Application (A1055) to permit the optional addition of short chain fructo-oligosaccharides (scFOS) to Infant Formula Products, Foods for Infants, & Formulated Supplementary Foods for Young Children

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STATUTORY DECLARATION

The undersigned being warned that knowing and wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 hereby declares that all statements made in this document of own knowledge are true; and all statements made on information and belief are believed to be true.

Executed in Westchester, Illinois, USA, the _____ day of August, 2010.

_____
Paul Zwijack, P.E.

ACKNOWLEDGMENT

STATE OF _____)
)SS.
COUNTY OF _____)

        I, _____, a Notary Public in and for and residing in said County and State, DO HEREBY CERTIFY THAT Paul Zwijack, P.E. of Corn Products International Inc., d/b/a GTC Nutrition, Inc., personally known to me to be the same person whose name is subscribed to the foregoing instrument, appeared before me this day in person and acknowledged that he signed and delivered said instrument as his own free and voluntary act and as the free and voluntary act of said company for the uses and purposes therein set forth.

GIVEN under my hand and notarial seal this _____ day of August, 2010.

_____
Notary Public

My Commission Expires:

_____

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Definitions

For the purposes of this Application and to ensure consistency with previous terminology used within Proposal P306 Addition of Inulin/FOS & GOS to Foods, and ensure clarity, the following definitions will be used:

Aspergillus niger	Includes strains known under the names *Aspergillus aculeatus, A. awamori, A. ficuum, A. foetidus, A. japonicus, A. phoenicis, A. saitor* and *A. usamii*.
Fructan	Polymers of fructose
Fructo-oligosaccharides (FOS)**	Fructans (fructose polymers with β (2→1) fructosyl-fructose linkages, where the polymerisation (DP) is less than or equal to five and is typically produced from enzymatic condensation of sucrose.
Inulin	Those fructans, with β (2→1) fructosyl-fructose linkages, where the DP range is typically between 2 and 60.
Inulin- derived substances	Used to collectively describe inulin, long-chain inulin and oligofructose. This term does not include those fructose polymers derived from sucrose
Invertase Enzyme (EC 3.2.1.26)	Also includes ß-fructofuranosidase (EC 3.2.1.26)
Long chain inulin	Those fructans with β (2→1) fructosyl-fructose linkages, where DP range is typically between 10 and 60 and there are no mono-or disaccharides present.
Oligofructans	A generic term inclusive of all oligosaccharide fructans with β (2→1) fructosyl-fructose linkages, independent of categorisation based on DP
Oligofructose	Describes those fructans, with β (2→1) fructosyl-fructose linkages, where the DP is less than 10 but greater than or equal to 2. Oligofructose is derived from inulin. Chicory inulin, for example, contains about 30% oligofructose.
Short chain fructo-oligosaccharides (scFOS)**	Fructans (fructose polymers with β (2→1) fructosyl-fructose linkages, where the DP is equal to or less than 5 and is typically produced from enzymatic condensation of sucrose.

** for the purposes of this application, and to ensure consistency of definition with Proposal 306 Addition of Inulin/FOS & GOS to Food, the terms FOS and scFOS will refer to the same substances, however scFOS will be preferably used to ensure clarity. This is not withstanding the more widely used definition of FOS referring to all fructans with β (2→1) fructosyl-fructose linkages (M. Roberfroid, 2007).

Abbreviations

AE	Adverse events	GRN	The file number FDA assigns to a GRAS notice
Ah	Hydrolyzing activitiy	GTC	Golden Technologies Company, Inc.
ALP	Alkaline phosphatase	HDL	High density lipoprotein
ALT	Alanine aminotransferase	HM	Human Milk
A. niger	*Aspergillus niger*	ITF	Inulin-type fructans
ARA	Arachidonic acid	ITT	Intent-to-treat
AST	Aspartate aminotransferase	kg-bw/d	Kilograms of body weight per day
At	Transfructosylating activity	LC-PUFA	Long chain polyunsaturated fatty acids
ATCC	American Type Culture Collection	LDL	Low density lipoprotein
CF	Control Formula	L/M	Lactulose/mannitol ratio
CFR	Code of Federal Regulations	MRSC	Mean Rank Stool Consistency
CHO	Chinese Hamster Ovary	NHANES	National Health and Nutrition Examination Survey
DHA	Docosahexaenoic acid	NIP	Nutrition Information Panel
DNA	Deoxyribonucleic acid	NOAEL	No Observed Adverse Effect Level
DP	Degree of polymerization	ppb	Parts per billion
EC	Enzyme Commission	ppm	Parts per million
EF	Experimental Formula	RODI	Reverse osmosis deionized water
EFSA	European Food Safety Authority	SAE	Serious adverse events
FCC	Food Chemical Codex	SCFA	Short-chain fatty acids
FDA	Food and Drug Administration	scFOS	Short-chain fructooligosaccharide
FISH	Fluorescent in Situ Hybridization	SDAY	Study Days
FOS	Fructooligosaccharide	SO	Soybean Oil
FOSHU	Food for Specified Health Use	SWI	Similac with Iron
FSANZ	Food Standards Australia New Zealand	UDS	Unscheduled DNA synthesis
FSFYC	Formulated Supplementary Foods for Young Children	USDA	United States Department of Agriculture
FSO	Fish oil and soybean oil mixture	USG	Urinary Specific Gravity
FTF	Transfructosylation		
GF2	1-Kestose		
GF3	Nystose		
GF4	Fructosyl-nystose		
GI	Gastrointestinal		
GOS	Galactooligosaccharides		
GRAS	Generally Recognized as Safe		

EXECUTIVE SUMMARY

This application seeks permission under the Australia New Zealand Food Standards Code, for the optional addition short-chain fructo-oligosaccharides (scFOS) to Infant Formula Products (Standard 2.9.1), Foods for Infants (Standard 2.9.2), and Supplementary Formulated Foods for Young Children (Standard 2.9.3 Division 4). This application proposes the maximum addition rates of scFOS to these food groups to be set to the same levels, but as an alternative to, those currently set for "inulin-derived substances":

- **Infant Formula products**   either (a) 110 mg per 100 kJ; or (b) 290 mg per 100 kJ in combination with galacto-oligosaccharides, where the scFOS is no more than 110 mg per 100 kJ (equivalent to 3g/L of formula).
- **Foods for Infants**   0.8 g/ 100 g either singularly or in combination with galacto-oligosaccharides, as consumed.
- **Supplementary formulated foods for young children**   1.6g / serving.

On this basis, the application proposes the term "inulin-derived substances" be replaced with the more globally recognised term "fructo-oligosaccharides".

Furthermore, this application seeks permission for approval of a new microbial source of invertase (also called ß-fructofuranosidase) (EC 3.2.1.26) enzyme from a natural, genetically unmodified strain of the fungus *Aspergillus niger* as a processing aid (enzyme) (Standard 1.3.3 Processing Aids). This specific invertase is used in the manufacture of scFOS. As scFOS has been available and in use in the general food supply in Australia and New Zealand since prior to 2000, the lack of inclusion of this enzyme in the 2007 review of enzymes used as processing aids, appears to have been an oversight.

scFOS has been used in infant formula products in Japan since the early 1990's and today is used in formula in the USA and the South East Asian regions. The structural, safety and toxicity profiles of scFOS are shown to be substantially equivalent to that of oligofructose, currently approved within the Food Standards Code for use in Infant Formula Products, Foods for Infants, and Supplementary Formulated Foods for Young Children. In addition the physiological effects of the two substances are shown to be similar.

The outcomes of published and unpublished clinical trials (Abbott Nutrition 2003 – 2009; submitted under confidence) in which infants were fed formula containing up to 3.0 g scFOS (or oligofructose)/L support the safe use of this level of added scFOS with no demonstrated adverse effects on water balance in the infant population. Results from studies in older infants and young children support the safe use, and the promotion of general well-being, in young children consuming of 3.0 g scFOS/L in supplementary formulated foods or follow-on formula.

1. General Information on the application

1.1 Purpose of the application

This application seeks permission under the Australia New Zealand Food Standards Code, for the optional addition short-chain fructo-oligosaccharides (scFOS) to Infant Formula Products (Standard 2.9.1), Foods for Infants (Standard 2.9.2), and Supplementary Formulated Foods for Young Children (Standard 2.9.3 Division 4). This application proposes the maximum addition rates of scFOS to these food groups to be set to the same levels, but as an alternative to, those currently set for "inulin-derived substances":

- **Infant Formula products**   either (a) 110 mg per 100 kJ; or (b) 290 mg per 100 kJ in combination with galacto-oligosaccharides, where the scFOS is no more than 110 mg per 100 kJ (equivalent to 0.3g/100ml of formula).
- **Foods for Infants**   0.8 g/ 100 g either singularly or in combination with galacto-oligosaccharides, as consumed.
- **Supplementary formulated foods for young children**   1.6g / serving.

It is not the intention of this application to increase the total level of allowable oligosaccharides currently permitted in the product groups specified above, and regulated within Chapter 2 Part 2.9 Special Purpose Foods. It is intended that scFOS would be used as an optional alternative to the inulin-derived substances, not incremental to the levels stipulated.

Regulatory approaches to meet the objectives of this application, may, by way of example, include the addition of specific clauses in relation to scFOS, as appropriate; or, replacement of the specific term "inulin-derived substances" with a more broadly defined terminology of FOS (i.e. to include all fructans with $\beta(2\rightarrow1)$ fructosyl-fructose linkages with DP≥2). Such an approach is an opportunity to realign the definition of FOS with that which is globally more accepted and recognised i.e. not restricted to scFOS.

The intended use of scFOS in Australia and New Zealand by the applicant is, in the first instance, for the manufacture of infant formula products, baby foods, and foods for young children. It is anticipated that some of those products may be manufactured for export. A number of countries importing infant formula require the products to be compliant with the food standards of the country of manufacture.

In addition, this application seeks permission for approval of a new microbial source of invertase (also called ß-fructofuranosidase) (EC 3.2.1.26) enzyme from a natural, genetically unmodified strain of the fungus *Aspergillus niger* as a processing aid (enzyme) (Standard 1.3.3 Processing Aids). This specific invertase is used in the manufacture of scFOS. scFOS has been commercially available and used in Australia and New Zealand since prior to 2000, and was included in the 1995 application for inulin and fructooligosaccharides as dietary fibre (Food Standards Australia New Zealand, 2000). This application will correct what appears to have been an oversight in the

2007 review of enzymes used as processing aids (Food Standards Australia New Zealand, 2007). That review P276 intended to include enzymes "not currently used in Australia and New Zealand", and given the history of use and availability of scFOS as ingredients in these countries, ideally the Invertase used in their manufacture would have been included.

Invertase (EC 3.2.1.26) sourced from *Saccharomyces cerevisiae* is currently listed as a permitted processing aid in Standard 1.3.3 of the Food Standards Australia New Zealand (FSANZ) Code.

Approval of this enzyme, from *A. niger*, would increase the options of the microbial source of invertase enzymes that may be used for processing aids. The proposed change to the table of Clause 17 of Standard 1.3.3, would read:

Enzyme	Source
Invertase EC 3.2.1.26	*Aspergillus niger* *Saccharomyces cerevisiae*

Submission of this application is made under Section 3.6 Standards - Related to the Composition of Food Products; Sub-section 3.6.2 Special Purpose Foods, and under Section 3.3 – Standards Related to Substances Added to Food; Sub-section 3.3.2 Processing Aids, of the Food Standards Australia New Zealand Application Handbook (1 July 2010).

1.2 Justification for the application

1.2.1 Background

In 2007, Proposal P306 Addition of Inulin/FOS & GOS to Foods was initiated by FSANZ, and resulted in variations to the Food Standards Code in 2008 permitting the addition of inulin-derived substances and Galacto-oligosaccharides (GOS) to Infant Formula Products, Foods for Infants, and Formulated Supplementary Foods for Young Children (FSFYC) up to specified levels. At the time of the Final Assessment Review (Food Standards Australia New Zealand (FSANZ), 2008a) specifically excluded scFOS (FOS) on the basis that at that time there was "insufficient evidence to assess either the safety or physiological effects of FOS". On that basis the addition of oligofructans was limited to "inulin-derived substances", however was inclusive of both long- and short-chain fractions. In September 2008, the Australia and New Zealand Food Regulation Ministerial Council ("Council") requested a "First Review" of the Final Assessment Report (Food Standards Australia New Zealand (FSANZ), 2008b). The primary grounds for the review were to address matters regarding (a) consistency of the draft variations with the objectives of the legislation that establishes FSANZ, (b) the assurance of the protection of public health and safety, (c) consistency with international food standards, and (d) the clarification of enforcement and compliance issues. Clarification was also sought as to why FSANZ recommended the use of oligofructose as an inulin-derived substance but not scFOS when oligofructose has an average chain length of less than 10 and can vary from a DP of 2-10. FSANZ reiterated scFOS produced from the enzymatic condensation of sucrose typically had a more narrow DP (3-5) than that of inulin-derived oligofructose, and furthermore it had not been sufficiently studied in infants and young children to allow any conclusions about the physiological effects of scFOS to be made. On that basis, FSANZ considered there was insufficient data to support the addition of scFOS to infant formula and special purpose foods for young children. In addition, FSANZ was not aware of the use of scFOS in the manufacture of infant formula products, infant foods, foods for young children or the general food supply in Australia or New Zealand, and hence the exclusion of scFOS was not considered problematic at that time.

This application will present data demonstrating the safety and efficacy of scFOS, that is functionally similar to the inulin derived oligofructose (DP2-10 as described by FSANZ (2008a,b), and suitable for use in infant formula, foods for infants and special purpose foods for young children. The comprehensive review of oligosaccharides for use in foods for infants and children completed as a part of the Final Assessment Review (Food Standards Australia New Zealand (FSANZ), 2008a), remains relevant to this application and that information will not be unnecessarily duplicated within this application.

1.2.2 Intended Nutrition and Technical Effect

Inulin type fructose polymers such as scFOS or oligofructose may be added to foods for technological reasons (e.g., emulsifier, thickener, stabilizer, and sweetener), or nutritional reasons (e.g., dietary fiber, prebiotic effects), intended to improve stool consistency. These substances may also be added to infant formula in circumstances where the baby may not be ingesting breast milk or to supplement breast milk. Breast feeding is known to afford infants significant protection from diarrhoeal diseases, respiratory tract infections, meningitis and necrotizing enterocolitis, and this has been attributed in part to the presence of fucosylated oligosaccharides which are collectively termed glycans (Morrow, Ruiz-Palacios, Jiang, & Newburg, 2005). A wide variety of oligosaccharides have been detected in human milk, some of which may contribute to the anti-infective and allergy-preventive properties of human milk. The carbohydrates in greatest abundance in human milk comprise lactose (~7% of the milk) and oligosaccharides (1-2%). Oligosaccharides with a degree of polymerization (DP) of 3-10 are found in human milk. These human milk oligosaccharides account for the third largest component of human milk, with the peak concentration of around 25 g/L in the colostrum during the first few weeks following birth and decline thereafter in normal human milk. Because of the presence of oligosaccharides in human milk, the non-digestible substances scFOS and oligofructose have been developed for addition to infant formula and foods to mimic the effects of oligosaccharides that occur naturally in breast milk. These substances are not absorbed in the small intestine and reach the large intestine essentially intact. Breastfed infants generally have softer stools compared with formula fed infants and this difference may be due in part to the presence of oligosaccharides in breast milk. Therefore, scFOS or oligofructose may be added to infant formula to provide a functionally similar component breastfed infants routinely obtain from human milk, and to soften stool consistency.

1.2.3 Intended Technological Need for the Processing Aid

Numerous enzymes from *A. niger* are approved as food processing aids by FSANZ in Standard 1.3.3, however, none are invertase enzymes (also called ß-fructofuranosidase). Invertase is an endocellular enzyme preparation. The specific invertase that is subject of this application is made by a natural, genetically unmodified strain of the fungus *Aspergillus niger (A. japonicus)*, and is used to make short-chain fructooligosaccharides (scFOS) from sucrose. Invertase enzyme from *A. niger* was selected for the production of scFOS from sucrose due to its high enzyme productivity and its stronger transfructosylating ability compared with its hydrolysing activity (Hidaka, 1988). *A. niger* ATCC 20611 presented the best At/Ah (transfructosylating activity/hydrolyzing activity) ratio and produced more mycelium fructosyltransferase than *Aspergillus oryzae* IPT-301by about 75% (Fernandez et al., 2007).

1.2.4 Safety of the Processing Aid

It is important to take into consideration the whole body of knowledge from the literature when testing industrial strains for any possible risk during the development of a fermentation process.

Production organisms that are chosen from strains that have been in use for many years and that have been examined for their ability to produce known toxins under the fermentation conditions are the safest to use. In addition, the products should be regularly checked to ensure that they meet the requirements of the health authorities (Schuster, Dunn-Coleman, Frisvad, & Van Dijck, 2002).

1.2.4.1 Information on the pathogenicity and toxicity of the source micro-organism

A. niger is generally regarded as a non-pathogenic fungus widely distributed in nature. The *A. niger* group have a long history of safe use in the fermentation industry. In addition, these species have never been identified to be the primary cause of any disease in man. Sporadic toxin formation under undefined conditions has not been observed under controlled fermentation conditions (Schuster, et al., 2002).

Apergillus species have been used for decades to produce food enzymes and citric acid. Specifically, *A. niger* has become a source of a variety of enzymes that are well established as technical aids in fruit processing, baking, and in the starch and food industries (Schuster, et al., 2002). *A. niger* is generally regarded as a safe organism as documented in a list of the Berufsgenossenschaft der Chemischen Industrie (1998), an organization responsible for occupational health and safety. Enzyme preparations from *A. niger,* and the *A. niger* organism itself, have repeatedly been reviewed and accepted by FAO/WHO experts listing them with an Acceptable Daily Intake of 'not specified' (FAO/WHO, 1972, 1978, 1981, 1987, 1990). The Commission of the European Communities has also expressed the opinion of safety for *A. niger* food enzymes (Commission of the European Communities 1992). *A. niger* is also considered non-pathogenic and non-toxic by the United States Food and Drug Administration (FDA) (Food and Drug Administration, 1998). The use of *A. niger* in fermentation facilities is unlikely to increase the risk of infection by *A. niger* (Environmental Protection Agency, 1997).

The *A. niger* group have a long history of safe use in the fermentation industry. In addition, these species have never been identified to be the primary cause of any disease in man. *A. niger* has only been able to colonize the human body as an opportunistic invader in a few cases and in almost all these cases the patients have a history of severe illness or immunosuppressive treatment. Sporadic toxin formation under undefined conditions has not been observed under controlled fermentation conditions (Schuster, et al., 2002). Oral doses of 50 mg/kg of *A. niger* failed to cause acute toxicity (Yoshizawa, Tsuchiya, Morooka, & Sawada, 1975). Humans are continuosly exposed to *A. niger* spores and vegetative forms on foodstuffs and in the air (Environmental Protection Agency, 1997).

A. niger strains produce a series of secondary metabolites, but only ochratoxin A is regarded as a mycotoxin. Only about 3–10% of the strains examined for ochratoxin A production have tested positive under favorable conditions. Thus, *A. niger* has a low frequency of Ochratoxin A production potential and is considered a safe production organism (Schuster, et al., 2002).

Several experimental studies of the pathogenic potential of *A. niger* have come to the conclusion that neither ingestion of large doses of spores (Nyiredy, Etter, Fesüs, & Mayer, 1975) nor inhalation of spores (Bhatia & Mohapatra, 1969)induces mycosis in experimental animals. *A. niger* was not even detected in the digestive tract at one day after ingestion. In addition, despite a long history and the intensive nature of *Aspergillus* research, no cases of *A. niger* been proven to produce aflatoxins or trichotecenes (Schuster, et al., 2002).

Although workers may have the risk of allergic hypersensitivity to inhaled spores, this can be handled in an industrial environment by minimizing the exposure of the workers to spore dust (Schuster, et al., 2002). Thus, it is concluded that *A. niger* is a safe production organism for industrial use provided the rules of good manufacturing practice are observed (Schuster, et al., 2002).

1.2.4.2 Information on the genetic stability of the source micro-organism

A. niger is relatively stable to spontaneous mutation compared with other *Aspergillus* species (Raper & Fennell, 1965).

1.2.5 Consumer Choice Issues

This Application proposes an extension of the types of oligofructans that may be added to foods for infants and young children, within the current regulatory limits set for permitted oligosaccharides. This will potentially result in new products, product range extensions, or product reformulations, any of which will provide increased product options for consumers. Infants and young children have varying responses to prebiotics in their foods, mostly positive. However, having a more diversified range of different product options available means that those infants who do not tolerate one type of prebiotic, may still benefit from consuming prebiotics, if able to change and a product is found to be product suitable for them.

1.2.6 Costs and Benefits for Industry, Consumers and Government

By far the largest volume of infant formula products manufactured in Australia and New Zealand is for export. Infant formula product, manufactured in Australia for export to a number of countries in South East Asia, contains added scFOS, and has been manufactured under contract to Meiji for a number of years. Approval of this application will assist the export of products, by enabling brand owners to globally align their product offerings, and continue the manufacture and export of their products from New Zealand; on the basis it is compliant with local regulations.

Incremental costs associated with the addition of scFOS to infant food products, are typically reflected in incremental price increases of the finished products, based on the added benefits of those products. The market for infant foods is typically differentiated into standard and premium (GOLD), with the premium products containing added optional ingredients that deliver nutritional benefits over and above base nutrition, such as prebiotics, long chain

polyunsaturated fatty acids etc. The premium products are typically priced higher than the standard product offerings, prebiotic-supplemented formula reported to carry a price premium of 15-20% over standard formula in supermarkets (Rao, Srinivasjois, & Patole, 2009).

The invertase enzyme from *A. niger* was specifically chosen for the production of scFOS from sucrose due to its high enzyme productivity and its stronger transfructosylating ability compared with its hydrolysing activity (Hidaka, Hirayama, & Sumi, 1988). In addition *A. niger* ATCC 20611 produced more mycelium fructosyltransferase when compared with *Aspergillus oryzae* IPT-301by about 75% (Fernandez, et al., 2007). If another invertase enzyme were used to produce scFOS that provided smaller yield, it would be more costly to produce scFOS, and the increased price would likely be reflected in incremental price increases of the finished products to consumers.

This application will not result in any additional compliance or regulatory costs to Government, as it is an extension to the range and definition of:

- Prebiotic substances already permitted for inclusion in foods for infants and young children.
- Microbial source of Invertase already permitted as a processing aid
- Microbial enzymes from *A. niger*

2 Information to Support the Application

2.1 Description of the substance (scFOS)

2.1.1 Introduction

The substance that is the subject of this application is short-chain fructooligosaccharide (scFOS), also referred to as FOS during the FSANZ evaluation and review process of Application P306 Addition of Inulin/FOS & GOS to Foods (Food Standards Australia New Zealand (FSANZ), 2008a, 2008b).

The identity of scFOS is reviewed below, and includes a current description of the production process for the scFOS (both powder and syrup forms), current product specifications, current batch data, and storage stability data.

2.1.2 Characteristics of scFOS

Food-grade oligosaccharides are most commonly manufactured by enzymatic processes. They are either "built up" from simple sugars, such as sucrose or lactose, by transglycosylation reactions, or formed by controlled hydrolysis of polysaccharides, such as inulin, starch or xylan (Crittenden & Playne, 1996). Whilst the terms oligofructose and fructooligosaccharide are generally considered to be synonymous names for the mixture of small inulin oligomers with DP_{max}<10 (where inulin is correctly a generic term that describes all β(1←2) linear fructan molecules) (Crittenden & Playne, 1996; M. Roberfroid, 2007), for the purposes of this document and consistency with FSANZ definitions, the terms will be differentiated based on the mode of manufacture. Roberfroid (2007) clearly identifies that although the inulin hydrolysate and the synthetic compound have slight differences in the ranges of DP, the same terminology is appropriate to describe both. scFOS and oligofructose have very similar physicochemical properties; the properties of the two substances are compared in Table 1.

The term scFOS is used to describe fructooligosaccharide that is produced by the action of β-fructofuranosidase (EC 3.2.1.26) from *Aspergillus niger* on sucrose syrup (the enzyme links additional fructose monomers to the sucrose molecule)(Food Chemicals Codex (FCC) 7th Ed., 2010b). scFOS is marketed under a variety of trade names, including Neosugar, Nutraflora, Meioligo[®], and Actilight[®].

Oligofructose, a related substance, refers to the ingredient that is produced from the partial enzymatic hydrolysis of inulin, and is marketed for example as Raftilose 95[®] (Beneo[™]) or Fibrulose ® (Cosucra Groupe Warcoing) (Table 1).

scFOS is composed of sucrose molecules (glucose-fructose disaccharides, GF) to which one, two, or three additional fructose units have been added by β-2,1-glycosidic linkages to the fructose unit of sucrose. The components that comprise scFOS and oligofructose include the following: 1-kestose - GF_2 (α-D-glucopyranoside-(1↔2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl , nystose - GF_3 (α-D-glucopyranoside-(1↔2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl-(1←2) β-D-fructofuranosyl), and fructosyl- nystose - GF_4 (α-D-glucopyranoside-(1↔2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl(1←2)-β-D-fructofuranosyl-(1←2)-β-D-fructofuranosyl). The scFOS ingredient, which is the subject of this application, is referred to as the GFn type of scFOS. Oligofructose derived from inulin contains both fructose chains and fructose chains with terminal glucose units; scFOS contains only fructose chains with glucose end units (Niness 1999; Shoaf et al. 2006). Raftilose P95, which is derived by partial hydrolysis of inulin, is comprised of approximately 75% of fructose-only chains (FFn) and approximately 25% of the GFn form (Shoaf et al. 2006).

SUCROSE

α-D-Glu-(1→2)-β-D-Fru

Transfructosylation by β-fructofuranosidase
(EC 3.2.1.26)

α-D-Glu-(1→2)-[β-D-Fru-(1→2)-]$_n$, where n = 2-4

OLIGOFRUCTANS
(Oligofructose, Fructooligosaccharide)

β-D-Fru-(1→2)-[β-D-Fru-(1→2)-]$_n$, where n = 1-9, and

α-D-Glu-(1→2)-[β-D-Fru-(1→2)-]$_n$, where n = 2-9

Controlled enzymatic hydrolysis using inulinase
(EC 3.2.1.7)

INULIN

α-D-Glu-(1→2)-[β-D-Fru-(1→2)-]$_n$, where n >10
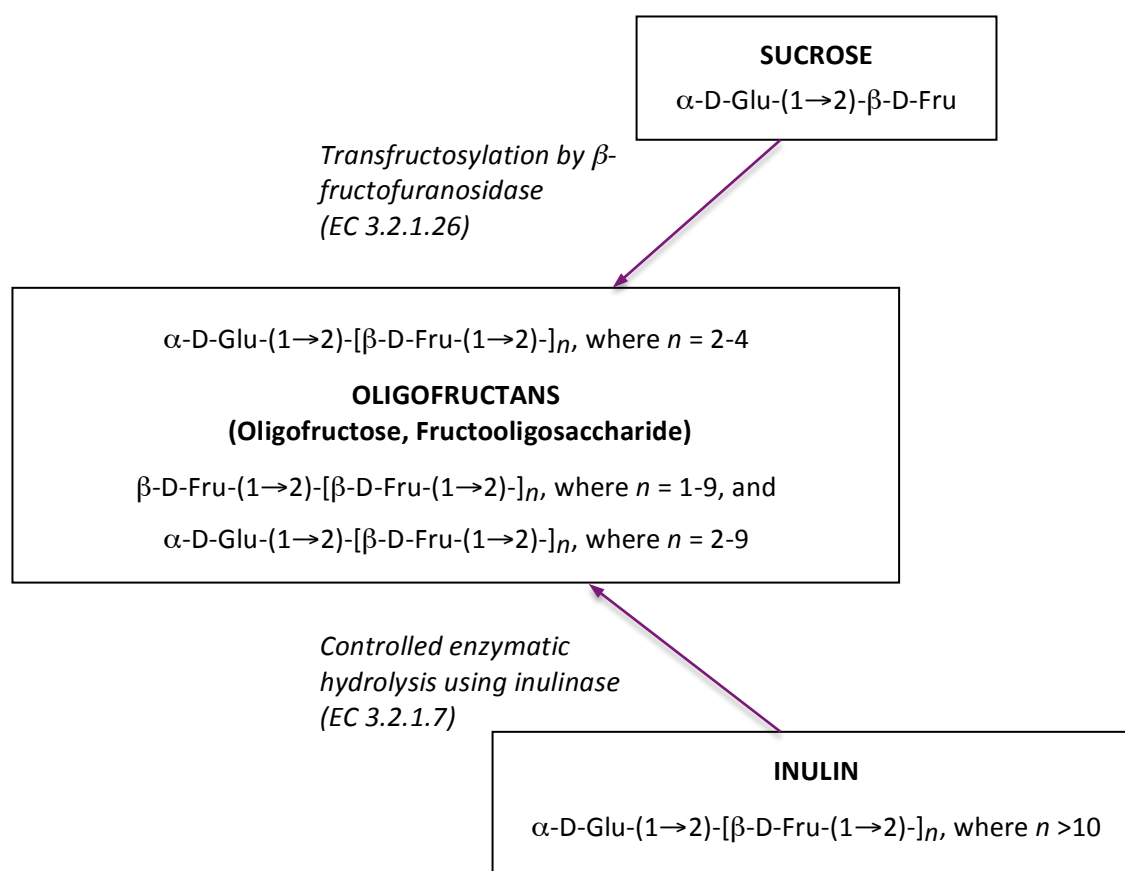
Figure 1: β(1←2) Oligosaccharides manufactured from sucrose or inulin
(Glu=glucose; Fru=fructose) (Adapted from Crittenden and Playne, 1996)

Figure 1 provides a schematic summary of the oligosaccharides relevant to this discussion that are manufactured from sucrose or inulin.

Fructans are characterized by the degree of polymerization (DP), which is a measure of the number of fructose molecules or saccharide units in the substance, the latter option used as

reference for this application. The DP ranges can vary for different fructans with some overlap in ranges. The average DP for scFOS is 3.5 (range is 2-5) and for oligofructose is 4 to 5 (range is 2-8) and both substances contain β-2,1 linkages between the fructose units. The specific DP values and range for oligofructose (DP<10) are in agreement with the more general definitions used by FSANZ (Food Standards Australia New Zealand (FSANZ), 2008a), however the DP for FOS (or more accurately scFOS) is best described as DP≤5 based on manufacturers information (Table 1). Further technical details are provided in the "Nutraflora Technicla Booklet July 2010" provided in support of this Application.

Table 1: Detailed Physicochemical Properties of scFOS and Oligofructose		
Parameter	scFOS (NutraFlora)	Oligofructose (Raftilose)
Common names	Fructo-oligosaccharide, FOS, short-chain FOS (scFOS)	Oligofructose
Trade names	Neosugar, NutraFlora, Meioligo; Actilight	Raftilose 95® (Beneo-Orafti) Fibrulose ®(Cosucra)
Manufacturer	GTC Nutrition Meiji	Orafti Cosucra Groupe Warcoing
Form	Powder or syrup	Powder
Source	Action of β-fructofuranosidase (EC3.2.1.26) (derived from *Aspergillus japonicus* ATCC 20611) on a sucrose syrup solution	Partial enzymatic hydrolysis of chicory inulin
Structure	2, 3, or 4 fructose units linked together, this molecule is linked to glucose	2 to 8 fructose units linked together, some of these molecules are terminated by a glucose unit
	$\alpha\text{-Glu-}(1\rightarrow2)\text{-}[\beta\text{-Fru-}(1\rightarrow2)]_{2-4}$ (100%)	$\alpha\text{-Glu-}(1\rightarrow2)\text{-}[\beta\text{-Fru-}(1\rightarrow2)]_{2-10}$ (~25%) $\beta\text{-Fru-}(1\rightarrow2)\text{-}[\beta\text{-Fru-}(1\rightarrow2)]_{1-10}$ (~75%)
Bond between fructose units	β-2,1	β-2,1
Range of DP	3-5	2-10
Glucose + fructose + sucrose	< 5%	< 6.8%
Oligosaccharide	> 95%	≥ 93.2%
Kestose (GF_2)	36.2 (30-42)	Not available
Nystose (GF_3)	49.1 (45-57)	Not available

Table 1: Detailed Physicochemical Properties of scFOS and Oligofructose		
Fructosyl-nystose (GF_4)	10.7 (5-15)	Not available
Molecular weight	Variable as by definition is a mixture of fructans (see above) Typically 627 g/mol	Not available
Solubility	Highly (≈80%) soluble in hot & cold water Almost insoluble in most organic solvents (Food Chemicals Codex (FCC) 7th Ed., 2010b)	Not available
Water activity (Nutraflora P-95)	0.1-0.2	Not available
pH (10% solution)	5.0-7.0	Not available
Calorific value	1.5 kcal/g	Not available

2.1.3 Identity

2.1.3.1 Chemical Composition

scFOS is a mixture of 1-fructose-(1-ß-fructofuranosyl)$_{n-1}$ sucrose oligomers; n may vary from 2 to 4. scFOS is prepared with ß-fructofuranosidase derived from *Aspergillus japonicus*. The Chemical Abstracts Service (CAS) Registry Number for fructooligosaccharide is 308066-66-2; this CAS No. is listed as an agricultural product in 7 CFR 205.606 (1-1-08). The monograph for scFOS is available in the Food Chemicals Codex (Food Chemicals Codex (FCC) 7th Ed., 2010b).

2.1.3.2 Common and Trade Names

Common or trade names for the substance that is the subject of this application include the following: fructooligosaccharide, FOS, short-chain fructooligosaccharide, scFOS, Neosugar, NutraFlora, NutraFlora® P-95, Meioligo®, and Actilight®. Table 2 shows the generic name, manufacturer, brand name and a description of a selection of some of the oligosaccharides that have been the subject of published research in relation to infant formula products.

Table 2: Oligosaccharides Commonly Used in Research				
Generic Term	Manufacturer	Brand Name	Description	DP
Fructo-oligosaccharide (FOS)	Eridania Beghin Say; GTC Nutrition	Actilight; NutraFlora	Short-chain Fructooligosaccharide	3-5
Oligofructose	Orafti	Raftilose P95/ Beneo P95	Hydrolysed inulin from chicory; 5% di- and	2-10

Table 2: Oligosaccharides Commonly Used in Research				
Generic Term	Manufacturer	Brand Name	Description	DP
	Cosucra	Fibrulose®	monosaccharides	
Long chain inulin	Orafti	Raftiline HP	Inulin from chicory with no mono- or disaccharides	10-60
Inulin	Orafti Cosucra Sensus	Raftiline Fibruline® Frutafit IQ	Inulin from chicory	2-60
Galacto-oligosaccharides (GOS)	Friesland Foods Domo	Vivinal	Galacto-oligosaccharides	3-8

2.1.3.3   Structure

The chemical structure of scFOS is shown in Figure 2.

Figure 2: Chemical Structure of scFOS
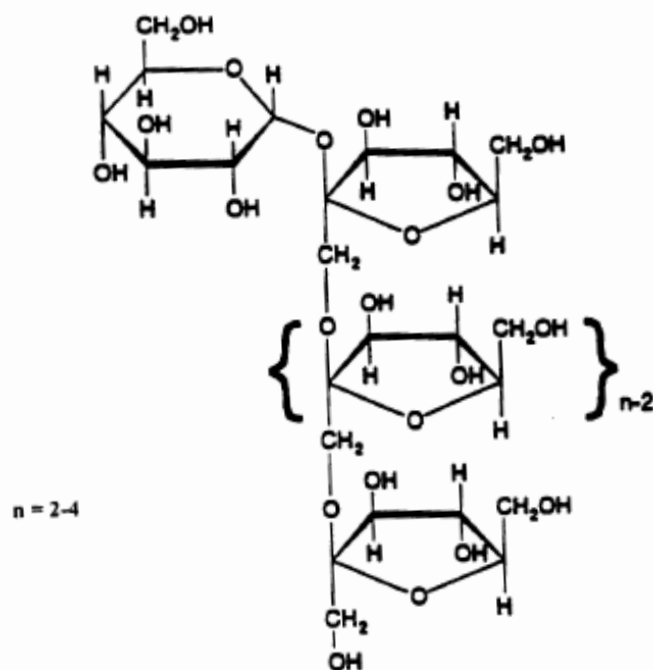
2.1.4 Production Process

2.1.4.1   Active Enzyme Manufacturing Process

2.1.4.1.1   Microbial Source of Active Enzyme

scFOS is produced by the action of ß-fructofuranosidase on a sucrose syrup solution. ß-fructofuranosidase is an endocellular enzyme preparation made by a natural, genetically unmodified strain of the fungus *A. japonicus* which is registered under the American Type Culture Collection (ATCC) number 20611.

A. japonicus ATCC 20611 is a specific species of *Aspergillus* that is commonly called *A. japonicus Saito.* It was originally-classified as *A. niger* until November 4, 1997 when ATCC renamed it *A. japonicus* based on its morphology (ENVIRON Corporation, 2000). However, within FSANZ Standard 1.3.3 Processing Aids, Clause 17 Permitted Enzymes

of Microbial origin, *Aspergillus niger* covers a wide range of strains, inclusive of *A. japonicus*. To ensure consistency with FSANZ Standard 1.3.3, this application is to permit the use of Invertase from *A. niger.* The use of Aspergillus spp. ATCC 20611 to produce the ß-fructofuranosidase (EC 3.2.1.26) used in the production of scFOS was described in detail in the original GRAS application in the USA, GRN 44 (ENVIRON Corporation, 2000). This application to permit the use of the ß-fructofuranosidase enzyme (EC 3.2.1.26) from *A. niger*, in addition to the currently permitted source (Invertase EC 3.2.1.26 from *Saccharomyces cerevisiae*), for the manufacture and use of foods and food ingredients in Australia and New Zealand underpins this application for the use of scFOS in infant and baby foods.

Aspergilli with brown to black-shaded spores constitute the *A. niger* group (Raper & Fennell, 1965). *Aspergilli* have been isolated all over the world. *A. niger* is a filamentous fungus that grows aerobically on organic matter. It can be found, in nature, in soil and litter, in compost and on decaying plant material (Schuster, et al., 2002). Reiss (1986) specified A. *niger* grows in a wide temperature range (6–47°C) with an optimum temperature at 35–37°C. The water activity limit for growth is relatively high compared with other *Aspergillus* species at 0.88. However, *A. niger is found* with a higher frequency in warm and humid places. *A. niger* is also able to grow over an extremely wide pH range: 1.4–9.8. In addition, the profuse production of conidiospores, which are distributed via the air, assure the ubiquitous occurrence of *A. niger in nature* (Rippel-Baldes 1955).

Aspergillus species are commonly used in food production and are approved by FSANZ (Standard 1.3.3, Clause 17) and the Food and Drug Administration (FDA) for a variety of uses. The *Aspergillus niger* group covers strains known under the names *Aspergillus aculeatus, A. awamori , A. ficuum, A. foetidus, A. japonicus, A. phoenicis, A. saitor* and *A. usamii* (Food Standards Australia New Zealand Code, 2009). All *A. niger* approved enzymes are listed in Table 3.

Table 3: *A. niger* Derived Enzymes Approved by FSANZ	
ENZYME FROM *A. NIGER*[1]	EC NUMBER
α–Amylase	EC 3.2.1.1
α–Arabinofuranosidase	EC 3.2.1.55
Asparaginase	EC 3.5.1.1
Carboxyl proteinase	EC 3.4.23.6
Catalase	EC 1.11.1.6
Cellulase	EC 3.2.1.4
Chymosin	EC 3.4.23.4
Endo-arabinase	EC 3.2.1.99
α-Galactosidase	EC 3.2.1.22
β-Galactosidase	EC 3.2.1.23
β-Glucanase	EC 3.2.1.6
Glucoamylase	EC 3.2.1.3

Table 3: *A. niger* Derived Enzymes Approved by FSANZ	
Glucose oxidase	EC 1.1.3.4
α-Glucosidase	EC 3.2.1.20
β-Glucosidase	EC 3.2.1.21
Hemicellulase endo-1,3-β-xylanase	EC 3.2.1.32
Hemicellulase endo-1,4-β-xylanase	EC 3.2.1.8
Hemicellulase multicomponent enzyme	EC 3.2.1.78
Hexose oxidase	EC 1.1.3.5
Inulinase	EC 3.2.1.7
Lipase, triacylglycerol	EC 3.1.1.3
Lysophospholipase	EC 3.1.1.5
Pectin lyase	EC 4.2.2.10
Pectinesterase	EC 3.1.1.11
Phospholipase A1	EC 3.1.1.32
Phospholipase A2	EC 3.1.1.4
3-Phytase	EC 3.1.3.8
4-Phytase	EC 3.1.3.26
Polygalacturonase or Pectinase multicomponent enzyme	EC 3.2.1.15
Transglucosidase	EC 2.4.1.24

[1]*Aspergillus niger* group covers strains known under the names *Aspergillus aculeatus, A. awamori , A. ficuum, A. foetidus, A. japonicus, A. phoenicis, A. saitor* and *A. usamii*.

Aspergillus niger is considered non-pathogenic and non-toxic by FDA (FDA 1998, as cited in ENVIRON 2000). *A. niger* is used in the production of numerous GRAS ingredients including glucono delta-lactone (21 CFR §184.1318), chymosin (21 CFR §184.1685), food-grade citric acid (21 CFR §173.280), and carbohydrase and cellulase for use in clam and shrimp processing (21 CFR §173.120). *A. oryzae* is used in the production of α-amylase (EC 3.2.1.1), which is a GRAS ingredient that can be used in the production of cereal flours (21 CFR §137.105, 21 CFR §137.200). In the 1960's, FDA authored an advisory opinion determining that carbohydrase and protease enzymes produced by *A. oryzae* are considered GRAS ingredients (Food and Drug Administration (FDA), 1998).

Aspergillus species are used in the production of several food ingredients recently determined to be GRAS, and a wide range is permitted under FSANZ Standard 1.3.3, Clause 17. For example, asparaginase enzyme preparation from *A. niger* (EC 3.5.1.1) is GRAS (GRN 214) for use in breads, other cereal based products and potato based products (Food and Drug Administration (FDA), 2007b). Asparaginase enzyme preparation from *A. oryzae* (EC 3.5.1.1) is GRAS (GRN 201) for similar uses (Food and Drug Administration (FDA), 2006b). Lipase enzyme (EC 3.1.1.3) preparations from *A. oryzae* (EC 3.1.1.3) (GRN 113) (Food and Drug Administration (FDA), 2003), and *A. niger* (GRN 111) (Food and Drug Administration (FDA), 2002), are GRAS for use in dairy-based flavouring preparations, cheeses, liquid and dried egg whites, bread, flour, bakery products not subject to a standard of identity, modified triglycerides,

hydrolyzed lecithin, edible fats and oils, and modified egg. *A. niger* and *A. oryzae* are used in the production of various GRAS enzyme preparations when encoding a gene from other species (for example, GRN 183, GRN 158 and GRN 142 (Food and Drug Administration (FDA), 2004, 2005, 2006a), respectively).

The use of *Aspergillus* spp. ATCC 20611 to produce ß-fructofuranosidase (invertase) used in the production of scFOS, is well documented (Nishizawa, Nakajima, & Nabetani, 2001). Yanai et al. (2001) have also reported on the characteristics of an extracellular fructofuranosidase from *A. niger* ATCC 20611. The transfructosylation (FTF) activity of this enzyme was increased compared with other known *Aspergillus* invertases. This strain was later reclassified as *Aspergillus japonicus* ATCC 20611 (Yanai, Nakane, Kawate, & Hirayama, 2001)

2.1.4.1.2   Production of Active Enzyme

The ß-fructofuranosidase enzyme (EC 3.2.1.26) of *A. niger*, specifically *A. japonicus* ATCC 20611 used in the production of scFOS is manufactured by Meiji Seika Kaisha, Ltd. The active enzyme is manufactured by Meiji Seika Kaisha Ltd., and is used by Meiji Seika, GTC Nutrition and Beghin Meiji to produce scFOS. The enzyme is subject to several quality control tests that are detailed below.

