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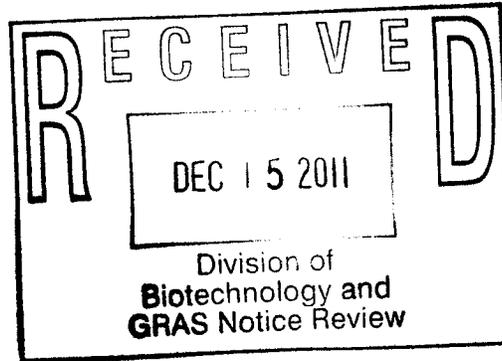


SENT VIA FEDEX

Mary Ditto, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

USA

Subject: GRAS Notice for Chitin-Glucan



November 17, 2011

Dear Dr. Ditto:

In accordance with 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting in triplicate, as the Notifier [KitoZyme s.a., Rue Haute Claire , 4, Parc Industriel des Hauts-Sarts, Zone 2, BE-4040, Herstal, Belgium], a Notice of the determination, on the basis of scientific procedures, that chitin-glucan produced from *Aspergillus niger*, as defined in the enclosed documents, is GRAS under specific conditions of use as a secondary direct food ingredient in the manufacture of alcoholic beverages, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, includes a comprehensive summary of the data available that has been reviewed by an independent panel of experts (the Expert Panel) qualified by scientific training and experience to evaluate the safety of chitin-glucan for use in alcoholic beverage manufacturing.

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

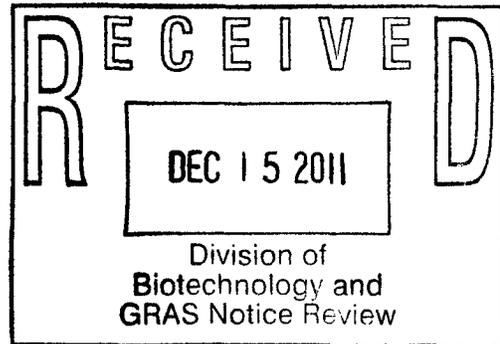
Véronique Maquet
Product Development Manager
KitoZyme S.A.

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KitoZyme sa



Chitin-Glucan GRAS Notice



Prepared for: Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Prepared by: Kitozyme S.A.
Rue Haute Claire, 4
Parc Industriel des Hauts-Sarts, Zone 2
BE-4040 Herstal
Belgium

September 30, 2011

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I. GRAS EXEMPTION CLAIM

A. Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

As defined herein, chitin-glucan, derived from *Aspergillus niger* (*A. niger*), has been determined by Kitozyme S.A. (Kitozyme) to be Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, and on the consensus opinion of an independent panel of Experts qualified by scientific training and expertise to evaluate the safety of chitin-glucan derived from *A. niger* under the conditions of intended use in food. Therefore, the use of chitin-glucan in food as described herein is exempt from the requirement of premarket approval (Section 409 of the *Federal Food, Drug and Cosmetic Act*).

Signed,

(b) (6)

Véronique Maquet, Ph.D.
Product Development Manager
Kitozyme

Date

November 18, 2011

B. Name and Address of Notifier

Kitozyme sa
Rue Haute Claire, 4
Parc Industriel des Hauts-Sarts, Zone 2
BE-4040 Herstal
Belgium

C. Common Name of the Notified Substance

Chitin-glucan

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D. Conditions of Intended Use in Food

Kitozyme intends to market chitin-glucan, derived from *A. niger*, as a secondary direct food ingredient (processing aid) for use in the production of alcoholic beverages in the United States at a use levels of between 50 to 500 g/hl during processing (Table I.D-1). Sediments containing chitin-glucan are removed from the wine, must, beer or spirits at the end of the treatment using physical separation processes such as racking, centrifugation, or filtration.

Food Category	Food-Use	Application	Use-Levels in Application¹
Beverages, Alcoholic	Beer	Clarification	100 g/100 L
	Wine	Clarification	100 g/100 L
		Fining	50 g/100 L
		Removal of trace metals	100 g/100 L
		Removal of potential mycotoxins	500 g/100 L
		Prevention of oxidation of color	100 g/100 L
		Reduction of heat labile proteins	100 g/100 L
		Aid to filtration	50 g/100 L
	Spirit	Removal of trace metals	100 g/100 L
		Improvement of flavor	50 g/100 L
	Must	Clarification/removal of off-flavor	100 g/100 L
		Removal of potential mycotoxins	500 g/100 L
		Prevention of oxidation of color	100 g/100 L

¹ **Note:** Chitin-glucan is used as a processing-aid during alcoholic beverage production and is subsequently removed by physical separation processes during manufacturing; therefore use levels do not constitute concentrations of chitin-glucan in the final product, which is absent chitin-glucan.

E. Basis for the GRAS Determination

Pursuant to 21 CFR §170.30, chitin-glucan derived from *A. niger*, has been determined by Kitozyme to be GRAS on the basis of scientific procedures (U.S. FDA, 2011). This GRAS determination is based on data generally available in the public domain pertaining to the safety of chitin-glucan for use in food, as discussed herein and in the accompanying documents, and on a consensus among a panel of Experts¹ who are qualified by scientific training and experience to evaluate the safety of chitin-glucan as a component of food.

¹ The Panel consisted of the below-signed qualified scientific Experts: Prof. Joseph F. Borzelleca, Ph.D., (Medical College of Virginia); Prof. Robert Nicolosi, Ph.D., (University of Massachusetts Lowell); and Prof. John Thomas, Ph.D., (Indiana University School of Medicine). A copy of the Expert Panel summary is located in Appendix A and is titled "Documentation Supporting the Generally Recognized as Safe (GRAS) Use of Chitin-Glucan Derived from *Aspergillus niger* as a Direct and Secondary Direct Food Ingredient for Use in a Variety of Foods".

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F. Availability of Information

Data and information that serve as the basis for this GRAS Notice will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of Kitozyme located at the following address:

Kitozyme S.A.
Rue Haute Claire, 4
Parc Industriel des Hauts-Sarts, Zone 2
BE-4040 Herstal
Belgium

Should the FDA have any questions or additional information requests regarding this Notice, Kitozyme also will supply these data and information.

II. DETAILED INFORMATION REGARDING THE IDENTITY OF THE SUBSTANCE

A. Identity

The common or usual name of this product is chitin-glucan. Kitozyme's chitin-glucan ingredient is an insoluble non-digestible fiber preparation derived from the cell walls of non-genetically modified *A. niger*. Chitin-glucan is composed predominantly of 2 types of polysaccharides, namely chitin (poly-N-acetyl-D-glucosamine) and *beta*-(1,3)-D-glucan (poly-D-glucose).

Common or Usual Name: Chitin-glucan

Chemical Name:

Chitin:

- Poly(N-acetyl-D-glucosamine)
- International Union of Pure and Applied Chemistry (IUPAC) name for chitin: N-[5-[[[3-acetylamino-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]methoxymethyl]-2-[[5-acetylamino-4,6-dihydroxy-2-(hydroxymethyl)oxan-3-yl]methoxymethyl]-4-hydroxy-6-(hydroxymethyl)oxan-3-yl]ethanamide

beta-(1,3)-D-glucan:

- Poly (D-glucose)
- IUPAC name for *beta*-D-glucan: 2-[4,5-dihydroxy-2-(hydroxymethyl)-6-[4,5,6-trihydroxy-2-(hydroxymethyl)oxan-3-yl]oxy-oxan-3-yl]oxy-6-(hydroxymethyl)oxan-3,4,5-triol

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Trade Names:

KiOfine-CG®

Chemical Abstracts Service (CAS) Number:

A CAS number for chitin-glucan is not available

Chemical and Physical Properties:

The chemical structure and the properties of chitin-glucan are summarized in Table II.A-1.

Name	Chitin-glucan
Molecular formula	$[C_6H_{10}O_5]_m - [C_8H_{13}NO_5]_n$
Chemical Structure	NA
Molecular Weight	NA – Insoluble particulate
Melting Point	Not applicable
Physical Form	Fine free-flowing powder
Color	Light yellowish to brown
Solubility	Not soluble in aqueous and organic solvents

B. Manufacturing Information

(i) Materials

Chitin-glucan is manufactured from the non-viable post-fermentation microbial biomass of non-genetically modified *A. niger*, and is sourced from manufacturers of food grade citric acid permitted for use in feed applications in the European Union. Commercial sources of *A. niger* used for production of chitin-glucan are subject to strict quality control by Kitozyme for compliance with internal specifications as described Table II.B-1. Sources of *A. niger* used for manufacture of chitin-glucan are authorized for sale for use in animal feed and conform to the European legislation (EC Directives 2002/32/EC, 2003/57/EC, and 2003/100/EC) regarding contaminants (heavy metals and aflatoxins), and pesticide residues (EC, 2002, 2003a,b). All raw materials and processing aids used in the manufacture of chitin-glucan are suitable food-grade materials and are used in accordance with applicable U.S. federal regulations, and/or are permitted for use in food as described in Table II.B-2 below.

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Analysis Parameter	Specification
Water Content (%)	≤10%
Ash (% dw)	≤2%
Protein (%)	≤10%
Lipids (%)	≤1%
Density (kg/m ³)	≤670
<i>Metals (ppm)</i>	
As	ND
Hg	ND
Pb	≤0.3
Cu	≤0.3 - 4.5
Cd	≤0.2
Zn	≤17 - 20
Bacteria cfu/g	≤1000
Yeasts and Molds cfu/g	≤10

dw = dry weight; ND = not detected

Material	Use	Regulatory Status
<i>Aspergillus niger</i> mycelium	Source of chitin-glucan	Permitted for use in the production of citric acid for use in food in the United States (21 CFR §173.280 – U.S. FDA, 2011).
Water	Solvent	N/A
Sodium Hydroxide	Processing-aid	In accordance with 21 CFR §184.1763, sodium hydroxide is permitted for use in food as a processing aid with no limitations other than cGMP (U.S. FDA, 2011).

cGMP = current Good Manufacturing Practices; N/A = Not applicable

(ii) Method of Manufacture

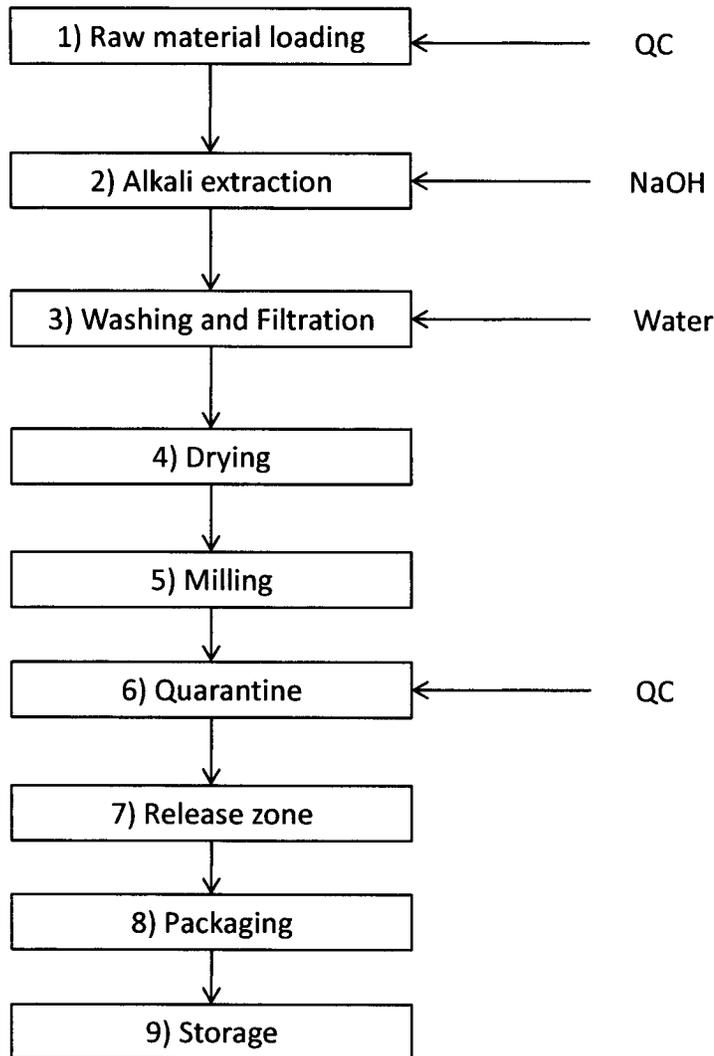
A schematic overview of the production process for chitin-glucan is presented in Figure II.B-1, and a general overview of the manufacturing process is described below. Each production run is documented in a lot record (designated the “Road-book”) as it is manufactured. For the manufacture of chitin-glucan, Kitozyme follows the Belgian legislation relative to auto-control in the food industry² and the auto-control guide established by the Belgian Federation for food supplements, dietary, and organic products (NAREDI) for the traceability, mandatory notification and risk analysis based on the Hazard Analysis and Critical Control Points (HACCP) method³.

² Arrêté Royal 27/04/2007; Arrêté Royal 24/10/2005; Arrêté Royal 22/01/2004; Arrêté Royal 14/11/2003 (AFSCA, 2011)

³ <http://www.naredi.be/engels/home.htm> (Naredi, 2001)

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Figure II.B-1 Schematic Overview of the Manufacturing Process for Chitin-Glucan



Upon receipt of the *A. niger* raw material, a sample is assessed for conformity to the requirements of Kitozyme (Table II.B-2) and the specifications of the supplier for microbiological content, heavy metal content, inorganics, and pesticides. Following inspection and demonstration of compliance with these limits, the material is loaded into the reaction tank, and is subjected to alkali hydrolysis (sodium hydroxide) for a controlled time period under constant stirring. The alkali soluble components (pigments, proteins, lipids, nucleotides, and other soluble intracellular metabolites) are removed through sequential washing and filtration steps until a target conductivity value is achieved. The alkali insoluble chitin and *beta*-(1,3)-glucan polysaccharides are then dried to produce a fine, free-flowing powder with a dry matter content >90%. Chitin-glucan is then milled and sieved to a granulation size of $\geq 95\%$ less than 500 μm , transferred to the quarantine storage zone for quality control testing, packaged using permitted food contact materials, and stored at room temperature in a secured storage zone.

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C. Product Specifications

The product specifications for chitin-glucan as supplied by Kitozyme are shown below in Table II.C-1. Analytical results from three non-consecutive lots of chitin-glucan demonstrate production of a consistent product in compliance with the product specifications (Table II.C-2).

Table II.C-1 Product Specifications for Chitin-Glucan		
Test Parameters/Test	Test Method	Tolerance/Specification
Physico-Chemical and Organoleptic		
Tapped density (g/cm ³)	EP2.9.34	0.7 to 1
Appearance	Visual observation	Fine free-flowing powder
Color	KZ PT-CQ-120	Light yellowish to brown
Odor	KZ PT-CQ-127	Odorless
Composition (% d.w.)		
Chitin-glucan	KZ PT-CQ-111	≥90
Ratio chitin-glucan	KZ PT-125	25:65 to 60:40
Loss on drying	KZ PT-CQ-100	≤10
Total ash	KZ PT-CQ-105	≤3
Soluble residues	KZ PT-CQ-126	≤5
Microbial and Heavy Metal Limits		
Aerobic plate count (cfu/g)	ISO 4833	≤1,000
Yeast/mould (cfu/g)	ISO 7954	≤100
Coliforms (cfu/g)	ISO 7251	≤100
Salmonella spp. (/25 g)	ISO 6579	Absent
Enterobacteriaceae (cfu/g)	ISO 215-28-2	≤10
Arsenic (mg/kg)	ICP-MS	≤1
Lead (mg/kg)	ICP-MS	≤1
Mercury (mg/kg)	ICP-MS	≤0.1
Cadmium (mg/kg)	ICP-MS	≤1

cfu = colony forming units; EP = European Pharmacopoeia; ICP-MS = inductively coupled plasma-mass spectrometry; ISO = International Organization for standardization; KZ PT-CQ = KitoZyme's internal procedure ; NMR = nuclear magnetic resonance; % d.w. = percent dry weight basis

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Table II.C-2 Summary of the Product Analysis for 3 Batches of Chitin-Glucan				
Test Parameters/Test	Specification	Manufacturing Batch		
		110210L3	L3000046	L3000047
Physico-Chemical and Organoleptic				
Tapped density (g/cm ³)	0.7 to 1	0.76	0.83	0.73
Appearance	Fine free-flowing powder	Fine free-flowing powder	Fine free-flowing powder	Fine free-flowing powder
Color	Light yellowish to brown	Light brown	Light yellowish	Light brown
Odor	Odorless	Odorless	Odorless	Odorless
Composition (% d/w)				
Chitin-glucan	≥90	93.6	93.7	94.1
Ratio chitin-glucan	25:75 to 60:40	Conform	Conform	Conform
Loss on drying	≤10	9.3	8.2	8.8
Total ash	≤3	2.5	2.8	2.3
Soluble residues	≤5	3.4	3.3	3.0
Microbial and Heavy Metal Limits				
Aerobic plate count (cfu/g)	≤1,000	conform	conform	Conform
Yeast/mould (cfu/g)	≤100	<10	<10	<10
<i>Salmonella</i> spp. (/25 g)	Absent	Absent	Absent	Absent
<i>Enterobacteriaceae</i> (cfu/g)	≤10	<10	<10	<10
Arsenic (mg/kg) (D.L. = 0.2)	≤1	0.2	0.2	0.2
Lead (mg/kg) (D.L. = 0.2)	≤1	0.2	0.2	0.2
Mercury (mg/kg) (D.L. = 0.01)	≤0.1	0.01	0.01	0.01
Cadmium (mg/kg) (D.L. = 0.05)	≤1	0.05	0.05	0.05

cfu = colony forming units; % d/w = percent dry weight basis

D. Additional Compositional Analysis

Analytical data characterizing the carbohydrate, protein, and lipid fractions of chitin-glucan are presented below. These analyses, described in Sections (i) through (iii), were conducted on chitin-glucan that was subject to hydrogen peroxide bleaching during manufacturing⁴, and are expected to be largely representative of the non-bleached material.

