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Suite 100, 50 Crowther Lane
Fredericton, New Brunswick, Canada
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Tel: +1.506.262.1348
Web: Chinovabioworks.com

3 March 2021

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

Dear Dr. Gaynor:

Re: GRAS Notice for Fiber Extracted from White Button Mushrooms

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Chinova Bioworks Inc., as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the company's conclusion that fiber extracted from white button mushrooms is GRAS on the basis of scientific procedures for use as an antimicrobial ingredient; the food use of fiber extracted from white button mushrooms, is therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*. Information setting forth the basis for Chinova Bioworks Inc.'s GRAS conclusion, as well as a consensus opinion of an independent panel of experts, also are enclosed for review by the agency.

I certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using Symantec Endpoint Protection 12.1.5.

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,

David Brown
Chief Operating Officer
Chinova Bioworks Inc.

dave@chinovabioworks.com
+1 506 999 2490

GRAS NOTICE FOR THE USE OF FIBER EXTRACTED FROM WHITE BUTTON MUSHROOMS AS AN ANTIMICROBIAL INGREDIENT IN FOOD AND BEVERAGE PRODUCTS

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
Chinova Bioworks Inc.
50 Crowther Lane, Suite 100
Fredericton, New Brunswick, Canada
E3C 0J1

DATE:
03 March 2021

GRAS Notice for the Use of Fiber Extracted from White Button Mushrooms as an Antimicrobial Ingredient in Food and Beverage Products

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GRAS Notice for the Use of Fiber Extracted from White Button Mushrooms as an Antimicrobial Ingredient in Food and Beverage Products

PART 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Chinova Bioworks (Chinova) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that Chinova's fiber extracted from white button mushrooms (*Agaricus bisporus*) is not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Chinova's view that fiber extracted from white button mushrooms (*A. bisporus*) is Generally Recognized as Safe (GRAS). In addition, as a responsible official of Chinova, the undersigned hereby certifies that all data and information presented in this Notice represent a complete and balanced submission that is representative of the generally available literature. Chinova considered all unfavorable, as well as favorable, information that is publicly available and/or known to Chinova and that is pertinent to the evaluation of the safety and GRAS status of Chinova's fiber extracted from white button mushrooms (*A. bisporus*) as an antimicrobial ingredient in food and beverage products as described herein.

Signed,

March 3, 2021

David Brown
Chief Operating Officer
Chinova Bioworks Inc.
dave@chinovabioworks.com

Date

1.1 Name and Address of Notifier

Chinova Bioworks Inc.
50 Crowther Lane, Suite 100
Fredericton, New Brunswick, Canada
E3C 0J1

1.2 Common Name of Notified Substance

White Button Mushroom Fiber; Mushroom Fiber; White Button Mushroom-derived Fiber; Mushroom-derived Fiber

1.3 Conditions of Use

Chinova's fiber extracted from white button mushrooms (*Agaricus bisporus*), comprised of chitosan and beta-glucan, is intended for use as an antimicrobial ingredient, as defined under 21 CFR §170.3(o)(2), in select food and beverage products in the U.S. A summary of the food categories and use levels in which the mushroom-derived fiber is intended for use is provided in Table 1.3-1 below. The proposed food uses for Chinova's fiber extracted from white button mushrooms (*A. bisporus*) are similar to those previously received GRAS status by the Flavor and Extract Manufacturers Association (FEMA) for use as an ingredient with flavor modifying properties (FEMA No. 4946) at levels up to 2,000 ppm. The FEMA-approved proposed uses of Chinova's fiber extracted from white button mushrooms are provided in Appendix A. The use levels of Chinova's fiber derived from white button mushrooms for use as an antimicrobial ingredient range from 0.01 to 0.150 g/100 g (equivalent to 100 to 1,500 ppm), which are much lower than the FEMA GRAS-approved use levels, which range from 1,500 to 2,000 ppm. Exposure to chitosan from flavoring and antimicrobial uses would not be additive, as the higher flavoring use levels would already be achieving the antimicrobial function.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Food Uses[a]	Proposed Use Levels (g/100 g)
Baked Goods and Baking Mixes	Bagels and English Muffins	0.100
	Bread (excluding sweet type breads and rolls)	0.100
	Cakes	0.100
	Light weight cakes	0.100
	Medium weight cakes	0.100
	Heavy weight cakes	0.100
	Cornbread, corn muffins, or tortillas	0.100
	Croissants	0.100
	Doughnuts (Donuts)	0.100
	French toast, pancakes, and waffles	0.100
	Muffins	0.100
	Pastries	0.100
	Pies	0.100
Beverages, Alcoholic	Cocktail drinks	0.040
Beverages and Beverage Bases	Energy drinks	0.040
	Enhanced or fortified waters	0.040
	Flavored or carbonated waters	0.040
	Soft drinks (regular and diet)	0.040
	Sport or electrolyte drinks, fluid replacement drinks	0.040
Cheeses	Cheese-based sauces	0.100
	Cottage cheese	0.100
	Cream cheese and cheese-based spreads	0.100
	Natural cheese	0.150
	Processed cheese or cheese mixtures	0.150
Coffee and Tea	Ready-to-drink coffees	0.015
	Ready-to-drink tea beverages	0.040