Methods to produce, purify and characterise the properties of the ß-fructofuranosidase (invertase) enzyme have been described in detail (Hidaka, et al., 1988; Hirayama, Sumi, & Hidaka, 1989). The enzyme preparation process is patented in numerous countries, including but not exclusively Japan, USA, Germany, Denmark, France, Great Britain, and the Netherlands. The fermentation method starts with sterilisation of the culture media and introduction into the batch of lyophilised spores. The fermentation process is temperature controlled and lasts for about 150 hours. The enzyme preparation is then concentrated by filtration and cryodessication of the liquid enzyme preparation. The process flow diagram for the general manufacturing process is shown below in Figure 3, with further specific details as provided in original manufacturing technical reports in Appendix IV.

Samples of the ß-fructofuranosidase enzyme are tested for proximate analyses, heavy metals, microbiological contamination, mycotoxins and enzyme activity. These results are reported in Certificates of Analysis (CoA), which are discussed here. Copies of CoA's from1989-1993 are included for reference in Appendix Va, and more recent examples (2010) are provided in the A1055 Supporting Document File submitted. The enzyme is tested for heavy metals as lead, as well as arsenic (as As_2O_3), lead, cadmium and mercury individually. The maximum heavy metal and lead levels reported in the Certificate of Analysis from 6 July 1990 is 9.6 ppm and 0.25 ppm, respectively. The enzyme is tested for microbiological contamination, including aerobic plate count,

coliforms, *E. coli*, coagulase positive *Staphylococci*, salmonella, and sulfite reducing anaerobic spores. The enzyme is tested for enzymatic activities including fructose-transferring activity, and activities of other enzymes such as amylase, protease and lipase. The transfer of fructose units is the desired enzymatic activity, and ranges from 1.02×10^6 to 1.12×10^6 unit/g. One unit of enzyme preparation is defined as the amount required to produce one micromole (1 μmol) of GF_2 (1-ketose) per hour from a 10 percent (weight/volume) sucrose solution at a temperature of 40 °C. The other enzyme activities (amylase, protease and lipase) are present at relatively low levels and considered insignificant in the final product. Test results for incidental residual enzymes are provided in Table 1 of Appendix III. The methodology to determine the invertase enzyme activity is also provided in Appendix III. The methodology described in Appendix III remains in use by manufacturers of the enzyme (Meiji Seika Kaisha Ltd). Samples of the ß-fructofuranosidase enzyme are screened for mycotoxins. As reported in GRN 44 (ENVIRON Corporation, 2000), analyses of samples conducted in 1989 and 1990 revealed no detectable levels of aflatoxin B_1, B_2, G_1, or G_2 (at a limit of detection of 5 ppb) or sterigmatocystin (at a limit of detection of 50 ppb). More recent mycotoxin screens conducted on samples of ß-fructofuranosidase enzyme in September and October of 2005 indicate that there were no detectable levels of aflatoxin B_1, B_2, G_1, or G_2 (at a limit of detection of 5 ppb), or sterigmatocystin or ochratoxin A (at a limit of detection of 0.05 ppm). Quality information and analysis certificates on the enzyme preparation can be found in Appendices V and VI.

Figure 3: Process Flow Diagram for the Manufacture of ß-fructofuranosidase
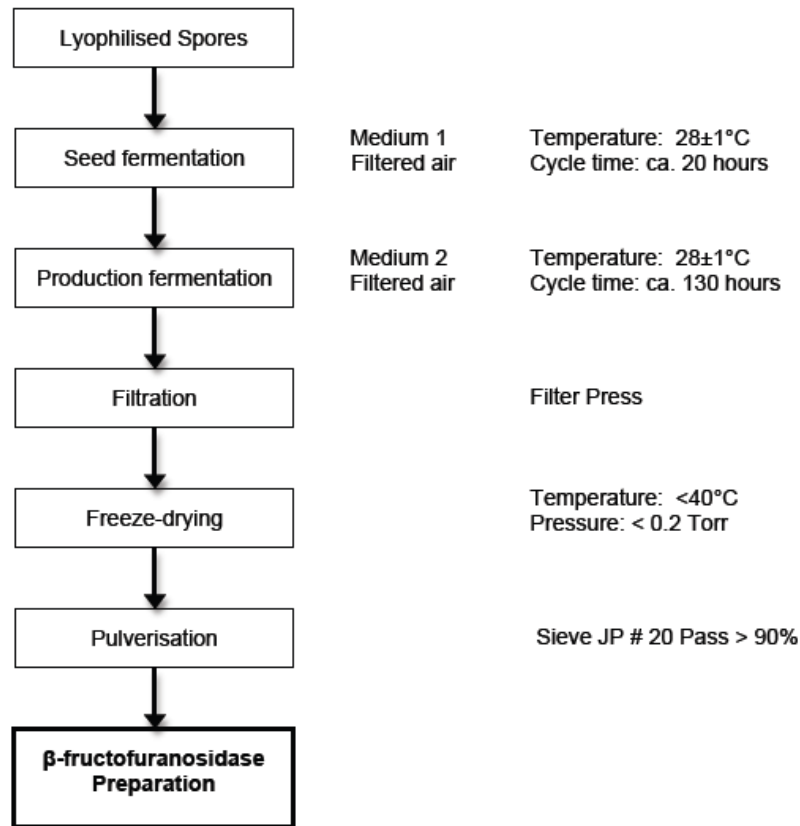
2.1.4.2   β-fructofuranosidase Enzyme Preparation Specification

Storage of the enzyme preparation at <5°C (cold room or refrigerator) is required.

Item	Specification
Colour	Pale brown powder
Odour	Characteristic odour
Form	Powder
Appearance	Free from visible contamination
Assay (activity)	>800,000 U/g
pH	6.0-7.0 (1g/100ml)
Loss on drying	Maximum 7% (1g, 105°C, 2 hrs, <3mmHg)
Particle size	JP#18 Pass: more than 90%
Heavy Metals (as Pb)	<5ppm
Microbiological	
Total Viable Count (including yeasts & molds)	<1,000/g
Coliforms	<30cfu/g
E.coli	Not detected /25g
Salmonella	Not detected /25g
Mycotoxins	

Aflatoxin B1	<5 ppm
Aflatoxin B2	<5 ppm
Aflatoxin G1	<5 ppm
Aflatoxin G2	<5 ppm
Sterimatocystin	<0.05 ppm

The β-fructofuranosidase enzyme preparation, is manufactured by Meiji Seika Kaisha Ltd., according to Good Manufacturing Practice (GMP).

The enzyme preparation meets the Joint Expert Committee on Food Additives (JECFA) criteria for enzyme preparations from microbial sources (Food and Agriculture Organization of the United Nations, 2006; Food and Agriculture Organization of the United Nations & World Health Organization, 2009).

Additionally the enzyme preparation also conforms to the criteria specified in the Food Chemicals Codex fro microbially-derived enzyme preparations (Food Chemicals Codex (FCC) 7th Ed., 2010a).

2.1.4.3   Enzyme Preparation Allergen Statement

The following statement is provided by the manufacturer (Meiji Seika Kaisha Ltd) of the β-fructofuranosidase enzyme preparation, used in the manufacture of scFOS. A digital copy is also provided in the A1055 Supporting Documents file submitted in support of this Application.

Meiji Seika Kaisha, Ltd. PHARMACEUTICALS GIFU PLANT

2890, Kitagata Kitagata-cho, Motosu-gun Gifu-ken, Japan

TEL:+81-58-324-4141 FAX:+81-58-323-2181

Allergenic Ingredients

Product: FRUCTOSYLFURANOSIDASE (FFCFF)

The table below indicates the presence (as added component) of the follwing allergens and products in this enzyme product.

Yes	No	Allergens	Description of components
	✓	Wheat	
	✓	Other cereals containing gluten	
	✓	Crustaceans	
	✓	Eggs	
	✓	Fish	
	✓	Peanuts	
✓		Soybeans	Corn salad oil*
	✓	Milk (including lactose)	
	✓	Nuts	
	✓	Celery	
	✓	Mustard	
	✓	Sesame Seeds	
	✓	Sulphur dioxide and sulfites >10 mg/ kg	
	✓	Lupine and products thereof	
	✓	Mollusk and products thereof	

*Corn salad oil : used for fermentation media component(0.25%)

Completed By : Quality control Director, Gifu Plant.
Meiji Seika Kaisha, Ltd.

M. Suzuki

Date: March 17, 2011

1/1

2.1.4.4   scFOS Manufacturing Process

Commercial production of scFOS is based on action of the enzyme ß-fructofuranosidase (EC 3.2.1.26), derived from *A. niger* ATCC 20611 (specifically *A. japonicus* ATCC 20611), on sucrose. The general process flow is outlined in Figure 4. The enzyme acts as both an invertase on sucrose molecules and a fructosyltransferase between sucrose molecules and fructofuranosyl-sucrose molecules (i.e., comprised of fructose chains with a terminal glucose). The invertase activity cleaves sucrose into glucose (G) and fructose (F) subunits. The fructofuranosidase activity transfers the fructose subunit from sucrose to a growing fructose chain to yield 1-kestose (GF_2), nystose (GF_3), and fructosyl-nystose (GF_4) molecules.

The enzyme acts as an invertase:
Sucrose   = Glucose (G) + Fructose (F)
And as a fructosylytransferase:
Sucrose (GF) + Sucrose (GF)   = 1-kestose (GF2) + Glucose
GF2 + Sucrose (GF)   = nystose (GF3) + Glucose
GF3 + Sucrose (GF)   = fructosyl-nytose (GF4) + Glucose

In summary:
GFn-1 + GF   = GFn + Glucose(n = 2, 3, 4)

The enzyme reaction is followed by purification steps and concentration of the reaction product. No enzyme or *A. niger* is present in the final scFOS product as shown by a negative test for protein on each lot. Furthermore, scFOS is not produced in Australia or New Zealand.

The only raw material used in the production of scFOS is sucrose. All process aids, including the invertase enzyme, used in the production are food grade.

Figure 4: General Production Process of NutraFlora Powder, NutraFlora Syrup and NutraFlora G Syrup
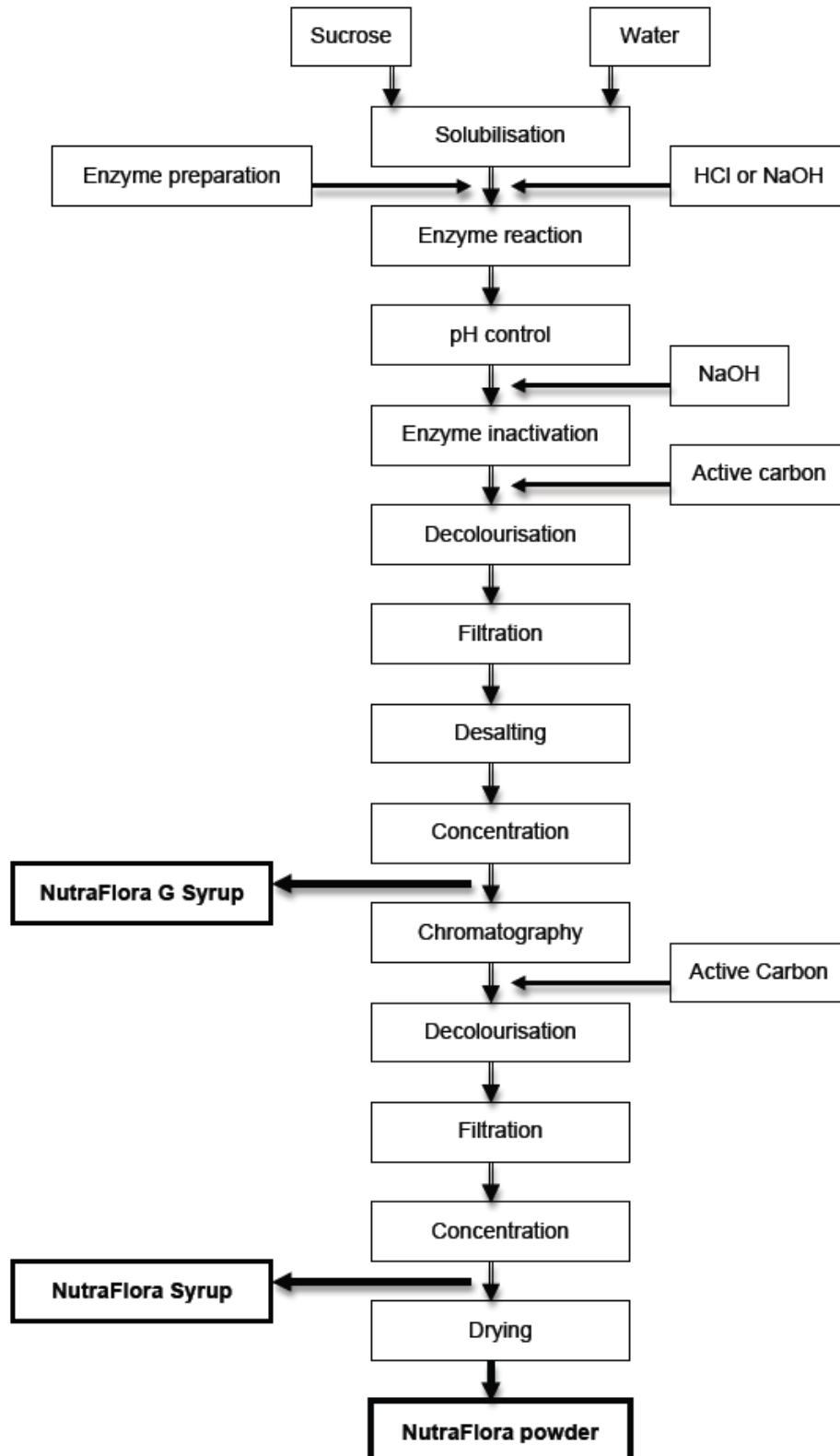
2.1.5 Finished Product Specifications

Specifications and stability data are presented for the Canadian-produced powder form, NutraFlora P-95, to illustrate the general characteristics of NutraFlora products. The syrup form of NutraFlora has the same product specifications on a dry basis, with a higher moisture content (~30%) and shorter shelf life (~3 months). Each batch of NutraFlora P-95 is analyzed for specific performance and quality parameters. The final product is analyzed for GF_2, GF_3, GF_4, ash, moisture, and several heavy metal and microbiological contaminants. The final product is also screened for protein using the Kjeldahl method to ensure there is no remaining detectable enzyme. The product specification is provided in Table 4.

A sample of NutraFlora P-95 was screened for the presence of organophosphate, chlorinated and n-methyl carbamate pesticides. At the limits of detection, no pesticides tested for were detected.

GTC Nutrition conducted a stability study to determine the shelf life of NutraFlora P-95. Three batches of NutraFlora P-95 were stored for two years, from September 2006 to October 2008. The batches were analyzed for moisture, pH, glucose, fructose, sucrose, GF_2, GF_3, GF_4 and total scFOS at intervals of approximately every three months. The total pH, glucose, fructose, sucrose, and scFOS and its components were stable throughout the study and remained within specifications. The moisture content increased slightly during the course of the study in all batches. The last sample of one batch was outside of specification, with a moisture content of 5.1% (the specification is 5.0%) (GTC Nutrition 2008). As stated on the product specification sheet, NutraFlora P-95 has a two year minimum shelf life from date of manufacture when sealed and stored under cool, dry conditions (25°C and 33% relative humidity).

Table 4: Nutraflora P-95 Manufacturing Specification			
Parameters	Specification	Units	Method of Analysis
Macrocomponents			
Glucose+Fructose+Sucrose	≤ 5	%, db	In-house HPLC method
Fructooligosaccharides	≥ 95	%, db	Total of GF
Protein	<0.5%	% w/w as - is	GTC CPC-SMA No P60 (digestion by Kjeldahl method)
Ash	≤ 0.1	% w/w	GTC CPC-SMA No A92 (conductivity)
Moisture	≤ 5	%	USP <921> Water Determination (KF)
Heavy Metals			
Arsenic (as As_2O_3)	≤0.1	ppm	ICP-MS
Cadmium (Cd)	≤0.01	ppm	ICP-MS
Lead (Pb)	″ 0.05	ppm	ICP-MS
Mercury (Hg)	″ 0.01	ppm	ICP-MS
Microbiological Components			
Enterobacteriaceae	<3	cfu/g	CMMEF 4th Edition
Anaerobic Thermophilic Spores	≤ 100	cfu/g	CMMEF 4th Edition
Aerobic Thermophilic Spores	≤ 100	cfu/g	CMMEF 4th Edition
Anaerobic Mesophilic Spores	≤300	cfu/g	CMMEF 4th Edition
Aerobic Mesophilic Spores	≤300	cfu/g	CMMEF 4th Edition
Abbreviations:			
db Dry basis			
w/w Weight for weight			

2.1.6 Allergen Statement

The following allergen statement for NutraFlora P-95 scFOS is issued by GTC Nutrition for commercial use. A digital copy is provided in the supporting documents file to this Application

NutraFlora® P-95, PIN 111001
Allergen Statement

Allergens	Present in the Product	Present in Other Products Manufactured on Same Line	Present in Other Products Manufactured on Same Plant
Wheat containing gluten, which include but are not limited to wheat, rye, barley, oats, spelt and buckwheat, their hybridized strains, and products of these. Corn, rice, sorghum, flax and products of these are NOT included.	NO	NO	NO
Crustaceans, Shellfish, and products of these, which include but are not limited to shrimp, prawns, crab, lobster, and crayfish.	NO	NO	NO
Mollusks and products of these, which include but are not limited to oysters, clams, scallops, and mussels.	NO	NO	NO
Eggs and egg products.	NO	NO	NO
Fish and fish products.	NO	NO	NO
Peanuts and products of these. Highly refined (refined, bleached and deodorized), peanut oil is NOT included.	NO	NO	NO
Soybeans and products of these, which include but are not limited to hydrolyzed vegetable protein and lecithin. Highly refined soybean oil is NOT included.	NO	NO	NO
Sesame Seeds and products of these.	NO	NO	NO
Milk and milk products, which include but are not limited to lactose, whey, casein and caseinates.	NO	NO	NO
Tree nuts and nut products, which include but are not limited to almonds, Brazil nuts, pecans, cashews, chestnuts, hazelnuts (filberts), pine nuts, pistachios, macadamia nuts, hickory nuts and walnuts.	NO	NO	NO
Sulphites in concentrations of 10 mg/kg (10 ppm) or more, measured as total sulphur dioxide. Sulphiting agents include but are not limited to sulphur dioxide, sodium sulphite, sodium and potassium bisulphite, and sodium and potassium metabisulphite.	NO	NO	YES

Plant Source	Yes	No	Plant Source Ingredient(s)
Hydrolyzed Plant Protein		✓	
Starch & Modified Starches		✓	
Flour		✓	
Gluten		✓	
Fat & Oil Products		✓	

FOOD INTOLERANT MATERIAL SOURCE

	Yes	No		Yes	No
Bananas		✓	Monosodium Glutamate (MSG)		✓
Carmine/Cochineal		✓	Mustard		✓
Celery		✓	Orange		✓
Cherries		✓	Pear		✓
Cinnamon		✓	Raspberries		✓
Corn		✓	Strawberries		✓
Grapefruit		✓	Sulphites - in concentrations of 10 mg/kg (10 ppm) or more, measured as total sulphur dioxide.		✓
Lactose		✓	Sunset Yellow – FD&C #6 – E110		✓
Lemons		✓	Tartrazine – FD&C Yellow #5 –E102		✓

Dietary Lifestyle: ☑ Suitable for Vegetarians   ☑ Suitable for Vegans

FOOD & SAFETY INFORMATION

☐ HACCP program in place	☑ An effective GMP program
☑ Non-accredited HACCP program	☑ Comply to government guidelines for pesticides, antibiotics and heavy metals
☑ An effective procedure to avoid cross- contamination of allergens	☑ Allergy education program for employees

Revision: August 2007
Supercedes: June 2006
Version: 002

Page 1 of 1

2.1.7 Analytical Methodology

Specific methods for the detection and quantitative measurement of total scFOS have been developed (Institute of Medicine of the National Academies, 2003; Ouarné, Guibert, Brown, & Bornet, 1999). The method has been used to analyse scFOS in infant and follow-on formulas.

The HPLC method is similar to AOAC method 997.08 (fructan analysis), which is based on the determination of glucose and fructose released by enzymatic hydrolysis of scFOS by invertase. Corrections are made for sucrose, free fructose, and free glucose in the sample.

Identification and assay procedures and criteria are also provided in the scFOS monograph of the Food Chemicals Codex (Food Chemicals Codex (FCC) 7th Ed., 2010b).

2.1.8 Stability of scFOS In Products

Based on data accumulated during the product development of infant formula products and foods for young children containing scFOS, compared to oligofructose, scFOS is more compatible with processing in that the ingredient withstands heat better and is more stable and undergoes less Maillard browning. Technical data is provided in the NutraFlora Technical Booklet submitted in the A1055 Supporting Documents file in support of this application.

In the United States, Abbott Nutrition markets Similac[®] Go & Grow[®] Soy-Based Infant Formula Powder for infants 9-24 months of age, containing scFOS (2.2 g/L) and Similac[®] Sensitive[®] Isomil Soy™ Infant Formula Powder for infants 0-12 months with scFOS (2.1 g/L). The scFOS has proven to be stable in both of these products and within desired specifications over the shelf-life of the product. Additionally, PediaSure[®] with Fiber (currently on market) containing 1.0 g scFOS/8 fl.oz. serving has also demonstrated proven stability over the shelf-life.

2.1.9 *In vitro* Fermentation Properties of Oligofructans

The physiological benefits of adding oligosaccharides to foods, including infant formula products and foods for infants, is due to the prebiotic properties, specifically the selected stimulation of bacterial growth in the large intestine and the modulation of bowel function through fermentation of the selected oligosaccharides (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004; Hernot et al., 2009). The fermentation properties of oligosaccharides are of interest particularly, as in addition to the beneficial effects (production of short chain fatty acids (SCFA)), the inevitable production of gases has the potential to be the source of adverse side effects such as discomfort due to meteorism

and bloating (Cummings, Macfarlane, & Englyst, 2001). Therefore there is a need to optimise SCFA production whilst minimising gas production.

In a comprehensive study, using human faecal inoculum in an *in vitro* fermentation model, Hernot *et al.* (2009) investigated the fermentation profiles, gas production volumes and rates, and microbiota modulation of a wide range of oligosaccharide compounds, including scFOS, oligofructose and galactooligosaccharides, with the objective of determining those oligosaccharides that produce the least gas, whilst maintaining SCFA production and beneficial effects on the microbiota. The oligofructan samples were loosely grouped according to DP; short chain (Nutraflora scFOS (GTC)), medium chain (Beneo P95 (Orafti) and Frutalose L90 (Sensus)), and long chain (Inulin and Beneo HP & ST (Orafti)). The galactooligosaccharide (Vivinal GOS) used, was classified as a medium chain oligosaccharide. The authors acknowledge that whilst the *in vitro* fermentation profiles for oligosaccharides do not take into account possible adaptation of human microflora after prolonged exposure to prebiotic oligosaccharides, the model is a useful tool to predict fermentation patterns *in vivo* (Hernot, et al., 2009). The advantage of the *in vitro* model is, it is standardised and, enables a comparative analytical study of multiple prebiotic substances, not be feasible *in vivo*.

Using this standardised *in vitro* model, the fermentation performance of the short- and medium- chain oligosaccharides (scFOS, oligofructose and GOS) was shown to be consistently different to that of the longer chain oligosaccharides (Hernot, et al., 2009). Rates of gas production were similar for Nutraflora scFOS, Beneo P95 and the Vivinal GOS. This group of oligosaccharides consistently produced the highest rates of total gas production, compared to the longer chain products. Within this group of products, the GOS produced a slightly smaller total gas volume over a 12 hour fermentation period, than the oligofructans of similar chain length. Fermentation of the short- and medium chain oligofructans (Nutraflora, Beneo P95 and Frutalose P90) and the Vivinal GOS resulted in the highest ($P<0.05$) production of SCFA at all time points (Hernot, et al., 2009). The largest increase in bifidobacteria was from the fermentation of the short- and medium-chain oligosaccharides (Vivinal GOS, Beneo P95 and Nutraflora scFOS) all with DP< 10, further demonstrating their substantially equivalent performance in relation to fermentation properties and the modulation of microbiota. This result is consistent with earlier work by Roberfroid and colleagues, who reported short chain oligosaccharides (DP 2-10) promote the growth of bifidobacteria by almost an order of magnitude, compared to higher DP oligosaccharides (DP>10) (M. B. Roberfroid, Van Loo, & Gibson, 1998).

2.2 Oligosaccharides in Infant Nutrition

A detailed review of the role of oligosaccharides in infant nutrition, and a detailed analysis of the intakes of oligosaccharides was included in the Final Assessment Report of Application P306 Addition of Inulin/FOS & GOS to Foods (Food Standards Australia New Zealand (FSANZ), 2008a), and it is not the intention of this application to repeat that review. The intent of the following overview is to provide a brief perspective for the potential contribution of scFOS to infant nutrition.

2.2.1 Introduction

In the first months of life, infants are exposed exclusively to the components in human milk, infant formula, or a combination of human milk and infant formula. Human milk does not contain ß- 2,1 fructans; it does, however, contain a high concentration of non-digestible oligosaccharides. After the introduction of weaning foods at approximately 4 to 6 months of life, infants are exposed to the vast array of components naturally present in foods, including ß-2,1 fructans, as well as unique ingredients that are used in the foods they consume.

Infants in Japan and Europe have been exposed to oligosaccharides including scFOS, galacto-oligosaccharide, and long chain-inulin, in infant formula for many years (Veereman, 2007; Yamamoto & Yonekubo, 1993). FOS has recently been added to follow-on formulas in the U.S., and other oligosaccharides, namely GOS, have recently been added to infant formulas. scFOS, oligofructose, and inulin are all added to many baby foods in the U.S., and may also be added to foods for the general population that infants consume.

2.2.2 Oligosaccharides in Human Milk

Human milk is regarded as the preferred source of nutrition for infant feeding. The American Academy of Pediatrics encourages exclusive use of human milk during the first six months of life, and in combination with solid foods from about six months through the first year of life and beyond as determined by mother and child (American Academy of Pediatrics, 2005).

Human milk is a complex substance consisting of thousands of constituents such as hormones, immune factors, and live cells in addition to macronutrients, vitamins, and minerals (Picciano, 2001). The concentration of oligosaccharides in human milk is approximately 7-13 g/L, making oligosaccharides the third largest solid constituent after lactose and fat (Boehm & Stahl, 2007). Human milk oligosaccharides are complex and diverse structures, and have a DP range of 3-10. Estimates of the number of unique oligo- and polysaccharide structures in human milk range from more than 200 to over 1000

(Boehm et al., 2005). The core oligosaccharide structure is characterized by the attachment of galactose and N-acetylglucosamine to lactose in ß-glycosidic linkages, while D-glucose, D-galactose, N-acetylglucosamine, L-fucose, and sialic acid (N-acetyl neuraminic acid) represent the key monomers in human milk oligosaccharides (Boehm & Moro, 2008). Human milk does not contain β-2,1 fructans. Exclusively breastfed infants, therefore, have not historically been exposed to these specific oligosaccharides, however, consumption of total oligosaccharides is roughly in the range of 5 to 10 g per day, assuming typical concentrations in human milk and an approximate human milk intake of 750 mL per day (Raiten, Talbot, & Waters, 1998).

2.2.3 Naturally Occurring Sources of β-2,1 Fructans

Natural sources of linear β-2,1 fructans are found in more than 36,000 plant species (Niness, 1999), including many foods which are commonplace in contemporary Western diets (Van Loo, Coussement, De Leenheer, Hoebregs, & Smits, 1995). Levels of inulin (DP predominantly 2 – 60), and oligofructose (DP 2 – 10), a subset of inulin, have been quantified in artichokes, asparagus, bananas, garlic, leeks, onions, wheat, rye, barley, and other foods (Van Loo, et al., 1995).

2.3 Safety of scFOS and Oligofructose

2.3.1 Introduction

scFOS is marketed and sold under the trade names NutraFlora, Meioligo, and Actilight for use as a prebiotic in a variety of food products around the world (*e.g.*, yogurt, baby food, ice cream, cereals, milk, muffins). NutraFlora is the same substance as Neosugar, originally developed by Meiji Seika Kaisha Ltd., and is manufactured by GTC Nutrition. In the USA, scFOS has GRAS status for use in a variety of foods, including baby foods, but excluding meat and poultry products and infant formula (Food and Drug Administration (FDA), 2000, 2007a); however, follow-on formula for infants with added fructooligosaccharide were available in the USA in 2008 (PBM Products 2008). There are other commercially available forms of oligosaccharides, including inulin and oligofructose (e.g., Raftilose 95, Beneo™) that have similar safety profiles. Historically, these fructooligosaccharide substances have been referred to interchangeably in the scientific literature and the available toxicological studies on all of these substances have been used to substantiate safety because they are so structurally similar and all have been shown to have similar physiological effects.

Safety studies conducted on scFOS (Neosugar) and oligofructose (Raftilose) are included in this application, in addition to a brief overview of the metabolism and physiological properties of scFOS in the gastrointestinal tract. Because of the potential for confusion regarding nomenclature of oligosaccharide substances, the specific test material is clearly identified and the studies are grouped by test material (e.g., scFOS - Neosugar, or Oligofructose - Raftilose). Importantly, there do not appear to be any differences in the safety profiles of these two oligosaccharides. Although many of the toxicological studies conducted with scFOS or oligofructose were conducted with young experimental animals, they were not designed to specifically evaluate the effects of scFOS in infants and children.

2.3.2 Metabolism and Physiological Properties of scFOS in the Gastrointestinal Tract

FOS belongs to a class of carbohydrates known as fructans that consist of linear chains of β-2,1 linked D-fructofuranose units with a terminal glucose moiety. The physiologic effects of scFOS are similar to those of a dietary fiber and include resistance to digestion by endogenous enzymes, fermentation by colonic microflora, shortened gastrointestinal transit time, increased faecal weight, reduction of faecal pH, predictable reduction in caloric value, reduction of plasma cholesterol and triglycerides, and reduction in glucose absorption. *In vitro* and *in vivo* studies in animals and humans have demonstrated that scFOS is not digested by the digestive enzymes of the alimentary canal; it passes unchanged into the colon where it is subsequently hydrolyzed and fermented by the microflora. Results from studies in humans indicate that approximately 89% of undigested

scFOS is fermented by the microflora into gasses, short-chain fatty acids (SCFA) and H_2, and 0.12% of ingested scFOS is recovered unchanged in the urine; none is recovered in the faeces[1] (Molis et al., 1996). The SCFA production is predominantly acetic acid, while propionic and butyric acids are generated in smaller amounts; the H_2 produced is absorbed and expired in the breath. Comparable results regarding non-digestibility and fermentation products have been reported for oligofructose[2] (Alles et al., 1996; Niness, 1999).

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 & Menzies, 1992). The gut of a newborn infant, especially a pre-term infant, is permeable to macromolecules, such as intact sugars and proteins (Weaver, KLaker, & Nelson, 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 take up larger nutritional molecules, as well as the development of systemic immune tolerance. However, this increased permeability can also have deleterious effects, such as increased uptake of infectious agents leading to the development of infection, inflammation, and systemic hypersensitivity (Insoft, Sanderson, & Walker, 1996). 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, MacPherson, & Hollander, 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, Kapembwa, Laker, Levin, & Griffin, 1990). With respect to infant intestinal development, in general, lactulose/mannitol (L/M) urinary ratios are highest in the first days of life, and then rapidly decrease (Colomé et al., 2007). In a single oral load study conducted to evaluate intestinal permeability as a marker of intestinal epithelial integrity in breast-fed or formula-fed infants (with or without prebiotics, nucleotides, or long-chain polyunsaturated fatty acids - LC-PUFA), Colomé et al. (2007) reported that formula-fed infants had urinary L/M ratios in the range of 0.268 to 0.341

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[1] Study subjects in the Molis et al. study were administered 20 g/day Actilight (Eridania-Beghin-Say, Paris), which consists of 44% 1-kestose (GF_2), 46% nystose (GF_3), and 10% 1-fructose-β-fructofuranosyl nystose (GF_4)

[2] Study subjects in the Alles et al. study were administered 5 or 15 g/day Raftilose 95

and breast-fed infants had a mean L/M ratio of 0.313). No significant difference was found in the intestinal permeability between breast-fed and standard cow's milk formula fed infants or in infants fed the formulas containing prebiotics, LC-PUFA, or nucleotides.

Mineral bioavailability could be influenced by several factors in the diet, the magnitude of which depends on inhibitors and promoters contained in a meal, and hence the diet composition (Lobo, Filho, Alvares, Cocato, & Colli, 2009). Oligofructose, inulin, and galactooligosaccharides are the most investigated dietary non-digestible oligosaccharides with respect to their effects on mineral bioavailability (Kruger, Gallaher, & Schollum, 2003; Lobo, et al., 2009; Scholz-Ahrens & Schrezenmeir, 2007). Data from animal and human studies show that β-2,1 fructans do not negatively affect mineral absorption or balance. Investigators have reported that scFOS ingestion decreases faecal excretion, increases apparent absorption, increases retention and increases urinary calcium (Ca) excretion, while other studies have reported increased absorption, increased or no change in retention, increased urinary excretion and decreased faecal excretion of magnesium (Mg) with scFOS ingestion (Baba et al., 1996; Delzenne, Aertssens, Verplaetse, Roccaro, & Roberfroid, 1995; A Ohta, Baba, Takizawa, & Adachi, 1994; Atsutane Ohta et al., 1995; Atsutane Ohta, Ohtsuki, Baba, Hirayama, & Adachi, 1998; A Ohta et al., 1995; A Ohta et al., 1994; A Ohta, Osakabe, Yamada, Saito, & Hidaka, 1993). Ohta *et al*. (1993, 1998) showed that the effect of scFOS on apparent absorption of Ca and Mg was dose dependent. In order to study the effects of an oligofructose (Raftilose 95) on nutrient digestion in both weanling and growing pigs, weanling pigs were fed diets containing 1% or 4% Raftilose 95 for up to 27 days and growing pigs diets containing 0.4% or 0.8% Raftilose 95 for 47 days (Houdijk et al., 1999). Results in the weanling group indicated there was an increase in nitrogen excretion, but no change in the ileal digestion of Ca, potassium (K), Mg, iron (Fe), copper (Cu), or zinc (Zn) 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. The authors concluded that dietary Raftilose 95 did not affect nutrient digestion in growing and weanling pigs. A study by Wolf et al. (1998) was conducted to evaluate the effect of FOS[3] incorporated at several dietary concentrations (0, 1, 3 and 5 g/100 g food -1, 3, 5%) on apparent mineral digestion and balance in growing rats (5 wks old). The primary objective was to determine the dose response effect of FOS on apparent digestion and balance of several minerals, including Ca, P, Mg, Na, Cl, K, Cu, Fe, Mn, and Zn. There was a statistically significant increase in Mg absorption as FOS concentration in the diet increased (an apparent 10.6% increase in Mg absorption for rats consuming 5% FOS as compared to control rats). Copper absorption was decreased by approximately 40% in animals fed 3 or 5% FOS. The

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[3] Source of >95% FOS was "Golden Technologies Company Inc. Westminster, CO; no other information provided about FOS

decreased Cu absorption may be explained by an increase in faecal microbial mass which would contain a relatively significant amount of Cu or by an increase in hepatic bile Cu excretion. With the exception of Mg and Cu, there was no effect of FOS on mineral absorption and balance of the other evaluated minerals in the growing rats (Wolf, Firkins, & Zhang, 1998).

A recent study by Lobo et al. (2009) was conducted to evaluate the effects of dietary lipid composition and oligofructose on mineral bioavailability in growing Wistar male rats (6 wks old). The rats were provided diets *ad libitum* containing four different treatments for 15 days, including 2 groups of 6 rats that received 10.87% Raftilose Synergy[4] – "oligofructose" (with 15% soybean oil (SO) or 11.5% fish oil and 3.5% soybean oil mixture (FSO)) in their diet. Two groups of control rats received either SO or FSO alone in their diets. Food consumption was measured daily and body weight was recorded every 48 hr; after 15 days, the liver and caecum with its contents were removed and weighed. The hind limbs and bones were harvested and stored for mineral analysis. Apparent mineral absorption and balance were calculated for Ca, Mg, Cu, Fe, and Zn. A significant decrease in total food intake and body weight gain was observed in rats administered oligofructose in their diets with SO or FSO as compared with controls. The body weight for the four treatment groups were as follows: SO - 85.2g; SO/ITF – 72.9g; FSO – 88g; FSO/ITF – 80.6g (the two ITF groups were significantly lower than the SO or FSO alone groups). Oligofructose administration was associated with an enlargement of the caecum (wall and contents), a significant decrease in caecal content pH, and a significant increase in faecal water content as compared to controls fed SO or FSO. Loose stools were observed in some of the rats in the FSO and oligofructose group within the first 5 days (number of rats not reported); however, consistency returned to normal during subsequent days. Dietary supplementation with 10% Raftilose Synergy for 15 d resulted in statistically significantly higher intestinal absorption of some macrominerals (Ca and Mg) and microminerals (Cu and Zn) in growing rats and these effects were reflected in statistically significant higher bone mineral content (Ca, Zn) and bone strength when FSO and/or SO were co-administered in the diet. The investigators concluded that dietary lipid composition may influence the effects of inulin-type fructans on mineral bioavailability and that a dietary combination of inulin-type fructans and lipids might be useful in maintaining skeletal integrity and improving bone health.

The available data on the effects of scFOS on mineral absorption and balance support the safe use of scFOS in infant formula at the anticipated exposure levels.

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[4] Raftilose Synergy is a 1:1 mixture of oligofructose (degree of polymerization [DP] ranging between 2 and 8, with a DP average of 4) and high-performance inulin (DP ranges between 10 and 65, with a DP average of 25), which is obtained by physically removing the lower-DP fraction from the native inulin, Orafti Active Food International, Tienen, Belgium.

2.3.3 Safety Toxicological Studies

Much of the information regarding toxicological studies with scFOS comes from a review in which the safety of inulin, oligofructose, and scFOS were evaluated (Carabin & Flamm, 1999). The authors concluded that results from toxicological studies on inulin-type fructans have not shown evidence of mortality, morbidity, target organ toxicity, reproductive or developmental toxicity, mutagenicity or carcinogenicity. Inulin, oligofructose, and scFOS are chemically similar entities demonstrating similar nutritional properties. According to Carabin and Flamm (1999), given the chemical and physiological similarities of inulin, oligofructose, and scFOS, the toxicological studies performed with scFOS and their results are predictive of the effects of inulin and oligofructose in demonstrating safety. Most of the studies reviewed by Carabin and Flamm (1999) were conducted with Neosugar, which is a mixture of fructooligosaccharides consisting of approximately 28% 1-kestose, 60% nystose, and 12% l-fructose-β-fructofuranosyl nystose (Tokunaga, Oku, & Hosoya, 1989). Neosugar has the same chemical structure as inulin, but has shorter chain length (up to four fructose units) and is produced by enzymatic synthesis from sucrose using fungal fructosyltransferase.

Standard toxicology studies conducted on Neosugar include a genotoxicity battery, developmental studies, a subchronic study, and a carcinogenicity study. These studies are briefly described below and summarized in Table 5 and Table 7. No specific safety issues were identified in these studies. Results from subchronic and carcinogenicity studies in rats demonstrate that there are no significant adverse effects up to 2,170 mg/kg/day for Neosugar (Clevenger et al., 1988; Tokunaga, Oku, & Hosoya, 1986). The No Observed Adverse Effect Level (NOAEL) for chronic administration of Neosugar is 2,170 mg/kg/day and the only effect noted was the occurrence of soft stools or diarrhoea after ingestion of large quantities of Neosugar (more than 5 percent in the diet of rats).

A few studies have been conducted to evaluate the tolerability and toxicity of oligofructose since the Carabin and Flamm review was published and they are summarized below. Four studies were conducted with Raftilose 95, including two toxicological studies that assessed the safety - an *in vitro* mutagenicity and an *in vivo* 13-week study in rats (Boyle et al., 2008). Two studies have been conducted to evaluate the effects of Raftilose 95 on faecal attributes, including an *in vivo* study in dogs conducted to evaluate nutrient digestibility, stool quality, and faecal protein catabolites (Propst, Flickinger, Bauer, Merchen, & Fahey, 2003) and an *in vivo* 2-week study in weanling pigs to evaluate the effects of various infant formulas, including Raftilose 95, on faecal attributes, including stool consistency, colour, odour, dry matter, organic matter, pH, and certain biochemical components (Barry et al., 2008).

The studies that have been conducted on scFOS (Neosugar) or other sources of scFOS, or oligofructose (Raftilose 95) are described below in separate sections to avoid confusion about the test material that was evaluated in each study. The studies are also summarized in Table 5, Table 6, Table 7 and Table 8.

2.3.3.1   In Vitro Toxicological Studies

2.3.3.1.1   In Vitro Toxicological Studies with scFOS (Neosugar)

An Ames assay with *Salmonella typhimurium* strains TA 1535, TA 1537, TA 1538, TA 98, and TA 100, and an *Escherichia coli* WP2 *uvr*A assay were conducted with Neosugar at concentrations of 50 to 5,000 µg/plate (Clevenger, et al., 1988)[5]. There was no increase in the frequency of mutations per plate in any bacterial strain with or without metabolic activation compared to controls. Therefore, under the conditions of both of these assays, scFOS did not possess mutagenic activity. In a mammalian cell mutation assay, mouse lymphoma L5178Y cells were exposed to 2,000 to 5,000 µg/ml of Neosugar for 3 hours in the presence or absence of an Aroclor-induced rat liver microsomal metabolic activation system (Clevenger, et al., 1988). Treatment with Neosugar did not produce an increase in the mutation frequency either with or without metabolic activation.

In an assay of DNA damage assessed by unscheduled DNA synthesis (UDS) in which HeLa S3 epithelioid cells were treated with Neosugar at concentrations ranging from 25 to 51,200 µg/ml, the percentage of cells undergoing DNA repair was quantified in the presence and absence of metabolic activation (Clevenger, et al., 1988). In the first of two experiments, UDS was significantly increased at the 1,600 µg/ml dose level, without metabolic activation. However, the single positive result occurred only at one dose level and no dose response was evident. Importantly, no significant increase was observed at any concentration either with or without metabolic activation in a repeat test. As shown in Table 5, in all three *in vitro* assays, Neosugar did not possess genotoxic potential under the conditions of the tests.

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[5]   Source of Neosugar was Meiji Seiki Kaisha, Ltd.; no other information provided about chemical composition

Table 5: *In Vitro* Toxicological Studies with scFOS			
Reference	Test Material	Study Design	Findings
Clevenger et al. 1988	Neosugar[a]	Ames Assay with *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537, and TA 1538, and an *Escherichia coli* WP2 uvrA assay in the presence or absence of an Aroclor-induced rat liver microsomal metabolic activation system at concentrations of 50 to 5,000 µg/plate	There was no increase in the frequency of mutations per plate in any bacterial strain with or without metabolic activation compared to controls
Clevenger et al. 1988	Neosugar	Mammalian cell mutation assay with mouse lymphoma L5178Y cells at concentrations of 2,000 to 5,000 µg/ml for 3 hours in the presence or absence of an Aroclor-induced rat liver microsomal metabolic activation system	No increase in the mutation frequency either with or without metabolic activation
Clevenger et al. 1988	Neosugar	Unscheduled DNA synthesis assay with HeLa S3 epithelioid cells treated with Neosugar at concentrations of 25 to 51,200 µg/ml in the presence or absence of an Aroclor-induced rat liver microsomal metabolic activation system	In the first of two assays, UDS significantly increased at 1,600 µg/ml, without metabolic activation (no dose response). No increase observed at any concentration either with or without metabolic activation in a repeat test.
[a]Neosugar from Meiji Seika Kaisha Ltd, Japan; no other information about chemical composition provided by Clevenger et al. 1988.			

2.3.3.1.2   In Vitro Toxicological Studies with Oligofructose (Raftilose 95)

An *in vitro* study was conducted to evaluate the ability of Raftilose 95 to induce reverse mutations in *S. typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and in *E. coli* tester strain WP2 *uvr*A in the presence and absence of rat liver S9 activation (Boyle, et al., 2008)[6]. Five dose levels were evaluated (100, 333, 1,000, 3,333, and 5,000 µg/plate) in triplicate. Raftilose did not induce a positive response at levels up to 5,000 µg/plate in the presence and absence of S9 activation with any of the tester strains used.

