Valle HFS 255

And

Gavin Thompson, Ph.D. ENVIRON (Tel: 703-516-2300)
Amy Guimaraes ENVIRON
Mary Murphy ENVIRON

Subject: Submission on behalf of Friesland Foods Domo's for galacto-oligosaccharides intended for use in certain foods and infant formula

Dr. Gaynor and I spoke to Dr. Thompson and his colleagues as a follow up to a previous voice mail message that we left for Dr. Thompson on October 26, 2007. We explained that we could not locate all the items in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1) and that, thus, the GRAS exemption claim as it currently exists is incomplete. We discussed the two items we could not locate with Dr. Thompson and his colleagues. The first item is a statement that the notifier is taking responsibility for the GRAS determination. The second item is a statement that the notifier agreed to the two procedures for making records available to FDA at FDA's request (i.e., at a specific site or by sending the requested records to FDA). We then explained that we would not consider the submission complete until we were in receipt of a complete GRAS exemption claim. Dr. Thompson agreed to submit three copies of a completed GRAS exemption claim.

FDA received the completed GRAS exemption claim on November 6, 2007, and considers this date to be the receipt date for the submission.

(b) (6)



Moraima J. Ramos Valle

(b) (5)



000150

November 5, 2007

Paulette Gaynor
GRAS Notification Program
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

11-06-07P03:57 RCVD

Re: GRAS Notification for Galacto-oligosaccharides (GOS)

Dear Dr. Gaynor:

On behalf of Friesland Foods Domo, ENVIRON International Corporation is pleased to submit this Notification of the Generally Recognized as Safe (GRAS) Determination for the use of galacto-oligosaccharides (GOS) in foods and infant formulas for term infants.

We are submitting (in triplicate) the GRAS Exemption Claim (dated November 5th, 2007) (attached). In response to consultation with you, we request that the attached statement supersede the statement (dated October 12th, 2007) that we previously submitted to you on behalf of Friesland Foods Domo.

The Statement of Consensus of an Expert Panel (dated March 30th, 2007) and the GRAS Determination (dated September 6th, 2007) that we previously submitted to you in triplicate are unchanged and, therefore, are not resubmitted.

If you have any questions, please contact us at +1 703 516 2300 or +1 703 589 8023 or gthompson@environcorp.com

Sincerely,

(b) (6)

Gavin Thompson
Principal Consultant

24-15175C

000151

GRAS Exemption Claim for Galacto-Oligosaccharides (GOS)

This is to notify you that Friesland Foods Domo claims that the use of galacto-oligosaccharides (GOS) prepared with a β -galactosidase derived from *Bacillus circulans* is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act because Friesland Foods Domo has determined such use to be Generally Recognized As Safe (GRAS). This determination and notification are in compliance with proposed Sec. 170.36 of Part 21 of the Code of Federal Regulations (21 CFR § 170.36) as published in the Federal Register, Vol. 62, No. 74, FR 18937, April 17, 1997.

A. NAME AND ADDRESS OF NOTIFIER

Friesland Foods Domo
P.O. Box 449
8000 AK Zwolle
The Netherlands

Contact:

Rob van Vliet
Manager, Quality Assurance
P.O. Box 449
8000 AK Zwolle
The Netherlands

B. COMMON OR USUAL NAME OF GRAS SUBSTANCE

The substance that is the subject of this GRAS determination is galacto-oligosaccharides (GOS) prepared with a β -galactosidase derived from *Bacillus circulans*. Other chemical names for this GOS are galactooligosaccharide, transgalactosylated oligosaccharide, transgalacto-oligosaccharide and oligogalactosyl-lactose. The common name for a product currently manufactured by Friesland Foods Domo that contains the GOS that is the subject of this GRAS determination is Vivinal® GOS (formerly known as Elix'or and "OLIGO").

C. INTENDED USE

GOS prepared with a β -galactosidase derived from *Bacillus circulans* is intended to be added to a variety of foods and also infant formulas for the routine feeding of term infants. The food categories to which GOS will be added and the maximum concentrations of GOS in foods and infant formulas are detailed in Table I-1 below.

| Table 1. Intended Uses of GOS | | | |
|---|--|---|--|
| Food Group | Food Group Category^a | Approximate serving size^b (g) | Maximum g GOS per serving^c |
| Bars | Bars | 40 | 5.0 |
| Dairy products | Yogurt | 227 | 7.5 |
| | frozen dairy desserts | 70 (1/2 cup) | 3.0 |
| Fruit drinks and waters/quenchers | fruit drinks (vitamin/mineral fortified) and energy drinks | 240 (240 mL) | 5.0 |
| | fitness water and thirst quenchers | 240 (240 mL) | 3.0 |
| Fruit preparations | fruit pie filling | 85 | 5.0 |
| | fruit prep | 40 (2 Tbsp) | 5.0 |
| | jelly/jam | 20 (1 Tbsp) | 5.0 |
| Infant formulas for term infants and baby foods | infant formula | 8 g per L | NA ^d |
| | infant meal replacement drinks | 250 | 3.0 |
| | baby juice | 120 (120 mL) | 3.0 |
| | baby yogurt drink | 125 (120 mL) | 3.0 |
| | baby dessert | 110 | 3.0 |
| | baby snack | 7 | 1.0 |
| Milk beverages | Milk | 244 (240 mL) | 5.0 |
| | milk drinks | 250 (240 mL) | 7.5 |
| | syrup flavoring for milk | 40 (2 Tbsp) | 5.0 |
| | meal replacement drinks | 250 (240 mL) | 5.0 |
| | milk substitutes | 245 (240 mL) | 5.0 |

^a In some food group categories, not all types of foods are intended for addition of GOS (e.g., addition of GOS is limited to nonfat and low fat milk only, not whole and reduced fat milk). The specific types of products intended for addition of GOS are detailed in Table III-3.

^b Serving sizes based on Reference Amounts Customarily Consumed (RACC) (21 CFR §101.12). Actual product serving sizes may differ slightly from these values.

^c GOS concentrations in foods or beverage as consumed.

^d Not applicable.

D. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of GOS (prepared with a β -galactosidase derived from *Bacillus circulans*) as an ingredient in foods and term infant formulas at the maximum levels described in Section C of this chapter is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of GOS from the intended uses specified above, as estimated by ENVIRON International Corporation (ENVIRON), 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 GOS is safe, and GRAS, under the intended conditions of use, the safety of the intake of GOS 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 GOS as an ingredient in foods and infant formulas has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

- 1) Results from *in vitro* and *in vivo* studies provide evidence that GOS is largely resistant to gastric acidity, hydrolysis by mammalian enzymes, and absorption in the gastrointestinal tract. The available evidence indicates that GOS is fermented in the lower gastrointestinal tract.
- 2) Human milk contains a complex mixture of oligosaccharides, and oligosaccharides account for the third largest solid constituent in human milk after lactose and fat. The total amount of complex oligosaccharides in mature human milk is typically estimated to range from 5 to 8 g/L, though higher levels have also been reported.
- 3) GOS are chains of galactose units, usually with a single terminal glucose molecule ((galactose(Gal))_n-glucose (Glu)). The type of β -glycosidic linkage between the monomer units is mainly 1→4 Gal, though other β -glycosidic linkages including 1→6 Gal, 1→2 Glc, 1→3 Glc, 1→4 Glc, 1→6 Glc, 1→2 Gal and 1→3 Gal may also be present.

- 4) GOS prepared with a β -galactosidase derived from *Bacillus circulans* is a well characterized oligosaccharide. It is a mixture of d-octasaccharides composed of 1-7 galactose units linked to a glucose molecule. The product reproducibly meets compositional standards and complies with limits on contaminants appropriate for food-grade ingredients. Product specifications are set to assure that GOS is suitable for use in food.
- 5) The β -galactosidase enzyme preparation derived from *Bacillus circulans* and used in the production of GOS is well characterized and reproducibly meets compositional and activity standards and complies with limits on contaminants appropriate for food-grade ingredients. Product specifications are set to assure that the β -galactosidase preparation is suitable as a processing aid. The starting organism, *Bacillus circulans*, is appropriately maintained and tested to assure purity of the organism. *Bacillus circulans* has no pathogenic activity, is not mutagenic, is negative for all tested toxins, has low acute toxicity, and has regulatory approvals in other countries.
- 6) Mean and 90th percentile 2-day average intakes of GOS by all individuals ages 2 and older who reported consumption of at least one food potentially fortified with GOS are 8.0 and 16.8 g/day, respectively. The mean estimated intake by infants 0-5 months old is 8.1 g/day and the mean GOS intake by infants 6-11 months old is 8.4 g/day. Teenage males have the highest estimated intake of GOS; their estimated mean and 90th percentile 2-day average intakes are 9.4 and 18.9 g/day, respectively. On a g/kg-bw/day basis, infants are estimated to have the highest intakes of GOS. The estimated 90th percentile 2-day average intake of GOS by infants 0-5 months old is 1.88 g GOS/kg-bw/day, and the estimated 90th percentile intake by infants 6-11 months of age is 1.55 g GOS/kg-bw/day. The estimated 90th percentile 2-day average intake of GOS by all users age 2 and older is 0.33 g GOS/kg-bw/day.
- 7) GOS has been tested for potential toxicity in two repeat dose 90-day studies in rats. Under the conditions of the tests, No Observed Adverse Effect Levels (NOAELs) were established at the highest doses tested due to the absence of adverse events. In one published study, GOS was administered to male and female Sprague Dawley CrI:CD¹(SD)IGS BR rats at doses of 0, 1.13 or 2.25 g GOS/kg-bw/day. The NOAEL of GOS in rats in this study was 2.25 g GOS/kg-bw/day. In an unpublished 90-day repeat dose study, GOS was administered to

GRAS Determination for GOS
Friesland Foods Domo

Wistar rats at doses of approximately 0, 1.6, 3.2, or 6.1 g GOS/kg-bw/day in male rats, and 0, 1.8, 3.6, or 6.9 g GOS/kg-bw/day in female rats. The NOAEL of GOS in rats in this study was 6.9 g GOS/kg-bw/day based on the highest dose tested in females. Other repeat dose and chronic studies of GOS consumption were conducted in rats, mice, pigs and dogs. Findings from these studies and the unpublished 90-day study corroborate the safety of GOS under the conditions of use.

- 8) A total of seven published studies and two unpublished studies of the effects of adult ingestion of GOS produced by Friesland Food Domo were reviewed, the studies represent six separate clinical trials. Results from published clinical studies involving the administration of GOS produced by Friesland Foods Domo to adults daily for time periods up to 3 weeks at doses of 8.1 to 20.8 g GOS per day (provided in 2 or 3 equivalent portions) indicate that consumption of GOS at these levels of intake is generally well tolerated, results from the unpublished studies corroborate this finding. The adverse effects associated with these levels of GOS intake were typically increased flatulence or gastrointestinal discomfort, and generally mild in nature. In one study, intake of 20.8 g GOS was reported to be well tolerated over a period of 5 days; GOS was consumed in lower doses in the preceding 4 days. In another study, intake of 20 g GOS daily for 4 days followed intake of lower doses of GOS for 4 days; gastrointestinal complaints were reported during GOS consumption, though they were considered to be mild in nature.
- 9) Nine additional studies (representing 10 study populations) of the effects of GOS consumption by adults were reviewed. The GOS used in these studies was from sources other than Friesland Foods Domo. In these studies GOS was administered for time periods ranging from 6 days to 4 weeks with doses ranging from 2.4 to 15 g GOS per day. Findings from these studies corroborate the safety of GOS consumption.
- 10) Results from a published study of term infants consuming 2.4 g GOS per L in infant formula daily for 6 months indicate that the supplemented formula was well tolerated. Results from two other studies in which infants consumed GOS-fortified formula for 3 or 18 weeks, or children consumed milk containing GOS for a period of 1 year, also indicate that the GOS-containing products were well tolerated. Additionally, results from 11 clinical studies in which term infants

GRAS Determination for GOS
Friesland Foods Domo

consumed infant formula containing a combination of GOS and fructo-oligosaccharide (FOS) at levels up to 7.2 g GOS per L to age 6 months indicate that the supplemented formulas are well tolerated by infants, produce no adverse effects such as diarrhea, reflux or increased incidence of crying, and support normal growth. Results from these studies also suggest that formulas with added GOS or GOS in combination with FOS may influence shifts in infant gut microflora that result in gut microflora more similar to the gut microflora of breast-fed infants.

- 11) Results from two clinical trials in a special population, preterm infants, indicate that formulas containing 9 g GOS per L and 1 g FOS per L were well tolerated and supported normal growth over the 2 or 4 week study periods. Findings from these studies in preterm infants corroborate safety of the GOS-supplemented formulas in term infants.

Determination of the GRAS status of GOS prepared with a β -galactosidase derived from *Bacillus circulans* under the intended conditions of use, has been made through the deliberations of A. Wallace Hayes, Ph.D., D.A.B.T., E.R.T., F.A.T.S. (Harvard School of Public Health, Boston, MA), David J. A. Jenkins, MD, Ph.D., D.Sc. (Professor, Canada Research Chair in Nutrition and Metabolism, Department of Nutritional Sciences), and Judith K. Jones, M.D., Ph.D. (President and CEO, The Degge Group, Ltd.). 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 GOS and the potential human exposure to GOS resulting from its intended use as an ingredient in foods and term infant formulas and have concluded:

There is no evidence in the available information on GOS prepared with a β -galactosidase derived from Bacillus circulans, that demonstrates, or suggests reasonable grounds to suspect a hazard to the public when GOS is used at levels that might reasonably be expected from the proposed applications. GOS prepared with a β -galactosidase derived from Bacillus circulans is GRAS for use in products as proposed by Friesland Foods Domo.

GRAS Determination for GOS
Friesland Foods Domo

Therefore, GOS prepared with a β -galactosidase derived from *Bacillus circulans* is safe, and GOS is GRAS at the proposed levels of addition to foods and infant formula. GOS prepared with a β -galactosidase derived from *Bacillus circulans* 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 sent to the U.S. Food and Drug Administration (FDA) upon request, or will be made available to FDA for reviewing and copying at reasonable times at the office of Gavin P. Thompson, Ph.D., Principal Consultant, ENVIRON International Corporation, 4350 North Fairfax Drive, Suite 300, Arlington, Virginia 22203. Telephone: 703-516-2300. Facsimile: 703-516-2393. Email: gthompson@environcorp.com

F. SIGNATURE

Friesland Foods Domo hereby makes and submits this notice of a GRAS Exemption Claim for Galacto-Oligosaccharides (GOS) under its intended conditions of use

(b) (6)

Rob van Vliet
Manager, Quality Assurance
Friesland Foods Domo

5 November 2007

Date

SUBMISSION END

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AM

**Ramos-Valle, Moraima**

From: Mary Murphy [mma@environcorp.com]
Sent: Thursday, January 31, 2008 2:38 PM
To: Ramos-Valle, Moraima
Cc: Gavin Thompson
Subject: GRN 236 - requested reference
Attachments: Yamashita and Kobata 1974.pdf

Dear Moraima,

Per our conversation, I am attaching a copy of Yamashita and Kobata 1974. Please do not hesitate to contact us if you have further questions during your review of GRN 236.

Regards,
Mary

Mary M. Murphy, M.S., R.D. | Science Manager

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1/31/2008

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Pages 000161-000167 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.



February 8, 2008

Moraima Ramos-Valle
Consumer Safety Officer
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
Room 2072
Mail Stop HFS-025
College Park, MD 20740-3835

RECEIVED
FEB 11 2008

BY:..... (b)(6)

Re: GRN 236: GRAS Notification for Galacto-oligosaccharides (GOS)

Dear Ms. Ramos-Valle:

On behalf of Friesland Foods Domo, ENVIRON International Corporation is contacting you regarding GRN 236, the GRAS Notification for the use of galacto-oligosaccharides (GOS) in foods and infant formula.

In response to our consultation with FDA, we herein submit the revised proposed use level of GOS in infant formula. The revised level is a maximum of 5 g GOS per liter infant formula. The intended uses of GOS in all categories of foods are unchanged from the uses specified in GRN 236.

We have enclosed in triplicate the revised pages of Chapter III of the safety assessment dossier to reflect this change in the intended use level and the resulting changes in the estimates of GOS intake by the U.S. population. These revised pages replace the corresponding pages in the GRAS Notification for GOS dated September 6th, 2007 as submitted to FDA and now identified as GRN 236. The revised pages of Chapter III are:

- 1) Section III. C. Intended Uses of Vivinal® GOS [pages 22-24]; and
- 2) Section III D. Estimated Intakes of GOS from the Intended Uses [pages 25-27]

Please do not hesitate to contact me if you have any additional questions or comments regarding GRN 236

Sincerely,

(b)(6)

Gavin Thompson
Principal Consultant

24-15175C

carbohydrates may be added to infant formulas for technical reasons or if they provide a “source of fermentable substrates for the gut microflora” (SCF 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® (SCF 2003).

A closely related oligosaccharide, fructo-oligosaccharide, has been determined to be GRAS for use in a variety of foods (FDA 2000). This oligosaccharide was determined to be safe when added to a variety of foods, including baby foods, at levels of 0.1-3.6%. Inulin, another non-digestible carbohydrate, has been determined to be GRAS for use in a variety of foods (FDA 2003).

C. Intended Uses of Vivinal® GOS

Friesland Foods DOMO intends to add GOS to a variety of foods and infant formula. The intended uses and levels of GOS per product are shown in REVISED Table III-3.

REVISED Table III-3. Intended Uses of GOS

| Food Group | Food Group Category | Examples of Foods in Category | Approximate serving size ^a (g) | Maximum g GOS per serving ^b |
|---|--|--|---|--|
| Bars | bars | snack bars, meal replacement bars, breakfast bars | 40 | 5.0 |
| Dairy products | yogurt | yogurt, excluding frozen yogurt | 227 | 7.5 |
| | frozen dairy desserts | frozen desserts such as ice creams and frozen yogurts, frozen novelties | 70 (½ cup) | 3.0 |
| Fruit drinks and waters/quenchers | fruit drinks (vitamin/mineral fortified) and energy drinks | fruit drinks (<100% real juice) identified as a vitamin and/or mineral fortified product; energy drinks | 240 (240 mL) | 5.0 |
| | fitness water and thirst quenchers | water with added vitamins/minerals, thirst quenchers | 240 (240 mL) | 3.0 |
| Fruit preparations | fruit pie filling | fruit fillings for pies | 85 | 5.0 |
| | fruit prep | fruit fillings in bars, cookies, yogurt, cakes, etc | 40 (2 Tbsp) | 5.0 |
| | jelly/jam | jellies, jams, fruit preserves, fruit butters | 20 (1 Tbsp) | 5.0 |
| Infant formulas for term infants and baby foods | infant formula for term infants | infant formula and follow-on formulas, includes ready-to-drink formula or formula prepared from powder or liquid concentrate | 5 g per L | NA ^c |
| | infant meal replacement drinks | meal replacement products such as Pediasure [®] | 250 | 3.0 |
| | baby juice | all types of juice identified as "baby" juices | 120 (120 mL) | 3.0 |
| | baby yogurt drink | yogurt and juice beverages identified as "baby" drinks | 125 (120 mL) | 3.0 |
| | baby dessert | fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts) | 110 | 3.0 |
| | baby snack | baby crackers, pretzels, cookies, snack items such as Gerber Graduates [®] finger foods | 7 | 1.0 |
| Milk beverages | milk | all acidophilus or fortified milks, nonfat and lowfat fluid milks, includes fluid milk and unreconstituted milk powder | 244 (240 mL) | 5.0 |
| | milk drinks | flavored milks including chocolate milk, coffee drinks, cocoa, smoothies (dairy or fruit based), other fruit and dairy combinations, kefir, includes ready-to-drink and powder mixes | 250 (240 mL) | 7.5 |
| | syrup flavoring for milk | syrups used to flavor milk beverages | 40 (2 Tbsp) | 5.0 |
| | meal replacement drinks | meal replacement beverages or diet beverages; includes ready-to-drink beverages and powder mixes | 250 (240 mL) | 5.0 |
| | milk substitutes | soy milk | 245 (240 mL) | 5.0 |

^a Serving sizes based on Reference Amounts Customarily Consumed (RACC) (21 CFR §101.12). Actual product serving sizes may differ slightly from these values.

^b GOS concentrations in foods or beverage as consumed.

^c Not applicable.

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D. Estimated Intakes of GOS from the Intended Uses

Estimates of potential intakes of GOS resulting from the intended uses of the oligosaccharide in select foods and infant formula 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). This NHANES data set provides nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States (NCHS 2006).

As part of the examination, trained dietary interviewers collect detailed information on all foods and beverages consumed by respondents in the previous 24 hour time period (midnight to midnight). A second dietary recall was administered by telephone 3 to 10 days after the first dietary interview, but not on the same day of the week as the first interview. A total of 9,043 respondents provided complete dietary intakes for the Day 1 recall, and 8,354 of the individuals provided a complete Day 2 recall.

The data files used to process the NHANES 2003-2004 dietary recalls include 6940 food codes, each identified by a unique number and descriptive name. The food codes represent single component foods or ingredients such as milk or vegetable oil; finished foods such as bread or margarine; and also food mixtures such as a grilled cheese sandwich. The database was reviewed, and all food codes (or portions of food codes) corresponding to one of the intended use categories of GOS were identified. Beverages containing milk as a component of a mixture, for example milk with chocolate syrup or a latte, also were identified, and the proportion of milk in each beverage (g per 100 g food code) was estimated using USDA survey files (USDA 2006). The weight per serving of each food code also was estimated using USDA survey files (USDA 2006).

Using the list of food codes and the NHANES 2003-2004 dietary recall data files from individuals with two complete days of dietary recall, ENVIRON estimated mean and 90th percentile 2-day average intakes of GOS from the individual product categories and also all categories combined. The 2-day average intakes represent the total estimated intakes of GOS during the two days of recall divided by two (i.e., $(\text{Intake}_{\text{Day 1}} + \text{Intake}_{\text{Day 2}})/2$). Intakes were calculated for subpopulations of infants (0-5 mo M+F, 6-11 mo M+F, 12-23 mo M+F), children (2-5 y M+F, 6-11 y M and F separately), teenagers (12-18 y M and F separately), adults (19+ y M and F separately), and all individuals ages 2+ y. Survey respondents were categorized into age groups based on ages reported at the time of the examination component in NHANES. The estimates were generated using survey

sample weights to adjust for differences in representation of subpopulations; results therefore are representative of the U.S. population.

Estimates of GOS intake from all uses in foods and infant formula combined are shown in REVISED Table III-4. As shown in the table, mean and 90th percentile 2-day average intakes of GOS by all individuals ages 2 and older who reported consumption of at least one food potentially fortified with GOS are 8.0 and 16.8 g/day, respectively. The mean estimated intake by infants 0-5 months old is 5.3 g/day and the mean GOS intake by infants 6-11 months old is 6.1 g/day. Teenage males have the highest estimated intake of GOS; their estimated mean and 90th percentile 2-day average intakes are 9.4 and 18.9 g/day, respectively. On a g/kg-bw/day basis, infants are estimated to have the highest intakes of GOS. The estimated 90th percentile 2-day average intake of GOS by infants 0-5 months old is 1.23 g GOS/kg-bw/day, and the estimated 90th percentile intake by infants 6-11 months of age is 1.18 g GOS/kg-bw/day. The estimated 90th percentile 2-day average intake of GOS by all users age 2 and older is 0.33 g GOS/kg-bw/day. The estimated intakes of GOS (g/day) by population and food group category are shown in Appendix 5.

REVISED Table III-4. Estimated 2-Day Average Intakes of GOS from All Proposed Uses in Foods and Infant Formula^a

| Population ^b | N ^c | Percent users ^d | 2-Day Average GOS Intakes Per User | | | |
|-------------------------|----------------|----------------------------|------------------------------------|-----------------|----------------------------|-----------------|
| | | | g GOS/d | | g GOS/kg-bw/d ^e | |
| | | | Mean | 90th Percentile | Mean | 90th Percentile |
| Infants, 0-5 mo | 101 | 100.0 | 5.3 | 7.7 | 0.88 | 1.23 |
| Infants, 6-11 mo | 172 | 99.5 | 6.1 | 10.1 | 0.71 | 1.18 |
| Infants, 12-23 mo | 229 | 85.6 | 5.3 | 11.2 | 0.46 | 1.01 |
| Children, 2-5 y | 621 | 92.3 | 7.8 | 18.2 | 0.45 | 1.01 |
| Boys, 6-11 y | 346 | 94.7 | 9.1 | 18.8 | 0.30 | 0.59 |
| Girls, 6-11 y | 404 | 92.5 | 8.9 | 16.4 | 0.28 | 0.51 |
| Teen males, 12-18 y | 693 | 79.1 | 9.4 | 18.9 | 0.16 | 0.38 |
| Teen females 12-18, y | 685 | 76.7 | 8.1 | 16.7 | 0.15 | 0.30 |
| Adult males, 19+ y | 1428 | 69.2 | 8.3 | 17.7 | 0.10 | 0.21 |
| Adult females, 19+ y | 1673 | 73.1 | 7.4 | 15.3 | 0.11 | 0.22 |
| Total population, 2+ y | 5850 | 75.0 | 8.0 | 16.8 | 0.15 | 0.33 |

^a Use level of GOS in foods and infant formula as specified in REVISED Table III-3

^b Breastfeeding infants and children were excluded from the sample population

^c Number of people consuming one or more foods containing GOS during the two 24-hour periods of dietary recall

^d Weighted percent

^e Analysis of intake in terms of g GOS/kg-bw/d was limited to people with a measured body weight

It is important to note that all estimates of intake presented in REVISED Table III-4 are likely overestimates of actual intakes of GOS 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 (REVISED Table III-3) was assumed to contain added GOS. 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 GOS, and not all products may contain the maximum proposed use levels of GOS.

The estimates of potential GOS intake by infants were based on the survey population of non-breastfeeding infants. The youngest infants (i.e., prior to introduction of weaning foods) in the sample population therefore are presumably consuming exclusively infant formula. Some infants, however, consume a combination of human milk and infant formula. The intakes of GOS by infants consuming a combination of human milk and formula would likely be lower than the estimates of GOS intake based on the population of exclusively formula fed infants.



February 19, 2008

02-20-08 P02:23 OUT

Moraima Ramos-Valle
Consumer Safety Officer
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
Room 2072
Mail Stop HFS-025
College Park, MD 20740-3835

Re: GRN 236: GRAS Notification for Galacto-oligosaccharides (GOS)

Dear Ms. Ramos-Valle:

On behalf of Friesland Foods Domo, ENVIRON International Corporation is submitting the enclosed revised exemption claim for GRN 236, the GRAS Notification for the use of galacto-oligosaccharides (GOS) in foods and infant formula.

In response to our consultation with FDA, we revised the proposed use level of GOS in infant formula to a maximum of 5 g GOS per liter infant formula. On February 8, 2008, we submitted revised sections of the safety dossier to reflect this change. The enclosed exemption claim (in triplicate), signed by Friesland Foods Domo, identifies the revised use level of GOS in infant formula.

Please do not hesitate to contact me if you have any additional questions or comments regarding GRN 236.

Sincerely,

(b)(6)

Gavin Thompson
Principal Consultant

24-15175CAFFD GRAS Exemption Claim cover ltr 2008-02-19.doc

GRAS Exemption Claim for Galacto-Oligosaccharides (GOS)

This is to notify you that Friesland Foods Domo claims that the use of galacto oligosaccharides (GOS) prepared with α -galactosidase derived from *Bacillus circulans* is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act because Friesland Foods Domo has determined such use to be Generally Recognized As Safe (GRAS). This determination and notification are in compliance with proposed Sec. 170.36 of Part 21 of the Code of Federal Regulations (21 CFR § 170.36) as published in the Federal Register, Vol. 62, No. 74, FR 18937, April 17, 1997.

A. NAME AND ADDRESS OF NOTIFIER

Friesland Foods Domo
P.O. Box 449
8000 AK Zwolle
The Netherlands

Contact:

Rob van Vliet
Manager, Quality Assurance
P.O. Box 449
8000 AK Zwolle
The Netherlands

B. COMMON OR USUAL NAME OF GRAS SUBSTANCE

The substance that is the subject of this GRAS determination is galactooligosaccharides (GOS) prepared with α -galactosidase derived from *Bacillus circulans*. Other chemical names for this GOS are galactooligosaccharide, transgalactosylated oligosaccharide, transgalacto-oligosaccharide and oligogalactosyl-lactose. The common name for a product currently manufactured by Friesland Foods Domo that contains the GOS that is the subject of this GRAS determination is Vivinal® GOS (formerly known as Elix'or and "OLIGO").

C. INTENDED USE

GOS prepared with a β -galactosidase derived from *Bacillus circulans* is intended to be added to a variety of foods and also infant formulas for the routine feeding of term infants. The food categories to which GOS will be added and the maximum concentrations of GOS in foods and infant formulas are detailed in Table II below.

| Table 1. Intended Uses of GOS | | | |
|--|--|---|--|
| Food Group | Food Group Category ^a | Approximate serving size ^b (g) | Maximum g GOS per serving ^c |
| Bars | Bars | 40 | 5.0 |
| Dairy products | Yogurt | 227 | 7.5 |
| | frozen dairy desserts | 70 (½ cup) | 3.0 |
| Fruit drinks and waters/quenchers | fruit drinks (vitamin/mineral fortified) and energy drinks | 240 (240 mL) | 5.0 |
| | fitness water and thirst quenchers | 240 (240 mL) | 3.0 |
| Fruit preparations | fruit pie filling | 85 | 5.0 |
| | fruit prep | 40 (2 Tbsp) | 5.0 |
| | jelly/jam | 20 (1 Tbsp) | 5.0 |
| Infant formulas for term infants and baby foods | infant formula | 5 g per L | NA ^d |
| | infant meal replacement drinks | 250 | 3.0 |
| | baby juice | 120 (120 mL) | 3.0 |
| | baby yogurt drink | 125 (120 mL) | 3.0 |
| | baby dessert | 110 | 3.0 |
| | baby snack | 7 | 1.0 |
| Milk beverages | Milk | 244 (240 ml) | 5.0 |
| | milk drinks | 250 (240 mL) | 7.5 |
| | syrup flavoring for milk | 40 (2 Tbsp) | 5.0 |
| | meal replacement drinks | 250 (240 mL) | 5.0 |
| | milk substitutes | 245 (240 mL) | 5.0 |
| ^a In some food group categories, not all types of foods are intended for addition of GOS (e.g., addition of GOS is limited to nonfat and low fat milk only, not whole and reduced fat milk). The specific types of products intended for addition of GOS are detailed in REVISED Table III-3. | | | |
| ^b Serving sizes based on Reference Amounts Customarily Consumed (RACC) (21 CFR §101.12). Actual product serving sizes may differ slightly from these values. | | | |
| ^c GOS concentrations in foods or beverage as consumed. | | | |
| ^d Not applicable. | | | |

(b)(6)

D. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of GOS (prepared with β -galactosidase derived from *Bacillus circulans*) as an ingredient in foods and term infant formulas at the maximum levels described in Section C of this chapter is based upon scientific procedures as described under 21 CFR§170.30(b). The intake of GOS from the intended uses specified above, as estimated by ENVIRON International Corporation (ENVIRON), 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 GOS is safe, and GRAS, under the intended conditions of use, the safety of the intake of GOS 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 GOS as an ingredient in foods and infant formulas has been determined to be safe through scientific procedures set forth under 21 CFR§170.30(b) based on the following:

- 1) Results from *in vitro* and *in vivo* studies provide evidence that GOS is largely resistant to gastric acidity, hydrolysis by mammalian enzymes, and absorption in the gastrointestinal tract. The available evidence indicates that GOS is fermented in the lower gastrointestinal tract.
- 2) Human milk contains a complex mixture of oligosaccharides, and oligosaccharides account for the third largest solid constituent in human milk after lactose and fat. The total amount of complex oligosaccharides in mature human milk is typically estimated to range from 5 to 8 g/L, though higher levels have also been reported.
- 3) GOS are chains of galactose units, usually with a single terminal glucose molecule ((galactose(Gal))_nglucose (Glu)). The type of β -glycosidic linkage between the monomer units is mainly 1→4 Gal, though other β -glycosidic linkages including 1→6 Gal, 1→2 Glc, 1→3 Glc, 1→4 Glc, 1→6 Glc, 1→2 Gal and 1→3 Gal may also be present.

- 4) GOS prepared with a β -galactosidase derived from *Bacillus circulans* is a well characterized oligosaccharide. It is a mixture of dioligosaccharides composed of 1-7 galactose units linked to a glucose molecule. The product reproducibly meets compositional standards and complies with limits on contaminants appropriate for food-grade ingredients. Product specifications are set to assure that GOS is suitable for use in food.
- 5) The β -galactosidase enzyme preparation derived from *Bacillus circulans* and used in the production of GOS is well characterized and reproducibly meets compositional and activity standards and complies with limits on contaminants appropriate for food-grade ingredients. Product specifications are set to assure that the β -galactosidase preparation is suitable as a processing aid. The starting organism, *Bacillus circulans*, is appropriately maintained and tested to assure purity of the organism. *Bacillus circulans* has no pathogenic activity, is not mutagenic, is negative for all tested toxins, has low acute toxicity, and has regulatory approvals in other countries.
- 6) Mean and 90th percentile 2-day average intakes of GOS by all individuals ages 2 and older who reported consumption of at least one food potentially fortified with GOS are 8.0 and 16.8 g/day, respectively. The mean estimated intake by infants 0-5 months old is 5.3 g/day and the mean GOS intake by infants 6-11 months old is 6.1 g/day. Teenage males have the highest estimated intake of GOS; their estimated mean and 90th percentile 2-day average intakes are 9.4 and 18.9 g/day, respectively. On a g/kg-bw/day basis, infants are estimated to have the highest intakes of GOS. The estimated 90th percentile 2-day average intake of GOS by infants 0-5 months old is 1.23 g GOS/kg-bw/day, and the estimated 90th percentile intake by infants 6-11 months of age is 1.18 g GOS/kg-bw/day. The estimated 90th percentile 2-day average intake of GOS by all users age 2 and older is 0.33 g GOS/kg-bw/day.
- 7) GOS has been tested for potential toxicity in two repeat dose 90-day studies in rats. Under the conditions of the tests, No Observed Adverse Effect Levels (NOAELs) were established at the highest doses tested due to the absence of adverse events. In one published study, GOS was administered to male and female Sprague Dawley Crl:CD[®](SD)IGS BR rats at doses of 0, 1.13 or 2.25 g GOS/kg-bw/day. The NOAEL of GOS in rats in this study was 2.25 g GOS/kg-bw/day. In an unpublished 90-day repeat dose study, GOS was administered to

Wistar rats at doses of approximately 0, 1.6, 3.2, or 6.1 g GOS/kgbw/day in male rats, and 0, 1.8, 3.6, or 6.9 g GOS/kgbw/day in female rats. The NOAEL of GOS in rats in this study was 6.9 g GOS/kgbw/day based on the highest dose tested in females. Other repeat dose and chronic studies of GOS consumption were conducted in rats, mice, pigs and dogs. Findings from these studies and the unpublished 90-day study corroborate the safety of GOS under the conditions of use.

- 8) A total of seven published studies and two unpublished studies of the effects of adult ingestion of GOS produced by Friesland Food Domo were reviewed; the studies represent six separate clinical trials. Results from published clinical studies involving the administration of GOS produced by Friesland Foods Domo to adults daily for time periods up to 3 weeks at doses of 8.1 to 20.8 g GOS per day (provided in 2 or 3 equivalent portions) indicate that consumption of GOS at these levels of intake is generally well tolerated; results from the unpublished studies corroborate this finding. The adverse effects associated with these levels of GOS intake were typically increased flatulence or gastrointestinal discomfort, and generally mild in nature. In one study, intake of 20.8 g GOS was reported to be well tolerated over a period of 5 days; GOS was consumed in lower doses in the preceding 4 days. In another study, intake of 20 g GOS daily for 4 days followed intake of lower doses of GOS for 4 days; gastrointestinal complaints were reported during GOS consumption, though they were considered to be mild in nature.
- 9) Nine additional studies (representing 10 study populations) of the effects of GOS consumption by adults were reviewed. The GOS used in these studies was from sources other than Friesland Foods Domo. In these studies GOS was administered for time periods ranging from 6 days to 4 weeks with doses ranging from 2.4 to 15 g GOS per day. Findings from these studies corroborate the safety of GOS consumption.
- 10) Results from a published study of term infants consuming 2.4 g GOS per L in infant formula daily for 6 months indicate that the supplemented formula was well tolerated. Results from two other studies in which infants consumed GOS fortified formula for 3 or 18 weeks, or children consumed milk containing GOS for a period of 1 year, also indicate that the GOS-containing products were well tolerated. Additionally, results from 11 clinical studies in which term infants

consumed infant formula containing a combination of GOS and fructo oligosaccharide (FOS) at levels up to 7.2 g GOS per L to age 6 months indicate that the supplemented formulas are well tolerated by infants, produce no adverse effects such as diarrhea, reflux or increased incidence of crying, and support normal growth. Results from these studies also suggest that formulas with added GOS or GOS in combination with FOS may influence shifts in infant gut microflora that result in gut microflora more similar to the gut microflora of breast-fed infants.

- 11) Results from two clinical trials in a special population, preterm infants, indicate that formulas containing 9 g GOS per L and 1 g FOS per L were well tolerated and supported normal growth over the 2 or 4 week study periods. Findings from these studies in preterm infants corroborate safety of the GOS-supplemented formulas in term infants.

Determination of the GRAS status of GOS prepared with β -galactosidase derived from *Bacillus circulans*, under the intended conditions of use, has been made through the deliberations of A. Wallace Hayes, Ph.D., D.A.B.T., E.R.T., F.A.T.S. (Harvard School of Public Health, Boston, MA); David J. A. Jenkins, MD, Ph.D., D.Sc. (Professor, Canada Research Chair in Nutrition and Metabolism, Department of Nutritional Sciences); and Judith K. Jones, M.D., Ph.D. (President and CEO, The Degge Group, Ltd.). 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 GOS and the potential human exposure to GOS resulting from its intended use as an ingredient in foods and term infant formulas and have concluded:

*There is no evidence in the available information on GOS prepared with a β -galactosidase derived from *Bacillus circulans* that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when GOS is used at levels that might reasonably be expected from the proposed applications. GOS prepared with a β -galactosidase derived from *Bacillus circulans* is GRAS for use in products as proposed by Friesland Foods Domo*

Therefore, GOS prepared with a β -galactosidase derived from *Bacillus circulans* is safe, and GOS is GRAS at the proposed levels of addition to foods and infant formula. GOS prepared with a β -galactosidase derived from *Bacillus circulans* 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.