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Food Uses[a]	Proposed Use Levels (g/100 g)
Condiments and Relishes	Ketchup	0.040
	Mustard	0.040
	Relish	0.080
Confections and frostings	Coatings	0.100
	Frostings and icings	0.040
Dairy Product Analogs	Imitation cheese	0.150
Fats and oils	Fat-based sauces	0.100
	Margarine and margarine-like spreads	0.100
	Mayonnaise and mayonnaise-type dressings	0.100
	Salad dressings	0.100
Gelatins, Puddings, and Fillings	Flans, custards, and other egg-based desserts	0.080
Grain Products and Pastas	Cereal and granola bars	0.020
	Energy bars or protein bars or meal replacement bars	0.020
	Macaroni and noodle products	0.020
Gravies and Sauces	Gravies	0.020
	Tomato-based sauces	0.020
	White sauces	0.100
Jams and Jellies	Jams, jellies, preserves, and marmalades	0.100
Milk Products	Plain or flavored yogurt	0.100
Processed Fruits and Fruit Juices	Fruit drinks and ades and smoothies	0.060
	Fruit juices	0.060
	Fruit nectars	0.060
	Fruit-based desserts	0.080
Plant Protein Products	Meat analogs	0.150
Processed Vegetables and Vegetable Juices	Vegetable juices	0.040
	Vegetable purees[b]	0.040
Soups and Soup Mixes	Prepared and canned soups	0.040
Sugar Substitutes	Sugar substitutes	0.100
Sweet sauces, toppings, and syrups	Sweet sauces, syrups, and toppings (including fruit-based)	0.100
	Cocoa syrups	0.100

CFR = *Code of Federal Regulations*; U.S. = United States.
[a] Chinova's mushroom-derived fiber is intended for use in unstandardized products when standards of identity, as established under 21 CFR §130 to 169, do not permit its addition.
[b] Food codes for vegetable mixtures and vegetable combinations (which are likely to be used to make purees) were included as a surrogate for 'vegetable purees'.

1.4 Basis for GRAS

Pursuant to 21 CFR §170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2020b), Chinova has concluded that the intended uses of Chinova's fiber extracted from white button mushrooms (*A. bisporus*), as described herein, are GRAS on the basis of scientific procedures (see Appendix B).

1.5 Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Chinova Bioworks Inc.
50 Crowther Lane, Suite 100
Fredericton, New Brunswick, Canada
E3C 0J1

Should the U.S. FDA have any questions or additional information requests regarding this Notification, Chinova will supply these data and information upon request.

1.6 Freedom of Information Act, 5 U.S.C. 552

It is Chinova's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the *Freedom of Information Act*, 5 U.S.C. 552.