The clastogenic potential of Raftilose 95 was evaluated based upon its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells in both the absence and presence of an Aroclor-induced S9 activation system (Boyle, et al., 2008). Doses tested included 313, 625, 1,250, 2,500, and 5,000 µg/mL and in the non-activated test system, treatment was for 4 h and for 20 h; in the S9 activated

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[6] Raftilose 95; Orafti, Tienen, Belgium is derived from chicory root inulin by enzymatic hydrolysis; contained 95% dry matter, and composed of glucose and fructose (0.1%), sucrose (5.6%) and oligofructose (94.3%)

test system, treatment time was for 4 h. A confirmatory assay was conducted by exposing CHO cells to Raftilose at dose levels of 1,250, 2,500, or 5,000 µg/mL, and to positive and solvent controls continuously for 20 h in the absence of S9 activation only. The clastogenic potential of Raftilose was measured by its ability to increase structural aberrations in a dose–responsive manner compared to the solvent control group. Raftilose was also evaluated for its ability to induce numerical chromosome aberrations. In the initial and confirmatory assay there was no toxicity observed at any dose level tested in both the absence and presence of S9 activation as measured by a >50% reduction in cell growth relative to the solvent control. The dose levels selected for evaluation of chromosomal aberrations in all treatment groups of the initial assay were 1,250, 2,500, and 5,000 µg/mL. No statistically significant increases in structural or numerical chromosome aberrations, relative to the solvent control, were observed in the non-activated or S9 activated groups, regardless of dose level.

As shown in Table 6, Raftilose 95 did not induce any positive responses in the Ames test, and the *in vitro* chromosomal aberration test showed no clastogenic effects. In several studies cited by Boyle et al. (2008), various fructooligosaccharides do not have any genotoxic or carcinogenic effects (Crittenden & Playne, 1996; Delzenne, 2003; Spiegel, Rose, Karabell, Frankos, & Schmitt, 1994). In humans and animals, dietary oligosaccharides suppress faecal activities of carcinogen-metabolizing enzymes (Boyle, et al., 2008). Colonic butyrate generated from oligofructose fermentation may also be Antimutagenic (Kaur & Gupta, 2002; Kleessen, Hartmann, & Blaut, 2001).

Table 6: *In Vitro* Toxicological Studies with Oligofructose			
Reference	**Test Material**	**Study Design**	**Findings**
Boyle et al. 2008	Raftilose 95[a]	Ames Assay with *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, and TA 1537 and *E. coli* tester strain WP2 *uvr*A in the presence and absence of rat liver S9 activation at concentrations of 100 to 5,000 µg/plate	No positive responses with or without metabolic activation
		Clastogenic potential evaluated using Chinese hamster ovary cells in the presence or absence of Aroclor-induced S9 at concentrations of 1,250 to 5,000 µg/ml. Cells were also evaluated for numerical chromosome aberrations.	No positive responses with or without metabolic activation; no numerical chromosome aberrations
[a]Raftilose 95 from Orafti, Tienen, Belgium – derived from chicory root inulin by enzymatic hydrolysis; contained 95% dry matter, and composed of glucose and fructose (0.1%), sucrose (5.6%) and oligofructose (94.3%)			

2.3.3.2   In Vivo Toxicological Studies

2.3.3.2.1   In Vivo Toxicological Studies with scFOS (Neosugar)

Takeda and Niizato (1982a) conducted an acute single dose study and three 6-week studies of scFOS (Neosugar). In the acute study, Neosugar[7] was administered by gavage to 4-week-old male and female JcL-IcR mice and 6-week-old male and 10-week-old female Sprague-Dawley rats in four groups of 6 (a total of 24 male and 24 female mice and 24 male and 24 female rats). No deaths, gross abnormalities, or changes in body weight were observed in mice given single oral doses of 3, 6, or 9 g/kg Neosugar and were observed for 7 days. The LD_{50} was determined to be greater than 9 g/kg bodyweight in mice and rats (Takeda & Niizato, 1982a).

In a 6-week gavage study, groups of 18 8-week-old male Wistar rats were administered daily doses of 1.5, 3, and 4.5 g/kg Neosugar or Neosugar G[8], while sucrose and glucose were the control substances (Takeda & Niizato, 1982b). Neosugar G contains a higher percentage of glucose than Neosugar, so it is not relevant to this safety assessment. On the 2nd, 4th, and 6th weeks, blood samples were obtained from 6 animals in each group. At sacrifice, liver, pancreas, adrenal glands, kidneys, brain, cerebellum, heart, lungs, spleen, pituitary gland, and testes were removed and evaluated for histopathology. There were no abnormalities or deaths during the study. A slight non-significant decrease in body weight was observed in the 3 and 4.5 g/kg Neosugar groups compared to controls. No consistent, treatment-related changes were seen in clinical chemistries. Distension of the caecum was observed in 4 rats in the 4.5 g/kg Neosugar treatment group at 4 weeks. It was concluded that there was no treatment-related toxicity at doses up to 4.5 g/kg Neosugar for 6 weeks.

A 6-week feeding study was conducted with groups of 18 6-7 week-old Male Wistar rats that were administered 5 or 10% Neosugar, 5 or 10% Neosugar G, or one of three control saccharides (sucrose, glucose, or sorbitol) in their diet *ad libitum* (Takeda & Niizato, 1982b). Blood samples were collected at the 2nd, 4th, and 6th weeks and immediately after collection of blood samples, the liver, adrenal, and pancreas were removed from some animals. At sacrifice (6th week), kidney, cerebrum, cerebellum, heart, lung, spleen, and testes were also obtained and examined histologically. Results revealed no treatment-related abnormalities or deaths. The sorbitol and Neosugar groups had lower body weights in the 1st to 5th week, but the growth trends

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[7] The composition of the Neosugar in the Takeda and Niizato 1982a and 1982b studies was trace of GF, 30.9%GF_2, 55% GF_3 and 14.1% GF_4 - from Meiji Seika Kaisha Ltd.

[8] The composition of the Neosugar G in the Takeda and Niizato 1982b and 1984 studies was 38%GF, 11% sucrose, 21% GF_2, 24% GF_3 and 6% GF_4 - from Meiji Seika Kaisha Ltd.

near completion of the study were similar to controls. Cholesterol levels of the Neosugar and Neosugar G groups were lower than control groups (no information on statistical significance provided). There were no significant treatment-related microscopic findings. Diarrhoea was observed in rats in the sorbitol control group on day 3 and in rats treated with Neosugar on about day 10 (no additional information was provided such as number of rats, dose group, duration of effect). Distension of the caecum was reported in the Neosugar and sorbitol control groups at 2 weeks and in the Neosugar, Neosugar G, and sorbitol control groups at 6 weeks (no other information was provided). The investigators concluded that the caecum distension was not treatment related because the effect was observed in the controls. It was concluded that there was no treatment-related toxicity at doses of up to 10% Neosugar in the diet for 6 weeks.

An 8-week study was conducted with rats to determine the effect of Neosugar on body weight gain, organ weight, serum lipids, faecal excretion and intestinal function (Tokunaga, et al., 1986). Groups of 6 Male Wistar rats (6-8 wks old) were administered either 10 or 20% Neosugar[9] (approximately 6 and 13 g/kg/day, respectively) in their diets. Rats developed diarrhoea after starting the Neosugar-containing diets, but this was not observed after 2 to 3 weeks. After 6 weeks of treatment, the body weight gain of the group receiving the 20% Neosugar diet was statistically significantly lower compared to the control group; however, there was no significant effect on body weight gain in the 10% Neosugar diet group. Daily food intake was similar in control and Neosugar-fed groups. Rats in both treatment groups exhibited a statistically significant increase in both wet weight and the ratio of caecum to colon weights. A greater effect was observed in the caecum than in the colon of rats in the 20% Neosugar group. The wet weight of the small intestine was statistically significantly increased in 20% Neosugar group but not the 10% Neosugar group. No significant increases in liver or kidney weight were observed and faecal wet weight was also statistically significantly increased in both treatment groups. Serum cholesterol levels were slightly but not statistically significantly reduced in the 20% Neosugar group. In addition, the serum triacylglycerol levels were statistically significantly decreased by Neosugar intake in both groups (however, the effect was greater in the 20% group). Faecal excretion of sterol is increased significantly by chronic intake of dietary fiber through the inhibition of intestinal cholesterol and bile acid absorption. A statistically significant increase in excretion of neutral sterols and acidic sterols was seen in both treatment groups. The concentration of volatile fatty acids per gram of wet faeces greatly increased in rats fed both dose levels of Neosugar; the greatest increase was in acetic acid, followed by propionic acid. The

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[9] The composition of the Neosugar from Meiji Seika Kaisha Ltd. was 28% GF_2, 60% GF_3 and 12% GF_4

results of this study suggest that Neosugar is not fully utilized by rats as an energy source and that the indigestible or unabsorbable nature of scFOS produced diarrhoea and a bulking effect in the intestinal lumen. The faecal volume was increased and intestinal transit time was shortened, similar to actions of a dietary fiber. In conclusion, no adverse effects of up to 20% (13 g/kg/day) Neosugar in the diet were observed in rats. The diarrhoea observed in rats fed the 10 or 20% diets stopped within 2-3 weeks.

In a chronic/carcinogenicity study in Fischer 344 rats (50/sex/group), Neosugar[10] was administered in diets at concentrations of 0, 8,000, 20,000, or 50,000 ppm (equivalent to approximately 341, 854, and 2,170 mg/kg/day, respectively, for male rats and 419, 1,045, and 2,664 mg/kg/day, respectively, for female rats) for 104 weeks (Clevenger, et al., 1988). No dose-related effects were noted on survival, growth, food consumption, feed efficiency, hematology parameters, blood chemistry parameters, organ weights, and non-neoplastic lesions. The incidence of rare and spontaneous tumours was comparable between control and Neosugar treated groups with the exception of pituitary adenomas in male rats. The increased incidence of this tumour was not considered to be treatment-related, however, because the incidence in all groups was within the historical control range of this spontaneous tumour and there was only equivocal evidence of a dose-response trend. The Cochran-Armitage trend test indicated a significant trend but logistic regression analysis indicated no dose-response relationship. Non-neoplastic lesions common in aging rats were observed in all groups, including controls. Neosugar did not affect the severity of lesions. Renal protein casts of moderate severity were found in 0, 4, 7, and 6 rats in the control, 8,000, 20,000, or 50,000 ppm groups. This finding lacked biological significance and relevance to the safety assessment of Neosugar, as renal protein casts are not uncommon in older male rats and there was no dose-response for this finding. For all other non-neoplastic lesions with differences from control values, a comparison to the historical control incidence revealed that the various lesions were common and highly variable in incidence in aging Fischer 344 rats. Consideration of the absence of dose-related effects and similar findings in historical control animals led to the conclusion that treatment with Neosugar did not affect the incidence of non-neoplastic lesions.

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[10] The composition of Neosugar evaluated in the Clevenger et al. 1988 study was 37% GF_2, 51% GF_3, 12% GF_4 from Mie Kariyo Co, Japan

Table 7: *In Vivo* Toxicological Studies with scFOS			
Reference	**Test Material**	**Study Design**	**Findings**
Takeda and Niizato 1982a	Neosugar	Single dose of 3, 6, or 9 g/kg administered by gavage to 4-week-old male and female JcL-IcR mice and 6-week-old male and 10-week-old female Sprague-Dawley rats in four groups of 6 (a total of 24 male and 24 female mice and 24 male and 24 female rats).	No deaths, gross abnormalities, or changes in body weight were observed in mice that were observed for 7 days. The LD_{50} was determined to be greater than 9 g/kg bodyweight in mice and rats.
Takeda and Niizato 1982b	Neosugar	6-week gavage study - groups of 18 8-week old male Wistar rats administered daily doses of 1.5, 3, and 4.5 g/kg Neosugar or Neosugar G; sucrose and glucose were the control substances. On the 2nd, 4th, and 6th weeks, blood samples obtained from 6 animals in each group. At sacrifice, liver, pancreas, adrenal glands, kidneys, brain, cerebellum, heart, lungs, spleen, pituitary gland, and testes were removed and evaluated for histopathology.	No abnormalities or deaths during the study. Slight decrease in body weight in the 3 and 4.5 g/kg groups compared to controls. No consistent, treatment-related changes in clinical chemistries. Distension of the caecum in 4 rats in the 4.5 g/kg Neosugar treatment group at 4 weeks. No treatment-related toxicity at doses up to 4.5 g/kg for 6 wks.
Takeda and Niizato 1982b	Neosugar	6-wk feeding study with groups of 18 male Wistar rats (ages 6-7 weeks) administered 5 or 10% Neosugar, 5 or 10% Neosugar G, or one of three control saccharides (sucrose, glucose, or sorbitol) in their diet *ad libitum*. Blood samples were collected at the 2nd, 4th, and 6th weeks; immediately after collection of blood samples, liver, adrenal, and pancreas were removed from some animals. At sacrifice at 6 weeks, kidney, cerebrum, cerebellum, hear, lung, spleen, and testes were obtained and examined histologically.	No treatment-related toxicity at doses of up to 10% in the diet for 6 weeks.

Table 7: *In Vivo* Toxicological Studies with scFOS			
Reference	**Test Material**	**Study Design**	**Findings**
Tokunaga et al. 1986	Neosugar	8-week study with 6 male Wistar rats per group (6-8 wks old) to determine the effect of Neosugar on body weight gain, organ weight, serum lipids, faecal excretion and intestinal function. Rats were administered either 10 or 20% Neosugar (approximately 6 and 13 g/kg/day, respectively) in their diets.	Rats developed diarrhoea after starting the Neosugar-containing diets, but this was not observed after 2 to 3 weeks. After 6 weeks of treatment, the body weight gain of the 20% group stat. significantly lower than control group; no significant effect on body weight gain in the 10% group. Daily food intake similar in control and Neosugar-fed groups. Rats in both treatment groups exhibited a statistically significant increase in both wet weight and the ratio of caecum to colon weights. Weight of the small intestine was stat. significantly increased in 20% Neosugar group. No significant increases in liver or kidney weight were observed. Faecal wet weight stat. significantly increased in both treatment groups. Serum cholesterol levels slightly reduced in the 20% group. Triacylglycerol levels stat. significantly decreased in both groups. Concluded no adverse effects of up to 20% (13 g/kg/day) Neosugar in the diet observed in rats.
Clevenger et al. 1988	Neosugar	Carcinogenicity study in Fischer 344 male and female rats (50/group) treated with dietary concentrations of 0, 8,000, 20,000, or 50,000 ppm (equivalent to approximately 341, 854, and 2,170 mg/kg/day, respectively, for male rats and 419, 1,045, and 2,664 mg/kg/day, respectively, for female rats) for 104 weeks.	No effects on survival, growth, food consumption, feed efficiency, haematology parameters, blood chemistry parameters, organ weights, and non-neoplastic lesions. The incidence of rare and spontaneous tumours was comparable between control and treated groups with the exception of pituitary adenomas in male rats. The increased incidence of this tumour was not considered to be treatment-related because the incidence in all groups was within the historical control range of this spontaneous tumour.

The composition of the Neosugar in the Takeda and Niizato 1982a and 1982b studies was trace of GF, 30.9% GF_2, 55% G_3 and 14.1% GF_4 - from Meiji Seika Kaisha Ltd.
The composition of the Neosugar in the Tokunaga et al. 1986 study was 28% GF_2, 60% GF_3 and 12% GF_4 - from Meiji Seika Kaisha Ltd
The composition of the Neosugar in the Clevenger et al. 1988 study was 37% GF_2, 51% GF_3, and 12% GF_4 from Meiji Seika Kaisha Ltd

2.3.3.2.2   In Vivo Toxicological Studies with Oligofructose (Raftilose)

Developmental and reproductive toxicity of Raftilose[11] was investigated rats treated with dietary concentrations of up to 20% (Henquin, 1988; Sleet & Brightwell, 1990)[12]. Henquin (1988) reported the results of a developmental toxicity study in 12 pregnant Wistar rats fed a diet containing 20% Raftilose during gestational days 1-21 and throughout lactation. They concluded that a diet containing 20% Raftilose has no significant effects on the course of pregnancy in rats or on the development of the foetuses and newborns. In the study by Sleet and Brightwell (1990), pregnant rats were pre-treated with 4.75% Raftilose in the diet on days 0 to 6 postcoitum, were then fed 5, 10, or 20% Raftilose from day 6 to day 15 postcoitum, and were sacrificed on day 20. No diarrhoea was observed in any of the test animals and no deaths were recorded. Treatment with Raftilose had no effect on the number of pups/litter, sex ratio, or viability of embryos and foetuses. Litter and foetal weights were not reduced; foetal weight of the 20% dose group was statistically significantly greater than that of the controls. Structural development of the foetuses was unremarkable. The investigators concluded that dietary supplementation with Raftilose at concentrations up to 20% did not negatively affect the pregnancy outcome or *in utero* development of rats.

A 13-week dietary study was conducted by Boyle et al. (2008) to evaluate the safety of Raftilose 95 in Sprague Dawley rats[13]. Groups of 40 male and female 7-week old rats were administered test diets that contained 0, 0.55, 1.65, 4.96, or 9.91% Raftilose *ad libitum*. All rats were observed for mortality, gross motor and behavioural activity and changes in appearance. Food consumption and body weight were measure daily from week -1 to week 13. Water consumption was measured daily for 3 consecutive days during weeks 1, 6, and 12. Blood samples were obtained during weeks -1, 1, 6, and 13 to measure several clinical chemistry and haematological parameters. Complete necropsies were performed and representative samples from each animal's organs and tissues were evaluated histologically. The bifidobacterial content of the faecal samples was also determined. Food consumption was significantly lower in animals fed the two highest doses; there were no toxicologically relevant clinical observations or changes in haematological parameters; total, HDL, and LDL cholesterol levels were significantly lower at several time points in animals fed Raftilose, particularly in male

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[11] Carabin and Flamm (1999) refer to the test substance as FOS, however, it appears that the studies were likely conducted with oligofructose (Raftilose).

[12] These are internal unpublished studies: Henquin, J. C. (1988). Reproduction Toxicity: Study on the Influence of Fructooligosaccharides on the Development of Fetal and Post-natal Rat. Raffinerie Tirlemontoise Internal Report.
Sleet R. and J. Brightwell. (1990). FS-Teratology Study in Rats. Raffinerie Tirlemontoise Internal Report.

[13] Raftilose 95 (Orafti, Tienen, Belgium) was composed of glucose and fructose (0.1%), sucrose (5.6%) and oligofructose (94.3%)

rats fed the highest levels. Lipid-lowering effects of oligofructose have been demonstrated in animals and humans by several investigators (Beylot, 2005). Boyle et al. (2008) concluded, that the NOAEL for oligofructose in the diet is 9.91%.

Propst et al. (2003) conducted a study to evaluate the effects of oligofructose and inulin on nutrient digestibility, stool quality, and faecal protein catabolites in adult dogs[14]. Dogs were administered gelatine capsules with doses of 1.5, 3, or 4.5 g/day for 98 days, to achieve doses equivalent to dietary concentrations of 0, 0.3, 0.6, or 0.9% Raftilose. Supplementation of Raftilose in dogs resulted in positive effects on indices known to be associated with gut health (e.g., nutrient digestibility, stool quality) in dogs consuming a meat-based diet. No decreases in ileal nutrient digestibility or negative effects on faecal volume or faecal score resulted from treatment with Raftilose. The authors concluded that beneficial effects were observed in dogs administered a dose of Raftilose equivalent to 0.9% in the diet; no adverse effects were reported.

Barry et al. (2008) conducted a study to evaluate the effects of ingredients of several commercially available infant formulas on faecal characteristics in 7-9 weanling pigs (14-17 days old) per group. Dietary treatments included a whey-dominant powdered diet with α-lactalbumin and 3.0 g/L Raftilose 95 (also known as "Beneo[®]") that was provided for 2 weeks *ad libitum* after reconstitution with water. The faecal characteristics and biochemical parameters that were evaluated included consistency, colour, odour, dry matter, organic matter, pH, biogenic amines, short and branched-chain fatty acids, phenols, indoles, and ammonia. There were no differences in the faecal characteristics between the piglets that ingested formula containing Raftilose or the control (whey formula) piglets. The authors concluded that faecal attributes observed in the piglets were consistent with normal, healthy gut function. Faecal consistencies were similar among all groups with piglets from all treatments producing faeces with an average score of soft (faecal dry matter at day 14 was 35.5% in the whey control group compared to 38.2% in the Raftilose treated group). The authors concluded that the use of an infant formula with the addition of Raftilose would produce faecal consistencies and attributes similar to formulas currently available to consumers, and would result in normal, healthy gut function in human infants.

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[14] Oligofructose - Raftilose P95 (Orafti, Tienen, Belgium), and inulin (Raftiline HPF; Orafti)

Table 8: *In Vivo* Toxicological Studies with Raftilose			
Reference	**Test Material**	**Study Design**	**Findings**
Henquin 1988 (unpublished study; described in Carabin and Flamm 1999)	Raftilose*	Developmental and reproductive toxicity of Raftilose with 12 pregnant Wistar rats treated with a dietary concentration of 20% during gestational days 1-21 and throughout lactation	Diet containing 20% Raftilose had no significant effects on the course of pregnancy in rats or on the development of the foetuses and newborns.
Sleet and Brightwell 1990 (unpublished study; described in Carabin and Flamm 1999)	Raftilose*	Developmental and reproductive toxicity with pregnant Crl CD (SD) BR rats pre-treated with 4.75% Raftilose in the diet on days 0 to 6 postcoitum and then fed 5, 10, or 20% Raftilose from day 6 to day 15 postcoitum and sacrificed on day 20.	No diarrhoea was observed in any of the test animals and no deaths. No effect on number of pups/litter, sex ratio, and viability of both the embryo and foetus. Litter and foetal weights were not reduced; foetal weight of the 20% dose group was statistically greater than controls. Structural development of the foetuses was unremarkable. Dietary supplementation with Raftilose at concentrations up to 20% did not negatively affect the pregnancy outcome or *in utero* development of rats.

Table 8: *In Vivo* Toxicological Studies with Raftilose			
Reference	**Test Material**	**Study Design**	**Findings**
Boyle et al. 2008	Raftilose 95	13-week dietary study to evaluate the safety of Raftilose 95 in SD rats. Groups of 40 male and female 7-week old rats were administered test diets that contained 0, 0.55, 1.65, 4.96, or 9.91%. All rats were observed for mortality, gross motor and behavioural activity, and changes in appearance. Food consumption and body weight were measure daily from week -1 to week 13. Water consumption was measured daily for 3 consecutive days during weeks 1, 6, and 12. Blood samples were obtained during weeks -1, 1, 6, and 13 to measure several clinical chemistry and haematological parameters. Complete necropsies were performed and representative samples from each animal's organs and tissues were evaluated histologically. The bifidobacterial content of the faecal samples was also determined.	Food consumption significantly lower in animals fed highest 2 doses; there were no toxicologically relevant clinical observations or changes in haematological parameters; total, HDL, and LDL cholesterol levels were significantly lower at several time points treated groups, esp. male rats fed the highest 2 doses. Concluded, that the NOAEL is 9.91%.
Propst et al. 2003	Raftilose	3-month study to evaluate the effects of Raftilose and inulin on nutrient digestibility, stool quality, and faecal protein catabolites in adult dogs. Dogs were administered gelatine capsules with doses of 1.5, 3, or 4.5 g/day, to achieve dietary concentrations equivalent to 0, 0.3, 0.6, or 0.9% Raftilose in the diet for 98 days.	Treatment resulted in positive effects on indices known to be associated with gut health in dogs consuming a meat-based diet. No decreases in ileal nutrient digestibility or negative effects on faecal volume or faecal score. Concluded that beneficial effects observed in dogs administered the dose of Raftilose equivalent to 0.9% in the diet; no adverse effects were reported.

Table 8: *In Vivo* Toxicological Studies with Raftilose			
Reference	**Test Material**	**Study Design**	**Findings**
Barry et al. 2008	Raftilose	2-week study to evaluate the effects of ingredients of several commercially available infant formulas on faecal characteristics in 7-9 weanling pigs (14-17 days old) per group. Dietary treatments included a whey-dominant powdered diet with α-lactalbumin and 3.0 g/L Raftilose 95 provided *ad libitum.* Faecal characteristics and biochemical parameters that were evaluated included consistency, colour, odour, dry matter, organic matter, pH, biogenic amines, short and branched-chain fatty acids, phenols, indoles, and ammonia.	No differences in the faecal characteristics between the piglets that ingested formula containing Raftilose or the control (whey formula) piglets and healthy gut function were attained in the weanling piglets consuming the experimental treatments. Faecal consistencies were similar among all groups with piglets from all treatments producing faeces with an average score of soft. Faecal attributes were consistent with normal health gut function in piglets.

2.3.3.2.3   *In Vivo Studies with Other Sources of FOS*

Anthony et al. (2006) conducted a 90-day oral gavage study with 6-week-old male and female rats treated with reverse osmosis deionised water (RODI), 2,500 or 5,000 mg/kg bw/day Vivinal® GOS syrup (45% GOS), or with FOS as a reference control (Mead Johnson Nutritionals – no other information about the formulation or source of FOS available). The FOS reference control was a mixture of 70% w/v FOS and 30% w/v lactose and glucose mixed with water. Although the dose level was not provided for FOS, the dose volume was 7.8 ml/kg bw, similar to the dose volume of the high dose GOS group at 5,000 mg/kg/day (the dose volume of the low dose GOS group was 3.9 ml/kg bw). Therefore, the dose of FOS was likely 5,000 mg/kg bw/day in this study. Body weights and food consumption were recorded on day 1 and weekly thereafter. Clinical chemistry and haematological parameters were evaluated along with urinalysis on day 90. Gross necropsies were conducted and organ weights recorded at the end of the study. Mean food consumption of the FOS control group and the high dose GOS group was significantly lower than the RODI control group. The occasional statistically significant haematological and clinical chemistry evaluations (e.g., mean eosinophil value for the FOS control group was higher than RODI control and mean glucose value was statistically lower for the FOS control males compared to the RODI water control group) were considered minor, inconsistent, not related to dose, and within the intra-laboratory historical control values. The NOAEL for GOS was reported

to be 5,000 mg/kg bw/day (the highest dose tested). Although this study was not designed to specifically evaluate the effects of FOS, the results provide support for a lack of adverse effects associated with FOS and a possible NOAEL of 5,000 mg/kg bw/day (Anthony, Merriman, & Heimbach, 2006).

2.3.3.3   Effects of scFOS or Oligofructose on Intestinal Barrier Function

In contrast to the potentially protective effects on faecal microflora in animals and humans consuming β-2-fructans observed in many studies, a single group of investigators have reported an increased risk for translocation of *Salmonella* in inoculated Wistar rats consuming Raftilose 95 or long chain inulin (Frutafit®, Sensus) in combination with a low-calcium diet (Bovee-Oudenhoven, Ten Bruggencate, Lettink-Wissink, & van der Meer, 2003; Ten Bruggencate, Bovee-Oudenhoven, Lettink-Wissink, Katan, & van der Meer, 2004; Ten Bruggencate, Bovee-Oudenhoven, Lettink-Wissink, & Van der Meer, 2003, 2005). Groups of 8 rats were fed restricted diets containing 3 g/100 g chow (3%) or 6 g/100 g (6%) of Raftilose for 2 weeks. After 2 weeks, the rats were orally inoculated with the pathogen *Salmonella enteriditis* and after 9 days, the rats were euthanized. Treatment with Raftilose was associated with faecal water cytotoxicity as tested *in vitro* with a suspension of red blood cells, and faecal mucin and urinary excretion of nitrate and nitrite were increased in rats consuming the β-2-fructans and low calcium diets (20 or 30 mmol $CaHPO_4$.$2H_2O$/kg diet). In animals consuming higher levels of calcium (100 mmol $CaHPO_4$.$2H_2O$/kg diet), however, these effects were not observed (Ten Bruggencate, et al., 2004). In a subsequent study (Ten Bruggencate, et al., 2005), intestinal permeability was observed to decrease in animals consuming a low-calcium diet and Raftilose. The effects of Raftilose or inulin on intestinal permeability and bacterial translocation in rats reported by Ten Bruggencate appear to be related to the low calcium diets (Guarner, 2007).

The same investigators also examined measures of intestinal barrier function in 34 healthy men consuming 20 g Raftilose 95 in combination with diets containing approximately 300 mg calcium (a low calcium diet) (Ten Bruggencate, Bovee-Oudenhoven, Lettink-Wissink, Katan, & van der Meer, 2006). In this placebo-controlled crossover study, Raftilose had no effects on faecal water toxicity or intestinal permeability. The authors concluded that consumption of Raftilose increased flatulence and intestinal bloating and doubled faecal mucin excretion, indicating mucosal irritation. However, Raftilose did not affect the cytotoxicity of faecal water and intestinal permeability and the increase in mucin excretion suggests mucosal irritation in humans, but the overall effects were more moderate than those in rats (Ten Bruggencate, et al., 2006).

These studies should be carefully evaluated, as there are some methodological issues to consider when drawing conclusions about the reported findings. Ten Bruggencate et al. (2005) stated "caution must be exercised when extrapolating findings from these animal experiments to humans because the intestinal permeability of rats is higher than that of humans." In addition, cytotoxicity of faecal water is usually tested *in vitro* using intestinal epithelial cells rather than red blood cells, which may be too susceptible to changes in pH by SCFA, whereas intestinal epithelial cells normally use organic acids as an energy source (Guarner, 2007). Translocation of gut bacteria is normally demonstrated by the detection of viable bacteria in mesenteric lymph nodes rather than by the urinary excretion of nitrites and nitrates. Faecal mucin excretion is not necessarily a marker of mucosal irritation; mucins are secreted in response to irritation as a repair mechanism, but other functions of mucins are lubrication to improve transit and nutrition of commensal bacteria (Hooper, Midtvedt, & Gordon, 2002). Fibres increase mucin secretion for lubrication purposes, and this is not associated with a deranged barrier function, but, on the contrary, deficiency of dietary fiber results in colonic mucosal fragility (Strugala, Allen, Dettmar, & Pearson, 2003).

Based on these studies, the available evidence in humans does not indicate that consumption of inulin or Raftilose as part of a balanced diet has any adverse effects on intestinal permeability, faecal water cytotoxicity, faecal ALP activity, the faecal concentration of mucin-type oligosaccharides, or presents an increased risk for bacterial translocation in humans.

2.4 Clinical Studies of scFOS or Oligofructose Fed to Infants and Toddlers

One of the primary reasons given by FSANZ for the exclusion of scFOS in Application P306 (Food Standards Australia New Zealand (FSANZ), 2008a, 2008b) was scFOS had not been sufficiently studied in infants and young children to allow any conclusions to be made regarding the physiological effects of scFOS. Furthermore FSANZ expressed concerns related to the potential for adverse effects on water balance, based on concerns raised previously during early reviews in the EU (Scientific Committee on Food, 2001a, 2001b; Scientific Panel on Dietetic Products Nutrition and Allergies, 2004). Those issues raised by the EU committees related to both categories of resistant short chain oligofructans, scFOS and oligofructose, and did not differentiate between the two compounds. The 2008 FSANZ review found that there was sufficient data to address the concerns regarding water balance for oligofructose, but not scFOS (Food Standards Australia New Zealand (FSANZ), 2008a, 2008b).

This section will present a number of clinical trials, which have been conducted to assess the suitability and potential benefits of scFOS or oligofructose (which are substantially equivalent) in the diets of infants and toddlers; findings from many of these studies have been reported in the peer-reviewed, published literature. In Japan, scFOS has been, and continues to be used extensively in infant formula and foods for young children, for many years, with the acceptable intake level of 4.2 g scFOS/day determined for infants less than one year of age based primarily on published safety and tolerance information from a large nationwide survey of infants in Japan (Yamamoto & Yonekubo, 1993). In that survey of 20,742 infants (up to 4.5 months of age), physical growth, nutritional intake, faecal properties and general health parameters for both breast milk- and infant formula-fed infants were reported. No adverse effects were reported for any of the health parameters surveyed. Based on the concentration of scFOS in infant formula in Japan at the time of the survey (3.2 g scFOS/L), infants were estimated to consume a mean and 90th percentile intake of 3.0 and 4.2 g/day of scFOS, respectively. A systematic review of randomized controlled trials evaluating the efficacy and safety of prebiotic supplementation in full-term infants (Rao, et al., 2009), made no differentiation of the oligofructan types in the selection criteria, including the 2006 study of Bettler and Euler (oligofructose supplemented) alongside other prebiotics. The conclusion of Rao et. al (2009) was that prebiotic supplemented formula is well tolerated by term neonates, and that it results in beneficial changes to bacterial microflora, and stool consistency that is similar to those of breastfed infants, without affecting weight gain. Several recent reviews of the roles of prebiotics in infant formula recognise scFOS as one of the range of prebiotics substances suitable for purpose (Braegger et al., 2011; Sherman et al., 2009)

In addition to currently published studies, Abbott Laboratories also has conducted several clinical studies in which scFOS was added to infant formula or milk-based beverages for older infants and toddlers. These studies have used commercially available oligosaccharide ingredients including scFOS or oligofructose, which are listed in Table 1

9. Results from this combined body of research on these ingredients provide information that can be used to assess the suitability of adding scFOS to infant formula and follow-on formula. Details of these studies, whilst submitted as Confidential Information at the time of this application, are intended for submission for publication in peer-reviewed journals, and conference presentations (Lasekan et al., 2010) in 2011 and beyond, at which time the published information becomes public.

2.4.1 scFOS or Oligofructose in Infant Formula

Several studies have been conducted in which the effects of scFOS or oligofructose in infant formula have been examined in the first weeks or months of life. Measures of faecal microflora and stool characteristics in these studies provide information on the potential benefits of ingestion, while measures of growth, tolerance, adverse events, and clinical chemistries provide evidence regarding the suitability of scFOS or oligofructose in infant formula consumed as a sole source of nutrition during the first months of life.

2.4.1.1   Abbott Nutrition Studies of scFOS in Infant Formula

Clinical trials have been conducted by Abbott Nutrition (USA) to assess the suitability and efficacy of scFOS in infant formula provided as a sole source of nutrition to term infants within their first four months of life (Table 9). Measures of faecal microflora and stool characteristics in these studies provide information on the potential benefits of ingestion, while measures of growth, tolerance, adverse events, and clinical chemistries provide evidence regarding the suitability of scFOS or oligofructose in infant formula consumed as a sole source of nutrition during the first months of life.

The source of the scFOS used in each of the studies was GTC Nutrition's NutraFlora P95, which is produced from the enzymatic activity of fructosyltransferase on sucrose. The studies include a two-week feeding intervention to assess the tolerance of formula containing scFOS and a 16-week study to assess growth of infants consuming formula containing scFOS (Abbott Laboratories, 2011d), a 4-week study to examine effects of the scFOS-supplemented formula on faecal microbiology (main study details submitted under Confidence), and a 35-day trial to assess the comparative gastrointestinal tolerance of soy-based infant formula containing scFOS (Abbott Laboratories, 2011a; Lasekan, et al., 2010).

Across all trials conducted by Abbott, where possible, measures were standardised. For example the ranking and scoring system for stool consistency was calculated as the mean rank stool consistency (MRSC). The mean rank stool consistency (MRSC) is calculated for each infant for each day by assigning a numerical value (rank) to each of the consistencies: watery=1; loose/mushy=2; soft=3; formed=4; and hard=5, multiplying the number of stools of each consistency with the numerical values, taking the sum and dividing by the total number of stools. The average of the daily mean ranks are computed to find the MRSC for the feeding period. The median MRSC is a summary statistic representative of the feeding group. The median MRSC for a feeding group is found by taking the middle ordered value of the MRSC of the infants in the group.

Abbott Nutrition follows the guidance developed by the American Academy of Pediatrics for the clinical testing of Infant Formulas with Respect to Nutritional Suitability for Term Infants. Based on early studies of infant growth, "the standard deviation of gain in weight on sex-specific and formula-specific basis for a 3-1/2 -month interval beginning during the first month of life is about 4.5 g/day. The number of subjects of a specified sex needed in each of two groups to detect a 3 g/day difference in weight gain ($p < 0.05$) with a power of 0.8 in a one- tailed test is therefore 28. If both sexes are studied, it will, of course, be necessary to take into account the sex-related difference in rate of gain" (Nelson, Rogers, Ziegler, & Fomon, 1989; USFDA Department of Health and Human Services, 1988).

When exclusively breast-fed infants are enrolled into a clinical trial, no distinction is made on how the infant is fed human milk (breast versus bottle). Furthermore, the studies did not consider if infants were firstborns. Beginning with the first feeding, parent(s) were asked to complete a Dietary Intake and Stool Record and record for each day until the next scheduled visit. At each study visit, study personnel provide verbal instructions on how to complete the forms, which contain written instructions and descriptions of stool categories. In addition, they are instructed to record characteristics (consistency, colour, and frequency) of their infant's stools, volume of formula consumed at each feeding, and incidence of spit-up and vomiting associated with feedings. The study personnel review completed forms at each study visit with the parent(s).

Table 9: Studies of scFOS in Infant Formula			
Reference (trial site)	Treatment Population Test Substance and Dose	Treatment Duration	Results of Ingestion*
(Abbott Laboratories, 2011d)	Term infants, 4-10 wk ± 3 d at recruitment Placebo-controlled, randomized, blinded, and parallel study Added to whey-enriched formula: • 1.5 g scFOS/L (n=22 ITT; 19 PP) • 3.0 g scFOS/L (n=20 ITT; 20 PP) • Control formula (n=21 ITT, 21 PP) Source: NutraFlora scFOS (GTC Nutrition Co.)	2 wk	Growth: -No effect on average weight gain/d or NCHS weight z-scores. Tolerance: -No effect on frequency and quantity of formula intake. -No effect on percent change from baseline in number of feedings with spit up and/or vomit. -No effect on stool frequency, ranked consistency, predominant consistency, color, and percent of stools with straining, odor, or gas. -Softer stool consistency during feeding period in all groups. Dropouts: -10 dropouts (scFOS0 = 1, scFOS1.5 = 5, scFOS3 = 4) -No difference in the proportion of treatment failures among groups; reasons include rejection of study formula (scFOS3 = 1) and adverse events (control = 1, scFOS1.5 = 2, scFOS3 = 3)—vomiting or spit up, diarrhoea, watery stools, fussiness, increased stool frequency, and weight loss. Microbiological Data: -No significant differences in bifidobacteria counts. -Clostridia counts decreased in the scFOS1.5 group compared to the control group. Other Endpoints: -No differences in AST or ALT plasma levels. -No ketones detected in urine before or after the feeding period. -Following the feeding period, GF_2 and GF_3 were detected in urine samples in a dose-dependent manner; not detected before FOS feeding, or in control group.

Table 9: Studies of scFOS in Infant Formula			
Reference (trial site)	Treatment Population Test Substance and Dose	Treatment Duration	Results of Ingestion*
(Abbott Laboratories, 2011d)	Term infants, 1-8 ± 3 d at enrollment Placebo-controlled, randomized, blinded, and parallel study Added to infant formula: • 3.0 g scFOS/L (n=50; 36 completed) • Control formula (n=52; 34 completed) • Human milk (n=25; 23 completed) Source: NutraFlora scFOS (GTC Nutrition Co.)	16 wk	Growth: -No differences in mean weight, length and head circumference; weight, length and head circumference gains; and weight, length and head circumference z-scores. Tolerance: -No difference in daily formula intake frequency and quantity, and caloric intake. -No difference in the percentage of feedings with spit up and/or vomit. -No difference in daily stool frequency except at d 28 when infants fed either of study formulas had a lower number of stools per day than infants fed HM. -Median stool consistency was harder for infants fed formulas compared to infants fed HM at d 28, 56, and 84. -Median rank stool consistency was lower in the scFOS3 group than in the control group at d28. -No difference in percent of stools with straining or dryness. Dropouts: -There was no statistical difference in the number of treatment failures between the formula groups. -Treatment failure (control = 6 and scFOS3 = 8). Adverse events: milk intolerance (control = 2 and scFOS3 = 4), colic (scFOS3 = 1), diarrhoea or watery stools (control = 2, scFOS3 = 1), constipation (control = 2, scFOS3 = 1), and gassiness (scFOS3 = 1). -A total of 20 protocol failures were reported (control = 12, scFOS3 = 6, HM = 2). Reasons: missed visits (control = 7, scFOS3 = 4, HM = 1), removed by investigator before study formula was started (control = 3, scFOS3 = 1), removed by investigator because of fever (control = 1), removed by parents due to failure to grow normally in absence of any organic symptom or other condition (scFOS3 = 1), consumed other foods (control =

Table 9: Studies of scFOS in Infant Formula			
Reference (trial site)	Treatment Population Test Substance and Dose	Treatment Duration	Results of Ingestion*
			1), unable to collect fecal specimens (HM = 1). Microbiological Data: - No differences between formula groups at any time points in fecal bifidobacteria, clostridia, and *C. difficile* counts. -No difference among the formula groups in changes in bifidobacteria, bacteroides, clostridia, and *C. difficile*. -At days 56, 84 and 112, lactobacilli counts and colonization rates were significantly higher in the scFOS3 group than in the control group. Other Endpoints: -No difference in plasma levels of AST and ALT. -No difference in indican/creatinine values. -Mean cholesterol levels lower in the scFOS group compared to the HM, but the same as the scFOS-free formula group. -Ketones were not detected in any urine samples. -GF_2 and GF_3 were not detected in any plasma samples. -GF_2 and GF_3 detected primarily in urine samples in the scFOS formula group.

2.4.1.1.1 Effects of Infant Formula Containing scFOS on Tolerance and Faecal Microbiota During 2 Weeks of Ingestion

Sixty-six infants were randomized to receive a whey-enriched infant formula containing 1.5 g scFOS/L, 3.0 g scFOS/L, or no added scFOS for a period of two weeks in this controlled trial designed to assess tolerance of and effects of scFOS on faecal microbiota (Abbott Laboratories, 2011d) . Infants were 4-10 weeks (± 3 days) old at recruitment. In the two weeks prior to the intervention period, all infants consumed a commercially available formula (Similac with Iron, SWI). Body weight was recorded at study entry and at the beginning and end of the two-week feeding intervention. At the beginning and end of the intervention period, faecal and urine samples were collected for analysis of microflora and scFOS, respectively. At the end of the study, blood samples were collected from a subsample of infants for analysis of scFOS, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). In both urine and blood, scFOS was assessed as ketose (GF_2) and nystose (GF_3). Throughout the baseline and intervention periods, parents kept daily records of formula intake, stool patterns (frequency, consistency, gas, odor, straining, color), and incidence of spit-up and vomiting.

A total of 63 infants entered the intervention period, with 22 infants in the 1.5 g scFOS/L group, 20 infants in the 3.0 g scFOS/L group, and 21 infants in the control group. All infants were full term, with a mean gestational age of 40 weeks per group. Infants in the 3.0 g scFOS/L group were older at study entry (49 ± 3 days) versus infants in the 1.5 g scFOS/L group (41 ± 3 days) or control group (39 ± 2 days). There were no differences in other demographic variables at study entry. The proportion of treatment failures did not differ significantly among the groups: 1 of 21 infants in the control group had vomit or spit up with every feeding and stools were watery; 2 of 19 infants (excluding the 3 protocol failures) in the 1.5 g scFOS/L group experienced intolerance on the study formula (excessive diarrhoea for 4 days and weight loss, or watery stools with each feeding), and 4 of 20 infants in the 3.0 g scFOS/L group experienced intolerance on the study formula (1 infant was gassy, fussy and rejected formula; and parents of 3 infants complained of infant diarrhoea, fussiness, excessive spit up and vomiting). All infants improved when they were switched to the control formula, SWI.

Measures of infant growth, formula intake, and tolerance are shown in Table 10 and Table 11. The frequency of feedings and the average formula intake per day were similar among the three groups during the intervention period. Volume and caloric intake based on body weight did not differ between the formula groups when adjusted

for gender (data not shown). There were no differences among the groups in average weight, weight gain per day, weight z-scores (data not shown), or percent change from baseline in number of feedings with spit up or vomit. Stool consistency was softer during the feeding period compared to baseline in all formula groups. Stool consistency, frequency, and predominant consistency were comparable between all groups, as were colour and percent of stools with straining, odour, or gas (data not shown).