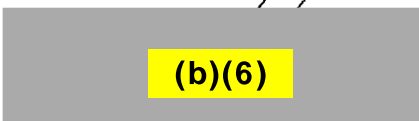
E. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be made available to FDA for reviewing and copying at reasonable times at the office of Gavin P.

Thompson, Ph.D., Principal Consultant, ENVIRON International Corporation, 4350 North Fairfax Drive, Suite 300, Arlington, Virginia 22203; Telephone: 703-516-2300; Facsimile: 703-516-2393; Email: gthompson@environcorp.com.


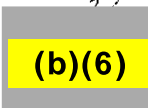
F. SIGNATURE

Friesland Foods Domo hereby makes and submits this notice of a GRAS Exemption Claim for Galacto-Oligosaccharides (GOS) under its intended conditions of use. .



(b)(6)

Rob van Vliet
Manager, Quality Assurance
Friesland Foods Domo

February 11, 2008
Date



(b)(6)



April 22, 2008

Moraima J. Ramos Valle
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RECEIVED
APR 24 2008

Re: GRAS Notice No. GRN 000236:
Galacto-oligosaccharides (GOS)

BY: (b)(6)

Dear Ms. Ramos Valle:

On behalf of Friesland Foods Domo, ENVIRON International Corporation is contacting you regarding the subject Notification of the Generally Recognized as Safe (GRAS) Determination for the use of galacto-oligosaccharides (GOS) in foods and infant formulas for term infants.

1) We are submitting the Appendices to the GRAS Determination for Galacto-oligosaccharides (GOS) in response to your request for these technical supporting documents. Paper sets (in triplicate) of the Appendices are enclosed; also a CD-ROM containing a set of the Appendices as PDF files is enclosed. We have provided full versions of all Appendices in accordance with your request. In addition, we have provided redacted versions of Appendix 1B and Appendix 2B; based on information from the Notifier, Friesland Foods Domo, we understand that these two appendices contain proprietary process technology information that the Notifier requests not be disclosed.

2) We have revised the food group category name "infant meal replacement drinks", to indicate that it represents a product designed for children. Our nomenclature clarification for this food group category does not result in any changes in our previously presented exposure estimates. We herein are submitting revised versions of the following tables of use and exposure information to incorporate the nomenclature change for this food group category:

i) In Table III-3 (pages 23 and 24) the food group category "infant meal replacement drinks" has been moved to the "milk beverages" food group and the category name has been changed to "meal replacement drinks for children";

ii) References to the population of individuals 12-23 months old in the text (page 25) and in Table III-4 (page 26) have been changed from "infants 12-23 mo." to "children 12-23 mo."; and

April 22, 2008

Moraima J. Ramos Valle
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

**Re: GRAS Notice No. GRN 000236:
Galacto-oligosaccharides (GOS)**

Dear Ms. Ramos Valle:

On behalf of Friesland Foods Domo, ENVIRON International Corporation is contacting you regarding the subject Notification of the Generally Recognized as Safe (GRAS) Determination for the use of galacto-oligosaccharides (GOS) in foods and infant formulas for term infants.

1) We are submitting the Appendices to the GRAS Determination for Galacto-oligosaccharides (GOS) in response to your request for these technical supporting documents. Paper sets (in triplicate) of the Appendices are enclosed; also a CD-ROM containing a set of the Appendices as PDF files is enclosed. We have provided full versions of all Appendices in accordance with your request. In addition, we have provided redacted versions of Appendix 1B and Appendix 2B; based on information from the Notifier, Friesland Foods Domo, we understand that these two appendices contain proprietary process technology information that the Notifier requests not be disclosed.

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i) In Table III-3 (pages 23 and 24) the food group category "infant meal replacement drinks" has been moved to the "milk beverages" food group and the category name has been changed to "meal replacement drinks for children";

ii) References to the population of individuals 12-23 months old in the text (page 25) and in Table III-4 (page 26) have been changed from "infants 12-23 mo." to "children 12-23 mo."; and

iii) In Revised Table 1 of the GRAS Exemption Claim the “infant meal replacement drinks” food group category has been moved to the “milk beverages” food group and renamed “meal replacement drinks for children”. We have added a footnote to Table 1 to indicate that Pediasure[®] is an example of a meal replacement drink for children.

These nomenclature changes for the food group category and the population group have been made in conformance with the definition of an infant as a person not more than 12 months old (21 C.F.R. 105.3(e)). Also, an explanatory footnote has been added to the revised tables of intended uses (Table III-3 and Table 1) to indicate that “baby foods” are foods designed for weaning infants and young children. Pages (in triplicate) containing these modifications follow immediately after this cover letter.

If you have any questions or comments, please contact me at +1 (703) 589 8023 or gthompson@environcorp.com or Mary Murphy at +1 (703) 516 2301 or mmurphy@environcorp.com.

Sincerely,

(b)(6)

Gavin Thompson
Principal Consultant

24-15175C

REVISED Table III-3. Intended Uses of GOS

| Food Group | Food Group Category | Examples of Foods in Category | Approximate serving size ^a (g) | Maximum g GOS per serving ^b |
|--|--|--|---|--|
| Bars | bars | snack bars, meal replacement bars, breakfast bars | 40 | 5.0 |
| Dairy products | yogurt | yogurt, excluding frozen yogurt | 227 | 7.5 |
| | frozen dairy desserts | frozen desserts such as ice creams and frozen yogurts, frozen novelties | 70 (½ cup) | 3.0 |
| Fruit drinks and waters/ quenchers | fruit drinks (vitamin/mineral fortified) and energy drinks | fruit drinks (<100% real juice) identified as a vitamin and/or mineral fortified product; energy drinks | 240 (240 mL) | 5.0 |
| | fitness water and thirst quenchers | water with added vitamins/minerals; thirst quenchers | 240 (240 mL) | 3.0 |
| Fruit preparations | fruit pie filling | fruit fillings for pies | 85 | 5.0 |
| | fruit prep | fruit fillings in bars, cookies, yogurt, cakes, etc. | 40 (2 Tbsp) | 5.0 |
| | jelly/jam | jellies, jams, fruit preserves, fruit butters | 20 (1 Tbsp) | 5.0 |
| Infant formulas for term infants and baby foods ^c | infant formula for term infants | infant formula and follow-on formulas; includes ready-to-drink formula or formula prepared from powder or liquid concentrate | 5 g per L | NA ^d |
| | baby juice | all types of juice identified as "baby" juices | 120 (120 mL) | 3.0 |
| | baby yogurt drink | yogurt and juice beverages identified as "baby" drinks | 125 (120 mL) | 3.0 |
| | baby dessert | fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts) | 110 | 3.0 |
| | baby snack | baby crackers, pretzels, cookies, snack items such as Gerber Graduates [®] finger foods | 7 | 1.0 |
| | | | | |
| Milk beverages | milk | all acidophilus or fortified milks; nonfat and lowfat fluid milks; includes fluid milk and unreconstituted milk powder | 244 (240 ml) | 5.0 |
| | milk drinks | flavored milks including chocolate milk, coffee drinks, cocoa, smoothies (dairy or fruit based), other fruit and dairy combinations, kefir; includes ready-to-drink and powder mixes | 250 (240 mL) | 7.5 |
| | syrup flavoring for milk | syrups used to flavor milk beverages | 40 (2 Tbsp) | 5.0 |
| | meal replacement drinks | meal replacement beverages or diet beverages; includes ready-to-drink beverages and powder mixes | 250 (240 mL) | 5.0 |
| | meal replacement drinks for children | meal replacement products for children, such as Pediasure [®] | 250 (240 mL) | 3.0 |
| | milk substitutes | soy milk | 245 (240 mL) | 5.0 |
| | | | | |

^a Serving sizes based on Reference Amounts Customarily Consumed (RACC) (21 CFR §101.12). Actual product serving sizes may differ slightly from these values.

^b GOS concentrations in foods or beverage as consumed.

^c Baby foods (including baby juice, baby yogurt drink, baby desserts, and baby snacks) are foods designed for weaning infants and young children (i.e., toddlers).

^d Not applicable.

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C. Estimated Intakes of GOS from the Intended Uses

Estimates of potential intakes of GOS resulting from the intended uses of the oligosaccharide in select foods and infant formula 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). This NHANES data set provides nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States (NCHS 2006).

As part of the examination, trained dietary interviewers collect detailed information on all foods and beverages consumed by respondents in the previous 24 hour time period (midnight to midnight). A second dietary recall was administered by telephone 3 to 10 days after the first dietary interview, but not on the same day of the week as the first interview. A total of 9,043 respondents provided complete dietary intakes for the Day 1 recall, and 8,354 of the individuals provided a complete Day 2 recall.

The data files used to process the NHANES 2003-2004 dietary recalls include 6940 food codes, each identified by a unique number and descriptive name. The food codes represent single component foods or ingredients such as milk or vegetable oil; finished foods such as bread or margarine; and also food mixtures such as a grilled cheese sandwich. The database was reviewed, and all food codes (or portions of food codes) corresponding to one of the intended use categories of GOS were identified. Beverages containing milk as a component of a mixture, for example milk with chocolate syrup or a latte, also were identified, and the proportion of milk in each beverage (g per 100 g food code) was estimated using USDA survey files (USDA 2006). The weight per serving of each food code also was estimated using USDA survey files (USDA 2006).

Using the list of food codes and the NHANES 2003-2004 dietary recall data files from individuals with two complete days of dietary recall, ENVIRON estimated mean and 90th percentile 2-day average intakes of GOS from the individual product categories and also all categories combined. The 2-day average intakes represent the total estimated intakes of GOS during the two days of recall divided by two (i.e., $(\text{Intake}_{\text{Day 1}} + \text{Intake}_{\text{Day 2}})/2$). Intakes were calculated for subpopulations of infants (0-5 mo M+F, 6-11 mo M+F), children (12-23 mo M+F, 2-5 y M+F, 6-11 y M and F separately), teenagers (12-18 y M and F separately), adults (19+ y M and F separately), and all individuals ages 2+ y. Survey respondents were categorized into age groups based on ages reported at the time of the examination component in NHANES. The estimates were generated using

survey sample weights to adjust for differences in representation of subpopulations; results therefore are representative of the U.S. population.

Estimates of GOS intake from all uses in foods and infant formula combined are shown in REVISED Table III-4. As shown in the table, mean and 90th percentile 2-day average intakes of GOS by all individuals ages 2 and older who reported consumption of at least one food potentially fortified with GOS are 8.0 and 16.8 g/day, respectively. The mean estimated intake by infants 0-5 months old is 5.3 g/day and the mean GOS intake by infants 6-11 months old is 6.1 g/day. Teenage males have the highest estimated intake of GOS; their estimated mean and 90th percentile 2-day average intakes are 9.4 and 18.9 g/day, respectively. On a g/kg-bw/day basis, infants are estimated to have the highest intakes of GOS. The estimated 90th percentile 2-day average intake of GOS by infants 0-5 months old is 1.23 g GOS/kg-bw/day, and the estimated 90th percentile intake by infants 6-11 months of age is 1.18 g GOS/kg-bw/day. The estimated 90th percentile 2-day average intake of GOS by all users age 2 and older is 0.33 g GOS/kg-bw/day. The estimated intakes of GOS (g/day) by population and food group category are shown in Appendix 5.

| REVISED Table III-4. Estimated 2-Day Average Intakes of GOS from All Proposed Uses in Foods and Infant Formula^a | | | | | | |
|---|----------------------|----------------------------------|---|------------------------|----------------------------------|------------------------|
| Population^b | N^c | Percent users^d | 2-Day Average GOS Intakes Per User | | | |
| | | | g GOS/d | | g GOS/kg-bw/d^e | |
| | | | Mean | 90th Percentile | Mean | 90th Percentile |
| Infants, 0-5 mo | 101 | 100.0 | 5.3 | 7.7 | 0.88 | 1.23 |
| Infants, 6-11 mo | 172 | 99.5 | 6.1 | 10.1 | 0.71 | 1.18 |
| Children, 12-23 mo | 229 | 85.6 | 5.3 | 11.2 | 0.46 | 1.01 |
| Children, 2-5 y | 621 | 92.3 | 7.8 | 18.2 | 0.45 | 1.01 |
| Boys, 6-11 y | 346 | 94.7 | 9.1 | 18.8 | 0.30 | 0.59 |
| Girls, 6-11 y | 404 | 92.5 | 8.9 | 16.4 | 0.28 | 0.51 |
| Teen males, 12-18 y | 693 | 79.1 | 9.4 | 18.9 | 0.16 | 0.38 |
| Teen females, 12-18 y | 685 | 76.7 | 8.1 | 16.7 | 0.15 | 0.30 |
| Adult males, 19+ y | 1428 | 69.2 | 8.3 | 17.7 | 0.10 | 0.21 |
| Adult females, 19+ y | 1673 | 73.1 | 7.4 | 15.3 | 0.11 | 0.22 |
| Total population, 2+ y | 5850 | 75.0 | 8.0 | 16.8 | 0.15 | 0.33 |
| ^a Use level of GOS in foods and infant formula as specified in REVISED Table III-3. | | | | | | |
| ^b Breastfeeding infants and children were excluded from the sample population. | | | | | | |
| ^c Number of people consuming one or more foods containing GOS during the two 24-hour periods of dietary recall. | | | | | | |
| ^d Weighted percent. | | | | | | |
| ^e Analysis of intake in terms of g GOS/kg-bw/d was limited to people with a measured body weight. | | | | | | |

A. INTENDED USE

GOS prepared with a β -galactosidase derived from *Bacillus circulans* is intended to be added to a variety of foods and also infant formulas for the routine feeding of term infants. The food categories to which GOS will be added and the maximum concentrations of GOS in foods and infant formulas are detailed in REVISED Table 1 below.

| REVISED Table 1. Intended Uses of GOS | | | |
|--|--|---|--|
| Food Group | Food Group Category^a | Approximate serving size^b (g) | Maximum g GOS per serving^c |
| Bars | bars | 40 | 5.0 |
| Dairy products | yogurt | 227 | 7.5 |
| | frozen dairy desserts | 70 (½ cup) | 3.0 |
| Fruit drinks and waters/quenchers | fruit drinks (vitamin/mineral fortified) and energy drinks | 240 (240 mL) | 5.0 |
| | fitness water and thirst quenchers | 240 (240 mL) | 3.0 |
| Fruit preparations | fruit pie filling | 85 | 5.0 |
| | fruit prep | 40 (2 Tbsp) | 5.0 |
| | jelly/jam | 20 (1 Tbsp) | 5.0 |
| Infant formulas for term infants and baby foods ^d | infant formula | 5 g per L | NA ^e |
| | baby juice | 120 (120 mL) | 3.0 |
| | baby yogurt drink | 125 (120 mL) | 3.0 |
| | baby dessert | 110 | 3.0 |
| | baby snack | 7 | 1.0 |
| Milk beverages | milk | 244 (240 mL) | 5.0 |
| | milk drinks | 250 (240 mL) | 7.5 |
| | syrup flavoring for milk | 40 (2 Tbsp) | 5.0 |
| | meal replacement drinks | 250 (240 mL) | 5.0 |
| | meal replacement drinks for children ^f | 250 (240 mL) | 3.0 |
| | milk substitutes | 245 (240 mL) | 5.0 |

^a In some food group categories, not all types of foods are intended for addition of GOS (e.g., addition of GOS is limited to nonfat and low fat milk only, not whole and reduced fat milk). The specific types of products intended for addition of GOS are detailed in REVISED Table III-3.

^b Serving sizes based on Reference Amounts Customarily Consumed (RACC) (21 CFR §101.12). Actual product serving sizes may differ slightly from these values.

^c GOS concentrations in foods or beverage as consumed.

^d Baby foods (including baby juice, baby yogurt drink, baby desserts, and baby snacks) are foods designed for weaning infants and young children (i.e., toddlers).

^e Not applicable.

^f Meal replacement drinks for children include products such as Pediasure®.



APPENDICES

COMPLETE

Part 1 of 2

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Appendix 1A

Materials Used in the Production of Vivinal[®] GOS

Contents

Appendix 1A: Materials Used in the Production of Vivinal[®] GOS

- Exhibit 1. Lactose
- Exhibit 2. Citric Acid Monohydrate
- Exhibit 3. Sodium Hydroxide
- Exhibit 4. Activated Carbon
- Exhibit 5. Cellulose
- Exhibit 6. Hydrochloric Acid
- Exhibit 7. Perlite

Appendix 1A Exhibit 1

Lactose

[Code of Federal Regulations]
[Title 21, Volume 2]
[Revised as of April 1, 2006]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR168]

[Page 546]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN SERVICES (CONTINUED)

PART 168_SWEETENERS AND TABLE SIRUPS--Table of Contents

Subpart B_Requirements for Specific Standardized Sweeteners and Table Sirups

Sec. 168.122 Lactose.

(a) Lactose is the carbohydrate normally obtained from whey. It may be anhydrous or contain one molecule of water of crystallization or be a mixture of both forms.

(b) The food shall meet the following specifications:

(1) The lactose content is not less than 98.0 percent, mass over mass (m/m), calculated on a dry basis.

(2) The sulfated ash content is not more than 0.3 percent, m/m, calculated on a dry basis.

(3) The pH of a 10.0-percent m/m solution is not less than 4.5 nor more than 7.5.

(4) The loss on drying for 16 hours at 120 [deg]C is not more than 6.0 percent, m/m.

(c) The name of the food is ``Lactose'' or, alternatively, ``Milk sugar''.

(d) The methods of analysis in paragraphs (d)(1), (d)(2), (d)(3), (d)(4), and (d)(5) of this section are to be used to determine whether the food meets the requirements of paragraphs (b)(1), (b)(2), (b)(3), and (b)(4) of this section. The methods are contained in ``Official Methods of Analysis of the Association of Official Analytical Chemists'', 14th Ed. (1984), including the 4th Supp. (1988), which is incorporated by reference in accordance with 5 U.S.C. 552(a). Copies of the material incorporated by reference may be obtained from the AOAC INTERNATIONAL, 481 North Frederick Ave., suite 500, Gaithersburg, MD 20877, or may be examined at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: <http://www.archives.gov/federal--register/code--of--federal--regulations/ibr--locations.html>.

(1) Lactose content, sections 31.064 to 31.071, ``Purity of Lactose, Liquid Chromatographic Method,'' First Action, 14th Ed. (1984), pp. 583 and 584.

(2) Lactose content, sections 31.064 to 31.071, ``Purity of Lactose, Liquid Chromatographic Method,'' ``Changes in Official Methods of Analysis,'' 14th Ed., 4th Supp. (1988), p. 212. This reference recognizes the change in status of the method from first action to final action.

(3) Sulfated ash content, section 31.014, ``Ash of Sugars and Sirups,'' Final Action, Sulfated Ash, 14th Ed. (1984), p. 575.

(4) pH, section 14.022, ``pH of Flour, Potentiometric Method, ''
Final Action, except that a 10-percent m/m solution of lactose in water
is used for the determination, 14th Ed. (1984), p. 252.

(5) Loss on drying at 120 [deg]C, section 31.070, 14th Ed. (1984),
p. 584.

[42 FR 14479, Mar. 15, 1977, as amended at 47 FR 11834, Mar. 19, 1982;
49 FR 10103, Mar. 19, 1984; 54 FR 24896, June 12, 1989; 55 FR 8459,
Mar. 8, 1990; 63 FR 14035, Mar. 24, 1998]

LACTOSE EDIBLE GRADE

| | |
|------------------------|------------------------------------|
| Name | α - lactose monohydrate |
| Type | washed lactose |
| Appearance | slightly yellow crystalline powder |
| Appearance of solution | slightly yellow, clear |
| Odour and taste | nearly odourless, slightly sweet |

Physical/Chemical properties

| | | |
|--|----------------|-----|
| Lactose monohydrate | ≥ 99.2 | % |
| Protein (KjN * 6.38) | ≤ 0.3 | % |
| Ash (sulphated / 800 ± 25 °C) | ≤ 0.3 | % |
| Total water (Karl Fischer) | ≤ 5.2 | % |
| Free moisture (24 h, room temp, vacuo, silica gel) | ≤ 0.2 | % |
| pH (10 % solution) | 6.0 - 6.6 | |
| Specific optical rotation (as monohydrate) | + 51.8 - 52.8° | |
| Heavy metals | ≤ 5 | ppm |

Microbiological data

| | | |
|------------------------------|--------------------|-----------------|
| Total plate count | (PCMA, 72 h, 30°C) | ≤ 3000 / g |
| Enterobacteriaceae | (VRBG, 24 h, 30°C) | < 10 / g |
| Escherichia coli | | absent in 1 g |
| Salmonellae | | absent in 25 g |
| Coagulase pos. staphylococci | | absent in 0.1 g |
| Yeasts | (OGGA, 96 h, 25°C) | ≤ 50 / g |
| Moulds | (OGGA, 96 h, 25°C) | ≤ 50 / g |

Particle sizes

| productnumber | CRYSTALS 234 | POWDER 236 | FINE 237 |
|---|-----------------|---------------|-------------|
| % remaining on sieve mesh > micron | % | % | % |
| 60 = > 250 | 15-30 | ≤ 5 | |
| 100 = > 150 | 45-75 | 5-25 | ≤ 2 |
| 200 = > 75 | ≥ 85 | 40-60 | ≤ 15 |
| 270 = > 53 | | | 10-25 |

Packaging: Deliveries in 25 kg polythene lined paper bags, big bags and bulk.

Storage: Keep in clean, dry conditions, 10° - 20°C, 60 % RH and away from strongly odourous materials

Appendix 1A

Exhibit 2

Citric Acid Monohydrate

[Code of Federal Regulations]
[Title 21, Volume 3]
[Revised as of April 1, 2006]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR184]

[Page 487]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN SERVICES (CONTINUED)

PART 184_DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE--Table of Contents

Subpart B_Listing of Specific Substances Affirmed as GRAS

Sec. 184.1033 Citric acid.

(a) Citric acid (C₆H₈O₇, CAS Reg. No. 77-92-9) is the compound 2-hydroxy-1,2,3-propanetricarboxylic acid. It is a naturally occurring constituent of plant and animal tissues. It occurs as colorless crystals or a white powder and may be anhydrous or contain one mole of water per mole of citric acid. Citric acid may be produced by recovery from sources such as lemon or pineapple juice; by mycological fermentation using *Candida* spp., described in Sec. 173.160 and 173.165 of this chapter; and by the solvent extraction process described in Sec. 173.280 of this chapter for the recovery of citric acid from *Aspergillus niger* fermentation liquor.

(b) The ingredient meets the specifications of the Food Chemicals Codex, 3d ed. (1981), pp. 86-87, and its third supplement (March 1992), pp. 107-108, which are incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, and the Center for Food Safety and Applied Nutrition (HFS-200), 5100 Paint Branch Pkwy., College Park, MD 20740, or may be examined at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: <http://www.archives.gov/federal--register/code--of--federal--regulations/ibr--locations.html>.

(c) In accordance with Sec. 184.1(b)(1), the ingredient is used in food with no limitations other than current good manufacturing practice.

(d) Prior sanctions for this ingredient different from the uses established in this section do not exist or have been waived.

[59 FR 63895, Dec. 12, 1994]

Citric acid solution 50%, SG60220001010

GENERAL INFORMATION

| | |
|----------------------------|---|
| Name product | Citric acid solution |
| General description | Organic acidifier, food grade |
| Legal declaration | Raw materials have to comply with Dutch and E.C. regulations for food grade products. |
| Remark | Ingredient must not be derived from genetically modified raw materials. Also free of foreign material and allergens that are not listed on the ingredient declaration |
| Resource Number | RN 60220001010 to be mentioned on each unit |
| Documents | Cert. of conformance |
| Production and expiry date | To be mentioned on each unit |
| Guaranteed shelflife | At least 12 months |
| Packaging | Box 1000 kg |
| Pallet type | N.A. |
| Bags/pallet | N.A. |
| Additional | N.A. |
| Storage conditions | Original unopened package below 25°C |

PRODUCT SPECIFICATION

| <u>Properties</u> | <u>Specification</u> | <u>Method of analysis</u> |
|-----------------------------|--------------------------------------|----------------------------|
| Chemical | | |
| Assay | 49 - 51 % | |
| pH | 1.5 - 2.5 | 0.5 % Solution FNZ 14.13 |
| Heavy metals (as Pb) | Max 5 ppm | USP 231 |
| Mercury | Max. 1 ppm | |
| Lead | Max 1 ppm | |
| Arsenic | Max. 1 ppm | |
| Calcium | Max. 100 ppm | |
| Magnesium | Max. 50 ppm | |
| Reaction on citrate | Positive | USP 191 |
| Ash (sulphated) | Max 0.05 % | FNZ-Method |
| Physical | | |
| Appearance | Clear practical colourless solution | Sensory evaluation method |
| Density | Ca. 1,234 – 1,248 kg/dm ³ | |
| Transmission (450 nm, 1 cm) | Min. 98% | |
| Absorbance (405 nm, 1 cm) | Max. 0,015 | |
| Flavour | Acidic | Sensory evaluation method |
| Aroma | Normal | Sensory observation method |

Appendix 1A

Exhibit 3

Sodium Hydroxide

[Code of Federal Regulations]
[Title 21, Volume 3]
[Revised as of April 1, 2006]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR184]

[Page 487]

TITLE 21--FOOD AND DRUGS

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(c) In accordance with Sec. 184.1(b)(1), the ingredient is used in food with no limitations other than current good manufacturing practice.

(d) Prior sanctions for this ingredient different from the uses established in this section do not exist or have been waived.

[59 FR 63895, Dec. 12, 1994]

| | | |
|---|---|--|
| Specification |  | Last changed date 28-12-2004 Page 1 van 1 |
| In case of document is printed, it concerns an uncontrolled copy. The authorised document is placed at Intranet | | |

Natronloog 33%

(SG60220004010)

SG504877

1. GENERAL INFORMATION

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

2. PRODUCT SPECIFICATION

| <u>Properties</u> | <u>Specification</u> | <u>Method of analysis</u> |
|---|----------------------|---------------------------|
| Chemical | | |
| Iron | Max 10 mg/kg * | SAM 104.04 |
| NAOH | 32 -34 % | SAM 284.01 |
| Chloride | Max 0.02 % * | SAM 269.03 |
| CO ₃ (as Na ₂ CO ₃) | Max 0.1 % | SAM 183.04 |
| Arsenicum | Max 1 mg/kg | |
| Lead | Max 0.15 mg/kg | |
| Mercury | Max 0.3 mg/kg | |
| Physical | | |
| Appearance | Liquid | Sensory evaluation method |
| Colour | Clear | Visual observation method |
| Aroma | Odourless | Sensory evaluation method |
| Microbiological | | |
| Salmonella | Absent in 25 ml | IDF 93B (1995) |
| Staphylococcus aureus | < 100.000 cfu/ml | IDF 60C (1997) |

3. CERTIFICATE OF ANALYSIS

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

Note: * = based on a 100% concentration

000203

Appendix 1A

Exhibit 4

Activated Carbon

[Code of Federal Regulations]
[Title 21, Volume 3]
[Revised as of April 1, 2006]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR173]

[Page 122-125]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN SERVICES (CONTINUED)

PART 173 SECONDARY DIRECT FOOD ADDITIVES PERMITTED IN FOOD FOR HUMAN CONSUMPTION--Table of Contents

Subpart A_Polymer Substances and Polymer Adjuvants for Food Treatment

Sec. 173.25 Ion-exchange resins.

Ion-exchange resins may be safely used in the treatment of food under the following prescribed conditions:

(a) The ion-exchange resins are prepared in appropriate physical form, and consist of one or more of the following:

- (1) Sulfonated copolymer of styrene and divinylbenzene.
- (2) Sulfonated anthracite coal meeting the requirements of ASTM method D388-38, Class I, Group 2, ``Standard Specifications for Classification of Coal by Rank,`` which is incorporated by reference. Copies are available from University Microfilms International, 300 N. Zeeb Rd., Ann Arbor, MI 48106, or available for inspection at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: <http://www.archives.gov/federal--register/code--of--federal--regulations/ibr--locations.html>.
- (3) Sulfite-modified cross-linked phenol-formaldehyde, with modification resulting in sulfonic acid groups on side chains.
- (4) Methacrylic acid-divinylbenzene copolymer.
- (5) Cross-linked polystyrene, first chloromethylated then aminated with trimethylamine, dimethylamine, di-ethylenetriamine, or dimethylethanol-amine.
- (6) Diethylenetriamine, triethylene-tetramine, or tetraethylenepentamine cross-linked with epichlorohydrin.
- (7) Cross-linked phenol-formaldehyde activated with one or both of the following: Triethylene tetramine and tetraethylenepentamine.
- (8) Reaction resin of formaldehyde, acetone, and tetraethylenepentamine.
- (9) Completely hydrolyzed copolymers of methyl acrylate and divinylbenzene.
- (10) Completely hydrolyzed terpolymers of methyl acrylate, divinylbenzene, and acrylonitrile.
- (11) Sulfonated terpolymers of styrene, divinylbenzene, and acrylonitrile or methyl acrylate.
- (12) Methyl acrylate-divinylbenzene copolymer containing not less than 2 percent by weight of divinylbenzene, aminolyzed with dimethylaminopro-pylamine.
- (13) Methyl acrylate-divinylbenzene copolymer containing not less than 3.5 percent by weight of divinylbenzene, aminolyzed with

dimethylaminopro-pylamine.

(14) Epichlorohydrin cross-linked with ammonia.

(15) Sulfonated tetrapolymer of styrene, divinylbenzene, acrylonitrile, and methyl acrylate derived from a mixture of monomers containing not more than a total of 2 percent by weight of acrylonitrile and methyl acrylate.

(16) Methyl acrylate-divinyl benzene di ethylene glycol divinyl ether terpolymer containing not less than 3.5 percent by weight of di vinyl benzene and not more than 0.6 percent by weight of di ethylene glycol divinyl ether, aminolyzed with di methyl amino propyl amine.

(17) Styrene-divinylbenzene cross-linked copolymer, first chloromethylated then aminated with dimethylamine and oxidized with hydrogen peroxide whereby the resin contains not more than 15 percent by weight of vinyl N,N-di methyl benzyl amine-N-oxide and not more than 6.5 percent by weight of nitrogen.

(18) Methyl acrylate-divinylbenzene-diethylene glycol divinyl ether terpolymer containing not less than 7 percent by weight of divinylbenzene

[[Page 123]]

and not more than 2.3 percent by weight of diethylene glycol divinyl ether, aminolyzed with di methyl amino propyl amine and quaternized with methyl chloride.

(19) Epichlorohydrin cross-linked with ammonia and then quaternized with methyl chloride to contain not more than 18 percent strong base capacity by weight of total exchange capacity [Chemical Abstracts Service name: Oxirane (chloromethyl)-, polymer with ammonia, reaction product with chloromethane; CAS Reg. No. 68036-99-7].

(20) Regenerated cellulose, cross-linked and alkylated with epi chloro hydrin and propylene oxide, then sulfonated whereby the amount of epi chloro hydrin plus propylene oxide employed does not exceed 250 percent by weight of the starting quantity of cellulose.

(b) Ion-exchange resins are used in the purification of foods, including potable water, to remove undesirable ions or to replace less desirable ions with one or more of the following: bicarbonate, calcium, carbonate, chloride, hydrogen, hydroxyl, magnesium, potassium, sodium, and sulfate except that: The ion-exchange resin identified in paragraph (a) (12) of this section is used only in accordance with paragraph (b) (1) of this section, the ion-exchange resin identified in paragraph (a) (13) of this section is used only in accordance with paragraph (b) (2) of this section, the resin identified in paragraph (a) (16) of this section is used only in accordance with paragraph (b) (1) or (b) (2) of this section, the ion-exchange resin identified in paragraph (a) (17) of this section is used only in accordance with paragraph (b) (3) of this section, the ion-exchange resin identified in paragraph (a) (18) of this section is used only in accordance with paragraph (b) (4) of this section, and the ion-exchange resin identified in paragraph (a) (20) of this section is used only in accordance with paragraphs (b) (5) and (d) of this section.

(1) The ion-exchange resins identified in paragraphs (a) (12) and (16) of this section are used to treat water for use in the manufacture of distilled alcoholic beverages, subject to the following conditions:

(i) The water is subjected to treatment through a mixed bed consisting of one of the resins identified in paragraph (a) (12) or (16) of this section and one of the strongly acidic cation-exchange

resins in the hydrogen form identified in paragraphs (a) (1), (2), and (11) of this section; or

(ii) The water is first subjected to one of the resins identified in paragraph (a) (12) or (16) of this section and is subsequently subjected to treatment through a bed of activated carbon or one of the strongly acidic cation-exchange resins in the hydrogen form identified in paragraphs (a) (1), (2), and (11) of this section.

(iii) The temperature of the water passing through the resin beds identified in paragraphs (b) (1) (i) and (ii) of this section is maintained at 30 [deg]C or less, and the flow rate of the water passing through the beds is not less than 2 gallons per cubic foot per minute.

(iv) The ion-exchange resins identified in paragraph (a) (12) or (16) of this section are exempted from the requirements of paragraph (c) (4) of this section, but the strongly acidic cation-exchange resins referred to in paragraphs (b) (1) (i) and (ii) of this section used in the process meet the requirements of paragraph (c) (4) of this section, except for the exemption described in paragraph (d) of this section.

(2) The ion-exchange resins identified in paragraphs (a) (13) and (16) of this section are used to treat water and aqueous food only of the types identified under Categories I, II, and VI-B in table 1 of Sec. 176.170(c) of this chapter: Provided, That the temperature of the water or food passing through the resin beds is maintained at 50 [deg]C or less and the flow rate of the water or food passing through the beds is not less than 0.5 gallon per cubic foot per minute.

(i) The ion-exchange resin identified in paragraph (a) (13) of this section is used to treat water and aqueous food only of the types identified under categories I, II, and VI-B in Table 1 of Sec. 176.170(c) of this chapter: Provided, That the temperature of the water or food passing through the resin bed is maintained at 50 [deg]C or less and the flow rate of the water or food passing through the bed is not less than 0.5 gallon per cubic foot per minute.

(ii) The ion-exchange resin identified in paragraph (a) (16) of this section is used to treat water and aqueous food only of the types identified under categories I, II, and VI-B in Table 1 of Sec. 176.170(c) of this chapter, Provided, that either:

(A) The temperature of the water or food passing through the resin bed is maintained at 50 [deg]C or less and the flow rate of the water or food passing through the bed is not less than 0.5 gallon per cubic foot per minute; or

(B) Extracts of the resin will be found to contain no more than 1 milligram/kilogram dimethylaminopropylamine in each of the food simulants, distilled water and 10 percent ethanol, when, following washing and pretreatment of the resin in accordance with Sec.

173.25(c) (1), the resin is subjected to the following test under conditions simulating the actual temperature and flow rate of use:

``The Determination of 3-Dimethylaminopropylamine in Food Simulating Extracts of Ion Exchange Resins,`` February 4, 1998, which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the Division of Petition Control (HFS-215), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740, or may be examined at the Center for Food Safety and Applied Nutrition's Library, 5100 Paint Branch Pkwy., College Park, MD 20740, or at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: <http://www.archives.gov/federal--register/code--of--federal--regulations/ibr--locations.html>.

(3) The ion-exchange resin identified in paragraph (a)(17) of this section is used only for industrial application to treat bulk quantities of aqueous food, including potable water, or for treatment of municipal water supplies, subject to the condition that the temperature of the food or water passing through the resin bed is maintained at **25** [deg]C or less and the flow rate of the food or water passing through the bed is not less than 2 gallons per cubic foot per minute.

(4) The ion-exchange resin identified in paragraph (a)(18) of this section is used to treat aqueous sugar solutions subject to the condition that the temperature of the sugar solution passing through the resin bed is maintained at 82 [deg]C (179.6 [deg]F) or less and the flow rate of the sugar solution passing through the bed is not less than 46.8 liters per cubic meter (0.35 gallon per cubic foot) of resin bed volume per minute.

(5) The ion-exchange resin identified in paragraph (a)(20) of this section is limited to use in aqueous process streams for the isolation and purification of protein concentrates and isolates under the following conditions:

(i) For resins that comply with the requirements in paragraph (d)(2)(i) of this section, the pH range for the resin shall be no less than 3.5 and no more than 9, and the temperatures of water and food passing through the resin bed shall not exceed **25** [deg]C.

(ii) For resins that comply with the requirements in paragraph (d)(2)(ii) of this section, the pH range for the resin shall be no less than 2 and no more than 10, and the temperatures of water and food passing through the resin shall not exceed 50 [deg]C.

(c) To insure safe use of ion-exchange resins, each ion-exchange resin will be:

(1) Subjected to pre-use treatment by the manufacturer and/or the user in accordance with the manufacturer's directions prescribed on the label or labeling accompanying the resins, to guarantee a food-grade purity of ion-exchange resins, in accordance with good manufacturing practice.

(2) Accompanied by label or labeling to include directions for use consistent with the intended functional purpose of the resin.

(3) Used in compliance with the label or labeling required by paragraph (c)(2) of this section.

(4) Found to result in no more than 1 part per million of organic extractives obtained with each of the named solvents, distilled water, 15 percent alcohol, and 5 percent acetic acid when, having been washed and otherwise treated in accordance with the manufacturer's directions for preparing them for use with food, the ion-exchange resin is subjected to the following test: Using a separate ion-exchange column for each solvent, prepare columns using 50 milliliters of the ready to use ion-exchange resin that is to be tested. While maintaining the highest temperature that will be encountered in use pass through these beds at the rate of 350-450 milliliters per hour the three test solvents distilled water, 15 percent (by volume) ethyl alcohol, and 5 percent (by weight) acetic acid. The first liter of effluent from each solvent is discarded, then the next 2 liters are used to determine organic extractives. The 2-liter sample is carefully evaporated to constant weight at 105 [deg]C; this is total extractives. This residue is fired in a muffle furnace at 850 [deg]C to constant weight; this is ash. Total extractives, minus ash equals the organic extractives. If the organic extractives are greater than 1 part per million of the solvent used, a blank should be run on the solvent and a correction

should be made by subtracting the total extractives obtained with the blank from the total extractives obtained in the resin test. The solvents used are to be made as follows:

Distilled water (de-ionized water is distilled).

15 percent ethyl alcohol made by mixing 15 volumes of absolute ethyl alcohol A.C.S. reagent grade, with 85 volumes of distilled de-ionized water.

5 percent acetic acid made by mixing 5 parts by weight of A.C.S. reagent grade glacial acetic acid with 95 parts by weight of distilled de-ionized water.