PART 2. § 170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

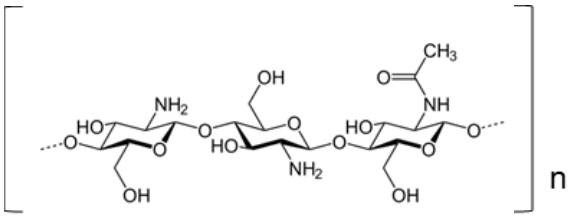
2.1 Identity of the Ingredient

2.1.1 Description of the Ingredient

Chinova's fiber (Chiber™) is a mixture of chitosan and *beta*-1,3-D-glucans. Chitosan is the main component, representing approximately 95% of the total volume, and is a soluble polymer derived from the cell walls of non-genetically modified *A. bisporus* (white button mushroom) biomass with a molecular weight (MW) range of 10 to 400 kDa[1]. Chitosan [(1,4)-2-amino-2-desoxy-*beta*-D-glucan] is a linear polycationic polysaccharide composed of glucosamine and *N*-acetyl glucosamine monomers linked together with a 1,4-β-linkage. Chitosan is a derivative of chitin, a naturally occurring carbohydrate polymer that is widely distributed in nature (*e.g.*, crustacean shells, fungal cell walls), where more than 60% of the acetyl groups are removed (*i.e.*, >60% deacetylation). The chemical structure of Chinova's fiber extracted from white button mushrooms is shown in Figure 2.1.1-1.

beta-1,3-D-Glucans are a major constituent of the cell walls of fungi and they are also present as structural components of many edible vegetables (Ko and Lin, 2004). *beta*-1,3-D-Glucans are composed of linear polysaccharide chains of varying average MW and can be linear (vegetable and *Aspergillus niger* sources) or branched (Baker's yeast) or both (mushrooms). Chinova's mushroom-derived fiber may contain up to 5% *beta*-1,3-D-glucans.

Figure 2.1.1-1 Chemical Structure of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*)



2.1.2 Trade Names

Chiber™

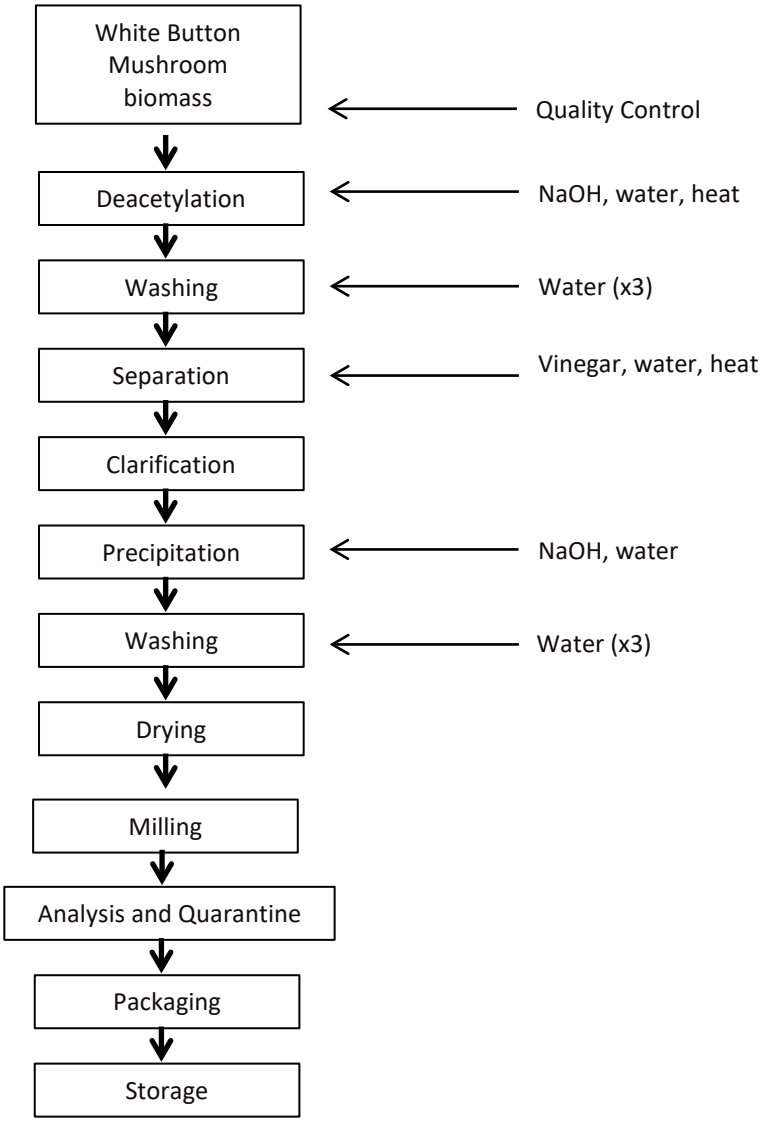
2.2 Method of Manufacture

Chinova's fiber extracted from white button mushrooms (*A. bisporus*) is manufactured in accordance with current Good Manufacturing Practices (cGMPs). The manufacturing process includes controls to ensure the quality of the final product prior to its release. A schematic of the production process is provided in Figure 2.2-1. All raw materials, processing aids, and food contact articles used in the manufacture of the mushroom-derived chitosan are food-grade and permitted for their respective uses in accordance with appropriate federal regulations, have been previously determined to be GRAS, or have been the subject of an effective food contact notification.