Results of plasma analysis of liver enzymes and urinary analysis of scFOS are shown in Table 12. Mean plasma concentrations of AST and ALT were normal and did not differ among the formula groups. Following consumption of the 1.5 g scFOS/L formula for two weeks, GF_2 was detected in 4 of 6 samples and GF_3 was detected in 5 of 6 samples, while all urine samples collected from infants consuming 3.0 g scFOS/L were found to contain the oligosaccharides. The concentration of GF_3 tended to be higher than the concentration of GF_2 (data not shown), and infants consuming formula containing 3.0 g scFOS/L tended to have higher concentrations of the oligosaccharides than infants consuming the formula with 1.5 g scFOS/L. The oligosaccharides were not detected in urine samples collected from infants prior to consumption of the scFOS-containing formulas, or from infants consuming the control formula. Ketones were not detected in urine from any infants before or after the feeding period.

There were no significant differences in faecal bifidobacteria counts among the formula groups after the 2-week feeding period. Faecal clostridia counts decreased in the population of infants fed formula containing 1.5 g scFOS/L as compared to infants fed the scFOS-free formula.

Results from this study indicate that formula containing 1.5 or 3.0 g scFOS/L was suitable for infants over the period of intervention. The addition of scFOS did not affect the proportion of treatment failures, formula intake or weight gains. All infants had looser stools during the intervention period, with 84 to 96% of parents identifying the infant's predominant stool consistency as watery or loose. This outcome may be attributed to the whey-enriched formula used in the study, as other investigators have reported that infants consuming a whey-predominant formula have more watery stools as compared to infants consuming a casein-predominant formula (Harrison, Graver, Vargas, Churella, & Paule, 1987; Malacaman, Abbousy, Crooke, & Nauyok, 1985). There were no differences among formula groups in the change in percent of feedings with spit-up from baseline (p>0.05). The seeming differences observed during the study period (13% for control vs 25% for the 3.0 g scFOS/L) are actually already present at baseline (8% for control vs 17% for the 3.0 g scFOS/L), which persisted throughout the study. Given the absence of a dose-response to the scFOS-

supplemented formulas, it is unclear that the spit-up following feedings experienced in this group can be attributed solely to the addition of scFOS. Although the difference among groups was not statistically significant, a higher number of infants in the 3.0 g scFOS/L group exited the study due to formula intolerance.

In summary, the whey-enriched formula used in this study confounds interpretation of the effects of the scFOS-containing formulas on stool consistency. Measures of growth, formula intake, and other indicators of tolerance, however, suggest that a whey-enriched formula containing up to 3.0 g scFOS/L is suitable for most infants.

2.4.1.1.2 Effects of Infant Formula Containing scFOS on Growth, Tolerance and Faecal Microbiota During the First 16 Weeks of Life

Following completion of the 2-week tolerance and efficacy study, Abbott Nutrition conducted a controlled, blinded, multi-centre, randomized study to assess the effects of formula containing 3.0 g scFOS/L on the growth, tolerance and faecal microbiota of infants during the first 16 weeks of life (Abbott Laboratories, 2011d). In this study, 102 formula-fed, term infants ages 1 to 8 (± 3) days were randomized to consume a formula with 3.0 g scFOS/L (n=50) or a control formula (n=52) during the study period. Based on Abbott Nutrition's clinical experience, a sample size of 50 per group was required to detect a difference of 3 g/day in weight gain between the two groups of infants with a power of 80% in a two-tailed test assuming a standard deviation of 5.3 g/day, incorporating the sex-related difference in rate of gain. A parallel arm of 25 infants receiving human milk as the primary feeding from birth to 16 weeks of age was followed. Weight, length, and head circumference were measured at study entry and at visits at 4, 8, 12, and 16 weeks of age. In addition to the growth measures, the investigator and/or primary care physician also assessed the status of each infant's health during each office visit. Prior to the four study visits, parents kept detailed 3-day records of infants' dietary intake, stool patterns (number, colour, consistency), and incidence of spit-up and vomit on forms provided by the study personnel. Parents were provided written guidelines for completion of the records, as well as complete verbal instructions. At each visit, stool samples were collected for analysis of anaerobic microflora, and urine samples were collected for measurement of ketones. Urine samples were also collected during the 12-week visit for analysis of the indican/creatinine ratio and bilirubin. During the 4- and 16-week study visits, blood samples were drawn for serum analysis of liver enzymes (ALT and AST), scFOS, and cholesterol. The primary study variable was growth, as assessed by measures of weight, length, and head circumference. The secondary study variables included formula intake, formula tolerance, stool microflora as assessed using standard microbiological techniques, and serum and urine biochemistries. The mean rank stool consistency (MRSC) is calculated for each infant for each day by assigning a numerical

value (rank) to each of the consistencies: watery≡1; loose/mushy≡2; soft≡3; formed≡4; and hard≡5, multiplying the number of stools of each consistency with the numerical values, taking the sum and dividing by the total number of stools. The average of the daily mean rank is computed to find the MRSC for the feeding period. The <u>median</u> MRSC is a summary statistic representative of the feeding group. The median MRSC for a feeding group is found by taking the middle ordered value of the MRSC of the infants in the group.

A total of 93 infants completed the study: 36 in the scFOS group, 34 in the control group, and 23 in the human milk group. There were 14 treatment failures in the study (scFOS = 8; control = 6), with no statistical difference in the number of treatment failures between the formula groups. Reasons for treatment failure included symptoms of milk intolerance (scFOS = 4; control = 2), colic (scFOS = 1), diarrhoea or watery stools (scFOS = 1; control = 2), constipation (scFOS = 1; control = 2), and gassiness (scFOS = 1). A total of 20 protocol failures was reported (scFOS = 6, control = 12, HM = 2). Reasons for protocol failure included: missed visits (scFOS = 4, control = 7, HM = 1), removed by investigator before study formula was started (scFOS = 1, control = 3,), removed by investigator because of fever (control = 1), removed by parents due to failure to grow normally in absence of any organic symptom or other condition (scFOS = 1), consumed other foods (control = 1), or unable to collect faecal specimens (HM = 1).

Measures of infant growth and formula intake are shown in Table 13 and measures of parent-reported tolerance are shown in Table 14. There were no differences among groups in average weight, length or head circumference measures; weight, length, or head circumference gains per day (data not shown); or weight, length, or head circumference z-scores. The daily frequency and volume of formula consumed did not differ between the formula groups, though through week 8 of the study, infants consuming human milk fed more frequently than infants consuming formula. Volume and caloric intake based on body weight did not differ between the formula groups when adjusted for gender (data not shown). There was no difference in the percentage of feedings with spit up or vomit across the feeding groups. Daily stool frequency among feeding groups was not different except at week 4, when infants fed either of the study formulas had fewer stools per day than infants consuming human milk. Following ingestion of the formula for approximately 4 weeks, infants consuming formula with 3.0 g scFOS/L had softer stools (assessed by mean rank consistency) than infants consuming the control formula. There were no differences across the formula groups in mean rank stool consistency (MRSC) during the remainder of the study, though infants fed formulas had firmer stools than infants fed human milk at weeks 4, 8, and 12. "Watery" was the predominant stool consistency reported in the monthly diaries by 10 to 23% of parents of infants fed the scFOS-supplemented formula or human milk, while 0 to 8% of infants

fed the control formula had a predominant stool consistency described as watery. The human milk group had a significantly lower median MRSC than the formula fed groups at day 28, day 56, and day 84 (ANOVA on ranked data; $p<0.05$) (Table 15). Also, the supplemented formula group had a significantly lower median MRSC than the non-supplemented (control) group at day 28 (ANOVA on ranked data; $p<0.05$). For this study, statistical analyses were not completed on the predominant stool consistencies reported from the parent diaries. However, the median MRSC and predominant stool consistency outcomes are highly correlated so most likely differences in median MRSC will also be reflected as differences in predominant stool consistency. In general, the predominant consistency distribution is shifted toward looser stools for the HM group relative to the supplemented group [37% (watery and loose) for the supplemented group, and 58% (watery and loose) for the HM fed group at week 1]. There also were no differences in the percent of stools with straining or dryness (data not shown).

Results from the assessments of plasma and urine are shown in Table 15. Plasma concentrations of AST and ALT were normal and did not differ between the formula groups. Infants fed human milk had higher AST, ALT and cholesterol activity than both formula fed groups, however all values were within the normal ranges for infants. The lower cholesterol levels in the formula fed infants can be expected as the formula provide lower levels of dietary cholesterol than does human milk. GF_2 and GF_3 were detected in approximately 30 to 80% of urine samples from infants consuming the scFOS-supplemented formula. The oligosaccharides were detected in urine from 1 infant fed the control formula at week 8 (GF_2), and one infant fed human milk at study entry (GF_2 and GF_3). GF_2 and GF_3 were not detected in any plasma samples (data not shown), and ketones were not detected in any urine samples.

Faecal bifidobacteria, clostridia, and *C. difficile* counts were similar among formula groups at all time points. At weeks 8, 12, and 16, lactobacilli counts and colonization rates were significantly higher in the scFOS formula group than in the control formula group.

Results from this study indicate that an infant formula containing 3.0 g scFOS/L is well tolerated. Although the study was not adequately powered to detect growth differences (American Academy of Pediatrics, 1998), the results from multiple anthropometric measures assessed in this trial suggest that formula containing up to 3.0 g scFOS/L supports normal infant growth in the first 4 months of life. The addition of scFOS to the formula resulted in softer stools compared to the control formula, but this difference was statistically significant only at 4 weeks. Overall, infants consuming human milk tended to have slightly softer and more frequent stools than infants consuming either of the formulas. The scFOS-containing formula had no adverse effects on plasma levels of liver enzymes and cholesterol, or urine total bilirubin. Quantifiable amounts of GF_2 and

GF$_3$ were detected in urine from infants consuming the scFOS-containing formula, though none was detected in blood. The finding of very small amounts of scFOS in urine is consistent with the 0.12% of ingested scFOS that was recovered unchanged in the urine of adults (Molis, et al., 1996). Findings from this study therefore indicate that formula containing 3.0 g scFOS/L is suitable for infants as a sole source of nutrition in the first months of life.

2.4.1.1.3 Comparative Gastrointestinal Tolerance of Soy-based Infant Formulas Containing scFOS in Healthy Term Infants

A randomized, double-blind, multi-center, parallel feeding trial (Abbott Laboratories, 2011a; Lasekan, et al., 2010) was conducted to assess the comparative gastrointestinal tolerance of soy-based infant formula with and without scFOS in healthy term infants. Growth, tolerance and the occurrence of adverse events were closely monitored throughout the study.

Infants 0-8 days of age were randomized to one of the study formulas, including a control formula and two other soy-based experimental formulas with scFOS with (EF1) and without (EF2) sucrose. The experimental formulas were supplemented with scFOS (NutraFlora FOS; GTC; Golden, CO) to provide 2.5 g/L and were fed for 35 days. The study was performed in accordance with the protocol, Good Clinical Practice, FDA regulations governing clinical study conduct, and the ethical principles that have their origin in the Declaration of Helsinki.

The primary outcome of the study was mean rank stool consistency (MRSC) over study days (SDAY) 1-35. The MRSC is calculated by assigning a score for stool consistency (1=watery, 2= loose/mushy, 3= soft, 4= formed, 5=hard), taking a daily mean, then computing the average of means over the period for each infant. The MRSC was statistically analysed using ANOVA with study centre and formula group (feeding) in the model. An adjustment was made to the alpha level for multiple comparisons among the feeding groups using Holm's step down procedure. The study design also included other measures of tolerance including stool patterns and spit-up associated with feedings. Anthropometric measurements were collected at the three study visits.

One deficiency cited by the European Food Safety Authority (European Food Safety Authority (EFSA), 2004) in their review of previous studies that fed FOS to infants in infant formula was that the studies did not monitor water balance (e.g., Euler et al. 2005). The key concern related to the potential impact on hydration status is related to the production of excess soft or watery stools upon introduction of a non-digestible oligosaccharide ingredient in the infant diet. Whilst formal physician assessment of water balance was not done in the earlier Abbott studies (Abbott Laboratories, 1993a,

1993b, 2005), all infants in those early studies were thoroughly examined by physicians trained to identify signs of dehydration. Furthermore, infants were weighed throughout these studies, and weight is a very objective measure of hydration status. Based on the concerns raised by EFSA, measures were taken in the 2009 Abbott study to specifically address hydration status. Formal physician assessment of infant hydration status was completed at two study visits, including the measurement of urine specific gravity (USG).

One hundred ninety-five term infants were enrolled. The resulting ITT group consisted of 188 infants with 186 infants evaluable at SDAY1, 142 infants evaluable on SDAY14, and 120 infants evaluable on SDAY35. There were no significant differences among the groups in study completion rates or premature discontinuation of study formula.

There were no differences in MRSC among the groups. Average MRSC in the evaluable population from enrolment to SDAY 35 was 2.6 ± 0.1 in the control group, 2.7 ± 0.1 in the EF1 group, and 2.5 ± 0.1 in the EF2 group. There also were no significant differences in the average number of stools and percentages of feedings with spit-up and/or vomit among the formula groups. There were no differences in growth measures among the groups.

There were no significant differences in physician assessment of hydration status or USG. One infant, in the EF2 group, had an elevated USG value of 1.0388 (>1.030) at 14, but not 35 days of age. The infant had no reported AE, SAE, diarrhoea, or watery stools and completed the study on the study formula. There were no statistical differences among the formula groups in incidence of AEs and SAEs and measures of hydration status. Although not statistically significant, there were 7 parental reports of loose watery stools in the EF1 group compared to 2 in the EF2 and 4 in the CF groups. Despite the number of parental reports in the EF1 group, the hydration status and USG in these subjects were normal.

Based on the results of this clinical trial, there were no differences in gastrointestinal tolerance of the infant formulas that were supplemented with scFOS compared to a formula without scFOS.

Table 10: Disposition, growth, and formula intake of infants participating in 2-week study of formula with added scFOS			
Formula Group [1]			
	Control	1.5 g scFOS/L	3.0 g scFOS/L
Enrolled (M/F) [completed]	23 (18/5) [20] [2]	22 (13/9) [17]	21 (11/10) [16] [2]
Treatment failures	1	2	4
Protocol failures	0	3	0
Mean ± SEM (n)			
Gestational age, wk	40.0 ± 0.2	40.0 ± 0.2	40.0 ± 0.2
Age at study entry, d	39 ± 2 [a]	41 ± 3 [a]	49 ± 3 [b]
Weight at study entry, g	4737 ± 206 (23)	4692 ± 157 (22)	4957 ±156 (21)
Weight at study end, g	5615 ± 180 (21)	5477 ± 164 (18)	5801 ± 169 (18)
Weight gain, g/d	28.2 ± 4.0 [a] (21)	29.2 ± 2.3 [a] (18)	25.4 ± 2.0 [a] (18)
Feeding volume, mL/d	830 ± 32 [a] (21)	826 ± 30 [a] (19)	862 ± 35 [a] (19)
Feeding frequency, No./d	5.9 ± 0.2 [a] (21)	6.6 ± 0.4 [a] (19)	6.1 ± 0.3 [a] (19)

[1] Study outcome means are based on data collected over the entire study period except when noted. Values with different superscripts are significantly different (p<0.05). All study formulas manufactured by Abbott Nutrition.

[2] 2 infants in the Control group and 1 in the 3.0 g scFOS/L group exited the study prior to initiation of the test formulas due to treatment or protocol failures; these infants were excluded from all analyses.

Reference: (Abbott Laboratories, 2011d)

Table 11: Tolerance and stool characteristics of infants in 2-week study of formula with added scFOS						
	Baseline by Formula Group [1]			Post 2-Week Treatment by Formula Group [1]		
Parameter	Control	1.5 g scFOS/L	3.0 g scFOS/L	Control	1.5 g scFOS/L	3.0 g scFOS/L
Tolerance - % feedings with, median						
Spit up	8	6	17	13	6	25
Vomit	1	0	1	0	0	3
Stool characteristics						
Number of stools/day, median	1.3	1.2	1.3	1.2	1.1	1.3
Consistency, median MRSC[2]	2.6	2.7	2.8	1.6	1.6	1.8
Predominant consistency, %						
Watery	5	5	5	48	53	42
Loose	36	38	15	48	42	42
Soft	41	48	65	5	0	16
Formed	14	10	10	0	0	0
Mixed or Missing[3]	5	0	5	0	5	0

[1] Values based on all non-protocol failures. No differences were detected between groups at p<0.05.

[2] Ranked on a 5-point scale: 1 = watery, 2 = loose/mushy, 3 = soft, and 4 = formed, and 5 = hard (hard category not included in results for this study).

[3] Predominant stool consistency result was a tie between two categories (mixed) or was missing for a subject.

Reference: (Abbott Laboratories, 2011d)

Table 12: Plasma liver enzyme and urinary scFOS concentrations of infants at end of 2-week study of formula with added scFOS			
	Formula Group		
Parameter	Control	1.5 g scFOS/L	3.0 g scFOS/L
	Mean ± SEM (n) [1]		
ALT, U/L [2]	32 ± 5^a (7)	30 ± 10^a (5)	37 ± 19^a (3)
AST, U/L [3]	46 ± 6^a (7)	47 ± 11^a (5)	46 ± 15^a (3)
	ppm range (quantifiable samples/total samples)		
GF$_2$	---[4]	39.6 – 56.6 (4/6)	59.2 – 114.0[5] (8/9)
GF$_3$	---	48.5 – 91.3 (5/6)	63.6 – 140.2 (9/9)

[1] Values at the same time point with unlike superscripts are different at p<0.05.

[2] Reference range of ALT for infants (newborn – 12 months of age) is 13-45 U/L (Tietz 1995).

[3] Reference range of AST for infants (newborn – 12 months of age) is 15-60 U/L (Tietz 1995).

[4] Not measured.

[5] GF$_2$ was detected in one sample but not quantifiable.

Reference: (Abbott Laboratories, 2011d)

Table 13: Disposition, growth, and formula intake of infants enrolled in 16-week study of formula with added scFOS			
	Feeding Group		
	Control Formula	3.0 g scFOS/L	Human Milk
Enrolled (M/F) [completed]	52 (25/27) [34]	50 (23/27) [36]	25 (13/12) [23]
Treatment failures	6	8	0
Protocol failures	12	6	2
	Mean ± SEM (n)		
Gestational age, wk	39.2 ± 0.2	39.5 ± 0.2	39.8 ± 0.2
Age at study entry, d	7 (1-18) [a]		0 (0 – 9) [b]
Weight at study entry, g	3334 ± 66 (47)	3474 ± 66 (49)	3545 ± 98 (25)
Head circumference at study entry, cm	35 ± 0.0 (42)	36 ± 0.0 (42)	36 ± 0.0 (25)
Length at study entry, cm	50 ± 0.0 (47)	50 ± 0.0 (49)	51 ± 0.0 (25)
Weight at study end, g	6426 ± 113 (33)	6629 ± 122 (36)	6471 ± 192 (23)
Weight gain, g/d	24.6 ± 1.0 (33)	23.1 ± 1.0 (36)	20.8 ± 2.0 (23)
Head circumference gain, mm/d	0.4 ± 0.0 (29)	0.3 ± 0.0 (29)	0.3 ± 0.0 (23)
Length gain, mm/d	1.0 ± 0.1 (33)	0.8 ± 0.1 (36)	0.9 ± 0.1 (23)
Feeding volume, mL/d	1040 ± 49 (34)	974 ± 45 (36)	NR
Feeding frequency, No./d	6.2 ± 0.4 (29)[a]	6.0 ± 0.3 (29)[a]	7.2 ± 0.5 (23)[b,2]

[1] Study outcome means are based on data collected over the entire study period except when noted. Values with different superscripts are significantly different (p<0.05). All study formulas manufactured by Abbott Laboratories.
[2] Infants fed human milk had significantly more feedings per day compared to formula groups at weeks 4, 8 and 12 (ANOVA on ranked data; p<0.05).
NR: Not reported or measured in the study.   Reference: (Abbott Laboratories, 2011d)

Table 14: Tolerance and stool characteristics of infants in 16-week study of formula with added scFOS												
	Week 4			Week 8			Week 12			Week 16		
Parameter	Control Formula	3.0 g scFOS/L	Human Milk	Control Formula	3.0 g scFOS/L	Human Milk	Control Formula	3.0 g scFOS/L	Human Milk	Control Formula	3.0 g scFOS/L	Human Milk
Tolerance - % feedings with, median												
Spit up	15	17	22	23	9	27	13	12	20	25	15	24
Vomit	0	0	0	0	0	0	0	0	0	0	0	0
Stool characteristics												
Number of stools/d, median	1.7[a]	2.0[a]	3.7[b]	1.0	1.0	2.0	1.3	1.0	1.3	1.0	1.0	0.8
Consistency, median MRSC[2]	3.0[a]	2.8[b]	2.0[c]	2.6[a]	2.5[a]	2.0[b]	2.4[a]	2.7[a]	2.0[b]	2.7	2.0	2.0
Predominant consistency, %												
Watery	0	10	13	5	11	23	8	11	10	6	23	10
Loose	17	21	61	38	26	64	36	24	71	21	14	48
Soft	48	48	22	41	39	14	36	39	10	44	37	33
Formed	24	7	0	3	0	0	0	8	0	6	3	0
Hard	5	2	0	0	0	0	0	0	0	0	0	0
Mixed or Missing[3]	7	12	4	14	24	0	19	19	10	24	23	10

[1] Values based on all available data. Values for parameters with different superscripts are significantly different (ANOVA on ranked data; p<0.05).

[2] Ranked on a 5-point scale: 1 = watery, 2 = loose/mushy, 3 = soft, 4 = formed, and 5 = hard.

[3] Predominant stool was a tie between two categories (mixed) or was missing for a subject.

Reference: (Abbott Laboratories, 2011d)

Parameter	Week 1	Week 4			Week 8	Week 12	Week 16		
	3.0 g scFOS/L	Control Formula	3.0 g scFOS/L	Human Milk	3.0 g scFOS/L	3.0 g scFOS/L	Control Formula	3.0 g scFOS/L	Human Milk
	Mean ± SEM (n) [1]								
ALT, U/L [3]	---[2]	30 ± 1 (39)	34 ± 2 (38)	38 ± 4 (22)	---	---	$32 ± 2^a$ (32)	$37 ± 3^a$ (33)	$49 ± 6^b$ (21)
AST, U/L [4]	---	$42 ± 2^a$ (39)	$43 ± 2^a$ (38)	$56 ± 4^b$ (22)	---	---	$46 ± 2^a$ (32)	$49 ± 3^a$ (33)	$65 ± 6^b$ (21)
Cholesterol, mg/dL [5]	---	$102 ± 3^a$ (39)	$110 ± 4^a$ (38)	$139 ± 5^b$ (22)	---	---	$117 ± 3^a$ (31)	$119 ± 4^a$ (33)	$148 ± 5^b$ (21)
	ppm range (quantifiable samples/total samples)								
GF_2	2.3 – 217.6 (9/24)	---	26.6 – 102.9 (5/6)	---	2.1 – 34.6 (7/9)	33.9 – 108.4 (4/6)	---	26.4 – 55.5 (2/2)	---
GF_3	5.2 – 130.3 $(8/24)^6$	---	12.6 – 110.6 (5/6)	---	1.5 – 26.7 $(5/9)^7$	7.1 – 64.2 $(3/6)^7$	---	--- $(0/2)^7$	---

Table 15: Plasma liver enzyme, plasma cholesterol, and urinary scFOS concentrations of infants during 16-week study of formula with added scFOS

[1] Values at the same time point with unlike superscripts are different (ANOVA; $p<0.05$).

[2] Not measured.

[3] Reference range of ALT for infants (newborn – 12 months of age) is 13-45 U/L (Tietz 1995).

[4] Reference range of AST for infants (newborn – 12 months of age) is 15-60 U/L (Tietz 1995).

[5] Reference range of cholesterol for infants newborn – 1 month of age is 45-198 mg/dL and for infants 2 months – 6 months of age is 60-218 (Soldin et al 1999).

[6] GF_3 was detected in one sample but not quantifiable.

[7] GF_3 was detected in two samples but not quantifiable.

Reference: (Abbott Laboratories, 2011d)

2.4.1.2   Other Studies of scFOS in Infant Formula

Guesry and colleagues (2000) conducted a clinical study in which 47 infants ages 7 to 20 days of life were randomized to consume a control infant formula or formula supplemented with 1.0, 2.0 or 3.0 g scFOS per day for a period of two weeks. Findings were presented in a meeting abstract. During the study, the infants' mothers recorded daily formula intake, stool number and characteristics (including loose stools), diaper rash, spitting up, vomiting, and colic. There was no difference in the frequency of diaper rash or colic. A significant increase in the number of stools was observed in the 3.0 g scFOS/d group compared to the other groups. No changes in faecal pH or bifidobacteria counts were observed. Observations related to growth parameters and reasons for dropouts were not reported in the abstract (Guesry, Bodanski, Tomsit, & Aeschlimann, 2000).

In a French study assessing the safety, tolerance, and protective effect of infant formulas containing mixtures of probiotics or probiotics and prebiotics (synbiotics), scFOS was used in combination with GOS (total GOS/scFOS 4g/L) (Chouraqui et al., 2008). Two hundred and eighty four (284) healthy term infants were enrolled in the prospective, controlled, double-blind, randomised trial. The infants were fed exclusively one of 4 trial formulas (2 probiotic, 2 synbiotic combinations) up to the age of 16 weeks. Safety and tolerance were assessed based on weight gain during the treatment period (primary outcome) as well as recumbent length, head circumference, digestive tolerance, and adverse events (secondary outcomes), which were evaluated at 2, 4, 8, 12, 16, and 52 wk of age. During the treatment period, difference in mean weight gain between control and study formula groups in both the intention-to-treat and per-protocol populations were within the predefined equivalence boundaries of ±3.9 g/d, indicating equivalent weight gain. Secondary outcomes did not show significant differences between groups during the treatment period. The study concluded infants fed formulas containing probiotics or synbiotics (GOS/scFOS) show a similar rate in weight gain compared with those fed a control formula and tolerate these formulas well (Chouraqui, et al., 2008).

2.4.2 Oligofructose or Oligofructose + Inulin in Infant Formula

A limited number of studies have been conducted to assess the suitability and potential benefits of adding oligofructose derived from inulin to formula for term infants within the first 3 months of life (Bettler & Euler, 2006; Euler, Mitchell, Kline, & Pickering, 2005), infants between approximately 3.5 and 6.5 months of life (Brunser, Figueroa, et al., 2006), and preterm infants within the first week of life (Kapiki et al., 2007).   The effects of formula containing a combination of oligofructose and lc-inulin on faecal microflora in the

4 weeks of life in term infants also have been examined ((Veereman-Wauters et al., 2008), as cited in FSANZ 2008a,b).

Euler and colleagues (2005) conducted a study to assess the effects of oligofructose (Raftilose® P95, Orafti; identified in the study as FOS) in infant formula on faecal microbiota. Healthy term infants ranging in age from 0.5 to 1.5 months were enrolled in the study. In this crossover study, infants were fed a whey-predominant infant formula containing either 1.5 g oligofructose /L (n=36) or 3.0 g oligofructose /L (n=34) for a period of one week, and all infants were fed formula with no added oligofructose another week. A week-long washout period preceded and followed each study period. A parallel group of infants fed human milk (n=17) was included in the analysis. Formula intake, stool frequency, stool size, stool consistency, and stool color were recorded by the parent for a 24-hour period before each weekly visit. At each visit, the parent or guardian was questioned about possible adverse events.

No differences in growth rate, weight or height were observed among the groups at the end of the trial. Adverse events were documented for the entire 5-week duration of the study. The investigators noted that there was no statistically significant difference in infant dropout rates between the human milk-fed group and either of the oligofructose groups. During the week of oligofructose supplementation, infants in both treatment groups experienced more events of flatulence, spit-up, and looser stools, and the incidence of events was lower in the 1.5 g oligofructose group than in the 3.0 g oligofructose group. The investigators reported that stool frequency decreased in the low dose group following the period of oligofructose ingestion as compared to the pre-oligofructose period, while frequency increased in the high dose group. Stool consistency changed minimally in the low dose group after oligofructose supplementation, while consistency was softer in the high dose group. Loose stools and overt diarrhoea were not reported for any infants. Satisfaction ratings for formula acceptability and tolerability declined at the visit after oligofructose supplementation; 67% were satisfied in the low-dose group vs. 59% in the high-dose group. Details regarding formula intake, stool color, and stool size were not presented. At baseline, the infants fed oligofructose had significantly higher counts of enterococci and bacteroides compared to levels in infants fed human milk; all other microorganism counts were similar across the feeding groups. After supplementation, mean bifidobacteria counts were greater in the 1.5 g oligofructose/L group than in either the human-milk or 3.0 g oligofructose /L group. Enterococci and clostridia counts for both oligofructose groups were higher compared to the human milk-fed infants at the end of supplementation. Seven days after the conclusion of supplementation, no significant differences in bifidobacteria levels among the groups remained. Levels of enterococci and bacteroides, however, were higher compared to human milk-fed infants at this same time point. Clostridia levels remained

higher for the 1.5 g oligofructose /L group compared to the human milk infants, but the difference was gone in the 3 g oligofructose /L group.

Bettler and Euler (2006) examined the growth, clinical chemistries, and adverse event occurrence of healthy term infants consuming formula supplemented with oligofructose (Raftilose® P95, Orafti; identified in the study as FOS) over a 12-week period. In this study, infants up to 14 days of age were randomized to receive 1.5 g oligofructose /L (n=98), 3.0 g oligofructose /L (n=101), or a control formula with no added oligofructose (n=98). Measures of growth were collected at baseline and at 4-week intervals during the study. Adverse events and infants' acceptance and tolerance of the formula were recorded at study visits during weeks 4, 8, and 12, and from telephone inquiries during weeks 2, 6, and 10. Clinical chemistries (albumin, blood urea nitrogen, calcium, magnesium, phosphorus, creatinine, triglycerides, low-density lipoprotein, and cholesterol) were assessed at baseline and at week 12. A total of 212 infants completed the study (67% of the control group, and 73% of each oligofructose group).

Infants in each of the three formula groups had normal weight, length and head circumference gains during the study. Infants in the 1.5 g oligofructose /L group were shorter on average than infants in the control formula group at week 8, though all measurements were within normal range. The 3.0 g oligofructose /L group had fewer formula-related adverse events than the other formula groups, and the 1.5 g oligofructose /L group had slightly more adverse events than the control group. The majority of the adverse events were described as mild and resolved without treatment. The investigators reported no differences in the incidence of diarrhoea, loose stools, dehydration, allergic reaction, or flatulence. Constipation was less frequent in the group that received 3.0 g oligofructose/L while vomiting was more common among the group receiving 1.5 g oligofructose/L, but neither was significant for events considered by the principal investigators to be formula related. Formula acceptability was similar among the three groups, with percent satisfaction ranging from 88 to 100% throughout the study. The investigators noted that the most common reasons for discontinuation were similar among the 3 study groups. In addition, the number of infants discontinuing the formula due to adverse events was similar across the 3 formula groups. Serum measures of proteins, minerals, and lipids did not differ among the formula groups at the completion of the study. Based upon the study findings, the investigators concluded that either oligofructose -containing formula is "safe and supports normal infant growth."

Brunser and colleagues (2006a) conducted a 13-week study to evaluate efficacy of oligofructose (Raftilose P95, Orafti; identified in the study as FOS) supplementation in modulating the composition of the faecal microbiota. Ninety infants approximately 3.5 months old were randomized to receive formula with 2 g oligofructose/L, Lactobacillus

johnsonni La1, or no added components. Another 26 infants were breastfed throughout the study. Mothers registered formula and food intake and possible associated adverse reactions in a standardized diet record. There were no differences in drop out rates among the 4 groups, and no withdrawals were associated with adverse reactions to the formulas. The number of adverse events per infant, including diarrhoeal episodes, was not significantly different between the four groups. No differences were observed in weight, height, weight for height, weight for age and height for age z-scores during the study. The average formula intake was similar among the groups, and all formulas were well tolerated. Culture methods show no effect of oligofructose -supplemented formula on faecal bifidobacteria, enterobacteria, C. perfringens, C. histolyticum, bacteroides, or enterococci compared to the control formula; results of a fluorescent in situ hybridisation (FISH) analysis also showed no effect of the formulas on faecal bifidobacteria.

Kapiki and colleagues (2007) assessed measures of growth, stool characteristics, and faecal flora composition in 36 preterm infants consuming formula with added "FOS" (4 g/L) and 20 preterm infants consuming formula with 4 g maltodextrin/L. The "FOS" used in the study was identified as inulin produced by partial enzymatic hydrolysis of chicory inulin, which is presumably an oligofructose. All infants were healthy, exclusively bottle-fed and enrolled in the study within 14 days of age. Daily records of formula intake, stool frequency, size, consistency score, and colour were maintained. Growth measurements were taken at baseline and study days 7 and 14. Faecal samples were also taken at baseline and at study day 7. Weight gain and arm circumference growth during the study was statistically higher in the control group compared to the oligofructose group, though length gain and head growth during the study did not differ between groups. Stool frequency was significantly higher in the oligofructose group, while stool consistency did not differ between the feeding groups. Both formulas were reported to be well tolerated. By day 7 of the study, infants in the oligofructose group had higher faecal counts of bifidobacteria and bacteroides and lower counts of E. coli and enterococci as compared to the control group.

Beneo Orafti recently assessed the growth, tolerance, and faecal microflora of a population of newborn infants consuming formula with added prebiotics, including oligofructose, over a period of 4 weeks; findings from the trial were presented in abstract form and additional details were summarized by FSANZ (Veereman-Wauters, et al., 2008). In this study, infants approximately 4 to 5 days of life were randomized to consume infant formula with 2 g oligofructose + 2 g lc-inulin/L (n=20), 4 g oligofructose + 4 g lc-inulin/L (n=20), 8 g GOS + lc-inulin (9:1)/L (n=20), or a control formula with no added prebiotics (n=20); a group of 26 infants fed human milk served as a reference control. The 1:1 combination of oligofructose and lc-inulin is produced by Orafti and referred to as Synergy1. Faecal samples were collected on days 1, 2 and 3; 12, 13 and 14; and 26, 27 and

28 and then averaged to represent week 1, week 2 and week 4 of supplementation, respectively. Information on regurgitation of formula, stool frequency, and stool consistency was recorded in study diaries (frequency of entries was not specified). The infants fed and grew normally during the study, and measures of weight, height, and food intake were comparable across the formula groups. No serious adverse events were observed. Vomiting and crying were similar in all groups and considered to be low. At the end of the 4-week feeding period, mean stool frequency of infants consuming the control formula or either of the formulas containing 8 g prebiotics/L decreased significantly from a mean of approximately 2.8 to 2.1 times per day. Mean stool frequency of infants consuming 4 g Synergy1/L was not significantly different from the baseline value. The mean stool frequency of infants consuming human milk was approximately 2.8 per day at baseline and approximately 4/day at the end of the study. Infants consuming the supplemented formulas had significantly softer stools at week 2, and the consistency at week 4 was approximately mid way between values from breast-fed infants and infants who received the control formula. The stool consistency of infants fed the control formula was significantly harder at weeks 2 and 4 as compared to week 1 values. Infants consuming human milk reportedly had more watery stools. The counts of total faecal bacteria increased in all formula groups with added prebiotics, while counts were unchanged in the control group. The supplemented formulas had no effects on faecal counts of lactobacilli, bacteroides and clostridia over time or across days. In both the 8 g Synergy1/L and 8 g GOS+lc-inulin/L groups, faecal bifidobacteria counts at weeks 2 and 4 were higher than counts at week 0+. Faecal bifidobacteria counts in the 4 g Synergy1/L group and the control group did not change significantly.

Overall, the study authors concluded that formula supplementation with the Synergy1 and GOS:FOS were well tolerated and led to an increase in faecal bifidobacteria. There were no apparent adverse effects at up to 8 g Synergy1/L or 8 g GOS+lc-inulin/L.

2.4.3 scFOS or Oligofructose in Follow-On Formula or Weaning Foods

scFOS, oligofructose, or combinations of oligofructose and lc-inulin have been added to the diets of older infants and toddlers to assess the effects of the non-digestible ingredient on a variety of endpoints including the composition of faecal microflora, prevalence of diarrhoea, general well-being, response to vaccinations, gastrointestinal effects, tolerance, and growth (Table 19). Abbott Laboratories conducted a study in which scFOS was added to milk-based beverages fed to children approximately 10 to 24 months of age. Other investigators have examined the effects of oligofructose or a combination of oligofructose and inulin in follow-on formulas or weaning foods for older infants or toddlers. In all of these studies, the scFOS or oligofructose-containing food was consumed

in addition to standard formula or typical weaning foods. Results from these studies provide additional information on the suitability and potential benefits of scFOS or oligofructose as part of a weaning diet.

2.4.3.1 Effects of Milk-Based Beverage with scFOS on Incidence and Severity of Diarrhoea in Toddlers

As a contribution to evidence to support the use and efficacy of scFOS in products suitable for Toddlers, Abbott Nutrition conducted a randomized, blinded, controlled study to assess the efficacy of a milk-based beverage supplemented with scFOS on incidence and diarrhoea in toddlers (Abbott Laboratories, 2011b). This study is also the subject of US Patent 5,827,526 "Use of Indigestible Oligosaccharides to Prevent Gastrointestinal Infections and Reduce Duration of Diarrhea in Humans" (Dohnalek, Ostrom, Hilty, & Lewis Center, 1998).

The study was clinical trial of 283 children ages 10 to 24 months at study entry attending day care in Santiago, Chile (Table 16). During a 16-week period, the children were assigned to consume a milk-based beverage with approximately 3.4 g scFOS/L (n=139) or the milk-based beverage with no added scFOS (n=144). Both the treatment and control beverages contained added nucleotides, and the beverages were fed *ad libitum* as the child's sole source of milk beverages. Children were encouraged to drink a minimum of 500 mL of the milk beverages each day at day care and at home. Solid foods were permitted. At study entry, children were monitored closely for diarrhoea and other significant medical illnesses. Research nurses visited the day care center weekly to ensure study compliance and identify episodes of diarrhoea and other illness. At weeks 1, 4, 8, 12, and 16 of the study, the children were evaluated for measures of tolerance, growth, and faecal microbiota. Day care center workers recorded study feeding intake; occurrences of stomach cramps and vomiting associated with feedings; stool frequency, consistency, and characteristics; the amount of gas; and constipation for each child on each day of attendance at the day care center. Parents maintained records of the same tolerance variables, as well as milk intake, during the 3 days prior to each study visit. Weight and length were measured at entry, and at the week 8 and week 16 visits. Faecal samples were collected at baseline, at week 16, and at one additional study visit during the trial for determination of faecal lactobacilli, bifidobacteria, rotavirus, and *Clostridium difficile*. Faecal specimens also were collected from children within two days of the start of an episode of diarrhoea and analyzed for faecal pathogens.

During two phases, a total of 242 children completed the study: 118 in the scFOS group, and 124 in the control group. A summary of the sample population and measures of infant growth is presented in Table C17. There were 3 treatment failures

in the scFOS group and 6 in the control group. The number of protocol failures in the scFOS and control groups was 18 and 14, respectively. Measures of weight and length were comparable in the two feeding groups throughout the study and normal in both groups. Children in the scFOS group had greater weight gains than children in the control group, though the gains were not statistically different.

A summary of the milk-based beverage intake, tolerance measures, and stool characteristics is shown in Table C18. There were no differences between feeding groups in volume or frequency of milk intake. The average intake of scFOS by children in the scFOS-containing milk beverages was approximately 2.5 g per day at each assessment. There also were no differences between groups in incidence and frequency of stomach cramps and/or vomiting with feedings. Children consuming the scFOS-containing milk had a lower mean rank stool consistency (i.e., softer stools) at week 1 as compared to the control milk group, and a higher percentage of watery stools or stools that were either watery or loose. There were no differences between groups in mean rank stool consistency at baseline or weeks 4, 8, 12, or 16. Children consuming the scFOS-supplemented milk had a greater increase in detectable levels of faecal bifidobacteria as compared to children consuming the control milk, though the beverages had no effects on faecal counts of Lactobacillus spp. The incidence of diarrhoea did not differ between the scFOS and control groups. Children consuming milk with added scFOS, however, had a shorter mean duration of acute diarrhoea episodes (3.91 vs. 4.88 days, p=0.036), lower incidence of otitis media (17 of 131 children vs. 33 or 134 children, p=0.023), and fewer courses of antibiotics to treat otitis media (p=0.039, relative risk of 0.501).

Table 16: Studies of scFOS in Follow-On Formula or Weaning Foods			
Reference (trial location)	Test Substance and Dose	Treatment Duration	Results of Ingestion*
(Abbott Laboratories, 2011b; Dohnalek, et al., 1998)	Toddlers, 10-24 mo Multicenter, controlled, randomized, trial Added to milk-based beverage (with nucleotides): • 3.5 g scFOS/L (n=139) [approximately 2.5 g/d] • Control milk (n=144) Source: NutraFlora scFOS (GTC Nutrition)	16 wk	Growth: -No difference in weight, length, or weight gain/d. Tolerance: -No difference in number of feedings/d or median volume consumed. -No difference in incidence and frequency of stomach cramps and/or vomiting. -Softer stools at week 1 and higher percent of watery and watery or loose stools; comparable stool consistency at weeks 4, 8, 12 and 6. Dropouts: -3 treatment failures in the scFOS group; 6 in the control group – all intolerance of study diet. Microbiological Data: -Greater increase in detection of faecal bifidobacteria; no change in lactobacilli. Other Endpoints: -Lower risk for diarrhoea, shorter duration of diarrhoea, lower incidence of otitis media, fewer courses of antibiotics to treat otitis media.

Table 17: Disposition and growth of infants enrolled in 16-week study of milk-based beverage with added scFOS		
	Feeding Group	
	Control Beverage	**scFOS Beverage[1]**
Enrolled [completed]	144 [124]	139 [118]
Treatment failures	6	3
Protocol failures	14	18
	Mean ± SEM	
Weight at: (g)		
Baseline	10,914 ± 120	10,936 ± 134
Midpoint	11,428 ± 127	11,437 ± 136
Study end	11,756 ± 131	11,786 ± 151
Weight gain at: (g/day)		
Midpoint	7.8 ± 0.6	9.6 ± 0.6
Study end	5.7 ± 0.6	6.4 ± 0.6
Length at: (cm)		
Baseline	79.6 ± 0.4	79.9 ± 0.4
Midpoint	81.6 ± 0.4	81.3 ± 0.4
Study end	83.1 ± 0.4	83.0 ± 0.4

[1] Beverage contained 3.4 g scFOS/L.