In addition to the organic extractives limitation prescribed in this paragraph, the ion-exchange resin identified in paragraph (a)(17) of this section, when extracted with each of the named solvents, distilled water, 50 percent alcohol, and 5 percent acetic acid, will be found to result in not more than 7 parts per million of nitrogen extractives (calculated as nitrogen) when the resin in the free-base form is subjected to the following test immediately before each use: Using a separate 1-inch diameter glass ion-exchange column for each solvent, prepare each column using 100 milliliters of ready to use ion-exchange resin that is to be tested. With the bottom outlet closed, fill each ion-exchange column with one of the three solvents at a temperature of 25 [deg]C until the solvent level is even with the top of the resin bed.

Seal each column at the top and bottom and store in a vertical position at a temperature of 25 [deg]C. After 96 hours, open the top of each column, drain the solvent into a collection vessel, and analyze each drained solvent and a solvent blank for nitrogen by a standard micro-Kjeldahl method.

(d)(1) The ion-exchange resins identified in paragraphs (a)(1), (a)(2), (a)(11), and (a)(15) of this section are exempted from the acetic acid extraction requirement of paragraph (c)(4) of this section.

(2) The ion-exchange resin identified in paragraph (a)(20) of this section shall comply either with:

(i) The extraction requirement in paragraph (c)(4) of this section by using dilute sulfuric acid, pH 3.5 as a substitute for acetic acid; or

(ii) The extraction requirement in paragraph (c)(4) of this section by using reagent grade hydrochloric acid, diluted to pH 2, as a substitute for acetic acid. The resin shall be found to result in no more than **25** parts per million of organic extractives obtained with each


of the following solvents: Distilled water; 15 percent alcohol; and hydrochloric acid, pH 2. Blanks should be run for each of the solvents, and corrections should be made by subtracting the total extractives obtained with the blank from the total extractives obtained in the resin test.

(e) Acrylonitrile copolymers identified in this section shall comply with the provisions of Sec. 180.22 of this chapter.

[42 FR 14526, Mar. 15, 1977, as amended at 46 FR 40181, Aug. 7, 1981; 46

FR 57033, Nov. 20, 1981; 49 FR 28830, July 17, 1984; 56 FR 16268, Apr.

22, 1991; 62 FR 7679, Feb. 20, 1997; 64 FR 14609, Mar. 26, 1999; 64 FR
56173, Oct. 18, 1999]

| | | |
|--|---|------------------------------|
| Specification |  | Last changed date 24-12-2004 |
| | | Page 1 van 1 |
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Actieve kool

(SG 60200000010)

SG504952

1. GENERAL INFORMATION

| | |
|----------------------------|---|
| Name product | Norit PN 2 |
| General discription | Steam activated carbon made from raw materials with vegetable origin for the purification of lactose |
| Legal declaration | Raw materials have to comply with Dutch and E.C. regulations for food products. |
| Remark | Ingredient must not be derived from genetically modified raw materials. Also free of foreign material and allergens that are not listed on the ingredient declaration |
| Resource Number | Number 504952 to be mentioned on each unit |
| Production and expiry date | To be mentioned on each unit |
| Shelf life | At least 1 year |
| Storage conditions | No special requirements |
| Packaging | Paper bags 20 kg |
| Documents | Packing list + certificate of analysis |
| Change control | Significant changes in raw materials, processing etc. need to be approved by BDI prior to implementation |

2. PRODUCT SPECIFICATION

Properties

Chemical

Molasse number
Moisture (W/W %)
PH

Specification

| | |
|-----------|----------|
| Max. 390 | NSTM (*) |
| Max.10 | NSTM (*) |
| 6.0 - 7.2 | NSTM (*) |

Physical

Appearance

Typical

(*) Norit standard test methods

3. CERTIFICATE OF ANALYSIS

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

Appendix 1A Exhibit 5

Cellulose

[Code of Federal Regulations]
[Title 21, Volume 3]
[Revised as of April 1, 2006]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR172]
[Page 105-106]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN
SERVICES (CONTINUED)

PART 172 FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN
CONSUMPTION--Table of Contents

Subpart I_Multipurpose Additives

Sec. 172.868 Ethyl cellulose.

The food additive ethyl cellulose may be safely used in food in
accordance with the following prescribed conditions:

(a) The food additive is a cellulose ether containing ethoxy
(OC<INF>2</INF>H<INF>5</INF>) groups attached by an ether linkage and
containing on an anhydrous basis not more than 2.6 ethoxy groups per
anhydroglucose unit.

(b) It is used or intended for use as follows:

- (1) As a binder and filler in dry vitamin preparations.
- (2) As a component of protective coatings for vitamin and mineral
tablets.
- (3) As a fixative in flavoring compounds.

[Code of Federal Regulations]
[Title 21, Volume 3]
[Revised as of April 1, 2006]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR172]

[Page 107]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN SERVICES (CONTINUED)

PART 172 FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN CONSUMPTION--Table of Contents

Subpart I_Multipurpose Additives

Sec. 172.870 Hydroxypropyl cellulose.

The food additive hydroxypropyl cellulose may be safely used in food, except standardized foods that do not provide for such use, in accordance with the following prescribed conditions:

(a) The additive consists of one of the following:

(1) A cellulose ether containing propylene glycol groups attached by an ether linkage which contains, on an anhydrous basis, not more than 4.6 hydroxypropyl groups per anhydroglucose unit. The additive has a minimum viscosity of 145 centipoises for 10 percent by weight aqueous solution at 25 [deg]C.

(2) A cellulose ether containing propylene glycol groups attached by an ether linkage having a hydroxypropoxy
(OC<INF>3</INF>H<INF>6</INF>OH)
content of 5 to 16 percent weight in weight (w/w) on an anhydrous basis, i.e., 0.1 to 0.4 hydroxypropyl groups per anhydroglucose unit. The common name for this form of the additive is low substituted hydroxypropyl cellulose.

(b) The additive is used or intended for use as follows:

(1) The additive identified in paragraph (a)(1) of this section is used or intended for use as an emulsifier, film former, protective colloid, stabilizer, suspending agent, or thickener, in accordance with good manufacturing practice.

(2) The additive identified in paragraph (a)(2) of this section is used or intended for use as a binder and disintegrator in tablets or wafers containing dietary supplements of vitamins and/or minerals. The additive is used in accordance with good manufacturing practice.

[46 FR 50065, Oct. 9, 1981]

[Code of Federal Regulations]
[Title 21, Volume 3]
[Revised as of April 1, 2006]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR172]
[Page 107-108]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN SERVICES (CONTINUED)

PART 172 FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN CONSUMPTION--Table of Contents

Subpart I_Multipurpose Additives

Sec. 172.872 Methyl ethyl cellulose.

The food additive methyl ethyl cellulose may be safely used in food in accordance with the following prescribed conditions.

(a) The additive is a cellulose ether having the general formula $[C_6H_{10}-x-y)O_5(CH_3)_x(C_2H_5)_y]_n$, where x is the number of methyl groups and y is the number of ethyl groups. The average value of x is 0.3 and the average value of y is 0.7.

(b) The additive meets the following specifications:

(1) The methoxy content shall be not less than 3.5 percent and not more than 6.5 percent, calculated as OCH_3 , and the ethoxy content shall be not less than 14.5 percent and not more than 19 percent, calculated as OC_2H_5 , both measured on the dry sample.

(2) The viscosity of an aqueous solution, 2.5 grams of the material in 100 milliliters of water, at 20 [deg]C, is 20 to 60 centipoises.

(3) The ash content on a dry basis has a maximum of 0.6 percent.

(c) The food additive is used as an aerating, emulsifying, and foaming agent, in an amount not in excess of that reasonably required to produce its intended effect.

[Code of Federal Regulations]
[Title 21, Volume 3]
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[Page 108]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN
SERVICES (CONTINUED)

PART 172 FOOD ADDITIVES PERMITTED FOR DIRECT ADDITION TO FOOD FOR HUMAN
CONSUMPTION--Table of Contents

Subpart I_Multipurpose Additives

Sec. 172.874 Hydroxypropyl methylcellulose.


The food additive hydroxypropyl methylcellulose (CAS Reg. No. 9004-65-3) may be safely used in food, except in standardized foods which do not provide for such use if:

(a) The additive complies with the definition and specifications prescribed in the National Formulary, 12th edition.

(b) It is used or intended for use as an emulsifier, film former, protective colloid, stabilizer, suspending agent, or thickener, in accordance with good manufacturing practice.

(c) To insure safe use of the additive, the container of the additive, in addition to being labeled as required by the general provisions of the act, shall be accompanied by labeling which contains adequate directions for use to provide a final product that complies with the limitations prescribed in paragraph (b) of this section.

[42 FR 14491, Mar. 15, 1977, as amended at 47 FR 38273, Aug. 31, 1982]

| | | |
|--|---|------------------------------|
| Specification |  | Last changed date 24-12-2004 |
| | | Page 1 van 1 |
| In case of document is printed, it concerns an uncontrolled copy The authorised document is placed at Intranet | | |

Alpha-Cel HKB200

(SG 60200000110)

SG504969

1. GENERAL INFORMATION

Name product
General discription
Legal declaration

Alpha-Cel HKB200 TM
100% purified powdered cellulose filter aid
Material has to comply with requirements in The Food Chemical Codex, 4th Edition, pages 96-97. Furthermore material must comply with relevant regulations for food grade products.

Remark

Ingredient must not be derived from genetically modified raw materials. Also free of foreign material and allergens that are not listed on the ingredient declaration

Resource number
Production and expiry date
Shelf life
Storage conditions
Packaging
Documents
Change control

Number 504969 to be mentioned on each unit
To be mentioned on each unit
At least 1 year
No special requirements
paper bags 23 kg
Packing list + certificate of analysis
Significant changes in raw materials, processing etc. need to be approved by BDI prior to implementation

2. PRODUCT SPECIFICATION

Properties **Chemical**

pH
Ash (total)
Heavy metals, as lead

Specification

5,0 – 7,5
< 0,5%
< 0,01 ppm

Method of analysis

10% solution

Physical

Appearance
Average particle length
Loose bulk Density
Packed bulk Volume

Fine white powder
100 µm
170 – 190 g/l
140 – 200 ml/50 gram

Typical Screen analysis

On > 40 mesh (420 µm)
Thru < 100 mesh (150 µm)
Thru < 200 mesh (75 µm)

0%
>95%
>80%

3. CERTIFICATE OF ANALYSIS

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

Appendix 1A

Exhibit 6

Hydrochloric Acid

[Code of Federal Regulations]
[Title 21, Volume 3]
[Revised as of April 1, 2006]
From the U.S. Government Printing Office via GPO Access
[CITE: 21CFR182]

[Page 473]

TITLE 21--FOOD AND DRUGS

CHAPTER I--FOOD AND DRUG ADMINISTRATION, DEPARTMENT OF HEALTH AND HUMAN
SERVICES (CONTINUED)

PART 182_SUBSTANCES GENERALLY RECOGNIZED AS SAFE--Table of Contents

Subpart B_Multiple Purpose GRAS Food Substances

Sec. 182.1057 Hydrochloric acid.

- (a) Product. Hydrochloric acid.
- (b) [Reserved]
- (c) Limitations, restrictions, or explanation. This substance is generally recognized as safe when used as a buffer and neutralizing agent in accordance with good manufacturing practice.

Hydro Chlorid Acid 30 %, SG010903

GENERAL INFORMATION

| | |
|----------------------------|--|
| Name product | Hydrochloric acid 30 % |
| General discription | Hydrochloric acid solution in water |
| Legal declaration | Raw materials have to comply with EC legislation for food additives (96/77/EC) and the Food Chemicals Code |
| Remark | Ingredient must be free of foreign material and allergens that are not listed on the ingredient declaration |
| Resource Number | SAP number 010903 to be mentioned on each unit |
| Documents | Delivery note, cert. of analysis, cleaning certificate (unless former load was the same product) |
| Production and expiry date | To be mentioned on certificate of analysis |
| Guaranteed shelflife | Indefinite, under correct storage conditions |
| Packaging | Bulk product, delivered by road tanker and stored in tank |
| Storage conditions | In well closed tank |
| Security full truck loads | Hoses carriers and outlet valves must be end capped and security tagged. Hatches or hatch covers must be security tagged. All security tag numbers must be recorded and available for inspection before unloading. All security tags must be intact on arrival. |

PRODUCT SPECIFICATION

| <u>Properties</u> | <u>Specification</u> | <u>Method of analysis</u> |
|--|-------------------------|---------------------------|
| Chemical | | |
| Specific gravity | 1149 kg/ m ³ | at 20 °C |
| HCL | 29 – 31 % | SAM 101.01 |
| Oxidizing substances (as Cl ₂) | Max 30 mg/kg | ISO 908 |
| Reducing substances (as SO ₂) | Max 70 mg/kg | |
| Sulphate | Max 0.5 % | |
| Iron | Max 5 mg/kg | SAM 104.07 |
| Lead | Max 1 mg/kg | |
| Arcenicum | Max 1 mg/kg | |
| Mercury | Max 1 mg/kg | |
| Organic comp. (Fluorcomp. excl.) | Max 5 mg/kg | |
| Benzene | Max 0.05 mg/kg | |
| Organic fluor compounds | Max 25 mg/kg | |
| Loss after drying | Max 0.1 % | SAM 117.07 |
| Physical | | |
| Appearance | Liquid | Sensory evaluation method |
| Colour | Clear to yellowish | Visual observation method |
| Aroma | Irritating odour | Sensory evaluation method |
| Flavour | Acidic | Sensory evaluation method |
| Microbiological | | |
| Salmonella | Absent in 25 ml | IDF 93B (1995) |
| Staphylococcus aureus | Max 100.000 CFU/ml | IDF 60C (1997) |

CERTIFICATE OF ANALYSIS

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

Appendix 1A Exhibit 7

Perlite

Code of Federal Regulations]
[Title 7, Volume 3]
[Revised as of January 1, 2006]
From the U.S. Government Printing Office via GPO Access
[CITE: 7CFR205.605]

[Page 422-423]

TITLE 7--AGRICULTURE

CHAPTER I--AGRICULTURAL MARKETING SERVICE \1\ (STANDARDS, INSPECTIONS, MARKETING PRACTICES), DEPARTMENT OF AGRICULTURE (CONTINUED)

PART 205_NATIONAL ORGANIC PROGRAM--Table of Contents

Subpart G_Administrative

Sec. 205.605 Nonagricultural (nonorganic) substances allowed as ingredients in or on processed products labeled as ``organic'' or ``made with organic (specified ingredients or food group(s)).''

The following nonagricultural substances may be used as ingredients in or on processed products labeled as ``organic'' or ``made with organic (specified ingredients or food group(s))'' only in accordance with any restrictions specified in this section.

(a) Nonsynthetics allowed:

Acids (Alginic; Citric--produced by microbial fermentation of carbohydrate substances; and Lactic).

Agar-agar.

Animal enzymes--(Rennet--animals derived; Catalase--bovine liver; Animal lipase; Pancreatin; Pepsin; and Trypsin).

Bentonite.

Calcium carbonate.

Calcium chloride.

Calcium sulfate--mined.

Carageenan.

Colors, nonsynthetic sources only.

Dairy cultures.

Diatomaceous earth--food filtering aid only.

Enzymes--must be derived from edible, nontoxic plants, nonpathogenic fungi, or nonpathogenic bacteria.

Flavors, nonsynthetic sources only and must not be produced using synthetic solvents and carrier systems or any artificial preservative.

Glucono delta-lactone--production by the oxidation of D-glucose with bromine water is prohibited.

Kaolin.

Magnesium sulfate, nonsynthetic sources only.

Nitrogen--oil-free grades.

Oxygen--oil-free grades.

Perlite--for use only as a filter aid in food processing.

Potassium chloride.

Potassium iodide.

Sodium bicarbonate.

Sodium carbonate.

Tartaric acid.

Waxes--nonsynthetic (Carnauba wax; and Wood resin).

Yeast--nonsynthetic, growth on petrochemical substrate and sulfite

waste liquor is prohibited (Autolysate; Bakers; Brewers; Nutritional; and Smoked--nonsynthetic smoke flavoring process must be documented).

(b) Synthetics allowed:

Alginates.

Ammonium bicarbonate--for use only as a leavening agent.

Ammonium carbonate--for use only as a leavening agent.

Ascorbic acid.

Calcium citrate.

Calcium hydroxide.

Calcium phosphates (monobasic, dibasic, and tribasic).

Carbon dioxide.

Cellulose--for use in regenerative casings, as an anti-caking agent (non-chlorine bleached) and filtering aid.

Chlorine materials--disinfecting and sanitizing food contact surfaces, Except, That, residual chlorine levels in the water shall not exceed the maximum residual disinfectant limit under the Safe Drinking Water Act (Calcium hypochlorite; Chlorine dioxide; and Sodium hypochlorite).

Ethylene--allowed for postharvest ripening of tropical fruit and degreening of citrus.

Ferrous sulfate--for iron enrichment or fortification of foods when required by regulation or recommended (independent organization).

Glycerides (mono and di)--for use only in drum drying of food.

Glycerin--produced by hydrolysis of fats and oils.

Hydrogen peroxide.

Lecithin--bleached.

Magnesium carbonate--for use only in agricultural products labeled ``made with organic (specified ingredients or food group(s)),'' prohibited in agricultural products labeled ``organic''.

Magnesium chloride--derived from sea water.

Magnesium stearate--for use only in agricultural products labeled ``made with organic (specified ingredients or food group(s)),'' prohibited in agricultural products labeled ``organic''.

Nutrient vitamins and minerals, in accordance with 21 CFR 104.20, Nutritional Quality Guidelines For Foods.

Ozone.

Pectin (low-methoxy).

Phosphoric acid--cleaning of food-contact surfaces and equipment only.

Potassium acid tartrate.

Potassium tartrate made from tartaric acid.

Potassium carbonate.

Potassium citrate.

Potassium hydroxide--prohibited for use in lye peeling of fruits and vegetables except when used for peeling peaches during the Individually Quick Frozen (IQF) production process.

Potassium iodide--for use only in agricultural products labeled ``made with organic (specified ingredients or food group(s)),'' prohibited in agricultural products labeled ``organic''.

Potassium phosphate--for use only in agricultural products labeled ``made with organic (specific ingredients or food group(s)),'' prohibited in agricultural products labeled ``organic''.

Silicon dioxide.

Sodium citrate.

Sodium hydroxide--prohibited for use in lye peeling of fruits and vegetables.

Sodium phosphates--for use only in dairy foods.

Sulfur dioxide--for use only in wine labeled ``made with organic grapes,`` Provided, That, total sulfite concentration does not exceed 100 ppm.

Tartaric acid.

Tocopherols--derived from vegetable oil when rosemary extracts are not a suitable alternative.

Xanthan gum.

(c)-(z) [Reserved]

[68 FR 61993, Oct. 31, 2003, as amended as 68 FR 62217, Nov. 3, 2003]

Perlite, SG504976

GENERAL INFORMATION

Name product
General description
Legal declaration

Remark

Resource number
Production date
Shelf life
Storage conditions
Packaging
Documents
Change control

Dicalite 4208
Thermally expanded vulcanic rock, aluminium silicate
Raw materials have to comply with Dutch and E.C. regulations for food products.
Ingredient must not be derived from genetically modified raw materials. Also free of foreign material and allergens that are not listed on the ingredient declaration
Number 504976 to be mentioned on each unit
To be mentioned on each unit
At least 1 year
No special requirements
paper bags 14,4 kg
Packing list + certificate of analysis
Significant changes in raw materials, processing etc. need to be approved by BDI prior to implementation

PRODUCT SPECIFICATION

Properties

Chemical

Silicon (%)
Aluminium (%)
Calcium (%)
Iron (%)
Manganese (%)
Potassium (%)
Sodium (%)

Specification

31 - 35
7.0 - 12.0
0.2 - 0.5
0.4 - 0.7
0.05 - 0.10
2.5 - 5.0
3.0 - 5.3

Method of analysis

Physical

Appearance
Particle shape
Colour
Flowrate
Cake density (lbs/ft³)
Float (ml/20 gr.)

dry powder
Multihedral plates
white to off-white
125 - 180
max. 11.3
max. 45

CERTIFICATE OF ANALYSIS

- Production date
- Permeability
- Cake density
- Float

Appendix 1B

Materials Used in the Production of Vivinal[®] GOS: Resin Specifications

PROPRIETARY PROCESS TECHNOLOGY

Contents

Appendix 1B: Resin Specifications

[PROPRIETARY PROCESS TECHNOLOGY]

Exhibit 1 Resin 1 Specifications

Exhibit 2 Resin 2 Specifications

Exhibit 3 Resin 3 Specifications

Appendix 1B Exhibit 1

Resin 1 Specifications

PROPRIETARY PROCESS TECHNOLOGY

**APPLEXION SAS**

264 avenue de la Mauldre
78680 EPONE - France

CERTIFICATE OF ANALYSIS

| | |
|-------------------------|------------------|
| PRODUCT NAME : | XA 748 Na |
| Our project reference : | 1342/10.6251 |
| Shipping units : | 2 250 l. |
| Batch number : | 02551494 |
| Net weight : | 1 822 kg |
| Manufacturing date : | 29.05.2006 |
| Expiration date : | 02.06.2009 |

ANALYSIS

| Test | Unit | Lower Limit | Upper Limit | Value |
|----------------------------|--------|-------------|-------------|-------|
| UNIFORMITY COEFFICIENT | Number | 0,00 | 1,80 | 1,32 |
| HARMONIC MEAN SIZE | mm | 0,590 | 0,84 | 0,667 |
| % < 0,300 MM | % | 0,00 | 1,00 | 0,00 |
| VOLUME TOTAL CAPACITY (NA) | eq/l | 1,80 | 999999,00 | 1,90 |
| % > 1,180 mm | % | 0,00 | 5,00 | 0,00 |
| MOISTURE HOLD CONT. (NA) | % | 47,00 | 54,00 | 49,70 |

Analytical results on this certificate conform to documented test plan.

APPLEXION
264, Avenue de la Mauldre
78681 EPONE CEDEX
Tél. : 01 30 90 50 00
Fax : 01 30 91 05 31
e-mail : applexionf@aol.com

25/10/2006

www.novasep.com



APPLEXION SAS

264 avenue de la Mauldre
78680 EPONE - France

Kosher status

1342/10.6251(3/3 EN)

Dear Sirs,

With respect to your inquiry concerning the Kosher status of

XA 748 Na RESIN

APPLEXION – NOVASEP Process has completed a review of the Kosher status of its Ion Exchange and Adsorbent Resins.

The review with the Orthodox Union revealed the following: Though most ion exchange or adsorbent resins are produced primarily from synthetic raw materials, some resins may use in their manufacture small amount of gelatin [of non-ruminant origin] in the early stages of their synthesis. These latter resins, including **XA 748 Na RESIN**, cannot be considered fully Kosher due to the presence of a [non-Kosher] material. The Kosher status of gelatin is currently under discussion.

However, according to Orthodox Union, **all APPLEXION – NOVASEP Process ion exchange and adsorbent resins would be most likely acceptable for use in producing Kosher foods**. Reason being that ion exchange and adsorbent resins are not sold as Food, but are only used as processing aids for food production. Ion exchange and adsorbent resins are not intended to remain in the food after the technical effect has been accomplished.

In accordance with the recommendations from the Orthodox Union, APPLEXION – NOVASEP Process recommends that the use of the resins be evaluated on a case to case basis, but will most likely be acceptable for production of most Kosher foods.

Epône, October 26, 2006

(b)(6)

P.PRINTEMPS

www.novasep.com



APPLEXION SAS

264 avenue de la Mauldre
78680 EPONE - France

Food and Drug Administration (FDA)

1342/10.6251(3/3 EN)

We hereby confirm that our ion exchange resin :

XA 748 Na RESIN

compositionally complies with **FDA regulation 21CFR 173.25** concerning foodstuffs intended for human consumption as per the following limitation :

The resin must be subjected to the pre-use treatment in accordance with the manufacturer's directions and meet the extractives limitations as prescribed in Paragraph (c) of FDA regulation 21CFR 173.25.

It is however your responsibility to abide by any clause of this (or other) regulation(s) that may apply to the specific use you make of our product.

This information is correct to the best of our current knowledge. We recommend that you verify on a regular basis with APPLEXION – NOVASEP Process, the latest food contact status of our product.

Epône, October 26, 2006

(b)(6)

P. PRINTEMPS

XA 748 Na

Acid Cation Exchange Resin

XA 748 Na is a macroporous cation exchange resin based on sulphonated crosslinked polystyrene. It has a moderate degree of crosslinking resulting in good regeneration efficiency. It is very resistant to osmotic shock and to mechanical attrition. XA 748 Na has a reduced amount of fines, allowing it to be used for the treatment of highly concentrated solutions. XA 748 Na is suited for condensate treatment, decalcification and demineralisation of sugar juices and treatment of oxidising solutions.

PROPERTIES

| | |
|--|-------------------------------------|
| Matrix _____ | Styrene divinylbenzene copolymer |
| Functional groups _____ | -SO ₃ ⁻ |
| Physical form _____ | Light grey beads |
| Ionic form as shipped _____ | Na ⁺ |
| Total exchange capacity ^[1] _____ | ≥ 1.8 eq/l (Na ⁺ form) |
| Moisture holding capacity ^[1] _____ | 47 - 54 % (Na ⁺ form) |
| Shipping weight _____ | 810 g/l |
| Specific gravity _____ | 1.20 to 1.24 (Na ⁺ form) |
| Particle size : | |
| Uniformity coefficient _____ | ≤ 1.8 |
| Harmonic mean size _____ | 500 - 840 µm |

[1]: Test methods are available on request.

Appendix 1B
Exhibit 2

Resin 2 Specifications

PROPRIETARY PROCESS TECHNOLOGY

**APPLEXION SAS**

264 avenue de la Mauldre
78680 EPONE - France

CERTIFICATE OF ANALYSIS

| | |
|-------------------------|---------------|
| PRODUCT NAME : | XA 904 |
| Our project reference : | 1342/10.6251 |
| Shipping units : | 2 250 l. |
| Batch number : | 02838603 |
| Net weight : | 1 507,50 kg |
| Manufacturing date : | 10.09.2006 |
| Expiration date : | 14.09.2009 |

ANALYSIS

| Test | Unit | Lower Limit | Upper Limit | Value |
|----------------------------|--------|-------------|-------------|-------|
| UNIFORMITY COEFFICIENT | Number | 0,00 | 1,80 | 1,47 |
| HARMONIC MEAN SIZE | mm | 0,550 | 0,750 | 0,603 |
| % < 0,300 MM | % | 0,00 | 1,00 | 0,2 |
| % > 1,180 mm | % | 0,00 | 1,00 | 0,00 |
| VOLUME TOTAL CAPACITY (NA) | eq/l | 1,25 | 999999,00 | 1,37 |
| MOISTURE HOLD CONT. (NA) | % | 57,00 | 63,00 | 60,40 |

Analytical results on this certificate conform to documented test plan.

APPLEXION
264, Avenue de la Mauldre
78681 EPONE CEDEX
Tél. : 01 30 90 50 00
Fax : 01 30 91 05 31
e-mail : applexion@aol.com

25/10/2006

www.novasep.com



APPLEXION SAS

264 avenue de la Mauldre
78680 EPONE - France

Kosher status

1342/10.6251(1/3 EN)

Dear Sirs,

With respect to your inquiry concerning the Kosher status of

XA 904 RESIN

APPLEXION – NOVASEP Process has completed a review of the Kosher status of its Ion Exchange and Adsorbent Resins.

The review with the Orthodox Union revealed the following: Though most ion exchange or adsorbent resins are produced primarily from synthetic raw materials, some resins may use in their manufacture small amount of gelatin [of non-ruminant origin] in the early stages of their synthesis. These latter resins, including **XA 904 RESIN**, cannot be considered fully Kosher due to the presence of a [non-Kosher] material. The Kosher status of gelatin is currently under discussion.

However, according to Orthodox Union, **all APPLEXION – NOVASEP Process ion exchange and adsorbent resins would be most likely acceptable for use in producing Kosher foods**. Reason being that ion exchange and adsorbent resins are not sold as Food, but are only used as processing aids for food production. Ion exchange and adsorbent resins are not intended to remain in the food after the technical effect has been accomplished.

In accordance with the recommendations from the Orthodox Union, APPLEXION – NOVASEP Process recommends that the use of the resins be evaluated on a case to case basis, but will most likely be acceptable for production of most Kosher foods.

Epône, October 26, 2006

(b)(6)

P. PRINTEMPS

www.novasep.com



APPLEXION SAS

264 avenue de la Mauldre
78680 EPONE - France

Food and Drug Administration (FDA)

1342/10.6251(1/3 EN)

We hereby confirm that our ion exchange resin :

XA 904 RESIN

compositionally complies with **FDA regulation 21CFR 173.25** concerning foodstuffs intended for human consumption as per the following limitation :

- > *The resin must be subjected to the pre-use treatment in accordance with the manufacturer's directions and meet the extractives limitations as prescribed in Paragraph (c) of FDA regulation 21CFR 173.25.*
- > *For use in industrial applications to treat bulk quantities of aqueous food, potable water, or municipal water, subject to the condition that the temperature is maintained at 25°C or less and the flow rate of the food or water passing through the bed is not less than 2 gallons per cubic foot per minute.*

It is however your responsibility to abide by any clause of this (or other) regulation(s) that may apply to the specific use you make of our product.

This information is correct to the best of our current knowledge. We recommend that you verify on a regular basis with APPLEXION – NOVASEP Process, the latest food contact status of our product.

Epône, October 26, 2006

(b)(6)

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XA 904

Weak Base Anion Exchange Resin

XA 904 is a macroreticular weak base anion exchange resin. Its very stable structure and limited reversible swelling make it very resistant to osmotic shock. The high degree of porosity of this resin provides efficient adsorption of large organic molecules and their desorption during regeneration, thus allowing excellent protection against organic fouling. XA 904 is intended primarily for the removal of strong acids from water following a strongly acidic cation exchange resin, and it provides excellent protection against organic fouling for the strong base anion exchange resin placed in the same vessel.

PROPERTIES

| | |
|-----------------------------|----------------------------------|
| Matrix | Styrene divinylbenzene copolymer |
| Functional groups | Tertiary amine |
| Physical form | Opaque spherical beads |
| Ionic form as shipped | Free base (FB) |
| Total exchange capacity | ≥ 1.25 eq/L (FB form) |
| Moisture holding capacity | 57 to 63 % (FB form) |
| Specific gravity | 1.040 to 1.060 (FB form) |
| Shipping weight | 670 g/L |
| Particle size : | |
| Uniformity coefficient | ≤ 1.80 |
| Harmonic mean size | 550 to 750 µm |
| Fine contents | < 0.300 mm : 1.0 % max |
| Coarse beads | > 1.180 mm : 1.0 % max |
| Maximum reversible swelling | FB → Cl ⁻ : 15 % |

Appendix 1B Exhibit 3

Resin 3 Specifications

PROPRIETARY PROCESS TECHNOLOGY

**APPLEXION SAS**

264 avenue de la Mauldre
78680 EPONE - France

CERTIFICATE OF ANALYSIS

| | |
|-------------------------|-----------------|
| PRODUCT NAME : | XA 90 CI |
| Our project reference : | 1342/10.6251 |
| Shipping units : | 3 000 l. |
| Batch number : | 02709867 |
| Net weight : | 2 100 kg |
| Manufacturing date : | 27.07.2006 |
| Expiration date : | 31.07.2009 |

ANALYSIS

| Test | Unit | Lower Limit | Upper Limit | Value |
|----------------------------|--------|-------------|-------------|-------|
| UNIFORMITY COEFFICIENT | Number | 0,00 | 1,90 | 1,37 |
| HARMONIC MEAN SIZE | mm | 0,530 | 0,800 | 0,640 |
| % < 0,300 MM | % | 0,00 | 2,50 | 0,1 |
| VOLUME TOTAL CAPACITY (NA) | eq/l | 1,00 | 999999,00 | 1,21 |
| % > 1,180 mm | % | 0,00 | 5,00 | 0,00 |
| MOISTURE HOLD CONT. (NA) | % | 54,00 | 61,00 | 55,00 |

Analytical results on this certificate conform to documented test plan.

APPLEXION
264, Avenue de la Mauldre
78681 EPONE CEDEX
Tél. : 01 30 90 50 00
Fax : 01 30 91 05 31
e-mail : applexionf@aol.com

25/10/2006

www.novasep.com



APPLEXION SAS

264 avenue de la Mauldre
78680 EPONE - France

Kosher status

1342/10.6251(2/3 EN)

Dear Sirs,

With respect to your inquiry concerning the Kosher status of

XA 90 CI RESIN

APPLEXION – NOVASEP Process has completed a review of the Kosher status of its Ion Exchange and Adsorbent Resins.

The review with the Orthodox Union revealed the following: Though most ion exchange or adsorbent resins are produced primarily from synthetic raw materials, some resins may use in their manufacture small amount of gelatin [of non-ruminant origin] in the early stages of their synthesis. These latter resins, including **XA 90 CI RESIN**, cannot be considered fully Kosher due to the presence of a [non-Kosher] material. The Kosher status of gelatin is currently under discussion.

However, according to Orthodox Union, **all APPLEXION – NOVASEP Process ion exchange and adsorbent resins would be most likely acceptable for use in producing Kosher foods**. Reason being that ion exchange and adsorbent resins are not sold as Food, but are only used as processing aids for food production. Ion exchange and adsorbent resins are not intended to remain in the food after the technical effect has been accomplished.

In accordance with the recommendations from the Orthodox Union, APPLEXION – NOVASEP Process recommends that the use of the resins be evaluated on a case to case basis, but will most likely be acceptable for production of most Kosher foods.

Epône, October 26, 2006

(b)(6)

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APPLEXION SAS

264 avenue de la Mauldre
78680 EPONE - France

Food and Drug Administration (FDA)

1342/10.6251(2/3 EN)

We hereby confirm that our ion exchange resin :

XA 90 CI RESIN

compositionally complies with **FDA regulation 21CFR 173.25** concerning foodstuffs intended for human consumption as per the following limitation :

The resin must be subjected to the pre-use treatment in accordance with the manufacturer's directions and meet the extractives limitations as prescribed in Paragraph (c) of FDA regulation 21CFR 173.25.

It is however your responsibility to abide by any clause of this (or other) regulation(s) that may apply to the specific use you make of our product.

This information is correct to the best of our current knowledge. We recommend that you verify on a regular basis with APPLEXION – NOVASEP Process, the latest food contact status of our product.

Epône, October 26, 2006

(b)(6)

P. PRINTEMPS

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XA 90 Cl

Strong Anionic Exchange Resin

XA 90 Cl is a strongly basic, type 2, macroreticular anion exchange resin used in mixed beds for the final polishing of fructose syrups. The large, fixed porosity of this resin provides far more complete removal of large organic molecules during adsorption and desorption cycles. The crosslinked polystyrenic matrix makes this resin particularly stable mechanically. When used in a mixed bed in the hydroxyl form with strong macroporous cationic resin, XA 90 Cl removes trace contaminants that cause odors, off-flavors, and color stability problems with stored syrups. This includes weak organic acids, nitrogen containing compounds, and HMF removal.

| PROPERTIES | |
|-----------------------------------|--|
| Matrix _____ | Macroreticular crosslinked polystyrene |
| Functional groups _____ | -N-(CH ₂) ₂ C ₂ H ₄ OH |
| Physical form _____ | Pale yellow, opaque beads |
| Ionic form as shipped _____ | Chloride |
| Total exchange capacity _____ | ≥ 1.0 eq/l. (Cl ⁻ form) |
| Moisture holding capacity _____ | 54 to 61 % (Cl ⁻ form) |
| Shipping weight _____ | 700 g/l. |
| Harmonic mean size _____ | 530 to 800 μm |
| Fine contents _____ | < 0.300 mm : 2.5 % max |
| Maximum reversible swelling _____ | Cl ⁻ → OH ⁻ : 15 % |
| Chemical resistance _____ | Insoluble in dilute solutions of acids or bases and common solvents. |

Appendix 1B

Materials Used in the Production of Vivinal[®] GOS: Resin Specifications

PROPRIETARY PROCESS TECHNOLOGY

[REDACTED]

Contents

Appendix 1B: Resin Specifications

[PROPRIETARY PROCESS TECHNOLOGY]

| | |
|-----------|--------------------------------------|
| Exhibit 1 | Resin 1 Specifications [REDACTED] |
| Exhibit 2 | Resin 2 Specifications [REDACTED] |
| Exhibit 3 | Resin 3 Specifications [REDACTED] |

Appendix 1B
Exhibit 1

Resin 1 Specifications

PROPRIETARY PROCESS TECHNOLOGY

[REDACTED]

Appendix 1B
Exhibit 2

Resin 2 Specifications

PROPRIETARY PROCESS TECHNOLOGY

[REDACTED]

Appendix 1B
Exhibit 3

Resin 3 Specifications

PROPRIETARY PROCESS TECHNOLOGY

[REDACTED]

Appendix 2A

β -Galactosidase and Biolacta[®] N5
from *Bacillus circulans*

Contents

Appendix 2A: β -Galactosidase and Biolacta[®] N5

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 - II. Production Process of Biolacta[®] N5
[Presented in Appendix 2B]
 - III. Safety Data of Biolacta[®] N5
 - IV. Basis for GRAS Determination
- Exhibit 2. Maintenance of Culture Strain
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- Exhibit 3. French Acknowledgement of *Bacillus circulans* (ATCC 31382) as Industrial Source for production of β -galactosidase
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[Presented in Appendix 2B]
- Exhibit 9. Stability of Biolacta[®] N5
- Exhibit 10. Daiwa's Assay Method for Measuring β -galactosidase Activity
- Exhibit 11. Daiwa's Assay Method for Measuring Aerobic Bacterial Count
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- Exhibit 13. Biolacta[®] N5 Batch Data, Product Specifications and Methods of Analysis
- Exhibit 14. Toxin Analysis of Biolacta[®] N5, stock powder
- Exhibit 15. Antibacterial Test, Biolacta[®] N5

- Exhibit 16. Acute Toxicity Test in Mice, Biolacta[®] N5
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- Exhibit 18. *In vitro* Mutagenicity Test, Biolacta[®] N5
- Exhibit 19. Chromosome Aberration Test in Chinese Hamster Ovary Cells, Biolacta[®] N5

Appendix 2A Exhibit 1

GRAS Status Summary for β -Galactosidase and Biolacta[®] N5

GRAS Status Summary for β -Galactosidase and Biolacta[®] N5

I. *BACILLUS CIRCULANS* AND B-GALACTOSIDASE

A. *Bacillus circulans*

1. Source of bacteria

The enzyme is obtained from the natural, non-GMO, non-pathogenic, bacterium *Bacillus circulans*. This species also produces other enzymatic actions that are applied in the food industry. The β -galactosidase that is used for the production of Vivinal[®] GOS is derived from the non-pathogenic *Bacillus circulans* strain ATCC 31382. The maintenance of *B. circulans* cell culture can be found in Exhibit 2.