(i) Carbohydrate Composition

Glycosyl Composition

The glycosyl composition of chitin-glucan was analyzed by gas chromatography coupled to mass spectrometry (GC-MS) after chitinase digestion (York *et al.*, 1986; Merkle and Poppe,

⁴ Chitin-glucan manufactured by Kitozyme for sale in food and supplement products within the European marketplace is subject to alkaline hydrogen peroxide bleaching. The manufacturing process of the bleached and non-bleached products is identical, with the exception of the addition of hydrogen peroxide during the alkali extraction stage for the bleached product.

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1994). Briefly, a chitinase digestion was applied to a sample of chitin-glucan (batch L09093CGL) to obtain a mixture of monosaccharides. The mixture was then derivatized in methylglycosides by acidic treatments and per-O-trimethylsilylation in TriSil (Pierce). The per-O-trimethylsilyl (TMS) methyl glycoside derivatives were analyzed by GC-MS, and the results were compared to a standard mixture of sugars derivatized under the same conditions as the sample to determine which glycosyl residues were present. The results are presented in Table II.D-1.

Glycosyl Residue	Mass (mg)	Mol%
Arabinose (Ara)	ND	-
Rhamnose (Rha)	ND	-
Fucose (Fuc)	ND	-
Xylose (Xyl)	ND	-
Glucuronic acid (GlcA)	ND	-
Galacturonic acid (GalA)	ND	-
Mannose (Man)	32.9	1.9
Galactose (GalA)	19.9	1.2
Glucose	1251.6	73.8
N-Acetyl Galactosamine (GalNAc)	ND	-
N-Acetyl Glucosamine (GlcNAc)	480	23.1
N-Acetyl Mannosamine (ManNAc)	ND	-
Sum	1784.4	-

- = not applicable; ND = not detected

Consistent with the fact that *beta*-1,3,-D-glucans are the predominant carbohydrate polymer in the cell wall of *A. niger*, glucose is the main sugar constituent of the material, with the remaining sugar content attributed to N-acetylglucosamine derived from chitin. In this sample, the chitin:glucan ratio is of 23.1:73.8. Small amounts of galactose and mannose also were detected in chitin-glucan.

Linkage Analysis

For glycosyl linkage analysis, a sample of chitin-glucan was premethylated, depolymerized, reduced, and acetylated. The resultant partially methylated alditol acetates (PMAAs) were then analyzed by GC-MS as described by York *et al.* (1986). The results are presented in Table II.D-2.

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Glycosyl Residue	Percentage Present
Terminally linked mannopyranosyl residue (t-Man)	0.76
Terminally linked glucopyranosyl residue (t-Glc)	7.31
Terminally linked galactofuranosyl residue (t-Galf)	1.18
Terminally linked mannofuranosyl residue (t-Manf)	0.42
4 linked xylopyranosyl residue (4-Xyl)	0.26
3 linked glucopyranosyl residue (3-Glc)	70.38
2 linked mannopyranosyl residue (2-Man)	0.17
4 linked mannopyranosyl residue (4-Man)	0.31
6 linked glucopyranosyl residue (6-Glc)	0.56
4 linked galactopyranosyl residue (4-Gal)	1.68
4 linked glucopyranosyl residue (4-Glc)	7.18
3,4 linked glucopyranosyl residue (3,4-Glc)	0.96
2,3 linked glucopyranosyl residue (2,3-Glc)	3.69
2,3,4 galactopyranosyl residue (2,3,4-Gal)	3.66
2,6 linked mannopyranosyl residue (2,6-Man)	0.50
4,6 linked glucopyranosyl residue (4,6-Glc)	0.09
2,3,4 linked glucopyranosyl residue (2,3,4-Glc)	0.53
3,4,6 linked glucopyranosyl residue (3,4,6-Glc)	0.06
2,3,6 linked glucopyranosyl residue (2,3,6-Glc)	0.31

Again, consistent with the high *beta*-1,3-D-glucan content of chitin-glucan, over 70% of the glycosyl residues present in the sample were 3-linked glucose residues. Seven percent of the residues were represented by terminal or 4-linked glucose linkages. The remainder (~15%) of glycosyl residues was a collection of different fractions, each less than 4% of the total.

(ii) Composition of the Protein Fraction

Chitin-glucan typically contains less than 4% protein. Conventional methods for determination of nitrogen/protein content are the Bradford protein assay, the Lowry method, the Kjeldhal method, and the bicinchoninic acid (BCA) method. However, because of the presence of the amino group on chitin, none of these methods can be used for protein analysis in chitin-glucan as the presence of the nitrogen interferes with these assays. Moreover, due to the conditions used for the extraction of chitin-glucan from the mycelium (use of sodium hydroxide for several hours), it is expected that any protein component from the source will be denatured and partially hydrolyzed resulting in small size peptides and/or amino acids.

For this reason, the protein components have been characterized by the identification of the amino acids resulting from total hydrolysis. The identification of amino acids is determined according to the method described in European Regulation EC N° 152/2009 by LAREAL Laboratory (FR) under the COFRAC accreditation (EC, 2009). Ten (10) batches of chitin-glucan

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extracted from 2 different sources of *A. niger* have been analyzed [i.e., 7 batches of chitin-glucan from supplier A and 3 batches of chitin-glucan from supplier B]. The 8 most abundant amino acids present in chitin-glucan produced from suppliers A and B, as well as the cumulated percentage of these 8 most abundant amino acids are reported in Table II.D-3. The data show that regardless of the source used for the production of chitin-glucan, the profile of amino acids is very reproducible and the composition of the protein fraction is very similar.

Amino Acid	Source of <i>A. niger</i> (%)	
	Supplier A	Supplier B
Leucine	12.5	10.8
Phenylalanine	10.0	10.7
Glutamic acid	8.5	8.9
Aspartic acid	8.2	8.3
Isoleucine	6.9	6.1
Valine	6.7	11.2
Alanine	6.6	6.3
Tyrosine	5.7	8.4
Cumulated percentage of the 8 most abundant amino acids	65.1	70.7

(iii) Composition of the Lipid Fraction

A typical production batch of chitin-glucan is composed of $\leq 1\%$ lipids. Standard food methods for lipid determination such as the Mojonnier ether extraction (AOAC method 989.05) or the Schmid-Bondzynski-Ratzlaff (SBR) method (ISO 1735:2004) are based on solvent extraction.

A review of the literature on lipids of *A. niger* mycelium from different strains shows that all strains contained phospholipids, glycolipids [monogalactosyl diacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG)], and neutral lipids (triglyceride, diglyceride, monoglyceride, sterols and pigments) (Chattopadhyay *et al.*, 1985, 1987). The main difficulty in the determination of these fractions in chitin-glucan is related to the low lipid content in chitin-glucan, which necessitates multiple solvent extractions to obtain a lipid fraction compatible with quantitative analysis. In addition, the determination of these compounds, with the exception of sterols, is not possible using standard methods. *A. niger* contains C16 to C18 saturated and unsaturated fatty acids (Chattopadhyay *et al.*, 1987). Small amounts of long chain (C20 to C24) and short chain (C10 to C14) saturated and unsaturated fatty acids are also present. Linoleic acid, oleic acid, and palmitic acid are the major fatty acids present in *A. niger*, while stearic acid and lignoceric acid are the minor ones.

To determine the composition of the lipid fraction in chitin-glucan, the following analyses were performed:

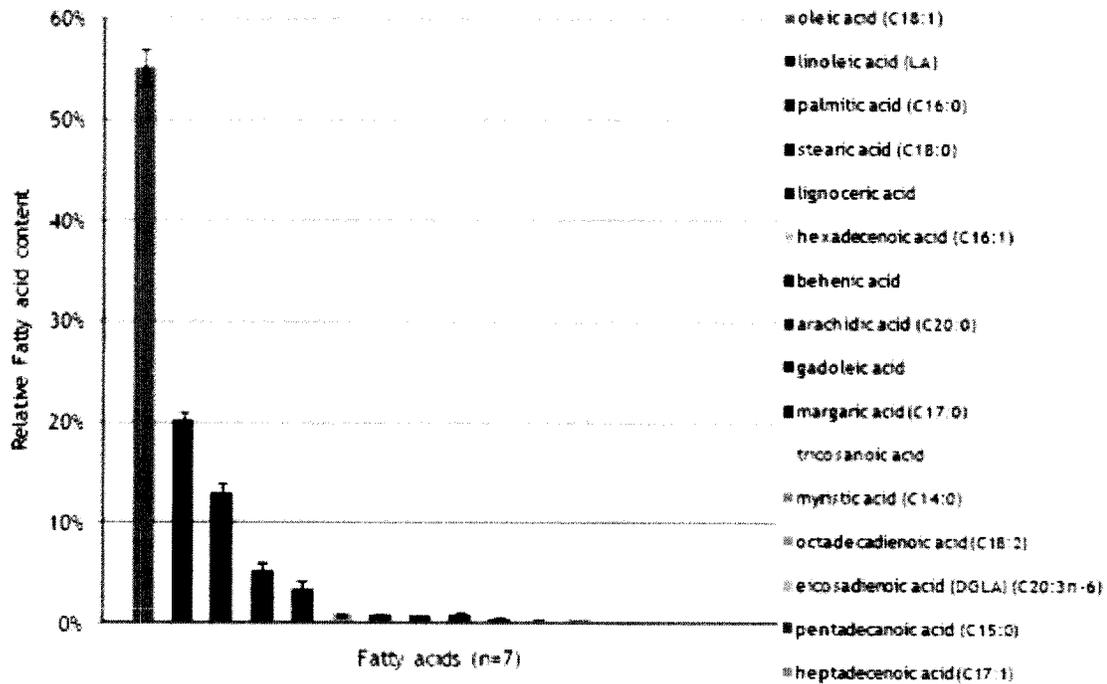
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- Total hydrolysis followed by fatty acid extraction according to the method described in Regulation EC N° 152/2009 (process B) by LAREAL laboratory (FR) under COFRAC accreditation (EC, 2009). The analysis of the fatty acid profile is based on the method NF EN ISO 5508-June 1995/NF EN ISO 5509-June 2000.
- Determination of the lipid content extracted by the Folch method (Folch *et al.*, 1957); preparation and analysis by gaseous phase chromatography of the methyl ester of fatty acids according to NF EN ISO 5509 and NF ISO 5508; determination of sterol content (individual and total sterols) according to NF EN ISO 12228. These analyses have been conducted by the French Institute of Fats and Oils (ITERG) laboratory, a COFRAC/ISO 17025 laboratory.

The results of the fatty acid profile analysis are presented in Figure II.D-1 and demonstrate the relative percentage of fatty acids detected in chitin-glucan. The values reported are the average \pm standard deviation of 7 different batches of chitin-glucan extracted from *A. niger* obtained from supplier A. Three (3) batches of chitin-glucan produced from supplier B also were assessed; however, in 2 of the batches the extracted lipid fraction was too small (less than 1%) for fatty acid determination. The results presented in Figure II.D-1 show that the 5 most abundant fatty acids are oleic acid (55.1%), linoleic acid (19.9%), palmitic acid (12.7%), stearic acid (5.1%), and lignoceric acid (3.3%). All of these fatty acids are common to the diet. The remaining fatty acids are present in relatively low percentage <1%. The fatty acid composition of the material is consistent with that expected in a material derived from *A. niger* as the 5 most abundant fatty acids in chitin-glucan compare to those which are reported in literature for the organism (Chattopadhyay *et al.*, 1985, 1987). The small standard deviation demonstrates that the fatty acid profile is very reproducible from batch to batch.

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Figure II.D-1 Composition of the Fatty Acid Fraction of Chitin-Glucan (*Aspergillus niger* from Supplier A)



Analyses for fungal sterols also was conducted; however, concentrations of sterols in the material were low (73.9 mg/100 g of chitin-glucan), with ergosterol accounting for the majority of the sterols (74%).

E. Stability of Chitin-Glucan

Bulk stability data were obtained using batches of chitin-glucan marketed in the European Union (EU), and therefore were determined using chitin-glucan that was subjected to hydrogen peroxide bleaching. Bleaching is not expected to impart significant chemical changes to the chemical composition of chitin nor *beta*-1,3-glucans and the stability data and was therefore considered representative of the non-bleached product.

A 36-month stability study of chitin-glucan conducted under the recommended storage conditions (*i.e.*, in the final closure container at 25±2°C) was initiated by Kitozyme in 2009, with the aim of establishing a shelf-life of 36 months. A minimum shelf-life of 24 months is expected for Kitozyme’s chitin-glucan ingredients. This expectation is based on the low water activity of the material and results of a stability studies presented below.

The testing conditions described in Table II.E-1 follows the International Conference on Harmonisation (ICH) Q1A (stability testing of new drug substances and products) guidance. It includes one lot of chitin-glucan with a sample size of 20 g. An initial long-term study at 25±2°C

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was set up for 36 months. A second study at $40\pm 2^{\circ}\text{C}$ and $75\pm 5\%$ relative humidity (RH) was set up under accelerated conditions for 12 months.

Storage Conditions	Temperature	Room Moisture	Test Intervals (T in month)
Accelerated conditions	$40 \pm 2^{\circ}\text{C}$	$75 \pm 5\%$ RH	T0, T3, T6,
Recommended storage conditions	$25 \pm 2^{\circ}\text{C}$	$60 \pm 5\%$ RH	T0, T6, T9, T12, T18, T24, T30, and T36

RH = relative humidity; T = time

The samples were stored in ICH climatic chambers. At different time intervals, samples were collected and tested for the parameters listed in Table II-E-2.

Test	Specification Limit	Method
Loss on drying (% w/w)	≤ 10	KZ PT-CQ-001
Water activity	< 0.4	Harmonized method ICC 121:1993 using Novasina 2000 equipment
Aerobic microbial count (cfu/g)	≤ 1000	ISO 4833
Total yeasts and molds count (cfu/g)	≤ 100	ISO 7954
<i>Escherichia coli</i> (cfu/g)	≤ 10	ISO 16649-2
<i>Listeria monocytogenes</i> (/25 g)	Absent	ISO 11290-1
<i>Enterobacteriaceae</i> (cfu/g)	≤ 10	ISO 21528-2
<i>Salmonella</i> spp. (/25 g)	Absent	ISO 6579

cfu = colony forming units; ICC = International Association for Cereal Science and Technology; ISO = International Organization for standardization; KZ PT-CQ = KitoZyme's internal procedure

The interim results from the first 12 months of the stability studies under recommended and accelerated conditions are presented in Appendix B. The results are all within the specification limits and demonstrate the absence of microbial growth. The shelf life of Kitozyme's chitin-glucan is estimated to be 24 months, with an expected shelf-life of 36 months.

III. SELF-LIMITING LEVELS OF USE

Under the intended conditions of use of Kitozyme's chitin-glucan, no self-limiting use levels are expected.

IV. BASIS FOR GRAS DETERMINATION

Kitozyme's GRAS self-affirmation of chitin-glucan under the proposed uses described herein was conducted using scientific procedures. Published and unpublished data and information supporting Kitozyme's GRAS determination is presented in Sections IV.A through IV.H. Current food uses and background exposure to chitin-glucan are discussed (Section IV.A), and exposure to chitin-glucan in the diet under the proposed food uses is presented in section IV.B. Information characterizing the metabolic fate of chitin-glucan is provided in Section IV.C. Product-specific toxicity studies have been performed to support the safety of *A. niger* derived chitin-glucan as manufactured by Kitozyme. These studies include acute, subacute and subchronic oral toxicity studies; *in vitro* genotoxicity data; studies evaluating nutritive effects of chitin-glucan in rodents and humans, and safety information obtained from investigations in which chitin-glucan was administered to hypercholesterolemic yet otherwise healthy human subjects (Sections IV.D through IV.G). These studies were performed using the bleached commercial product currently marketed in the EU. To support the applicability of studies conducted with bleached chitin-glucan to the food use of the non-bleached material, information detailing the effects of hydrogen peroxide bleaching on chemical composition and toxicity of processed fiber also is presented. Finally, the safety of *A. niger* as a raw material for use in the production of Kitozyme's chitin-glucan preparations, including information relevant to potential allergenicity concerns, also were considered and is presented in Section IV.H.