[1] Chitosan in this molecular weight range is considered low molecular weight chitosan (LMWC).

The white button mushroom biomass is initially inspected for conformity to the internal raw material standards [heavy metal content, moisture content, microbiology (total aerobic plate count, yeast, and molds) and visual appearance] and upon approval is combined with liquid sodium hydroxide and water in a vessel and heated. The thermal alkali process removes acetyl groups from the chitin fiber and simultaneously hydrolyzes proteins and saponifies lipids from the biomass. The deacetylated biomass is removed from the alkali solution, and repeatedly rinsed with water. The biomass is then exposed to a solution of food-grade vinegar wherein chitosan is separated from the mushroom biomass into solution. The solution is then clarified *via* centrifugation. The clarified liquid is adjusted to a slightly higher than neutral pH using sodium hydroxide, precipitating the fiber out of solution. The precipitated fiber is collected by centrifugation, and repeatedly washed with water. The collected dewatered fiber is dried using a drum dryer. The dried fiber is milled into a fine powder and held for quality control analysis. Subject to approval from the control analysis, the fiber is then packaged and stored.

Figure 2.2-1 Schematic Overview of the Manufacturing Process for Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*)



2.3 Product Specifications

Food-grade chemical and microbiological specifications have been established for Chinova's fiber extracted from white button mushrooms (*A. bisporus*) (Table 2.3-1). All methods of analysis are internationally recognized [*e.g.*, International Organization for Standardization (ISO)] or equivalent, or have been developed internally and validated by Chinova.

Table 2.3-1 Product Specifications for Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*)

Specification Parameter	Specification Limit	Method of Analysis
Identification	Positive	FTIR, H-NMR
Color of powder	White to beige	Validated Internal (visual)
Appearance of 1% solution of 1% HAc	Clear	Validated Internal (visual)
Degree of deacetylation (mol%)	≥80	Validated Internal
Molecular weight average (kDa)	10 to 400	HPLC
Moisture (%, w/w)	≤10	Validated Internal
Total ash (%, w/w)	≤3	Validated Internal
Solubility (%, w/w)	≥99.5	Validated Internal
Heavy Metals		
Arsenic (ppm)	≤0.2	ISO 11885 (ICP-OES)
Lead (ppm)	≤1.0	ISO 11885
Cadmium (ppm)	≤0.2	ISO 11885
Mercury (ppm)	≤0.2	ISO 11885
Microbiological Parameters		
Aerobic microbial count (CFU/g)	≤100	ISO 4833 Part 2 2013
Yeast and mold count (CFU/g)	≤100	ISO 21527-2
Escherichia coli (CFU/10 g)	Absent	ISO 7251
Salmonella (CFU/25 g)	Absent	AOAC 2013.01

AOAC = Association of Official Agricultural Chemists; CFU = colony-forming units; FTIR = Fourier-transform infrared spectroscopy; H-NMR = proton nuclear magnetic resonance; HAc = acetic acid; HPLC = high-performance liquid chromatography; ICP-OES = inductively coupled plasma-optical emission spectrometry; ISO = International Organization for Standardization; kDa = kilodaltons; ppm = parts per million.

2.4 Product Analysis

Analysis of 5 production lots of Chinova's fiber extracted from white button mushrooms (*A. bisporus*) demonstrates that the manufacturing process, as described in Section 2.2, produces a consistent product that meets the established product specifications. A summary of the chemical and microbiological analyses is provided in Table 2.4-1.