2. The Genus

The *Bacillus* genus is a group of gram positive bacteria. They are rod-shaped in young cultures, produce endospores and have marked acidity from glucose. Within the genus is large variability in carbohydrate product of fermentation, although for some species it can be almost all lactase (Holt et al. 1994).

3. *Circulans* tend to produce 4' GOS

Rabiu et al (2001) determined that GOS can be made of differing β -glycosidic linkages depending on the enzyme source. In another study, GOS derived from *Bacillus circulans* and *Cryptococcus laurentii* was found to predominantly produce GOS with 1 \rightarrow 4 Gal linkages (Sako et al. 1999).

4. Recognition of the Commercial Use of the Bacteria

The beta-galactosidase (lactase) enzyme preparation used in the production of Vivinal[®] GOS is found in the intestine of young mammals, but also in certain strains of yeast, molds, and bacteria. The enzyme has been approved for use in countries outside of the U.S.

Exhibits 3 and 4 are approvals for the use of the enzyme in France and Japan, respectively. Daiwa Kasei has obtained approval to manufacture the β -Galactosidase as a food additive in Japan.

Enzymes used commercially are derived from bacteria (*Bacillus circulans*), molds (*Candida pseudotropicalis*, *Aspergillus niger* or *oryzae*) and yeasts (*Kluyveromyces lactis*

or *fragilis* or *marxianus*). The FDA has affirmed several of these lactase enzyme preparations as GRAS (e.g. 21 CFR 184.1387, 21 CFR 184.1388).

Moreover, according to the Code of Federal Regulations, Title 21, Volume 3, other enzymes produced from the *Bacillus* genus have GRAS status in the U.S. These include: *Bacillus stearothermophilus* (§184.1012), *Bacillus subtilis* (§173.115 and §184.1148), *Bacillus amyloliquefaciens* (§184.1148), *Bacillus cereus* (§173.150) and *Bacillus coagulans* (§184.1372).

Bacillus circulans strain ATCC 31382 is listed as a microorganism accepted as a harmless contaminant present in food by The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and The Association of Microbial Food Enzyme Producers (AMFEP). (See Exhibit 5).

B. β -galactosidase

1. Identity

The enzyme used in the manufacturing of galactooligosaccharides is β -galactosidase (lactase). The enzyme is found in the intestine of young mammals, where it is responsible for the hydrolysis of lactose into glucose and galactose. The glucose and galactose then can be absorbed by the cells lining the small intestine. The enzyme is also found in certain strains of yeasts, molds and bacteria.

The systematic name of β -galactosidase is β -D-galactoside galactohydrolase (IUBMB Enzyme Nomenclature: EC 3.2.1.23, Chemical Abstracts Service Registry No. 9031-11-2). Other names for the enzyme include: lactase, β -lactosidase, maxilact, hydrolact, β -D-lactosidase, S 2107, lactozym, trilactase, β -D-galactanase, oryzatym and sumiklat.

2. Enzyme Properties

β -galactosidase is a hydrolase enzyme that works by transferring non-reducing β -D-galactose residues from β -D-galactosides (e.g. lactose) to water. Under conditions of high lactose concentration the enzyme utilizes lactose as an alternate acceptor to water resulting in the formation of galactooligosaccharides. During the reaction, the lactose forms an active intermediate with the enzyme. This intermediate can react with available sugar or water, respectively, to give an oligosaccharide or galactose and a free glucose, respectively. Thus, the enzyme can perform both polymerization and hydrolysis with the equilibrium dependent on both the type of enzyme and the reaction conditions.

One lactase unit (LU) is defined as the amount of enzyme which liberates 1 μ mol of glucose per minute from lactose (final concentration 10%) at the early stage of the reaction at a temperature of 40°C and a pH of 6.0.

3. Enzyme Product Characterization

Table I-1 below is a summary of β -galactosidase enzyme preparation composition based

| Table I-1. Batch Analysis of β -Galactosidase Composition | | | | | | | |
|---|----------------|-------------|--------------|--------------|--------------|--------------|--------------------|
| | Results | | | | | | |
| | | | Lot | Number | | | |
| | LT0502200.10sp | LT0606154 | LT0607144.01 | LT0607205.11 | LT0607205.12 | Mean | Standard Deviation |
| Chemical Composition (g/100g) | | | | | | | |
| Protein | 68.9 | 68.1 | 69.4 | 70 | 70 | 69.1 | 0.80 |
| Fat/Oil | <.1 | <.1 | <.1 | <.1 | <.1 | <.1 | 0 |
| Carbohydrates | 10.1 | 9.8 | 9.2 | 8.8 | 8.9 | 9.48 | 0.57 |
| Total Organic Solids | 79 | 77.9 | 78.6 | 78.8 | 78.9 | 78.58 | 0.44 |
| Moisture | 4.2 | 4.4 | 4.5 | 4.6 | 4.5 | 4.44 | 0.15 |
| Ash | 16.8 | 17.7 | 16.9 | 16.6 | 16.6 | 16.92 | 0.45 |
| Lead ($\mu\text{g/kg}$) | <40 | <40 | <40 | <40 | <40 | <40 | 0 |
| Total Components | 100 | 100 | 100 | 100 | 100 | 99.94 | |
| Source: Daiwa Kasei | | | | | | | |

on typical batch data. The ash component represented in the table is sodium chloride. Documentation of the presented data can be found in Exhibit 6.

4. Intended use and use level of enzyme

The enzyme preparation, a component of Biolacta[®] N5, is only used in the production of GOS. Therefore, the enzyme is not present in the final GOS product. The enzyme is inactivated by acid and heat reactions and then removed by a filtration step.

II. PRODUCTION PROCESS OF BIOLACTA® N5

[Presented in Appendix 2B]

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III. SAFETY DATA OF BIOLACTA[®] N5

The Mycotoxin Research Association analyzed Biolacta[®] N5 using the B.G.E. Josefsson and T.E. Moller method. They concluded that the β -galactosidase preparation does not contain aflatoxin B1, ochratoxin A, sterigmatocystin, zearalenone or T-2 toxin. The certificate of analysis is presented in Exhibit 14.

The Japan Food Laboratories found no antibacterial activity present in the Biolacta[®] N5 stock powder. (See Exhibit 15).

The Japan Food Research Laboratories also investigated the acute oral toxicity of Biolacta[®] N5 stock powder in mice in accordance with The Organization of Economic Co-operation and Development (OECD) guidelines for testing of chemicals. Oral administration of 2000 mg/kg bodyweight caused no deaths, apparent symptoms, abnormal necropsy findings or effects on body weight gain. From these results, it was concluded that the Biolacta[®] N5 stock powder has no acute oral toxicity in mice. (See Exhibit 16).

By performing a micronucleus test, TNO Nutrition and Food Research Institute (Division of Toxicology) concluded that there was no indication of chromosomal damage and/or damage to mitotic apparatus in bone marrow cells of mice treated by intraperitoneal injection of Biolacta[®] N5 at a dose level of 2000 mg/kg bw. (See Exhibit 17).

The Biolacta[®] N5 stock powder was examined for mutagenic activity in the pre-incubation Ames Salmonella microsome assay using four strains of *Salmonella typhimurium* and one strain of *Escherichia coli*. The assays were performed in duplicate both with and without the rat-liver metabolic activation system. No evidence showing a mutagenic potential of the test substance was obtained in this bacteria test system at the dose levels used. (See Exhibit 18).

The test substance Biolacta[®] N5 stock powder was examined for its potential to induce structural chromosome aberrations in Chinese hamster ovary cells in both the absence and presence of a metabolic activation system (S-9 mix). The Biolacta[®] N5 stock powder was not clastogenic under the conditions tested in this study. (See Exhibit 19).

IV. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of β -galactosidase derived from *Bacillus circulans* as a processing aid in the production of GOS is based upon scientific procedures as described under 21 CFR §170.30(b). The use of β -galactosidase derived from *Bacillus circulans* for the intended use 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). There is no evidence in the available information that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when it is used at levels that might reasonably be expected from the proposed applications. β -galactosidase derived from *Bacillus circulans* is GRAS for use in products as proposed by Friesland Foods Domo.

It 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.

Appendix 2A Exhibit 2

Maintenance of Culture Strain

[Presented in Appendix 2B]

Appendix 2A

Exhibit 3

French Acknowledgment of *Bacillus circulans*
(ATCC 31382) as Industrial Source for
production of β -galactosidase

Pages 000263-000264 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 2A
Exhibit 4

Japanese Recognition of β -galactosidase
as a Food Additive

[[Home](#)]

List of Existing Food Additives

This list of food additives from natural origin is compiled and published by the Ministry of Health and Welfare on April 16, 1996.

These additives are listed here in alphabetic order. The number preceding the name of each additive is the sequence number given to the corresponding additive in the original Japanese list.

| No. | Name | Note |
|-----|--------------------------------------|--|
| 316 | Absinth extract | A substance composed mainly of sesquiterpenes obtained from the whole absinth grass. |
| 16 | α -Acetolactate decarboxylase | - |
| 15 | N-Acetylglucosamine | - |
| 8 | Achromopeptidase | - |
| 200 | Acid phosphatase | - |
| 199 | Acid clay | - |
| 6 | Actinidine | - |
| 89 | Activated acid clay | - |
| 88 | Active carbon | A substance obtained by carbonizing and activating carbon-containing substances. |
| 9 | Acylase | - |
| 18 | 5'-Adenylic acid | - |

| | | |
|-----|--------------------------------------|--|
| 482 | Forsythia extract | A substance composed mainly of phyllyrin obtained from forsythia fruits. |
| 379 | Fractionated lecithin | A substance composed mainly of sphingomyelin, phosphatidyl inositol, phosphatidyl ethanolamine and phosphatidyl choline obtained from "vegetable lecithin" or "yolk lecithin". |
| 369 | Fructosyl transferase | - |
| 370 | Fructosyl transferase-treated stevia | A substance composed mainly of fructosylstevioside obtained from a "stevia extract". |
| 363 | L-Fucose | - |
| 362 | Fukuronori extract | A substance composed mainly of polysaccharides obtained from FUKURO-NORI (<i>Gloiopeltis furcata</i> POSTEL et RUPR). |
| 353 | Furcellaran | A substance composed mainly of polysaccharides obtained from the whole algae of furcellaria (<i>Furcellaria fastigiata</i> HUD). |
| 96 | α -Galactosidase | - |
| 97 | β -Galactosidase (Lactase) | - |
| 414 | Gallic acid | - |
| 409 | Garden balsam extract | A substance obtained from the whole grass of garden balsam. |



第 1 章

「既存添加物名簿」及び 「既存添加物名簿収載品目リスト」等の解説

」

5'イワセイKK

既存添加物名簿番号 (97)

| 品 名 | 名 称 | β-ガラクトシダーゼ |
|----------|-----|---|
| | 別 名 | ラクターゼ |
| 簡略名又は類別名 | | カルボヒドラーゼ |
| 英 名 | | β-Galactosidase (Lactase) |
| 基原・製法・本質 | | 動物の臓器より、冷時～微温時水で抽出して得られたもの、又は糸状菌 (<i>Aspergillus oryzae</i> , <i>Penicillium multicolor</i> , <i>Rhizopus oryzae</i>), 細菌 (<i>Bacillus circulans</i> , <i>Streptococcus</i>) 若しくは酵母 (<i>Kluyveromyces fragilis</i> , <i>Kluyveromyces lactis</i> , <i>Saccharomyces</i>) の培養液より、冷時～室温時水で抽出して得られたもの、室温時自己消化処理して得られたもの、冷時～室温時濃縮したもの、冷時エタノール、含水エタノール若しくはアセトンで処理して得られたもの、又は硫酸アンモニウム等で分面した後、脱塩処理して得られたものである。 |
| 用 途 | | 酵素 |
| 概 要 | | 本酵素は、微生物、動物、植物などに広く存在する。 動物又は糸状菌、細菌、酵母から得られた加水分解酵素である。 乳糖をグルコースとガラクトースに分解する。 酵素安定剤としてグリセリンを含有させる。 |
| 性 状 | | 白～淡黄～褐色の粉末、粒、塊又は無～淡褐色の液体である。 |
| 品 質 特 性 | | 牛乳およびホエー中の乳糖の β-D-ガラクトシド結合を分解する。またガラクトシル転移作用もある。 本酵素の至適 pH、至適温度は基原により異なる。 |
| 溶 解 性 | | 水に可溶、エタノールに不溶である。 |
| 使用上の注意 | | 使用条件を製品説明書等で確認の上使用すること。 開封後はなるべく早く使用すること。 |
| 保存上の注意 | | 冷暗所で密閉保存する。 |
| 主な使用対象食品 | | 牛乳、乳製品（加工乳、アイスクリーム、ヨーグルト）等 |
| 備 考 | | JECFA：規格有り (Carbohydrase, <i>Asp. niger</i> , var. 等由来) 米国：CFR 173.120 (Carbohydrase), CFR 184.1388 (Lactase, <i>Kluyveromyces lactis</i> 由来) 参考：酵素番号：EC3.2.1.23, β-D-Galactosidase |

既存添加物名簿

| 品 名 | 名 称 |
|----------|-----|
| 簡略名又は類別名 | 別 名 |
| 英 名 | |
| 基原・製法・本質 | |
| 用 途 | |
| 概 要 | |
| 性 状 | |
| 品 質 特 性 | |
| 溶 解 性 | |
| 使用上の注意 | |
| 保存上の注意 | |
| 主な使用対象食品 | |
| 備 考 | |

Appendix 2A
Exhibit 5

Recognition from the Association of Microbial
Food Enzyme Producers of *Bacillus circulans*
being a Harmless Food Contaminant

Pages 000271-000272 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 2A

Exhibit 6

β -galactosidase Batch Data and Specifications

Concerning the composition of lactase concentrate

The analysis of five batches lactase concentrate (LT0502255.10SP, LT0606154, LT0607144.01, LT0607205.11, LT0607205.12) gives the following results.

Results of the analysis in the five batches of lactase concentrate.

| Lot (Lactase concentrate) | LT0502255.10SP | LT0606154 | LT0607144.01 | LT0607205.11 | LT0607205.12 |
|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| | (Quantity / 100 grams) | (Quantity / 100 grams) | (Quantity / 100 grams) | (Quantity / 100 grams) | (Quantity / 100 grams) |
| Energy (Calories) | 306 | 303 | 306 | 306 | 307 |
| Moisture (g) | 4.2 | 4.4 | 4.5 | 4.6 | 4.5 |
| Protein (g) | 68.9 | 68.1 | 69.4 | 70.0 | 70.0 |
| Fat / Oil (g) | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Carbohydrates (g) | 10.1 | 9.8 | 9.2 | 8.8 | 8.9 |
| Ashes (g) | 16.8 | 17.7 | 16.9 | 16.6 | 16.6 |

BIOLACTA N5 enzyme preparation (Lactase concentrate : 30%、 Lactose : 70%)

Results of Heavy metals in the five batches of lactase concentrate.

Test items : Heavy metals(as Pb)

| Lot | Specification | Results |
|----------------|----------------------------|----------------------------|
| LT0502255.10SP | not more than 40 μ g/g | not more than 40 μ g/g |
| LT0606154 | not more than 40 μ g/g | not more than 40 μ g/g |
| LT0607144.01 | not more than 40 μ g/g | not more than 40 μ g/g |
| LT0607205.11 | not more than 40 μ g/g | not more than 40 μ g/g |
| LT0607205.12 | not more than 40 μ g/g | not more than 40 μ g/g |

Appendix 2A Exhibit 7

Optimal Conditions for Hydrolysis to Occur

[Presented in Appendix 2B]

Appendix 2A Exhibit 8

Manufacturing Conditions for Biolacta[®] N5

[Presented in Appendix 2B]

Appendix 2A Exhibit 9

Stability of Biolacta[®] N5

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA. 520-3203 JAPAN

PHONE : +81-748-75-1194
F A X : +81-748-75-0312

Stability of BIOLACTAN5

| Lot No. | Initial activity | Store temperature 5°C | | | | | | | |
|---------|------------------|------------------------|-------|------|-------|-------|--------|-----------------|---------------|
| | | Remanning activity , % | | | | | | Drying loss , % | |
| | | 1 | 2 | 3 | 6 | 9 | 12 | 15 comb | Initial Final |
| P4FB091 | 5,330 | 100.9 | 98.7 | 99.1 | 100.9 | 100.8 | 102.4 | 101.9 | 1.8 1.3 |
| P4FB092 | 5,530 | 97.6 | 95.3 | 96.1 | 96.6 | 95.3 | (87.9) | 95.1 | 2.1 2.1 |
| P4FC791 | 5,630 | 97.5 | 99.8 | 97.5 | 97.9 | 97.7 | 96.4 | 92.9 | 1.4 1.5 |
| P4FC792 | 5,420 | 98.9 | 100.0 | 99.6 | 99.3 | 96.5 | 93.7 | 93.0 | 1.5 1.4 |
| Average | | 98.7 | 98.5 | 98.2 | 98.7 | 97.5 | 97.5 | 95.7 | |

| Lot No. | Initial activity | Store temperature 25°C | | | | | | | |
|---------|------------------|------------------------|------|------|------|------|------|-----------------|---------------|
| | | Remanning activity , % | | | | | | Drying loss , % | |
| | | 1 | 2 | 3 | 6 | 9 | 12 | 15.9 | Initial Final |
| P4FB091 | 5,330 | 93.1 | 93.8 | 94.1 | 91.9 | 91.6 | 90.8 | 89.9 | 1.8 2.1 |
| P4FB092 | 5,530 | 89.5 | 88.8 | 89.9 | 91.0 | 85.5 | 81.3 | 81.1 | 2.1 1.8 |
| P4FC791 | 5,630 | 93.8 | 95.6 | 94.0 | 94.8 | 92.4 | 90.9 | 89.9 | 1.4 1.2 |
| P4FC792 | 5,420 | 91.7 | 93.7 | 94.5 | 93.4 | 89.9 | 89.5 | 91.1 | 1.5 1.3 |
| Average | | 92.1 | 93.0 | 93.2 | 92.8 | 89.9 | 88.9 | 88.8 | |

Appendix 2A

Exhibit 10

Daiwa's Assay Method for Measuring β -galactosidase Activity

B 1 1 1 – 1 ASSAY METHOD OF LACTASE ACTIVITY, LU

Principle

When lactose is hydrolyzed by lactase, it is converted into glucose and galactose. The lactase activity is determined by measuring the amount of liberated glucose.

Definition of activity unit

One lactase unit (LU) is defined as the amount of enzyme that liberates 1 μ mol of glucose per min at the early stage of the reaction at 40°C, pH 6.0.

Reagents and solutions

- (1) 1 mol/L Acetic acid solution
Dilute 60 g of acetic acid with distilled water and bring to 1,000 mL.
- (2) 1 mol/L sodium acetate solution
Dissolve 136 g of sodium acetate trihydrate in distilled water and bring to 1,000 mL.
- (3) 1 mol/L acetate buffer (pH 6.0)
Add 1 mol/L acetic acid solution to 1 mol/L sodium acetate solution to give a pH of 6.0.
- (4) 10% Triton X-100 solution
Dissolve 10 g of Triton X-100 in distilled water by heating and bring to 100 mL.
- (5) Diluent
Dissolve 11.69 g of sodium chloride in distilled water, add 100 mL of 1 mol/L acetate buffer and 1.0 mL of 10% Triton X-100 solution, and add distilled water to bring to 1,000 mL.
- (6) 1.5 mol/L hydrochloric acid
Dilute 135 mL of hydrochloric acid to 1,000 mL with distilled water.
- (7) 1.5 mol/L sodium hydroxide solution
Dissolve 63 g of sodium hydroxide in distilled water and bring to 1,000 mL.
- (8) Substrate(12% lactose solution)
Accurately weigh 12.63 g of lactose monohydrate into a 200 mL Erlenmeyer flask, add about 80 mL of distilled water, and heat the flask in a boiling water bath to dissolve. Then cool it with a running water, quantitatively transfer the mixture into a 100 mL volumetric flask, add 10 mL of 1 mol/L acetate buffer (pH 6.0), and bring to volume with distilled water. Prepare before use.
- (9) Test preparation
Prepare a solution from the enzyme preparation so that 1 mL of the final dilution will contain between 0.5 and 3.0 lactase units(LU). Weigh the enzyme preparation, quantitatively transfer it into the volumetric flask of appropriate size, dissolve in diluent, dilute to volume with diluent, and mix.

Procedure

(Test)

Pipet 5 mL portion of the substrate into a 18 ϕ \times 180mm test tube and preincubate it in a water bath at 40 \pm 0.5 °C for 10 min. Rapidly pipet 1 mL of the test preparation into the equilibrated substrate and then mix by swirling, starting the stopwatch at zero time. Allow to stand at 40 \pm 0.5 °C for exactly 10 min, add 1 mL of 1.5 mol/L sodium hydroxide solution, and immediately mix by swirling.

Allow to stand at 40 \pm 0.5 °C for 5 min and immerse the test tube in a ice water.

(Blank)

Pipet 5 mL portion of the substrate into the same size test tube, add 1 mL of 1.5 mol/L sodium hydroxide solution, and then mix by swirling. Allow to stand at 40 ± 0.5 °C for 10 min, add 1 mL of the test preparation, and immediately mix by swirling. Allow to stand at 40 ± 0.5 °C for 5 min and immerse the test tube in a ice water.

Add 1 mL of 1.5 mol/L hydrochloric acid to each test tube, mix by swirling, and allow to stand in a ice water for a few minutes. Immediately determine glucose content for each reaction mixture according to ASSAY METHOD OF GLUCOSE.

Calculation

Calculate the activity of the enzyme preparation taken for analysis as follows:

$$\text{LU/g} = \frac{G_T - G_S}{0.18} \times 8 \times \frac{1}{10} \times \frac{1}{W} = \frac{G_T - G_S}{0.225 \times W}$$

In which G_T is glucose content, in mg/mL, for Test; G_B is glucose content, in mg/mL, for Blank; 0.18 is the amount of glucose, in mg, equivalent to 1 μ mol; 8 is the total volume, in mL, of the reaction mixture; 10 is the reaction time, in min; W is the weight, in g, of the enzyme preparation contained in 1 mL of the test preparation.

DAIWA KASEI K. K.

Appendix 2A Exhibit 11

Daiwa's Assay Method for Measuring Aerobic Bacterial Count

V 1 1 2 Test for Total Aerobic Micro-organisms

Media

1. Soybean-Casein Digest Agar Medium (SCD Agar Medium)

| | | |
|-------------------------------|-------|----|
| Pancreatic digest of casein | 15.0 | g |
| Papaic digest of soybean meal | 5.0 | g |
| Sodium chloride | 5.0 | g |
| Agar | 15.0 | g |
| Purified water | 1,000 | mL |

Autoclave at 121 °C for 15 minutes. (Final pH : 7.3 ± 0.2)

Procedure

The following operation should be carried out in a clean bench.

1. Preparation of the test sample

Aseptically weigh 10 g of sample into 90 mL of sterile physiological saline to obtain 1 : 10 dilution. Add one drop of sterile antifoam (such as polyalkylene glycol), and mix sufficiently. Dilute further, if necessary, the fluid so that 1 mL will be expected to yield between 20 and 200 colonies.

2. Cultivation technique

Pipet 1 mL of final dilution of the sample onto each of two Petri dishes. Add to each dishes 15 to 20 mL of SCD Agar Medium previously cooled to 48 - 50 °C. Mix the sample with the agar by tilting and rotating the dishes, and allow the contents to solidify with the dishes standing on a horizontal surface. Invert the dishes, and incubate at 30 - 32 °C for 72 hours.

3. Numeration

Examine the Petri dishes for growth, count the number of colonies, and express the average for the two dishes in terms of the number of microorganisms per g of sample. If the average of the numbers of colonies for two dishes representing the initial 1 : 10 dilution of the sample is less than 20, express the result as " less than 200 microorganisms per g of sample ".

Appendix 2A

Exhibit 12

Daiwa's Assay Method for Measuring Presence of Coliforms

V 2 1 2 Test for Coliforms

Media

1. Enrichment Agar Medium (Desoxycholate Agar)

| | |
|-----------------------------|----------|
| Peptone | 10 g |
| Lactose | 10 g |
| Sodium desoxycholate | 1 g |
| Sodium chloride | 5 g |
| Dibasic potassium phosphate | 2 g |
| Ferric ammonium citrate | 2 g |
| Neutral red | 0.033 g |
| Agar | 15 g |
| Purified water | 1,000 mL |

Heat gently to boil and boil for 1 minute , cool to 50°C (Final pH : 7.2 ± 0.2)

2. Selection Agar Medium (EMB Agar)

| | |
|-----------------------------|----------|
| Peptone | 10 g |
| Lactose | 10 g |
| Dibasic potassium phosphate | 2 g |
| Eosin Y | 0.4 g |
| Methylene blue | 0.065 g |
| Agar | 18 g |
| Purified water | 1,000 mL |

Autoclave at 121 °C for 15 minutes. (Final pH : 6.8 ± 0.2)

3. Confirmation Broth Medium (Lactose Broth)

| | |
|-----------------|----------|
| Beef extract | 3 g |
| Peptone | 10 g |
| Lactose | 5 g |
| Bromthymol blue | 0.024 g |
| Purified water | 1,000 mL |

Autoclave at 121 °C for 15 minutes. (Final pH : 6.9 ± 0.2)

4. Growth Agar Medium (Nutrient Agar)

| | |
|-----------------|----------|
| Beef extract | 5 g |
| Peptone | 10 g |
| Sodium chloride | 5 g |
| Agar | 15 g |
| Purified water | 1,000 mL |

Autoclave at 121 °C for 15 minutes. (Final pH : 7.0 ± 0.2)

Procedure

The following operation should be carried out in a clean bench.

1. Preparation of the test sample

Aseptically weigh 10 g of sample into 90 mL of sterile physiological saline to obtain 1 : 10 dilution. Add one drop of sterile antifoam (such as polyalkylene glycol), and mix sufficiently.

2. Cultivation technique

Pipet 1mL of final dilution of the sample onto each of two Petri dishes.

Add to each dishes 15 to 20 mL of enrichment agar medium previously cooled to 45 - 50 °C. Mix the sample with the agar by tilting and rotating the dishes, and allow the contents to solidify with the dishes standing on a horizontal surface. Invert the dishes, and incubate at 35 - 37 °C for 20 ± 2 hours. If typical dark-red colonies are present, pick one of the representative colonies by means of an inoculating loop and streak it on the surface of selection agar medium. (If suspect colonies are present, pick two or more colonies.) Incubate at 35 - 37 °C for 24 ± 2 hours.

If typical coliform colonies are present, transfer one of the typical colonies to growth agar medium and incubate at 35 - 37 °C for 22 ± 2 hours ; perform Gram stain. At the same time, transfer the same colony to confirmation broth medium and incubate at 35 - 37 °C for 36 ± 12 hours ; examine for gas formation.

3. Numeration

If the culture on growth agar medium is Gram-negative nonsporeforming rods and the typical colony produces gas in confirmation medium, express the result as " positive " considering that coliforms are detected.

Appendix 2A Exhibit 13

Biolacta[®] N5 Batch Data, Production Specifications and Methods of Analysis

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F A X : +81-748-75-0312

Batch data of Biolacta N5 (macronutrients)

| | Quantity/100 grams BIOLACTA N5 | | | | |
|--------------------|--------------------------------|-----------|--------------|--------------|--------------|
| | Provided before | LT0606154 | LT0607144.01 | LT0607205.11 | LT0607205.12 |
| Energy (Calories) | 357 | 356 | 357 | 357 | 358 |
| Moisture (g) | 4.9 | 5.0 | 5.0 | 5.0 | 5.0 |
| Protein (g) | 20.7 | 20.4 | 20.8 | 21.0 | 21.0 |
| Fat/Oil (g) | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| Carbohydrates (g) | 69.4 | 69.3 | 69.1 | 58.9 | 69.0 |
| Total Sugars (g) | 66.3 | 66.5 | 66.5 | 66.3 | 66.3 |
| Dietary Fibers (g) | 3.0 | 2.8 | 2.6 | 2.6 | 2.7 |
| Ashes (g) | 5.1 | 5.3 | 5.1 | 5.1 | 5.0 |
| Sodium (mg) | 1.8 | 2.0 | 1.85 | 1.83 | 1.81 |

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Product Specification

December 15, 2006

Address 4-19, HIE-CHO KONAN SHIGA 520-3203, JAPAN
Manufacturer DAIWA KASEI K. K.
TEL +81-748-75-1191 FAX +81-748-75-0312

| | | |
|--------------|--|--------------------|
| Product: | BIOLATA N5 | Product code: 7002 |
| Description: | A bacterial Lactase from <i>Bacillus circulans</i> . A light brown powder. | |

Specification:

| Test Items | Specification | Assay method |
|---|--|---|
| Lactase activity | not less than 5,000IU/g | Daiwa's method |
| *Heavy metals (as Pb) | not more than 40 μ g/g | JP (method 2) |
| *Arsenic (as As ₂ O ₃) | not more than 3 μ g/g | AAS method |
| Viable bacteria count | not more than 10 ⁴ /g | Daiwa's method (SCD agar) |
| Coliforms | not more than 30 /g | Daiwa's method (DCA agar) |
| Salmonella | Negative in 25g | Daiwa's method (FDA/BAM 8 th) |
| E. coli | Negative in 25g | Daiwa's method (FDA/BAM 8 th) |
| Notes | *It is tested using concentrated material before standardization. | |
| Composition | 30% Lactase, 70% Lactose | |
| Expiration | 6 months under room temperature after shipment under the sealed condition. | |
| Packing | 25.5kg / Fiber drum (in 1.5kg/PE \times 17) | |
| Handling | Attached MSDS | |

DAIWA KASEI K. K.

BIOLACTA N5 standardization from the enzyme concentrate

| Concentrate Lot No. | Enzyme concentrate , % | Lactose , % |
|---------------------|------------------------|-------------|
| LT0502255.10SP | 30.6 | 69.4 |
| LT0606154 | 30.4 | 69.6 |
| LT0607144.01 | 27.7 | 72.3 |
| LT0607205.11 | 28.2 | 71.2 |
| LT0607205.12 | 28.6 | 71.4 |

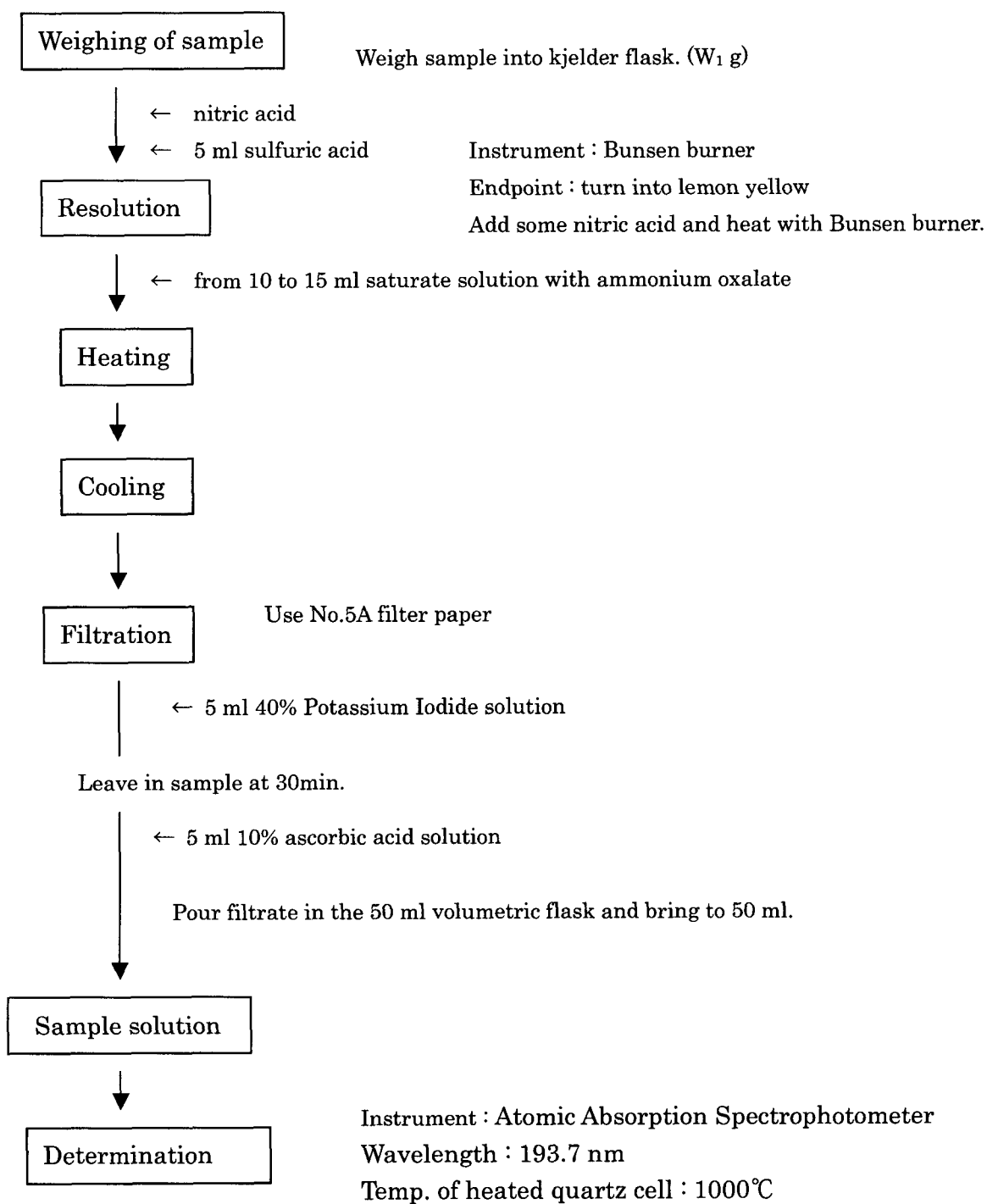
BIOLACTA N5 batch data of analysis

| Lot No. Test items | Specification | P6HD001 | P6JA201 | P6JD101 | P6LA501 | P6kb301 |
|--|---------------|-----------|-----------|-----------|-----------|-----------|
| Lactose hydrolyzing Activity (LU/g) | \leq 5,000 | 5,750 | 5,770 | 5,630 | 5,670 | 5,600 |
| ONPG hydrolyzing activity (LSU/g) | Only report | 5,060 | 5,100 | 5,070 | 4,660 | 4,780 |
| Arsenic [as As] (μ g/g) | \geq 3 | \geq 3 | \geq 3 | \geq 3 | \geq 3 | \geq 3 |
| Heavy metals [as Pb] (μ g/g) | \geq 40 | \geq 40 | \geq 40 | \geq 40 | \geq 40 | \geq 40 |
| Aerobic microorganisms (CFU/g) | 10 | 10 | 10 | 10 | 10 | 10 |
| Coliforms (CFU/g) | \geq 30 | \geq 30 | \geq 30 | \geq 30 | \geq 30 | \geq 30 |
| Salmonella | Negative | Negative | Negative | Negative | Negative | Negative |
| E. coli | Negative | Negative | Negative | Negative | Negative | Negative |

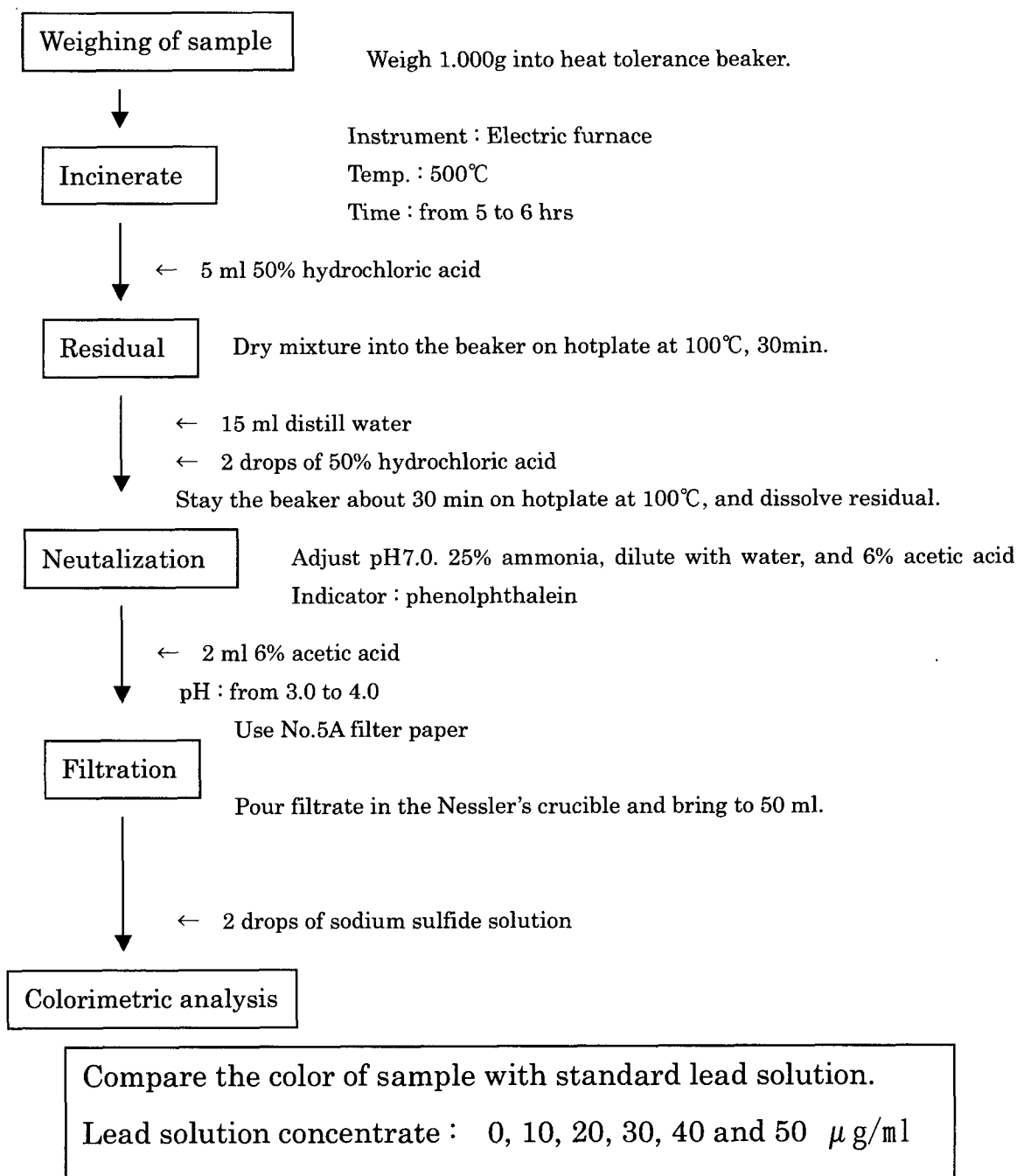
\leq : Not less than, \geq : Not more than, Negative : Negative in 25g