Moreover, these data were reviewed by a Panel of Experts, qualified by scientific training and experience to evaluate the safety of chitin-glucan as a food ingredient. The Panel reached a unanimous consensus that chitin-glucan was GRAS, based on scientific procedures, for use as a secondary direct food ingredient in the manufacture of alcoholic beverages as described herein. A summary of the Expert Panel's GRAS evaluation is presented in Appendix A.

A. Current Uses and Background Exposure to Chitin-Glucan in the Diet

Resolutions permitting the use of chitin-glucan (KiOfine-CG) in winemaking as a fining agent and contaminant treatment have been granted by the International Organisation of Vine and Wine (OIV/OENO 336B/2009; 367/2009; 337B/2009; 338B/2009; 339B/2009) (OIV, 2009), and corresponding approval for use in wine products marketed within the European Union has been issued by the European Commission (EU, 2011a).

Chitin-glucan manufactured by Kitozyme has been granted Novel Food approval by the European Commission, for use in food supplement products in the European Union (EC, 2011)

Background exposure to chitin-glucan from consumption of food products currently available within the U.S. food supply is not expected.

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B. Estimated Intake of Chitin-Glucan

Chitin-glucan has several functional purposes in the treatment of wine. During the settling process, whereby particles settle and form a sediment, chitin-glucan can be used as a fining agent to decrease the levels of colloids and turbidity in musts and wine (Borner *et al.*, 2005, 2007a,b, 2008a,b). In wine, chitin-glucan can be used for stabilization following alcoholic fermentation and prior to bottling. Chitin-glucan also was demonstrated to remove heavy metal contaminants (*i.e.*, lead, iron, cadmium) and mycotoxins (*i.e.*, ochratoxin A) from wines (Borner and Teissedre, 2008).

The individual proposed technological uses and use-levels for chitin-glucan are summarized in Table IV.B-1. Individual food uses were grouped in food-use categories according to Title 21, Section §170.3 of the CFR (U.S. FDA, 2011).

Table IV.B-1 Summary of the Individual Proposed Technological Uses, Use-Levels, and Maximum Residues for Chitin-Glucan in the U.S.

Food Category	Food-Use	Application	Use-Levels in Application ¹
Beverages, Alcoholic	Beer	Clarification	100 g/100 L
	Wine	Clarification-	100 g/100 L
		Fining	50 g/100 L
		Removal of trace metals	100 g/100 L
		Removal of potential mycotoxins	500 g/100 L
		Prevention of oxidation of color	100 g/100 L
		Reduction of heat labile proteins	100 g/100 L
		Aid to filtration	50 g/100 L
		Spirit	Removal of trace metals
	Improvement of flavor		50 g/100 L
	Must	Clarification/removal of off-flavor	100 g/100 L
		Removal of potential mycotoxins	500 g/100 L
		Prevention of oxidation of color	100g/100 L

¹ Note: Chitin-glucan is used as a processing-aid during alcoholic beverage production and is subsequently removed by physical separation processes during manufacturing; therefore use levels do not constitute concentrations of chitin-glucan in the final product, which is absent chitin-glucan.

Regardless of the technological purpose, the sediments, which contain the chitin-glucan, are removed from the wine, must, beer or spirit at the end of the treatment by physical separation processes such as racking, centrifugation, or filtration. Since chitin-glucan is insoluble at all pH levels and in both aqueous and organic solutions, residual chitin-glucan will not be present in treated products. To demonstrate this, insoluble sediments filtered from finished wine processed using chitin-glucan (40 g/100 L) were collected washed with water, dried, and analyzed by infrared (IR) spectroscopy for identification of residual chitin-glucan. The IR spectrum did not contain the peaks characteristic to chitin-glucan (see Appendix C for certificate of analyses of results).

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Thus, based on the absent exposure to chitin-glucan under the proposed food uses described in Table IV.B-1, calculation of estimated intakes was not deemed necessary in the assessment of the safety of the material for the proposed food uses in wine/alcoholic beverage processing.

C. Absorption, Distribution, Metabolism and Excretion

No published studies evaluating the absorption, distribution, metabolism, or excretion of chitin-glucan derived from *A. niger* were identified. Chitin-glucan is an insoluble dietary fiber and is composed predominantly of 2 polysaccharides, *beta*-glucan (consisting of repeating units of D-glucose linked *via beta*-1,3 linkages) and chitin (composed of repeating units of N-acetyl-D-glucosamine linked *via beta*-1,4 linkages). Although the nature by which the *beta*-glucans and chitin are associated in the cell wall of *A. niger* is not completely understood, the *beta*-glucan and chitin polymers are closely associated during synthesis of the cell wall, which results in strong hydrogen bonding between adjacent molecules and produces a largely non-covalently associated material that is highly water insoluble, and highly resistant to acid digestion at ambient temperatures. The human digestive tract can efficiently hydrolyze glucose polymers linked by *alpha*-glycosidic linkages, such as those found in starch and glycogen; however, with the exception of lactose which is hydrolyzed by *beta*-galactosidase, humans cannot digest sugars linked by *beta*-glycosidic bonds (Wisker *et al.*, 1985). Thus, given the *beta*-linked structure of the polymers and highly insoluble physico-chemical properties, chitin-glucan is not expected to be digested by human enzymes to any significant extent, and absorption of the material will not occur. Since studies pertaining to *A. niger* or other fungal derived chitin-glucan products were not available, relevant data pertaining to the metabolism of the individual polysaccharides, *beta*-glucan and chitin, are discussed.

(i) beta-Glucan

beta-Glucan derived from *A. niger* consists of repeating units of D-glucose linked by *beta*-1,3 linkages. No metabolism studies were identified on *beta*-glucan from *A. niger*; however, the metabolism of mixtures containing structurally similar *beta*-glucan materials, such as yeast-derived *beta*-glucans (branched *beta*-1,3/1,6-glucans) and cereal grains such as oat or barley fractions (containing a mixture of linear *beta*-1,3- and 1,4-glucans) has been investigated. Studies regarding the metabolism of *beta*-1,3-glucan-containing mixtures are presented below to provide an understanding of how *beta*-glucans from *A. niger* are expected to be metabolized based on the similarity in linkages.

In vivo studies conducted on pigs following the ingestion of oats have demonstrated the partial depolymerization of *beta*-glucan as it passes through the upper gastrointestinal tract, based on the observed progressive decrease in molecular weight (Johansen *et al.*, 1993, 1997). In human studies, approximately 20% of oat *beta*-glucan is degraded in the upper intestinal tract of ileostomy patients who had consumed dietary fiber containing *beta*-glucans (Englyst and

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Cummings, 1985; Åman *et al.*, 1995; Sundberg *et al.*, 1996). Based on the limited capacity of humans and pigs to digest *beta*-linked fibers, it is expected that the observed partial depolymerization of the glucans administered in these studies is due to metabolism by the gut microflora as gut bacteria loads in the upper intestine of ileostomy patients and in pigs is likely higher than that of healthy human subjects. Due to the lower levels of bacteria present in the upper gastrointestinal tract of healthy human subjects, it is expected that the *beta*-glucan would be subjected to even less depolymerization in healthy subjects.

As the absorption of insoluble fiber does not occur in the upper gastrointestinal tract, the majority of the *beta*-glucan polymers are expected to travel intact to the colon. Degradation of oat *beta*-glucan occurs primarily in the large intestine and any remaining intact *beta*-glucan is excreted in the feces (Bach Knudsen *et al.*, 1993; Johansen *et al.*, 1997; Bach Knudsen and Canibe, 2000). *beta*-Glucan from yeast is fermented by colonic microflora from rats and humans, resulting in the production of short-chain fatty acids (*i.e.*, acetate, propionate, and butyrate) (McBurney, 1991; Nakamura *et al.*, 2001a,b; Yoshida *et al.*, 2005). In human studies, the detection of increased levels of hydrogen in the breath of subjects who had ingested diets containing oat and barley *beta*-glucans indicated that the material was being fermented in the colon (Behall *et al.*, 1998; Lifschitz *et al.*, 2002).

As short-chain fatty acids are quickly absorbed in humans (McNeil *et al.*, 1978), determining the relative levels of short-chain fatty acids is difficult; thus, the production of short-chain fatty acids from the fermentation of *beta*-glucan has been investigated in *in vitro* studies. *beta*-Glucan-containing fibers from barley, oats, and an unspecified source were fermented *in vitro* with human or swine fecal inocula (Casterline *et al.*, 1997; Fardet *et al.*, 1997; Monsma *et al.*, 2000). Approximately 50% of the total production of short-chain fatty acids was identified as acetate and the remainder consisted of mainly propionate and butyrate, produced in equal amounts (Casterline *et al.*, 1997; Fardet *et al.*, 1997; Monsma *et al.*, 2000). Acetate and propionate accounted for up to 80% of the short-chain fatty acid composition in cannulated pigs administered diets enriched with oat *beta*-glucans (Bach Knudsen *et al.*, 1993; Bach Knudsen and Canibe, 2000). Acetate, propionate, and butyrate have been reported to account for at least 93% of the total short-chain fatty acids produced from ileal digesta obtained from swine fed diets containing oat *beta*-glucan (Monsma *et al.*, 2000). Following their production, the short-chain fatty acids are subsequently absorbed and metabolized by the body through well-characterized pathways to produce carbon dioxide, methane, and hydrogen (Karppinen *et al.*, 2000).

(ii) Chitin

Chitin is composed of repeating units of N-acetyl-D-glucosamine linked by *beta*-1,4 linkages. No metabolism studies were identified on chitin derived from *A. niger*; however, information concerning the potential metabolism of chitin is discussed.

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Although it has been generally assumed that humans cannot digest chitin, at least 2 functional human chitinases have been identified. The expression of the first enzyme, chitotriosidase, was demonstrated in macrophages by Northern blot analysis and in lymph node, bone marrow, and lungs by dot blot analysis using an RNA Master Blot (Boot *et al.*, 1995, 2005). Based on its tissue expression, chitotriosidase is unlikely to be involved in the digestion of ingested chitin. The second human chitinase was termed human acidic mammalian chitinase (AMCase) and its expression was demonstrated in the stomach and lung (Boot *et al.*, 2001, 2005). Human AMCase has an acidic pH optimum, is extremely acid stable, and is present and active in human gastric juice; thus, it may be involved in the degradation of chitin-containing foods (Boot *et al.*, 2001; Paoletti *et al.*, 2009). The activity of AMCase in gastric juice samples from 48 Italian subjects (26 males, 22 females) aged 23 to 76 years with symptoms of dyspepsia and postprandial pains ranged from 0.013 to 36.270 nmol/mL/hour (Paoletti *et al.*, 2009). Only 14.6% of the subjects were considered to have high AMCase activity (range of 3.540 to 36.270 nmol/mL/hour), whereas 75% had moderate activity (0.178 to 2.800 nmol/mL/hour) and 10.4% had negligible activity (0.013 to 0.086 nmol/mL/hour). Fly wings used as chitin-containing substrates that were incubated with human gastric juice with AMCase activity of 19.4 nmol/mL/hour at 37°C for up to 8 hours showed degradation on the borders and surfaces of the wings (Paoletti *et al.*, 2009). The N-acetylglucosamine content of the gastric juice following incubation with the wings was low; however, when allosamidin was used as a negative control to inhibit chitinase activity, no N-acetylglucosamine was detected. The authors concluded that the AMCase was responsible for the observed degradation of the fly wings. As the degradation was limited, the authors further concluded that the function of AMCase in gastric juice may be to soften the chitin, rather than provide extensive digestion. Therefore, although humans may possess an active chitinase in the digestive system, given the low prevalence of high activity and the observed effects on the fly wings, it is unlikely that the activity of the chitinases would be high enough to fully digest the chitin in chitin-glucan and the majority of the chitin is expected to pass through the upper gastrointestinal system relatively intact.

Endochitinase, *beta*-N-acetylglucosaminidase, chitosanase, and chitinase are enzymes that are involved in the degradation of chitin (Simunek *et al.*, 2001). Bacterial isolates from fecal samples from horse, takin, pig, and goat exhibited activity from each of the afore-mentioned enzymes when grown on chitin. Simunek *et al.* (2001) also demonstrated that the presence of other carbohydrates influences the production of chitinase: chitinase activity was increased in the presence of hemicelluloses and decreased in the presence of starch. Thus, it is expected that other components of the diet will affect the degradation of chitin. A chitinolytic bacterial strain has been isolated from human feces (Simunek *et al.*, 2002). This bacterium was identified as *Clostridium paraputrificum* and was reported to have endochitinase and *beta*-N-acetylglucosaminidase activity. *In vitro* cultivation of the bacteria with colloidal chitin resulted in the production of hydrogen, carbon dioxide, acetate, and lactate, as well as minute quantities of propionate and butyrate. Following 2 days of cultivation, greater than 90% of the chitin was degraded (initial quantity not reported in abstract). Thus, bacteria in the human colon may be

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able to degrade chitin; however, as mammals are not known to metabolize chitin as a nutrient (Funkhouser and Aronson, 2007), it is expected that the degradation of chitin would be limited. Any chitin that is not degraded or fermented in the colon is expected to be excreted intact in the feces.

(iii) Summary

Humans cannot digest sugars linked by *beta*-glycosidic linkages (Wisker *et al.*, 1985). Results from human and animal studies have indicated that *beta*-glucan are not absorbed in the upper gastrointestinal tract, and during passage through the colon these materials are subject to fermentation by the indigenous microflora resulting in the production of innocuous products (*i.e.*, short-chain fatty acids) (McBurney, 1991; Nakamura *et al.*, 2001a,b; Yoshida *et al.*, 2005). As *beta*-glucans are not absorbed and are expected to produce innocuous products, there is no risk of systemic toxicity and no safety concern regarding the metabolism of *beta*-glucan is anticipated. Likewise, the metabolism of chitin is not expected to pose any safety concern as chitin is expected to be excreted intact in the feces, given the limited activity of chitinases in the human digestive system (Boot *et al.*, 2001, 2005; Paoletti *et al.*, 2009) and the limited evidence on the microbial fermentation of chitin. Therefore, as chitin-glucan comprises *beta*-glucan and chitin, there is no risk of systemic toxicity and no safety concern is expected from the metabolic products from either polysaccharide.

D. Toxicological Studies

As discussed, authorizations permitting the use of chitin-glucan in wine making and as a Novel food for use in supplement products in the European Union have been granted by the European Commission (EC, 2011; EU, 2011b). Chitin-glucan currently marketed within the European marketplace is subjected to an alkaline hydrogen peroxide bleaching step during manufacturing, a processing step that is widely utilized for the bleaching of grain products world-wide. Animal toxicity, *in vitro* genotoxicity, and additional rodent and human studies evaluating the nutritive use of chitin-glucan described below in Sections IV.D through IV.G were conducted using chitin-glucan test materials that are representative of the ingredient marketed within the European Union, and therefore was subjected to bleaching during manufacturing. Since introduction of a non-bleached product is proposed for the U.S., marketplace, information characterizing the effect of peroxide bleaching on dietary fiber was reviewed, and studies evaluating the biological and toxicological properties of bleached and non-bleached dietary fiber (*beta*-glycans) are compared. Based on this information Kitozyme concluded that changes imparted to chitin-glucan subjected to bleaching would be quantitatively small, and largely cosmetic in nature. In the absence of significant material changes to the chemical composition of the principal constituents of the material, published safety studies conducted with Kitozyme's bleached chitin-glucan were therefore considered relevant to the safety of the non-bleached product under the proposed conditions of use in food.

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(i) Effect of Hydrogen Peroxide Bleaching on Chemical Composition and Toxicity of Processed Fiber

The use of hydrogen peroxide for bleaching of dietary fiber has a long-history of safe use in food world-wide, and is a process that is widely used by the grain industry (Saunders *et al.*, 2007). The bleaching action of hydrogen-peroxide is attributed to its ability to oxidize colored pigments, and treatment of chitin-glucan with hydrogen-peroxide is not expected to produce significant material changes to the chemical structure *beta*-linked carbohydrate polymers. The effect of hydrogen peroxide bleaching on the composition of processed oat hulls was evaluated by López-Guisa *et al.* (1988). In this study, processed oat hulls were washed with water and then treated with food-grade hydrogen peroxide in an alkaline environment at pH 10 to 12 for 1 to 2 hours at elevated temperatures (50 to 100°C). Hydrogen peroxide bleaching of processed oat hulls was reported to free soluble carbohydrates bound to fiber, increase the soluble fiber neutral sugars by 3%, and decreases the lignin content of the material by 6% (López-Guisa *et al.*, 1988). Overall changes in fiber composition were largely limited to slight increases (<5%) in the concentrations of soluble neutral sugars and soluble fiber (Table IV.D-1).