Table 2.4-1 Summary of the Product Analysis for 5 Lots of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*)

Specification Parameter	Specification Limit	Manufacturing Lot No.				
		CH20180623A1	CH20180604A1	CH20180514A1	CH20180518A1	CH20180502A1
Identification	Positive	Positive	Positive	Positive	Positive	Positive
Color of powder	White to beige	White	White	White	White	White
Appearance of 1% solution of 1% HAc	Clear	Clear	Clear	Clear	Clear	Clear
Degree of deacetylation (mol%)	≥80	94	92	93	93	95
Molecular weight average (kDa)	10 to 400	60±5	60±5	60±5	60±5	60±5
Loss on drying (%, w/w)	≤10	6.6	6.1	6.9	7	6.4
Total ash (%, w/w)	≤3	1.6	1.7	1.8	1.6	1.8
Solubility (%, w/w)	≥99.5	100	100	100	100	100
Heavy Metals						
Arsenic (ppm)	≤0.2	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Lead (ppm)	≤1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
Cadmium (ppm)	≤0.2	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Mercury (ppm)	≤0.2	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Microbiological Parameters						
Aerobic microbial count (CFU/g)	≤100	<5	<5	<5	<5	<5
Yeast and mold count (CFU/g)	≤100	<5	<5	<5	<5	5
Escherichia coli (CFU/10 g)	Absent	Absent	Absent	Absent	Absent	Absent
Salmonella (CFU/25 g)	Absent	Absent	Absent	Absent	Absent	Absent

CFU = colony-forming units; HAc = acetic acid; kDa = kilodaltons; ppm = parts per million.

2.5 Compositional Equivalence of Chinova's Fiber Extracted from White Button Mushrooms (*A. bisporus*) to Crustacean-Derived Chitosan

A compositional analysis of Chinova's fiber extracted from white button mushrooms (*A. bisporus*) was conducted to demonstrate that the mushroom-derived chitosan is equivalent to crustacean-derived chitosan, as well as to a chitosan reference standard described in the United States Pharmacopoeia (USP) monograph of chitosan[2]. The method of identification for chitosan, as referenced in the USP monograph, is infrared absorption (Method 197A – Spectrophotometric identification tests). The results of the infrared spectroscopy analysis are described in Section 2.5.1 below. In addition, the chitosan derived from *A. bisporus*, crustacean-derived chitosan and USP monograph reference chitosan were analyzed by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy (see Section 2.5.2). The results of the infrared and

[2] "*Chitosan in an unbranched binary polysaccharide consisting of N-acetyl-D-glucosamine and D-glucosamine units linked in a β(1-4) manner. Chitosan is obtained by partial deacetylation of chitin, which is extracted from the shells of edible shrimps and crabs suitable for human use. Its degree of deacetylation is NLT 70.0% and NMR 95%*" (USP, 2020).

^{1}H-NMR spectroscopy demonstrate that chitosan derived from *A. bisporus* is compositionally identical to chitosan derived from crustacean sources.

2.5.1 Infrared Spectroscopy Analysis

Fourier-transform infrared spectroscopy (FTIR) is the most commonly used method for identification of chitosan (Kumirska *et al*., 2010). Samples prepared for chitosan from *A. bisporus*, crustacean-derived chitosan, and USP monograph reference chitosan were analyzed by FTIR. The FTIR spectra demonstrate that Chinova's fiber derived from white button mushrooms (*A. bisporus*) is chemically identical to crustacean-derived chitosan products, including the USP monograph reference chitosan (Figure 2.5.1-1). The peak shown in each spectrum at approximate wavelength of 2,300 cm^{-1} is associated with carbon dioxide from the environment and is not associated with the chitosan sample.

Figure 2.5.1-1 Fourier-Transform Infrared Spectra for Chitosan from *Agaricus bisporus* and Crustacean Sources