Pages 000291-000292 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

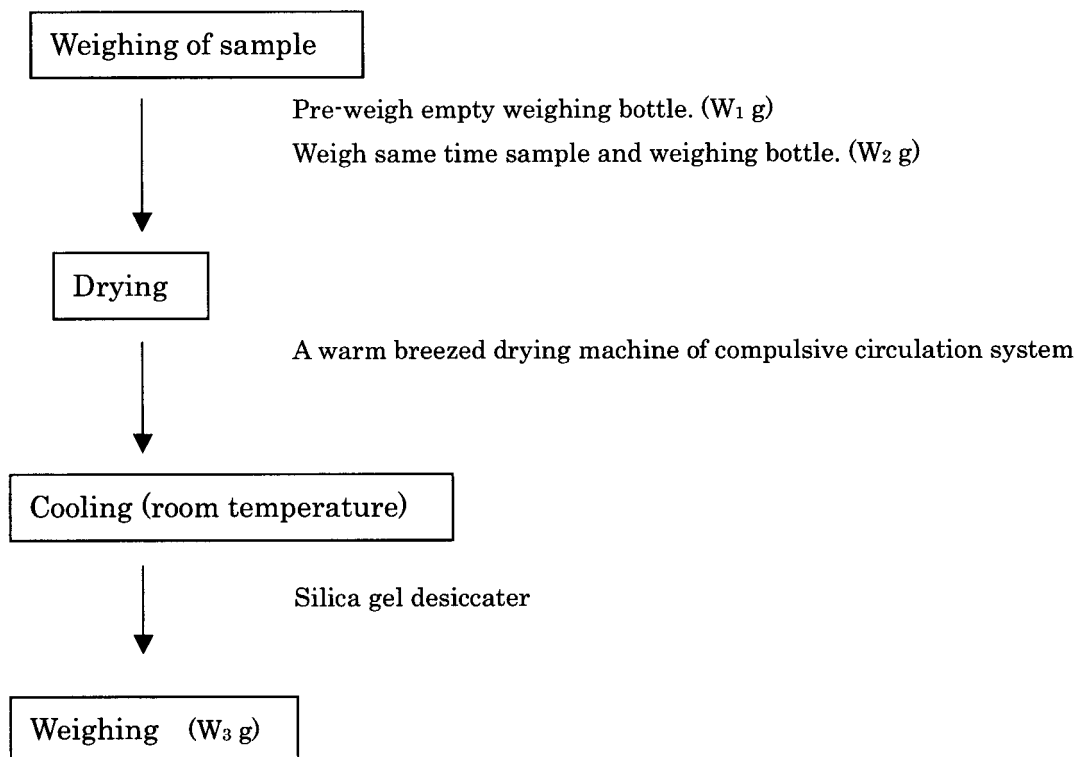
Analysis schema of Arsenic
(Atomic absorption spectrophotometry - method of hydride-)



Analysis schema of Heavy Metals (Sodium sulfide method)



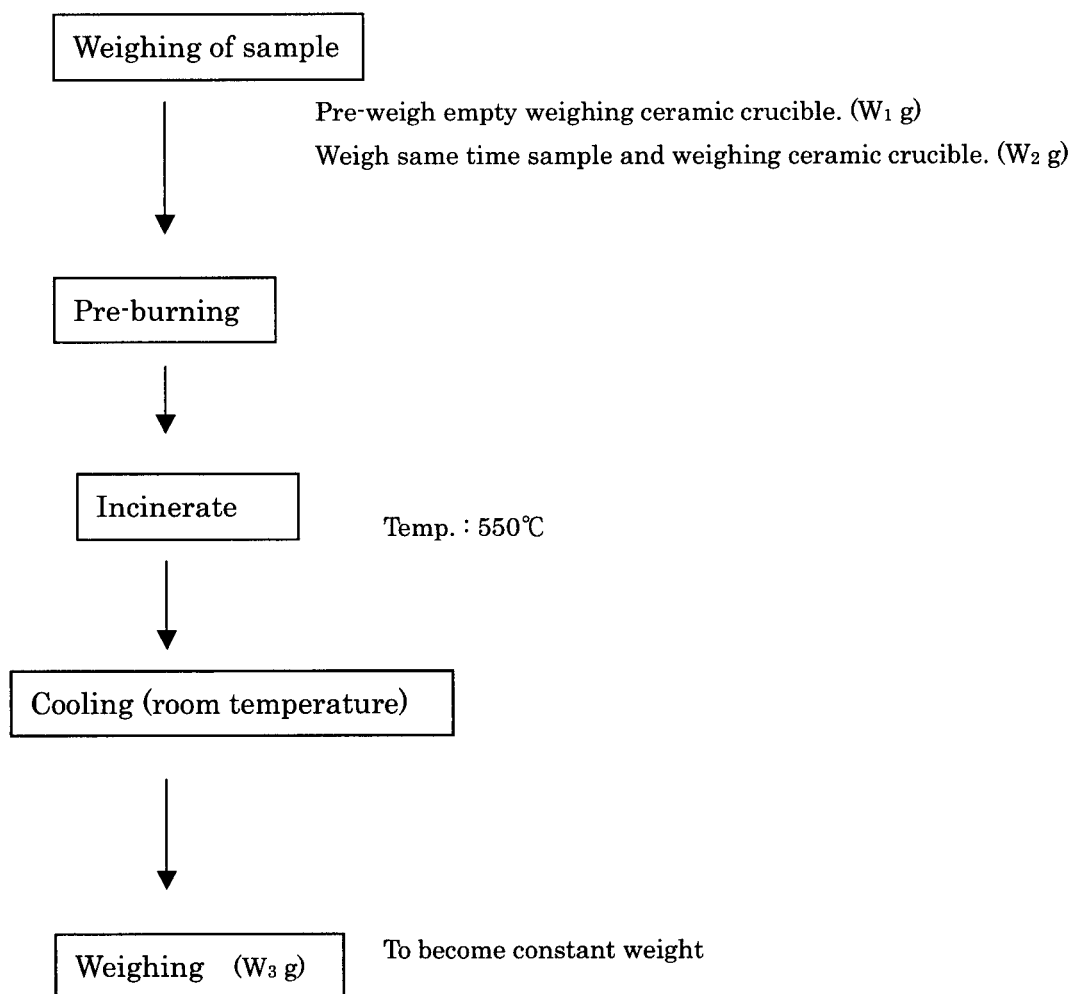
Analysis schema of Moisture
(105°C, 5hrs drying method)



Calculation :

$$\text{Moisture (g/100g)} = (W_2 - W_3) / (W_2 - W_1) \times 100$$

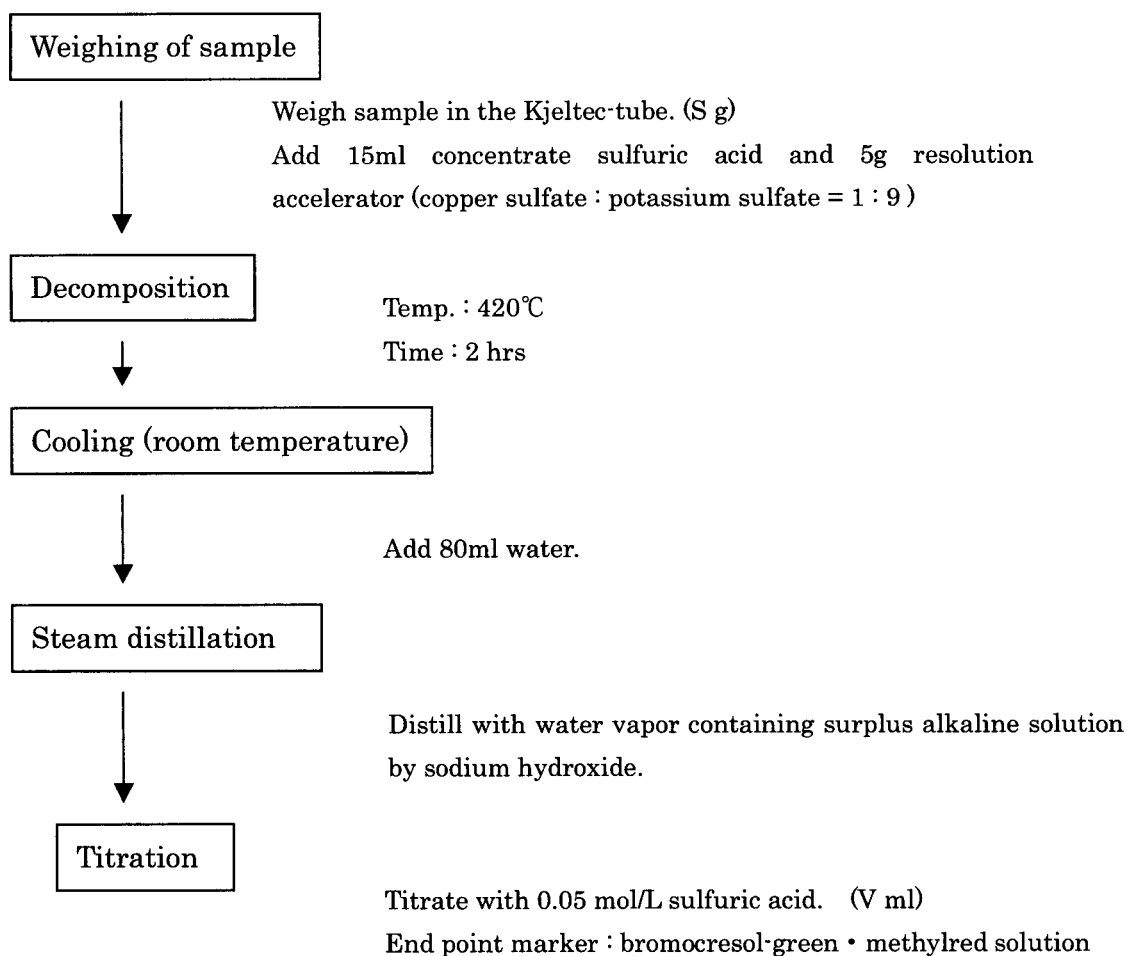
Analysis schema of Ash
(550°C, burning method)



Calculation :

$$\text{Ash (g/100g)} = (W_3 - W_1) / (W_2 - W_1) \times 100$$

Analysis schema of Protein (Kjeldahl method)



Instrument : KJELTEC AUTO SAMPLER SYSTEM Analyzer

Calculation :

$$\text{Protein (g/100g)} = (V - B) \times F \times 0.0014 \times K \times 100 / S$$

V : Titration volume (test) (ml)

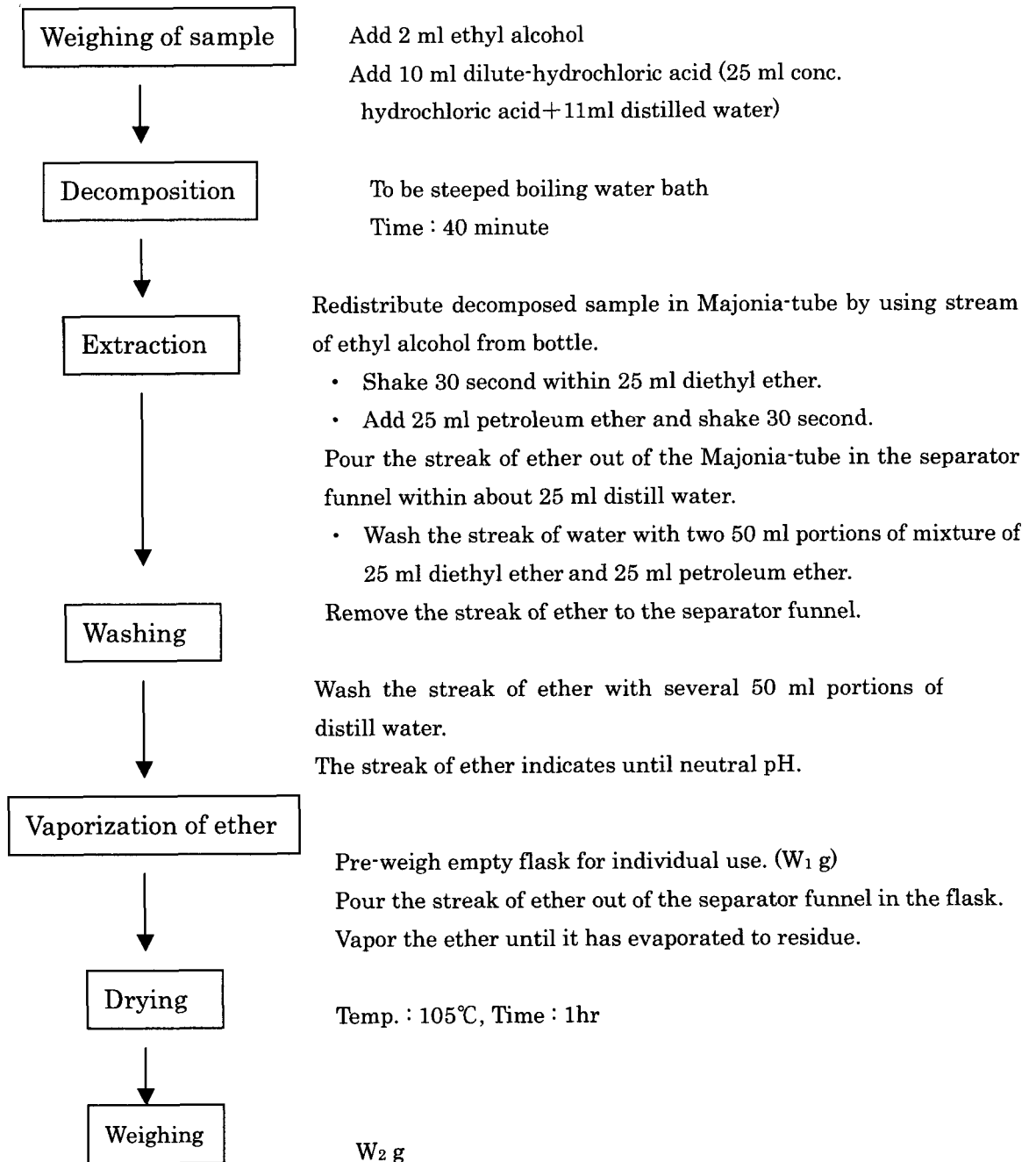
B : Titration volume (blank) (ml)

F : Factor of 0.05 mol/L sulfuric acid

K : 6.25 ; Conversion constant

S : Weighing of sample (g)

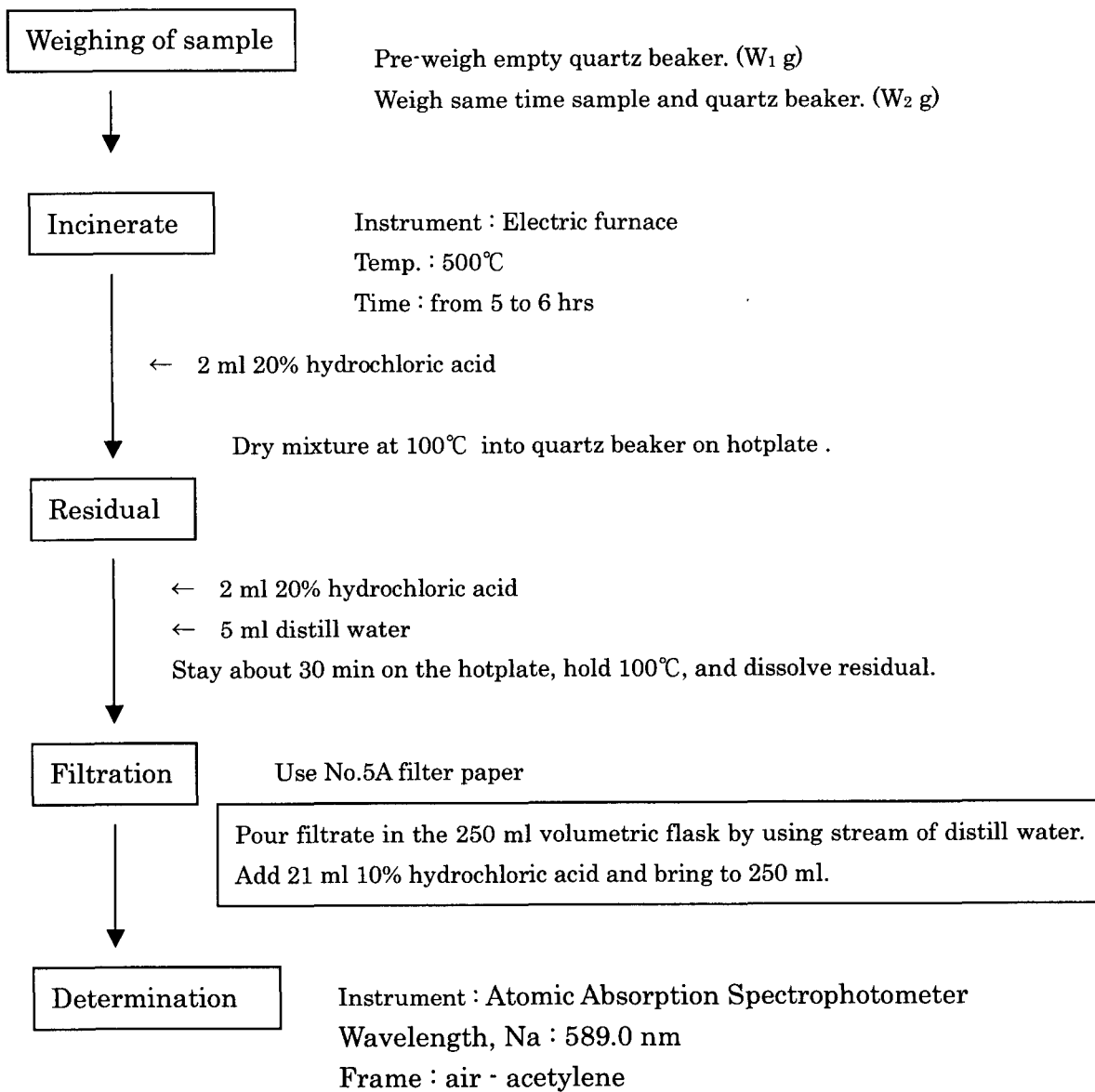
Analysis schema of Fat/Oil
(The method of acid decomposition)



Calculation :

$$\text{Fat/Oil (g/100g)} = (W_2 - W_1) / S \times 100$$

Analysis schema of Sodium (Atomic absorption spectrophotometry)



Calculation :

$$\text{Sodium (g/100g)} = A \times D \times F/S$$

A : Reading of determination

D : Dilution rate

F : Factor of Na solution

S : Sample weight

Appendix 2A

Exhibit 14

Toxin Analysis of Biolacta[®] N5, stock powder



MYCOTOXIN RESEARCH ASSOCIATION
(M. R. A.)

4th Floor, Daidoanyu Bldg.,
92, Yamashita-cho, Naka-ku, Yokohama
231 JAPAN

Myco No. 2 - 1945

Date: Feb. 25, 1991

C E R T I F I C A T E

Article Examined : BIOLACTA[®], Stock powder.
[A technical grade stock powder of a β -galactosidase produced
by Bacillus circulans]

Name and Address of

Applicant : DAIWA KASEI K.K. .
7-12 UENOMACHI 5-CHOME TENNOJIKU, OSAKA 543

Kind of Examination : Qualitative Analysis of Aflatoxin B₁, Ochratoxin A,
Sterigmatocystin, Zearalenone, and T-2 Toxin

Method of Analysis :

We showed in accompanying sheet

Result of Examination:

Receiving No. and Date: No.02-6366 and January 14, 1991

I hereby certify that the result of examination was as
stated above.

(b)(6)

(Dr. Shigeru Oshifuchi)

For Director
Mycotoxin Research Association
Designated by the Minister of
Health & Welfare



MYCOTOXIN RESEARCH ASSOCIATION
(M. R. A.)

4th Floor, Daidounyu Bldg.,
92, Yamashita-cho, Naka-ku, Yokohama
231 JAPAN

Method of Analysis : For Aflatoxins, Ochratoxin A, Sterigmatocystin,
and Zearalenone

- B.G. EGON JOSEFSSON and TORD E. MOLLER
Screening Method for the Detection Aflatoxins,
Ochratoxin, Patulin, Sterigmatocystin, and
Zearalenone in Cereals
Journal of The AOAC 60(6):1369-1371, 1977

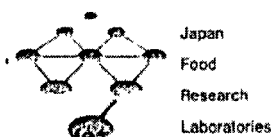
for T-2 Toxin

- CHOONG W. CHUNG, et al
Rabbit Skin Test for Estimation of T-2 Toxin
and Other Skin-Irritating Toxins in Contaminated
Corn
Journal of The AOAC 57(5):1121-1127, 1974
- R.M. EPPLEY
Screening Method for Zearalenone, Aflatoxin, and
Ochratoxin
Journal of The AOAC 51(1):74-78, 1968

| | | |
|-------------------------|--------------------------|----------|
| Result of Examination : | Aflatoxin B ₁ | Negative |
| | Ochratoxin A | Negative |
| | Sterigmatocystin | Negative |
| | Zearalenone | Negative |
| | T-2 Toxin | Negative |

Appendix 2A Exhibit 15

Antibacterial Test, Biolacta[®] N5



Japan Food Research Laboratories

AUTHORIZED BY THE JAPANESE GOVERNMENT

HEAD OFFICE : 52-1 MOTOMYOGI CHO, SHIBUYA KU, TOKYO
OSAKA BRANCH : 3-1, TOYOTSU CHO, SUITA-SHI, OSAKA
NAGOYA BRANCH : 5-13 4-CHOME OSU, NAKA-KU, NAGOYA
KYUSHU BRANCH : 1-12 SHIMOGOFUKU MACHI, HAKATA KU, FUKUOKA-SHI

ANALYSIS CERTIFICATE

No.OS54010334-4

March 8, 1991

Requested by : DAIWA KASEI K. K.
7-12 UEHONMACHI 5-CHOME TENNOJIKU, OSAKA 543

Received : January 12, 1991

Antibacterial Activity Test

1. Sample

BIOLACTA[®], stock powder

A technical grade stock powder of a
 β -galactosidase produced by Bacillus circulans.

2. Purpose

To test antibacterial activity of the sample.

3. Outline

The test was carried out in accordance with the method
described in Food Additives and Contaminants Committee, 1982,
LONDON "REPORT ON THE PREVIEW OF ENZYME PREPARATIONS" APENDIX W,
General Purity Criteria Applicable to Enzyme Preparations, 9.

4. Results

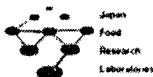
Results are shown in Table 1.

Table 1 Antibacterial activity of the sample

| Test organism | Disk | | | | | |
|--|------|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| <u>Staphylococcus aureus</u> ATCC 6538 | - | - | - | - | - | - |
| <u>Escherichia coli</u> ATCC 11229 | - | - | - | - | - | - |
| <u>Bacillus cereus</u> ATCC 2 | - | - | - | - | - | - |
| <u>Bacillus circulans</u> ATCC 4516 | - | - | - | - | - | - |
| <u>Streptococcus pyogenes</u> ATCC 12344 | - | - | - | - | - | - |
| <u>Serratia marcescens</u> ATCC 14041 | - | - | - | - | - | - |

- ; Inhibition zone was not detected.

- continued -



- page 2 -

5. Methods

1) Test organisms

Staphylococcus aureus ATCC 6538
Escherichia coli ATCC 11229
Bacillus cereus ATCC 2
Bacillus circulans ATCC 4516
Streptococcus pyogenes ATCC 12344
Serratia marcescens ATCC 14041

2) Culture plates

Make a test plate of each organism by preparing a 1:10 dilution of a 24 hours Trypticase Soy Broth culture in Trypticase Soy Agar [TSA] (for Streptococcus pyogenes ATCC 12344 a 1:20 dilution).

Pour 15 ml of plain TSA into a Petri dish and allow the medium to harden. Overlay with 10 ml of seeded TSA and let solidify.

3) Disk preparation

Make a 10 % solution of the enzyme preparation by adding 1 g of the sample to 9 ml of sterile distilled water.

Mix thoroughly with a Vortex mixer to obtain a homogeneous suspension.

Autoclave the paper disks (ϕ 13 mm), then saturate them with the enzyme by application of 0.1 ml of a 10 % solution of the enzyme to the disk surface. Prepare 6 disks for the enzyme : place a disk on the surface of the 6 inoculated agar plates.

4) Incubation

Keep the 6 plates in the refrigerator overnight to obtain proper diffusion. Incubate the plates at 37 °C for 24 hours. Examine the plates for any inhibition zones that may have been caused by the enzyme preparation.

- The end -

Japan Food Research Laboratories

(b)(6)

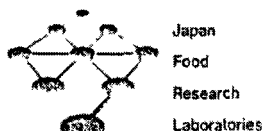
K. Takasu

Inspector

000305

Appendix 2A Exhibit 16

Acute Toxicity Test in Mice, Biolacta[®] N5



Japan Food Research Laboratories

AUTHORIZED BY THE JAPANESE GOVERNMENT

HEAD OFFICE : 52-1, MOTOMYOGI-CHO, SHIBUYA-KU, TOKYO
OSAKA BRANCH : 3-1, TOYOTSU-CHO, SUITA-SHI, OSAKA
NAGOYA BRANCH : 5-13, 4-CHOME, OSU, NAKA-KU, NAGOYA
KYUSHU BRANCH : 1-12, SHIMOGOFUKU-MACHI, HAKATA-KU, FUKUOKA-SHI

ANALYSIS CERTIFICATE

No. OS54010334-2

March 8, 1991

Requested by : DAIWA KASEI K. K.

7-12 UEHONMACHI 5-CHOME TENNOJIKU, OSAKA 543

Received : January 12, 1991

Acute Toxicity Test in Mice of BIOLACTA[®], stock powder

Abstract

The acute oral toxicity of BIOLACTA[®], stock powder in mice was investigated in accordance with OECD GUIDELINES FOR TESTING OF CHEMICALS(1987).

Oral administration of 2,000 mg/kg caused no deaths, apparent symptoms, abnormal necropsy findings or effects on body weight gain.

From these results, it was considered that BIOLACTA[®], stock powder has no acute oral toxicity in mice.

Test period

January 28, 1991 ~ March 8, 1991

Materials and Methods

1) Test substance

BIOLACTA[®], stock powder

A technical grade stock powder of a

β -galactosidase produced by Bacillus circulans.

2) Preparation of test solution

Test substance was dissolved in purified water.

Concentration of the solution was 200 mg/ml.

3) Experimental animals

Each 10 male and 10 female mice of the ICR strain bred by Japan SLC, INC. were used. The mice were obtained at 4 weeks old and acclimated to the laboratory conditions for 1 week. They were kept in groups according to sex in plastic cages at a temperature of 23 ± 2 °C with 12 hours light-dark cycle. There were 10 mice per cage. Conventional laboratory diets and fresh water were fed ad libitum.

- continued -

4) Procedure (Limit test)

The mice were fasted prior to substance administration for about 4 hours. Following a period of fasting, they were weighed. The males ranged from 24.9 to 32.1 g and the females were between 24.2 and 27.7 g. The test substance was administered orally at the dose level of 2,000 mg/kg to the mice by gavage using a stomach tube.

Clinical examination was made frequently on the day of administration and once a day during the following period. The mice were weighed weekly.

At the end of the test (14 days), they were sacrificed and necropsy of all mice was carried out.

Results

1) Clinical examination

No abnormal clinical signs were observed in both males and females and no mice were dead.

2) Body weight (Table 1, 2)

No decrease of body weight was detected in both males and females.

3) Necropsy

No abnormalities were found in both males and females.

Discussion

Oral administration of 2,000 mg/kg caused no abnormalities and no deaths in mice.

Consequently, it was considered that BIOLACTA[®], stock powder has no acute oral toxicity in mice and the lethal dose is more than 2,000 mg/kg.

- The end -

Japan Food Research Laboratories

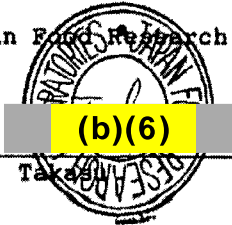

(b)(6)
K. Takashi Inspector

Table 1 Body weight (male)

[g]

| Mouse No. | Before treatment | After treatment | |
|---------------|------------------|-----------------|----------------|
| | | 7 days | 14 days |
| 1 | 32.1 | 39.4 | 42.6 |
| 2 | 27.7 | 34.1 | 37.2 |
| 3 | 29.9 | 33.7 | 36.1 |
| 4 | 31.7 | 34.3 | 36.9 |
| 5 | 30.4 | 37.0 | 40.4 |
| 6 | 28.1 | 36.6 | 38.6 |
| 7 | 27.0 | 35.7 | 38.0 |
| 8 | 25.3 | 32.5 | 35.2 |
| 9 | 24.9 | 33.4 | 35.2 |
| 10 | 27.9 | 36.9 | 40.5 |
| mean \pm SD | 28.5 \pm 2.5 | 35.4 \pm 2.1 | 38.1 \pm 2.5 |

Table 2 Body weight (female)

[g]

| Mouse No. | Before treatment | After treatment | |
|---------------|------------------|-----------------|----------------|
| | | 7 days | 14 days |
| 1 | 27.3 | 29.4 | 29.6 |
| 2 | 24.2 | 25.3 | 27.2 |
| 3 | 27.0 | 29.4 | 30.6 |
| 4 | 26.5 | 29.3 | 32.7 |
| 5 | 26.2 | 28.8 | 29.7 |
| 6 | 27.7 | 30.2 | 32.3 |
| 7 | 25.6 | 27.4 | 29.5 |
| 8 | 25.3 | 27.7 | 29.5 |
| 9 | 25.0 | 26.5 | 29.3 |
| 10 | 24.5 | 30.4 | 31.4 |
| mean \pm SD | 25.9 \pm 1.2 | 28.4 \pm 1.7 | 30.2 \pm 1.6 |

Appendix 2A

Exhibit 17

Micronucleus Test in Mice, Biolacta[®] N5

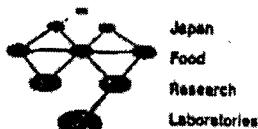
Pages 000311-000343 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 2A
Exhibit 18

In vitro Mutagenicity Test, Biolacta[®] N5

194704月07日(木) 08:56 宛先 600131545756625

発信 CHUGAI BOYER



Japan Food Research Laboratories
AUTHORIZED BY THE JAPANESE GOVERNMENT

Annex 1

3/25

HEAD OFFICE : 52-1, NOTOYOTOGI-CHO, SHIBUYA-KU, TOKYO
OSAKA BRANCH : 13-1, TOYOTSU-CHO, SUITA-SHI, OSAKA
NAGOYA BRANCH : 5-13, 4-CHOME, OBU, NAKA-KU, NAGOYA
KYUSHU BRANCH : 1-12, SHIMOGOPUKU-MACHI, HAKATA-KU, FUKUOKA-SHI
TAMA BRANCH : 111-10, 8-CHOME, NAGAYAMA, TAMA-SHI, TOKYO

REPORT

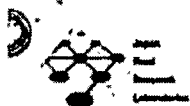
No. OS56060963-2
August 18, 1993

In Vitro Microbiological Mutagenicity Tests to Assess the
Potential Mutagenic Effect of BIOLACTA*, Stock powder

Requested by : DAIWA KASEI K.K.
7-12, Uehonmachi 5-chome, Tennoji-ku, Osaka 543

Received : June 23, 1993

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I, the undersigned, hereby declare that the work described in this report was performed under my supervision, as Study Director, in compliance with Guidelines for the Standards of Mutagenicity Test Using Microorganisms (Notification No. 77 of Labour Standards Bureau, Ministry of Labour) except for minor items, none of which is considered to have an impact on the validity of the data or the interpretation of the results in the report.

The tests described in this report were carried out from June 23, to August 18, 1993.

This is a translation of the original report, No. OS56060963-1, written in Japanese.

Study Director

(b)(6)

Hidetaka Arita D.V.M. Ph.D.
Department of Biological Safety Research
Japan Food Research Laboratories
Tama Laboratory



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In Vitro Microbiological Mutagenicity Tests to Assess the
Potential Mutagenic Effect of BIOLACTA®, Stock powder.

Abstract

BIOLACTA®, Stock powder was examined for a mutagenic activity in the pre-incubation Ames *Salmonella* microsome assay, using four strains of *Salmonella typhimurium*, TA1535, TA1537, TA98, and TA100 and a strain of *Escherichia coli*, WP2 *uvr* A. The assays were performed in duplicate, both with and without the rat-liver metabolic activation system. Slight increases in the revertant colony number were observed in *S. typhimurium* TA98 and TA100, not exceeding twice the negative controls. Such increases were larger in the presence of the metabolic activation than in the absence.

The increases were considered to have been caused by the influence of histidine or peptides or by some mutagens contained by the test substance, which is a proteinaceous material. The amount of free histidine (9 mg/100 g), however, was not enough to increase the revertant colonies. An extract of the test substance with dimethylsulfoxide was not mutagenic in TA100 with metabolic activation. Many mutagenic chemicals are soluble in organic solvents, so it was suggested that the test substance did not contain any mutagenic chemical.

Accordingly, the increased revertant colonies may have been caused by such decreased components of protein-like peptides that the tester strains can utilize. It was concluded that no evidence showing a mutagenic potential of the test substance was obtained in this bacterial test system at the dose levels used.

Method in detail

The method used was as described in Experimental Procedure.

Test substance : BIOLACTA®, Stock powder

A technical grade stock powder of a lactase
produced by *Bacillus circulans*.

Appearance : Light brown powder

Storage conditions : At room temperature.

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Test 1

Solvent : Sterilized deionized water

Preparation of a test solution : The test substance was dissolved to a concentration of 50 mg/ml.

Dose levels

Dose-range finding test :

5,000, 1,000, 500, 100, 50, and 10 µg/plate

Mutation test :

5,000, 2,500, 1,250, 625, 313, and 156 µg/plate

Test 2

Solvent : Dimethylsulfoxide

Preparation of a test solution : The test substance was suspended to a concentration of 100 mg/ml. The suspension was shaken for about an hour at room temperature. After shaken, the suspension was centrifuged for 10 min at 3,000 rpm at 20°C. The supernatant (the extract) was used for the test.

Dose levels : 200, 100, and 50 µl/plate

Results and Discussion

The revertant colony counts in test 1 are shown in Tables 1, 2, 3, and 4. The test substance was not toxic towards the tester strains at the dose levels used. Slightly more revertant colonies, but not more than twice, than those of the negative control and dose-related increases were observed in *S. typhimurium* TA98 and TA100. The increases after metabolic activation were more than those without it.

Two explanations may be given to the increased revertant colonies by the test substance ; (i) effects of free histidine or peptides contained by the test substance which is a proteinaceous material, and (ii) those of some mutagens contained by the test substance. To verify either of the explanations, additional experiments were carried out ; (i) measuring the amount of free histidine in the test substance, and (ii) mutagenicity tests with TA100 of a dimethylsulfoxide extract of the test substance, since most mutagenic chemicals are soluble while peptides are insoluble in dimethylsulfoxide.



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Free histidine in the test substance is 9 mg/100 g. At the highest dosage (5,000 µg/plate), the amount of free histidine from the test substance is about 3% of that in the top agar, therefore free histidine does not seem to contribute in increasing revertant colonies.

The extract of the test substance showed no mutagenic activity (Table 5). Accordingly, the increased revertant colonies may have been caused by such decreased components of protein-like peptides that the tester strains can utilize. It is concluded that no evidence showing a mutagenic potential of the test substance was obtained in this bacterial test system.

Positive control chemicals such as 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, N-ethyl-N'-nitro-N-nitrosoguanidine, 9-aminoacridine, and 2-aminoanthracene markedly increased the revertant colonies.

Experimental procedure

1. Materials

1-1 Bacterial strains

The *Salmonella typhimurium* strains used in this laboratory were obtained from Dr. Bruce N. Ames of the University of California at Berkeley and the *Escherichia coli* strain from Dr. Matsushima of the University of Tokyo.

The *S. typhimurium* strains are all histidine auxotrophs. Some additional genetic markers serve to make the test more sensitive for certain types of mutagens.

The DNA repair mutation (*uvrB*) eliminates excision repair, an error-free repair pathway for DNA damage induced by UV light and certain chemical mutagens. The *uvrB* mutation is part of a deletion mutation extending into the gene for biotin biosynthesis. Therefore, the biotin requirement is indicative of the deletion of this region.

The *rfa* mutation leads to a defective lipopolysaccharide coat and makes the strain more permeable to many large molecules. Strains TA98 and TA100 also contain a resistance transfer factor (plasmid pKM101). This factor, which confers ampicillin resistance, enhances the operation of an error-prone repair system.



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The *E. coli* strain is a tryptophan auxotroph and also defective in the DNA-repair capacity (*uvrA*).

When these histidine or tryptophan-dependent cells are grown on minimal glucose agar plates containing a trace of histidine or tryptophan, only such cells that have reverted to histidine or tryptophan independence (*his*⁺ or *trp*⁺) are able to form colonies. A small amount of histidine or tryptophan allows all the bacteria to undergo a little multiplication; in many cases, this growth is essential for mutagenesis to occur.

The (*his*⁺ or *trp*⁺) revertants are easily visible as colonies against the slight background growth. The spontaneous mutation frequency of each strain is relatively constant.

All tester strains are kept frozen at -80°C in Nutrient broth No. 2 (OXOID) supplemented with 8% sterile dimethylsulfoxide.

The strains are tested routinely for their genetic markers and other characteristics, such as response to positive control chemicals and the number of spontaneous revertants.

For use in tests, subcultures were grown in Nutrient broth No. 2 (OXOID) at 37°C for about 8 hours under gentle shaking.

1-2 S9 Mix

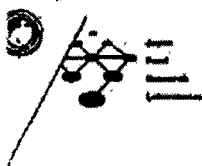
The metabolic activation mixture for each experiment consists of, for 1 ml :

| | | |
|----------------------------------|-----|------|
| S9 fraction | 0.1 | ml |
| MgCl ₂ | 8 | μmol |
| KCl | 33 | μmol |
| G-6-P | 5 | μmol |
| NADPH | 4 | μmol |
| NADH | 4 | μmol |
| Sodium phosphate buffer (pH 7.4) | 100 | μmol |

S9 fraction was purchased from KIKKOMAN and stored at -80°C.