Reference: (Abbott Laboratories, 2011b)

Table 18: Feeding statistics, tolerance and stool characteristics of children in 16-week study of milk-based beverage with added scFOS										
	Week 1		Week 4		Week 8		Week 12		Week 16	
Parameter	Control	scFOS	Control	scFOS	Control	scFOS	Control	scFOS	Control	scFOS
Feeding volume, mL/d	736 ± 17	710 ± 17	713 ± 17	757 ± 18	770 ± 17	730 ± 19	745 ± 17	745 ± 19	750 ± 18	766 ± 18
Feeding frequency, No./d	3.5 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.5 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.5 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.6 ± 0.1
Tolerance - % feedings with										
Stomach cramps	12.4 ± 2.2	13.4 ± 2.4	10.3 ± 2.1	10.5 ± 2.0	9.1 ± 2.1	6.9 ± 1.8	4.5 ± 1.4	4.1 ± 1.3	0.9 ± 0.7	5.4 ± 4.0
Vomit	0.9 ± 0.4	0.3 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.2	0.5 ± 0.2	0.2 ± 0.1	1.0 ± 0.4	0.2 ± 0.2	0.2 ± 0.2
Stomach cramps & vomit	13.3 ± 2.2	13.7 ± 2.3	10.5 ± 2.1	10.8 ± 2.0	9.3 ± 2.1	7.3 ± 1.8	4.6 ± 1.4	5.0 ± 1.4	1.1 ± 1.0	5.6 ± 4.0
Stool characteristics										
Number of stools/d, median	2.0	2.2	2.0	2.0	2.0	1.7	2.0	2.0	2.0	1.7
Number of stools/d	2.0 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	2.2 ± 0.1	1.9 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.7 ± 0.1
Consistency										
Mean	3.2 ± 0.1[a]	3.0 ± 0.1[b]	3.2 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.5 ± 0.1
Median	3.2	3.0	3.1	3.2	3.3	3.3	3.4	3.3	3.5	3.6
Predominant, %										
Watery	5.7 ± 1.4[a]	10.6 ± 2.0[b]	5.1 ± 1.3	4.3 ± 1.0	1.9 ± 0.8	4.4 ± 1.2	2.7 ± 0.9	2.2 ± 0.8	2.2 ± 0.8	1.3 ± 0.5
Loose/Mushy	13.6 ± 2.0	16.5 ± 2.0	13.9 ± 2.0	13.2 ± 2.1	16.2 ± 2.2	10.8 ± 1.9	10.2 ± 1.7	11.4 ± 1.9	14.2 ± 2.4	8.7 ± 1.6
Soft	43.8 ± 3.1	40.7 ± 3.0	42.9 ± 3.3	39.3 ± 3.2	37.5 ± 3.0	37.6 ± 3.3	39.5 ± 3.1	35.6 ± 3.2	33.3 ± 3.0	35.8 ± 3.1
Formed	33.0 ± 3.1	27.6 ± 1.0	37.2 ± 3.5	39.0 ± 2.9	39.4 ± 3.2	41.8 ± 3.3	41.8 ± 3.4	44.7 ± 3.2	43.0 ± 1.9	46.2 ± 3.4
Hard	4.0 ± 1.3	3.7 ± 1.0	2.9 ± 0.8	4.0 ± 1.2	4.9 ± 1.3	5.5 ±1.4	5.6 ± 1.3	5.6 ± 1.5	7.0 ± 1.9	6.7 ± 1.6
Watery or loose/mushy	19.4 ± 2.4[a]	27.1 ± 2.7[b]	19.0 ± 2.4	17.4 ± 2.4	18.2 ± 2.4	15.2 ± 2.3	12.9 ± 2.0	13.6 ± 2.1	14.2 ± 2.4	10.0 ± 1.7

[1] Beverage contained 3.4 g scFOS/L.

[2] Values with different superscripts are significantly different at the p≤0.05.

Reference: (Abbott Laboratories, 2011b)

2.4.3.2   Oligofructose or Inulin in Follow-On Formula or Weaning Foods

Brunser and colleagues (2006b) also conducted a 3-week trial of 130 children 1 to 2 years of age who had just completed 1 week of amoxicillin treatment. The toddlers received more than 500 mL/day of either a standard formula (n=66) or formula with 3.15 g oligofructose and 1.35 g inulin/L (n=64). Ingestion of at least 2.25 g per day of the oligofructose and inulin combination in infant formula had no effect on gastrointestinal symptoms including incidence of flatulence, restlessness, cramping pain, crying, vomiting or episodes of diarrhoea over the 3-week period. The treatment formula had no effect on daily stool frequency or consistency. Higher levels of faecal bifidobacteria were found in the treatment group compared to the control group after 1 week of prebiotic administration though this difference was gone by week 3. The treatment had no effect on levels of faecal *E. coli* (Brunser, Gotteland, et al., 2006).

Duggan and colleagues (2003) recruited infants from a community with a high burden of gastrointestinal infections in Peru to assess the impact of oligofructose in infant cereal (with or without added zinc) on the prevalence of diarrhoea in two trials. Infants 6 to 12 months of age were randomized to consume infant cereal with or without added oligofructose (0.55 g/15 g cereal) for a period of 6 months (Duggan et al., 2003). A standard cereal was used in the first trial, and a zinc-fortified cereal (1 mg/15 g cereal) was used in the second. In each trial, infants in the oligofructose group consumed on average 0.67 g oligofructose/day. No differences in daily cereal intakes were observed between the control and oligofructose groups, and growth parameters including weight and height were comparable between feeding groups. No dropouts were reported to be related to the oligofructose supplementation. The treatment had no effect on diarrhoea prevalence, number of hospitalizations, visits to the local clinic, use of antibiotics, or immune response to an influenza immunization.

In a multicenter, randomized, controlled trial, Bettler and colleagues (2006) examined the effects of formula containing *Bifidobacterium lactis* (probiotic) or a combination of *B. lactis* and 1.5 g oligofructose/L in toddlers 12 to 34 months old. During the 4-week study, there was no difference across formula groups in the number and kinds of formula-related adverse events including gastrointestinal adverse events. Formula acceptance was similar across groups. Mean body weights increased similarly for each formula group. There was an increased frequency of detection of *B. lactis* in the synbiotic group compared to the control group at day 7 and day 28 compared to control, but no difference compared to the probiotic group. No significant differences were found for faecal concentrations of bifidobacteria, bacteroides, streptococci, and lactobacilli in the synbiotic group compared to the other formula groups. Clostridia

counts were greater in the synbiotic group compared to the probiotic group, but the same as the control group at day 28.

In another study, Moore and colleagues (2003) randomized 56 infants, 16-42 weeks of age, to receive 0.75 g oligofructose (identified in the study as FOS; assumed to be oligofructose based on Veereman 2007) or maltodextrin/25 g serving of cereal for a period of 4 weeks. The average daily intake of oligofructose from the cereal was 1.05 g, with some infants consuming up to 3 g oligofructose/day from this source. Stool frequency increased in the oligofructose-supplemented infants and stools were softer, but no diarrhoea was reported. Additionally, there were no differences between groups in crying, spitting-up, or colic. There were no reported serious adverse events or a difference in non-serious adverse events between groups. No differences were found for stool pH. Changes in weight and length were similar between groups (Moore et al., 2003).

Saavedra and colleagues (Saavedra & Tschernia, 2002; Saavedra et al., 1999; Tschernia et al., 1999) also examined the effects of adding oligofructose to infant cereals. In this 6 month study, 140 infants 4-24 months of age were randomized to receive cereal with or without 0.55 g oligofructose/15 g cereal. Average oligofructose intake from the cereal was 1.1 g/day. The cereal was reported to be well tolerated. Oligofructose intake had no effect on frequency of reported diarrhoea, stool frequency, stool consistency, diaper rash or flatulence. The infants in the oligofructose group had improved GI status with decreased bowel movement discomfort, vomiting, regurgitation, and diarrhoea severity. Oligofructose intake resulted in normal growth and was associated with a lower incidence of febrile events, runny nose, antibiotic use, medical attention seeking, and daycare absenteeism. Based on their research, these study investigators concluded that infants and young children can consume up to 0.8 g oligofructose/kg body weight per day without experiencing any adverse gastrointestinal effects. The investigators also noted that delivery of the oligofructose in a semi-solid food (i.e., cereal) may have contributed to the tolerance of these levels of oligofructose.

Waligora-Dupriet and colleagues (2007) randomized 35 infants and toddlers ages 7 to 19 months to consume a supplement providing 2 g oligofructose or 2 g maltodextrin in food or beverage each day for a period of 3 weeks. The oligofructose was well-tolerated, and had no effect on feeding refusal, abdominal pain, and frequency and consistency of stools. The number of infectious diseases requiring antibiotic treatment, episodes of flatulence, diarrhoea, vomiting, and fever were lower in the FOS group compared to the control group. All subjects reportedly exhibited normal growth. Faecal bifidobacteria counts tended to increase in the oligofructose group as

compared to the control group, while clostridia counts decreased. Oligofructose had no effects on faecal enterococci, enterobacteria or bacteroides (Waligora-Dupriet et al., 2007) .

In an Indonesian based study, 8-month old infants consuming cereal with added oligofructose + inulin in a 70:30 combination (1 g/25 g infant cereal) had higher IgG antibody levels than infants consuming a control cereal, and mild reactions after the measles vaccination (e.g., fever, runny nose) were more often observed (Firmansyah, Pramita, Carrie Fassler, Haschke, & Link-Amster, 2000). The investigators also reported no difference between groups in growth or overall health status.

2.4.4 Summary of Studies of scFOS or Oligofructose in Infant Formula, Follow-On Formula, or Weaning Foods

A study reporting the health effects of scFOS consumption from infant formula by infants in Japan revealed that the level of scFOS in infant formula (3.2 g scFOS/L) was well tolerated and had no adverse effects on health (Yamamoto & Yonekubo, 1993). The estimated mean and 90th percentile consumption levels of scFOS from infant formula were 3.0 and 4.2 g scFOS/day, respectively. Since that time, the effects scFOS or oligofructose in infant formula on infant growth, tolerance, stool characteristics, and faecal microflora have been assessed in studies detailed in the published literature and studies conducted by Abbott Laboratories. Infants were fed scFOS or oligofructose up to a nominal concentration of 3.0 g/L in term infant formula for periods of 1 to 16 weeks in seven studies. In another study the effects of 4.0 g oligofructose/L in formula for preterm infants was assessed for 2 weeks. Results from these trials provide additional evidence to evaluate the safety and suitability of scFOS in infant formula and follow-on formula. One additional study was conducted to examine the effects of a combination of oligofructose and lc-inulin; results from this study therefore provide supportive evidence of the suitability of oligofructose in formula for term infants.

scFOS and oligofructose also have been added to follow-on formula or weaning foods to assess the effects of the oligosaccharides on faecal microflora, incidence of diarrhoea, tolerance, and general well-being. In one study, older infants and toddlers consumed an average of 2.5 g scFOS/day for 16 weeks. Older infants and toddlers consumed in the range of 0.7 to 1.1 g added oligofructose/day for a period of 6 months in two studies, and in other studies infants consumed in the range of 1 to 2 g added oligofructose daily for periods of 3 or 4 weeks. Measures of tolerance, growth, food intake, and adverse events in these studies provide information to determine the appropriateness of adding scFOS to follow-on formula. Results from two additional studies in which young children

consuming a combination of oligofructose and lc-inulin in follow-on formula provide corroborative evidence.

Anthropometric data collected in studies of infants consuming formula supplemented with scFOS or oligofructose in the first months of life indicate that the formulas support normal growth. The growth of term infants consuming up to 3.0 g scFOS/L during the first 16 weeks of life was comparable to the growth of infants consuming human milk or formula without the added scFOS (Abbott Laboratories, 2003 - 2009). Results from a study designed to assess growth also demonstrate that infants consuming up to 3.0 g oligofructose/L over a period of 12 weeks (beginning within the first two weeks of life) exhibit normal growth (Bettler & Euler, 2006). Measures of growth also were collected over periods of 1, 2, 4 or 13 weeks in smaller trials in which up to 3.0 g scFOS or oligofructose/L or up to 8.0 g oligofructose + lc-inulin/L was added to infant formula and the results suggest that the supplemented formulas support normal growth. Anthropometric measures of older infants and toddlers consuming scFOS or oligofructose also indicate that the supplemented formulas or weaning foods support normal growth.

Measures of serum markers of protein status, mineral status, kidney function, liver function, and lipids were within normal ranges in the growth studies, and provide additional evidence of the safety of the scFOS and oligofructose ingredients in formula (Bettler & Euler, 2006).

The tolerance of scFOS and oligofructose in formula was carefully monitored in studies to assess the suitability of the ingredients in formula. Measures of tolerance included occurrence of adverse events, formula intake and acceptability, occurrence of spit up and vomiting, and stool characteristics. A summary of the tolerance endpoints collected in the studies of scFOS or oligofructose in infant formula is presented in Table 9.

Across all studies in infants within the first 3 to 4 months of life, there were no significant differences among feeding groups in participant dropout rates or adverse event reports. Infants experienced increased spit-up during a week of consuming formula with added oligofructose (Euler et al. 2005), though formulas containing 3.0 g scFOS/L or 3.0 g oligofructose/L were not associated with more spit-up or vomiting in longer studies (Abbott Laboratories, 2003 - 2009; Bettler & Euler, 2006). The addition of up to 3.0 g scFOS/L or 3.0 g oligofructose/L infant formula had no effect on frequency of formula feedings and the total intake of formula as compared to intake of a comparable control formula.

Measures of water balance in infants are based on determining water intake (in feed) and water losses (urinary, faecal, evaporative and incidental (e.g. vomit)) (Bergmann, Ziegler, & Fomon, 1974; Ziegler & Fomon, 1971), and may include measures of osmolality (feed and urine). A number of these measures are reported in these studies and provide evidence of no adverse effects on water balance in healthy infants. This is further supported by the most recent study with scFOS (Abbott Laboratories, 2003 - 2009), which specifically addressed measures of water balance, including urinary specific gravity. These findings are similar to those recently reported (Fanaro et al., 2009) for formula containing GOS of similar DP at 5g/L, where no evidence of adverse effects on water balance was found.

Mean stool consistency was monitored in studies, typically by means of ranking the stool consistency on a 4- or 5-point scale at selected times during the study. Parents were given a stool diary with definitions of the stool consistency rankings, and instructed by study personnel on how to record stool consistency. Infants consuming 3 g scFOS/L in infant formula had softer stools after 4 weeks (Abbott Laboratories, 2003 - 2009). Stools became softer following a week of formula with 3.0 g oligofructose/L (Euler, et al., 2005), and infants consuming formula with 3.0 g oligofructose/L experienced less constipation than infants consuming formula with 1.5 g oligofructose/L or no added oligofructose (Bettler & Euler, 2006). Infants consuming formula containing either 4 or 8 g/L of a 1:1 combination of oligofructose and lc-inulin (Synergy1) had softer stools than infants consuming formula with no added prebiotics (Veereman-Wauters, et al., 2008). In other studies, however, the addition of scFOS or oligofructose to infant formula had no effect on stool consistency (Abbott Laboratories, 2003 - 2009; Kapiki, et al., 2007).

The occurrence of diarrhoea was monitored in studies to determine if addition of the oligosaccharide was tolerated. In no study was the occurrence of diarrhoea or watery stools significantly higher among infants consuming scFOS- or oligofructose-supplemented infant formulas as compared to infants consuming control formulas, or human milk. Throughout extended periods of monitoring in these studies (i.e., 12, 13 or 16 weeks), no concerns regarding water balance in the infants were noted.

Stool frequency increased after one week of consuming formula with 3.0 g oligofructose/L, while frequency decreased following consumption of formula with 1.5 g oligofructose/L (Euler, et al., 2005). Stool frequency also increased in infants consuming 3 g scFOS added to formula per day as compared to infants consuming 1 g lactose (Guesry, et al., 2000), and in preterm infants consuming 4 g oligofructose/L formula as compared to infants consuming formula with 4 g maltodextrin/L (Kapiki, et al., 2007). The addition of 4 g Synergy1/L to formula had no effect on stool frequency over a 4 week period, while addition of either 8 g Synergy1 or 8 g GOS+lc-inulin/L was associated with decreased stool

frequency (Veereman-Wauters, et al., 2008). In other studies, the addition of approximately 1.5, 2 or 3 g scFOS/L to formula had no effect on stool frequency in term infants (Abbott Laboratories, 2003 - 2009).

Infants consuming infant formula or a combination of human milk and infant formula have harder and fewer stools than infants fed exclusively human milk (Alarcon, Tressler, Mulvaney, Lam, & Comer, 2002; Lloyd et al., 1999). Some studies have reported that the addition of scFOS or oligofructose to formula results in softer stools, which are more similar to those of a breastfed infant. A stool softening effect has been reported for other oligosaccharides added to infant formula. Ben et al. (2004) observed softer stools in infants consuming formula with 2.4 g galacto-oligosaccharides (GOS) added per liter formula (Ben et al., 2004). Formulas containing up to 8.0 g/L of a combination of GOS and long-chain inulin also have been associated with softer stools as compared to a control formula in a dose-dependent manner (Moro et al., 2006; Moro et al., 2002).

Results from studies in older children provide additional support for a stool softening effect of added scFOS and tolerance of intakes of approximately 2 or more grams per day. Older infants and toddlers consumed an average of 2.5 g scFOS/day for 16 weeks from a milk-based beverage containing approximately 3.4 g scFOS/L; the addition of scFOS to the diet was well-tolerated, and children were at lower risk for diarrhoea and experienced a shorter duration of diarrhoea (Abbott Laboratories, 2003 - 2009). Children consuming 2 g oligofructose/day as a supplement in food or beverages had less flatulence, diarrhoea, vomiting and fever (Waligora-Dupriet, et al., 2007). Daily intake of approximately 1.1 g oligofructose was associated with less severe diarrhoeal episodes and less absenteeism from daycare (Saavedra & Tschernia, 2002). Chronically constipated children 2 to 5 years old had softer stools after consuming 0.6 g/kg/bw serving of scFOS (Abbott Laboratories, 2003 - 2009), while children ages 10-13 reportedly tolerated up to 9 g oligofructose consumed on a single occasion (unpublished data as cited in Carabin and Flamm 1999). Results from these studies indicate that scFOS or oligofructose may support the well-being of older infants and toddlers.

The available evidence suggests that scFOS or oligofructose in infant formula has no consistent or little effect on fecal microflora under the studied conditions of use. Consumption of up to 3 g scFOS/L or 3 g scFOS/day has not been found to consistently affect fecal bifidobacteria or lactobacilli counts in term infants in several studies based upon analyses with microbiological techniques (Abbott Laboratories, 2003 - 2009; Brunser, Figueroa, et al., 2006; Euler, et al., 2005; Guesry, et al., 2000), or molecular techniques (Brunser, Gotteland, et al., 2006). Faecal bifidobacteria counts in preterm infants consuming 4 g oligofructose/L for 2 weeks, however, were higher than counts in

infants consuming a control formula (Kapiki, et al., 2007), and fecal lactobacilli counts and colonization rates were significantly higher among infants consuming 3 g scFOS/L infant formula as compared to infants consuming a control formula (Abbott Laboratories, 2003 - 2009). In pediatric studies the addition of scFOS or oligofructose also has not been found to have a consistent effect on the reduction of faecal enteropathogens such as *C. difficile* (Abbott Laboratories, 2003 - 2009; Brunser, Figueroa, et al., 2006; Euler, et al., 2005).

2.4.5 scFOS and Laxation in Children

Chronic childhood constipation in one of the most common pasediatric conditions (Hyman et al., 2006; Youssef, Langseder, Verga, Mones, & Rosh, 2005). Estimates suggest up to 10% of children suffer from chronic constipation, although only about 3% seek medical advice (Roma, Adamidis, Nikolara, Constantopoulos, & Messaritakis, 1999). Chronic constipation affects quality of life. Chronic stool retention may result in recurrent abdominal pain, urinary tract pathology, and faecal soiling in children, resulting in psycosocial difficulties along with the physical trauma (Youssef, et al., 2005). Childhood constipation is typically treated with a combination of toilet training and oral laxatives (Bekkali, Bongers, Van den Berg, Liem, & Benninga, 2007; NASPGHAN Constipation Guideline Committee, 2006), and whilst fibre has often been implicated in the cause, its use as a treatment is often limited due to palatability issues.   Fructans such as scFOS, share many of the same physiological properties as fibre and have well documented stool softening effects in adult humans when administered at 0.8g/kg-bw/day (Abbott Laboratories, 2011c).

The objective of a study conducted by Abbott Laboratories (Abbott Laboratories, 2011c) to investigate the efficacy of scFOS as a treatment option for childhood constipation, was to determine the dose of scFOS at which 50% of children with a history of simple constipation produced a stool with pudding-like or watery consistency. Fifty-five children (aged 2-5 years) were enrolled into a double-masked, two group, placebo controlled, parallel, multi-centre, acute dosage titration study. The children were randomly assigned in a 3:2 fashion to receive either scFOS or sucrose after blocking on sex. Following a 3-d baseline period, each child received 0.2 g/kg body weight of scFOS or sucrose once daily at breakfast. The dose was increased in 0.2 g/kg/d increments every other day just until each child produced a pudding-like or watery stool. The maximum possible dosage was 0.8 g/kg/d. Parents recorded bowel function and subjective tolerance.

An intent-to-treat analysis of the data indicated that the effective dose of scFOS was 0.79 g/kg/d (95% CI of 0.58 to 1.12 g/kg/d). The minimum dose at which stool consistency of the scFOS group softened to a greater extent ($P = 0.004$) from baseline compared with the sucrose group occurred at the 0.6 g/kg/d dose. However, administration of scFOS did not change ($P > 0.05$) the frequency of bowel movements from baseline compared with

sucrose regardless of dose. Similar results were obtained when data were analyzed from only subjects deemed to be protocol evaluable.

The study concluded scFOS was effective at softening stools in children at a dose comparable to that reported for adults without affecting the frequency of bowel movement (Abbott Laboratories, 2011c).

2.4.6 Conclusions of Safety and Clinical Studies

The results of toxicological studies indicate that fructooligosaccharides such as scFOS (Neosugar) and oligofructose (Raftilose 95) are not mutagenic, teratogenic, or carcinogenic, and do not produce significant adverse effects in animals following acute or chronic administration of up to 20 percent (20%) in the diet. The effects that were noted in toxicological studies (i.e., increased weight of the small intestines, caecum, and colon) are consistent with the gastrointestinal changes caused by ingestion of high levels of any non-digestible material. Human tolerance to the consumption of scFOS has been established in a number of clinical trials and study results indicate that scFOS, ingested at up to 20 g/day in adults, appears to be safe and well tolerated. A study reporting the effects of scFOS consumption by infants in Japan revealed that 3.2 g scFOS/L infant formula is well tolerated and has no adverse effects based on measures of physical growth, nutritional intake, faecal properties, and general health parameters. Furthermore, results from published and unpublished clinical trials in which infants were fed formula containing up to 3.0 g scFOS (or oligofructose)/L also support the safe use of this level of added scFOS with no demonstrated effect on water balance in the infant population. Results from studies in older infants and young children also support the safe use of 3.0 g scFOS/L in young children consuming the scFOS in supplementary formulated foods or follow-on formula, and up to 0.8 lg-bw/day when used for therapeutic purposes as a laxative agent.

	Short-Chain Fructooligosaccharides							Oligofructose				
Table 19: Summary of Tolerance Endpoints in Studies of Infants Consuming scFOS or Oligofructose in Infant Formula[1]												
	Clinical Trials Conducted by Abbott Laboratories 1993 - 2009											
Reference	**Guesry et al. 2000**	**(Abbott Laboratories, 2011d)**		**Abbott Laboratories (main data submitted under Confidentiality)**		**(Abbott Laboratories, 2011d)**	**(Abbott Laboratories, 2011a)**		**Euler et al. 2005**		**Bettler & Euler 2006**		**Brunser, Figueroa et al. 2006**
Feeding Duration	2 wk	2 wk		4 wk		16 wk	5 wk		1 wk		12 wk		13 wk
Dose of scFOS or oligofructose	1, 2, or 3 g/d	1.5 g/L	3.0 g/L	2.0 g/L	3.0 g/L	3.0 g/L	2.5 g/L	2.5 g/L	1.5 g/L	3.0 g/L	1.5 g/L	3.0 g/L	2.0 g/L
Study base formula	Not specified	Whey-enriched formula		Milk-based formula		Similac with Iron (Abbott)	Soy formula with sucrose	Soy formula without sucrose	S-26® Gold (Wyeth Nutrition)		S-26® Gold (Wyeth Nutrition)		Nan 2® (Nestle)
Analytic sample size	11 or 12 (PP)	19 (PP)	20 (PP)	18 completed	23 completed	50 enrolled, 36 completed	67 enrolled, 55 completed	63 enrolled, 54 completed	28 (PP)	30 (PP)	98 (ITT)	101 (ITT)	32 (ITT)
Stool Characteristics												
Stool frequency	↑ in 3 g/day	NS	NS	NS	NS	NS	NS	NS	[↓][3]	[↑]	--[4]	--	--
Stool consistency (↓ indicates softer stools)	--	NS	NS	[↓]	[↓]	NS [↓ at d28]	NS	NS	NS	↓	--	--	--

Table 19: Summary of Tolerance Endpoints in Studies of Infants Consuming scFOS or Oligofructose in Infant Formula[1]

	Short-Chain Fructooligosaccharides								Oligofructose				
		Clinical Trials Conducted by Abbott Laboratories 1993 - 2009											
Reference	Guesry et al. 2000	(Abbott Laboratories, 2011d)		Abbott Laboratories (main data submitted under Confidentiality)		(Abbott Laboratories, 2011d)	(Abbott Laboratories, 2011a)		Euler et al. 2005		Bettler & Euler 2006		Brunser, Figueroa et al. 2006
Diarrhoea	--	NS[5]	NS[5]	NS	[↓][5]	NS[5]	NS	NS	No incidents		NS	NS	NS
Flatulence	--	NS	NS	--	--	--	NS	NS	↑	↑	NS	NS	--
Other tolerance measures													
Emesis/vomiting	--	NS	NS	--	--	NS	NS	NS	--	--	NS	NS	--
Spit-ups	--	NS	NS	[↓][6]	NS[6]	NS	NS	NS	↑	↑	--	--	--
Formula intake													
Frequency	--	NS	NS	NS	NS	NS	NS	NS	--	--	--	--	--
Quantity	--	NS	NS	NS	[↑]	NS	NS	NS	--	--	--	--	NS
Formula satisfaction	--	--	--	[↑]	[↑]	--	NS	NS	[↓]	[↓]	NS	NS	--
Dropouts													
Total dropout rate	--	5/22 vs. 1/21	4/20 vs. 1/21	NS	NS	14/50 vs. 18/52	12/64	9/62	8/36	4/34	26/98 vs. 32/98	27/101 vs. 32/98	12/32 vs. 10/33
Differences in dropouts	--	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

			Short-Chain Fructooligosaccharides			Oligofructose		
Table 19: Summary of Tolerance Endpoints in Studies of Infants Consuming scFOS or Oligofructose in Infant Formula[1]								
			Clinical Trials Conducted by Abbott Laboratories 1993 - 2009					
Reference	**Guesry et al. 2000**	**(Abbott Laboratories, 2011d)**	**Abbott Laboratories (main data submitted under Confidentiality)**	**(Abbott Laboratories, 2011d)**	**(Abbott Laboratories, 2011a)**	**Euler et al. 2005**	**Bettler & Euler 2006**	**Brunser, Figueroa et al. 2006**
Reasons for dropouts	--	Vomiting or spit up, diarrhoea, watery stools, fussiness, increased stool frequency, and weight loss, protocol violation	Adverse events, formula intolerance, protocol violation	Colic, diarrhoea, watery stools, constipation, gassiness, protocol violation, milk intolerance	Adverse events, intolerance	Adverse events (3 and 1, respectively), loss to follow-up, protocol violation	Adverse events (18 and 14, respectively), failure to return, protocol violation, physician/family request	Illness, antibiotic use, voluntary withdrawal or non-compliance; no adverse reactions to formulas

[1] Compared to control formula, significance evaluated at p < 0.05 level

[2] Analytically determined concentrations of scFOS in the formulas were 2.4 and 3.4 g scFOS/L, respectively.

[3] [ ] indicates significance level not presented

[4] -- no data available

[5] Data presented for percent watery stools; no data available for diarrhoea

[6] Spit-up and vomiting were considered the same in this study

3   Dietary Exposure

3.1.   Target Populations

This intention of this application is for the permission for the addition of scFOS to Infant Formula Products (Standard 2.9.1), Foods for Infants (Standard 2.9.3) and Supplementary Formulated Foods for Young Children (Standard 2.9.3 Division 4), which specifically cover the age ranges of birth to 12 months, and 1 to 3 years. It is envisaged that the addition of scFOS to other foods for general consumption, should it be required, would be permitted under the general food regulations, similar to inulin-derived substances for that purpose.

3.2   Dietary Intake in Infants and Young Children

3.2.1   Estimated Intake of scFOS from the Proposed Use in Formula in Australia and New Zealand

Detailed dietary modelling was completed to determine the baseline dietary exposure of infants and young children in Australia and New Zealand to oligosaccharides in the Final Review of Application P306. That dietary exposure data has been used to determine the estimated mean and 95th percentile dietary intakes of all oligofructans and GOS in the target population. The maximum level of scFOS proposed within this application is the same level as is currently allowed in the Food Regulations. On that basis there is no change to the dietary intake estimates for the age groups from that previously calculated. A summary of those intakes is shown in Table 20 and Table 21. To provide comparative information, estimated intakes based on a lower addition rate (2.5g/L versus the proposed 3.0g/L of this application) are provided.

Population groups	Age	50th percentile body weight (kg)	Estimated energy requirement (kJ/kg bw/day)	Estimated intake of formula# (mL/day)	Combined assessment: Oligofructans and GOS as total based on maximum permitted concentrations		Separate assessment: Oligofructans only based on maximum permittedconcentrations		GOS only based on maximum permitted concentrations	
					Mean (g/day)					
					Baseline	Maximum combined	Baseline	Maximum	Baseline	Maximum
Australia	3 months	6.4	343	800	0	6.4	0	2.4	0	6
	9 months	8.9	335	545	5	9.4	4	5.6	1	5
	1 year	9.6	345	425	7	10.4	5	6.3	1	5
New Zealand	3 months	6.4	343	800	0	6.4	0	2.4	0	6
	1-3 years	9.6	NA	280	17	19.2	12	12.8	5	7

Table 20: Estimated mean dietary intake of all oligofructans and GOS for *combined* and *separate assessments* for Australia and New Zealand population groups

Energy content of cow's milk based infant formula = 274 kJ/100 g
NA Not applicable

Population groups	Age	50th percentile body weight	Estimated energy requirement	Estimated intake of formula#	Combined assessment		Separate assessment			
					Oligofructans and GOS as total based on maximum permitted concentrations		Oligofructans only based on maximum permitted concentrations		GOS only based on maximum permitted concentrations	
		(kg)	(kJ/kg bw/day)	(mL/day)	95th percentile (g/day)					
					Baseline	*Maximum combined*	*Baseline*	*Maximum*	*Baseline*	*Maximum*
Australia	3 months	6.4	343	800	0	16.0	0	6.0	0	16
	9 months	8.9	335	545	12	23.5	9	14.0	3	14
	1 year	9.6	345	425	17	26.0	14	15.8	4	12
New Zealand	3 months	6.4	343	800	0	16.0	0	6.0	0	16
	1-3 years	9.6	NA	280	42	48.0	31	32	12	18

Table 21: Estimated 95th percentile dietary intake of oligofructans and GOS for *combined* and *separate* assessments for Australia and New Zealand population groups

Energy content of cow's milk based infant formula = 274 kJ/100 g

NA Not applicable

3.2.2 Estimated Intake of scFOS from the Proposed Use in Formula in the USA

Estimates of potential intakes of scFOS resulting from addition of scFOS to infant formulas and follow-on formulas at the proposed levels of use 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 was also the source of consumption data used to estimate intakes of naturally occurring oligosaccharides and scFOS added to foods.

The NHANES data set provides nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States (National Center for Health Statistics (NCHS), 2006). As part of the examination component, 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. Proxy interviews were conducted for respondents less than six years of age. 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 scFOS from naturally occurring sources, existing GRAS uses, and the proposed intended use in infant formulas and follow-on formulas.

The food codes for infant formulas reported by NHANES respondents are identified by brand name and form (e.g., prepared from powder or ready-to-feed). All formulas were included in the estimates of intake. We assumed that the total amount of infant formula consumed by NHANES participants is representative of the total amount of scFOS-supplemented infant formula or follow-on formula which would be consumed by infants of comparable ages, and that the concentration of scFOS (2.5 g per L formula) is the same in all formulas. Potential intakes of scFOS from background sources, existing GRAS uses, and the intended use in infant formulas and follow-on formulas were calculated for three subpopulations of infants (0 through 5 months, 6 through 8 months, and 9 through 11 months) who consumed infant formulas and who did not breastfeed. The 2-day average intakes represent the estimated theoretical intakes of scFOS (and the related substance, oligofructose) from naturally occurring sources, existing GRAS uses, the proposed uses in infant and follow-on formula, and from all sources combined on each of the two days of recall divided by two (i.e., (IntakeDay 1 + IntakeDay 2)/2). 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 scFOS intake from infant formula and follow-on formula based on the proposed uses of the ingredient are presented in Table 22. As shown, infants in the first six months of life are estimated to consume the highest levels of scFOS from the proposed use of scFOS in infant formula. The mean estimated intakes of scFOS from formula by formula consumers 0 through 5 months, 6 through 8 months, and 9 through 11 months of age are 2.4, 2.1, and 1.8 g/day, respectively. The estimated 90th percentile intakes of scFOS from formula by formula consumers 0 through 5 months, 6 through 8 months, and 9 through 11 months of age are 3.1, 2.8, and 2.7 g/day, respectively.

Table 22: Estimated 2-Day Average Intake of scFOS from Proposed Use of 2.5 g scFOS/L in Infant Formula and Follow-on Formula				
Age Group[a]	scFOS Intake			
	g/day		g/kg bw/day	
	Mean	90th Percentile	Mean	90th Percentile
0 - 5 months (n = 101)	2.4	3.1	0.40	0.57
6 - 8 months (n = 86)[b]	2.1	2.8	0.26	0.38
9 - 11 months (n = 82)	1.8	2.7	0.20	0.28
Data source: NCHS (2006) [a] Sample population limited to infants who consumed infant or follow-on formula. [b] A bodyweight was not available for one infant in the 6 – 8 mo group; estimates of intake in g/kg bw/day based on 85 infants.				

Estimates of oligofructose intake from naturally occurring sources, theoretical intakes of scFOS from current US GRAS sources, estimates of scFOS from the proposed uses in formula, and theoretical estimates of total potential exposures to oligofructose and scFOS from all sources combined are shown in Table 23.

The estimates of potential scFOS 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 only infant formula. The intakes of scFOS by infants consuming a combination of human milk and formula would likely be lower than the estimates of scFOS intake based on the population of exclusively formula fed infants.

Table 23: Theoretical 2-Day Average Intake of scFOS from Background Sources, Existing GRAS Uses, and Proposed Use in Infant Formula and Follow-on Formula

Age Group[a]	FOS Source	scFOS Intake			
		g/day		g/kg bw/day	
		Mean	90th Percentile	Mean	90th Percentile
0 - 5 months (n = 101)	Background[c]	<0.05	0.1	0.01	0.01
	Existing US GRAS uses	0.4	1.0	0.06	0.14
	Proposed use in infant/follow-on formula[d]	2.4	3.1	0.40	0.57
	Total scFOS intake	2.8	4.6	0.47	0.47
6 - 8 months (n = 86) [b]	Background	0.2	0.4	0.02	0.05
	Existing US GRAS uses	1.3	2.3	0.15	0.26
	Proposed use in infant/follow-on formula	2.1	2.8	0.26	0.38
	Total scFOS intake	3.5	4.7	0.43	0.43
9 - 11 months (n = 82)	Background	0.5	1.1	0.05	0.11
	Existing US GRAS uses	2.6	4.6	0.27	0.47
	Proposed use in infant/follow-on formula	1.8	2.7	0.20	0.28
	Total scFOS intake	4.9	7.2	0.52	0.52

Data source: NCHS (2006)

[a] Sample population limited to infants who consumed infant or follow-on formula.

[b] A bodyweight was not available for one infant in the 6 – 8 mo group; estimates of intake in g/kg bw/day based on 85 infants.

[c] Intake of oligofructose from naturally occurring sources.

[d] Assumed 2.5 g scFOS/L formula.

3.3 Use of scFOS in Infant Formula, Foods for Infants and Foods for Young Children

3.3.1 International Regulatory Status of Short Chain Fructo-oligosaccharides in Foods

Regulatory bodies in the European Union and Australia/New Zealand have developed specific opinions or regulations regarding the use of FOS in infant formula. These statements or regulations are listed in Table 24 and described below. In the USA the status of ingredients is based on notification under the US GRAS system and for infant formula subsequent compliance with 21 CFR 106 Infant Formula Quality Control Procedures and 21 CFR 107 Infant Formula, which include requirements for premarket assessment prior to reformulation and the addition of new ingredients. Information for the USA is included in the table for comparison. A similar system of GRAS and premarket approval is required in Canada. A similar system is required in Canada. Where appropriate regulations for inulin and GOS are included in the summary table, as these non-digestible carbohydrates are frequently used in combination in foods for infants, children, and adults. This section is not a comprehensive review of all the worldwide regulations or guidelines for these ingredients.

In Japan, scFOS has been permitted for use in infant formula since the early 1990's (Yamamoto & Yonekubo, 1993), with no limitation.

A number of developing countries, both in Asia and other parts of the world, default to CODEX Alimentarius Standards. The CODEX Standards (*CODEX Stan 72-1891 (rev. 2007) Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants* and *CODEX Stan 156-1987 Standard for Follow-up Formula*) include clauses for the addition of "Optional Ingredients". These ingredients may be added on the basis they are suitable for use in the product, are scientifically shown to be efficacious and are present at sufficient levels to ensure the intended effects are achieved. On this basis the use of scFOS would be viewed as permissible in those countries defaulting to the CODEX standard.

Throughout various countries, oligosaccharides are referred to by various terms, including fructooligosaccharide (FOS), short-chain fructooligosaccharide (scFOS), oligofructose, inulin, long-chain inulin (lc-inulin) and high molecular weight oligofructose. These terms have no standard definitions, and some of the terms are used interchangeably to describe substances that vary substantially in chain length (M. Roberfroid, 2007). Because of this nomenclature issue, there may be some confusion about what specific ingredient various opinions, guidelines, or regulations are intended for in some cases.

Table 24: Food Additive Regulations for FOS, Inulin, and GOS				
Country	Substance	Approved Uses	Maximum Use Level	Reference
USA	scFOS (Nutraflora, Actilight, Meioligo, Neosugar)	Infant & Follow-on Formula (0-12 months)	2.5g/L	As marketed
		Toddler (12-24 mo) foods	1 g/svg	FDA 2000, 2007
		General food supply	1 g/svg	
	Inulin (Frutafit)	Baby foods	1 g/svg	FDA 2003b, 2008a
		General food supply	1-15%	
	GOS (Vivinal)	Term infant formula	5 g/L	FDA 2008b
		Baby foods	1-3 g/svg	
		General food supply	3-7.5 g/svg	
	GOS (GTC)	Term infant formula & follow-on Formula	7.2 g/L	(Food and Drug Administration (FDA), 2009b)
		Baby, infant & toddler foods	0.86-1.28 g/svg	(Food and Drug Administration (FDA), 2009a)
		General food supply	1.28 g/svg	(Food and Drug Administration (FDA), 2009a)
E.U.	FOS[a]	Not in infant formula	NA	Scientific Committee on Food 2003; EFSA 2004
		Follow-on formula	As established by generally accepted scientific data	Commission Directive 2006/141/EC (Article 6)
	LC-inulin and GOS	Infant formula	8 g/L of 90:10 mixture of GOS and LC-inulin	Commission Directive 2006/141/EC (Annex I (9) and Annex II (7)
		Follow-on formula		

Table 24: Food Additive Regulations for FOS, Inulin, and GOS				
Country	Substance	Approved Uses	Maximum Use Level	Reference
Australia/New Zealand	scFOS[b]	Not in infant formula	NA	FSANZ 2008a,b
	Inulin derived substances[b]	General food supply	No specific limits	
	Inulin derived substances and/or GOS, singularly or combined, in any ratio	Infant and follow-on formula	3 g/L	
		Infant foods	0.8 g/100 g	
		Formulated supplementary foods	1.6 g/svg (8 g/L)	

[a] No differentiation between scFOS and oligofructose.

[b.] FSANZ uses the term FOS to describe fructose polymers with average DP<4 that are typically produced from enzymatic condensation of sucrose; defines inulin derived substances as a mixture of polymers of fructose and includes inulin, long-chain inulin and oligofructose, but does not include fructose polymers enzymatically produced from sucrose by enzymatic action; and defines GOS as a mixture of those substances produced from lactose by enzymatic action.

3.3.2 Use of scFOS in Infant Formula, Foods for Infants and Foods for young Children

Meiji pioneered the use of scFOS in infant formula during the early 1990's and today in Japan it is widely used (Yamamoto & Yonekubo (1993)) in products for infants and young children. Meiji manufactures infant formula in Japan, and under contract in Australia. The markets for Meiji nutritional products include Japan, Pakistan, China, Vietnam, & Taiwan (Figure 4 and Figure 5).

http://www.meinyu.co.jp/english/trust/index.html (accessed 17 October 2009).

Figure 5: Meiji nutritional products for export.

Products for sale in Japan that are known to contain scFOS include:

- Meiji Hohoemi (infant formula for infants up to 9 months) with 3.2g scFOS /litre
- Meiji Milfy (infant formula for infants with cows milk allergy)
- Meiji Step (follow-on formula for infants 9 months and over)

Figure 6: Meiji Hohoemi, Milfy and Step Infant Formula Products

In Spain Blemil Plus AE (follow-on formula for infants 6 months and older) and Natur 2 (follow-on formula for infants 4 months and older) both contain scFOS.

In the United States, Abbott Nutrition markets Similac® Go & Grow® Soy-Based Infant Formula Powder for infants 9-24 months of age, containing scFOS (2.2 g/L) and Similac® Sensitive® Isomil Soy™ Infant Formula Powder for infants 0-12 months with scFOS (2.1 g/L) (Abbott Laboratories, 2010). NutraFlora is also added to medical foods for children available in the USA and Canada. Vital Jr.™ and PediaSure® Enteral Formula With Fiber and scFOS® are enteral products designed for sole-source or supplemental nutrition for children ages 1 to 13 years (Abbott Nutrition, 2009). Each product contains 3 - 4 g scFOS/L, and each product has a caloric density of 1 kcal/mL.

Figure 7: Similac® Sensitive® Isomil Soy™ Infant Formula Pack and Nutrition Information Panel

NUTRIENTS PER 100 CALORIES (5 FL OZ, PREPARED AS DIRECTED)

PROTEIN	2.45	g	WATER	133	g
FAT	5.46	g	LINOLEIC ACID	1000	mg
CARBOHYDRATE	10.4	g			

VITAMINS

VITAMIN A	300	IU	NIACIN	1350	mcg
VITAMIN D	60	IU	FOLIC ACID (FOLACIN)	15	mcg
VITAMIN E	1.5	IU	PANTOTHENIC ACID	750	mcg
VITAMIN K	11	mcg	BIOTIN	4.5	mcg
THIAMIN (VIT. B_1)	60	mcg	VITAMIN C		
RIBOFLAVIN (VIT. B_2)	90	mcg	(ASCORBIC ACID)	9	mg
VITAMIN B_6	60	mcg	CHOLINE	12	mg
VITAMIN B_{12}	0.45	mcg	INOSITOL	5	mg

MINERALS

CALCIUM	105	mg	COPPER	75	mcg
PHOSPHORUS	75	mg	IODINE	15	mcg
MAGNESIUM	7.5	mg	SELENIUM	1.8	mcg
IRON	1.8	mg	SODIUM	44	mg
ZINC	0.75	mg	POTASSIUM	108	mg
MANGANESE	25	mcg	CHLORIDE	62	mg

INGREDIENTS: 39.2% CORN SYRUP SOLIDS, 14.7% SOY PROTEIN ISOLATE, 11.5% HIGH OLEIC SAFFLOWER OIL, 9.6% SUGAR (SUCROSE), 8.4% SOY OIL, 7.7% COCONUT OIL; **LESS THAN 2% OF:** C. COHNII OIL', M. ALPINA OIL‡, BETA-CAROTENE, LUTEIN, LYCOPENE, FRUCTOOLIGOSACCHARIDES, CALCIUM PHOSPHATE, POTASSIUM CITRATE, POTASSIUM CHLORIDE, MAGNESIUM CHLORIDE, SODIUM CHLORIDE, ASCORBIC ACID, CHOLINE CHLORIDE, L-METHIONINE, TAURINE, ASCORBYL PALMITATE, FERROUS SULFATE, m-INOSITOL, MIXED TOCOPHEROLS, ZINC SULFATE, d-ALPHA-TOCOPHERYL ACETATE, L-CARNITINE, NIACINAMIDE, CALCIUM PANTOTHENATE, CUPRIC SULFATE, THIAMINE CHLORIDE HYDROCHLORIDE, VITAMIN A PALMITATE, RIBOFLAVIN, PYRIDOXINE HYDROCHLORIDE, FOLIC ACID, POTASSIUM IODIDE, POTASSIUM HYDROXIDE, PHYLLOQUINONE, BIOTIN, SODIUM SELENATE, VITAMIN D_3 AND CYANOCOBALAMIN. **CONTAINS SOY INGREDIENTS.**

*CONTAINS NO DAIRY INGREDIENTS, MANUFACTURED ON DAIRY EQUIPMENT.
†SOURCE OF DOCOSAHEXAENOIC ACID (DHA)
‡SOURCE OF ARACHIDONIC ACID (ARA)
SOY PROTEIN ISOLATE IS A SOURCE OF NUCLEOTIDES.