	Total Neutral Sugars	Uronic Acids	Klason Lignin	Total in Fraction	Total Dietary Fiber
<i>Processed oat hull</i>					
Soluble	0.5	0.8	tr	0.6	-
Non-soluble	60.4	1.5	17.5	79.4	80.0
<i>Bleached oat hull fiber</i>					
Soluble	3.5	0.2	0.0	4.0	-
Non-soluble	69.1	1.1	11.4	81.7	85.7

Data adapted from López-Guisa *et al.* (1988); tr=trace;

The toxicity of bleached *versus* non-bleached dietary fiber sources has been reported in the literature. Kehoe *et al.* (1990) administered bleached (*via* hydrogen peroxide) oat hull fiber to male and female Cri:CD® Br rats (~6 weeks of age) at dietary levels of 5, 10, or 15% for 13 weeks, while non-bleached oat hull fiber (15% in the diet) was used as a comparator. Body weight, feed consumption, clinical chemistry and hematology, organ weights, and macroscopic and microscopic evaluations were conducted. No differences in the toxicity profile of bleached *versus* non-bleached fiber were reported by the authors. Similarly, López-Guisa *et al.* (1988) reported the results of feeding studies comparing bleached and non-bleached dietary fiber sources using male Sprague-Dawley rats (265±7.8 g). Animals were randomized to groups consuming cellulose, processed oat hulls, coated (starch) oat hulls, or bleached oat hulls at dietary concentrations of 5, 10, or 15% for a period of 6 weeks. Body weight and feed consumption were evaluated. Histopathology of the spleen, pancreas, stomach, duodenum, ileum and colon, kidneys, and liver were conducted on all groups at the end of the study. No

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differences in the toxicology profiles of animals consuming the bleached *versus* non-bleached fiber were reported by the authors. The similarity in toxicity profiles reported in animals consuming high dietary concentrations of bleached *versus* non-bleached dietary fiber shows that hydrogen peroxide bleaching does not impart physico-chemical changes to *alpha* and *beta*-linked linear glycans that result in biologically distinct effects following consumption of these materials at high dietary concentrations. As such, studies conducted with bleached chitin-glucan are expected to be relevant to the risk assessment of the non-bleached material under the proposed food and technological uses.

(ii) Acute Toxicity

The acute oral toxicity of *A. niger* derived chitin-glucan was investigated in rats in a good laboratory practice (GLP)-compliant study conducted according to the OECD Guideline for Testing of Chemicals No. 425 and the U.S. Environmental Protection Agency (EPA) Guideline No. 870.1100 (U.S. EPA, 2002; Richeux, 2005; OECD, 2008a). Female Sprague-Dawley rats (1/dose-level, except for the high-dose level, which contained 3 animals) received commercial food grade chitin-glucan, manufactured by Kitozyme, at single doses of 990, 1,750, 3,100, or 5,000 mg/kg body weight. Chitin-glucan was diluted in olive oil and was administered by gavage at a volume of 5 mL/kg body weight for the 990 mg/kg body weight dose-group and at a volume of 20 mL/kg body weight for the remaining dose-groups. No mortalities were reported during the study and no compound-related clinical signs were observed. Body weight and body weight gain remained normal throughout the study period. After 14 days, a necropsy was conducted on each animal. No compound-related changes were reported following macroscopic examination. As no mortalities occurred during the study, the LD₅₀ of chitin-glucan could not be determined. The LD₅₀ of chitin-glucan in rats following oral exposure is therefore greater than 5,000 mg/kg body weight, the highest dose tested.

A 7-day range finding study was conducted with *A. niger* derived chitin-glucan in male rats to determine the appropriate dose levels for a subsequent 28-day repeat-dose toxicity study (Lina, 2006). Four (4) groups of 4 male Wistar Crl:WI (WU) rats were administered diets containing 0 (control), 1 (low-dose), 5 (mid-dose), or 10% (high-dose) chitin-glucan (commercial food grade material as manufactured by Kitozyme), corresponding to 0, 970, 4,700, and 9,500 mg/kg body weight/day, for a 7-day study period. No mortalities and no clinical signs were reported in any group during the study. No compound-related changes were reported in body weight, food intake, or water intake (visually inspected). At necropsy, no compound-related changes were reported in any animal following macroscopic examination. The relative kidney weight was significantly decreased in the high-dose group compared to the control group; however, no dose-response was observed and the authors reported that the toxicological relevance of this finding is unknown. In addition, the absolute and relative weight of the filled cecum was significantly increased in the high-dose group. The authors interpreted this finding as a physiological response due to the high dose of chitin-glucan administered. Based on the results

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of the study, the authors proposed dose levels of 0, 1, 5, and 10% chitin-glucan in the diet for the subsequent 4-week study.

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Table IV.D-2 Summary of Acute and Short-term Toxicity Studies on Kitozyme's Chitin-Glucan						
Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Test Article	Dose (mg/kg bw/d)	Parameters Measured Related to Safety	Reported Effects^{a,b}	Reference
Rat (Sprague-Dawley), F, 1/group, except in high-dose group, which had 3 animals	Oral (gavage) Single administration	Chitin-glucan (Kitozyme)	Group 1: 990 Group 2: 1,750 Group 3: 3,100 Group 4: 5,000	<ul style="list-style-type: none"> • Clinical signs • Mortality • Body weight and body weight gain • Macroscopic examination 	<ul style="list-style-type: none"> • No mortalities and no compound-related clinical signs • Normal body weight and body weight gain • No compound-related changes reported following macroscopic examination 	Richeux, 2005
Rat [Wistar Cri:WI (WU)], M, 4/group	Oral (in the diet) 7 days	Chitin-glucan (Kitozyme)	Group 1: 0 (0%) Group 2: 970 (1%) Group 3: 4,700 (5%) Group 4: 9,500 (10%)	<ul style="list-style-type: none"> • Clinical signs • Mortality • Body weight • Food intake • Water intake • Macroscopic examination • Organ weights 	<ul style="list-style-type: none"> • No mortalities and no compound-related clinical signs • NSD in body weight • NSD in food intake or water intake (visually inspected) • No compound-related changes reported following macroscopic examination • ↓ relative kidney weight [Group 4] • ↑ absolute and relative weight of the filled cecum [Group 4] 	Lina, 2006

↓ = decreased; ↑ = increased; F = female animals; M = male animals; NSD = no significant differences
^a unless stated otherwise, all reported effects are statistically significantly different relative to control group(s)
^b numbers in [] correspond to the dose group(s) in which the reported effects were observed

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(iii) Subacute and Subchronic Toxicity

A 28-day GLP-compliant toxicity study was performed with *A. niger* derived chitin-glucan in rats in-line with the OECD Guideline for Testing of Chemicals No. 407 (OECD, 2008b; Lina, 2006). Groups of male and female Wistar rats [CrI:WI(WU)] (5/sex/group) received commercial food grade chitin-glucan manufactured by Kitozyme, at dose levels of 0 (control), 1 (low-dose), 5 (mid-dose), or 10% (high-dose) in the diet for a period of 4 weeks. The mean daily intake of chitin-glucan was estimated to be 0.8, 4.0, and 8.2 g/kg body weight/day (males) and 0.8, 3.9, and 7.8 g/kg body weight/day (females), respectively, for the low-, mid-, and high-dose groups. General clinical signs (abnormalities, signs of ill health, or reactions to the test article) were recorded at least once per day. Detailed clinical observations (e.g., piloerection, changes in the eye, gait including posture, presence of clonic or tonic movements, stereotypes, and bizarre behavior) were recorded weekly during the study period up to and including Week 3, and at Week 4, behavioral observations (functional observational battery and motor activity assessment) were performed on all rats. Body weight measurements for each animal were recorded prior to the study initiation and on Days 0, 7, 14, 21, 27, and 28. Food intake was measured over successive periods of 3 to 4 days during the study period. Any possible differences in water intake were monitored by visual inspection. On Day 28, all rats were necropsied and blood samples were obtained for the assessment of hematological parameters (hemoglobin, packed cell volume, red blood cell count, reticulocytes, total and differential white blood cell count, prothrombin time, and thrombocyte count). In addition, the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated. At the end of the study period, clinical chemistry parameters also were examined [alkaline phosphatase activity (ALP), aspartate aminotransferase activity (ASAT), alanine aminotransferase activity (ALAT), gamma glutamyl transferase activity (GGT), total protein, albumin, ratio of albumin to globulin, urea, creatinine, fasting glucose, total bilirubin, total cholesterol, triglycerides, phospholipids, inorganic phosphate, calcium, chloride, potassium, and sodium]. The weights of the adrenals, brain, epididymides, heart, cecum (full and empty), kidneys, liver, spleen, testes, and thymus were recorded. Macroscopic and microscopic examination of all gross lesions, adrenals, axillary lymph nodes, brain, cecum, colon, epididymides, gut associated lymphoid tissue including Peyer's patches, heart, small intestine, kidneys, liver, lungs, mesenteric lymph nodes, peripheral nerve, esophagus, ovaries, prostate, rectum, spinal cord, spleen, sternum with bone marrow, stomach, testes, thymus, thyroid, trachea/bronchi, urinary bladder, uterus with cervix, and vagina were performed on all animals. The mammary gland, mandibular lymph nodes, and seminal vesicles or coagulating glands were examined macroscopically only. No significant compound-related changes were reported in general condition, survival, growth, food intake, or neurobehavioral testing. The findings from pathological examinations were reported to be common for rats of this strain and age and occurred at random incidences. Several differences in hematology and clinical chemistry parameters were noted; however, these differences were not considered to be toxicologically significant due to the lack of a dose-response, the

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occurrence in only one sex, the minor differences in magnitude, and the absence of accompanying relevant changes in organ weight or histopathology. No changes of toxicological significance were reported in any dose-group; thus, the authors determined the no-observed-adverse-effect level (NOAEL) for rats over a 28-day period to be 10% of chitin-glucan in the diet [equivalent to 7.8 (females) and 8.2 (males) g/kg body weight/day], the highest dose tested.

A subchronic oral toxicity study was conducted with *A. niger* derived chitin-glucan in rats in a GLP-compliant study by Jonker *et al.* (2010). This study was conducted in accordance with the OECD Guideline for Testing of Chemicals No. 408 (OECD, 1998). Groups of male and female Wistar rats (20/sex/group) [CrI:WI(WU)] were administered 0 (control), 1 (low-dose), 5 (mid-dose), or 10% (high-dose) commercial food grade chitin-glucan manufactured by Kitozyme, in the diet for a period of 13 weeks. These doses were equivalent to 0, 632, 3,217, and 6,589 mg/kg body weight/day, respectively, for males and 0, 684, 3,437, and 7,002 mg/kg body weight/day, respectively, for females. All animals were observed at least once per day for clinical signs (*i.e.*, general signs, hunched posture, piloerection and sparsely haired areas of the skin, paleness and cataract of the eye, and kinks in the tail). Body weight measurements and food intake data were recorded weekly. Water intake was measured daily during 5-day periods at Weeks 1, 6, and 12. Ophthalmoscopic observations were recorded for all rats prior to the administration period and only in control and high-dosed rats at the end of the study period. Neurobehavioral tests, such as functional observation battery tests (*i.e.*, assessment of grip strength and sensory reactivity to various stimuli and spontaneous motor activity measurements) were conducted on 10 animals/sex/group during Week 13. In addition, detailed clinical observations, such as the presence of clonic or tonic movements, stereotypes, and bizarre behavior were observed. Hematology and clinical chemistry analyses were conducted on 10 animals/sex/group on Days 8, 45 (males) or 44 (females), and 91 (at termination of the administration period). The hematology parameters evaluated were red blood cells, hemoglobin, packed cell volume, reticulocytes, total and differential white blood cells, prothrombin time, and thrombocytes. In addition, MCV, MCH, and MCHC were calculated. At necropsy, bone marrow slides were prepared for bone marrow cytology; however, the slides were not examined as no relevant effects on the hematopoietic system were reported. The clinical biochemistry parameters assessed included ALP, ASAT, ALAT, GGT, sorbitol dehydrogenase activity (SDH), total bilirubin, total protein, albumin, ratio of albumin to globulin (calculated), glucose, total cholesterol, phospholipids, triglycerides, creatinine, urea, inorganic phosphate, calcium, chloride, potassium, and sodium. Urine was collected from 10 rats/sex/group during Week 13 and the parameters evaluated were volume, density, appearance, semi-quantitative (dipstick) measurements (*i.e.*, pH, protein, glucose, ketones, urobilinogen, bilirubin, and occult blood), and microscopy of the sediment (*i.e.*, red and white blood cells, epithelial cells, amorphous material, crystals, casts, bacteria, worm eggs, and sperm cells). The weights of the adrenals, brain, epididymides, heart, cecum (full and empty), kidneys, liver, ovaries, spleen, testes, thymus, thyroid with parathyroids, and uterus were measured. A macroscopic examination was conducted on all tissues and organs listed above in the 28-day study, and

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additionally on the aorta, exorbital lachrymal glands, eyes, femur with joint, Harderian gland, nasal turbinates, oviducts, pancreas, parathyroid, salivary glands (parotid, sublingual, and submaxillary), pituitary, skeletal muscle (thigh), skin (flank), and Zymbal's gland. A histopathological examination using light microscopy was performed on all tissues and organs described above for macroscopic examination (except for exorbital lachrymal glands and Zymbal's gland) for animals in the control and high-dose groups. No mortalities were reported during the study period.

Food intake in high-dose rats was significantly increased with no changes in body weight, in comparison to control rats. The author considered this finding to be of no toxicological relevance due to the lower energy content of the high-dose diet compared to the control diet. Significant increases in water intake were observed at several time-points in all male dose-groups administered chitin-glucan and in females of the mid- and high-dose groups; however, due to the absence of a dose-response relationship and the absence of any pathological condition known to increase water intake, this effect was considered to be of no toxicological significance by the author. A significant increase in the absolute weight of the full and empty cecum of mid- and high-dose males and high-dose females, and a significant increase in the full and empty cecum weights relative to body weight in the high-dose males and females were reported compared to controls but were not considered to be toxicologically relevant by the authors as cecal enlargement is a common finding in rats fed large amounts of poorly digestible or non-absorbable carbohydrates. Several significant changes in hematology were noted at various time-points in male and female rats administered chitin-glucan in comparison to control diets (discussed in detail in Table IV.D-3); however, as there were no dose-response relationships and/or the effects were no longer present at the end of the study period, these changes were not considered to be related to chitin-glucan. A significant increase in the number of thrombocytes was observed in high-dose females at Week 14. This finding also was considered to be of no toxicological relevance since the value was within the historical control range and occurred in female rats only. In addition, numerous statistically significant, sporadic changes in clinical biochemistry were reported in male and female rats administered chitin-glucan compared to controls (as shown in Table IV.D-3). Similarly, these changes were not considered to be compound-related since the effects were not confirmed at the end of the study period and/or no dose-response relationship was observed. No compound-related changes in urinary parameters were reported, although significantly higher pH values in mid- and high-dose males and a significantly increased urine volume in high-dose females were reported. The change in urinary pH levels was considered by the authors not to be of clinical significance since this change was limited to males and no dose-response was observed. The increase in urine values in high-dose females was attributed to 2 females that displayed pyelitis, which was not compound-related, upon microscopic examination of the kidneys. No compound-related gross findings at necropsy and no compound-related histopathological changes were observed. A statistically increased incidence of Rathke's pouch remnant's (cysts) in the pituitary of high-dose males (5/20) was reported compared to controls (0/20). This finding was not

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considered to be compound-related as Rathke's pouch cysts are considered to be congenital aberrations in adult male Wistar rats (MacKenzie and Boorman, 1990) and the authors considered the difference in incidence to reflect normal biological variation. Thus, no adverse compound-related changes were reported in general condition, appearance, neurobehavior, growth, food and water intake, ophthalmoscopy, hematology and clinical chemistry values, urinalysis, organ weights, or pathological findings. Based on the results of the study, the authors concluded that under the conditions of the study, the NOAEL for chitin-glucan was 10% in the diet, which was equivalent to an overall estimated daily intake of 6.6 and 7.0 g/kg body weight in male and female rats, respectively, the highest dose tested.