The appended data were as follows :

| | |
|---------------|---|
| Species | rat |
| Strain | Sprague-Dawley |
| Sex | male |
| Age | 7 weeks old |
| Weight range | 190 ~ 239 g |
| Inducer | phenobarbital (PB) & 5,6-benzoflavone (5,6-BF) |
| Dose | PB = 30 + 60 + 60 + 60 mg/kg & 5,6-BF = 80 mg/kg |
| Date prepared | June 4, 1993 |
| Lot No. | RAA-293 |



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1-3 Top agar

The top agar consists of, for 100 ml :

| | |
|--------------------|-------|
| Bacto agar (DIFCO) | 0.6 g |
| NaCl | 0.5 g |

The top agar was autoclaved and mixed with a 0.1 volume of sterile 0.5 mM histidine HCl·H₂O - 0.5 mM biotin solution and 0.5 mM tryptophan solution for the four *Salmonella* strains and *E. coli* strain.

1-4 Minimal glucose agar plate

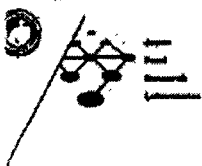
The minimal glucose agar medium was purchased from NISSHIN FLOUR MILLING Co., Ltd.

Date prepared : April 19, 1993 (Lot No. BS010DI)

The minimal glucose agar medium consists of, for 1 L :

| | |
|--|--------|
| MgSO ₄ ·7H ₂ O | 0.2 g |
| Citric acid·H ₂ O | 2 g |
| K ₂ HPO ₄ | 10 g |
| NH ₄ H ₂ PO ₄ | 1.92 g |
| NaOH | 0.66 g |
| Glucose | 20 g |
| Agar | 15 g |

Each plate contained about 30 ml of the minimal glucose agar medium.



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1-3 Top agar

The top agar consists of, for 100 ml :

| | |
|--------------------|-------|
| Bacto agar (DIFCO) | 0.6 g |
| NaCl | 0.5 g |

The top agar was autoclaved and mixed with a 0.1 volume of sterile 0.5 mM histidine HCl·H₂O - 0.5 mM biotin solution and 0.5 mM tryptophan solution for the four *Salmonella* strains and *E. coli* strain.

1-4 Minimal glucose agar plate

The minimal glucose agar medium was purchased from NISSHIN FLOUR MILLING Co., Ltd.

Date prepared : April 19, 1993 (Lot No. BS010DI)

The minimal glucose agar medium consists of, for 1 L :

| | |
|--|--------|
| MgSO ₄ ·7H ₂ O | 0.2 g |
| Citric acid·H ₂ O | 2 g |
| K ₂ HPO ₄ | 10 g |
| NH ₄ H ₂ PO ₄ | 1.92 g |
| NaOH | 0.66 g |
| Glucose | 20 g |
| Agar | 15 g |

Each plate contained about 30 ml of the minimal glucose agar medium.



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2. Procedure

The following procedure was carried out on each tester strain.

(a) Without metabolic activation

A 0.1-ml portion of the test solution at each concentration was added to one set of sterile 12 x 75 mm glass tubes.

Sterile 0.1 M sodium phosphate buffer (pH 7.4) [0.5 ml] and 0.1 ml of a bacterial suspension were added to each tube. The tubes were stirred and placed under shaking for 20 minutes in a 37°C water bath. Then, 2 ml of top agar was added to each tube. The contents were vortexed and poured onto the surface of a minimal glucose agar plate.

After the top agar had solidified, the plates were incubated for 48 hours at 37°C. The *his*⁺ and *trp*⁺ revertant colonies were counted.

(b) With metabolic activation

The method was as described in (a) except for that 0.5 ml of a S9 mix was added to each tube in place of sterile buffer.

A concurrent sterility, negative control, and positive control tests were run in every experiment.

3. Statistical analysis

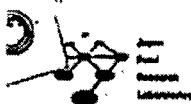
No statistical analysis was performed.

The number of the colonies appearing on each plate was tabulated.

4. Assessment of results

The mean number of revertant colonies for all treatment groups was compared with those obtained for negative and positive control groups. The effect of metabolic activation was assessed by comparing the results obtained in the presence and absence of the S9 mix for each treatment group.

A compound is deemed to provide evidence of a mutagenic potential if (1) a statistically significant dose-related increase in the number of revertant colonies is obtained in two separate experiments, and (2) the number of revertant colonies is larger than twice the concurrent solvent control value.



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- page 6/7 -

2. Procedure

The following procedure was carried out on each tester strain.

(a) Without metabolic activation

A 0.1-ml portion of the test solution at each concentration was added to one set of sterile 12 x 75 mm glass tubes.

Sterile 0.1 M sodium phosphate buffer (pH 7.4) (0.5 ml) and 0.1 ml of a bacterial suspension were added to each tube. The tubes were stirred and placed under shaking for 20 minutes in a 37°C water bath. Then, 2 ml of top agar was added to each tube. The contents were vortexed and poured onto the surface of a minimal glucose agar plate.

After the top agar had solidified, the plates were incubated for 48 hours at 37°C. The *his*⁺ and *trp*⁺ revertant colonies were counted.

(b) With metabolic activation

The method was as described in (a) except for that 0.5 ml of a S9 mix was added to each tube in place of sterile buffer.

A concurrent sterility, negative control, and positive control tests were run in every experiment.

3. Statistical analysis

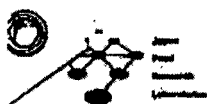
No statistical analysis was performed.

The number of the colonies appearing on each plate was tabulated.

4. Assessment of results

The mean number of revertant colonies for all treatment groups was compared with those obtained for negative and positive control groups. The effect of metabolic activation was assessed by comparing the results obtained in the presence and absence of the S9 mix for each treatment group.

A compound is deemed to provide evidence of a mutagenic potential if (1) a statistically significant dose-related increase in the number of revertant colonies is obtained in two separate experiments, and (2) the number of revertant colonies is larger than twice the concurrent solvent control value.



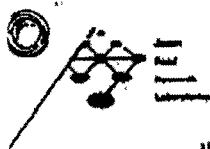
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- page 7/7 -

5. References

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2. McCann, J., Choi, E. and Ames, B.N. Proc. Nat. Acad. Sci. USA 75, 5135 (1975)
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6. The Ministry of Labour, Japan : The Standards of Mutagenicity Test Using Microorganisms, The industrial Safety and Health Law, The Labour Standards Bureau (1991)

- The end -



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No.0556060963

Table 1 Result

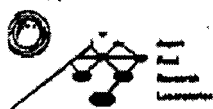
Test specimen : BIOLACTA[®], Stock powder

| Specimen Substance conc. S9Mix μg/plate | | The number of revertant colony(colonies/plate) | | | | |
|---|----------|--|---------|---------|-----------------|---------|
| | | Base-pair substitution type | | | Frameshift type | |
| | | TA100 | TA1535 | WP2uvrA | TA98 | TA1537 |
| Negative control | | | | | | |
| 0 | — | 89 | 6 | 24 | 20 | 8 |
| | | 91 | 3 | 21 | 18 | 11 |
| | | (90) | (5) | (23) | (18) | (8) |
| Test specimen | | | | | | |
| 10 | — | 86 | 8 | 24 | 13 | 8 |
| | | 99 | 1 | 27 | 17 | 4 |
| | | (93) | (5) | (26) | (15) | (6) |
| 50 | — | 100 | 3 | 30 | 19 | 8 |
| | | 119 | 8 | 19 | 17 | 8 |
| | | (110) | (8) | (25) | (18) | (7) |
| 100 | — | 89 | 6 | 28 | 22 | 6 |
| | | 101 | 8 | 21 | 18 | 8 |
| | | (95) | (7) | (24) | (20) | (7) |
| 500 | — | 101 | 2 | 30 | 18 | 7 |
| | | 100 | 7 | 24 | 18 | 5 |
| | | (101) | (5) | (27) | (18) | (6) |
| 1000 | — | 123 | 12 | 24 | 12 | 9 |
| | | 116 | 13 | 30 | 12 | 5 |
| | | (120) | (13) | (27) | (12) | (7) |
| 5000 | — | 122 | 4 | 25 | 25 | 9 |
| | | 122 | 2 | 16 | 27 | 8 |
| | | (122) | (3) | (21) | (26) | (9) |
| Positive Chemicals | | | | | | |
| control | μg/plate | AF-2 | ENNG | AF-2 | AF-2 | 9-AA |
| | | 0.01 | 5 | 0.01 | 0.05 | 80 |
| w/o | colonies | 1115 | 5516 | 390 | 346 | 4368 |
| S9 Mix | /plate | 1190 | 5270 | 332 | 359 | 4345 |
| | | (1153) | (5393) | (361) | (353) | (4357) |

AF-2 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide

ENNG N-ethyl-N'-nitro-N-nitrosoguanidine

9-AA 9-aminoacridine



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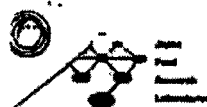
No. DS56060963

Table 2 Result

Test specimen : BIOLACTA*, Stock powder

| Specimen Substance conc. S9Mix μg/plate | | The number of revertant colony(colonies/plate) | | | | |
|---|----------|--|---------|-----------------|---------|--------|
| | | Base-pair substitution type | | Frameshift type | | |
| | | TA100 | TA1535 | WP2uvrA | TA98 | TA1537 |
| Negative control | | | | | | |
| 0 | + | 92 | 10 | 20 | 27 | 24 |
| | | 96 | 8 | 25 | 20 | 19 |
| | | (94) | (9) | (23) | (24) | (22) |
| Test specimen | | | | | | |
| 10 | + | 88 | 7 | 17 | 20 | 17 |
| | | 105 | 6 | 20 | 24 | 15 |
| | | (97) | (7) | (19) | (22) | (16) |
| 50 | + | 102 | 8 | 13 | 27 | 16 |
| | | 87 | 4 | 30 | 22 | 14 |
| | | (95) | (6) | (22) | (25) | (15) |
| 100 | + | 108 | 10 | 27 | 35 | 26 |
| | | 132 | 10 | 20 | 34 | 19 |
| | | (120) | (10) | (24) | (35) | (23) |
| 500 | + | 109 | 10 | 17 | 22 | 22 |
| | | 124 | 8 | 26 | 25 | 17 |
| | | (117) | (9) | (22) | (24) | (20) |
| 1000 | + | 112 | 5 | 32 | 35 | 18 |
| | | 122 | 14 | 18 | 21 | 17 |
| | | (117) | (10) | (25) | (28) | (18) |
| 5000 | + | 140 | 11 | 25 | 28 | 25 |
| | | 146 | 9 | 22 | 39 | 25 |
| | | (143) | (10) | (24) | (34) | (25) |
| Positive control | | | | | | |
| Chemicals | 2-AA | 2-AA | 2-AA | 2-AA | 2-AA | 2-AA |
| μg/plate | 1 | 2 | 10 | 0.5 | 2 | |
| colonies | 2272 | 166 | 529 | 309 | 145 | |
| S9 Mix | 1867 | 170 | 486 | 228 | 154 | |
| /plate | (2070) | (168) | (508) | (269) | (150) | |

2-AA 2-aminoanthracene



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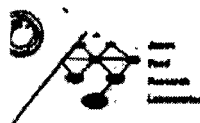
No.0556060963

Table 2 Result

Test specimen : BIOLACTA®, Stock powder

| Specimen Substance conc. S9Mix μg/plate | | The number of revertant colony(colonies/plate) | | | | |
|---|----------|--|---------|-----------------|---------|---------|
| | | Base-pair substitution type | | Frameshift type | | |
| | | TA100 | TA1535 | WP2uvrA | TA98 | TA1537 |
| Negative control | | | | | | |
| 0 | + | 92 | 10 | 20 | 27 | 24 |
| | | 96 | 8 | 25 | 20 | 19 |
| | | (94) | (9) | (23) | (24) | (22) |
| Test specimen | | | | | | |
| 10 | + | 88 | 7 | 17 | 20 | 17 |
| | | 105 | 6 | 20 | 24 | 15 |
| | | (97) | (7) | (19) | (22) | (16) |
| 50 | + | 102 | 8 | 13 | 27 | 16 |
| | | 87 | 4 | 30 | 22 | 14 |
| | | (95) | (6) | (22) | (25) | (15) |
| 100 | + | 108 | 10 | 27 | 35 | 26 |
| | | 132 | 10 | 20 | 34 | 19 |
| | | (120) | (10) | (24) | (35) | (23) |
| 500 | + | 109 | 10 | 17 | 22 | 22 |
| | | 124 | 8 | 26 | 25 | 17 |
| | | (117) | (9) | (22) | (24) | (20) |
| 1000 | + | 112 | 5 | 32 | 35 | 18 |
| | | 122 | 14 | 18 | 21 | 17 |
| | | (117) | (10) | (25) | (28) | (18) |
| 5000 | + | 140 | 11 | 25 | 28 | 25 |
| | | 146 | 9 | 22 | 39 | 25 |
| | | (143) | (10) | (24) | (34) | (25) |
| Positive Chemicals | | | | | | |
| control | μg/plate | 2-AA | 2-AA | 2-AA | 2-AA | 2-AA |
| | | 1 | 2 | 10 | 0.5 | 2 |
| with colonies | | 2272 | 166 | 529 | 309 | 145 |
| S9 Mix /plate | | 1867 | 170 | 486 | 228 | 154 |
| | | (2070) | (168) | (508) | (269) | (150) |

2-AA 2-aminoanthracene



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No.0556080963

Table 3 Result

Test specimen : BIOLACTA[®], Stock powder

| Specimen Substance conc. S9 Mix μg/plate | | The number of revertant colony(colonies/plate) | | | | |
|--|----------|--|---------|-----------------|--------|---------|
| | | Base-pair substitution type | | Frameshift type | | |
| | | TA100 | TA1535 | WP2uvrA | TA98 | TA1537 |
| Negative control | | | | | | |
| 0 | - | 92 | 3 | 20 | 22 | 6 |
| | | 99 | 4 | 25 | 15 | 10 |
| | | (96) | (4) | (23) | (19) | (8) |
| Test specimen | | | | | | |
| 156 | - | 115 | 8 | 15 | 23 | 12 |
| | | 93 | 4 | 20 | 25 | 5 |
| | | (104) | (6) | (18) | (24) | (9) |
| 313 | - | 122 | 7 | 25 | 17 | 13 |
| | | 67 | 4 | 23 | 23 | 9 |
| | | (105) | (6) | (24) | (20) | (11) |
| 625 | - | 100 | 6 | 27 | 21 | 8 |
| | | 102 | 8 | 35 | 17 | 3 |
| | | (101) | (7) | (31) | (19) | (6) |
| 1250 | - | 100 | 7 | 27 | 18 | 13 |
| | | 97 | 11 | 28 | 23 | 9 |
| | | (99) | (9) | (28) | (21) | (11) |
| 2500 | - | 114 | 9 | 23 | 20 | 9 |
| | | 87 | 5 | 26 | 21 | 7 |
| | | (101) | (7) | (25) | (21) | (8) |
| 5000 | - | 130 | 7 | 26 | 22 | 6 |
| | | 118 | 9 | 34 | 23 | 11 |
| | | (124) | (8) | (30) | (23) | (9) |
| Positive Chemicals | | | | | | |
| control | μg/plate | AF-2 | ENNG | AF-2 | AF-2 | 9-AA |
| w/o | colonies | 0.01 | 5 | 0.01 | 0.05 | 80 |
| S9 Mix | /plate | 1009 | 5295 | 370 | 337 | 5776 |
| | | 933 | 5391 | 297 | 355 | 4748 |
| | | (971) | (5343) | (334) | (346) | (5262) |

AF-2 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide

ENNG N-ethyl-N'-nitro-N-nitrosoguanidine

9-AA 9-aminoacridine

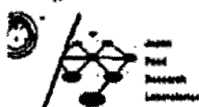
No.0556060863

Table 4 Result

Test specimen : BIOLACTA®, Stock powder

| Specimen Substance conc. S9Mix μg/plate | | The number of revertant colony(colonies/plate) | | | | |
|---|--------|--|--------|---------|-----------------|--------|
| | | Base-pair substitution type | | | Frameshift type | |
| | | TA100 | TA1535 | WP2uvrA | TA98 | TA1537 |
| Negative control | | | | | | |
| 0 | + | 81 | 2 | 20 | 29 | 23 |
| | | 88 | 3 | 24 | 21 | 14 |
| | | (85) | (3) | (22) | (25) | (18) |
| Test specimen | | | | | | |
| 156 | + | 88 | 11 | 30 | 29 | 18 |
| | | 99 | 11 | 21 | 27 | 31 |
| | | (94) | (11) | (26) | (28) | (25) |
| 313 | + | 93 | 6 | 22 | 28 | 15 |
| | | 105 | 3 | 32 | 29 | 17 |
| | | (99) | (5) | (27) | (29) | (16) |
| 625 | + | 103 | 7 | 22 | 25 | 26 |
| | | 109 | 7 | 26 | 23 | 25 |
| | | (106) | (7) | (24) | (24) | (26) |
| 1250 | + | 93 | 9 | 30 | 39 | 18 |
| | | 107 | 10 | 32 | 35 | 21 |
| | | (100) | (10) | (31) | (37) | (20) |
| 2500 | + | 120 | 7 | 22 | 33 | 28 |
| | | 113 | 3 | 29 | 43 | 24 |
| | | (117) | (5) | (26) | (38) | (26) |
| 5000 | + | 167 | 11 | 31 | 37 | 39 |
| | | 145 | 15 | 27 | 34 | 31 |
| | | (156) | (13) | (29) | (36) | (35) |
| Positive control | | | | | | |
| Chemicals | | 2-AA | 2-AA | 2-AA | 2-AA | 2-AA |
| μg/plate | | 1 | 2 | 10 | 0.5 | 2 |
| colonies | | 1488 | 242 | 495 | 236 | 245 |
| S9 Mix | /plate | 1354 | 266 | 621 | 359 | 229 |
| | | (1421) | (254) | (558) | (298) | (237) |

2-AA 2-aminoanthracene



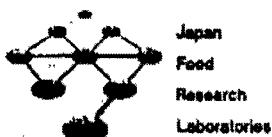
No.0556060963

Table 5 Result

Test specimen : The extract of BIOLACTA®, Stock powder

| Specimen | The number of revertant colony(colonies/plate) | |
|-----------------------|--|---------|
| Substance conc. S9Mix | Base-pair substitution type | |
| μl/plate | TA100 | |
| <hr/> | | |
| Negative control | | |
| 0 | + | 108 |
| | | 95 |
| | | (102) |
| Test specimen | | |
| 50 | + | 96 |
| | | 94 |
| | | (95) |
| 100 | + | 93 |
| | | 104 |
| | | (99) |
| 200 | + | 96 |
| | | 106 |
| | | (102) |
| <hr/> | | |
| Positive control | Chemicals | 2-AA |
| with S9 Mix | μg/plate colonies /plate | 1 |
| | | 1799 |
| | | 1141 |
| | | (1470) |

2-AA 2-aminoanthracene



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KYUSHU BRANCH : 1-12, SHIMOGOFUKU-MACHI, HAKATA-KU, FUKUOKA-SHI

17/25

ANALYSIS CERTIFICATE

No. OS54010334-2
March 8, 1991

Requested by : DAIWA KASEI K. K.
7-12 UEHONMACHI 5-CHOME TENNOJIKU, OSAKA 543

Received : January 12, 1991

Acute Toxicity Test in Mice of BIOLACTA[®], stock powder

Abstract

The acute oral toxicity of BIOLACTA[®], stock powder in mice was investigated in accordance with OECD GUIDELINES FOR TESTING OF CHEMICALS(1987).

Oral administration of 2,000 mg/kg caused no deaths, apparent symptoms, abnormal necropsy findings or effects on body weight gain.

From these results, it was considered that BIOLACTA[®], stock powder has no acute oral toxicity in mice.

Test period

January 28, 1991 ~ March 8, 1991

Materials and Methods

1) Test substance

BIOLACTA[®], stock powder

A technical grade stock powder of a
 β -galactosidase produced by Bacillus circulans.

2) Preparation of test solution

Test substance was dissolved in purified water.
Concentration of the solution was 200 mg/ml.

3) Experimental animals

Each 10 male and 10 female mice of the ICR strain bred by Japan SLC, INC. were used. The mice were obtained at 4 weeks old and acclimated to the laboratory conditions for 1 week. They were kept in groups according to sex in plastic cages at a temperature of 23 ± 2 °C with 12 hours light-dark cycle. There were 10 mice per cage. Conventional laboratory diets and fresh water were fed ad libitum.

- continued -



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- page 2 -

4) Procedure (Limit test)

The mice were fasted prior to substance administration for about 4 hours. Following a period of fasting, they were weighed. The males ranged from 24.9 to 32.1 g and the females were between 24.2 and 27.7 g. The test substance was administered orally at the dose level of 2,000 mg/kg to the mice by gavage using a stomach tube.

Clinical examination was made frequently on the day of administration and once a day during the following period. The mice were weighed weekly.

At the end of the test (14 days), they were sacrificed and necropsy of all mice was carried out.

Results

1) Clinical examination

No abnormal clinical signs were observed in both males and females and no mice were dead.

2) Body weight (Table 1, 2)

No decrease of body weight was detected in both males and females.

3) Necropsy

No abnormalities were found in both males and females.

Discussion

Oral administration of 2,000 mg/kg caused no abnormalities and no deaths in mice.

Consequently, it was considered that BIOLACTA[®], stock powder has no acute oral toxicity in mice and the lethal dose is more than 2,000 mg/kg.

- The end -

Japan Food Research Laboratories

(b)(6)

K. Takahashi

Inspector

000363



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Table 1 Body weight (male)

| Mouse No. | Before treatment | After treatment | |
|---------------|------------------|-----------------|----------------|
| | | 7 days | 14 days |
| 1 | 32.1 | 39.4 | 42.6 |
| 2 | 27.7 | 34.1 | 37.2 |
| 3 | 29.9 | 33.7 | 36.1 |
| 4 | 31.7 | 34.3 | 36.9 |
| 5 | 30.4 | 37.0 | 40.4 |
| 6 | 28.1 | 36.6 | 38.6 |
| 7 | 27.0 | 35.7 | 38.0 |
| 8 | 25.3 | 32.5 | 35.2 |
| 9 | 24.9 | 33.4 | 35.2 |
| 10 | 27.9 | 36.9 | 40.5 |
| mean \pm SD | 28.5 \pm 2.5 | 35.4 \pm 2.1 | 38.1 \pm 2.5 |

Table 2 Body weight (female)

| Mouse No. | Before treatment | After treatment | |
|---------------|------------------|-----------------|----------------|
| | | 7 days | 14 days |
| 1 | 27.3 | 29.4 | 29.6 |
| 2 | 24.2 | 25.3 | 27.2 |
| 3 | 27.0 | 29.4 | 30.6 |
| 4 | 26.5 | 29.3 | 32.7 |
| 5 | 26.2 | 28.8 | 29.7 |
| 6 | 27.7 | 30.2 | 32.3 |
| 7 | 25.6 | 27.4 | 29.5 |
| 8 | 25.3 | 27.7 | 29.5 |
| 9 | 25.0 | 26.5 | 29.3 |
| 10 | 24.5 | 30.4 | 31.4 |
| mean \pm SD | 25.9 \pm 1.2 | 28.4 \pm 1.7 | 30.2 \pm 1.6 |



22/25

**MYCOTOXIN RESEARCH ASSOCIATION
(M. R. A.)**

4th Floor, Daidoanyu Bldg.,
92, Yamashita-cho, Naka-ku, Yokohama
231 JAPAN

Myco No. 2 - 1945

Date: Feb. 25, 1991

C E R T I F I C A T E

Article Examined : BIOLACTA[®], Stock powder.
[A technical grade stock powder of a β -galactosidase produced
by Bacillus circulans]

Name and Address of

Applicant : DAIWA KASEI K.K. .
7-12 UEHONMACHI 5-CHOME TENNOJIKU, OSAKA 543

Kind of Examination : Qualitative Analysis of Aflatoxin B₁, Ochratoxin A,
Sterigmatocystin, Zearalenone, and T-2 Toxin

Method of Analysis :

We showed in accompanying sheet

Result of Examination:

Receiving No. and Date: No.02-6366 and January 14, 1991

I hereby certify that the result of examination was as
stated above.

(b)(6)

(Dr. Shigeru Oshifuchi)

For Director

Mycotoxin Research Association

Designated by the Minister of

Health & Welfare



23/25

**MYCOTOXIN RESEARCH ASSOCIATION
(M. R. A.)**

4th Floor, Daikanyu Bldg.,
92, Yamashita-cho, Naka-ku, Yokohama
231 JAPAN

Method of Analysis : for Aflatoxins, Ochratoxin A, Sterigmatocystin,
and Zearalenone

- B.G. EGON JOSEFSSON and TORD E. MOILLER
Screening Method for the Detection Aflatoxins,
Ochratoxin, Patulin, Sterigmatocystin, and
Zearalenone in Cereals
Journal of The AOAC 60(6):1369-1371, 1977

for T-2 Toxin

- CHOONG W. CHUNG, et al
Rabbit Skin Test for Estimation of T-2 Toxin
and Other Skin-Irritating Toxins in Contaminated
Corn
Journal of The AOAC 57(5):1121-1127, 1974
- R. M. EPPLEY
Screening Method for Zearalenone, Aflatoxin, and
Ochratoxin
Journal of The AOAC 51(1):74-78, 1968

| | | |
|--------------------------------|--------------------------|----------|
| Result of Examination : | Aflatoxin B ₁ | Negative |
| | Ochratoxin A | Negative |
| | Sterigmatocystin | Negative |
| | Zearalenone | Negative |
| | T-2 Toxin | Negative |



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 NAGOYA BRANCH : 5-13, 4-CHOME, OSU, NAKA-KU, NAGOYA
 KYUSHU BRANCH : 1-12, SHIMOGOFUKU-MACHI, HAKATA-KU, FUKUOKA-SHI

24/25

ANALYSIS CERTIFICATE

No. OS54010334-4
 March 8, 1991

Requested by : DAIWA KASEI K. K.
 7-12 UEHONMACHI 5-CHOME TENNOJIKU, OSAKA 543

Received : January 12, 1991

Antibacterial Activity Test

1. Sample

BIOLACTA[®], stock powder
 A technical grade stock powder of a
 β -galactosidase produced by Bacillus circulans.

2. Purpose

To test antibacterial activity of the sample.

3. Outline

The test was carried out in accordance with the method
 described in Food Additives and Contaminants Committee, 1982,
 LONDON "REPORT ON THE PREVIEW OF ENZYME PREPARATIONS" APENDIX W,
 General Purity Criteria Applicable to Enzyme Preparations, 9.

4. Results

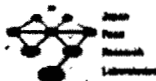
Results are shown in Table 1.

Table 1 Antibacterial activity of the sample

| Test organism | Disk | | | | | |
|--|------|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| <u>Staphylococcus aureus</u> ATCC 6538 | - | - | - | - | - | - |
| <u>Escherichia coli</u> ATCC 11229 | - | - | - | - | - | - |
| <u>Bacillus cereus</u> ATCC 2 | - | - | - | - | - | - |
| <u>Bacillus circulans</u> ATCC 4516 | - | - | - | - | - | - |
| <u>Streptococcus pyogenes</u> ATCC 12344 | - | - | - | - | - | - |
| <u>Serratia marcescens</u> ATCC 14041 | - | - | - | - | - | - |

- ; Inhibition zone was not detected.

- continued -



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- page 2 -

C. Methods

1) Test organisms

Staphylococcus aureus ATCC 6538
Escherichia coli ATCC 11229
Bacillus cereus ATCC 2
Bacillus circulans ATCC 4516
Streptococcus pyogenes ATCC 12344
Serratia marcescens ATCC 14041

2) Culture plates

Make a test plate of each organism by preparing a 1:10 dilution of a 24 hours Trypticase Soy Broth culture in Trypticase Soy Agar [TSA] (for Streptococcus pyogenes ATCC 12344 a 1:20 dilution).

Pour 15 ml of plain TSA into a Petri dish and allow the medium to harden. Overlay with 10 ml of seeded TSA and let solidify.

3) Disk preparation

Make a 10 % solution of the enzyme preparation by adding 1 g of the sample to 9 ml of sterile distilled water.

Mix thoroughly with a Vortex mixer to obtain a homogeneous suspension.

Autoclave the paper disks (ϕ 13 mm), then saturate them with the enzyme by application of 0.1 ml of a 10 % solution of the enzyme to the disk surface. Prepare 6 disks for the enzyme : place a disk on the surface of the 6 inoculated agar plates.

4) Incubation

Keep the 6 plates in the refrigerator overnight to obtain proper diffusion. Incubate the plates at 37 °C for 24 hours. Examine the plates for any inhibition zones that may have been caused by the enzyme preparation.

- The end -

Japan Food Inspection Laboratories

(b)(6)

K. Takagi Inspector

000368

Appendix 2A Exhibit 19

Chromosome Aberration Test in Chinese Hamster Ovary Cells, Biolacta[®] N5

Pages 000370-000414 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 2B

β -Galactosidase and Biolacta[®] N5
from *Bacillus circulans*

PROPRIETARY PROCESS TECHNOLOGY

Contents

Appendix 2B: β -Galactosidase and Biolacta[®] N5

[PROPRIETARY PROCESS TECHNOLOGY]

- Exhibit 1. GRAS Status Summary for β -Galactosidase and Biolacta[®] N5
II. Production Process of Biolacta[®] N5
- Exhibit 2. Maintenance of Culture Strain
- Exhibit 7. Optimal Conditions for Hydrolysis to Occur
- Exhibit 8. Manufacturing Conditions for Biolacta[®] N5

Appendix 2B
Exhibit 1

GRAS Status Summary for
 β -Galactosidase and Biolacta[®] N5
Section II

PROPRIETARY PROCESS TECHNOLOGY

II. PRODUCTION PROCESS OF BIOLACTA[®] N5

1. Biolacta[®] N5

The commercial name of the β -galactosidase preparation used in the manufacturing process of Vivinal[®] GOS is Biolacta[®] N5. Biolacta[®] N5 is manufactured by Daiwa Kasei in Japan. Properties of the Biolacta[®] N5 β -galactosidase preparation used in the production of Vivinal[®] GOS are presented in Table II-1.

| Table II-1. Properties of the Biolacta [®] N5 β -galactosidase Preparation | |
|---|--|
| Property | Biolacta [®] N5 |
| Optimal pH | 6.0 (40°C, 10 min, 10% lactose, 10 mmol/L Britton-Robinson buffer) |
| pH-Stability | 5.0 – 9.5 (treated at 30°C for 60 min., 47.5 mmol/L Britton-Robinson buffer) |
| Optimal Temperature | 65°C (pH 6.0, 10 min, 10% lactose, 50 mmol/L acetic acid-sodiumacetate buffer) |
| Thermal Stability | Below 55°C (pH. 6.0, 50 mmol/L acetic acid-sodiumacetate buffer) |
| Solubility | readily soluble in water and insoluble in organic solvent |

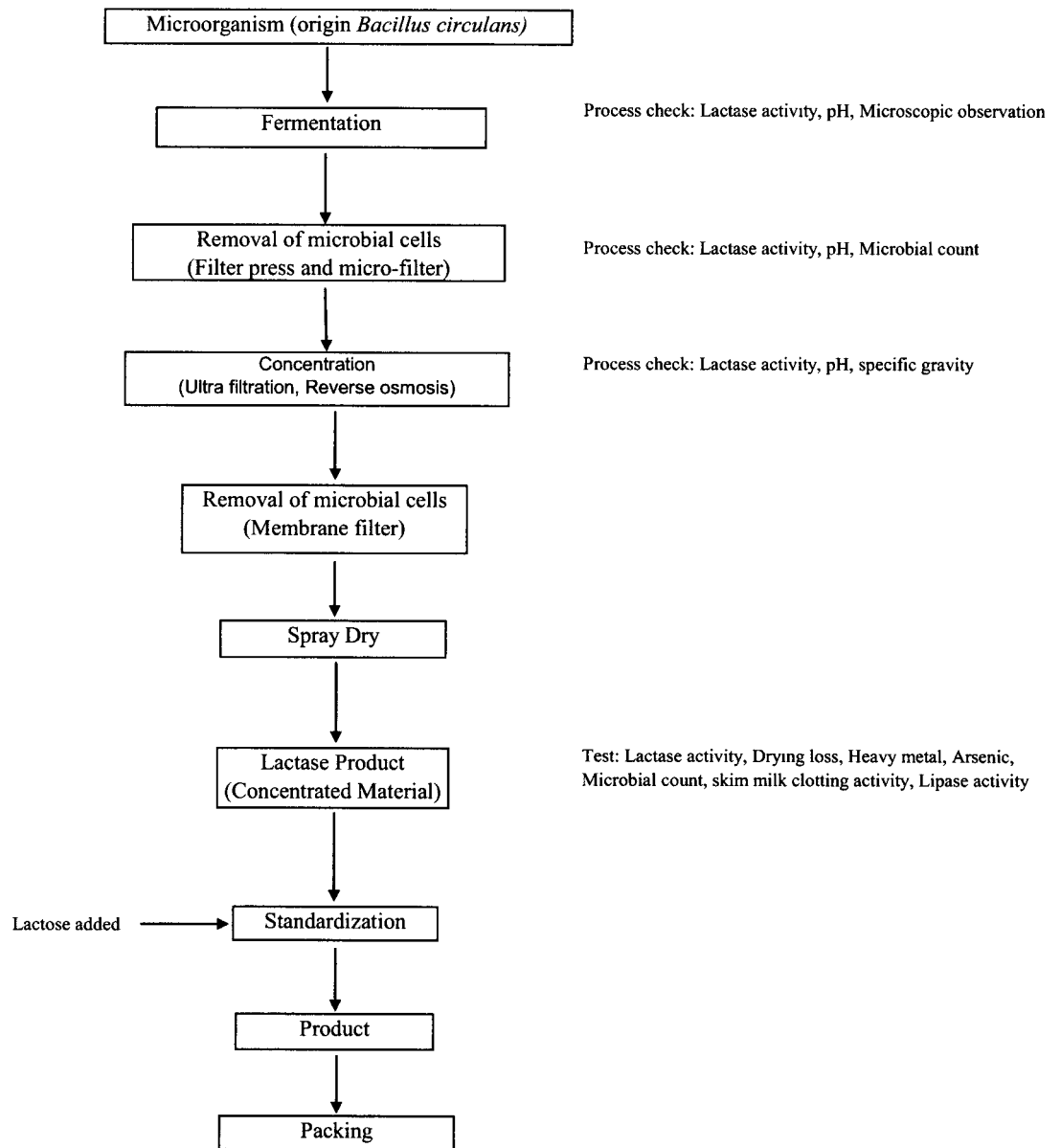
2. Description of any potential side reactions

The β -galactosidase enzyme is a hydrolase that can transfer non-reducing β -D-galactose residues from β -D-galactosides (e.g. lactose) to water. Under the conditions applied in the production process of Vivinal[®] GOS (high lactose concentrations) the enzyme utilizes lactose as an alternative acceptor (instead of water) resulting in the formation of galacto oligosaccharides. Under other reaction conditions, the enzyme could perform hydrolysis as opposed to polymerization reactions resulting in lower molecular weight (shorter-chain) GOS. Optimum pH for hydrolysis to occur ranges from 5.5 to 6.5 while optimum temperature for hydrolysis is 60°C (See Exhibit 7).

3. Biolacta[®] N5 Production Process

Biolacta[®] N5 is produced by means of bacterial fermentation. The process comprises a cultivation step followed by several filtration and purification steps. Figure 1 below is a flow chart representation of the production process for Biolacta[®] N5. A food grade fermentation medium (defatted soybean meal containing lactose) is used in the manufacturing of β -galactosidase. β -galactosidase is an extracellular enzyme. It is isolated from other proteins and enzymes present in the medium or cell content by filterpress and a microfilter. Isolation of the enzyme is ensured by measuring enzyme activity. Further details pertaining to the temperatures, pH levels and other parameters involved in the production process are presented in Exhibit 8.

Figure 1. Production Process of Biolacta[®] N5



Stability of Biolacta[®] N5 over a period of 15 months is demonstrated in Exhibit 9.

4. Methods used to establish the identity and to evaluate the purity of Biolacta® N5

Assay methods utilized to identify and evaluate the purity of the Biolacta® N5 preparation are presented in Table II-2. Daiwa's methods, as referred to in the table, are also further elucidated in Exhibits 10-12.

| Table II-2. Methods for Evaluating Purity of Biolacta® N5 | | |
|--|-------------------------|---------------------------|
| Test items | Specification | Assay method |
| Lactase activity | $\geq 5,000\text{LU/g}$ | Daiwa's method |
| Drying loss | $\leq 6.0\%$ | JP (1g, 80°, 3h) |
| *Heavy metals (as Pb) | $\leq 40\mu\text{g/g}$ | JP (method 2) |
| *Arsenic (as As_2O_3) | $\leq 3\mu\text{g/g}$ | JP (method 3) |
| Viable bacteria count | $\leq 10^4/\text{g}$ | Daiwa's method (SCD agar) |
| Coliforms | $\leq 30/\text{g}$ | Daiwa's method (DCA agar) |

*Tested using concentrated material before standardization; Biolacta is 30% lactase and 70% lactose.

The Total Organic Solids (TOS) in Biolacta® N5 make up 90% of the product. Excluding lactose, 23.7% of the product is TOS (protein and fiber). Biolacta® N5 has not been observed to have other enzymatic activities. Table II-3 below is a summary of Biolacta® N5's composition based on typical batch data. Exhibit 13 provides documentation of this batch data along with specifications.