Figure 8: Similac® Go & Grow® Pack and Nutrition Information Panel

NUTRIENTS PER 100 CALORIES (5.1 FL OZ, PREPARED AS DIRECTED)

PROTEIN	2.80	g
FAT	5.40	g
CARBOHYDRATE	10.3	g
WATER	133	g
LINOLEIC ACID	1000	mg

VITAMINS

VITAMIN A	300	IU
VITAMIN D	60	IU
VITAMIN E	3	IU
VITAMIN K	11	mcg
THIAMIN (VIT. B_1)	60	mcg
RIBOFLAVIN (VIT. B_2)	90	mcg
VITAMIN B_6	60	mcg
VITAMIN B_{12}	0.45	mcg
NIACIN	1350	mcg
FOLIC ACID (FOLACIN)	15	mcg
PANTOTHENIC ACID	750	mcg
BIOTIN	4.5	mcg
VITAMIN C (ASCORBIC ACID)	12	mg
CHOLINE	12	mg
INOSITOL	5	mg

MINERALS

CALCIUM	195	mg
PHOSPHORUS	130	mg
MAGNESIUM	7.5	mg
IRON	2	mg
ZINC	0.75	mg
MANGANESE	25	mcg
COPPER	75	mcg
IODINE	15	mcg
SELENIUM	1.8	mcg
SODIUM	44	mg
POTASSIUM	120	mg
CHLORIDE	62	mg

INGREDIENTS: 38.6% CORN SYRUP SOLIDS, 16.4% SOY PROTEIN ISOLATE, 10.9% HIGH OLEIC SAFFLOWER OIL, 9.6% SUGAR (SUCROSE), 8.1% SOY OIL, 7.4% COCONUT OIL, 3.0% CALCIUM PHOSPHATE; **LESS THAN 2% OF:** C. COHNII OIL‡, M. ALPINA OIL‡, BETA-CAROTENE, LUTEIN, LYCOPENE, FRUCTOOLIGOSACCHARIDES, POTASSIUM CITRATE, SODIUM CHLORIDE, CALCIUM CARBONATE, MAGNESIUM CHLORIDE, ASCORBIC ACID, L-METHIONINE, POTASSIUM CHLORIDE, CHOLINE CHLORIDE, TAURINE, FERROUS SULFATE, ASCORBYL PALMITATE, m-INOSITOL, MIXED TOCOPHEROLS, ZINC SULFATE, d-ALPHA-TOCOPHERYL ACETATE, L-CARNITINE, NIACINAMIDE, CALCIUM PANTOTHENATE, CUPRIC SULFATE, THIAMINE CHLORIDE HYDROCHLORIDE, VITAMIN A PALMITATE, RIBOFLAVIN, PYRIDOXINE HYDROCHLORIDE, FOLIC ACID, POTASSIUM IODIDE, PHYLLOQUINONE, BIOTIN, VITAMIN D_3, CYANOCOBALAMIN, SODIUM SELENATE AND POTASSIUM HYDROXIDE. **CONTAINS SOY INGREDIENTS.**

*CONTAINS NO DAIRY INGREDIENTS. MANUFACTURED ON DAIRY EQUIPMENT.
†SOURCE OF DOCOSAHEXAENOIC ACID (DHA)   ‡SOURCE OF ARACHIDONIC ACID (ARA)

U.S. Patents 5,221,545; 6,136,858; 6,596,767; 7,090,879; D576,035 and D578,401

Figure 9: Similac® Go & Grow Pack and Nutritional Information Panel

Also in the USA, PBM products manufacture and sell Bright Beginnings Soy Pediatric Drink (Figure 9), a ready-to-drink supplement specially formulated to meet the nutritional needs of children who may be allergic to cow's milk protein or lactose-intolerant, containing scFOS. (PBM Products, 2010). It contains a total of 3g dietary fibre per 237mL (8 fl. oz) serve.

Figure 10: Bright Beginnings Soy Pediatric Drink

scFOS is an ingredient in a number of foods for infants and children around the world, with a selection of examples summarised in Table 25, and product pack images shown in Appendix 1.

Table 25: Products for infants and young children containing scFOS ingredient			
Type of Product	**Country**	**Products Enriched with scFOS (Trade names: NutraFlora, Meioligo, Actilight)**	**Manufacturer**
Infant Cereal	Spain	Blevit Plus (infant cereal without gluten)	Laboratorios Ordesa
	Spain	Hero Baby (infant cereals 2 types - 8 Cereales con Miel and Multicereales)	Hero
	Spain	Santuri Sandoz (infant cereal)	Nutrition and Santé
	France	Modilac Douceur Veloutée (infant cereal)	Soldilac
Baby Food Puree	USA	Beech Nut baby food (Apple, Banana, Carrot, & Sweet Potato flavours)	Beech Nut
Baby Yoghurt	USA	Horizon Organic Baby Yogurt 4 oz cups (Peach, Pear, and Vanilla flavours)	White Wave
Medical Nutrition – Young Children	USA	PediaSure & Vital Jr	Abbott Nutrition
	Canada	PediaSure	Abbott Nutrition
Children's Cereals	USA	Wild Puffs (several flavours)	Barbara's Bakery
	USA	Eating Right Kids, Puffed Cocoa Cereal	Safeway
	USA	Eating Right Kids, Toasted O's,	Safeway
	USA	Eating Right Kids, Fruity Ringlets Cereal	Safeway
	USA	Monkeybrains Oatmeal	Shrky LLC

3.3.3 Use of scFOS in the General Food Supply

3.3.3.1 Australia and New Zealand

In New Zealand and Australia scFOS is available within the general food supply as supplements. These products are available through retail outlets and on-line and are sold as being suitable for children and adults.

Several nutritional type dietary supplementary foods available in Australia may also contain scFOS (Figure 10). For example, the Ensure Pudding range (3g scFOS/ 4oz (125g) serving) and Enrich Plus beverages (13g scFOS/litre) from Abbott Laboratories, available primarily through hospitals, and to a limited extent from pharmacies.

Figure 11: Examples of supplements and food-type supplements available in Australia and New Zealand containing scFOS

3.3.3.2   Rest of the World

The use of scFOS as a prebiotic ingredient in a wide range of food products for older children and adults is steadily increasing around the world. A summary of selected applications, representing products that may possibly also be consumed by young children, is summarised in Table 26. It is impractical to summarise all the products available globally, however additional information and products containing scFOS can be found on the NutraFlora website (GTC Nutrition, 2009).

In Japan, FOS (including) scFOS has been included in the approved FOSHU list of functional ingredients for a number of years (Chow, 2002; Ohama, Ikeda, & Moriyama, 2006). Given the dominance of Meiji within the Japanese food industry, scFOS is one of the key ingredients sources of oligofructans for functional prebiotic ingredient and supplement use.

Table 26: Selected foods containing scFOS			
Class of Product	**Country**	**Products Enriched with scFOS (Trade names: NutraFlora, Meioligo, Actilight)**	**Manufacturer**
Medical Nutrition	USA, Canada & Japan	Ensure, various flavours	Abbott Nutrition
	USA, Canada & Japan	Ensure Plus, various flavours	Abbott Nutrition
	USA & Canada	Glucerna, various flavours	Abbott Nutrition
Cultured Dairy	USA	Horizon Organic Low-fat Blended Yoghurt 6 oz cups - Blueberry, Lemon, Peach, Raspberry, Strawberry, and Strawberry	White Wave

Class of Product	Country	Products Enriched with scFOS (Trade names: NutraFlora, Meioligo, Actilight)	Manufacturer
		Table 26: Selected foods containing scFOS	

Class of Product	Country	Products Enriched with scFOS (Trade names: NutraFlora, Meioligo, Actilight)	Manufacturer
		Banana	
	USA	Horizon Organic Fat-Free, Fruit-on-the-bottom Yoghurt 6 oz cups - Blueberry, Cherry, Peach, Raspberry, Strawberry, Mixed Berry	White Wave
	USA	Horizon Organic Whole Milk Yoghurt Vanilla 32 oz tubs	White Wave
	USA	Horizon Organic Fat Free Yoghurt 32 oz tubs. Plain and Vanilla	White Wave
	USA	BeneFIT nonfat yoghurt 4oz cups - Blueberry, Raspberry, Strawberry, and other flavours	Walmart
	USA	Eating right Yoghurt 4 oz cups various flavours	Safeway
	Mexico	Masau Yoghurt beverage	Masau
Cereal	USA	Puffins, Multigrain	Barbara's Bakery
Bakery	USA	De Wafelbakkers frozen pancakes and waffles - Blueberry, Apple Cinnamon, Seven Grain, Oat Bran, etc.	DeWafelbakkers
Frozen Desserts	USA	Julie's Organic low fat Yoghurt Bars	Oregon Ice Cream
	USA	Eating Right Vanilla Ice Cream Cups	Safeway
	Colombia	Mimo's Soy Frozen Dessert	Mimo's
Beverages	USA	Vitasoy Plus, Soy Beverage – plain flavour	Vitasoy
	USA	NuVim	NuVim
	USA	Naked Juice (various flavours)	Pepsico

In addition to foods scFOS (NutraFlora) is widely available to the general public in dietary supplements (Figure 11).

Figure 12: Examples of supplements available in the USA containing scFOS

Pictorial examples of various products from around the world, containing scFOS are provided in Appendix I, and examples of product information brochures form the USA in Appendix II. These examples provided with the intent of demonstrating safe and extensive use of scFOS. As these examples are from outside Australia and New Zealand, the images may not meet the Australia New Zealand Food Standards code in relation to labelling etc. The images and product representations as depicted, are not intended for use in Australia or New Zealand.

4. Information related to impact on the food industry

4.1 Projected cost to the food industry

Permission for the optional addition of scFOS to Infant Formula Products, Foods for Infants, and FSFYC is not anticipated to have any major impact on the market for these products in Australia and New Zealand. Possible outcomes of the permission to add scFOS may include the reformulation of some existing items, or the launch of some new products.

The market for Infant Formula Products in Australia and New Zealand is segmented into standard formula, premium formula and small volumes of specialised formula (e.g. for metabolic, immunological, renal, hepatic and malabsorptive conditions). The addition of ingredients such as oligosaccharides, long-chain polyunsaturated fatty acids etc. is typically limited to the premium and specialised formulas due to the added cost of manufacture and higher cost of product at the point of sale due to the inclusion of those ingredients. The addition of scFOS to infant formula will not have any significant impact on the market size or structure, however may ultimately extend consumer choice options through the introduction of new, or reformulation of existing product offerings.

Similarly the new products in the category of Foods for Infants and FSFYC may be developed or reformulated, expanding consumer options. There are incremental manufacturing costs associated with the addition of the scFOS as an ingredient, which would typically be passed on to the consumer.

4.2 Impact on international trade

The permitted addition of scFOS to Infant Formula Products, Foods for Infants, and FSFYC will have a positive effect on international trade. It will remove current constraints to the export of these products to countries requiring compliance to local regulations in the country of manufacture, thus permitting new manufacture for increased export opportunities. Furthermore, it will allow manufacturers and brand owners to acquire Certificates of Free Trade form authorities in Australia and New Zealand in regard to the products manufactured for export, and remove the need for administrative processes currently required to enable export.

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Appendix I: Product Images

Infant Formulas

Infant Cereal

Pureed Baby Food

Cultured Dairy

Kid's Cereals

Frozen Dairy

Beverages & Bakery

Medical Nutritional Products

Appendix II: Product Information
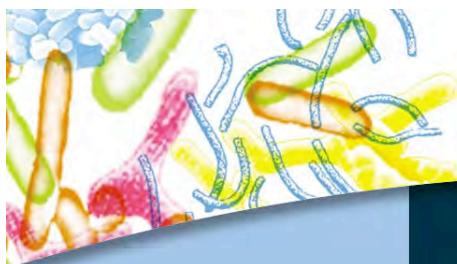

Ensure Plus®

See what all the *plus* is about

- Supports a healthy immune system
- Enhances calcium absorption
- Promotes digestive tract health
- Helps maintain regularity

*12 fl oz of Ensure Plus per day are needed to get 3 g NutraFlora to enhance calcium absorption.

Ensure Plus® with NutraFlora® scFOS PREBIOTIC SOLUBLE FIBER

NutraFlora® creates positive

Prebiotics Primer

The balance of gut bacteria contributes to normal intestinal function, digestion, and immune health.

Prebiotics are nondigestible ingredients that stimulate the growth of bacteria. In essence, prebiotics serve as food for bacteria.

Some prebiotics can be broken down by digestive enzymes in the saliva, stomach, and small intestine. Prebiotics that break down early in the digestive tract serve as food for both good *and* bad bacteria.

NutraFlora is a rare form of prebiotic in that it feeds only the good bacteria. When beneficial species of bacteria increase, pathenogenic bacteria tend to decrease.

Start With the GI Tract

Approximately 70% of human immunity is found in the digestive tract

The intestines represent 400 square meters of Gut Associated Lymphoid Tissue (GALT)

- Home to 100 trillion bacterial cells=3 pounds of bacteria

The gut microflora break down fermentable fibers and other carbohydrates that are not digested in the upper GI tract
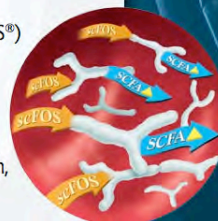
- This breakdown produces fatty acids that are important for supporting a healthy intestinal barrier

▶ Enter NutraFlora

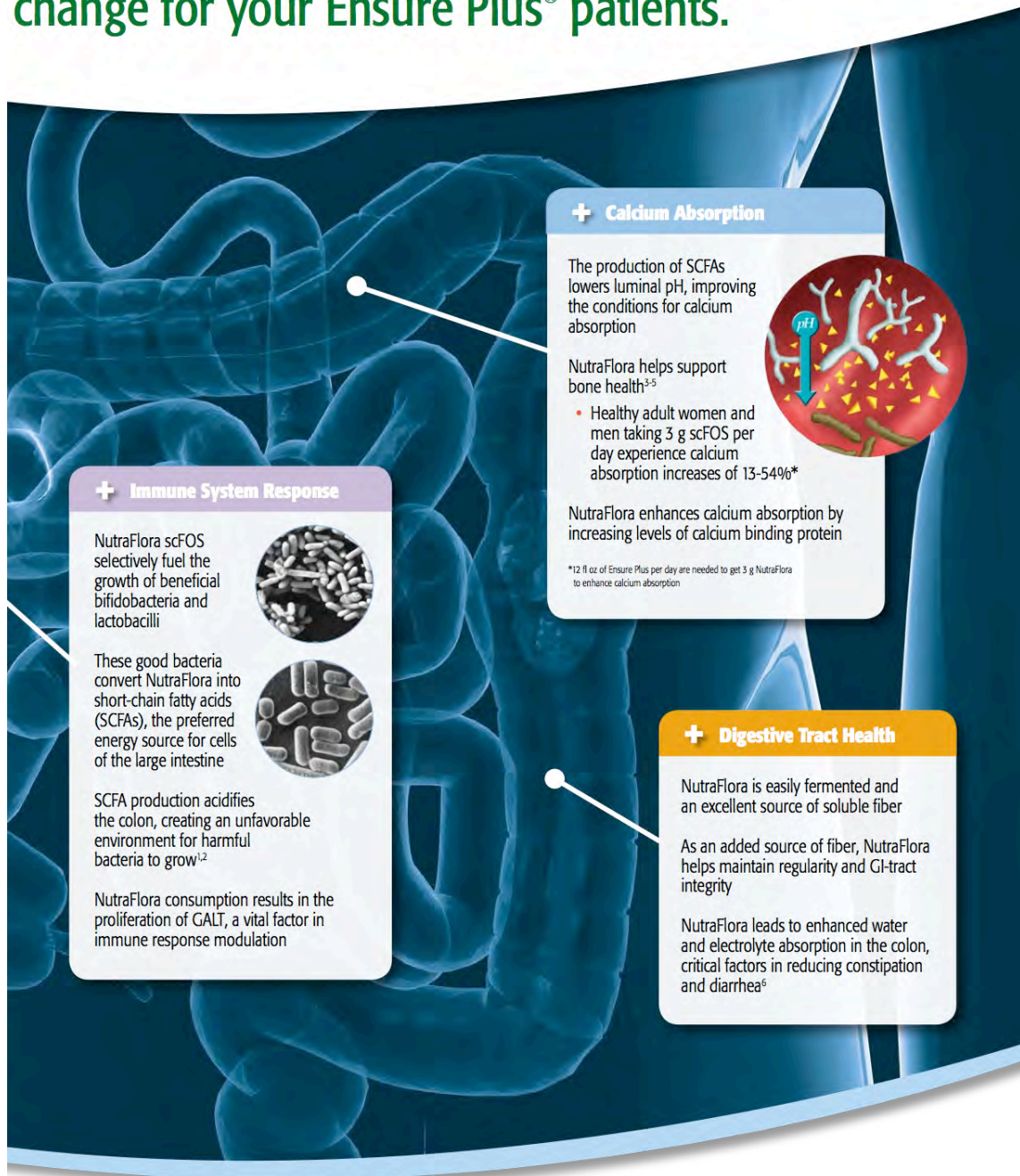NutraFlora short-chain fructooligosaccharides (scFOS®) are a natural prebiotic soluble fiber

NutraFlora scFOS work in the large intestine. They are not digested in the mouth, stomach, or small intestine

NutraFlora scFOS are food for good bacteria, and only good bacteria, in the large intestine

change for your Ensure Plus® patients.

+ Calcium Absorption

The production of SCFAs lowers luminal pH, improving the conditions for calcium absorption

NutraFlora helps support bone health[3-5]

- Healthy adult women and men taking 3 g scFOS per day experience calcium absorption increases of 13-54%*

NutraFlora enhances calcium absorption by increasing levels of calcium binding protein

*12 fl oz of Ensure Plus per day are needed to get 3 g NutraFlora to enhance calcium absorption

+ Immune System Response

NutraFlora scFOS selectively fuel the growth of beneficial bifidobacteria and lactobacilli

These good bacteria convert NutraFlora into short-chain fatty acids (SCFAs), the preferred energy source for cells of the large intestine

SCFA production acidifies the colon, creating an unfavorable environment for harmful bacteria to grow[1,2]

NutraFlora consumption results in the proliferation of GALT, a vital factor in immune response modulation

+ Digestive Tract Health

NutraFlora is easily fermented and an excellent source of soluble fiber

As an added source of fiber, NutraFlora helps maintain regularity and GI-tract integrity

NutraFlora leads to enhanced water and electrolyte absorption in the colon, critical factors in reducing constipation and diarrhea[6]

The New Taste of Tolerance

The New Shape of Tolerance

Vital jr.

Therapeutic Semi-Elemental Nutrition for Children

Taste Is The Difference

Do you have patients that could begin oral intake, but refuse to drink other semi-elemental formulas?
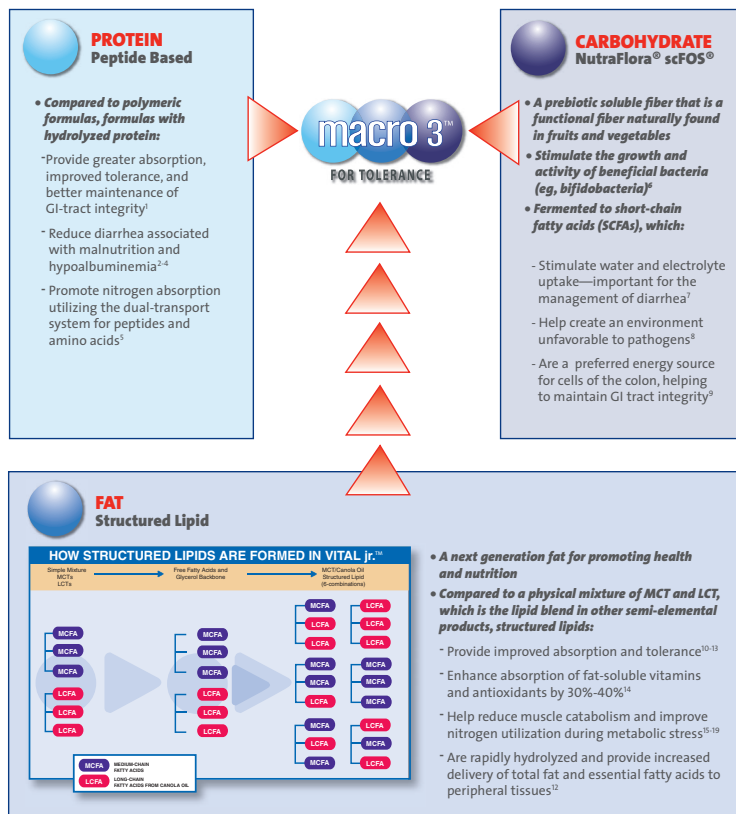
- Vital jr.™ is available in two delicious "Kid-Approved" flavors — Vanilla and Strawberry — to help oral compliance

- The flavors were tested with children, and THEY said Vital jr. tastes great

- Vital jr. received the 2007 ChefsBest™ Award for Best Taste

* The ChefsBest Award for Best Taste is awarded to the brand rated highest overall among leading brands by independent professional chefs. This award is only given if there is a statistically significant difference between products in a category.

Tolerance Is The Difference

macro 3 **is an advanced PROTEIN, FAT, and CARBOHYDRATE blend specifically designed for excellent tolerance and absorption**

PROTEIN
Peptide Based

- *Compared to polymeric formulas, formulas with hydrolyzed protein:*

  - Provide greater absorption, improved tolerance, and better maintenance of GI-tract integrity[1]

  - Reduce diarrhea associated with malnutrition and hypoalbuminemia[2-4]

  - Promote nitrogen absorption utilizing the dual-transport system for peptides and amino acids[5]

CARBOHYDRATE
NutraFlora® scFOS®

- *A prebiotic soluble fiber that is a functional fiber naturally found in fruits and vegetables*
- *Stimulate the growth and activity of beneficial bacteria (eg, bifidobacteria)[6]*
- *Fermented to short-chain fatty acids (SCFAs), which:*

  - Stimulate water and electrolyte uptake—important for the management of diarrhea[7]

  - Help create an environment unfavorable to pathogens[8]

  - Are a preferred energy source for cells of the colon, helping to maintain GI tract integrity[9]

macro 3™
FOR TOLERANCE

FAT
Structured Lipid

HOW STRUCTURED LIPIDS ARE FORMED IN VITAL jr.™

Simple Mixture MCTs LCTs → Free Fatty Acids and Glycerol Backbone → MCT/Canola Oil Structured Lipid (6-combinations)

| MCFA | MEDIUM-CHAIN FATTY ACIDS |
| LCFA | LONG-CHAIN FATTY ACIDS FROM CANOLA OIL |

- *A next generation fat for promoting health and nutrition*
- *Compared to a physical mixture of MCT and LCT, which is the lipid blend in other semi-elemental products, structured lipids:*

  - Provide improved absorption and tolerance[10-13]

  - Enhance absorption of fat-soluble vitamins and antioxidants by 30%-40%[14]

  - Help reduce muscle catabolism and improve nitrogen utilization during metabolic stress[15-19]

  - Are rapidly hydrolyzed and provide increased delivery of total fat and essential fatty acids to peripheral tissues[12]

Safety Is The Difference

Introducing Vital jr. in the prefilled container designed with kids and clinicians in mind

MINIMIZE FORMULA HANDLING
- Cap has recessed port and is covered by a protective "No IV" peel-off sticker

HELPS REDUCE RISK OF TOUCH-CONTAMINATION
- Meets HACCP recommendations[20]
- Usable up to 48 hours after initial spike when clean technique and only one new feeding set is used

EXCLUSIVE ROSS SEMIRIGID CONTAINER
- Easy to distinguish from an IV-type bag
- Easy to handle and store
  - Reduces preparation time
  - Stands upright to save storage space

EXCLUSIVE 500-mL SIZE
- Size is more appropriate for use in some pediatric patients and may reduce waste

The Vital *jr.*™ Difference for Oral and Tube Feeding

Vital jr. is designed for children ages 1-13 with malabsorption and maldigestion and for patients with:

- Celiac Disease
- Inflammatory Bowel Disease
- Cystic Fibrosis
- Pancreatic Disorders
- Chronic Diarrhea
- Short-Bowel Syndrome

NUTRITIONAL INFORMATION

	Per 8 fl oz	Per 500 mL
Calories	237	500
Protein, g	7.1	15.0
Total Fat, g	9.6	20.0
Total Carbohydrate, g	31.7	67.0
Dietary Fiber, g	0.71	1.5
L-Carnitine, mg	4.0	8.5
Nutrient Density (Cal/mL)	1.0	1.0
Protein (% Cal)	12	12
Carbohydrate (% Cal)	53	53
Fat (% Cal)	35	35
Kosher	Yes	Yes
Lactose-Free	Yes	Yes
Gluten-Free	Yes	Yes
Osmolality (mOsm/kg H_2O)	390	390

Use under medical supervision.

The only pediatric semi-elemental product:

- With "Kid-Approved" Taste
- With Macro 3™ for Tolerance
- In the 500-mL Prefilled Container
- Indicated for Children 1 to 13 Years of Age*
- With 50% MCT *PLUS* Structured Lipid for Easier Absorption
- With Certified Kosher Status

*Meets or exceeds 100% of DRIs for protein, vitamins, and minerals for children 1 to 8 years of age in 1000 mL; for children 9 to 13 years of age in 1500 mL.

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AVAILABILITY

8-fl-oz cans; 24/case

Vanilla	#59762
Strawberry	#59760

500-mL Ready-To-Hang®; 8/case

Vanilla	#50096

NutraFlora® and scFOS® are not trademarks of Abbott Laboratories.
PREBIOTIC • SOLUBLE FIBER

ROSS NUTRITION
GROWING STRONG KIDS

Appendix III: Microbial Study of the Enzyme Preparation

Note: Original reports as reproduced were marked Confidential at the time of publication, and the type was unable to be removed. These documents are purposefully included in this Public document.

METHOD FOR MEASUREMENT OF THE ENZYME ACTIVITY

I. Preparation of reagents

(I) Preparation of buffer solution McIlvain Buffer)

a) Dissolve 35.8g of $Na_2HPO_4.12H_2O$ or 17.8g of $Na_2HPO_4.2H_2O$ in distilled water and fill accurately up to 1000 ml,

b) Dissolve 21,0g of citric acid in distilled water and fill accurately up to 1000 ml.

c) Add b) into a) while stirring to make pH 5. 00 ± 0.01 using pH meter at 25°C.

(2) Preparation of substrate solution

Disolve 25,0 g of sucrose, which has been previously dried at 105 °C for 6 hours, in distilled water and fill acurately up to 100 ml.

(3) Preparation at enzyme suspension

a) Weigh about 1 g of enzyme powder accurately in 100 ml beaker and add 50ml at McIlvain Buffer

b) After agitation for 10 to 16 min using a magnetic stirrer, transfer the suspension with rinsing buffer into a 100ml volumetric flask and fill accurately up to100 ml with McIlvain Buffer.
..... (the first dilution x 100)

c) Transfer b) to a 200ml beaker and agitate it furthermore using a magnetic stirrer.

d) Accurately weigh 1 ml of the enzyme suspension

c) under agitation using a pipette into a 100 ml volumetric flask and fill it up to 100 ml with McIlvain Buffer.

Tech. App. V -25

Note: it is better

I) to mill the enzyme powder previously, and
2) to dilute the enzyme by two steps
in order to make accurate enzyme suspension.

2. Measurement of enzyme activity

(1) Accurately weigh 2,0ml of pH 5.0 McIlvain Buffer and 1.0 ml of the enzyme suspension in a L-shape test tube (Monod tube) and mix well.

The enzyme suspension should be homogeneously suspended.

(2) After preincubation of (1) 40 ± 0. 5 °C for 2 minutes in a Momod shaker, add accurately 2,0 ml of substrate solution preincubated at 40 ± 0. 5 °C, and then perform enzyme reaction at 40 ± 0. 5 °C during 1 hour with moderate shaking stroke (30 rpm).

(3) Terminate enzyme reaction by heatinq in boiling water for 10 minutes.

Centrifuge the reaction mixture under 2,000 G for 15 minutes or filtrate the reaction mixture through 0,45 μm membrane filter to get supematant solution.

4) Perform the quantitative analysis of 1-kestose (GF2) in the reaction mixture by HPLC under the following condition, and calculate the percentage of GF2 area by integrator.

HPLC conditions :
Column : PNH2-10 (shimadzu)
Mobile solvent : CH3CN:H20 - 65 :35 (Standard)
Solvent flow rate : 1,1 ml/min.
Detector : Ri (Refractive Index detector)
Sensitivity of detector: 8×10^{-5} RIUFS
Injection volume : 10 μl

Tech. App. V -26

Note: it is better

I) to mill the enzyme powder previously, and
2) to dilute the enzyme by two steps
in order to make accurate enzyme suspension.

2. Measurement of enzyme activity

(1) Accurately weigh 2,0ml of pH 5.0 McIlvain Buffer and 1.0 ml of the enzyme suspension in a L-shape test tube (Monod tube) and mix well.

The enzyme suspension should be homogeneously suspended.

(2) After preincubation of (1) 40 ± 0. 5 °C for 2 minutes in a Momod shaker, add accurately 2,0 ml of substrate solution preincubated at 40 ± 0. 5 °C, and then perform enzyme reaction at 40 ± 0. 5 °C during 1 hour with moderate shaking stroke (30 rpm).

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Mobile solvent : CH3CN:H20 - 65 :35 (Standard)
Solvent flow rate : 1,1 ml/min.
Detector : Ri (Refractive Index detector)
Sensitivity of detector: 8×10^{-5} RIUFS
Injection volume : 10 μl

Tech. App. V -26

TECHNICAL DATA ON THE ENZYME PREPARATION

1-ACTIVE COMPONENT

1.1- Active enzyme:

β-D-fructofuranosidase (or invertase as a generic name), β-D-fructosyl-transferase, EC 3.2.1.26,

1.2- Activity of the enzyme preparation:

About 1 000 000 units/g enzyme preparation (at least 800 000 units/g).

One unit of the enzyme preparation is defined as the amount required to produce 1 μmol of 1-kestose (GF2) per hour from a 10% (w/v) sucrose solution under a temperature of 40°C (refer to appendixes Tech. App. IV for the specifications and to Tech. App. V for the employed method).

1.3- List of subsidiary enzymatic activities:

Since many active enzymes are present in the cells of the producing micro-organism, and since the β-fructofuranosidase is an endo-cellular enzyme, other enzymatic activities have been researched on two different batches (refer to Table 1 for the results and to appendixes Tech. App. III for the analysis certificates and Tech. App V for the employed methods)·

Under the condition of use (sucrose as substrate, anaerobic conditions and heating of the FOS for the enzyme inactivation), this subsidiary activities are of no significance in the final product.

Table 1: Levels of subsidiary activities in the enzyme preparation

	Date of production	10/15/86	10/15/86	12/14/89	12/14/89	12/14/89	1993	3/10/93
	Batch number	201	201	190	1900	1900	2301	2401
	date of analysis	12/89	12/17/93	12/89	1/29/90	12/17/93	12/17/93	12/17/93
activity (units/g)	Fructose transferring activity*	1.06 10	nm	nm	1.02 10	nm	1.10 10	$1.19\ 10^6$
	Amylase	11	13	14	nm	12	9	12
	Protease	1900	1945	720	nm	763	805	1127
	Lipase	7	90	60	nm	96	113	98

nm: not measured

* 1 unit/g is defined as the amount required to produce 1 μmol of 1-kestose (GF2) per hour from a 10% (w/v) sucrose solution under a temperature of 40°C.

Appendix IV: Manufacturing Process of the Enzyme Preparation

Note: *Original reports as reproduced were marked Confidential at the time of publication, and the type was unable to be removed. These documents are purposefully included in this Public document.*

2- MICROBIAL SOURCE MATERIAL:

The enzyme preparation is issued from a culture of *Aspergillus niger* which is not genetically modified.

The strain is registered under the ATCC number 20611.

The strain of *Aspergillus niger* is considered as non-pathogenic and non-toxic in man or other animals by the US government (FDA) for the preparation of a number of enzymes (ATCC, 1987).

3- MANUFACTURING PROCESS: method of manufacture and purification procedure

The enzyme preparation has been patented in several countries : Germany, Denmark, France, Great Britain, Netherland, Japan, USA...

All methods to produce β-fructofuranosidase are published in Hidaka et al. (1988)[1] and Hirayama et al. (1989)[2]. The culture conditions of the *Aspergillus niger* and the enzyme preparation are described below.

The strain *Aspergillus niger* (ATCC 20611, not genetically modified), has been chosen because it produces the best yields of the enzymatic preparation.

A quality control is regularly done to ensure the absence of (1) strain drift, (2) toxin production and (3) foreign micro-organisms (refer to appendix Tech. App. II to Tech. App. V).

3.1- Enzyme preparation:

Refer to figure 1.

Fermentation method: sterilisation of culture media (described below), introduction into the batch of lyophilised spores,

Temperature: minimum temperature 25°C, maximum temperature 30°C

Duration of the cultivation: about 150 hours.

Concentration of the enzyme preparation by filtration and cryodessication of the liquid enzyme preparation.

This lyophilised enzyme preparation is imported from Meiji-Seika-Kaisha (Japan).

---

[1] Hidaka H., Hirayama M. And Sumi N (1988). A fructo-oligosaccharide producing enzyme from Aspergillus niger ATCC 20611, Agric. Biol. Chem. 52(5): 1181-1187.
[2] Hirayama M., Sumi N. And Hidaka H. (1989) Purification and properties of a fructo-oligosaccharides producing -fructofuranosidase from Aspergillus niger ATCC 20611, Agric. Biol. Chem. 53(3): 667-673.

Figure 1: Process flow sheet for the manufacturing of fructofuranosidase

Lyophilized tube	
↓	
Seed fermentation	Medium 1 Filtered air Temperature: 28±1°C Cycle time: Ca. 20 hours
↓	
Production fermentation	Medium 2 Filtered air Temperature: 28±1°C Cycle time: about 130 hours
↓	
Filtration	Filter press
↓	
Freeze dry	Freeze dryer Temperature:<40°C Pressure: <0.2 Torr
↓	
Pulverization	Pulverizer JP#20 Pass > 90%
↓	
Fructofuranosidase preparation	
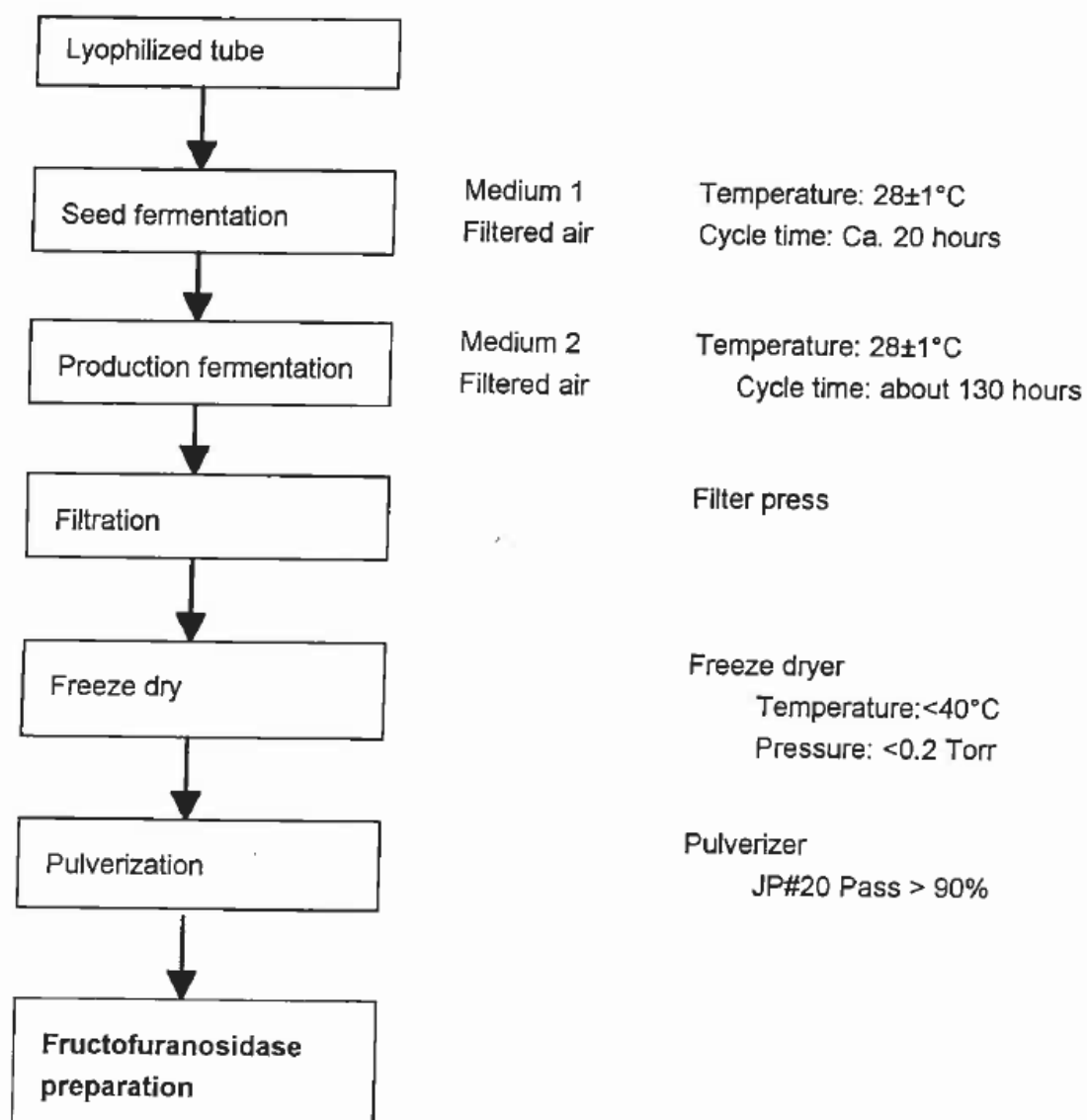
3.2- Culture media:

medium 1

Sucrose HA	5.0 %
Bouillon (Powder)	2.0 %
Silicone KM-72	appropriate
Sodium carboxymethyl-cellulose	0.5 %

medium 2

Sucrose HA	5.0% w/v, (weight/volume)
yeast's extracts	6.5% w/v
sucro-esters of fatty acids	0.5% w/v
Sodium carboxymethyl-cellulose	0.5% w/v
Corn salad oil	0.25% w/v
water	up to 100% w/v
Silicone KM-72	0.5 %
Adekanol LG-109	appropriate
Sodium hydroxide solution	appropriate

3.3- Enzyme preparation specification

The activity of the enzyme preparation is about 1 000 000 units/g.

One unit of the enzyme preparation is defined as the amount required to produce 1 µmol of 1-kestose (GF2) per hour from a 10% (w/v) sucrose solution under a temperature of 40°C.

Table 2: Manufacturer's specifications for the enzyme preparation

water loss by total dehydration	<7%
heavy metals	<10mg/kg
arsenic	<1mg/kg
mycotoxins and sterigmatocystin	absence (<5mg/kg)
antibiotic activity	none
mesophylic total count	<50 000/g
coliforms	<30/g
Salmonella	absence/25g

Manufacturer's statement about production controls, end-product checks and analysis reports are presented below.

4- CARRIERS AND OTHER ADDITIVES AND INGREDIENTS

No carriers or other additives and ingredients are added to the enzyme preparation.

5- USAGE

5.1- technological function of the enzyme

The enzyme preparation acts as an invertase:

Sucrose (GF)   ------>   Glucose + Fructose

and as a fructosyltransferase:

Sucrose + GF   ------>   1-kestose (GF2) + Glucose

GF2 +GF   ------>   nystose (GF3) + Glucose

GF3 + GF   ------>   fructosyl-nystose (GF4) + Glucose

in summary :

GFn-1 + GF   ------>   GFn + Glucose   (n= 2, 3, 4)
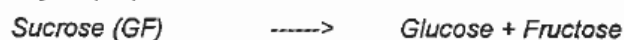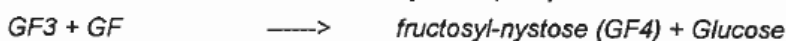
Biochemical reactions are described in figure 2

5.2- Type of foodstuffs in which the enzyme is intended to be used

Preparation of fructo-oligosaccharides from sucrose through the transfructosylating action of the notified enzyme preparation.

5.3- Maximum amount of enzyme preparation to be used in the production of scFOS

Not more than 0.5 g of enzyme preparation per kg of sucrose.

figure 2: Principle of β-fructofuranosidase action on sucrose

sucrose

glucose

glucose

glucose

sucrose

kestose

sucrose

nystose

sucrose

fructosyl-nystose

action of the enzyme
β– fructofuranosidase
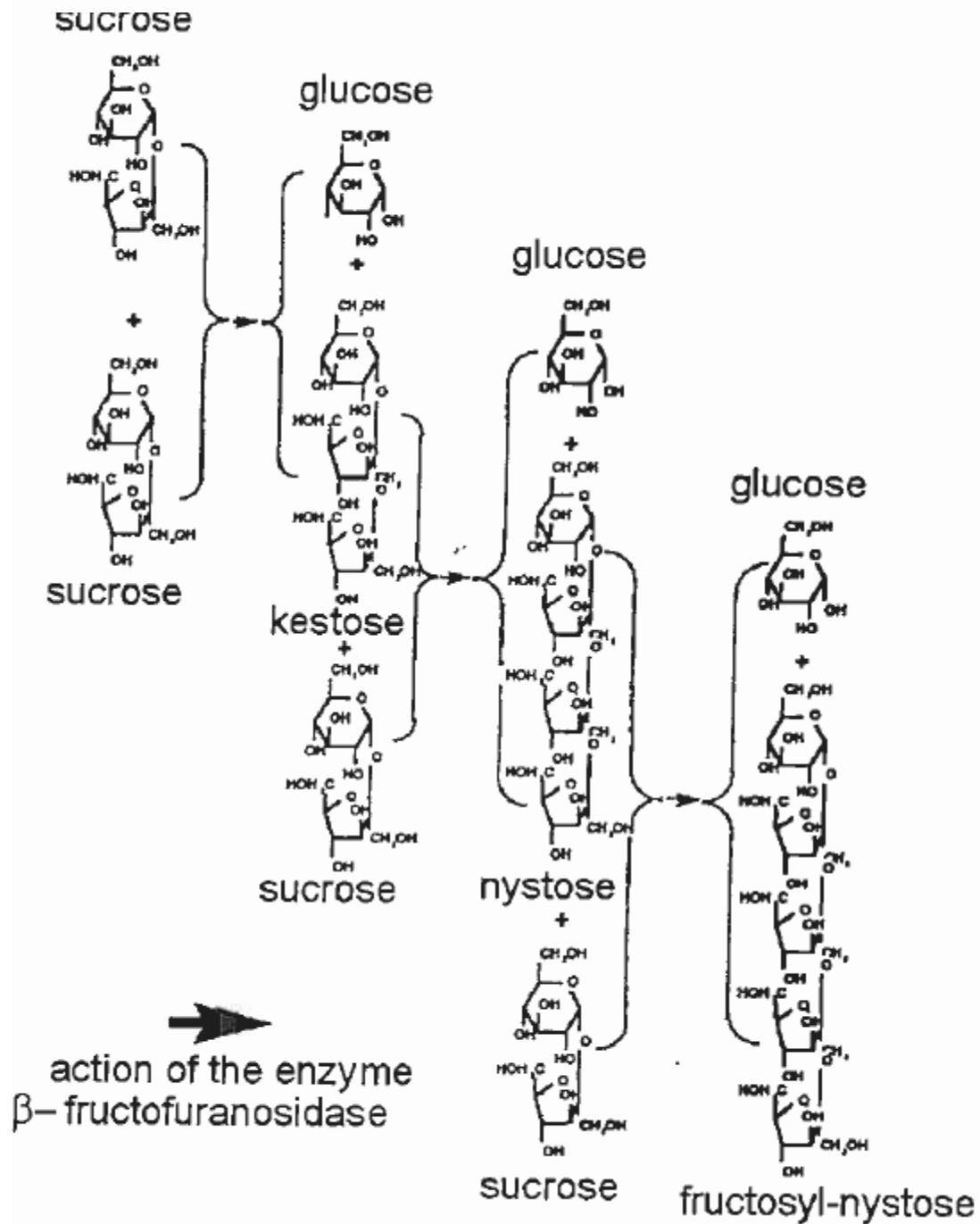
Appendix V: Quality Controls of the Enzyme Preparation

       a.   Enzyme preparation specification

       b.   In production controls and final checks of the enzyme preparation

       c.   Raw materials specifications

       d.   Typical results from manufacturing lots

Note: Original reports as reproduced were marked Confidential at the time of publication, and the type was unable to be removed. These documents are purposefully included in this Public document.