Table IV.D-3 Summary of Toxicity Studies Conducted with Kitozyme's Chitin-Glucan						
Species (Strain), Sex, and Number of Animals	Study Duration	Test Article Used and Route of Administration	Dose (mg/kg bw/d)	Observations		Reference
Rat Wistar WU [Cr:WI(WU)] n = 5/sex/group	28 days	Bleached chitin-glucan (Kitozyme) (in the diet)	Group 1: (0%) (control) Group 2: (1%) 800 Group 3: (5%) 4,000 (♂); 3,900 (♀) Group 4: (10%) 8,200 (♂); 7,800 (♀)	General condition/survival	<ul style="list-style-type: none"> No compound-related clinical signs observed and no mortalities reported during study period. NSD in neurobehavior 	Lina, 2006
				Food and water intake	<ul style="list-style-type: none"> NSD in food intake No changes in water intake 	
				Body weight	<ul style="list-style-type: none"> NSD in body weight 	
				Organ and tissue effects	<ul style="list-style-type: none"> ↑ absolute and relative weight of the empty cecum [♂ of Group 4 and ♀ of Groups 3 and 4] No compound-related changes following macroscopic or microscopic examination 	
				Hematology, clinical chemistry, and urinalysis	<p>Hematology:</p> <ul style="list-style-type: none"> ↑ hemoglobin and packed cell volume [♂ of Groups 2, 3 and 4] ↓ MCV [♀ of Groups 2, 3, and 4] ↑ RBC and ↓ MCH [Group 4 ♀] ↓ percentage of basophils [♂ of Groups 3 and 4] (NSD in absolute number of basophils) <p>Clinical chemistry:</p> <ul style="list-style-type: none"> ↓ bilirubin [♂ of Groups 3 and 4] ↓ phospholipids and ↑ inorganic PO₄ [Group 4 ♀] 	
NOAEL	<ul style="list-style-type: none"> 10% (equivalent to 8,200 and 7,800 mg/kg body weight/day in males and females, respectively) 					

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Table IV.D-3 Summary of Toxicity Studies Conducted with Kitozyme's Chitin-Glucan						
Species (Strain), Sex, and Number of Animals	Study Duration	Test Article Used and Route of Administration	Dose (mg/kg bw/d)	Observations		Reference
Rat Wistar [Cr:WI(WU)] 20/sex/group 000067	13 weeks	Bleached chitin-glucan (Kitozyme) (in the diet)	Group 1: 0% (control)	General condition/survival	<ul style="list-style-type: none"> No mortalities during study period. NSD in general condition, appearance, or neurobehavior of all animals No compound-related ocular changes reported. 	Jonker <i>et al.</i> , 2010
			Group 2: (1%) 632 (♂) and 684 (♀)	Food and water intake	<ul style="list-style-type: none"> NSD in mean feed consumption in either sex at the 28 or 90 day intervals NSD in water intake at week 1, 6 or 13 	
			Group 3: (5%) 3,217 (♂); 3,437 (♀)	Body weight	<ul style="list-style-type: none"> NSD in body weight on day 28 or 90 	
			Group 4: (10%) 6,589 (♂); 7,002 (♀)	Organ and tissue effects	<ul style="list-style-type: none"> ↑ relative weight of the full and empty cecum in high dose ♂ and ♀ [+18%, P<0.01] ↑ incidence of Rathke's pouch remnant's (cysts) [5/20 in Group 4 ♂ vs. 0/20 in control] No compound-related changes observed following gross pathology or histopathology. 	
				Hematology, clinical chemistry, and urinalysis	<p>Hematology¹:</p> <ul style="list-style-type: none"> ↑ in thrombocytes at week 13 [+17%, P<0.05, Group 4 ♀ vs. control] <p>Clinical Biochemistry:</p> <ul style="list-style-type: none"> ↑ in ASAT at week 13 [+37%, P<0.001, Group 2 ♀ vs. control] <p>Urinalysis²:</p> <ul style="list-style-type: none"> ↑ pH at Week 13 [+0.8 pH units, p<0.01♂ of Groups 3 and 4 vs. control] 	

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Table IV.D-3 Summary of Toxicity Studies Conducted with Kitozyme's Chitin-Glucan						
Species (Strain), Sex, and Number of Animals	Study Duration	Test Article Used and Route of Administration	Dose (mg/kg bw/d)	Observations		Reference
				NOAEL	<ul style="list-style-type: none"> • 10% (equivalent to 6,589 and 7,002 mg/kg body weight/day in ♂ and ♀, respectively) 	

↑ = increased; ↓ = decreased; ASAT = aspartate aminotransferase; ♀ = female animals; ♂ = male animals; MCV = Mean corpuscular volume; MCH = Mean corpuscular hemoglobin; NOAEL = no-observed-adverse-effect level; NSD = no significant difference; RBC = red blood cell.

¹ One animal showed a high percentage of neutrophils. The authors considered this finding to be related to the pyelitis observed in one of its kidneys. After exclusion of this animal, the percentage of neutrophils for females in Group 4 was not significantly different from the control group.

² Two animals showed high values for urine volume and low values for density. The authors considered this finding to be related to the pyelitis observed in their kidneys. After exclusion of these 2 animals, the urine volume and density for females in Group 4 were not significantly different from the control group.

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(iv) Developmental and Reproductive Toxicity Studies

No developmental or reproductive toxicity studies of chitin-glucan were identified in the published literature.

E. Genotoxicity Mutagenicity

(i) In Vitro: Ames Assay

The genotoxicity/mutagenicity of *A. niger* derived chitin-glucan manufactured by Kitozyme was assessed using the AMES assay in a GLP-compliant study performed in accordance with OECD Guideline for Testing of Chemicals No. 471 (OECD, 1997; de Cristóbal, 2005). Chitin-glucan was tested at 5 concentrations (25, 75, 250, 750, and 2,500 µg/plate) in strains of *Salmonella* Typhimurium (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* WP2 (pKM 101) in the presence and absence of metabolic activation [*i.e.*, rat liver microsome fraction (S9)]. Negative (spontaneous reversion rate) and positive controls (*i.e.*, 2-nitrofluorene, sodium azide, 9-aminoacridine, 4-nitroquinoline-N-oxide, and 2-aminoanthracene) were included in this experiment. In addition, a sterility test (to examine microbiological contamination of the test article and of the S9 mix), a cytotoxicity test, and a solubility test were conducted. No contamination and no cytotoxic effects were observed. Due to the low solubility of chitin-glucan, the highest dose used was 2,500 µg/plate. Each bacterial strain was tested in triplicate, both in the presence and absence of S9. After incubating the plates at 37°C for a period of 48 hours, the number of colonies per plate was counted. No dose-response was observed in the bacterial strains tested with chitin-glucan and there were no increases in the number of revertant colonies per plate for any of the strains tested with any concentration of chitin-glucan in the presence or absence of S9. Thus, the results indicated that chitin-glucan is not mutagenic. In addition, the results for the positive and negative controls were reported to be within acceptable limits when compared to historical data.

F. Other Relevant Animal Studies

A 4-week study was performed to determine the effect of *A. niger* derived chitin-glucan (Kitozyme) in mice consuming a high-fat diet (Neyrinck *et al.*, 2011). Male C57b16/J mice (8/group) were fed a control diet, a high-fat diet, or a high-fat diet supplemented with 10% chitin-glucan [equivalent to 15,000 mg/kg body weight/day (U.S. FDA, 1993)] for a period of 4 weeks. The consumption of chitin-glucan attenuated the increase in body weight gain, total serum cholesterol, serum high-density lipoprotein cholesterol (HDL), and hepatic triglycerides that was observed in the animals consuming the high-fat diet. Finally, normalization of the fecal microflora genomic profile towards that of mice consuming the control chow diet was observed in animals consuming chitin-glucan. An exception was noted for *Bifidobacteria* spp., which were

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significantly increased by ~1 log unit relative to controls ($P < 0.05$) when assessed in the total cecal content.

The effects of chitin-glucan (Kitozyme) on several biochemical parameters of oxidation were evaluated in hamsters fed a high-fat diet (Berecochea-Lopez *et al.*, 2009; Deschamps *et al.*, 2009). Three groups of male Syrian golden hamsters (12/group) were fed a diet enriched with cholesterol and poor in antioxidants (vitamin C, vitamin E, and selenium) for 12 weeks. The hamsters also received daily gavage doses of tap water (control) or a suspension of chitin-glucan at a dose of 21.4 or 42.8 mg/kg body weight/day. After 12 weeks, significantly increased levels of superoxide dismutase (SOD) and glutathione peroxidase were reported in the chitin-glucan groups in a dose-dependent manner compared to the control group. Significantly decreased levels of triglycerides and thiobarbituric acid reactive substances (TBRS) were observed in both chitin-glucan groups and significantly decreased levels of cardiac superoxide anions in the high-dose group were reported compared to the high-fat diet control group. The administration of chitin-glucan also significantly decreased aortic cholesterol and aortic fatty streak accumulation (AFSA). No toxic effects or clinical signs as a result of the consumption of chitin-glucan (at either dose-level) were reported during the 12-week study period. The authors noted an improvement in the antioxidant status of hamsters.

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Table IV.F-1 Summary of Short-Term Studies Conducted with Chitin-Glucan						
Species (Strain), Sex, and Number of Animals	Study Duration	Test Article Used and Route of Administration	Dose (mg/kg bw/d)	Observations^{a,b}		Reference
Mouse (C57b16/J) ♂, n = 8/group	4 weeks	Bleached chitin-glucan (KiOnutrime-CG) (in the diet)	Group 1: (0%) (control) Group 2: (0%) (+ high-fat diet) Group 3: (10%) 15,000 (+ high-fat diet)	General condition/survival	<ul style="list-style-type: none"> • Not reported 	Neyrinck <i>et al.</i> , 2011
				Food and water intake	<ul style="list-style-type: none"> • ↓ feed efficiency compared to mice fed a high-fat diet [Group 3 vs. Group 2] 	
				Body weight	<ul style="list-style-type: none"> • Attenuation of body weight gain induced by consumption of high-fat diet 	
				Organ and tissue effects	<ul style="list-style-type: none"> • Attenuated decrease in cecal weight induced in animals consuming the high-fat diet [Group 3] 	
				Hematology, clinical chemistry, and urinalysis	<ul style="list-style-type: none"> • Attenuation of ↑ serum TC, serum HDLC, and hepatic triglycerides induced in animals consuming the high-fat diet [Group 3] • Attenuation of adverse changes in serum TC, serum HDLC, and hepatic triglycerides • Normalization of high-fat induced hyperglycemia 	
Gut microflora	<ul style="list-style-type: none"> • Normalization of the gut microflora genomic profile towards that of control mice • ↑ in genomic markers for <i>Bifidobacteria</i> spp. relative to controls (~1 log ↑, P<0.05). 					

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Table IV.F-1 Summary of Short-Term Studies Conducted with Chitin-Glucan						
Species (Strain), Sex, and Number of Animals	Study Duration	Test Article Used and Route of Administration	Dose (mg/kg bw/d)	Observations^{a,b}		Reference
Hamster (Syrian golden), ♂, 12/group	12 weeks	Bleached chitin-glucan (KiOnutrime-CG) (suspended in water) (gavage)	Group 1: 0 (control) Group 2: 21.4 Group 3: 42.8	General condition/survival	• No toxic effects or clinical signs reported [Groups 2 and 3]	Berecochea-Lopez <i>et al.</i> , 2009; Deschamps <i>et al.</i> , 2009
				Food and water intake	• NSD in food intake	
				Body weight	• NSD in body weight	
				Organ and tissue effects	• Not examined	
				Hematology, clinical chemistry, and urinalysis	Clinical chemistry: • ↓ TG [Groups 2 and 3] • NSD in TC and HDLC • ↑ SOD [Groups 2 and 3] • ↑ glutathione peroxidase activity [Groups 2 and 3] • ↓ cardiac superoxide anion [Group 3] • ↓ TBRS [Groups 2 and 3] • ↓ aortic cholesterol [Groups 2 and 3] • ↓ AFSA [Groups 2 and 3]	

↑ = increased; ↓ = decreased; ♂ = male animals; ♀ = female animals; AFSA = aortic fatty streak accumulation; HDLC = high-density lipoprotein cholesterol; NE = not evaluated; NR = not reported; NSD = no significant difference; SOD = superoxide dismutase; SDH = sorbitol dehydrogenase; TBRS = thiobarbituric acid reactive substances; TC = total cholesterol; TG = triglyceride

¹ Study report indicated a total of 18 hamsters divided in 3 groups of 8 hamsters per group; however, the discussion is presented only on 2 groups. Due to this discrepancy, the number of animals per group is unclear.

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G. Human Studies

Chitin-glucan

In addition to its utility as a processing-aid in for use in alcoholic beverage manufacture, the nutritional use of chitin-glucan manufactured by Kitozyme has been investigated in 2 human studies. A single-blinded pilot study was conducted with a group of male adult volunteers (mean age of 32 years; mean body mass index of 24 kg/m²) with slight hypercholesterolemia (mean cholesterol of 1.78 g/L) to investigate the metabolic effects of chitin-glucan consumption (Deschamps *et al.*, 2009). Twenty (20) volunteers received 1 capsule 3 times per day, providing a total of 4.5 g of chitin-glucan (manufactured by Kitozyme) per day for a period of 4 weeks. A control group (10 subjects) received a placebo (kaolin). On Days 0, 14, and 28, cholesterol, triglycerides, glucose, and antioxidant parameters [glutathione and oxidized-low-density lipoprotein (LDL)] were measured. Although no statistically significant between group differences in anti-oxidant enzyme activity (oxidized LDL, oxidized glutathione) were observed, treatment with chitin-glucan showed a decrease of oxidized-LDL in the treatment group, compared to the baseline. All hepatic (γ GT, TGO, TGP) and renal (creatinine, urea) parameters were normal. The investigator noted that minor gastrointestinal side-effects common to consumption of dietary fiber were reported in some individuals in the treatment group. These effects were moderate, subjective symptomatology that did not adversely affect the subjects. Based on the study results, the investigator concluded that this interventional food study with chitin-glucan fiber shows that the material is innocuous at a dose of 4.5 g per day.

In a second randomized, double-blind, placebo-controlled, multi-center study, Bays *et al.* (2011) evaluated administration of *A. niger*-derived chitin-glucan to a group of slightly hypercholesterolemic yet healthy human subjects (n = 135; 63 males, 72 females) between the ages of 21 and 70 years (mean age of 50.7 years) with a body mass index between 21.0 and 35.0 kg/m² (overall mean = 27.9 kg/m²) and fasting serum LDL-cholesterol between 118 and 190 mg/dL (overall mean = 148.6 mg/dL). Subjects were randomized to 1 of 4 study groups (n = 33 to 35/group): 4.5 g chitin-glucan/day, 1.5 g chitin-glucan/day, 1.5 g chitin-glucan in combination with 135 mg olive extract/day, or placebo (rice flour). Subjects received their respective study products as 3 divided doses daily for a period of 6 weeks. Parameters assessed included fasting serum oxidized LDL-cholesterol, LDL cholesterol, total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, glucose, insulin, F2-isoprostanes, and blood pressure at baseline, and after 4 and 6 weeks of study product consumption. Vital signs, standard blood chemistry panel, and adverse events also were evaluated.

No clinically significant changes in vital signs were observed in any subject. There were no statistically significant differences in electrolytes, hematology, blood chemistry, renal function, and liver function parameters reported among groups. Musculoskeletal system and general infections (*i.e.*, gastroenteritis, yeast infections, upper respiratory tract infections, *etc.*) were

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reported (group not specified); however, these were not considered to be treatment-related by the investigators and there were no significant differences in the incidence of these adverse events in the test groups compared to the placebo group ($P = 0.773$). The only adverse events that were considered by the investigators to be probably related to treatment were gastrointestinal (GI) complaints, which were reported in 1 subject in the 4.5 g chitin-glucan/day group, 1 subject in the 1.5 g chitin-glucan plus olive extract group, and 2 subjects in the placebo group. No serious adverse events occurred during the course of the study. Based on the results of this study, the investigators concluded that the consumption of chitin-glucan at doses up to 4.5 g per day for 6 weeks was safe, well-tolerated, and without any significant adverse events compared to the placebo group

H. Information Pertaining to the Safety of *Aspergillus niger* in Food Uses

(i) Historical Use of *Aspergillus niger* in Food

A. niger has a long history of safe use in the food industry. It has been used in the production of citric acid since 1919, and since the 1960s as a source of a variety of enzymes for use in food production (Röhr *et al.*, 1983; Schuster *et al.*, 2002). For example, amyloglucosidases from *A. niger* are used in the production of glucose syrup and alcohol, pectin esterases, endo- and exo-polygalacturonidases, and pectin lyases are used in the production of wine and fruit juice, hemicellulases are used in baking, and glucose oxidase and catalase are used to remove glucose and oxygen from foods (Schuster *et al.*, 2002).

In the United States, citric acid produced by *A. niger* fermentation is GRAS affirmed for use in food [limited to current Good Manufacturing Practices (cGMP)] (21 CFR §184.1033 – U.S. FDA, 2011), and non-pathogenic, non-toxicogenic strains of *A. niger* are permitted sources of carbohydrase and cellulase for use in clam and shrimp processing (21 CFR §173.120 – U.S. FDA, 2011). The FDA also has evaluated a number of GRAS Notices for the use of carbohydrase, catalase, cellulase, glucose oxidase, pectinase, protease, lactase, lipase, and phospholipase A2 from *A. niger* (U.S. FDA, 2002a,b, 2003, 2005, 2006). Based on the available information, the FDA determined that they had no questions concerning the manufacturers' conclusions that each of the afore-mentioned *A. niger*- derived enzyme preparations were GRAS under their intended conditions of use.

Numerous enzyme preparations from *A. niger* have undergone safety evaluations by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), including *A. niger* sources of carbohydrase, amyloglucosidase, endo-1,3(4)- β -glucanase, hemi-cellulase, pectinase, and protease (JECFA, 1988, 1990). In the initial evaluation, an acceptable daily intake (ADI) of 0 to 1 mg total organic solids (TOS)/kg body weight/day was established based on NOAELs of 100 mg TOS/kg body weight/day in several 90-day rat studies (JECFA, 1988). In 1990, the enzyme preparations were reconsidered and the ADI was changed to "not specified" "[i]n view

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of the fact that [*A.*] *niger* is a common organism in food, that many strains have had a long history of use as an enzyme source, and that the numerous studies of various preparations from various strains have demonstrated no hazard to human health” (JECFA, 1990). JECFA also has evaluated the safety of enzyme preparations from genetically modified strains of *A. niger* and has similarly assigned ADIs of “not specified” (JECFA, 1990, 2009).