USP – Crustacean Chitosan

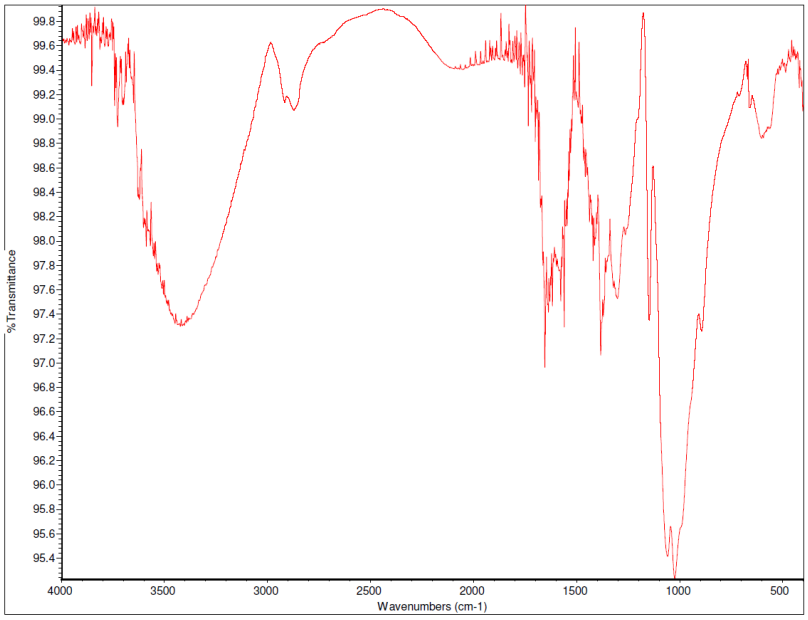
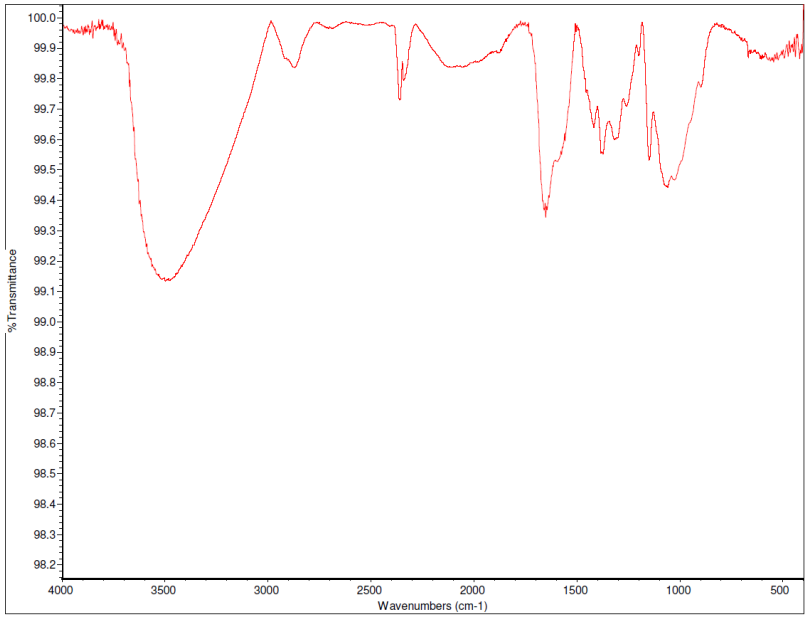
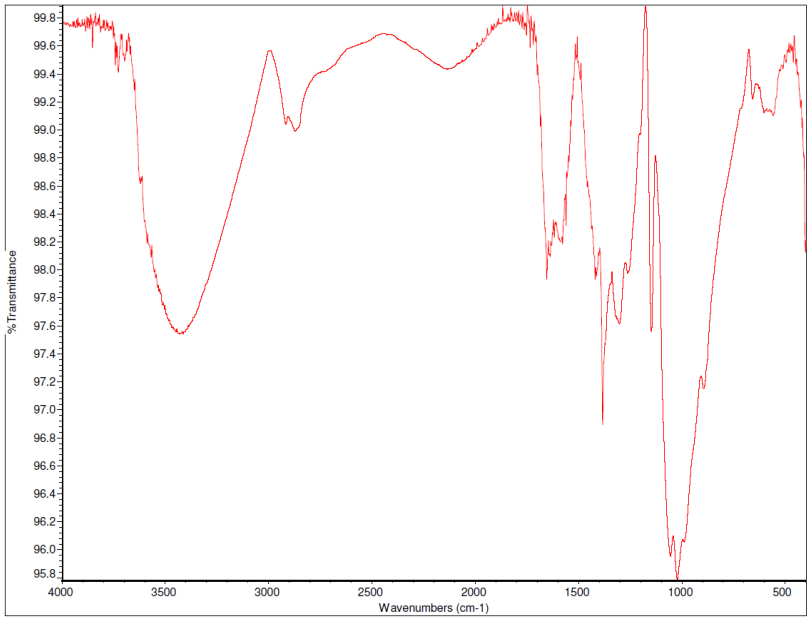


Figure 2.5.1-1 **Fourier-Transform Infrared Spectra for Chitosan from *Agaricus bisporus* and Crustacean Sources**

Commercial – Crustacean Chitosan



Chinova's Product – White Button Mushroom (*A. bisporus*) Fiber



USP = United States Pharmacopeia.