Table II-3. Batch Analysis Data of Biolacta® N5 Composition

| | Results | | | | | | | | |
|-----------------------------------|-------------|--------------|--------------|--------------|----------|-------------|-------------|-----------------|-------------------------------|
| | Lot Number | | | | | | | | |
| | P6IID001 | P6JA201 | P6JD101 | P6LA501 | P6kb301 | Mean | Std | Specification | Analytical Method |
| Lactase Activity (LU/g) | 5,750 | 5,770 | 5,630 | 5,670 | 5,600 | 5,684 | 74 | ≥ 5,000 LU/g | Daiwa |
| ONPC Hydrolyzing Activity (LSU/g) | 5,060 | 5,100 | 5,070 | 1,660 | 1,780 | 3,734 | 1,839 | Analyzed only | N/A |
| *Heavy Metals (µg/g) | ≤40 | ≤40 | ≤40 | ≤40 | ≤40 | ≤40 | 0 | ≤40 µg/g | JP (method 2) |
| *Arsenic (µg/g) | ≤3 | ≤3 | ≤3 | ≤3 | ≤3 | ≤3 | 0 | ≤3 µg/g | AAS |
| Aerobic Microorganisms (CFU/g) | 10 | 10 | 10 | 10 | 10 | 10 | 0 | ≤10 CFU/g | Daiwa (SCD agar) |
| Coliforms (CFU/g) | ≤30 | ≤30 | ≤30 | ≤30 | ≤30 | ≤30 | 0 | ≤30 CFU/g | Daiwa (DCA agar) |
| Salmonella (in 25 g) | Negative | Negative | Negative | Negative | Negative | -- | -- | Negative in 25g | Daiwa FDA/BAM 8 th |
| E. coli | Negative | Negative | Negative | Negative | Negative | -- | -- | Negative in 25g | Daiwa FDA/BAM 8 th |
| | Lot Number | | | | | | | | |
| Macronutrients (g/100g) | LT0606154 | LT0607144.01 | LT0607205.11 | LT0607205.12 | -- | Mean | Std | | |
| Protein | 20.4 | 20.8 | 21.0 | 21.0 | | 20.8 | 0.28 | -- | Jpn Food Res Lab |
| Fat/Oil | <0.1 | <0.1 | <0.1 | <0.1 | | <0.1 | 0 | -- | Jpn Food Res Lab |
| Carbohydrates | 69.3 | 69.1 | 68.9 | 69.0 | | 69.1 | 0.2 | -- | -- |
| Total Sugars (Lactose) | 66.5 | 66.5 | 66.3 | 66.3 | | 66.4 | 0.1 | -- | Jpn Food Res Lab |
| Dietary Fibers | 2.8 | 2.6 | 2.6 | 2.7 | | 2.7 | 0.1 | -- | AOAC 985.29 |
| Total Organic Solids (TOS) | 89.7 | 89.9 | 89.9 | 90 | | 89.9 | 0.13 | -- | -- |
| Moisture | 5.0 | 5.0 | 5.0 | 5.0 | | 5.0 | 0 | -- | Jpn Food Res Lab |
| Ash | 5.3 | 5.1 | 5.1 | 5.0 | | 5.1 | 0.13 | -- | Jpn Food Res Lab |
| Sodium | 2.0 | 1.9 | 1.8 | 1.8 | | 1.9 | 0.09 | -- | Jpn Food Res Lab |
| Total Components | 100 | 100 | 100 | 100 | | 100 | 0 | | |

Source: Daiwa Kasei; N/A = Not Applicable

* Tested using concentrated material before standardization

Appendix 2B Exhibit 2

Maintenance of Culture Strain

PROPRIETARY PROCESS TECHNOLOGY

DAIWA KASEI K. K.
4-19, HIE-CHO, KONAN, SHIGA, 520-3203 JAPAN

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F A X : +81-748-75-0312

Maintenance of the master cell culture of production strain for β -galactosidase

The master cell culture of production strain for β -galactosidase is identified by its unique number, and it is stored frozen at $-75^{\circ}\text{C} \sim -85^{\circ}\text{C}$.

Maintenance of the culture is conducted at 5 years intervals, according to following procedures:

1. Plating out

- 1.1 Remove the vial of master cell culture from storage and thaw the contents rapidly in a 38°C water bath
- 1.2 Streak a loopful of the contents in the vial onto nutrient agar plate and cultivate for 3 days at 38°C .
- 1.3 Select 21 of isolated colonies on the plate and identify each colony by marking unique number on the bottom of petri dish.
- 1.4 Pick up bacteria cells from each colony with sterilized toothpick and transfer to fresh nutrient agar plate. Mark each colony number on the bottom of petri dish to identify each transferred culture. After 3 days incubation at 38°C , store it in a refrigerator.

2. Enzyme productivity

- 2.1 Inoculate a loopful of cell from the colonies obtained in 1.3 to 100 ml of seed medium¹⁾ in 500 ml flasks and cultivate for 24 hrs at 38°C , 110 strokes/minute (see culture).
- 2.2 At the same time, inoculate master cell culture into a flask and the same condition as a control.
- 2.3 Inoculate 1 ml of each seed culture to 100 ml of production medium²⁾ in 500 ml Erlenmeyer flask and cultivate for 3 days at 38°C .
- 2.4 After 3 days, assay β -galactosidase activity of the cultured fluid.

— Each culture should show activity of 97 to 103 % as compare with 100 % for a control.

Note : If one or more cultures show lower activity than 97 % of that of a control, select the culture prepared in 1.4, which has show normal enzyme productivity. Disperse the cell in physiological saline. And do over again plating out from 1.2.

3. Preparation of frozen culture

- 3.1 Inoculate a loopful of cells from one of the plate (see 1.4) to 100 ml of seed medium¹⁾ in 500 ml flask and cultivate at 38°C , 110 strokes/minute.
- 3.2 After 24 hrs, dispense 0.5 ml aliquots of seed culture into autoclaved vials containing 0.5 ml of protective medium (20% glycerin). Store the vials frozen at $-75^{\circ}\text{C} \sim -85^{\circ}\text{C}$ (approximately 200 vials).

DAIWA KASEI K. K.
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4. Inspection of frozen culture

4.1 Enzyme productivity

4.1.1 Sample 10 vials of frozen culture at random, and prepare seed cultures (see 2.1).

4.1.2 Inoculate 1 ml of each 24-hr-old culture to production medium and cultivate (see 2.3).

4.1.3 After 3 days, assay the enzyme activity of the culture.

—The activity of each culture should be in a range of 97 to 103 % of the mean value.

Note : If one or more cultures show lower activity than 97 % of that of a control, do over again plating out.

4.2 Subculture

4.2.1 Using a vial of frozen culture, prepare seed culture (see 2.1).

4.2.2 Subculture 1 ml of a 24 hr-old culture to seed medium and production medium, and cultivate (see 2.1 and 2.3). Repeat this procedure twice at 24 hrs intervals

4.2.3 Inspect microbial purity in seed culture by mean of plate culture (see 2.1), and assay the enzyme activity of production cultures.

Foreign microbes should not be detected on the agar plate.

The enzyme activity in production culture should not decline during repeated subculturing.

5. Identification

The lot of frozen culture which has passed the inspection, is stored as a new master cell culture.

The lot is identified by color of screw cap vial and the lot number, which is marked on the surface of each vial.

| | | |
|-------------------------------------|------------------------------------|--------|
| 1) Composition seed medium | Resolved soybean | 3.0 % |
| | meat extract | 2.6 % |
| | Yeast extract | 1.0 % |
| | Lactose | 0.3 % |
| | Autoclaved for 12 minutes at 125°C | |
| 2) Composition of production medium | Lactose | 7.8 % |
| | De fattyed soy bean | 3.8 % |
| | Corn steep liquor | 2.4 % |
| | Ammonium diphosphate | 0.4 % |
| | Calcium carbonate | 0.3 % |
| | Ammonium sulfate | 0.05 % |
| | Soy bean oil (anti foam) | 0.75 % |
| | Autoclaved for 12 minutes at 121°C | |

Appendix 2B

Exhibit 7

Optimal Conditions for Hydrolysis to Occur

PROPRIETARY PROCESS TECHNOLOGY

11.7 Datenblatt der Firma Daiwa Kasei K.K., Osaka, zur β -Galactosidase aus
Bacillus circulans

LACTASE
BIOLACTA

This lactase is of *Bacillus circulans* and is characterized by the following features :

1. High affinity for the natural substrate, lactose, compared with other lactases (Table 1)
2. Optimum pH range : 5.5 - 6.5 (Fig. 1)
3. Stable pH range : 4.5 - 10.0 (Fig. 2)
4. Optimum temperature : 60°C (Fig. 3)

Activity units

LU : One LU of lactase hydrolyzes 1 μ mole of lactose per minute at 40°C, pH 6.0.

ONPGU : One ONPGU of lactase hydrolyzes 1 μ mole of o-nitrophenyl- β -D-galactopyranoside (ONPG) per minute at 40°C, pH 6.0.

Application to Milk or Whey

The optimum working temperature of this lactase is around 50°C (Fig.5), as is expected from Fig. 3 and 4, but it is practically applicable enough at 60°C (Fig. 6) and 5°C (Fig. 7).

Three LU of this lactase can hydrolyze 70 per cent of the lactose in 1 ml of fresh milk in about 40 minutes at 60°C and in about 8 hours at 5°C.

Specification

| Grade | Unit | Form | |
|--------------|------------|--------|---------------|
| BIOLACTA FNS | 5,000 LU/g | Powder | Protease Free |
| BIOLACTA NS | 5,000 LU/g | Powder | |

Table 1 LU/ONPGU Ratios of Lactases

| Origin of lactases | B. circulans | Asp. oryzae | Asp. niger | Pen. citrinum | E. coli | Sacch. lactis |
|--------------------|--------------|-------------|------------|---------------|---------|---------------|
| LU/ONPGU | 1 | 1/4 | 1/3 | 1/4 | 1/28 | 1/4 |

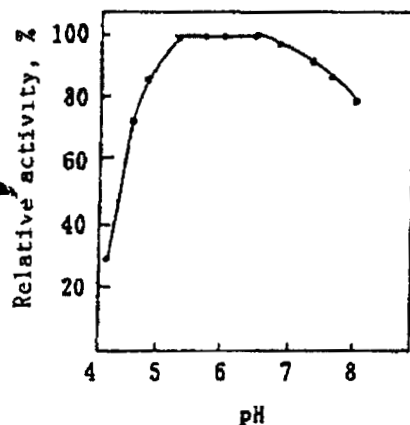


Fig. 1 pH - Activity

Substrate : lactose

Buffer : citrate-phosphate
(McIlvaine's)

Reaction : 40°C , 15 min

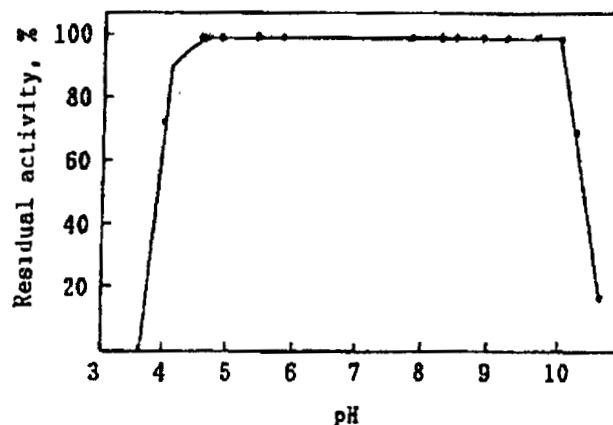


Fig. 2 pH - Stability

Buffer : pH 2.6 - 8.3 , citrate-phosphate
pH 8.5 - 10.2 , glycine-NaOH

Incubation : 30°C , 1 hr

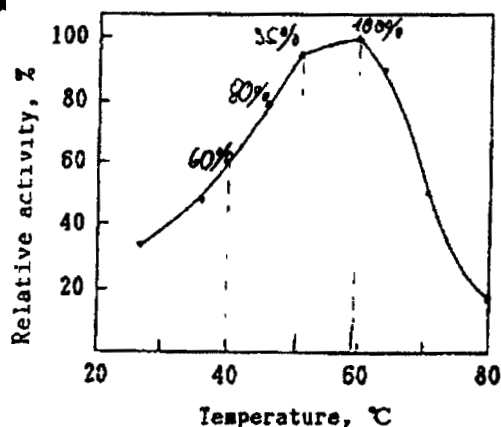


Fig. 3 Temperature - Activity

Substrate : lactose

Buffer : 0.1M acetate, pH 6.0

Reaction : 15 min

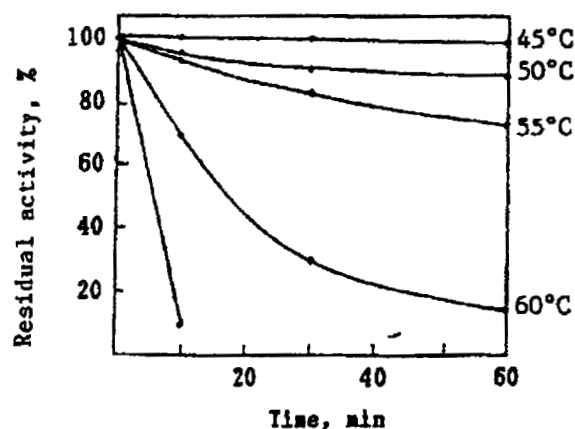


Fig. 4 Temperature - Stability

Incubation : in 0.1M acetate
buffer, pH 6.0

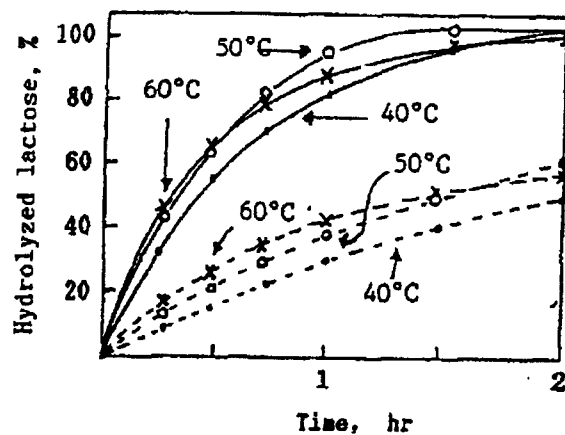


Fig. 5 Application to Whey

Substrate : whey powder in 0.1M acetate buffer, pH6.0
lactose content 4.9 %
Lactase level :
—— 5 LU per ml of substrate
----- 1 LU per ml of substrate
Temperature : 40°C, 50°C, and 60°C

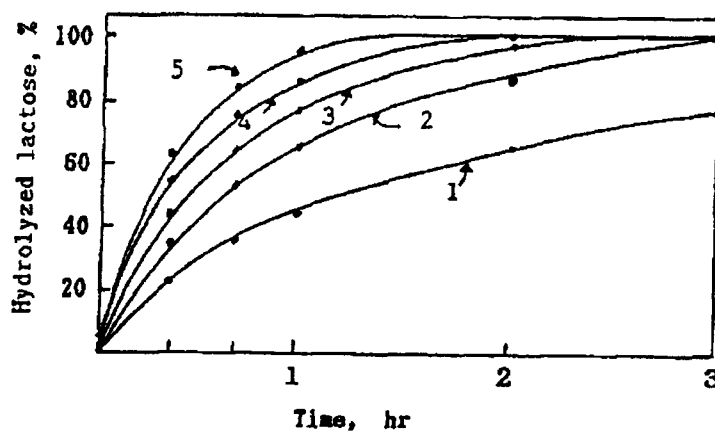


Fig. 6 Application to Milk at 60°C

Substrate : fresh milk
Lactase level :
1, 2, 3, 4, and 5
LU per ml of milk

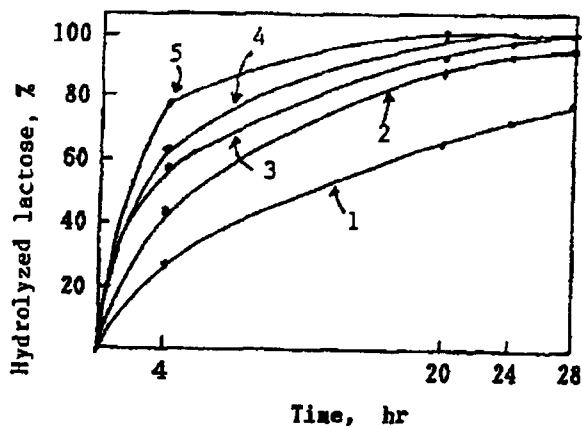


Fig. 7 Application to Milk at 5°C

Substrate : fresh milk
Lactase level : 1, 2, 3, 4, and 5
LU per ml of milk

Appendix 2B
Exhibit 8

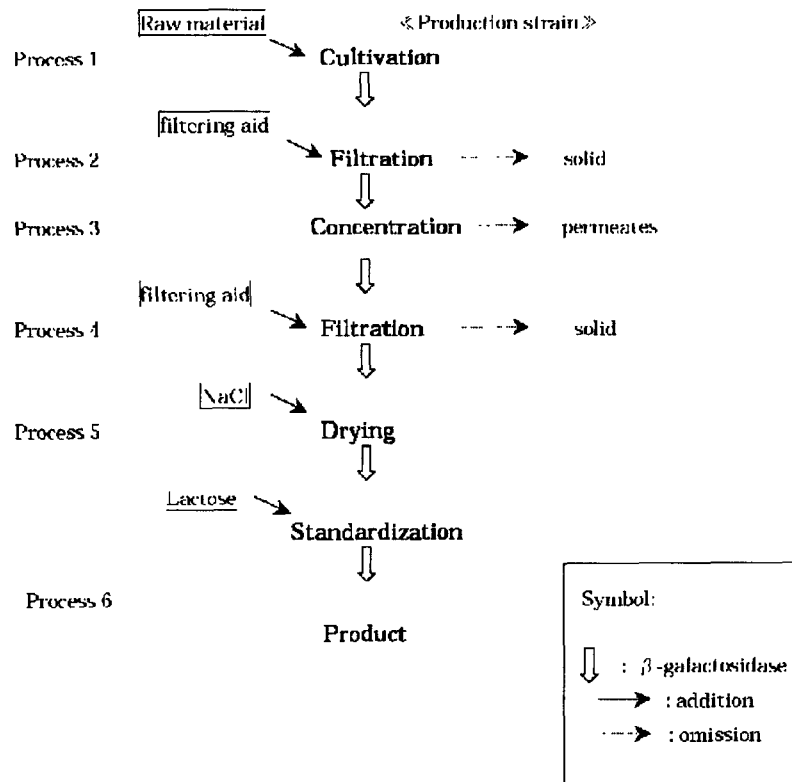
Manufacturing Conditions for Biolacta[®] N5
PROPRIETARY PROCESS TECHNOLOGY

Manufacturing process of *Bacillus circulans* β -Galactosidase

Process scheme

β -galactosidase is produced by means of bacterial fermentation. The process was comprised a cultivation step, followed by several filtration and purification steps. An outline of the manufacturing process, including information on fermentation media and conditions is given. The production process is depicted in the figure below.

Fig.1 Process scheme



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PHONE : +81-748-75-1194
F A X : +81-748-75-0312

Table 1. Production control β galactosidase

| Process | | Control point | Standard | Materials |
|------------------|----------------------------------|--|---|--|
| 1. Cultivation | Seed culture & Pre-culture | Temperature | 38.0 \pm 0.5°C | Resolved soybean |
| | | pH | To 7.0 from 7.5 | Meat extract |
| | | Microscopic observation, subculture | Microbial contaminant should not be detected | Yeast extract |
| | | | | Lactose |
| | Main culture | Temperature | 38.0 \pm 0.5°C | Lactose, Defatted |
| | | pH | 7.5 \pm 0.3 | Soybean, Corn steep |
| | | Microscopic observation | Microbial contaminant should not be detected | Liquor, Soybean oil Water, (NH ₄) ₂ SO ₄ (NH ₄)H ₂ PO ₄ , Na ₂ CO ₃ , CaCO ₃ |
| 2. Filtration | 1 st Filtration | Temperature | To 15°C from 7°C | Perlite, Na ₂ HPO ₄ |
| | | pH | To 5.5 from 5.0 | CaCl ₂ , NaOH |
| | | Clearness | Microscopic | |
| | 2 nd filtration | Pressure | \leq 2 kg/cm ² | Perlite, Diatomaceous earth |
| | | Microbial count | \leq 10 ² CFP/ml | Diatomaceous earth |
| | Sterile filtration (ceramic) | Temperature | To 20°C from 10°C | |
| 3. Concentration | Ultra filtration | pH | To 7.0 from 6.0 | Acetic acid |
| | | Clearness | Microscopic | |
| 4. Filtration | Sterile filtration (ceramic) | Microbial count | \leq 10 ² CFP/ml | Perlite |
| | | Temperature | \leq 5°C | Diatomaceous earth |
| | Membrane filtration | Microbial count | $<$ 10 CFP/ml | |
| 5. Drying | Spray drying | Air temperature | Inlet 155°C | |
| | | | Outlet 85°C | |

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Table 2. Raw materials and processing aids for the production of *B. galactosidase*

| Substance | Grade | Function |
|--|-----------------|----------------|
| Resolved soybean | food | Nutrient |
| Meat extract | Food(kosher) | Nutrient |
| Yeast extract | Food | Nutrient |
| Lactose | Food | Nutrient |
| Defatted soybean | Food | Nutrient |
| Corn steep liquor | Food additive | Nutrient |
| Soybean oil | Food | Nutrient |
| (NH ₄) ₂ SO ₄ | Food additive | Nutrient |
| Na ₂ CO ₃ | Food additive | Nutrient |
| (NH ₄) ₂ HPO ₄ | Food additive | Nutrient |
| CaCO ₃ | Food additive | Nutrient |
| Water | Food ingredient | Nutrient |
| CaCl ₂ | Food additive | Processing aid |
| Na ₂ HPO ₄ | Food additive | Processing aid |
| NaOH | Food additive | Processing aid |
| Acetic acid | Food additive | Processing aid |
| Perlite | Filter aid | Processing aid |
| Diatomaceous earth | Filter aid | Processing aid |

Appendix 2B

β -Galactosidase and Biolacta[®] N5
from *Bacillus circulans*

PROPRIETARY PROCESS TECHNOLOGY

[REDACTED]

Contents

Appendix 2B: β -Galactosidase and Biolacta[®] N5-

- Exhibit 1. GRAS Status Summary for β -Galactosidase and Biolacta[®] N5
II. Production Process of Biolacta[®] N5
[PROPRIETARY PROCESS TECHNOLOGY]
[REDACTED]
- Exhibit 2. Maintenance of Culture Strain
[PROPRIETARY PROCESS TECHNOLOGY]
[REDACTED]
- Exhibit 7. Optimal Conditions for Hydrolysis to Occur
[PROPRIETARY PROCESS TECHNOLOGY]
[REDACTED]
- Exhibit 8. Manufacturing Conditions for Biolacta[®] N5
[PROPRIETARY PROCESS TECHNOLOGY]
[REDACTED]

Appendix 2B
Exhibit 1

GRAS Status Summary for
 β -Galactosidase and Biolacta[®] N5
Section II

PROPRIETARY PROCESS TECHNOLOGY

[REDACTED]

II. PRODUCTION PROCESS OF BIOLACTA® N5

[REDACTED]

Appendix 2B
Exhibit 2

Maintenance of Culture Strain

PROPRIETARY PROCESS TECHNOLOGY

[REDACTED]

Appendix 2B
Exhibit 7

Optimal Conditions for Hydrolysis to Occur
PROPRIETARY PROCESS TECHNOLOGY

[REDACTED]

Appendix 2B
Exhibit 8

Manufacturing Conditions for Biolacta[®] N5

PROPRIETARY PROCESS TECHNOLOGY

[REDACTED]

Appendix 3

Vivinal[®] GOS

Contents

- Exhibit 1. Documentation of Vivinal® GOS Batch Data and Specifications
- Exhibit 2. Methods of Analysis for Heavy Metals in Vivinal® GOS
- Exhibit 3. Methods of Analysis for Components of Vivinal® GOS
- Exhibit 4. Adapted Vivinal® GOS Extraction Procedure
- Exhibit 5. Viscosity Method of Analysis
- Exhibit 6. Consistency of Vivinal® GOS Production

Appendix 3 Exhibit 1

Documentation of Vivinal[®] GOS Batch Data and Specifications

CERTIFICATE OF ANALYSIS

| | |
|--------------------|--------------------------------------|
| Product | : Vivinal® GOS |
| Product code | : 502668 |
| Order no. | : 1001185 |
| Customer's ref.no. | : 1024768/L-Art. 2001189 |
| Destination | : |
| Quantity | : 2.400 kg |
| Batchnumber | : 611698 |
| Date of production | : 08-12-2005 |
| Retest date | : 08-06-2007 |
| Contact person | : Sales Support Friesland Foods Domo |

Description : galacto-oligosaccharide syrup

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

| <u>Chemical/ physical:</u> | <u>Specification</u> | <u>Results</u> | <u>Method of analysis</u> |
|----------------------------|----------------------|----------------|--|
| Dry matter | 74 - 76 % | 74.0 % | IDF 26A (1993), 2½ h 102±2°C |
| Galacto-oligosaccharides | min. 57 % on DM | 58.4 % | AOAC vol 85 (2002), method 2001.02 |
| Nitrogen | max. 0.016 % on DM | 0.000 % | IDF 20B (1993), Kjeldahl |
| Sulphated ash | max. 0.3 % on DM | 0.13 % | AOAC 17ed.(2000) 930.30,sulphated ≤550°C till constant weight |
| Lactose anhydrous | max. 23 % on DM | 20.3 % | AOAC vol 85 (2002), method 2001.02 |
| Glucose anhydrous | max. 22 % on DM | 20.2 % | AOAC vol 85 (2002), method 2001.02 |
| Galactose | min. 0.8 % on DM | 1.3 % | AOAC vol 85 (2002), method 2001.02 |
| Viscosity (25°C) | 1000 – 5000 cPs | 2059 | HAAKE |
| Nitrite | max. 2 ppm on DM | 0.08 | IDF 97A (1984), spectrofotometric |
| pH | 3.2 - 3.8 | 3.51 | ISO 10523 (1994), potentiometric (10% w/w) |

Microbiological:

| | | | |
|------------------------|-----------------|---------|--|
| Total plate count 30°C | max. 3000 cfu/g | 3 / g | IDF 100B (1991), PCMA 72h 30°C |
| Enterobacteriaceae | absent in 1 g | absent | BDI 23, VRBG 24h 30°C |
| E. coli | absent in 5 g | absent | IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C |
| Yeasts | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Moulds | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Staphylococci coag.pos | absent in 1 g | absent | IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C |
| Salmonellae | absent in 25 g | absent | IDF 93B (1995) |

Borculo, 17-01-2006

Manager QS

000443

CERTIFICATE OF ANALYSIS

Product : **Vivinal®** GOS
Product code : 502668
Order no. : 1005159
Customer's ref.no. : call-off 87
Destination :
Quantity : 2.400 kg
Batchnumber : 612924
Date of production : 09-02-2006
Retest date : 09-08-2007
Contact person : Sales Support Friesland Foods Domo

Description : galacto-oligosaccharide syrup

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

| Chemical/ physical: | Specification | Results | Method of analysis |
|----------------------------|----------------------|----------------|--|
| Dry matter | 74 - 76 % | 75.1 % | IDF 26A (1993), 2½ h 102±2°C |
| Galacto-oligosaccharides | min. 57 % on DM | 60.8 % | AOAC vol 85 (2002), method 2001.02 |
| Nitrogen | max. 0.016 % on DM | 0.006% | IDF 20B (1993), Kjeldahl |
| Sulphated ash | max. 0.3 % on DM | 0.16 % | AOAC 17ed.(2000) 930.30,sulphated ≤550°C till constant weight |
| Lactose anhydrous | max. 23 % on DM | 18.5 % | AOAC vol 85 (2002), method 2001.02 |
| Glucose anhydrous | max. 22 % on DM | 19.7 % | AOAC vol 85 (2002), method 2001.02 |
| Galactose | min. 0.8 % on DM | 1.1 % | AOAC vol 85 (2002), method 2001.02 |
| Viscosity (25°C) | 1000 – 5000 cPs | 2074 | HAAKE |
| Nitrite | max. 2 ppm on DM | 0.06 | IDF 97A (1984), spectrofotometric |
| pH | 3.2 - 3.8 | 3.3 | ISO 10523 (1994), potentiometric (10% w/w) |

Microbiological:

| | | | |
|------------------------|-----------------|---------|--|
| Total plate count 30°C | max. 3000 cfu/g | < 1 / g | IDF 100B (1991), PCMA 72h 30°C |
| Enterobacteriaceae | absent in 1 g | absent | BDI 23, VRBG 24h 30°C |
| E. coli | absent in 5 g | absent | IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C |
| Yeasts | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Moulds | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Staphylococci coag.pos | absent in 1 g | absent | IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C |
| Salmonellae | absent in 25 g | absent | IDF 93B (1995) |

Borculo, 14-03-2006

(b)(6)

Manager QS

000444

CERTIFICATE OF ANALYSIS

| | |
|--------------------|--------------------------------------|
| Product | : Vivinal® GOS |
| Product code | : 502668 |
| Order no. | : 1006059 |
| Customer's ref.no. | : call-off 5 |
| Destination | : |
| Quantity | : 3.600 kg |
| Batchnumber | : 613006 |
| Date of production | : 13-02-2006 |
| Retest date | : 13-08-2007 |
| Contact person | : Sales Support Friesland Foods Domo |

Description : galacto-oligosaccharide syrup

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

| <u>Chemical/ physical:</u> | <u>Specification</u> | <u>Results</u> | <u>Method of analysis</u> |
|----------------------------|----------------------|----------------|--|
| Dry matter | 74 - 76 % | 74.7 % | IDF 26A (1993), 2½ h 102±2°C |
| Galacto-oligosaccharides | min. 57 % on DM | 60.0 % | AOAC vol 85 (2002), method 2001.02 |
| Nitrogen | max. 0.016 % on DM | 0.006% | IDF 20B (1993), Kjeldahl |
| Sulphated ash | max. 0.3 % on DM | 0.19 % | AOAC 17ed.(2000) 930.30,sulphated ≤550°C till constant weight |
| Lactose anhydrous | max. 23 % on DM | 18.7 % | AOAC vol 85 (2002), method 2001.02 |
| Glucose anhydrous | max. 22 % on DM | 20.1 % | AOAC vol 85 (2002), method 2001.02 |
| Galactose | min. 0.8 % on DM | 1.2 % | AOAC vol 85 (2002), method 2001.02 |
| Viscosity (25°C) | 1000 – 5000 cPs | 1918 | HAAKE |
| Nitrite | max. 2 ppm on DM | 0.06 | IDF 97A (1984), spectrofotometric |
| pH | 3.2 - 3.8 | 3.5 | ISO 10523 (1994), potentiometric (10% w/w) |

Microbiological:

| | | | |
|------------------------|-----------------|---------|--|
| Total plate count 30°C | max. 3000 cfu/g | 19 / g | IDF 100B (1991), PCMA 72h 30°C |
| Enterobacteriaceae | absent in 1 g | absent | BDI 23, VRBG 24h 30°C |
| E. coli | absent in 5 g | absent | IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C |
| Yeasts | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Moulds | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Staphylococci coag.pos | absent in 1 g | absent | IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C |
| Salmonellae | absent in 25 g | absent | IDF 93B (1995) |

Borculo, 09-03-2006

Manager QS

000445

CERTIFICATE OF ANALYSIS

| | |
|--------------------|--------------------------------------|
| Product | : Vivinal® GOS |
| Product code | : 502668 |
| Order no. | : 1013848 |
| Customer's ref.no. | : |
| Batchnumber | : 614405 |
| Date of production | : 26-04-2006 |
| Retest date | : 26-10-2007 |
| Contact person | : Sales Support Friesland Foods Domo |

Description : galacto-oligosaccharide syrup

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

| <u>Chemical/ physical:</u> | <u>Specification</u> | <u>Results</u> | <u>Method of analysis</u> |
|----------------------------|----------------------|----------------|--|
| Dry matter | 74 - 76 % | 75.3 % | IDF 26A (1993), 2½ h 102±2°C |
| Galacto-oligosaccharides | min. 57 % on DM | 60.1 % | AOAC vol 85 (2002), method 2001.02 |
| Nitrogen | max. 0.016 % on DM | 0.009 % | IDF 20B (1993), Kjeldahl |
| Sulphated ash | max. 0.3 % on DM | 0.2 % | AOAC 17ed.(2000) 930.30,sulphated ≤550°C till constant weight |
| Lactose anhydrous | max. 23 % on DM | 18.0 % | AOAC vol 85 (2002), method 2001.02 |
| Glucose anhydrous | max. 22 % on DM | 20.7 % | AOAC vol 85 (2002), method 2001.02 |
| Galactose | min. 0.8 % on DM | 1.2 % | AOAC vol 85 (2002), method 2001.02 |
| Viscosity (25°C) | 1000 – 5000 cPs | 2221 | HAAKE |
| Nitrite | max. 2 ppm on DM | 0.4 | IDF 97A (1984), spectrophotometric |
| pH | 3.2 - 3.8 | 3.4 | ISO 10523 (1994), potentiometric (10% w/w) |

Microbiological:

| | | | |
|------------------------|-----------------|---------|--|
| Total plate count 30°C | max. 3000 cfu/g | 3 / g | IDF 100B (1991), PCMA 72h 30°C |
| Enterobacteriaceae | absent in 1 g | absent | BDI 23, VRBG 24h 30°C |
| E. coli | absent in 5 g | absent | IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C |
| Yeasts | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Moulds | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Staphylococci coag.pos | absent in 1 g | absent | IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C |
| Salmonellae | absent in 25 g | absent | IDF 93B (1995) |

Borculo, 22-05-2005

Manager QS

000446

CERTIFICATE OF ANALYSIS

Product : **Vivinal®** GOS
Product code : 502668
Order no. : 1034840
Customer's ref.no. : all.wk 48/call-off 38
Batchnumber : 618025
Date of production : 17-11-2006
Retest date : 17-05-2008
Contact person : Sales Support Friesland Foods Domo

Description : galacto-oligosaccharide syrup

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

| Chemical/ physical: | Specification | Results | Method of analysis |
|----------------------------|----------------------|----------------|--|
| Dry matter | 74 - 76 % | 75.0 % | IDF 26A (1993), 2½ h 102±2°C |
| Galacto-oligosaccharides | min. 57 % on DM | 60.4 % | AOAC vol 85 (2002), method 2001.02 |
| Nitrogen | max. 0.016 % on DM | 0.002% | IDF 20B (1993), Kjeldahl |
| Sulphated ash | max. 0.3 % on DM | 0.19 % | AOAC 17ed.(2000) 930.30,sulphated ≤550°C till constant weight |
| Lactose anhydrous | max. 23 % on DM | 17.4 % | AOAC vol 85 (2002), method 2001.02 |
| Glucose anhydrous | max. 22 % on DM | 21.0 % | AOAC vol 85 (2002), method 2001.02 |
| Galactose | min. 0.8 % on DM | 1.3 % | AOAC vol 85 (2002), method 2001.02 |
| Viscosity (25°C) | 1000 – 5000 cPs | 1677 | HAAKE |
| Nitrite | max. 2 ppm on DM | 0.0 | IDF 97A (1984), spectrophotometric |
| pH | 3.2 - 3.8 | 3.6 | ISO 10523 (1994), potentiometric (10% w/w) |

Microbiological:

| | | | |
|------------------------|-----------------|---------|--|
| Total plate count 30°C | max. 3000 cfu/g | 1 / g | IDF 100B (1991), PCMA 72h 30°C |
| Enterobacteriaceae | absent in 1 g | absent | BDI 23, VRBG 24h 30°C |
| E. coli | absent in 5 g | absent | IDF 170A-1 (1999), LSTB 48h 37°C, ECB 48h 44°C |
| Yeasts | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Moulds | max. 50 cfu/g | < 1 / g | IDF 94B (1990), OGYE 5 days 25°C |
| Staphylococci coag.pos | absent in 1 g | absent | IDF 60C (1997), GCB 48h 37°C, BPA 48h 37°C |
| Salmonellae | absent in 25 g | absent | IDF 93B (1995) |

Borculo, 04-12-2006

(b)(6)

Manager QS

000447

Appendix 3 Exhibit 2

Methods of Analysis for Heavy Metals in Vivinal[®] GOS

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Appendix 3
Exhibit 3

Methods of Analysis for Components of
Vivinal[®] GOS

Pages 000484-000506 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.



BORCULO DOMO *Ingredients*

Determination: Enterobacteriaceae (DOMO 23)

Scope: Powders
Coconut oil
Glucose syrup
Creamer mix
Lecithin

Materials:

- Sterile 10 ml pipette
- Sterile PVC-bottle
- Sterile Petri dish (diam. 14 cm)

Medium: Violet Red Bile Glucose Agar (VRBGA)

Procedure: sample bottle.

- * Weigh an amount of appr. 2.5 g of powder in a tared
- * Place the bottle under the dilution machine (Synerga)
- * Synerga doses Ringersolution of 45°C up to 10^{-1}
- * Close the bottle and don't shake the solution, so the powder particles slowly sink away in the solution.
N.B.: do shake hard-soluble powders (like Hiprotal) directly after addition of the Ringer solution, because these powders will not dissolve at a later stage.
- * Place the bottle in a water bath of 45°C for 5 minutes.
- * Place the bottle in a water bath of 37°C for 1 - 1.5 hours.
- * Pipette 10 ml of the solution (10^{-1}) in a Petri dish and add at least 40 ml of medium of 45°C to the dish.
- * Mix directly and let solidify.

Incubation: 18 - 24 hours at 30°C.

Interpretation: Count all the developed purple/red colonies (= number per gram of product)

Pages 000508-000534 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 3
Exhibit 5

Viscosity Method of Analysis

Method Measurement on the Haake VT500

Author(s): **Trainee Physics & Chemistry**

Group: **Physics & Chemistry**

Key Words: **viscosity Haake**

4 April 2007

1 The method

1.1 Introduction

Viscometry

The viscosity of a fluid can be measured indirectly. It is a calculation between the amount force needed to deform a fluid.

There are some different kinds of viscous behaviour. There are Newtonian and non-Newtonian fluids. A Newtonian fluid has a constant viscosity at different shear rates.

The viscosity of a non-Newtonian fluid increases or decreases when the shear rate increases. This is called dilatancy or pseudoplastic.

1.2 The apparatus

The VT500 is a viscotester from Haake. The viscotester measures the viscosities of fluids. The VT includes a computer program and this program is a ramping program for a controlled measurement.

2 Reagents

Materials/requirements:

- Calibrated thermometer (0°C-100°C)
- Refrigerated circulator
- Cup and bob (MV en MVII St)
- VT500
- Validated viscometer
- Glycerol
- Glycerine

3 Preparation

3.1 Sample preparation

N/a

3.2 Calibration

For calibration, a measurement with glycerol must be done. The viscosity must be the same at all different shear rates.