B- ENZYME PREPARATION

1- ENZYME PREPARATION SPECIFICATION

The enzyme preparation of β-fructofuranosidase is stored in a cold room or in a fridge (<5°C). The enzyme preparation specifications are described in table below.

enzyme preparation specifications

item	specifications
colour	pale brown powder
odour	characteristic odour
form	powder
appearance	free from visible evidence of contamination
assay	not less than 800 000 U/g
pH	6.0~7.0 (1g/100ml)
loss of drying	not more than 7.0% (1g, 105°C, 2hrs, <3mmHg)
particle size	JP#18 Pass : more than 90%
heavy metals	less than 50ppm
arsenic	less than 10 ppm

2- ENZYME PREPARATION SPECIFICATION CONTROLS

Exemple of an anlysis certificate realized for each batch of the enzyme preparation.

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CERTIFICATE OF ANALYSIS

Jan. 19, 1993

Product: FURAHOSIDASE

Lot No.:   FFB - 20087

Mfg. Date: Nov. 21, 1989

Testing Date: Jan. 19, 1993

ANALYSIS:

Description: Conforms

Activity: 1,070,000   u/g

Loss on drying: 5.5   %

Heavy metals: Not more than 10 ppm

Arsenic: Not more than   1 ppm

MEIJI SEIKA KAISHA, LTD.

S. Minami

S. MINAMI, Manager

Quality Control Section
Ashigara Plant

FFB 51

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3- RAW MATERIALS SPECIFICATION

The used raw materials are in accordance with the required specification for human nutrition to limit at the maximum, the presence of chemical or microbial contaminants in the food ingredient.

3.1) Sugar HA

Specifications
A clean, white, odourless granule
Identity : To pass test
Loss on drying : Maximum 0.1%
Content (Sugar) : Minimum 99.5%

Tests
Appearance
Clean and free from visible evidence of contamination.
Identity
1. Description test
Colour : white
Odour : odourless
Form : granule

2. Chemical test
Dissolve 10mg of the sample into 1ml of water.
When 2ml of 0.2% anthrone sulfate solution is added to the sample, a green colour is produced.
Loss on drying
Accurately weigh about 1g of the sample into a tared weighing bottle and dry for 4 hours in an oven at 105°C. Cool in a desiccator, reweigh and calculate the percent of loss on drying..
Content (Sugar)
Accurately weigh 1.0g of the sample and dissolve it into 100ml of water. Measure the optical rotation of the solution at 20°C with a polarimeter tube 100mm in length using Na lamp as the light source. Then determine the content (sugar) by means of the measured value of the optical rotation.

Tech. App. IV -8

3.2) Bouillon (Powder)

Specifications
A clean, pale brown powder with characteristic odour.
Identity   :   To pass test
pH   :   6.5~7.5

Tests
Appearance
Clean and free from visible evidence of contamination.
Identity
Description test
Colour : Pale brown
Odour   : characteristic odour
Form   : powder
pH
Transfer 2g of the sample into a 200ml beaker, add 100ml of distilled water.
Determine the pH of the solution using a pH meter

3.3) Adekanol LG-109

Adekanol LG-109 is a kind of defoamer which consists of polyoxyalkylenepolyether.

Specifications
A clear, colourless, oily liquid with faintly characteristic odour.
Identity   :   To pass test
pH   :   6.0~8.0
Moisture(water) :   Maximum 0.5%
Intensity of colour: APHA Maximum 100

Tests
Appearance
Clear and free from visible evidence of contamination.
Identity
1. Description test
Colour : colourless
Odour   : faintly characteristic odour
Form   : oily liquid
2. Chemical test
When a drop of the sample is dissolved in 2ml of water and 1ml of 10% cobalt
nitrate-ammonium thiocyanate solution is added, a blue precipitate is formed.
pH
Transfer 60ml of aqueous isopropanol solution (10:6 by volume) into a 100ml
beaker and adjust the pH to 7.0 under stirring by means of a magnetic stirrer until
pH is stable. The pH should be checked one minute after stopping to stir at any
time. N/100 hydrochloric acid or n/100 sodium hydroxide is used for the
adjustment of the pH. Transfer this solution into the 100ml flask into which 10g of
the sample has been weighed. Continue to stir for the further 5 minutes period
and read the pH.
Moisture
Apply Karl Fisher Method.

Tech. App. IV -10

ASAHI DENKA KOGYO K.K.

Confidential

MATERIAL SAFETY DATA SHEET

ISSUE DATE: July. 2.1991

H M I S
HEALTH: 0
FLAMMABILITY: 1
REACTIBITY: 1
PERSONAL PROTECTION
EQUIPMENT: B

SECTION I

Manufacturer's Name
ASAHI DENKA KOGYO K. K.

Address
3 - 14. NIHONBASHI - MUROMACHI
2 - CHOME, CHUO - KU, TOKYO
103, JAPAN
TEL 81-3-5255-9016

Distributor

Emergency Telephone Number
TEL

Chemical Name & Structure
N.A.

Trade Name: ADEKANOL LG-109
Chemical family: Defoamer   Formula: N.A.

SECTION II INGREDIENTS/IDENTITY

(1) HAZARDOUS INGREDIENTS

(2) EPA SECTION 313 SUPPLIER NOTIFICATION

MATERIAL	CAS	%	OSHA PEL	ACGIH TLV	MATERIAL	CAS	%
N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

SECTION III PHYSICAL DATA

Boiling Point	N.D.A.	°F	°C	Specific Gravity ($H_2O=1$)	1.022 (25°C)
Vapor Pressure (mm Hg)	N.D.A.			Percent Volatile by Volume (%)	N.D.A.
Vapor Density (Air=1)	N.D.A.			Evaporation Rate (BUTYL ACETATE=1)	N.D.A.
Melting Point	< 0°C			Solubility in water	Moderate (20°C)
Appearance & Odor	Colorless liquid with mild odor				

SECTION IV FIRE AND EXPLOSION HAZARD DATA

Flash Point (Method Used)	218°C (C.O.C.)	Flammable Limits	N.D.A.	LEL N.D.A.	UEL N.D.:
Extinguishing Media	Dry chemical, CO_2, foam				
Special Fire Fighting Procedures	None				
Unusual Fire and Explosion Hazards	None				

N.A.= NOT APPLICABLE   N.D.A.= NO DATA AVAILABLE

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SECTION V REACTIVITY DATA

Stability	Unstable		Conditions to avoid
	Stable	×	High temperature exceeding 80 ℃

Incompatibility (Materials to Avoid)	Strong oxidizing agents

Hazardous Decomposition or Byproducts	CO on incomplete combustion

Hazardous Polymerization	May Occur		Conditions to avoid
	Will Not Occur	×	None

SECTION VI HEALTH HAZARD DATA

Route(s) of Entry: Inhalation? No   Skin? Yes   Ingestion? No

Health hazards: Acute oral toxicity : Slightly toxic $LD_{50}=$ >15000mg/Kg (Mouse)

Carcinogenicity: NTP? No   IARC Monographs? No   OSHA Regulates? No

Signs and Symptoms of Exposure: None normally encountered

Medical Conditions Generally Aggravated by Exposure: Unknown

Emergency and First Aid Procedures:

Eye: Immediately flush eyes with plenty of water for at least 15 minutes.

Skin: Wash with soap and water.

Inhalation: Call a physician.

SECTION VII PRECAUTIONS FOR SAFE HANDLING AND USE

Step to Be Taken in Case Material is Released or Spilled: Remove all sources of ignition.

Wasted Disposal Method: Controlled burning.

Precautions to Be Taken in Handling and Storing: Keep away from heat and flame.

Store in a cool place and protect from direct sunlight.

Other Precautions: None

SECTION VIII CONTROL MEASURES

Respiratory Protection (Special Type): Not normally required.

Ventilation	Local Exhaust Not normally required.		Special	None
	Mechanical (General) Desirable		Other	None

Protective Gloves Rubber	Eye Protection Safety glasses

Other Protective Clothing or Equipment: None

Work/Hygienic Practices   Wash thoroughly after handling.

SIGNATURE: *Kaoru Komiya*
TITLE   Manager

We believe the statements, technical information and recommendations contained herein are reliable, but they are given without warranty or guarantee of any kind, express or implied, and we assume no responsibility for any loss, damage or expense, direct or consequential, arising out of their use.

Tech. App. IV -12

3.4) Sodium Hydroxide Solution Technical

Specifications
A colourless or slightly yellow, odourless or practically odourless, viscous liquid, which is clear or may be somewhat turbid and may contain small quantities of suspended matter or sediment.
Identity   :   To pass test
Chloride   :   Maximum 0.5% NaCl
Assay   :   Maximum 45% NaOH

Tests
Appearance
Clear or somewhat turbid and may contain small quantities of suspended matter or sediment.
Identity
1. Description test
    Colour :   colourless or at most slightly yellow
    Odour :   odourless or practically odourless
    Form   :   viscous liquid
2. Chemical test
    A. When a litmus-paper is dipped in the sample of 1% solution, it changes blue.
    B. When the sample is heated in a colourless flame, a yellow flame is produced

Chloride and Assay
Proceed as described in JIS K 1200.

3.5) Yeast extract

Specifications
A dark brown paste with characteristic odour.
Identity   :   To pass test
Loss on drying :   30±2%
Total nitrogen :   4.0~5.5%

Tests
Appearance
Clean and free from visible evidence of contamination.
Identity
1. Description test
    Colour :   dark brown
    Odour :   yeast like odour
    Form   :   paste
2. Chemical test
    Suspend about 50mg of the sample in 5ml of water.
    Add 3ml of 0.2% ninhydrin pyridine-acetone (1:10) solution to this suspension and heat, a blue to blue purple colour is produced.

Loss on drying
Accurately weigh about 1g of the sample into a tared weighing bottle and dry for 4 hours in an oven at 105°C. Cool in a desiccator, reweigh and calculate the percent of loss on drying.

Total nitrogen
Accurately weigh 0.5g of sample into a 300ml decomposing flask and add 1.5g of anhydrous cupric sulfate and 10ml of conc. Sulphuric acid. Incline the decomposing flask to 45°C, heat gently until foams disappear, then boil vigorously and continue to boil further 30 minutes after solution is clear.

Tech. App. IV -13

After cooling, add 150ml of distilled water and a few granules of zinc. Add 40ml of 50% sodium hydroxide aqueous solution to the dissolved solution and distil the solution. Receive the distillate into a flask containing 20ml of 4% boric acid until the volume reaches 100ml. Titrate the distillate with 0.1N sulphuric acid using bromocresol green-methyl red as indicator.

Calculate the weight of nitrogen form the volume of 0.1N sulphuric acid.

$$\text{Weight of total nitrogen(\%)} = \frac{0.1N\text{-}H_2SO_4 \text{ ml X 1.4 X F X 100}}{\text{weigh of sample (g) X 1000}}$$

F: factor of 0.1N H_2SO_4

3.6) Silicone KM-72

Silicone KM-72 is a kind of defoamer which consists of silicone and emulsifier.

Specifications

A clean, odourless cream with milky colour.

Identity   : To pass test

Non-volatile matter : 33.5~37.5%

Defoaming ability   :   Maximum 500ml

Tests

Appearance

Clean and free from visible evidence of contamination.

Identity

1. Description test

Colour : milky white

Odour : odourless

Form : cream

2. Chemical test

Suspend 0.4g of the sample in 20ml of 0.5N-sodium hydroxide solution. When 12ml of 10% ammonium chloride is added to the suspension and heat, a white gelatinous precipitate is formed. It is insoluble in diluted hydrochloric acid.

Non-volatile matter

Accurately weigh 1.5g of the sample in a glass dish with a diameter of 60mm ,and a depth of about 10mm, and heat for 3 hours at 105°C keeping the dish horizontal in a thermostatical electric oven. After cooling in a desiccator reweigh the dish and calculate the weight of volatile matter in percentage.

Defoaming ability

To 0.6g of sample, accurately weighed in a 50ml beaker, add 100ml of 0.2% sodium oleate solution little by little and transfer the sample into 1 litre cylinder in the thermostatic (254±0.5°C) water tank of an apparatus for defoaming ability test. Lead air, previously regulated to 1 litre/min., into the cylinder and bubble. Read the height of the bubbles after 5 minutes, which can express the defoaming ability.

Tech. App. IV -14

Shin Etsu

Shin-Etsu Chemical Co.,Ltd.

6-1, Ohtemachi 2-cnome Chiyoda-ku, Tokyo, Japan.
TEL:
FAX.
DATE Sep. 1,1994   NO. C-4096E

To : Meiji Seika Kaisha , LTD

Certificate

We hereby certify that the following product manufactured by
Shin-Etsu Chemical Co., Ltd.is silicone defoaming agent .
Each ingredients are listed on the positive list of the FDA .

Product : KM-72

Yuji Hinoto
General Manager
Quality Assurance Department
Gunma Complex

Tech. App. IV -15

ShinEtsu

Shin-Etsu Chemical Co.,Ltd.

6-1. Ohtemachi 2-chome, Chiyoda-ku, Tokyo, Japan
TEL:
FAX:
DATE Sep. 1,1994   C-4095E

To : Meiji Seika Kaisha , LTD.

Certificate
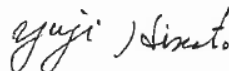
We hereby certify that Silicone defoaming agent for food, KM72, manufactured by Shin-Etsu Chemical Co., Ltd. is emulsion consisting of the following food additives and conforms to the Japanese Food Sanitation Law.

Formulation of KM72 :

Silicone resin (*1)	30.0%
Sorbitan fatty acid ester	3.5%
Glycerine fatty acid ester	2.4%
Sodium Carboxymethylcellulose	0 3%
Water	63.8%

(*1) The viscosity of base oil, dimethylpolysiloxane, is 100 to 1100 centistokes at 25 ℃.

Your kind consideration and arrangements will be greatly appreciated.

Yuji Hinoto
Manager
Quality Assurance Department
Gunma Complex

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3.7) Sodium Carboxymethyl cellulose

Specifications
A clean, white to pale brown, odourless powder
Identity   :   To pass test
pH   :   6.0~8.0
Loss on drying   :   Maximum 10.0%

Tests
Appearance
Clean and free from visible evidence of contamination.
Identity
1. Description test
Colour   :   white to pale brown
Odour   :   odourless
Form   :   powder
2. Chemical test
Dissolve 0.5g of the sample into 50ml of water and heat it for 20 minutes at 60~70°C and make a homogeneous solution.
Then cool and use it as sample solution.
1)Add 4ml of water into the 1ml of sample solution. Then add 1 drop of the diluted sample solution into ,0.5ml of chromotropic acid solution and heat in a water bath, a red purple colour is produced.
2)Add 10ml of acetone into the sample solution and mix by stirring, white precipitate is produced.
pH
Determine the pH of the sample solution, described in "chemical test" using a pH meter.
Loss on drying
Accurately weigh about 1g of the sample into a tared weighing bottle and dry for 4 hours in an oven at 105°C. Cool in a desiccator, reweigh and calculate the percent of loss on drying.

3.8) Sucrose Esters of Fatty Acids

Specifications
A yellowish brown paste with characteristic odour
Identity   :   To pass test
Dry substance   :   40~42
Heavy metal   :   Not more than 20ppm
Arsenic   :   Not more than 1ppm
Tests
Appearance
Clean and free from visible evidence of contamination.
Identity
1. Description test
Colour   :   yellowish brown
Odour   :   Alcoholic odour
Form   :   paste
2. Chemical test
Accurately weigh about 2g of the sample on tared weighing stainless plate and add 5ml of Isobutanol. Then dry for 1 hour by Ultra red Dryer. Cool in a desiccator, reweigh and calculate the percent of loss on drying

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Heavy metal
    By method II of Japanese Pharmacopeia.
Arsenic
    By method III of Japanese Pharmacopeia

3.9) Corn Salad Oil

Specifications
A pale yellow, odourless or practically odourless, oily liquid
Identity   :   To pass test
Water   :   Maximum 0.1%
Specific gravity (25°C/25°C)   :   0.915~0.921

Tests
    Appearance
        Clean and free from visible evidence of contamination.
    Identity
        Colour :   pale yellow
        Odour   :   odourless or practically odourless
        Form   :   oily liquid
    Water
        Determined by the Karl Fisher method.
    Specific gravity (25°C/25°C)
        Determined by the hydrometer method.

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Table AV-1: Typical NutraFlora P-95 Test Results				
Parameters	**Specification**	**Units**	**Typical**	**Method of Analysis**
Macrocomponents				
Glucose+Fructose+Sucrose	≤ 5	%, db	1	In-house HPLC method
Sucrose			3	In-house HPLC method
Fructooligosaccharides	≥ 95	%, db	96	Total of GF
GF$_2$		%, db	36	In-house HPLC method
GF$_3$		%, db	50	In-house HPLC method
GF$_4$		%, db	11	In-house HPLC method
Protein	<0.5%	% w/w as - is	0	GTC CPC-SMA No P60 (digestion by Kjeldahl method)
Ash	≤ 0.1	% w/w	0.01	GTC CPC-SMA No A92 (conductivity)
Moisture	≤ 5	%	3.8	USP <921> Water Determination (KF)
Heavy Metals				
Arsenic	≤0.1	ppm	<0.01	ICP-MS
Cadmium	≤ 0.01	ppm	< 0.001	ICP-MS
Lead	" 0.05	ppm	< 0.01	ICP-MS
Mercury	" 0.01	ppm	< 0.005	ICP-MS
Microbiological Components				
Standard Plate Count		cfu/g	10-50	GTC CPC-SMA No I-A
Mold & Yeast		cfu/g	<10	GTC CPC-SMA No II-A
Enterobacteriaceae	<3	cfu/g	0	CMMEF 4th Edition
Anaerobic Thermophilic Spores	≤ 100	cfu/g	0	CMMEF 4th Edition
Aerobic Thermophilic Spores	≤ 100	cfu/g	<2	CMMEF 4th Edition
Anaerobic Mesophilic Spores	≤ 300	cfu/g	0	CMMEF 4th Edition
Aerobic Mesophilic Spores	≤ 300	cfu/g	0.00	CMMEF 4th Edition
Salmonella			Negative	AOAC 966.08
Staphylococcus aureus			Negative	AOAC 966.08
E.coli			Negative	AOAC 966.24
Abbreviations:				

Abbreviations:

AOAC   Association of Official Analytical Chemists

db   Dry basis

CMMEF   Compendium of Methods for the Microbiological Examination of Foods

HPLC   High Performance Liquid Chromatography

ND   Not detected

USP   United States Pharmacopeia

w/w   Weight for weight

Appendix VI: Experimental Results and Analysis Certificates of the Enzyme Preparation

Appendix VI-1 Reports of Japan Food Research Laboratories Studies

MEIJI SEIKA KAISHA, LTD. NEW MATERIALS DIVISION

6 July 1990

Analysis Table of Furanosidase(β-Fructofuranosidase)

Product Batch No. Reffered No. Manufacturing Date Testing Date	PFF-201 FFB-20017 '86/10/15 '89/12	PFF-1900 FF-00190 '89/12/14 '89/12	PFF-1900 PFF-1900 '89/12/14 '90/ 1/29	PFF-2001 PFF-2001 '90/ 2/23 '90/ 6/19	PFF-2101 PFF-2101 '90/ 3/31 '90/ 6/19
Moisture(%)	7.1	5.9			
Protein(%,f=6.25)	37.9	36.2			
Fat(%)	9.8	8.3			
Fiber(%)	10.2	15.1			
Ash(%)	5.3	9.9			
Non-fibrous carbohydrate(%)	29.7	24.6			
Arsenic(ppm,as As_2O_3)	0.3	0.2			
Heavy metals(ppm,as Pb)	9.6	6.2			
Lead(ppm)	0.25	0.16			
Cadmium(ppm)	0.03	0.03			
Mercury(ppm)	ND(<0.01)	ND(<0.01)			
Aflatoxin(B_1,B_2,G_1,G_2)	ND(<5ppb)	ND(<5ppb)			
Sterigmatocystin	ND(<5ppb)	ND(<5ppb)			
Aerobic plate count	3.6×10^4/g	<300 /g	2.0×10^1/g	1.2×10^3/g	2.2×10^1/g
Coliform organisms count	0.91 /g	<0.3 /g	2.3/g	9.3/g	9.3/g
Escherichia coli	(+)/25g	(-)/25g	(-)/25g	(-)/25g	(-)/25g
Coagulase positive Staphilococci	(-)/ 1g	(-)/ 1g	(-)/ 1g	(-)/ 1g	(-)/ 1g
Salmonella	(-)/25g	(-)/25g	(-)/25g	(-)/25g	(-)/25g
Sulfite reducing anaerobic spores	3.2×10^4/g	(-)/ 1g	6/g	(-)/ 1g	1/g
Antibacterial activity	ND	ND			
Enzymatic activities (unit/g)					
Fructose-transferring activity	1.06×10^6		1.02×10^6	1.08×10^6	1.12×10^6
Amylase	11	14			
Protease	1900	720			
Lipase	7	60			

Tech. App. III -3

Japan Food Research Laboratories
AUTHORIZED BY THE JAPANESE GOVERNMENT

HEAD OFFICE : 52-1, MOTOYOYOGI-CHO, SHIBUYA-KU, TOKYO
OSAKA BRANCH : 3-1, TOYOTSU-CHO, SUITA-SHI, OSAKA
NAGOYA BRANCH : 5-13, 4-CHOME, OSU, NAKA-KU, NAGOYA
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ANALYSIS CERTIFICATE

No. 42111913-003

Requested by : MEIJISEIKA KAISHA LTD.

Sample : Furanosidase

Labeled : FFB-20017

Date of Assay : Dec. 9, 1989

Laboratory No. 1

Received : Nov. 22, 1989

This is to certify that the following result(s) have been obtained
according to our analysis on the above-mentioned sample(s) submitted
by the applicant.

R E S U L T S

Moisture [Air oven method] : .... 7.1 %
Protein (N x 6.25) : .... 37.9 %
Fat [with acid hydrolysis] : .... 9.8 %
Fiber : .... 10.2 %
Ash : .... 5.3 %
Non-fibrous carbohydrate : .... 29.7 %
Arsenic (as As_2O_3) : .... 0.3 ppm
Heavy metals (as Pb) : .... 9.6 ppm
Lead : .... 0.25 ppm
Cadmium : .... 0.03 ppm
Mercury : .... not detected
                        (detectable content 0.01 ppm)
Aflatoxin B_1 : .... not detected
                        (detectable content 5 ppb)
Aflatoxin B_2 : .... not detected
                        (detectable content 5 ppb)
Aflatoxin G_1 : .... not detected
                        (detectable content 5 ppb)
Aflatoxin G_2 : .... not detected
                        (detectable content 5 ppb)
Sterigmatocystin : .... not detected
                        (detectable content 5 ppb)

– continued –

Tech. App. III -4
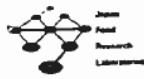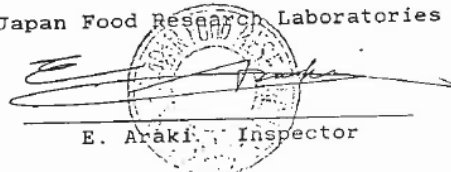
```
Aerobic plate count : ..................... 3.6 x 10^4/g
⎡ Coliform organisms count : ................ 0.91/g
⎢ Escherichia coli : ........................ positive/25g
*1 ⎢ Coagulase positive Staphylococci : ........ negative/1g
⎣ Salmonella : .............................. negative/25g
*2  Sulfite reducing anaerobic spores : ....... 3.2 x 10^6/g
```

*1 by the method proposed from the client.
*2 heat-shocked at 70℃, 20 min.

– The end –

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E. Araki   Inspector

Tech. App. III -5

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KYUSHU BRANCH : 1-12, SHIMOGOFUKU-MACHI, HAKATA-KU, FUKUOKA-SHI

CERTIFICATE

No. 42111913-005
Dec. 27, 1989

Requested by : MEIJI SEIKA KAISHA, LTD.   Laboratory No. 1

Received : Nov. 22, 1989

TEST FOR ANTIBACTERIAL ACTIVITY

1. Sample
   Furanosidase (FFB-20017)

2. Purpose of the test
   To test antibacterial activity of the sample.

3. Outline of test
   The test was carried out in accordance with the methods described
   in TESTS FOR ANTIBACTERIAL ACTIVITY of "GENERAL STANDARDS FOR ENZYME
   REGULATIONS, Second edition" by THE ASSOCIATION OF MICROBIAL FOOD
   ENZYME PRODUCERS.
   Medium and paper disk were used as follows:
       Broth Medium, TRYPTO-SOY BROTH (EIKEN CHEMICAL CO., LTD.);
       Agar Medium, TRYPTO-SOY AGAR (EIKEN CHEMICAL CO., LTD.);
       Paper disk, AA DISCS, 13.0 mm in diameter (WHATMAN LTD.).

4. Test results
   Test results are shown in Table 1.
   As shown by the results, it was interpreted that antibacterial
   activity of the sample was negative.

- continued -
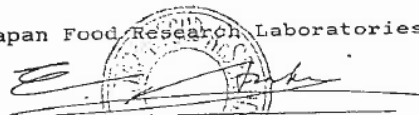
Tech. App. III -6

Table 1   Antibacterial activity of the sample

Test organisms	Clear zone around a disk
Bacillus cereus ATCC 2	not detected
Bacillus circulans IFO 13628 (ATCC 4516)	not detected
Escherichia coli ATCC 11229	not detected
Serratia marcescens ATCC 14041	not detected
Staphylococcus aureus IFO 13276 (ATCC 6538)	not detected
Streptococcus pyogenes ATCC 12344	not detected

-The end -

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Tech. App. III -7
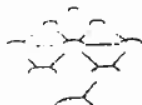
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KYUSHU BRANCH : 1·12, SHIMOGOFUKU·MACHI, HAKATA·KU, FUKUOKA·SHI

ANALYSIS CERTIFICATE

No. 42120486-003

Requested by : MEIJISEIKA KAISHA LTD.   Date of Assay : Dec. 19, 1989

Sample : Furanosidase   Laboratory No. 1

Labeled : FF-00190   Received : Dec. 5, 1989

This is to certify that the following result(s) have been obtained according to our analysis on the above-mentioned sample(s) submitted by the applicant.

R E S U L T S

Moisture [Air oven method] : .... 5.9 %
Protein (N x 6.25) : .... 36.2 %
Fat [with acid hydrolysis] : .... 8.3 %
Fiber : .... 15.1 %
Ash : .... 9.9 %
Non-fibrous carbohydrate : .... 24.6 %
Arsenic (as As_2O_3) : .... 0.2 ppm
Heavy metals (as Pb) : .... 6.2 ppm
Lead : .... 0.16 ppm
Cadmium : .... 0.03 ppm
Mercury : .... not detected
                        (detectable content 0.01 ppm)
Aflatoxin B_1 : .... not detected
                          (detectable content 5 ppb)
Aflatoxin B_2 : .... not detected
                          (detectable content 5 ppb)
Aflatoxin G_1 : .... not detected
                          (detectable content 5 ppb)
Aflatoxin G_2 : .... not detected
                          (detectable content 5 ppb)
Sterigmatocystin : .... not detected
                          (detectable content 5 ppb)

– continued –

Tech. App. III -8

Aerobic plate count : .... not more than 300/g

Coliform organisms count : .... negative
(not more than 0.3)/g

*1 Escherichia coli : negative/25g
Coagulase positive Staphylococci : negative/1g
Salmonella : negative/25g

*2 Sulfite reducing anaerobic spores : negative/1g

*1 by the method proposed from the client.
*2 heat-shocked at 70°C, 20 min.

– The end –

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Tech. App. III -9

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ANALYSIS CERTIFICATE

No. 43011051-002

Requested by : Meiji Seika Kaisha Ltd.

Date of Assay : Jan. 29, 1990

Sample : Furanosidase

Laboratory No. 1

Labeled : PFF-1900

Received : Jan. 19, 1990

This is to certify that the following result(s) have been obtained according to our analysis on the above-mentioned sample(s) submitted by the applicant.

R E S U L T S

Aerobic plate count : .... 2.0×10^4/g
*1 Coliform organisms count : 2.3/g
*1 Escherichia coli : negative/25g
*1 Coagulase positive Staphylococci : negative/1g
*1 Salmonella : negative/25g
*2 Sulfite reducing anaerobic spores : 6/g

*1 by the method proposed from the client
*2 heat-shocked at 70℃, 20 min.

— The end —

Japan Food Research Laboratories

T. Kamibe, Inspector

Tech. App. III -12
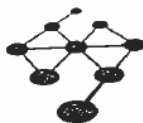
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NAGOYA BRANCH : 5-13, 4-CHOME, OSU, NAKA-KU, NAGOYA
KYUSHU BRANCH : 1-12, SHIMOGOFUKU-MACHI, HAKATA-KU, FUKUOKA-SHI

ANALYSIS CERTIFICATE

No. 43060440-004
June 19, 1990

Requested by : Meiji Seika Kaisha Ltd.
4-16, Kyobashi 2-chome,
Chuo-ku, Tokyo 104

Sample : Furanosidase

Description : PFF-2001

Received : June 6, 1990

This is to certify that the following result(s) have been obtained according to our analysis on the above-mentioned sample(s) submitted by the applicant.

RESULTS

Aerobic plate count : .... 1.2×10^3/g
*1 Coliform organisms count : 9.3/g
*1 Escherichia coli : negative/25g
*1 Coagulase positive Staphylococci : negative/1g
*1 Salmonella : negative/25g
*2 Sulfite reducing anaerobic spores : negative/1g

*1 by the method proposed from the client
*2 heat-shocked at 70°C, 20 min.

– The end –

Japan Food Research Laboratories

E. Araki, Inspector

Tech. App. III -13
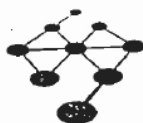
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KYUSHU BRANCH : 1-12, SHIMOGOFUKU-MACHI, HAKATA-KU, FUKUOKA-SHI

ANALYSIS CERTIFICATE

No. 43060440-006
June 19, 1990

Requested by : Meiji Seika Kaisha Ltd.
4-16, Kyobashi 2-chome,
Chuo-ku, Tokyo 104

Sample : Furanosidase

Description : PFF-2101   Received : June 6, 1990

This is to certify that the following result(s) have been obtained
according to our analysis on the above-mentioned sample(s) submitted
by the applicant.

R E S U L T S

Aerobic plate count : .... 2.2×10^4/g
*1 Coliform organisms count : 9.3/g
*1 Escherichia coli : negative/25g
*1 Coagulase positive Staphylococci : negative/1g
*1 Salmonella : negative/25g
*2 Sulfite reducing anaerobic spores : 1/g

*1 by the method proposed from the client
*2 heat-shocked at 70℃, 20 min.

- The end -

Japan Food Research Laboratories

E. Arai   Inspector

Tech. App. III -14

Meiji

MEIJI SEIKA KAISHA, LTD.
4-16, KYOBASHI 2 CHOME, CHUO-KU, TOKYO, 104 JAPAN.

TELEPHONE : 03-3273-3441, 3442
FAX : 03-3271-5617

December 17, 1993

Protease, Amylase and Lipase activities
in Fructofuranosidase

1. Activities
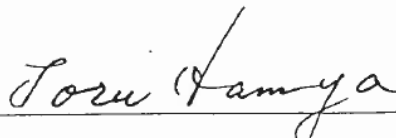
FFase Lot No.	Product. date	Protease unit/g	Amylase unit/g	Lipase unit/g
PFF- 201	'86/10/15	1945	13	90
PFF-1900	'89/12/14	,763	12	96
PFF-2301	'93/	805	9	113
PFF-2401	'93/ 3/10	1127	12	98

a) FFase activity of PFF-2301: 1.10×10^{6}
b) FFase activity of PFF-2401: 1.19×10^{6}

2. Analysis

Testing date : December 17, 1993
Conducted by : Toru Hamaya
Bio Science Laboratories,
Meiji Seika Kaisha, Ltd.

signature *Toru Hamaya*

Tech. App. III -15

Appendix VI-2 Report of INSERM U87 Study

English Translation of Original French Report

<u>Note:</u> *Original reports as reproduced were marked Confidential at the time of publication, and the type was unable to be removed. These documents are purposefully included in this Public document.*

REPORT

ON THE MICROBIOLOGICAL AND MYCOTOXICAL STUDY OF AN ENZYMATIC

PREPARATION OF THE *Aspergillus niger* USED FOR NEOSUGAR PRODUCTION

studies achieved at the INSERM Unit n°87

Groupe de Recherche sur la Toxicologie des Aliments et des Boissons
Institut de Physiologie
2, rue François Magendie
31400 Toulouse

The aim of this work was to know if the enzymatic preparation BÉGHIN-SAY Company intends to use for the NEOSUGAR production, and provided by a Japanese Company, implies living microorganisms (particularly *Aspergillus niger*) or not; and secondly to quantify an eventual production of mycotoxins (aflatoxins) by *Aspergillus niger* when the enzymatic preparation is prepared or during the NEOSUGAR producing reaction.

This mould is indeed given in literature as a mycotoxin producer and particular of aflatoxins (who's structures are represented in Annex B), (CHRISTENSEN *et al.*, 1968; SEMENIUK *et al.*, 1971; WYLLIE *et al.*, 1976; BOTTON *et al.*, 1985).

The work thus involves 3 different aspects:

I - a microbial study of the enzymatic preparation to determine, in a qualitative and quantitative way, the microorganism(s) present in the enzymatic preparation.

II - a group of other studies (pilot reactors, aflatoxin production tests and enzymatic studies) based on the results obtained during the microbiological study.

III - a biochemical study (dosage of the aflatoxins and the sterigmatocystin) of the enzymatic preparation, the sucrose, and various samples taken at different stages of the NEOSUGAR producing reaction (at the end of the reaction, after active charcoal addition and after filtration).

This study was achieved on samples taken during a reaction done according to the Japanese and BÉGHIN-SAY protocol.

Tech. App. III -17

I- MICROBIOLOGICAL STUDY OF THE ENZYMATIC PREPARATION

Previously to this study, we assembled a bibliography on the fine morphologic structure of *Aspergillus niger* and its possibilities to produce different toxic metabolites (dossier joined).

Moreover, we realised an observation of the enzymatic preparation on the optical microscope, to guide our future investigations. The results of this observation are presented on photographs 1 and 2.

On these photographs we can clearly see entire or fragmented mycelian filaments. We did not find sporiferous organs nor spores in these preparations.

These observations make us think that the enzymatic preparation may have been prepared in a fermentor under quite violent shaking, according to the fragmentation of mycelium. The drying of the mycelium was done in a quick way and not at a very advanced level because we didn't observe deterioration of the mycelian structures subsequently to drying.

1- Viability study of the myceline preparation

We putted 1g of preparation in suspension in 10 ml physiological water and realised a dilution range from 10^{-1} to 10^{-6}.

For each dilution, including the enzyme preparation, we seeded different culture media with 0.25 ml of inoculum.

The following media were considered:
* *Actinomycets* medium.
* Martin medium without Bengal pink and antibiotics. This culture medium is very lowly selective and admits isolation of numerous microorganisms.
* Martin mediums additionned with antibiotics which is a highly selective medium convenient for the isolation of mould and yeast.
* Brian and M.E.A. media convenient for the isolation of moulds of the genus *Aspergillus*.
* Salty malt medium, convenient for the isolation and characterisation of the *Aspergillus*.
* Csapeck medium, selective by its acidity, convenient for the characterisation of fongic species.
* And finally the M.Y.20 and M.40.Y. media who are rich in sugars and badly convenient for the isolation of osmophil mushrooms.

The composition of these different culture media is given in Appendix V.

RESULTS:
Three different strains of microorganisms were shown in the preparation.
*1 Mushroom strain.
*2 Bacteria strains.

If the presence of other moulds seem improbable to us, the presence of supplementary bacterial strains is quite envisageable and even probable.

2- Qualitative and quantitative study of the mould strain

1-The mould strain:
The strain presents a slow growth thallus on the Csapeck medium, the mycelium is white, colourless on the reverse side. The biseriated conial heads are devided in several dark brown colours.

Tech. App. III -18

Photograph 1: Enzymatic powder
(magnification X 600. Photographic enlargement).
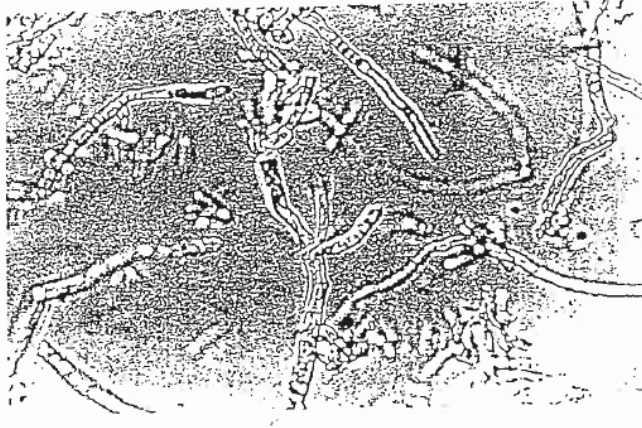
Photograph 2: Enzymatic powder
(magnification X 1000. Photographic enlargement).
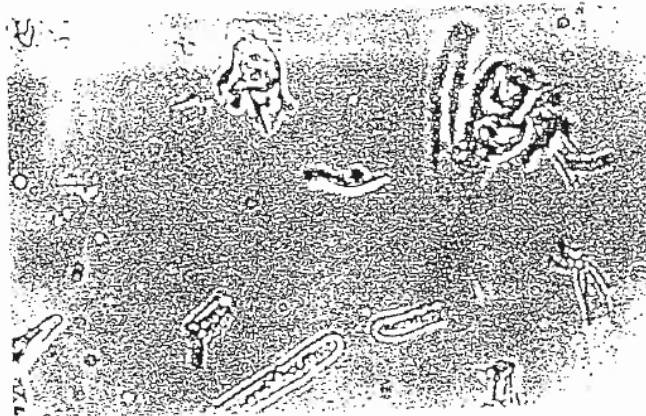
Tech. App. III -19

We then undertook an optical microscope examination of the main morphological characterisations of the fongic strain (after setting up in Aman lactophénol).

Results are presented on photograph 3, 4 and 5.

The fongic strain presents smooth conidiophors of about 0.5 mm, brown in their superior part with globulous, verrucous, 4μm brown conidia. The 5μm are brownish. The fongic strain observed presents all the characteristics of *Aspergillus niger* as defined by RAPPER *et al.* in 1965 and as shown on figure1.

Thus, the submitted enzymatic preparation encloses viable mycelian fragments belonging to *Aspergillus niger*.

At this stage of the study, a question rises up:

"Is the isolated strain the one used to obtain the powder?"

We'll try to answer this question in the second part.

From a quantitative point of vue, we could, thanks to the dilution range, determine that 1g of enzymatic preparation contained 48.10^7 living fongic elements.

2-The bacterial strains:

The qualitative characterization of the two bacterial strains needed the use of a certain number of tests:

* Observation of the bacterial strains with optical or electronical microscope,
* Specific colorations:
    Gram coloration,
    Malachite green coloration, specific to bacterial spores,
    P.A.S. coloration to show the polysaccharides,
* Biochemical tests:
    Mobility test,
    Indol production test,
    API galleries use
        API 20 A (identification system for anaerobic bacteria)
        API 20 B (study system for aerobic heterotrophous bacteria)
        API 20 E (study system for gram negative bacillus).

From a quantitative point of vue, we only did a global estimation of the number of bacteria by gram of enzymatic preparation by preparing as formerly a dilution scale going from 10^{-1} to 10^{-11}.

RESULTS:

**FIRST BACTERIAL STRAIN: (KRIEG *et al.*, 1984)

This bacterial strain presents long chains of lenticular cells. The bacterial gram+ cells are not mobile and do not contain spores.

The colonies are very small: their diameter is inferior to 1mm and their growth depends on the presence of fermentable sugars.

The P.A.S. coloration showed an intense production of polysaccharides.

From a biochemical point of vue, this bacterial strain doesn't possess catalase, doesn't produce indol, doesn't hydrolyse the proteins (the test with gelatine is negative) and doesn't reduce the nitrates. All the sugars except rhamnose, glycerol, sorbitol and inositol are fermented. There is then a production of lactic acid and CO_2. This strain doesn't hydrolyse arginine, can be optionally anaerobic and is non-pathogenic. This microorganism could be identified as *Leuconostoc mesenteroïdes*.

Photograph 6, taken with an optical microscope, gives a good image of this bacterium.

Photographs 7 and 8, taken with an electronical microscope, are more precise in showing the bacterial cells.

Tech. App. III -20

Photograph 3: Mycelian filaments
(magnification X 400. Photographic enlargement)
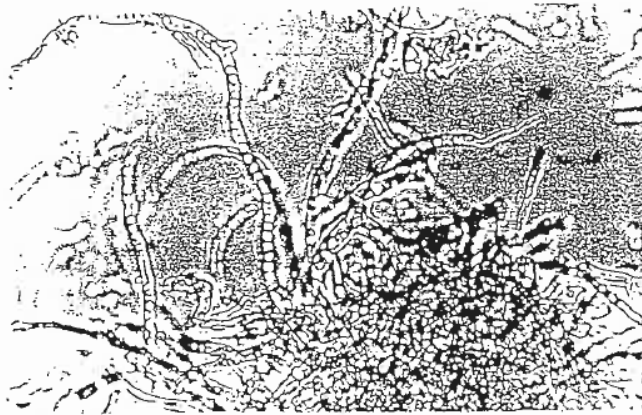
Photograph 4: Mycelian filaments and sporiferous head
(magnification X 1000. Photographic enlargement)
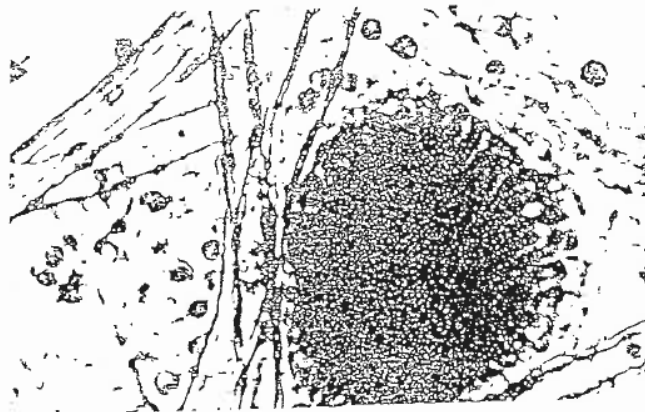
Tech. App. III -21

Photograph 5: *Sporiferous head*
(magnification X 1000. Photographic enlargement)
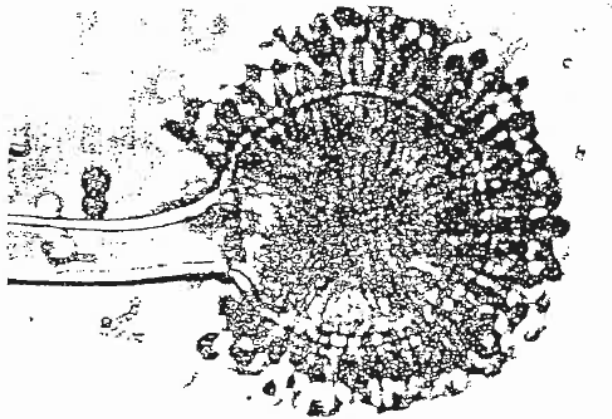
Figure 1: *Aspergillus niger; details of sporiferous head*
(BOTTON et al., 1985).
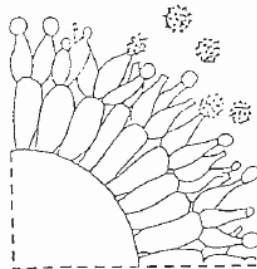
Tech. App. III -22

Photograph 6: Leuconostoc mesenteroïdes in long chains (young culture) (microscopical observation, magnification X 1000. Photographic enlargement).
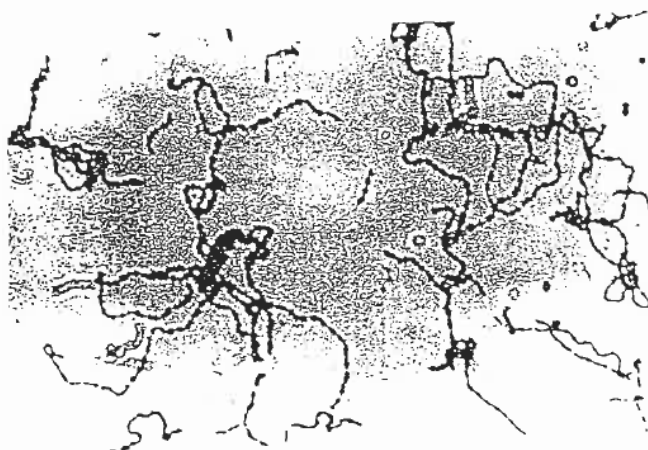
Photograph 7: Global vue of a chain of Leuconostoc mesenteroïdes (magnification X 10 000).
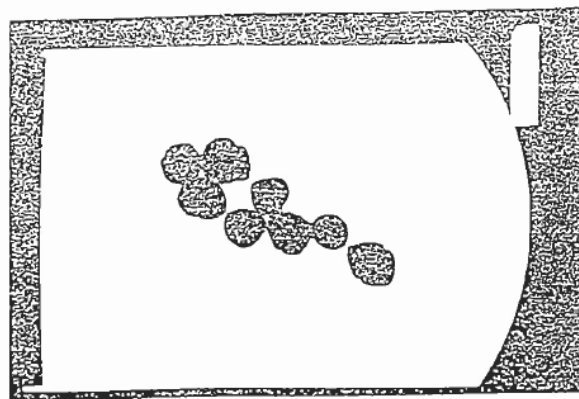
Tech, App. III -23

** <u>SECOND BACTERIAL STRAIN</u>: (KRIEG *et al.*, 1984)

This bacterial strain presents ovoid cells, in pairs, tetrads, heaps or chains of different length. The bacterial cells are gram+, are not mobile and do not contain spores.