(ii) Pathogenicity of *Aspergillus niger*

Kitozyme’s chitin-glucan is manufactured from non-viable sources of *A. niger*, and the material is subject to extensive processing conditions that are not conducive to the survival of any viable cells remaining within the material. Analytical data establishing the absence of significant quantities of viable mold or bacterial counts in the final product are presented. Although the presence of viable *A. niger* in the final product is highly unlikely, consistent with a scientific procedures approach to the safety of chitin-glucan, consideration of the pathogenicity of the source material (*A. niger*) is presented below.

In general, *A. niger* is considered to be non-pathogenic to animals and humans (U.S. EPA, 1997; Schuster *et al.*, 2002). No adverse effects and no gross lesions were reported following the administration of 0.5 to 1 million *A. niger* spores/day *via* pipette into the crop of hybrid broiler chicks for 10 days, followed by 4 days recovery, and then the administration of 5 to 40 million spores/day for an additional 10 days (Nyireddy *et al.*, 1975). *A. niger* was not isolated from samples taken from the proventriculus, gizzard, anterior segment of the small intestine, large intestine, cecum, or rectum of birds euthanized at Days 1, 3, or 10; however, *A. niger* was isolated from the crop of birds killed at Day 3. The authors concluded that the “oral ingestion of even very large amounts of such fungal stages involves practically no risk of mycosis or mycotoxicosis”.

Opportunistic infection in humans by *A. niger* has been reported but in most cases the individuals were severely ill or immunocompromised (Schuster *et al.*, 2002). *A. niger* has been reported to cause aspergillosis following inhalation of the spores and otomycoses of the outer ear following colonization of the ear (Schuster *et al.*, 2002); however, no reports of infections following oral exposure were identified. Similarly, a comprehensive review of *A. niger* pathogenicity by the United States Environmental Protection Agency’s (EPA) did not identify *A. niger* colonizations or pathogenic conditions occurring in humans originating from oral exposure (U.S. EPA, 1997). A few reports of infections by *A. niger* following unknown or routes of exposure other than inhalation were identified in the published literature, and a summary of the identified reports is provided below in Table IV.H-1.

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Table IV.H-1 Summary of Reported Cases of <i>Aspergillus niger</i> Infections						
Subject Characteristics	Route of Exposure	Dose and Duration	Description of Pathology	Method of Identification	Implications	Reference
<i>Animals</i>						
Cow	NR in abstract	NR in abstract	Subclinical mastitis caused by <i>A. niger</i>	NR in abstract	Unknown	EI-Bassiony <i>et al.</i> , 2009 [abstract only]
<i>Humans</i>						
Subject with pulp necrosis and periradicular lesion of the root canal	Unknown	Unknown	<i>A. niger</i> was isolated from the infected root canal	Macroscopic - colony texture, color of the front and reverse side, pigment production, colony size, growth time, exudate production or not Microscopic - examination of mycelia fragments	<i>A. niger</i> may be an opportunistic fungal pathogen present in endodontic infections; however, it is the interplay of different microorganism species that is the causative agent. Further studies are required to delineate the role of filamentous fungi in periradicular disease.	Gomes <i>et al.</i> , 2010
35-year-old female immunosuppressed patient on continuous ambulatory peritoneal dialysis	Suspected <i>via</i> catheter	Unknown	Fungal systemic infection	Isolation of <i>A. niger</i> from peritoneal dialysis fluid	Fungal peritonitis is rare and immunosuppression was a factor in the appearance of the infection.	Bibashi <i>et al.</i> , 1993
39-year-old female	Possibly operative contamination, pulmonary etiology, or primary implant contamination	Unknown	<i>A. niger</i> isolated from fluid within and fibrous capsules surrounding bilateral inflatable silicone breast implants	Culture identification	Fungal infection and colonization following mammary implants is rare and likely due to contamination during surgery.	Williams <i>et al.</i> , 1983
Neutropenic patients with proven or suspected systemic fungal infection	Unknown	Unknown	<i>A. niger</i> was the causative agent in 1 case. Although the site of infection with <i>A. niger</i> was not reported, the most common site of infection in the study was the lung	Culture of fungi from deep tissues or sterile body fluids or cytological or histological examination of sites of infection	Not enough information regarding <i>A. niger</i> infection to determine.	van't Wout <i>et al.</i> , 1991

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(iii) Toxicogenicity of *Aspergillus niger*

Although strains of *A. niger* have been reported to synthesize a number of secondary metabolites with poorly characterized toxicity profiles, including malformins, naphtho- γ -pyrones, aurasperone D, nigerazines, nigragillin, oxalic acid, pyranonigrin, and kotanins, the production of mycotoxins known to be adversely associated with human health is limited to ochratoxins and fumonisins (Schuster *et al.*, 2002; van Dijck *et al.*, 2003; Blumenthal, 2004; EFSA, 2007; Frisvad *et al.*, 2007). Ochratoxin A is a nephrotoxic and carcinogenic compound, and fumonisins have been shown to be hepatotoxic and carcinogenic in rats (Bennett and Klich, 2003; Blumenthal, 2004); therefore, the capacity to synthesize these compounds would render strains of *A. niger* unsuitable for use in food manufacture. The production of mycotoxins by *A. niger* is strain specific, and literature reports of ochratoxin production by isolates of *A. niger* indicate that the prevalence of ochratoxin production is limited; ranging between 1.7 to 18.5% (mean of 7.8%) of strains tested in 6 individual studies (Abarca *et al.*, 2001). In addition, the production of various secondary metabolites and mycotoxins appears to be influenced by the conditions of fermentation, and an inverse relationship exists between stressful environmental conditions which favor mycotoxin production and fermentation conditions that favor growth and enzyme production (Olempska-Beer *et al.*, 2006). Although it is recognized that various species within the *Aspergillus* genus can synthesize aflatoxins, to date, the production of aflatoxins has not been confirmed in any strain of *A. niger* (Schuster *et al.*, 2002).

Previously, JECFA had required that aflatoxin B1, ochratoxin A, sterigmatocystin, T-2 toxin (a trichothecene toxin), and zearalenone not be detectable in all enzyme preparations from fungal sources (JECFA, 1989). However, at the 55th meeting, the Committee recognized that the list of mycotoxins was not relevant to all food enzyme preparations from fungal sources and also agreed that it was impractical and unwarranted to list all known mycotoxins of potential concern. At the 57th meeting, the Committee agreed “that enzyme preparations derived from fungal sources be evaluated for those mycotoxins that are known to be produced by strains of the species used in the production of the enzyme preparation or related species” (JECFA, 2001).

As defined by Pariza and Foster (1983), a non-toxicogenic strain of microorganism can be defined “as one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure.” As discussed, strains of *A. niger* have been safely used in the production of food grade citric acid since 1919. Throughout this period, manufacturers of citric acid have selected proprietary strains, and developed controlled fermentation processes to produce citric acid that is food grade. Based on this long-history of safe use in the food industry, *A. niger* strains used in the production of citric acid are generally recognized as non-toxicogenic organisms. This fact was recognized by the FDA, and no strain specific qualifications, or specifications on the *A. niger* strains used in the

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production of citric acid were imposed by the FDA during promulgation of 21 CFR §173.120 (U.S. FDA, 2011).

Since the source material used in the manufacture of Kitozyme chitin-glucan is obtained from manufacturers of food grade citric acid, mycotoxins are not expected to be present. Nevertheless, Kitozyme has analyzed both the raw *A. niger* source material obtained from citric acid manufactures, and the final product, for the presence of several mycotoxins including ochratoxins, aflatoxins, and fumonisins. As shown in Table IV.H-2 below, no mycotoxins were detected in the raw material or final product at the limits of detection. Kitozyme conducts periodic testing of the raw materials used in the production of chitosan for assurance that the materials used in the production of Kitozyme *A. niger*-derived products are suitable for food uses.

Mycotoxin (µg/kg)	<i>A. niger</i> biomass	Chitin-glucan Lot No. L09093CGL
Aflatoxin B1	<0.1	<0.1
Aflatoxin B2	<0.1	<0.1
Aflatoxin G1	<0.1	<0.1
Aflatoxin G2	<1	<1
Ochratoxin	<1	<1
Fumonisin B1	<100	<100
Fumonisin B2	<100	<100

*Raw material used in the production of chitin-glucan

(iv) Allergenicity of *Aspergillus niger*

Several known allergens have been identified in *A. niger* and they are listed in Table IV.H-3. Asp n 18 (*beta*-Xylosidase) is used in the food industry and has allergenic activity when inhaled, although no allergenicity was reported when orally ingested (EFSA, 2010). 3-Phytase B is authorized as a feed additive in the EU (EFSA, 2010).

Asp n 18 (Beta-Xylosidase, MG 105 000)
Asp n 18 (vacuolar serin protease, MG 34 000)
Pectinase (MG 35 000)
Glucoamylase (MG 66 000)
Xylanase
Phytase
Cellulase
Flaviatase
3-phytase B

^a Referenced in EFSA, 2010

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Seaton and Wales (1994) conducted an 8-year follow-up study on the clinical reactions (mainly occupational asthma) reported by workers of a manufacturing plant producing citric acid from fermentation of molasses with *A. niger*. The authors concluded that *A. niger* is a weak antigen and simple hygiene measures (such as partial enclosure, exhaust ventilation, and using respirators when accessing the area) were sufficient to protect the workforce.

Reports of allergenicity for glucosamine hydrochloride derived from fermentation process with *A. niger*, have not been previously reported (EFSA, 2009). Although no proteins were detected in the final product, the European Food Safety Agency (EFSA) noted in an Opinion of *A. niger*-derived glucosamine hydrochloride as a novel food ingredient that the possibility of allergenicity could not be fully excluded (EFSA, 2009). Similarly, in a Scientific Opinion on the safety of Kitozyme's chitin-glucan derived from *A. niger* as a novel food ingredient, EFSA concluded that the "allergenic risk cannot be ruled out, but is expected not to be higher than the consumption of other *A. niger* derived products" (EFSA, 2010). As such, the allergenicity potential of Kitozyme's chitin-glucan ingredient derived from *A. niger* cannot be completely ruled out, though the risk is also likely to be minimal. Kitozyme noted that the known *A. niger* antigens are likely to be degraded during the extraction process of chitin-glucan due to their large sizes, and mass balance analysis suggests the final product contains low concentrations of proteins (~2 to 3%).

(v) Conclusions on the Safety of *Aspergillus niger* as a Source Material for Chitin-glucan

Kitozyme's chitin-glucan is manufactured from non-viable post-fermentation biomass of *A. niger* obtained exclusively from manufacturers of food grade citric acid. *A. niger* is a permitted source organism for the production of citric acid in the United States, and the production of citric acid *via A. niger* fermentation has a long history of safe use worldwide. Evidence for pathogenicity of *A. niger* is limited to sporadic cases of opportunistic infection, typically *via* parenteral routes in immunocompromised or unhealthy subjects. Although literature reports indicate that select strains of *A. niger* are known to produce ochratoxins and fumonisins, this phenotype is not common to the species. In addition, it has been shown that manufacturing conditions of fermentation are not conducive to mycotoxin production, which typically requires stressful growth inhibitory environmental conditions for optimal mycotoxin production. The absence of mycotoxins in food products derived from *A. niger* sources is further supported *via* analytical data provided by Kitozyme, which demonstrate the absence of ochratoxins, fumonisins, and aflatoxins in the raw *A. niger* biomass, and similar absence of ochratoxins in the final product. Finally, the absence of secondary metabolites of toxicological significance as carry-over products is supported by the lack of toxicity in animal studies of *A. niger* derived *beta*-glucan (Jonker *et al.*, 2010).

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It may be concluded from the above that the strains of *A. niger* used in the production of citric acid are non-pathogenic and non-toxicogenic, and are therefore safe for use in the production of Kitozyme chitin-glucan.

I. Summary and Basis for GRAS

KitoZyme proposes to market chitin-glucan, derived from *Aspergillus niger*, under the trade name KiOfine-CG[®], in the U.S. as an secondary direct food ingredient (processing-aid) for fining and decontamination applications during the manufacture of alcoholic beverages.

Chitin-glucan is manufactured from the non-viable post-fermentation microbial biomass of *A. niger* obtained from manufacturers of food grade citric acid. The *A. niger* strains used during citric acid production are non-genetically modified, and are permitted for use in the production of citric acid in the United States under 21 CFR §173.280 – U.S. FDA, 2011. The *A. niger* raw material used in the production of chitin-glucan is subject to strict quality specifications imposed by Kitozyme ensuring the absence of microbial contamination and heavy metals. Chitin-glucan is produced in-line with cGMP, using manufacturing processes and processing-aids commonly used in the food industry. The final product consists of the digested, milled cell walls of *A. niger* and is an insoluble fungal fiber composed predominantly of 2 types of polysaccharides, namely chitin (poly-N-acetyl-D-glucosamine) and *beta*-(1,3)-D-glucan. Small amounts of protein, lipids, and innocuous minerals also are present in the material. KitoZyme has developed food grade specifications for the product, which characterize the material to 100% purity and contain appropriate limits for heavy metal and microbial contaminants. Batch analyses for three non-consecutive lots of chitin-glucan demonstrate compliance with the product specifications, and additional analytical data demonstrating the consistency of the manufacturing process and the capacity of the source material (*A. niger* mycelium) to produce a consistent product from batch to batch are presented.

Chitin-glucan is stable at ambient storage conditions, and a shelf-life of 2 years has been established for the product. Chitin and *beta*-glucans are inert and stable materials, and the production of unknown degradation products under the proposed conditions of use is not expected.

Chitin-glucan as marketed under the trade name KiOfine-CG, is intended to be used for technological purposes as a processing aid in the manufacture of alcoholic beverages. The intake of chitin-glucan from all intended technological uses was estimated based on its properties, the processing of the final products, and analytical results from a final wine product that had been treated with chitin-glucan. Chitin-glucan is insoluble at all pH levels and in both aqueous and organic solutions, and the ingredient is effectively removed from solution using standard physical separation processes employed by the wine and beverage industries. Wine processed with chitin-glucan has been analyzed for residual chitin-glucan using Fourier transmission infrared spectroscopy, and chitin-glucan was absent from the finished product.

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The estimated intake of chitin-glucan from all intended technological uses as a processing-aid can be considered as negligible.

Chitin-glucan is an insoluble *beta*-glycan fiber that is not subject to human digestive processes, therefore absorption and exposure of the systemic circulation to chitin-glucan will not occur. Although small quantities of acid stable chitinases have been detected in human gastric juice, the metabolic activity of these enzymes appears to be limited and significant hydrolysis of the dietary *beta*-glycan polymers is unlikely. Based on monosaccharide analysis of the material, putative hydrolysis products generated during gastric transit would consist of innocuous compounds common to the diet including glucose, mannose, and galactose. Small quantities of N-acetylglucosamine also may be produced; however, this metabolite also is known to be innocuous, even when consumed at high dietary concentrations (Lee *et al.*, 2004; Takahashi *et al.*, 2009). Chitin-glucan is expected to travel intact throughout the gastrointestinal tract to the colon and be subject to limited metabolism by the endogenous microflora of the large intestine. Microbial fermentation of chitin-glucan would result in the production of normal metabolites of fermentation which include the production of the short-chain fatty acids, and H₂, CO₂, and CH₄ gases. Exposure to these metabolites is expected on a daily basis from the consumption of a non-digestible dietary fiber.

Chitin-glucan manufactured by Kitozyme, and marketed within the European Union is subject to alkali bleaching during manufacturing. Hydrogen peroxide bleaching of dietary fiber has a long-history of safe-use world-wide and bleaching of oat hulls is common practice by grain processors. A review of the literature for information detailing the effects of alkali hydrogen peroxide bleaching of *beta*-glycan fibers indicate that the effect of the bleaching process is largely limited to effects on natural colors (de-pigmentation), removal of natural flavors within the material, and freeing of soluble fiber bound sugars. A small amount of *beta*-glycan hydrolysis may occur; however, overall, only marginal increases (<5%) in total soluble fiber have been reported. Studies comparing the consumption of *beta*-glycan (oat) fiber processed in the presence or absence of hydrogen peroxide bleaching indicate that the biological and toxicological properties of bleached and non-bleached fiber are comparable (López-Guisa *et al.*, 1988; Kehoe *et al.*, 1990). Therefore, product specific oral toxicology and genotoxicity studies conducted with chitin-glucan subject to alkali hydrogen peroxide bleaching during manufacturing were considered relevant to non-bleached chitin-glucan, which is proposed for marketing in the United States.