4 Operating procedure

1. Start the VT500 by switching on the green button
2. Connect the sensor system with rotor to the VT500
3. Select a sensor (number 3)
4. Fill the sample into the measuring system
5. Insert the measuring cup into temperature vessel and fasten it
6. Allow the sample to warm up until the preset temperature value is reached. This can be controlled by pushing on the button "D" till the °C display is flashing (a red light)
7. Push the zero button
8. Turn the speed to 1 (the smallest speed)
9. Start the motor
10. Set the display to viscosity by pushing the D-button until mPas or Pas is flashing
11. Read the display and note the data.
12. Set the display on shear rate by pushing the D-button again till 1/s is flashing.
13. Read the data and note it.
14. Now set the speed on 2 and repeat steps 10 till 14.
15. Do this with all the speeds but stop when the torque is above the 2,00 Ncm
16. When all measurements are done clean the measuring system with soap. !!!!! Don't use a scourer or brush!!!!!!
17. When all data is entered in the pc a graph will be shown. (On the x-axis is the shear rate and the viscosity is the y-axis).

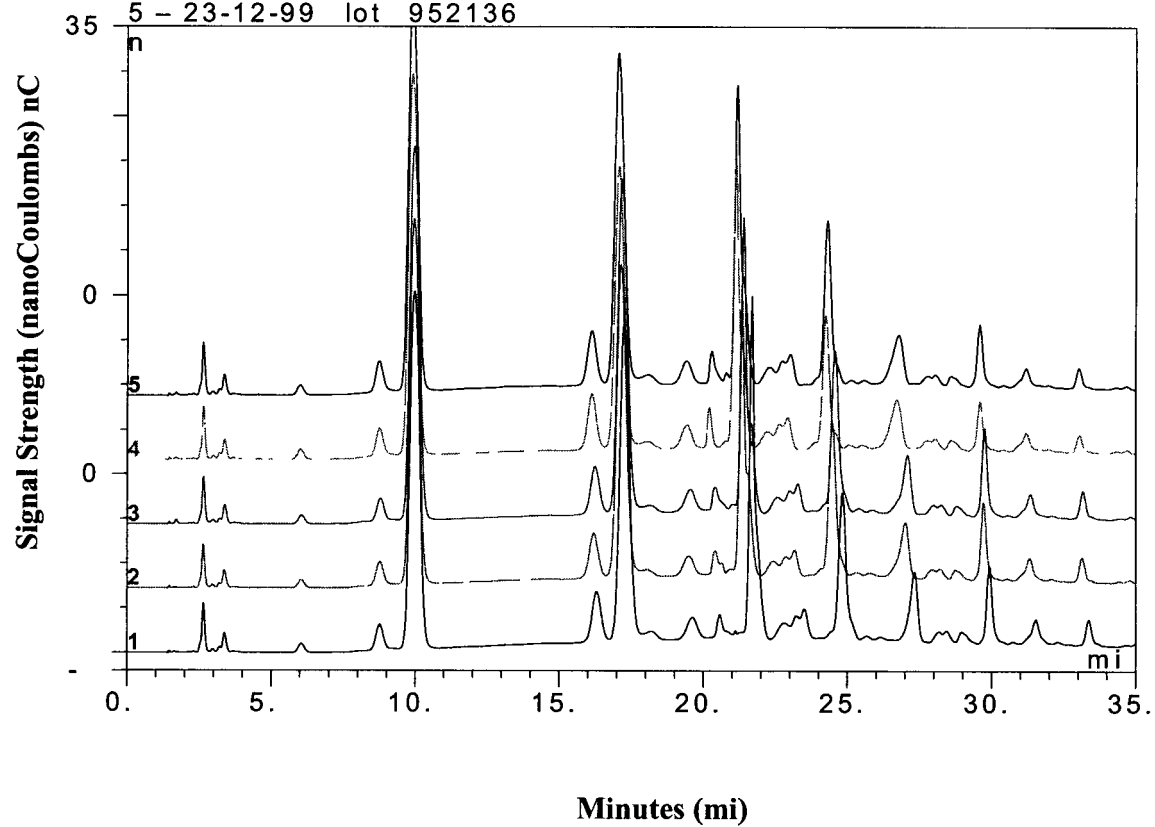
If the sample is Newtonian the graph will be a horizontal line. If the line goes up or down the sample is either shear thickening or shear thinning.

Appendix 3
Exhibit 6

Consistency of Vivinal[®] GOS Production

Consistency of Vivinal® GOS Production

1 - 09-02-02 lot 206112
2 - 24-12-01 lot 152048
3 - 06-07-01 lot 027129
4 - 11-02-00 lot 006162
5 - 23-12-99 lot 952136





APPENDICES

COMPLETE

Part 2 of 2

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BY:.....

Appendix 4

Approved Uses of Vivinal[®] GOS in Europe

ENVIRON

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Contents

- Exhibit 1. Dutch Government Novel Foods Exemption Letter
- Exhibit 2. Italian Ministry of Health Approval Letter for the use of GOS as a Soluble Fiber
- Exhibit 3. UK Confirmation for the Marketing of GOS
- Exhibit 4. Official Journal of the European Union Acknowledging Addition of GOS to Infant Formulas and Follow-on Formulas

Appendix 4
Exhibit 1

Dutch Government Novel Foods
Exemption Letter

Appendix I

Ministerie van Volksgezondheid, Welzijn en Sport

Borculo Whey Products
Ir. H.J.A.R. Timmermans
Postbus 46
7270 AA BORCULO

| | | | |
|----------------------|------------------|----------------|------------|
| Ons kenmerk | Inlichtingen bij | Doorkiesnummer | Rijswijk |
| GZB/VVB/961159 | R. Top | 3406963 | 6 mei 1996 |
| Onderwerp | | Bijlage(n) | Uw brief |
| Nieuw voedingsmiddel | | - 1 - | |

Hierbij doe ik u de ontheffing toekomen van art. 2 van het Warenwetbesluit Toelating nieuwe voedingsmiddelen met betrekking tot uw aanvraag voor het produkt galacto-oligo-sacchariden voor gebruik in levensmiddelen. Zoals u kunt zien bevat de ontheffing een eis ten aanzien van de etikettering van de produkten die als zodanig verkocht worden. Ik ben echter ook voornemens om op korte termijn een regeling af te kondigen die een dergelijke etikettering vereist voor de levensmiddelen waarin galacto-oligo-sacchariden zijn verwerkt. Ik ga ervan uit dat u uw afnemers hiervan op de hoogte stelt.

Tot slot wijs ik u erop, dat op grond van artikel 7:1 van de Algemene wet bestuursrecht degene wiens belang rechtstreeks bij een besluit is betrokken daartegen binnen zes weken na de dag waarop het aangevallen besluit bekend is gemaakt, een bezwaarschrift kan indienen bij het bestuursorgaan dat het besluit heeft genomen. Een dergelijk bezwaarschrift dient u te adresseren aan de Centrale Directie Wetgeving en Juridische Zaken van het Ministerie van VWS, Postbus 5406, 2280 HK Rijswijk.

De Directeur Gezondheidsbeleid,

(b)(6)

mr. S. van Hoogstraten

561159-Top.gku

Postbus 5406
2280 HK Rijswijk
Telefoon (070) 340 79 11
Fax (070) 340.78.34

Bezoekadres:
Sir W. Churchilllaan 368
Rijswijk

Correspondentie uitsluitend
richten aan het postadres
met vermelding van de
datum en het kenmerk van
deze brief

Telex Rijswijk
31680 vws'w nl
32347 vws'w p

000544

VVB

Voor de Staatscourant:

betr:
Nieuw voedingsmiddel

Kenmerk
GZB/VVB/961158

Rijswijk
8 mei 1996

De staatssecretaris van Volksgezondheid, Welzijn en Sport

handelende in overeenstemming met de Ministers van Economische Zaken en van Landbouw, Natuurbeheer en Visserij;

Gelezen het verzoek van de firma Borculo Whey Products te Borculo van 24 juli 1995;

Gelet op artikel 16, tweede en vierde lid, van de Warenwet;

Gezien het advies van de Adviescommissie Warenwet van 19 maart 1996 met nummer 14982/(7)5 en het advies van de Voorlopige Commissie Veiligheid Nieuwe Voedingsmiddelen van 17 januari 1996 met nummer A95007;

BESLUIT:

Aan de firma Borculo Whey Products, te Borculo, wordt ontheffing verleend van artikel 2 van de Warenwetregeling Toelating nieuwe voedingsmiddelen, voor zover het betreft het produkt galacto-oligo-sacchariden, onder de voorwaarde dat op de verpakking van de waar de volgende vermelding is aangebracht: overmatig gebruik kan een laxerend effect hebben.

Deze ontheffing zal in de Staatscourant worden geplaatst.

De Staatssecretaris van Volksgezondheid,
Welzijn en Sport,
namens deze,
De Directeur Gezondheidsbeleid,

(b)(6)

mr. S. van Hoogstraten

961158-000000

000545

Copy of the letter from Department of Health, Welfare and Sport
(GZB/VVB/961159)

Department of Health, Welfare and Sport

H.J.A.R. Timmermans
Borculo Whey Products
Postbus 46
7270 AA Borculo
The Netherlands

Our reference :GZB/VVB/961159
Subject :New food ingredient
For information :R. Top
Direct number :+31 (0)70 - 3406963
Enclosure(s) :1

Rijswijk, 6 May 1996
Your letter: -

With reference to your request concerning the use of galacto-oligosaccharides in food products, I enclose the exemption from section 2 of the Commodities Act Decree on the Permitted Use of New Foodstuffs. As you can see, the exemption includes a labelling requirement when the substance is sold as a separate product. In the near future, I also intend issuing a regulation making such labelling a requirement for food products that contain galacto-oligosaccharides. I assume you will inform your customers of this intention.

In closing, I draw your attention to the fact that under section 7:1 of the Administrative Law (General Principles) Act, any party whose interests are directly involved in a resolution may lodge an objection to the administrative body that passed the resolution within six weeks after the date on which the resolution was made known. Such an objection should be directed to the Central Legislation and Legal Affairs Department of the Ministry of Health, Welfare and Sport, Postbus 5406, 2280 HK Rijswijk, The Netherlands.

The Director of Health Policy

S. van Hoogstraten

For the Government Gazette:

Subject :New food ingredient
Reference :GZB/VVB/961158

Rijswijk, 8 May 1996

The Secretary of State for Health, Welfare and Sport

acting in agreement with the Ministers for Economic Affairs and for Agriculture,
Nature Management and Fisheries;

having considered the request submitted by the company Borculo Whey Products of Borculo dated 24 July 1995;

pursuant to section 16, subsections 2 and 3, of the Commodities Act;

in view of the recommendation of the Commodities Act Advisory Committee dated 19 March 1996, number 14982/(7)5, and the recommendation of the Provisional Committee on the Safety of New Foodstuffs dated 17 January 1996, number A95007;

has issued this **RESOLUTION**:

The company Borculo Whey Products of Borculo is granted exemption from section 2 of the Commodities Act Decree on the Permitted Use of New Foodstuffs in respect of galacto-oligosaccharides, subject to the condition that the external packaging of the substance contains the following warning: excessive use may have a purgative effect.

This exemption will be published in the Government Gazette.

For the Secretary of State for Health, Welfare and Sport,
The Director of Health Policy

S. van Hoogstraten

Appendix 4
Exhibit 2

Italian Ministry of Health
Approval Letter for the use of
GOS as a Soluble Fiber

3.MAR.1998 16:16

DR GINO FUMAGALLI

NR.053 P.1/3



DR. GINO FUMAGALLI S.p.A.

Capitale Sociale L. 250.000.000
20092 CINISELLO BALBANO (Milano)
Viale Brianza 160 - Tel. 02/6128812 (5 linee)
Telefax 02/6124219 - Telex 332098 FUMOR I

To: BORCULO WHEY PRODUCTS From: Marco Fumagalli
Attention: Mr. Frank Fox Company: Dr. Gino Fumagalli SPA
Fax n.: 0031545256787 Fax n.: +39-2-6124210
Date: 3 March 1998
subject: web page
total pages: 3 (if you don't receive all the message please call n. 02-6128812)

RE: ELIX'OR APPROVAL.

Dear Mr. Fox,

I am glad to send you the copy of the official approval by the Italian Health Ministry.

Starting from now the galacto-oligosaccharides can be used and labelled in Italy as "soluble fibre".

If you need a complete translation in english of the text, don't hesitate to ask it.

Best regards.

(b)(6)

DR. GINO FUMAGALLI SPA
Marco Fumagalli

COLLA MISTURA: Tel. 02/6128812 Fax 02/6124210 Telex 332098 FUMOR I

000549



Ministero della Sanità

Spett.le
Dr. Gino FUMAGALLI Spa
Viale Brianza, 160
20092 Cinisello Balsamo-MI

600,12 | 254 AG | 620

18 FEB 1998

OGGETTO: Galatto oligo saccaridi.

In riferimento alla richiesta qui pervenutaci, concernente l'oggetto, si comunica che la Commissione Consultiva per i prodotti destinati ad una alimentazione particolare, operante presso lo scrivente Dipartimento, si è pronunciata in merito all'impiego negli alimenti del galatto oligo saccaridi in data 02/10/97.

Per completezza d'informazione, si allega il citato parere.

Si resta a disposizione per eventuali ulteriori chiarimenti.

(b)(6)

IL DIRETTORE GENERALE DEL DIPARTIMENTO

(b)(6)



Ministero della Sanità

DIPARTIMENTO DEGLI ALIMENTI NUTRIZIONE E SANITA' PUBBLICA VETERINARIA
UFFICIO XII

GALATTO-OLIGOSACCARIDI

Le caratteristiche chimico-fisiche dei GOS, alla luce della attuale conoscenza, inducono a valutare la possibilità di una loro collocazione nel contesto della fibra alimentare solubile. Esistono però marcate differenze di comportamento fisiologico e di effetti rispetto a fibre idrosolubili polisaccaridiche.

In particolare, nel tratto intestinale superiore i GOS non manifestano caratteristiche sazianti, ipoglicemizzanti e ipolipidizzanti, mentre nel tratto intestinale inferiore manifestano attività probiotica, oltre a riequilibrare l'ecosistema contrastando lo stabilirsi di specie patogene.

Sotto l'aspetto normativo si rileva che secondo le attuali disposizioni comunitarie e del Codex Alimentarius sull'etichettatura nutrizionale dei prodotti alimentari, la "fibra alimentare" è una voce non ancora ben definita sul piano strutturale ed analitico.

Per quanto sopra si ritiene che i GOS possano essere assimilati alla fibra alimentare solubile ai fini dell'etichettatura nutrizionale, in linea col parere espresso per altri oligosaccaridi indigeribili (FOS).

Roma, 2.10.97

gos

MINISTERO DELLA SANITA' - SEZIONE SANITA' PUBBLICA



TOSI & G. S. R. L.
Servizi del latte, del siero e dei cereali

Via Risorgimento, 32
20059 VIMERCATE (MI) - Italy
Tel. 039/608.29.10-13 - Fax 039/608.28.95

22/11/01

Vimercate, IL

Spett.le BORCULO DOMO INGREDIENTS

tot. n° of pages (this one enclosed): 1

To the attention of: **Margriet Schoterman**

OBJECT: DECLARATION ITALIAN MINISTRY OF HEALTH

Dear Margriet

Herewith I send you the English translation of the declaration for the use of GOS as soluble fibre.

II

GALACTO-OLIGOSACCHARIDES

The chemical-physical features of GOS, based on the today knowledge of the matter, let us insert them among the dietary soluble fibres.

However GOS show big differences compared with other water soluble polysaccharides.

Particularly, in the upper part of the intestine, GOS don't have satiating, hypoglycemic and hypolipidogenic effects; in the colon they show prebiotic activity and they balance the flora equilibrium.

Based on the today EEC regulations and based on the Codex Alimentarius of the nutritional labels of food ingredients, the term "DIETARY FIBRE" is not well defined yet.

For all these reasons, the non-digestible oligosaccharides, such as GOS and FOS, can be declared in the label as "dietary fibre".

Rome, 2 October 1997

II

Please, feel free to contact me for any question.

Thank you for the replies on Vivinal GOS.

Kind Regards

Marcin Marco

(b)(6)

Appendix 4 Exhibit 3

UK Confirmation for the Marketing of GOS



Joint Food Safety and Standards Group

*Ergon House, 17 Smith Square, London, SW1P 3JR
Tel: 0171-238-6180 Fax: 0171-238-6382
E-mail: e.j.osullivan@fssg.maff.gov.uk*



Your reference:

Our reference: NFB 18

Mrs M Schoterman
Development and Application
Borculo Whey Products
Postbus 46
7270 AA Borculo
The Netherlands

1 July 1998

Dear Mrs Schoterman

GALACTO-OLIGOSACCHARIDE - ELIX'OR

Thank you for your letter of 8 June 1998, in which you explained your present situation concerning your galacto-oligosaccharide product Elix'or®.

We apologise for the delay in dealing with your initial enquiry, but we can now confirm that your product Elix'or® does fall outside the scope of Regulation 258/97 for Novel Foods and Novel Food Ingredients. Therefore, your product can be freely marketed within the UK.

In your letter you also enquired about labelling your product with the words 'contains fibre' or similar wording. I regret that we are unable to provide nutritional labelling advice in response to enquiries about specific products. This responsibility lies with local authorities who enforce food law in the UK. In your case we recommend that your importer or your UK agent for your product refers your enquiry to their Home Authority or, in the absence of a Home Authority, the local authority Trading Standards Department or Environmental Health Department. However, if you do not have a representative in the UK please contact us again and we will pass your enquiry on the relevant branch within the Ministry who will endeavour to follow up your enquiry with the relevant authority.

You also stated in your letter that the Dutch and Belgian Governments have already declared that galacto-oligosaccharides can be considered as dietary fibre. The situation in the UK is under review, but fibre, for the purposes of the Regulations, means dietary fibre defined as non starch polysaccharides. This is in line with the Committee on the Medical Aspects of Food Policy (COMA) recommendations in the 'Dietary Reference Values for Food Energy and Nutrients for the United Kingdom' (report available from the Stationery Office Publications Centre Tel: 0171 873 9090).

I have been advised that at this time galacto-oligosaccharides are excluded from the current definition of dietary fibre for nutritional labelling purposes.

I hope that this information is of assistance in the marketing of your product within the UK. If we can be of further assistance please do not hesitate to contact us again.

Yours sincerely

(b)(6)

Elizabeth O'Sullivan
Additives and Novel Foods, Branch 'C'
MAFF

000555

Appendix 4
Exhibit 4

Official Journal of the European Union
Acknowledging Addition of GOS to
Infant Formulas and Follow-on Formulas

Pages 000557-000589 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 5

Estimated 2-Day Average Intakes of GOS by Food Category from all Proposed Uses in Foods and Infant Formula

| Estimated 2-Day Average Intakes of GOS by Food Category from the Proposed Uses in Food and Infant Formula | | | | | |
|--|--------------------------|----------------------------------|--------------------------------------|---------------------|--------------------------------|
| Population^a | Users^b | Percent Users^c | Food Group Category | Mean (g/day) | 90th Percentile (g/day) |
| Infants, 0-5 mo | 101 | 100.0 | infant formula | 4.8 | 6.2 |
| | 19 | 16.8 | baby juice | 2.9 | 5.7 |
| | 3 | 1.3 | baby dessert | 0.8 | 1.5 |
| | 4 | 3.5 | baby snack | 1.0 | 1.2 |
| | 1 | 1.2 | frozen dairy desserts | 0.0 | 0.0 |
| | 101 | 100.0 | All Categories Combined | 5.3 | 7.7 |
| Infants, 6-11 mo | 3 | 0.9 | milk | 0.6 | 1.3 |
| | 3 | 1.9 | milk drinks | 1.4 | 1.9 |
| | 10 | 2.6 | drinks - fruit/energy drinks | 1.4 | 2.2 |
| | 1 | 0.3 | drinks - fitness water/quencher | 0.6 | 0.6 |
| | 168 | 98.5 | infant formula | 4.0 | 5.6 |
| | 90 | 45.0 | baby juice | 2.6 | 5.9 |
| | 1 | 1.0 | baby yogurt drink | 1.3 | 1.3 |
| | 51 | 31.4 | baby dessert | 1.4 | 3.8 |
| | 41 | 29.6 | baby snack | 1.2 | 1.7 |
| | 13 | 6.0 | yogurt | 1.5 | 2.8 |
| | 13 | 5.4 | frozen dairy desserts | 1.2 | 2.8 |
| | 6 | 2.4 | fruit prep/pie filling/jelly jam | 0.7 | 1.2 |
| | 172 | 99.5 | All Categories Combined | 6.1 | 10.1 |
| Children, 12-23 mo | 14 | 6.5 | milk | 4.2 | 6.9 |
| | 40 | 14.7 | milk drinks | 4.4 | 15.6 |
| | 21 | 6.3 | syrup flavoring for milk | 2.6 | 4.8 |
| | 3 | 0.5 | meal replacement drinks for children | 15.1 | 21.8 |
| | 1 | 0.2 | milk substitutes | 21.2 | 21.2 |
| | 82 | 26.8 | drinks - fruit/energy drinks | 3.4 | 7.9 |
| | 14 | 6.6 | drinks - fitness water/quencher | 4.4 | 7.5 |
| | 18 | 4.8 | infant formula | 1.8 | 4.0 |
| | 28 | 10.1 | baby juice | 5.0 | 7.9 |
| | 14 | 4.3 | baby dessert | 0.7 | 1.5 |
| | 12 | 3.1 | baby snack | 1.1 | 2.3 |
| | 52 | 28.8 | yogurt | 2.4 | 4.5 |
| | 57 | 24.7 | frozen dairy desserts | 1.4 | 2.9 |
| | 12 | 5.0 | bars | 2.2 | 2.7 |
| | 52 | 21.4 | fruit prep/pie filling/jelly jam | 1.5 | 2.5 |
| | 229 | 85.6 | All Categories Combined | 5.3 | 11.2 |
| Children, 2-5 y | 98 | 16.4 | milk | 6.3 | 12.8 |
| | 179 | 30.3 | milk drinks | 5.2 | 10.3 |
| | 62 | 8.7 | syrup flavoring for milk | 2.7 | 7.2 |
| | 2 | 0.1 | meal replacement drinks | 1.3 | 2.6 |
| | 9 | 1.3 | meal replacement drinks for children | 1.9 | 2.6 |

| Estimated 2-Day Average Intakes of GOS by Food Category from the Proposed Uses in Food and Infant Formula | | | | | |
|--|--------------------------|----------------------------------|--------------------------------------|---------------------|--------------------------------|
| Population^a | Users^b | Percent Users^c | Food Group Category | Mean (g/day) | 90th Percentile (g/day) |
| | 7 | 1.3 | milk substitutes | 7.2 | 15.4 |
| | 262 | 30.0 | drinks - fruit/energy drinks | 6.1 | 14.5 |
| | 38 | 4.7 | drinks - fitness water/quencher | 2.5 | 4.5 |
| | 1 | 0.1 | infant formula | 4.4 | 4.4 |
| | 6 | 0.4 | baby juice | 4.6 | 8.6 |
| | 3 | 0.3 | baby dessert | 2.7 | 4.0 |
| | 3 | 1.0 | baby snack | 0.7 | 1.7 |
| | 123 | 22.7 | yogurt | 2.8 | 4.0 |
| | 224 | 36.3 | frozen dairy desserts | 2.1 | 3.2 |
| | 23 | 4.8 | bars | 2.3 | 3.5 |
| | 191 | 31.4 | fruit prep/pie filling/jelly jam | 2.3 | 7.0 |
| | 621 | 92.3 | All Categories Combined | 7.8 | 18.2 |
| Boys, 6-11 y | 53 | 15.5 | milk | 6.9 | 12.9 |
| | 147 | 39.0 | milk drinks | 6.7 | 11.3 |
| | 23 | 6.9 | syrup flavoring for milk | 2.7 | 4.1 |
| | 1 | 0.1 | meal replacement drinks | 7.4 | 7.4 |
| | 1 | 0.0 | meal replacement drinks for children | 3.0 | 3.0 |
| | 2 | 0.3 | milk substitutes | 1.3 | 2.5 |
| | 154 | 31.0 | drinks - fruit/energy drinks | 5.0 | 9.5 |
| | 38 | 11.9 | drinks - fitness water/quencher | 2.8 | 5.9 |
| | 38 | 10.5 | yogurt | 3.0 | 7.8 |
| | 141 | 39.4 | frozen dairy desserts | 2.6 | 5.7 |
| | 17 | 9.8 | bars | 3.0 | 5.4 |
| | 111 | 34.6 | fruit prep/pie filling/jelly jam | 3.6 | 9.4 |
| | 346 | 94.7 | All Categories Combined | 9.1 | 18.8 |
| Girls, 6-11 y | 89 | 28.6 | milk | 4.5 | 9.1 |
| | 158 | 35.9 | milk drinks | 6.0 | 11.3 |
| | 24 | 4.6 | syrup flavoring for milk | 2.2 | 6.2 |
| | 2 | 0.5 | meal replacement drinks | 2.7 | 3.1 |
| | 2 | 1.1 | milk substitutes | 4.2 | 10.0 |
| | 165 | 26.7 | drinks - fruit/energy drinks | 5.9 | 10.5 |
| | 35 | 12.7 | drinks - fitness water/quencher | 4.0 | 8.3 |
| | 2 | 0.1 | baby juice | 3.5 | 4.7 |
| | 42 | 9.4 | yogurt | 2.4 | 3.7 |
| | 182 | 42.9 | frozen dairy desserts | 3.0 | 5.6 |
| | 21 | 9.4 | bars | 2.8 | 5.4 |
| | 123 | 29.9 | fruit prep/pie filling/jelly jam | 2.6 | 4.8 |
| | 404 | 92.5 | All Categories Combined | 8.9 | 16.4 |
| Teen males, 12-18 y | 168 | 20.7 | milk | 5.8 | 13.7 |
| | 193 | 20.4 | milk drinks | 6.8 | 13.0 |
| | 42 | 4.8 | syrup flavoring for milk | 4.0 | 7.6 |
| | 6 | 0.3 | meal replacement drinks | 4.2 | 8.8 |

| Estimated 2-Day Average Intakes of GOS by Food Category from the Proposed Uses in Food and Infant Formula | | | | | |
|--|--------------------------|----------------------------------|----------------------------------|---------------------|--------------------------------|
| Population^a | Users^b | Percent Users^c | Food Group Category | Mean (g/day) | 90th Percentile (g/day) |
| | 3 | 0.4 | milk substitutes | 3.5 | 3.6 |
| | 248 | 21.4 | drinks - fruit/energy drinks | 7.7 | 17.1 |
| | 109 | 17.5 | drinks - fitness water/quencher | 5.7 | 12.0 |
| | 28 | 3.6 | yogurt | 4.3 | 6.8 |
| | 225 | 29.0 | frozen dairy desserts | 3.6 | 6.3 |
| | 40 | 6.7 | bars | 3.4 | 7.0 |
| | 171 | 18.7 | fruit prep/pie filling/jelly jam | 3.2 | 7.6 |
| | 693 | 79.1 | All Categories Combined | 9.4 | 18.9 |
| Teen females, 12-18 y | 159 | 22.8 | milk | 6.1 | 12.6 |
| | 188 | 19.6 | milk drinks | 6.3 | 13.6 |
| | 29 | 3.4 | syrup flavoring for milk | 3.3 | 4.7 |
| | 7 | 0.6 | meal replacement drinks | 4.0 | 8.0 |
| | 5 | 0.7 | milk substitutes | 7.4 | 15.1 |
| | 253 | 20.8 | drinks - fruit/energy drinks | 6.4 | 13.1 |
| | 54 | 7.3 | drinks - fitness water/quencher | 2.9 | 4.9 |
| | 56 | 6.5 | yogurt | 4.2 | 6.8 |
| | 237 | 28.2 | frozen dairy desserts | 3.2 | 6.6 |
| | 53 | 8.2 | bars | 3.5 | 4.6 |
| | 160 | 17.4 | fruit prep/pie filling/jelly jam | 2.5 | 4.8 |
| | 685 | 76.7 | All Categories Combined | 8.1 | 16.7 |
| Adult males, 19+ y | 401 | 19.6 | milk | 5.4 | 12.1 |
| | 200 | 11.1 | milk drinks | 7.4 | 14.2 |
| | 32 | 2.5 | syrup flavoring for milk | 3.1 | 6.2 |
| | 40 | 2.1 | meal replacement drinks | 7.6 | 14.3 |
| | 39 | 2.4 | milk substitutes | 4.0 | 7.8 |
| | 348 | 12.6 | drinks - fruit/energy drinks | 7.2 | 17.5 |
| | 112 | 5.0 | drinks - fitness water/quencher | 6.0 | 11.3 |
| | 94 | 4.6 | yogurt | 3.4 | 6.6 |
| | 534 | 28.1 | frozen dairy desserts | 3.6 | 6.5 |
| | 102 | 7.4 | bars | 3.4 | 6.2 |
| | 478 | 23.0 | fruit prep/pie filling/jelly jam | 3.8 | 8.6 |
| | 1428 | 69.2 | All Categories Combined | 8.3 | 17.7 |
| Adult females, 19+ y | 521 | 24.5 | milk | 4.4 | 8.6 |
| | 278 | 12.8 | milk drinks | 7.4 | 15.0 |
| | 66 | 3.1 | syrup flavoring for milk | 2.5 | 4.7 |
| | 54 | 2.4 | meal replacement drinks | 4.7 | 7.0 |
| | 63 | 3.0 | milk substitutes | 4.3 | 9.9 |
| | 387 | 14.3 | drinks - fruit/energy drinks | 5.7 | 12.1 |
| | 44 | 1.8 | drinks - fitness water/quencher | 6.5 | 18.0 |
| | 1 | 0.0 | baby snack | 3.1 | 3.1 |
| | 217 | 9.5 | yogurt | 3.2 | 5.6 |
| | 649 | 29.3 | frozen dairy desserts | 3.0 | 5.7 |

| Estimated 2-Day Average Intakes of GOS by Food Category from the Proposed Uses in Food and Infant Formula | | | | | |
|---|--------------------------|----------------------------------|--------------------------------------|---------------------|--------------------------------|
| Population^a | Users^b | Percent Users^c | Food Group Category | Mean (g/day) | 90th Percentile (g/day) |
| Total population, 2+ y | 133 | 7.4 | bars | 3.6 | 6.4 |
| | 517 | 23.5 | fruit prep/pie filling/jelly jam | 2.8 | 5.3 |
| | 1673 | 73.1 | All Categories Combined | 7.4 | 15.3 |
| | 1489 | 21.8 | milk | 5.1 | 10.8 |
| | 1343 | 16.1 | milk drinks | 6.9 | 13.4 |
| | 278 | 3.5 | syrup flavoring for milk | 2.8 | 5.6 |
| | 112 | 1.8 | meal replacement drinks | 5.9 | 13.4 |
| | 10 | 0.1 | meal replacement drinks for children | 1.9 | 2.6 |
| | 121 | 2.2 | milk substitutes | 4.3 | 9.9 |
| | 1817 | 16.5 | drinks - fruit/energy drinks | 6.3 | 14.5 |
| | 430 | 5.1 | drinks - fitness water/quencher | 5.1 | 11.3 |
| | 1 | 0.0 | infant formula | 4.4 | 4.4 |
| | 8 | 0.0 | baby juice | 4.4 | 8.6 |
| | 3 | 0.0 | baby dessert | 2.7 | 4.0 |
| | 4 | 0.1 | baby snack | 0.9 | 1.7 |
| | 598 | 8.0 | yogurt | 3.2 | 6.1 |
| | 2192 | 30.2 | frozen dairy desserts | 3.1 | 6.1 |
| | 389 | 7.5 | bars | 3.4 | 5.8 |
| | 1751 | 24.0 | fruit prep/pie filling/jelly jam | 3.2 | 7.0 |
| | 5850 | 75.0 | All Categories Combined | 8.0 | 16.8 |
| ^a Breastfeeding infants and children were excluded from the sample population ^b Number of people consuming one or more foods containing GOS during the two 24-hour periods of dietary recall ^c Weighted percent Values of 0.0 represent GOS intake of <0.05. The estimates of GOS intake from infant formula are based on 5 g GOS per liter formula (as consumed). | | | | | |

Appendix 6

Digestibility of Vivinal[®] GOS



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SUPPLEMENT 3

Lund July 5th, 1994

FAX 00931-545756625

BORCULO Whey Products
Drs Robert Kammelar
Postbus 46, 7270 AA Borculo
The Netherlands

Report

Hydrolysis of galacto-oligosaccharides by human intestinal enzymes and acid

Oligosaccharide preparations

Three oligosaccharide preparations have been delivered to us for these experiments: I, dated on 20/12/93 (1.9g), II (1.5g) and III (0.23g) dated 11/3/94 and sent to us. The following data on mono- and disaccharide content were obtained:

Table 1. Substrate purity

| g/100g | I | II | III |
|------------------------|---------|------|------|
| <u>From Borculo</u> | | | |
| Galactose | 1.2 | 0.09 | 0.11 |
| Glucose | 0.3 | 0.03 | 0.67 |
| Lactose | 0.0 | 2.61 | 1.63 |
| <u>Our analyses</u> | | | |
| Galactose ¹ | 0.07 | 0.06 | |
| Glucose ² | < 0.003 | 0.3 | |

¹ Determined with the galactose dehydrogenase - NAD system used for the hydrolysis experiments.

² Determined with the glucose oxidase, peroxidase system used in the hydrolysis experiments.

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From our analyses, preparation I was most pure regarding glucose content and similar to II regarding galactose content. Preparation I was therefore chosen for the hydrolysis experiments. Obviously, our enzymatic methods and your chromatography system give somewhat different values of galactose and glucose at the very low levels present.

Hydrolysis by human intestinal enzymes

The conditions for the hydrolysis tests were set according to the disaccharidase assay of Dahlqvist (1), using 28 mM final substrate concentration and Na-maleate buffer pH 6.0, temperature 37°C. Assuming that the preparations contained mainly trisaccharides, the final oligosaccharide concentration in the hydrolysis experiments was set to 15 mg/ml, corresponding to 28 mM at molecular weight 540. The same molar lactose concentration was used for comparison.

The initial experiments were performed on a pool (A) of human small intestinal biopsy homogenates with normal disaccharidase activities. The lactase activity of this pool was 22 units/g protein (one unit hydrolyses 1 μ mole of substrate per minute). Using this pool A, liberation of glucose and galactose was followed during prolonged incubations up to 6 hours. Lactose hydrolysis was studied for comparison. Glucose determination was performed with a glucose oxidase-peroxidase reagent (1) and galactose determination with a galactose dehydrogenase-NAD⁺ system, using fluorimetric detection of reduced coenzyme (2).

Table 2. Hydrolysis of oligosaccharide preparation I and lactose by human intestinal mucosa (pool A).

| <p> μg/10 μl homogenate (3.62 mg protein/ml homogenate) </p> | Oligosaccharide | | Lactose | |
|--|-----------------|-----------|---------|-----------|
| | glucose | galactose | glucose | galactose |
| 30 minutes | 0.5 | 0.3 | 4.3 | 4.0 |
| 60 " | 0.7 | 0.8 | 8.4 | 6.5 |
| 120 " | 1.1 | 1.6 | 16.6 | 20.5 |
| 180 " | 1.7 | 2.7 | 28.9 | 29.0 |
| 240 " | 1.7 | 3.4 | 30.4 | 42.0 |
| 360 " | 2.0 | 5.0 | 46.0 | 64.0 |

The liberation of glucose and galactose from the oligosaccharide preparation is less than 10% of that from lactose. Whereas the glucose liberation levels

off during the incubation, the galactose liberation proceeds in an essentially linear way throughout the incubation time of 6 hours.

Ten different biopsies with normal disaccharidase activities were then analysed with the following results, expressed as units/g protein.

Table 3. Enzyme activity (units/g protein) towards oligosaccharide preparation I and lactose in 10 human intestinal biopsies with normal disaccharidase activities including lactase (incubation time 2 hours at 37°C).

| Biopsy no. | Oligosaccharidase | | Lactase <i>(based on glucose - 0.6)</i> |
|------------|-------------------|----------------|--|
| | Glucose lib. | Galactose lib. | |
| 1 | 1.1 | 1.8 | 19.4 |
| 2 | 1.4 | 2.8 | 29.7 |
| 3 | 1.5 | 2.6 | 33.3 |
| 4 | 1.1 | 2.4 | 29.1 |
| 5 | 1.5 | 1.7 | 18.2 |
| 6 | 2.1 | 2.6 | 29.2 |
| 7 | 3.1 | 4.4 | 44.2 |
| 8 | 2.5 | 4.0 | 41.6 |
| 9 | 2.0 | 2.5 | 39.2 |
| 10 | 0.9 | 1.2 | 13.6 |

As shown in Table 3, the oligosaccharidase activity was 5-10% of the lactase. More galactose than glucose was liberated. Since there was no detectable lactose in the oligosaccharide preparation, this activity seems to represent true hydrolysis of the oligosaccharides.

Acid stability

The pH stability was tested at pH 1, 2, 3, 4 and 5. A 0.2 M acetic acid solution was adjusted to the relevant pH values with HCl or NaOH. Equal parts of an oligosaccharide I solution (60 mg/ml) and solution with the respective pH was incubated at 37°C for 2 hours. There was no detectable liberation of either glucose or galactose (duplicate determinations) at any one of the tested pH values. Therefore, incubations were not performed for 1 and 4 hours.

In summary, human intestinal biopsy homogenates were able to hydrolyse the galacto-oligosaccharides at a very slow rate, with liberation of more galactose than glucose at prolonged incubation. The activity amounted to 5-10% of the lactase activity. This means that, in practice, the absorption of

glucose and galactose from ingested galacto-oligosaccharides would be negligible. Due to the low activity, the kinetic analyses with Vmax and Km determinations could not be performed. Instead, the progress of glucose and galactose liberation was followed during prolonged incubation. These experiments indicate that a limited amount of glucose is liberated relatively early, whereas the galactose liberation proceeds in a linear way with time even at prolonged incubation. The oligosaccharides were stable against acid hydrolysis at pH 1-5 during at least 2 hours at 37°C.

(b)(6)

Nils-Georg Asp, M.D. professor
Head of Department

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Appendix 7

Subchronic Oral Toxicity Study Of Vivinal[®] GOS in Rats

Pages 000601-000881 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Appendix 8

Human Tolerance Studies of Vivinal[®] GOS

Contents

- Exhibit 1. Tolerance of Galacto-oligosaccharides in Men, van Dokkum 1995
- Exhibit 2. Effects of Fructo-oligosaccharides and Galacto-oligosaccharides on Breath Hydrogen Excretion and Gastrointestinal Wellbeing, Alles & Schoterman 1999

Appendix 8
Exhibit 1

Tolerance of Galacto-oligosaccharides in
Men, van Dokkum 1995

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Appendix 8
Exhibit 2

Effects of Fructo-oligosaccharides and
Galacto-oligosaccharides on Breath
Hydrogen Excretion and Gastrointestinal
Wellbeing, Alles & Schoterman 1999

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