The colonies are very small: their diameter is inferior to 1mm and their growth depends on the presence of fermentable sugars.

The P.A.S. coloration showed an intense production of polysaccharides which is however much less intense than the former bacterial strain.

From a biochemical point of vue, this bacterial strain doesn't possess catalase, doesn't produce indol, doesn't hydrolyse the proteins (the test with gelatine is negative) and doesn't reduce the nitrates. All the sugars except rhamnose are fermented. There is then a production of lactic acid and CO_2. This strain doesn't hydrolyse arginine, can be optionally anaerobic and is non-pathogenic. This microorganism was identified as *Streptococcus sp.*.

Photograph 9, taken with an optical microscope, gives a good image of this bacterium.

Photographs 10, 11, 12 and 13, taken with an electronical microscope, are more precise in showing the bacterial cells.

From a quantitative point of vue, we could determine, thanks to a dilution range, that one gram of enzymatic preparation enclosed 13.10^{10} living bacterial cells.

<u>CONCLUSION:</u>

From the enzymatic preparation, we isolated three different strains of microorganisms:

* One strain of *Aspergillus niger*.
* One strain of *Leuconostoc mensenteroïdes*.
* One strain of *Streptococcus sp.*.

But it is not impossible that the preparation may contain other bacterial strains.

The presence of two bacterial strains associated to *Aspergillus niger* is witnessing problems of contamination in the production of the enzymatic preparation and might bring up later technological problems.

These bacterial strains have a high development rate and are shown to produce polysaccharides. Now, according to the use of this enzymatic preparation to produce NEOSUGAR from sucrose, these microorganisms might find themselves in conditions favourable to intense polysaccharides production. This could have bad consequences by lowering of NEOSUGAR production efficiency and by bringing up problems of filter and tube blocking by polysaccharides.

Tech. App. III -24

Photograph 8: Detail of a chain (magnification X 24 000).
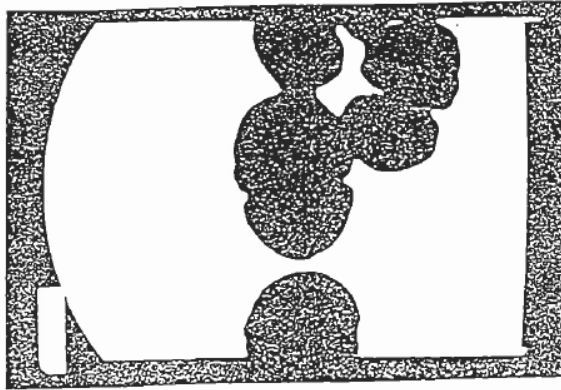
Photograph 9: Streptococcus sp. in pairs, tetrads or in small chains (young culture) (magnification X 1000. Photographic enlargement).

Tech. App. III -25

confidential
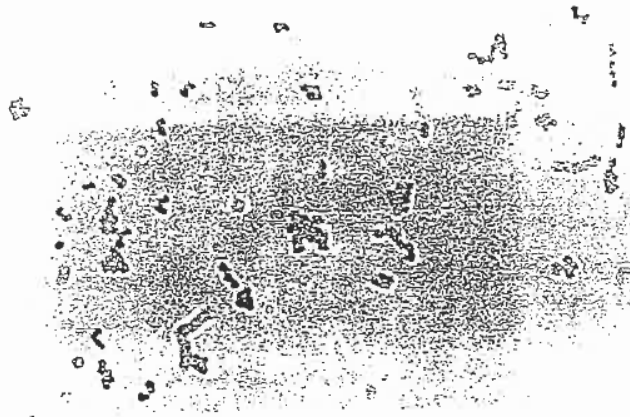
Photograph 10: Streptococcus sp. in pair (magnification X 20000).
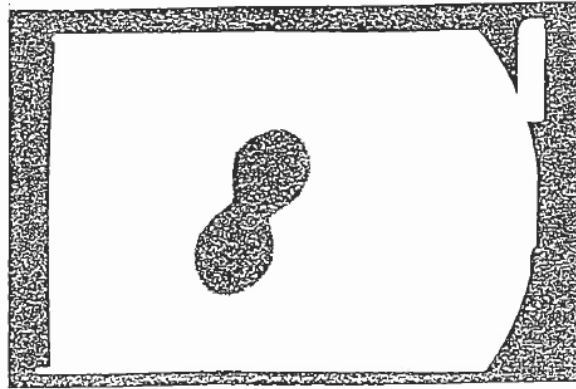
Photograph 11: Streptococcus sp. in tetrad (magnification X 18000).

Tech. App. III -26
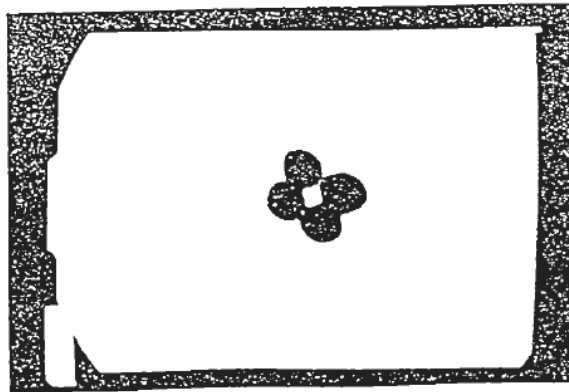
Photograph 12: Streptococcus sp. in chain (magnification X 20000).
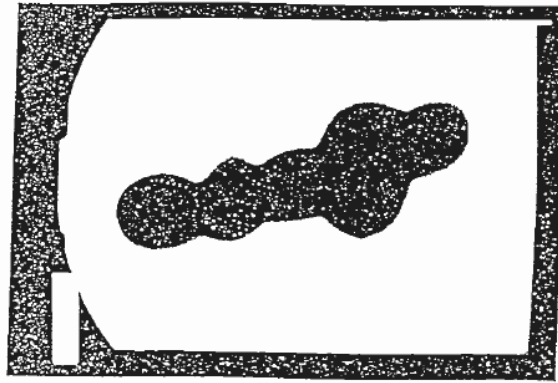
Photograph 13: Streptococcus sp. in heap (magnification X 20000).

Tech. App. III -27
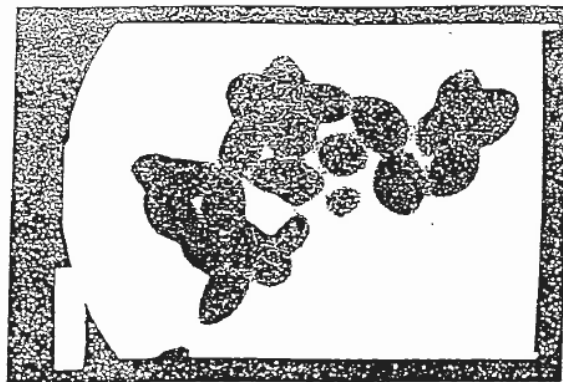
II-ANNEX STUDIES

In this second part, we made a serie of analysis to complete the former results. Thus, we will consider:
  *If the strain can be the one from which the preparation is obtained,
  *If the *Aspergillus niger* strains isolated from the preparation can produce aflatoxins,
  *and finally, if the strains isolated during the former study resist to the conditions necessary (temperature, osmotic pressure, anaerobiosis) for the industrial reaction to produce NEOSUGAR.

1- Is the isolated fongic strain the one used to obtain the enzymatic preparation?:

It was interesting to know if the *Aspergillus niger* strain isolated from the enzymatic preparation could be the one used by Japanese to prepare their powder. We may indeed imagine that the Japanese, to protect the strain that produces the interesting enzymatic system have additionned another strain without any industrial interest and easy to isolate in the fongic preparation.
This study was realised to compare numerous enzymatic activities found in the preparation with the *Aspergillus niger* strain.
That is why we used API ZYM galleries. The API ZYM system is a micromethod of semi-quantitative research of enzymatic activities. The tested enzymatic activities are presented in Annex A.
Two bacterial strains being present in the preparation, we realised a "blank" by testing the enzymatic activities of each of these strains.

RESULTS:
The detailed results are presented in the 4 following cards:

CONCLUSION:
The bacterial strains isolated from the preparation have negligible enzymatic activities with regard to the mushrooms and the powder.
The powder and the *Aspergillus niger* strain present the same enzymatic activities.
There are therefore good chances that the *Aspergillus niger* strain isolated from the preparation provided by the Japanese is the one that was used to prepare the enzymatic preparation used to produce NEOSUGAR.
But, taking in account the insufficient specificity of the tests, it is not possible to have an absolute certitude.

2- study of the production of aflatoxin by the fongic strain isolated in the preparation.

We prepared 3 erlenmeyers containing 1g cork preparation and 50 ml PAI toxinogen medium (whose composition is given in Appendix V). Two of the three containers were seeded with 1ml of a spore suspension and fongic mycelium. The third container wasn't seeded and was used as a "blank". After 15 days of culture in dark conditions and room temperature (25°C), we extracted and purified aflatoxins in the blank and in the fongic cultures according to techniques developed at the laboratory (LEITAO *et al.*, 1987a; LEITAO *et al.*, 1987b).
The dosage of aflatoxins was realised by thin layer chromatography and high performance liquid chromatography (LEITAO *et al.*, 1987a; LEITAO *et al.*, 1988a).

RESULTS:
None of the two chromatographies did reveal the presence of aflatoxins in the fongic cultures.
The results obtained by liquid chromatography are shown in figures 2, 3 and 4.

Tech. App. III -28

apí ZYM

Bactérie 1

Leuconostoc mesenteroides

Date 29|06|88
Incubation 30°C

apí ZYM

Bactérie 2

Streptococcus sp.

Date 29|06|88
Incubation 30°C

apí ZYM

Date 30°C 24heures

apí ZYM

A. niger

Date 30°C 24heures

Tech. App. III -29

Figure 2: Aflatoxin standards (gain 100).
aflatoxin B1: 0.005 µg/ml
aflatoxin B2: 0.001 µg/ml
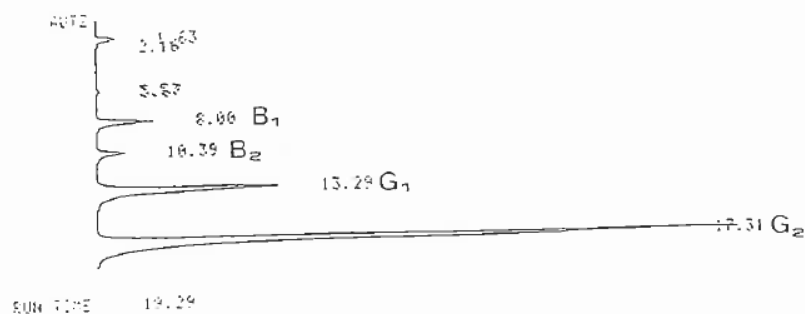aflatoxin G1: 0.01 µg/ml
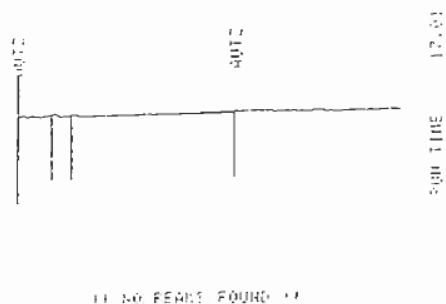aflatoxin G2: 0.05 µg/ml

Figure 3: Blank (gain 100).

Figure 4: Extract of Aspergillus niger culture (gain 100).

Tech. App. III -30

CONCLUSION

The *Aspergillus niger* strain isolated from the enzymatic preparation does not produce aflatoxins.

3- Study of the resistance of microorganism strains under NEOSUGAR producing experimental conditions.

The aim of this study is to examine eventual resistance of different microorganisms isolated from the preparation in NEOSUGAR producing conditions (temperature, osmotic pressure, anaerobiosis). We also envisaged the behaviour of these microorganisms in the case the experimental conditions wouldn't be maintained (loss of anaerobiosis or aerobiosis, no temperature elevation to 80°C...).

Tests were realised in tubes containing 30 ml of a sucrose solution with a concentration of 750g dry matter per litre.

The test tubes were seeded either with preparation or with the *Aspergillus niger* strain, or with Leuconostoc strain, or with Streptococcus strain.

The tubes were then treated as follow:

* Culture at 70°C during 20 hours in anaerobiosis and 30 minutes at 80°C.
* Culture at 70°C during 20 hours in anaerobiosis, but no temperature elevation at the end of the reaction.
* Culture at 70°C during 20 hours (10 hours in anaerobiosis and 10 hours in aerobiosis) and finally, 30 minutes at 80°C.
* Culture at 70°C during 20 hours (10 hours in anaerobiosis and 10 hours in aerobiosis) and finally, but no temperature elevation at the end of the reaction.
* Culture at 70°C during 20 hours in aerobiosis and 30 minutes at 80°C.
* Culture at 70°C during 20 hours in aerobiosis, but no temperature elevation at the end of the reaction.

After each of these treatments, we seeded Petri boxes containing appropriate culture media for these microorganisms (Brian medium for the preparation and the fongic strain, Déméter medium, whose composition is given in Appendix IV, for the preparation and the bacterial strains). We thus could estimate the growing of the different microorganisms.

RESULTS - CONCLUSIONS

The fongic strain isolated or contained in the cultivated preparation in strict anaerobiosis, anaerobiosis and aerobiosis, or in aerobiosis, for 20 hours at 70°C and at a high osmotic pressure, wasn't killed under these experimental conditions and subsequently grows on the surface of the culture media in the Petri boxes.

On the contrary, temperature elevation up to 80°C during 30 minutes at the end of the reaction destroys those microorganisms; hence, this temperature elevation at the end of the reaction to destroy the enzymatic system is sufficient to assure good sterility of the final product. It eliminates the microorganisms who might have developed themselves in the sucrose solution during the NEOSUGAR producing reaction.

Consequently, the eliminated microorganisms will not give rise to deterioration of the final product in case of a certain period of time before use.

It seems important to us to insist on this point: the temperature elevation during 30 minutes at 80°C at the end of the reaction is an important technological element for a good later conservation of the product.

Tech. App. III -31

III-BIOCHEMICAL STUDY - DOSAGE OF MYCOTOXINS.

1- Dosage of the sterigmatocystin.

Sterigmatocystin (whose structure is presented in annex B) is not a mycotoxin normally produced by *Aspergillus niger*. This molecule is an intermediate in the aflatoxin production metabolism. Since the enzymatic preparation used in the NEOSUGAR producing reaction is obtained from a culture of *Aspergillus niger*, it seems to be necessary to look if, under the culture conditions used in Japan, the mushroom wouldn't develop a toxic metabolism, finding itself blocked at an intermediate stage, stage which could be a formation of sterigmatocystin.

Moreover, sterigmatocystin is a mycotoxin which is not excreted in the culture medium by the mushrooms producing it but, on the contrary, stays in the mycelium.

Consequently, we dosed the sterigmatocystin in the enzyme preparation, the sucrose and the different samples collected at different stages of the NEOSUGAR producing reaction (end of the reaction, after active charcoal addition, after filtration).

This study was realised on samples collected during a reaction done by Mr. GUIBERT according to the Japanese protocol.

The extraction, purification and dosage techniques (thin layer chromatography and high performance liquid chromatography) used for the sterigmatocystin were developed at the laboratory (LEITAO et al., 1988a; LEITAO et al., 1988b).

RESULTS
The results are presented in figures 5, 6, 7, 8, 9, and 10

CONCLUSION
None of the analysed samples contained sterigmatocystin. So, if the *Aspergillus niger* used for the preparation of the enzymatic preparation developed a toxic metabolism, it wasn't blocked at a middle stage.

2- Dosage of the aflatoxins.

Aflatoxins are mycotoxins extremely persistent during industrial and biological transformations and are finally retrieved in the transformed products if the basic products were contaminated by these molecules.

To see if aflatoxins had been produced in the enzymatic preparation by *Aspergillus niger* and thus if there is a toxicity risk in the final product of the reaction (NEOSUGAR), we dosed the aflatoxins in the enzymatic preparation, the sucrose and the different samples collected at different stages of the NEOSUGAR producing reaction. (end of the reaction, after active charcoal addition, after filtration).

This study was realised on samples collected during a reaction done by Mr. GUIBERT according to the BÉGHIN-SAY protocol.

The extraction, purification and dosage techniques (thin layer chromatography and high performance liquid chromatography) used for the sterigmatocystin were developed at the laboratory (LEITAO et al., 1987; LEITAO et al., 1988a).

RESULTS:
* JAPANESE EXPERIMENTAL PROTOCOL:
The results are presented in figures 11, 12, 13, 14, 15 and 16
* BÉGHIN-SAY EXPERIMENTAL PROTOCOL:
The results are presented in figures 17, 18, 19, 20, 21 and 22.

Tech. App. III -32

Figure 5: Sterigmatocystin standard (1 µg/ml; sensibility 0.01)

AUTZ

10.74 ster.

RUN TIME   14.69
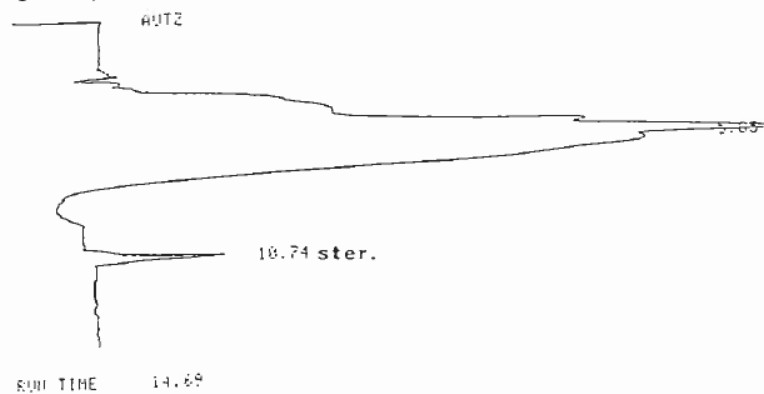
Figure 6: Search of Sterigmatocystin in the sucrose by H.P.L.C. (sensibility 0.01).

AUTZ

RUN TIME   13.16
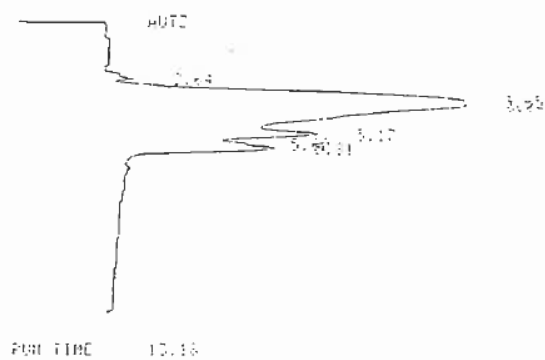
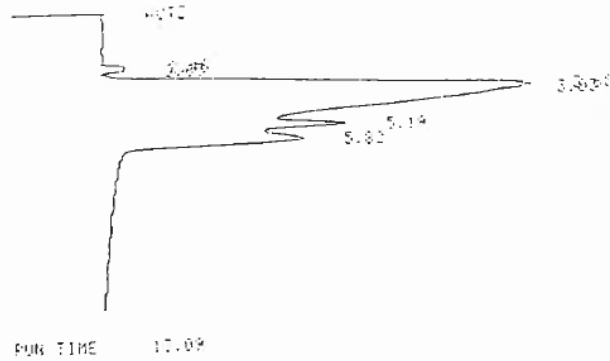Figure 7: Search of Sterigmatocystin in the powder by H.P.L.C. (sensibility 0.01).


AUTZ

3.22
4.09

RUN TIME   12.03

Tech. App. III -33
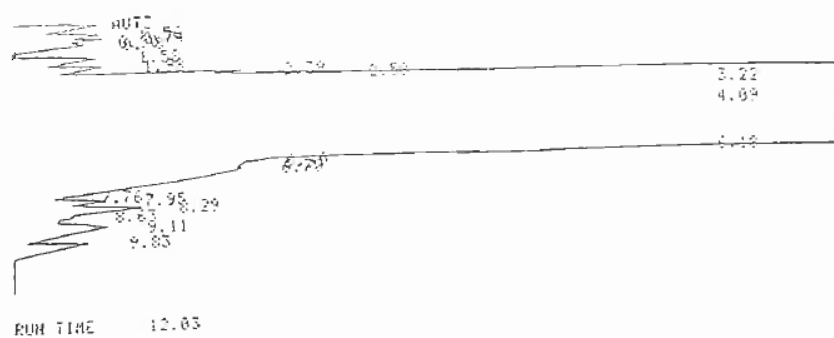
Figure 8: Search of Sterigmatocystin in a sample collected at the end of the reaction by H.P.L.C. (sensibility 0.01).
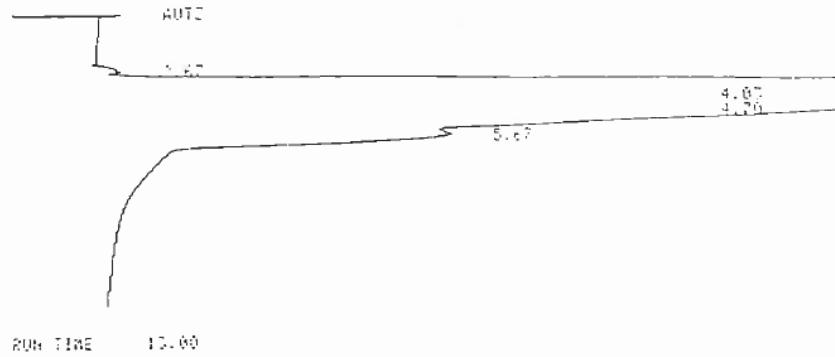
Figure 9: Search of Sterigmatocystin in a sample collected after addition of active charcoal by H.P.L.C. (sensibility 0.01).

Figure 10: Search of Sterigmatocystin in a sample collected after filtration by H.P.L.C. (sensibility 0.01).
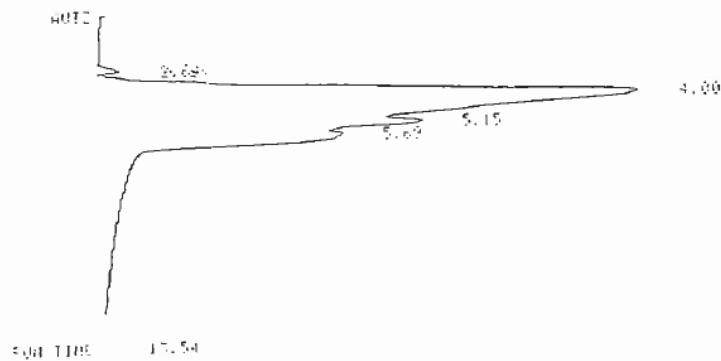
Tech. App. III -34

Figure 11:   Aflatoxin standards (gain 100).
aflatoxin B1: 0.005 µg/ml
aflatoxin B2: 0.001 µg/ml
aflatoxin G1: 0.01 µg/ml
aflatoxin G2: 0.05 µg/ml
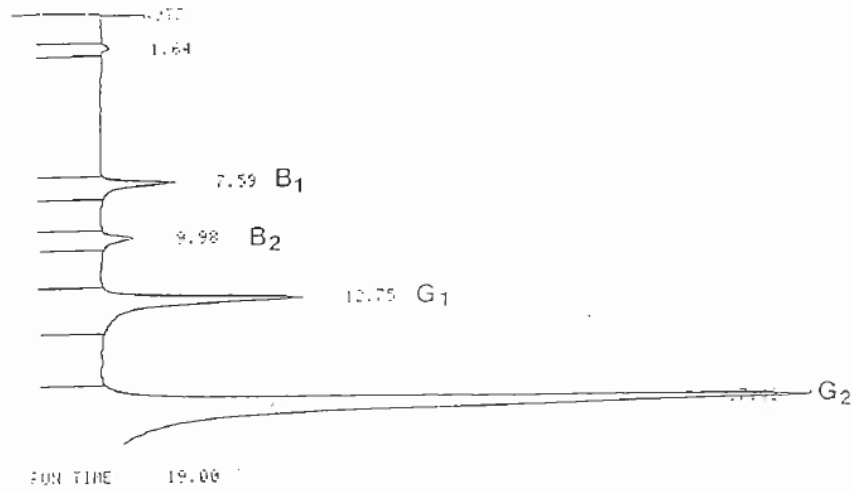
Figure 12:   Search for aflatoxins in the sucrose by H.P.L.C. (gain 100).

Tech. App. III -35
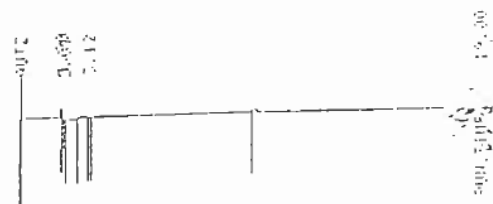
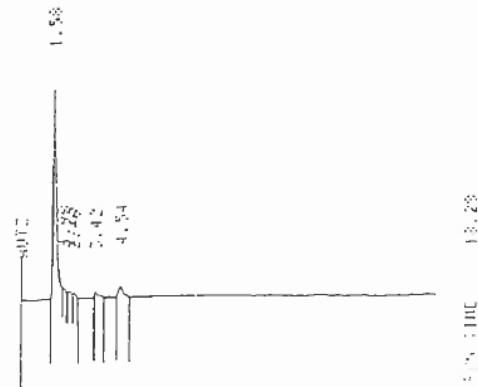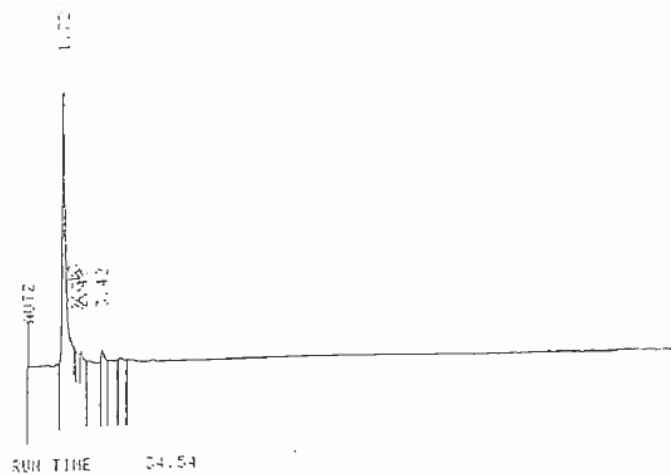Figure 13: Search for aflatoxins in the powder by H.P.L.C. (gain 100).
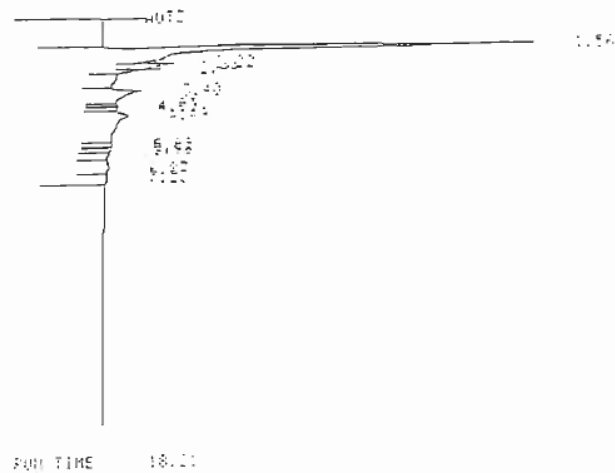
Figure 14: Search for aflatoxins in a sample collected at the and of the reaction by H.P.L.C. (gain 100).


Figure 15: Search for aflatoxins in a sample collected after addition of active charcoal by H.P.L.C. (gain 100).
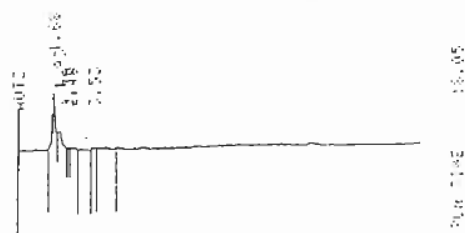
Tech. App. III -36

Figure 16: Search for aflatoxins in a sample collected after filtration by H.P.L.C. (gain 100).
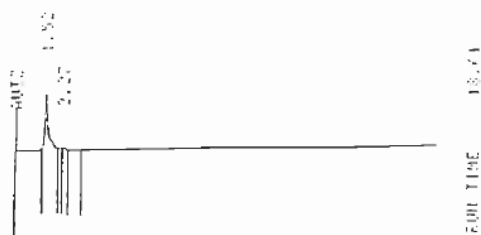
Figure 17: Aflatoxin standards (gain 100).
aflatoxin B1: 0.005 µg/ml
aflatoxin B2: 0.001 µg/ml
aflatoxin G1: 0.01 µg/ml
aflatoxin G2: 0.05 µg/ml
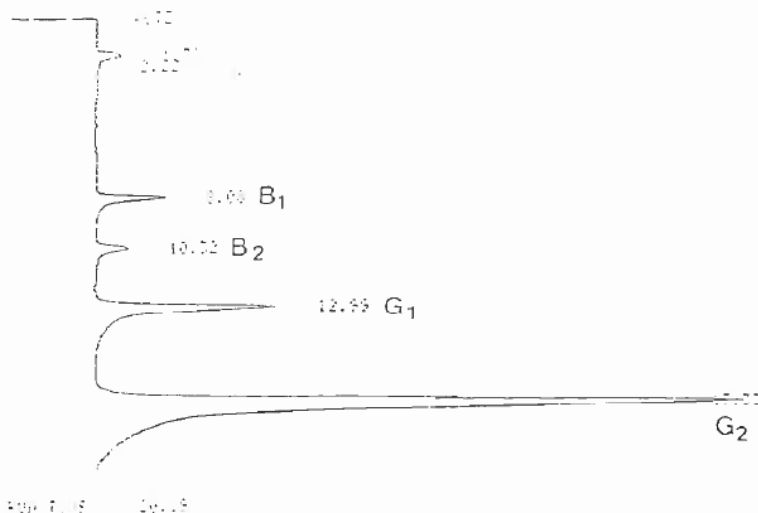
Figure 18: Search for aflatoxins in the sucrose by H.P.L.C. (gain 100).
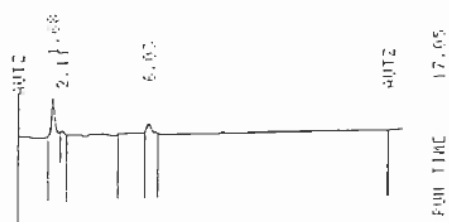
Tech. App. III -37

Figure 19:   Search for aflatoxins in the powder by H.P.L.C. (gain 100).

Figure 20: Search for aflatoxins in a sample collected at the and of the reaction by
H.P.L.C. (gain 100).

RUN TIME   34.54

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Figure 21:Search for aflatoxins in a sample collected after addition of active charcoal by H.P.L.C. (gain 100).

Figure 22:Search for aflatoxins in a sample collected after filtration by H.P.L.C. (gain 100).
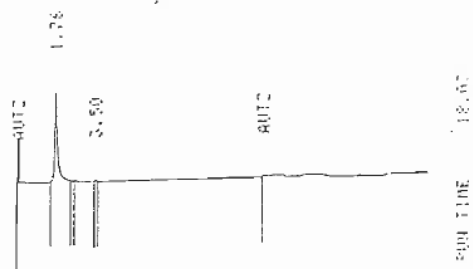
Tech. App. III -39

CONCLUSION:

None of the analyzed samples contained aflatoxins, nor did the initial products (sucrose, enzymatic preparation) nor the *bruto* (or more or less purified) reaction products. This counts independently of the protocol used to realise the enzymatic reaction.

The *Aspergillus niger* used for the preparation of the enzymatic preparation did not develop toxinogen metabolisms, either during the preparation of it nor during the enzymatic reaction.

III-40

GENERAL CONCLUSION

I - The "enzymatic preparation" we have examined contains living organisms. We isolated:

A strain of mould belonging to the specie *Aspergillus niger* which is probably the only mould specie present.

Two bacterial strains, one belonging to *Leuconostoc mesenteroïdes*, the other to *Streptococcus sp.*.

There are probably other bacterial strains in the powder. It is impossible for us to tell what interest or what trouble their presence might bring.

II - The *Aspergillus niger* strain was cultivated in a particularly favourable liquid medium for toxicogenesis. Despite this, it didn't produce aflatoxins.

Moreover, taking in account a possible industrial camouflage, we examined if the *Aspergillus niger* strain we isolated and the one whose mycelian fragments constitutes the essential part of the enzymatic preparation were identical. The tests were in favour of a same identity but did not allow an absolute conclusion. This could only be possible by starting from the beginning the preparation of the enzymatic preparation from the strain we isolated (macro-culture; powdering-drying; NEOSUGAR manufacturing with efficiency measures).

The bacteria do not seem to provoque toxicity problems to the consumer. But they might cause some technological difficulties (particularly Leuconostoc) by the production of polysaccharides increasing the viscosity of the solutions (filtration, circulation in the pipes, etc...).

The *Aspergillus niger* strain and the bacterial strains are still alive at the end of the enzymatic reaction. It seems essential to us to strictly respect the conditions of the final stage of the manufacturing protocol (80°C during 30 minutes) with the aim, the enzymatic reaction being stopped, to eliminate definitively these microorganisms. If this is not respected, the stability of the final products will not be sure and these microbial strain will be implanted in the installations that will be used for later manufacturing, which should then be periodically sterilised.

III - The search for mycotoxins able to be produced by *Aspergillus niger* was done.

We did not find the presence of sterigmatocystin, toxical intermediate for the formation of aflatoxins, in the enzymatic preparation, nor the raw material, nor in some samples collected during the NEOSUGAR producing process.

We searched for the 4 aflatoxins: B1, B2, G1 and G2 in the "enzymatic preparation", the raw material and at different production stages.

We did not find aflatoxins, in any sample.

Our following observations concern two points:

1 - The presence of bacteria:

We ignore if these bacteria play a part in the enzymatic reaction.

If not, it would be convenient to get rid of them and to realise an "enzymatic preparation" totally clear from these microorganisms.

If they do, it would be preferable to cultivate the different microorganisms separately and then to unify them, to have a good control of the mixing. This of course suppose a complete study of the bacterial population present in the "enzymatic preparation".

III-41

2 - The toxicological aspect:
We searched for eventual production of sterigmatocystin and the 4 aflatoxins: B1, B2, G1 and G2 and concluded of their absence.

But we know that *Aspergillus niger* can produce other mycotoxins like flavosperone (BOTTON *et al.*, 1985) or the malformines (CURTIS *et al.*, 1974).
These compounds less known than the aflatoxins present a relatively high toxicity and might be at the origin of mycotoxicoses. They have been at the origin of breeding accidents (SCOTT, 1964)
Moreover, *Aspergillus niger* produces a lot of oxalic acid and kojic acid. These metabolites present a non negligible toxicity for breeding animals.
Works (Wilson *et al.*, 1961; Scott, 1964; Willie *et al.*, 1976) have shown that the production of great quantities of oxalic acid in food by *Aspergillus niger* may have toxic effects.
The conditions of the "enzymatic reaction" are too particular (T°, osmotic pressure) to allow the extrapolation of the physiology of mould from usual culture conditions.

Consequently, if this work makes possible to solve certain toxicological problems, it doesn't permit to eliminate them all.
But it draws the attention on technological aspects, to reduce some difficulties and to permit some improvement of the proceeding.

Toulouse, July the 27th, 1988

G. DE SAINT-BLANQUAT

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[1]*Since the redaction of this report, this paper has been published in Journal of Liquid Chromatography 11: 2285-2293*
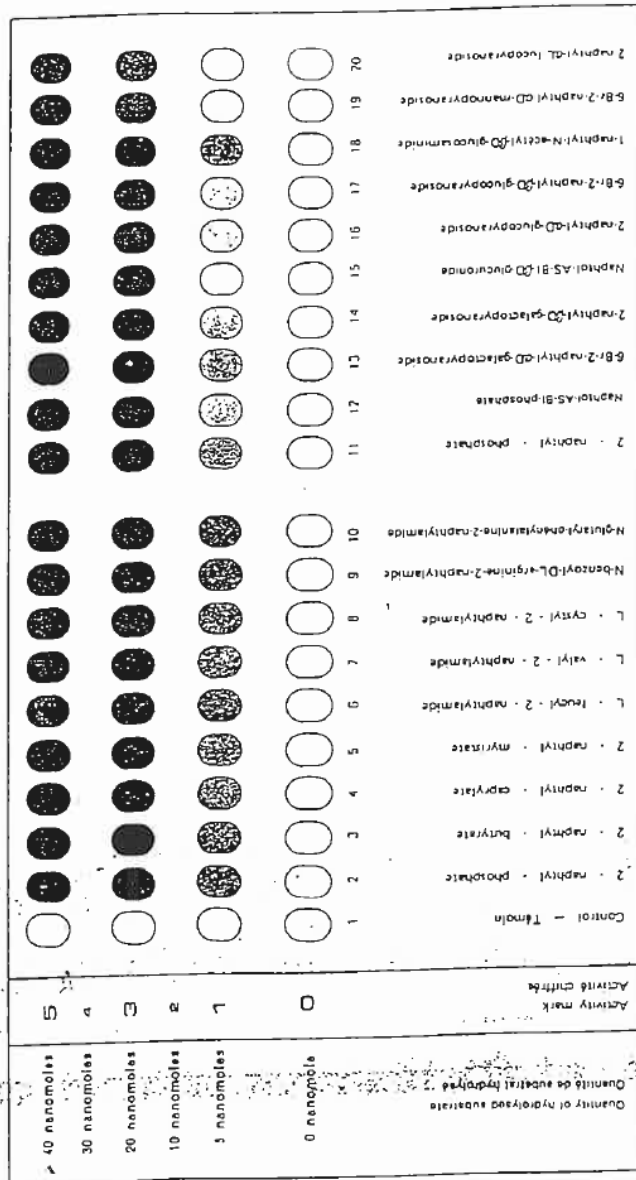
Tech. App. III -43

confidential

ANNEX-A-

INTERPRÉTATION DES TESTS

N°	ENZYME RECHERCHÉE	SUBSTRAT	pH	Positive	Négative
1	Témoin			Incolore ou couleur de l'échantillon si celui-ci a une coloration importante	
2	Phosphatase alcaline	2-naphtyl phosphate	8,5	Violet	
3	Estérase (C 4)	2-naphtyl butyrate	6,5	Violet	
4	Estérase Lipase (C 8)	2-naphtyl caprylate	7,5	Violet	
5	Lipase (C 14)	2-naphtyl myristate	-	Violet	
6	Leucine arylamidase	L-leucyl-2-naphtylamide	-	Orange	
7	Valine arylamidase	L-valyl-2-naphtylamide	-	Orange	
8	Cystine arylamidase	L-cystyl-2-naphtylamide	-	Orange	
9	Trypsine	N-benzoyl-DL-arginine-2-naphtylamide	8,5	Orange	
10	α Chymotrypsine	N-glutaryl-phénylalanine-2-naphtylamide	7,5	Orange	
11	Phosphatase acide	2-naphtyl phosphate	5,4	Violet	
12	Naphtol-AS-BI-phosphohydrolase	Naphtol-AS-BI-phosphate	-	Bleu	
13	α galactosidase	6-Br-2-naphtyl-αD-galactopyranoside	-	Violet	
14	β galactosidase	2-naphtyl-βD-galactopyranoside	-	Violet	
15	β glucuronidase	Naphtol-AS-BI-βD-glucuronide	-	Bleu	
16	α glucosidase	2-naphtyl-αD-glucopyranoside	-	Violet	
17	β glucosidase	6-Br-2-naphtyl-βD-glucopyranoside	-	Violet	
18	N-acetyl-β glucosaminidase	1-naphtyl-N-acétyl-βD-glucosaminide	-	Marron	
19	α mannosidase	6-Br-2-naphtyl-αD-mannopyranoside	-	Violet	
20	α fucosidase	2-naphtyl-αL-fucopyranoside	-	Violet	

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scFOS Application – GTC Nutrition
August 2010

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API ZYM

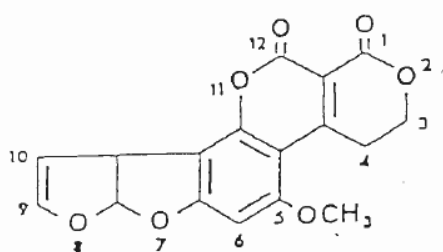READING SCALE – ECHELLE DE LECTURE
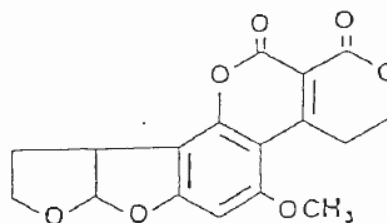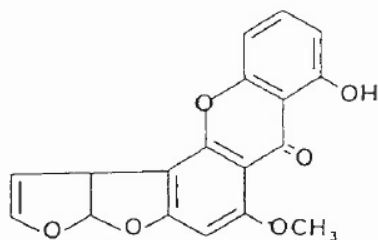
Tech. App. III -45

ANNEX-B-
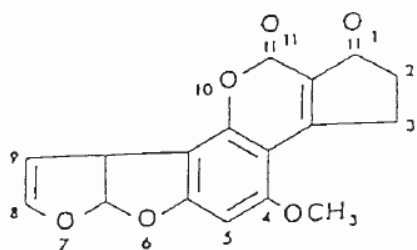
Aflatoxine B₁
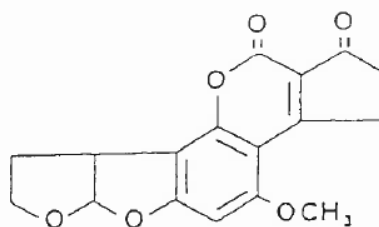
Aflatoxine B₂

Aflatoxine G₁

Aflatoxine G₂

Stérigmatocystine

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