Chitin-glucan is of low toxicity. No mortalities, no compound-related clinical signs, no effects on body weight gain, and no compound-related macroscopic changes were observed following the acute administration of up to 5,000 mg chitin-glucan/kg body weight by gavage to female Sprague-Dawley rats. The consumption of diets containing up to 10% chitin-glucan (equivalent to 8,200 and 7,800 mg/kg body weight/day in male and female rats, respectively) by Wistar rats for 28 days did not result in any compound-related clinical signs, abnormalities in

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neurobehavior, alterations upon macro- or microscopic examinations, or significant differences in food or water intake, body weights, or hematological, biochemical, or urinalysis results; a NOAEL of 10% of chitin-glucan in the diet [equivalent to 7.8 (females) and 8.2 (males) g/kg body weight/day] was determined. In a current Good Laboratory Practice (cGLP) and OECD (No. 408) compliant study, subchronic administration of chitin-glucan to the diets of Wistar rats was without evidence of toxicity (Jonker *et al.*, 2010). During this study, chitin-glucan was provided at dietary concentrations of up to 10% (equivalent to 6,589 and 7,002 mg/kg body weight/day for males and females, respectively) for a 13 week period, and no compound-related changes were reported in general condition, appearance, neurobehavior, growth, ophthalmoscopy, hematology and clinical chemistry values, urinalysis, organ weights, or macroscopic or histopathological findings. Food intake in high-dose rats was significantly increased with no changes in body weight, in comparison to control rats; however, the authors considered this finding to be of no toxicological relevance due to the lower energy content of the high-dose diet compared to the control diet. A significant increase in the absolute weight of the full and empty cecum of mid- and high-dose males and high-dose females, and a significant increase in the full and empty cecum weights relative to body weight in the high-dose males and females also were observed, an effect that was attributed to consumption of the test material. However, this finding has been reported by other authors, where large doses of fungal derived fibers were similarly administered to rodents during subchronic toxicity evaluations (Feletti *et al.*, 1992; Babíček *et al.*, 2007). In addition, increased cecum weights associated with the consumption of indigestible carbohydrates (sorbitol, mannitol, cyclitol, caramel and polydextrose) is a well established phenomenon in rodents, and is not considered to have toxicological relevance to humans (WHO, 1987). Based on the results of the study, the authors concluded that under the conditions of the study, the NOAEL for chitin-glucan was 10% in the diet, which was equivalent to an overall estimated daily intake of 6.6 and 7.0 g/kg body weight in male and female rats, respectively, the highest concentration tested. Finally, chitin-glucan also was non-mutagenic when tested in an Ames assay.

Human investigations evaluating the nutritive use of chitin-glucan, manufactured by Kitozyme, also were reviewed. Consistent with observations in rodents, chitin-glucan was well tolerated by a group of 20 healthy human subjects (mean age 32 years) administered chitin-glucan at a dose of 4.5 g/day for a duration of 4 weeks. The safety of chitin-glucan was further evaluated in larger group healthy volunteers. One-hundred and thirty-five slightly hypercholesterolemic yet healthy male and female subjects (aged 21 to 70 years) were randomized to one of four groups receiving 4.5 g of chitin-glucan, 1.5 g of chitin-glucan, 1.5 g of chitin glucan in combination with an olive oil extract, or placebo (rice flour) daily for 6 weeks. No statistically significant differences physical examination parameters (body weight, diastolic or systolic blood pressure, heart rate) or safety related clinical chemistry and hematology endpoints were observed following completion of the treatment phase. No statistically significant differences in between group incidences of adverse events were reported, and no serious adverse events occurred during the course of the study.

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Chitin-glucan is produced from the non-viable post-fermentation biomass of *A. niger* obtained from producers of food grade citric acid. Strains of *A. niger* used for citric acid production have a long-history (>90 years) of safe use in food world-wide. In the U.S., *A. niger* is a permitted fermentation organism for use in the production of citric acid (21 CFR §173.280 – U.S. FDA, 2011) and carbohydrase and cellulase derived from non-toxigenic, non-pathogenic strains of *A. niger* are permitted for use in clam and shrimp processing (21 CFR §173.120 – U.S. FDA, 2011). Carbohydrase, catalase, cellulase, glucose oxidase, pectinase, protease, lactase, lipase, and phospholipase A2 from *A. niger*. Carbohydrase, catalase, cellulase, glucose oxidase, pectinase, protease, lactase, and lipase from *A. niger* are GRAS for use in food production in the U.S.; these GRAS determinations have been Notified to the FDA without objection from the Agency (GRN 89, 111, 132, 158, 183 – U.S. FDA, 2002a,b, 2003, 2005, 2006). JECFA also has evaluated the safety of a number of enzyme preparations from *A. niger* and assigned an ADI of not specified (JECFA, 1988, 1990, 2009). The history of use, and safety of *A. niger* for use by the food industry has been comprehensively reviewed in the literature by a number of experts, supporting general recognition of the species as safe and suitable for use in the production of food ingredients (Schuster *et al.*, 2002; van Dijck *et al.*, 2003; Blumenthal, 2004; Olempska-Beer *et al.*, 2006). Finally, during the JECFA's most recent evaluation of enzyme preparations derived from *A. niger*, an ADI of not specified was determined based on the view that *A. niger* is a common organism in food, that many strains of the species have had a long history of use as an enzyme source, and that the numerous studies of enzyme preparations derived from the organism have demonstrated no hazard to human health (JECFA, 1990). Based on the aforementioned information, it was determined that strains of *A. niger* used in the production of food grade citric acid would be GRAS for use in the production of chitin-glucan.

Kitozyme's GRAS self-affirmation was evaluated by a Panel of Experts, qualified by relevant experience and scientific training to evaluate the safety of chitin-glucan under the proposed food uses; the Panel reached a unanimous consensus that these uses were GRAS and would similarly be considered GRAS by other qualified Experts. Finally, general recognition of safety is further supported by recent authorizations by the European Commission and the International Organisation of Vine and Wine, which permit the aforementioned uses of *A. niger* derived chitin-glucan for use in wine production in the European Union.

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J. Conclusions

Based on the data and information summarized above, it may be concluded that the proposed uses of chitin-glucan (KiOfine-CG) derived from *Aspergillus niger*, meeting appropriate food-grade specifications and produced consistent with cGMP, is Generally Recognized as Safe (GRAS) based on scientific procedures.

Therefore, the use of Kitozyme's chitin-glucan ingredient as a secondary direct food ingredient in the production of alcoholic beverages as described herein is exempt from the requirement of premarket approval (Section 409 of the *Federal Food, Drug and Cosmetic Act*).

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Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	Section §	Section Title
170—Food additives	170.3	Definitions
	170.30	Eligibility for classification as generally recognized as safe (GRAS)
173—Secondary direct food additives permitted in food for human consumption	173.120	Carbohydrase and cellulase derived from <i>Aspergillus niger</i>
	173.280	Solvent extraction process for citric acid
184—Direct food substances affirmed as generally recognized as safe	184.1033	Citric acid
	184.1763	Sodium hydroxide

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APPENDIX A

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Expert Panel Report on the Generally Recognized as Safe (GRAS) Use Chitin-Glucan as a Direct and Secondary Direct Food Ingredient in Multiple Food Categories

September 12th, 2011

Kitozyme S.A. (Kitozyme) proposes to market chitin-glucan, derived from *Aspergillus niger* (*A. niger*), under the trade name KiOnutrime-CG[®] in the United States (U.S.) for use as a food ingredient in a variety of traditional food and beverage products. Chitin-glucan also is intended for use as a secondary direct food ingredient (*i.e.* processing-aid), marketed under the trade name KiOfine-CG[®] in the United States (U.S.) for the production of alcoholic beverages, fruit musts, and fruit juices.

Kitozyme SA convened an Expert Panel (the "Panel") of independent scientists qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether the proposed uses of chitin-glucan in the proposed foods described in Table A-1 and A-2 (see Attachment A) are safe and suitable and would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of: Dr. Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Dr. Robert Nicolosi, Ph.D. (University of Massachusetts Lowell), and Dr. John A. Thomas, Ph.D. (Indiana University School of Medicine). *Curricula vitae* for each Panel member are included in Attachment B.

The Expert Panel, independently and collectively, critically evaluated a dossier which included details pertaining to the method of manufacture and product specifications, supporting analytical data, conditions of intended use in food, estimated exposure under the proposed uses, and a comprehensive assessment of the available scientific literature through April 2011 pertaining to the safety of the material. Stability data and product specific safety studies of Kitozyme's chitin-glucan were conducted using chitin-glucan subject to bleaching¹, which differs from the material that is the subject of this GRAS determination which is an unbleached product. To support the GRAS use of the non-bleached material, the Panel was presented with information describing the effects of hydrogen peroxide bleaching on the chemical composition and toxicity of processed fiber. In addition, the Expert Panel evaluated other information deemed appropriate or necessary.

¹ Chitin-glucan (KiOnutrime-CG) manufactured by Kitozyme has been granted Novel Food approval by the European Commission, for use in food supplement products in the European Union (EU, 2011a). Resolutions permitting the use of chitin-glucan (KiOfine-CG) in winemaking have been granted by the International Organisation of Vine and Wine (OIV/OENO 336-337-338-339A/2009), and the European Commission (OIV, 2009; EU, 2011b). These products currently marketed in the European Union are subject to alkali hydrogen peroxide bleaching during manufacturing. Chitin-glucan proposed for sale in the United States is unbleached.

Following an independent critical evaluation of the data, the Expert Panel unanimously concluded that the intended uses described herein for chitin-glucan, meeting appropriate food-grade specifications as described in the supporting dossier [**Documentation Supporting the Generally Recognized as Safe (GRAS) Use of Chitin-Glucan Derived from *Aspergillus niger* as a Direct and Secondary Direct Food Ingredient for Use in a Variety of Foods**] and manufactured consistent with current Good Manufacturing Practice (cGMP), are GRAS based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is provided below.

Chitin-glucan is manufactured using the microbial biomass of *A. niger* (*A. niger* mycelium) that remains after the manufacture of food grade citric acid; this source material is obtained from strains permitted for use in the production of citric acid for use in food in the United States (21 CFR §173.280 – U.S. FDA, 2011) and the European Union. Kitozyme imposes strict quality control measures on the *A. niger* raw material used in production of chitin-glucan which ensures the absence of microbial and mycotoxin contamination and heavy metals. Chitin-glucan is produced consistent with cGMP, using suitable manufacturing processes and processing-aids commonly employed by the food industry. Briefly, the *A. niger* biomass is loaded into a reaction tank and is processed *via* an alkali extraction method, which results in solubilization of proteins and fats. The soluble components are then removed from the reaction mixture using several wash and filtration steps, and the material is then subject to flash drying and milling to produce the final product. The final ingredient is an insoluble particulate material composed predominantly of 2 types of polysaccharides, namely chitin (poly-N-acetyl-D-glucosamine) and *beta*-1,3-glucan. Small amounts of protein and lipids also are present in the final material, at ≤6% and ≤1%, respectively. Kitozyme has developed food grade specifications for the product, which characterize the material to 100% purity and contain appropriate limits for heavy metal and microbial contaminants. Analytical results from 3 non-consecutive lots of chitin-glucan demonstrate compliance with product specifications.

Additional analytical data characterizing the carbohydrate, protein, and lipid composition of bleached chitin-glucan also have been performed by Kitozyme. Studies on the stability of bleached chitin-glucan also were reviewed. Based on this information the Panel concluded that chitin-glucan is highly stable at ambient storage conditions; a shelf life of 24 months has been estimated by Kitozyme. Information supporting the stability of bleached chitin-glucan in various smoothie beverage products also was reviewed by the Expert Panel and the concentration of chitin-glucan did not change during the 5-week test period.

The Expert Panel understands that chitin-glucan is proposed for use as a direct food ingredient in beverages and beverage bases, breakfast cereals, grain products and pastas, milk products, and processed fruits and fruit juices (Table A-1). Estimates for the intake of Kitozyme's chitin-glucan ingredient were based on the proposed food-uses and use-levels in conjunction with food consumption data included in the 2003-2004 and 2005-2006 NHANES (CDC, 2006, 2009;

USDA, 2009), which provides the most appropriate data for evaluating food use and food consumption patterns in the U.S. Under the conditions of intended use and on an all-user basis, the mean intake of chitin-glucan by the total U.S. population from all proposed food-uses was estimated to be 2.1 g/person/day or 43 mg/kg body weight/day. The heavy consumer (90th percentile) all-user intake of chitin-glucan by the total U.S. population from all proposed food-uses was estimated to be 4.3 g/person/day or 99 mg/kg body weight/day. Among the individual population groups, on an absolute basis the highest mean and 90th percentile all-user intakes of chitin-glucan were observed among male teenagers, with values of 3.1 and 6.0 g/person/day, respectively. On a per kilogram body weight basis, the greatest mean and 90th percentile all-user intakes of chitin-glucan were observed among infants, with values of 146 and 288 mg/kg body weight/day, respectively.

Chitin-glucan also is proposed for use as a processing aid (clarification, fining, filtration-aid, impurity removal) in the manufacture of alcoholic beverages, fruit musts, and processed fruits and fruit juices (Table A-2). Chitin-glucan is insoluble at all pH levels and in both aqueous and organic solutions, and is therefore easily removed from beverage products through the application of standard physical separation processes. Therefore, the use of chitin-glucan as a processing-aid during the production of various alcoholic and non-alcoholic beverage products would not result in measureable quantities of chitin-glucan in the product. The Expert Panel reviewed the infrared (IR) spectroscopy data obtained from wine treated with chitin-glucan and chitin-glucan was not found in the finished product. The Expert Panel concluded that the estimated intake of chitin-glucan from the proposed use as a processing-aid would be negligible, and would not add to the cumulative exposure estimated for its use as a direct food ingredient (Table A-1).

Chitin-glucan is an insoluble fungal polysaccharide complex. The material, and its principal constituent molecules (chitin and *beta*-1,3-glucan), are resistant to acid hydrolysis, therefore degradation of the material in the stomach will not occur. The Expert Panel also noted that with the exception of *beta*-galactosidases that hydrolyze lactose, humans are unable to hydrolyze beta-linked sugars through enzymatic processes. Thus, based on the insolubility of the material, and the beta-linked polymer structure of the constituent molecules, digestion and absorption of chitin-glucan will not occur. The Expert Panel noted that small quantities of acid stable chitinases have been detected in human gastric juice (Boot *et al.*, 2001, 2005; Paoletti *et al.*, 2009); however, the metabolic activity of these enzymes appears to be limited and significant hydrolysis of the material is unlikely. Putative hydrolysis products generated from chitin would consist of N-acetylglucosamine and chitin polymers of various molecular weights; production of small quantities of these compounds during consumption of chitin-glucan is of limited toxicological significance (Lee *et al.*, 2004; Takahashi *et al.*, 2009). The Expert Panel concluded that chitin-glucan would travel intact through the gastrointestinal tract to the colon, and would not be digested or absorbed. However, transit of chitin-glucan to the colon would subject the material to fermentation by colonic microflora. The Expert Panel reviewed a number

of studies characterizing the fermentation of chitin, as well as *beta*-(1,3)-D-glucans derived from yeasts and oat, by rat and human colonic microflora. A chitinolytic bacterial strain has been isolated from human feces (Simunek *et al.*, 2002); however, mammals are not known to metabolize chitin as a nutrient (Funkhouser and Aronson, 2007), and it is expected that any degradation of orally consumed chitin-containing materials would be limited. The Expert Panel concluded that chitin-glucan would be excreted in the feces intact or subject to limited microbial fermentation resulting in the production of normal metabolites of fermentation which include the production of the short-chain fatty acids (*i.e.*, acetate, propionate, butyrate, lactate), and H₂, CO₂, and CH₄ gases. Exposure to these metabolites is expected to occur on a daily basis from the consumption of a non-digestible dietary fiber.

The Expert Panel reviewed a number of product specific toxicology studies including acute, short-term (7- and 28-day), and subchronic (90-day) toxicity studies of bleached chitin-glucan manufactured by Kitozyme. These studies were conducted using Good Laboratory Practice and in accordance with OECD guidelines for the testing of chemicals².

No mortalities, no compound-related clinical signs, no effects on body weight gain, and no compound-related macroscopic changes were observed following the acute administration of up to 5,000 mg chitin-glucan/kg body weight by gavage to female Sprague-Dawley rats.

The consumption of diets containing up to 10% bleached chitin-glucan (equivalent to 8,200 and 7,800 mg/kg body weight/day in male and female Wistar rats, respectively) for 28 days did not result in any compound-related clinical signs, abnormalities in neurobehavior, alterations upon macro- or microscopic examinations, or significant differences in food or water intake, body weights, or hematological, biochemical, or urinalysis results. A no-observed-adverse-effect level (NOAEL) of 10% of chitin-glucan, the highest concentration fed, equivalent to 7.8 (females) and 8.2 (males) g/kg body weight/day, was established.

Bleached chitin-glucan produced by Kitozyme was provided at dietary concentrations of up to 10% (equivalent to 6,589 and 7,002 mg/kg body weight/day) to male and female Wistar rats, for 13 weeks (Jonker *et al.*, 2010). No compound-related changes were reported in general condition, appearance, neurobehavior, growth, ophthalmoscopy, hematology and clinical chemistry, urinalysis, organ weights, or macroscopic or histopathological findings. Food intake in high-dose rats was significantly increased with no changes in body weight, in comparison to control rats; however, the authors considered this finding to be of no toxicological relevance due to the lower energy content of the high-dose diet compared to the control diet. A significant

² Good laboratory practice (GLP)-compliant study conducted according to the OECD Guideline for Testing of Chemicals No. 425 (OECD, 2008a) and the U.S. Environmental Protection Agency (EPA) Guideline No. 870.1100 (U.S. EPA, 2002).

Good laboratory practice (GLP)-compliant study conducted in-line with the OECD Guideline for Testing of Chemicals No. 407 (OECD, 2008b)

Good laboratory practice (GLP)-compliant study conducted according to OECD Guideline for Testing of Chemicals No. 408 (OECD, 1998).

increase in the absolute weight of the full and empty cecum of mid- and high-dose males and high-dose females, and a significant increase in the full and empty cecum weights relative to body weight in the high-dose males and females also were observed, an effect that was attributed to consumption of the test material. This finding has been reported where large doses of fungal derived fibers were similarly administered to rodents during subchronic toxicity evaluations (e.g., Feletti *et al.*, 1992; Babícek *et al.*, 2007). In addition, increased cecum weights associated with the consumption of indigestible carbohydrates (sorbitol, mannitol, caramel and polydextrose) is a well established phenomenon in rodents, and is not considered to have toxicological relevance to humans (WHO, 1987). Jonker *et al.* (2010) concluded that under the conditions of the study, the NOAEL for chitin-glucan was 10%, the highest concentration fed, which was equivalent to an overall estimated daily intake of 6.6 and 7.0 g/kg body weight in male and female rats, respectively. The Expert Panel concluded that the NOAEL determined by the authors was appropriate.

The mutagenicity of bleached chitin-glucan manufactured by Kitozyme was evaluated at 5 concentrations (25, 75, 250, 750, and 2,500 µg/plate) in strains of *Salmonella* Typhimurium (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* WP2 (pKM 101) in the presence and absence of metabolic activation [*i.e.*, rat liver microsome fraction (S9)]. This study was conducted using Good Laboratory Practice and in accordance with OECD guidelines for the testing of chemicals³. No increases in the number of revertant colonies per plate for any of the strains tested in the presence or absence of S9 were observed. The authors concluded that chitin-glucan was not mutagenic. A critical review of the literature failed to provide evidence suggestive that chitin-glucan represents a mutagenic hazard. The Expert Panel concluded that chitin-glucan was not mutagenic.

Two additional animal studies conducted using Kitozyme's bleached chitin-glucan also were critically evaluated and discussed by the Expert Panel (Berecochea-Lopez *et al.*, 2009; Neyrinck *et al.*, 2011). These studies were designed to evaluate the effects of the short-term dietary administration of chitin-glucan to mice and Syrian Golden hamsters; toxicology based endpoints were not included in the study designs. The results of these studies did not raise safety concerns for the Expert Panel.

Two clinical studies conducted with bleached chitin-glucan manufactured by Kitozyme were evaluated by the Expert Panel. The first was a published single-blinded pilot study conducted in adult male subjects (mean age of 32 years, mean cholesterol of 1.78 g/L, mean body mass index of 24 kg/m²) administered 1 capsule of chitin-glucan 3 times daily for 4 weeks (total daily dose of 4.5 g; 20 subjects) (Deschamps *et al.*, 2009). A control group (10 subjects) received a placebo (kaolin). The Expert Panel noted that information on the safety of chitin-glucan (e.g., reports of adverse events) was not presented in the study.

³ Good laboratory practice (GLP)-compliant study conducted according to the OECD Guideline for Testing of Chemicals No. 471 (OECD, 1997).

In a non-controlled dietary intervention study, chitin-glucan was well tolerated by a group of 20 healthy human subjects (mean age 32 years) administered chitin-glucan at a dose of 4.5 g/day for a duration of 4 weeks; however, no specific safety endpoints were included in this study design (Deschamps *et al.*, 2009). In a study by Bays *et al.* (2010), the safety and tolerability of chitin-glucan was evaluated as secondary endpoints within a randomized, double-blind, placebo-controlled, multi-center study conducted with hypercholesterolemic yet otherwise healthy male and female adult subjects. One-hundred and thirty-five volunteers (aged 21 to 70 years) were randomized to 1 of 4 groups receiving 4.5 g of chitin-glucan, 1.5 g of chitin-glucan, 1.5 g of chitin glucan in combination with an olive oil extract, or placebo (rice flour) daily for 6 weeks. No statistically significant differences in physical examination parameters (body weight, diastolic or systolic blood pressure, heart rate) or safety-related clinical chemistry and hematology endpoints were observed following completion of the treatment phase. No statistically significant differences in between group incidences of adverse events were reported, and no severe adverse events attributed to chitin-glucan consumption were observed.

The Expert Panel understood that the studies used to support the safety of Kitozyme's chitin-glucan ingredient were performed using the bleached material currently marketed within the European Union. However, it was concluded that data provided by these studies were relevant to the risk assessment of the non-bleached material under the proposed food and technological uses. The use of hydrogen peroxide for bleaching of dietary fibers has a long-history of safe use in food world-wide, and is a process that is widely used by the grain industry (Saunders *et al.*, 2007). The bleaching action of hydrogen-peroxide is attributed to its ability to oxidize colored pigments, and neutralize off-flavors; treatment of chitin-glucan with hydrogen-peroxide is not expected to produce significant material changes to the chemical structure of the beta-linked carbohydrate polymers. For example as reported by Lopez-Guiza *et al.* (1988), chemical effects of alkaline hydrogen peroxide processing of oat hulls fiber were limited minor increases (<5% increase) in the concentrations of soluble neutral sugars and soluble fiber. The toxicity of bleached versus non-bleached dietary fiber sources has also been reported in the literature (Kehoe *et al.*, 1990; Lopez-Guiza *et al.*, 1988). Animals consuming bleached dietary fiber had similar toxicity profiles compared to those consuming non-bleached dietary fiber, suggesting hydrogen peroxide bleaching does not impart physico-chemical changes to *alpha* and *beta*-linked linear glycans that result in biologically distinct effects, even following consumption of these materials at high dietary concentrations (up to 15% supplementation in diets).

The microbial biomass of *A. niger* remaining from the production of food-grade citric acid is considered a safe and suitable raw material for the manufacture of Kitozyme's chitin-glucan. Information characterizing the pathogenicity and toxigenicity of *A. niger* was reviewed by Kitozyme. *A. niger* is generally regarded as a non-pathogenic microorganism (Schuster *et al.*, 2002; van Dijck *et al.*, 2003; Blumenthal, 2004; Olempska-Beer *et al.*, 2006). One study evaluating the administration of viable *A. niger* spores (5 to 40 million spores/day) in the feed (organism pipetted onto feed) of hybrid broiler chicks for 10 days followed by 4 days recovery

and then the administration for another 10 days was reported by Nyiredy *et al.* (1975). The authors concluded that the “oral ingestion of even very large amounts of such fungal stages involves practically no risk of mycosis or mycotoxicosis”. Using Dialog[®], a comprehensive review of the literature through June 2010 was conducted using the search terms *Aspergillus* and *niger* with bacteremia, infect, infectious, infection, pathogen and, pathogenicity, pathogenic. Reports of infectivity attributed to direct or indirect oral consumption of *A. niger* by humans were not identified. The Expert Panel concurs with the generally accepted position that *A. niger* is non-pathogenic.

Kitozyme noted that although strains of *A. niger* have been reported to synthesize a number of secondary metabolites with poorly characterized toxicology profiles, including malformins, naphtho- γ -pyrones, aurasperone D, nigerazines, nigragillin, oxalic acid, pyranonigrin, and kotanins, the production of mycotoxins known to be adversely associated with human health is limited to ochratoxins and fumonisins (Schuster *et al.*, 2002; van Dijck *et al.*, 2003; Blumenthal, 2004; EFSA, 2007; Frisvad *et al.*, 2007). As defined by Pariza and Foster (1983), “A non-toxicogenic microorganism is one that does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure”. As discussed, strains of *A. niger* have been safely used in the production of food grade citric acid since 1919. Since then, manufacturers of citric acid have selected proprietary strains, and developed controlled fermentation processes to produce citric acid that is food grade. Based on this long-history of safe use in the food industry, *A. niger* strains used in the production of citric acid are widely considered safe and suitable for this purpose. This fact was recognized by the U.S. Food and Drug Administration (FDA), and no strain specific qualifications, or specifications on the *A. niger* strains used in the production of citric acid were imposed by the FDA during promulgation of 21 CFR §173.120 and 21 CFR §184.1033 (U.S. FDA, 2011). Since the source material used in the manufacture of Kitozyme’s chitin-glucan is obtained from manufacturers of food grade citric acid, mycotoxins are not expected to be present. Accordingly, Kitozyme has demonstrated the absence of several mycotoxins including ochratoxins, aflatoxins, and fumonisins in both the raw *A. niger* source material and final chitin-glucan product.

In addition to use as a fermentation organism, *A. niger* is widely used by the food industry world-wide as a source of enzymes. Carbohydrase and cellulase derived from non-toxicogenic, non-pathogenic strains of *A. niger* are permitted for use in clam and shrimp processing (21 CFR §173.120 – U.S. FDA, 2011). Carbohydrase, catalase, cellulase, glucose oxidase, pectinase, protease, lactase, and lipase from *A. niger* are GRAS for use in food production in the U.S.; these GRAS determinations have been Notified to the FDA without objection from the Agency (GRN 89, 111, 132, 158, 183 – U.S. FDA, 2002a,b, 2003, 2005, 2006). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) also has evaluated the safety of a number of enzyme preparations from *A. niger* and assigned an acceptable daily intake of not specified (JECFA, 1988, 1990, 2009). The history of use, and safety of *A. niger* for use by the food industry has been comprehensively reviewed in the literature by a number of experts, including

the FDA, supporting the conclusion that the use of *A. niger* in food production is safe and suitable (Schuster *et al.*, 2002; van Dijck *et al.*, 2003; Blumenthal, 2004; Olempska-Beer *et al.*, 2006). During the JECFA's most recent evaluation of enzyme preparations derived from *A. niger*, an ADI of "not specified" was determined based on the view that *A. niger* is a common organism in food, that many strains of the species have had a long history of use as an enzyme source, and that the numerous studies of enzyme preparations derived from the organism have demonstrated no hazard to human health (JECFA, 1990).

The Expert Panel concluded that provided that the raw material source of *A. niger* used in the production of chitin-glucan was obtained from commercial manufacturers of food grade citric acid in accordance with 21 CFR §173.120 and 21 CFR §184.1033 (U.S. FDA, 2011), this raw material would be considered safe and suitable for use in the manufacture Kitozyme's chitin-glucan.

Conclusion

We, the Expert Panel, have independently and collectively critically evaluated the data summarized above and conclude that the intended uses and use levels of chitin-glucan, as direct and secondary direct food ingredient in multiple food categories, when produced using suitable and safe sources of *A. niger* obtained from manufacturers of food grade citric acid, and manufactured and used in accordance with current good manufacturing practice, are Generally Recognized as Safe (GRAS), based on scientific procedures.

It is our opinion that other qualified experts would concur with this conclusion.

(b) (6)

Prof. Joseph F. Borzelleca, Ph.D.
Virginia Commonwealth University
School of Medicine

19 September 2011
Date

(b) (6)

Prof. Robert J. Nicolosi, Ph.D., M.S., CNS
University of Massachusetts Lowell

13 September 2011
Date

(b) (6)

Prof. John A. Thomas, Ph.D.
University of Indiana School of Medicine

9/16/11
Date

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Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	Section §	Section Title
101—Food labeling	101.12	Reference amounts customarily consumed per eating occasion
173—Secondary direct food additives permitted in food for human consumption	173.120	Carbohydrase and cellulase derived from <i>Aspergillus niger</i>
	173.280	Solvent extraction process for citric acid
184—Direct food substances affirmed as generally recognized as safe	184.1033	Citric acid

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ATTACHMENT A

Individual Proposed Food-Uses and Use-Levels for Chitin-Glucan in the U.S.

Table A-1 Summary of the Individual Proposed Food-Uses and Use-Levels for Kitozyme's chitin-Glucan (KiOnutrime-CG) in the U.S.

Food Category	Proposed Food-Uses	KiOnutrime-CG Level (g/serving)	RACC* (g or mL)	Use-Levels (%)
Beverages and Beverage Bases	Energy Drink Shots [^]	1.1	60	1.83
	Energy Drink Beverages [^]	1.1	240	0.46
	Sports and Isotonic Beverages	1.1	240	0.46
	Enhanced Waters	1.1	240	0.46
	Meal Replacement Beverages (non milk-based)	1.1	240	0.46
Breakfast Cereals	Ready-to-Eat Breakfast Cereals	1.1	15 to 55	2.00 to 7.33
Grain Products and Pastas	Cereal and Energy Bars (Grain-Based Bars/Breakfast Bars/Health Bars/Granola-type Bars, etc.)	1.1	40	2.75
Milk Products	Milk-Based Meal Replacement Beverages	1.1	240	0.46
	Smoothies	1.1	240	0.46
	Yogurt Drinks	1.1	240	0.46
	Yogurt	1.1	225	0.49
Processed Fruits and Fruit Juices	Fruit Juice	1.1	240	0.46
	Fruit Juice Drinks and Ades	1.1	240	0.46

* RACC = Reference Amounts Customarily Consumed per Eating Occasion (21 CFR §101.12 - U.S. FDA, 2011). When a range of values is reported for a proposed food-use, particular foods within that food-use may differ with respect to their RACC.

[^] No food codes for energy drink shots were identified, thus food codes for energy-type drinks were used as surrogates. As the same food codes were included for both Energy Drink Shots and Energy Drink Beverages, the intakes were calculated using the use-level for Energy Drink Shots only to provide a more conservative estimate of intake without duplicating food codes in the assessment.

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Table A-2 Summary of the Individual Proposed Technological Uses, Use-Levels, and Maximum Residues for Kitozyme's Chitin-Glucan (KiOfine-CG) in the U.S.

Food Category	Food-Use	Application	Use-Levels in Application	Residues ¹ in final product (ppm)
Beverages, Alcoholic	Beer	Clarification	100 g/100 L	not determined
	Wine	Clarification-	100 g/100 L	not detected
		Fining	50 g/100 L	not detected
		Removal of trace metals	100 g/100 L	not detected
		Removal of potential mycotoxins	500 g/100 L	not detected
		Prevention of oxidation of color	100 g/100 L	not detected
		Reduction of heat labile proteins	100 g/100 L	not detected
		Aid to filtration	50 g/100 L	not detected
		Spirit	Removal of trace metals	100 g/100 L
	Improvement of flavor		50 g/100 L	not determined
Fresh Fruits	Must	Clarification/removal of off-flavor	100 g/100 L	not determined
		Removal of potential mycotoxins	500 g/100 L	not determined
		Prevention of oxidation of color	100g/100 L	not determined
Processed Fruits and Fruit Juices	Fruit Juices	Clarification/removal of off-flavor	100 g/100 L	not determined

¹ 500 mL of wine that had been treated with 40 g/100 L chitin-glucan and had the sediments removed was filtered, and the solid residue that collected on the filter was washed with water, dried, and analyzed by infrared (IR) spectroscopy for identification of residual chitin-glucan

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ATTACHMENT B

Expert Panel *Curriculum vitae*

APPENDIX B

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Table B-1 Testing Program for the Stability Study of KiOnutrime-CG								
Test	Specification Limit	Temperature and Test Interval						
		T0	25°C			40°C		
			T3	T6	T12	T3	T6	T12
Loss on drying (% w/w)	≤10	5.86	5.97	5.95	6.15	6.16	6.61	6.78
Water activity	<0.4	0.228	0.218	0.222	0.238	0.252	0.262	0.338
Aerobic microbial count (cfu/g)	≤100	<10	<10	<10	<10	<10	<10	<10
Total yeasts and molds count (cfu/g)	≤100	<10	<10	<10	<10	<10	<10	<10
<i>Escherichia coli</i> (cfu/g)	≤10	<10	<10	<10	<10	<10	<10	<10
<i>Listeria monocytogenes</i> (/25 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
<i>Enterobacteriaceae</i> (cfu/g)	≤10	<10	<10	<10	<10	<10	<10	<10
<i>Salmonella</i> spp. (/25 g)	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

APPENDIX C



CERTIFICAT D'ANALYSE

Original

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NOM : Echantillon kitozyme FTIR	ANALYSE N° : 10/131
ORIGINE : Kitozyme	PROTOCOLE : SOP 124
INFORMATIONS : SYLE0005-153	DATE DE RECEPTION : 16/09/2010
	DATE DU DEBUT D'ANALYSE : 20/09/2010

<u>Contrôle</u>	<u>Spécification</u>	<u>Résultat</u>
Comparaison de spectres FTIR : Chitine glucan Lot MAPE2-4 et SYLE0005-153	Positive	Négatif

Conclusions : Les positions et les dimensions relatives des bandes du spectre de l'échantillon et du spectre de référence ne sont pas concordantes. Le produit n'est donc pas conforme aux spécifications.

<u>Date</u> :	<u>Date</u> :	<u>Date</u> :	<u>Date</u> :
<u>Analyste</u>	<u>Responsable Q.C.</u>	<u>Responsable Q.A.</u>	<u>Directeur du Laboratoire</u>
<u>Nom</u> : S. Houari	<u>Nom</u> : E. Ziemons	<u>Nom</u> : F. Lecomte	<u>Nom</u> : Ph. Hubert

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SUBMISSION END