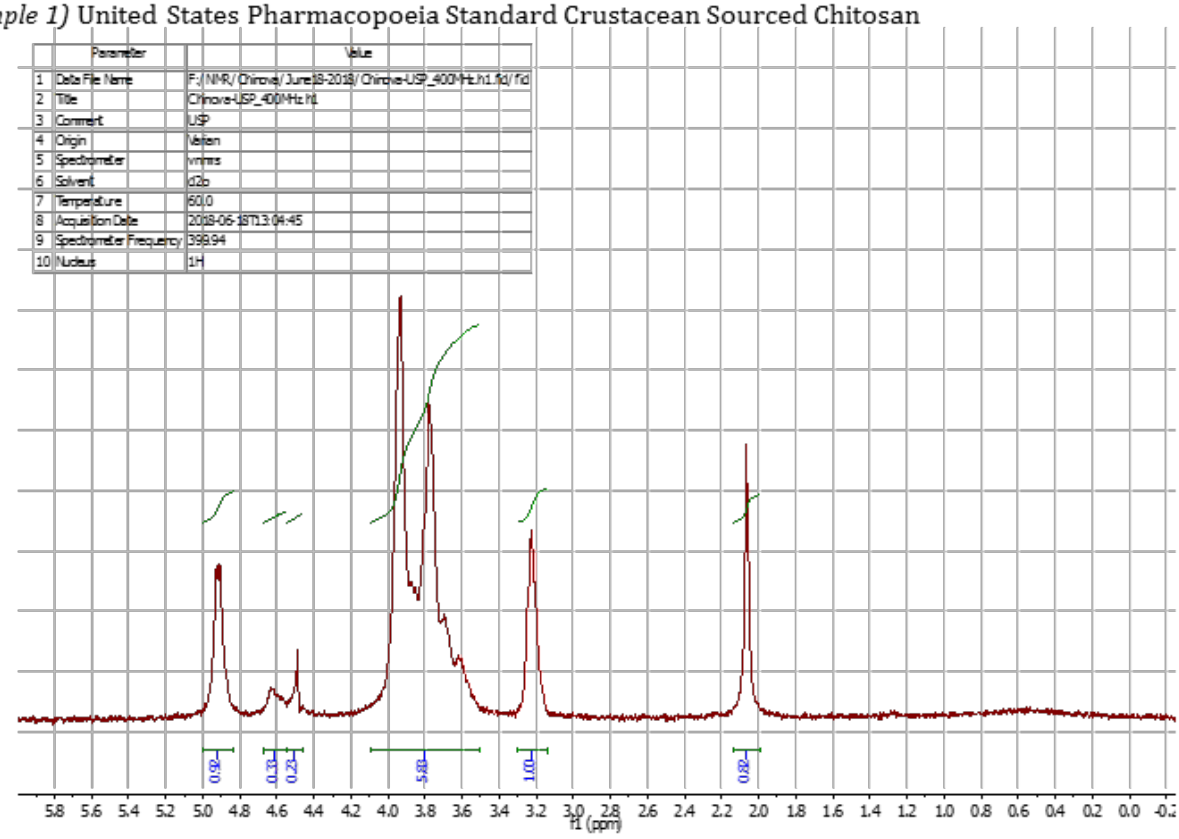
2.5.2 Nuclear Magnetic Resonance Spectroscopy

Samples of Chinova's fiber from white button mushrooms (*A. bisporus*), crustacean-derived chitosan, and USP monograph reference chitosan were analyzed by ^{1}H-NMR spectroscopy to provide information regarding the degree of deacetylation (DDA) of the compound and the compositional equivalency. The spectra shown in Figure 2.5.2-1 demonstrate that chitosan derived from white button mushrooms is compositionally equivalent to crustacean-derived chitosan products, including the USP monograph reference chitosan.

Figures 2.5.2-1 Nuclear Magnetic Resonance Spectra for Chitosan from *Agaricus bisporus* and Crustacean Sources

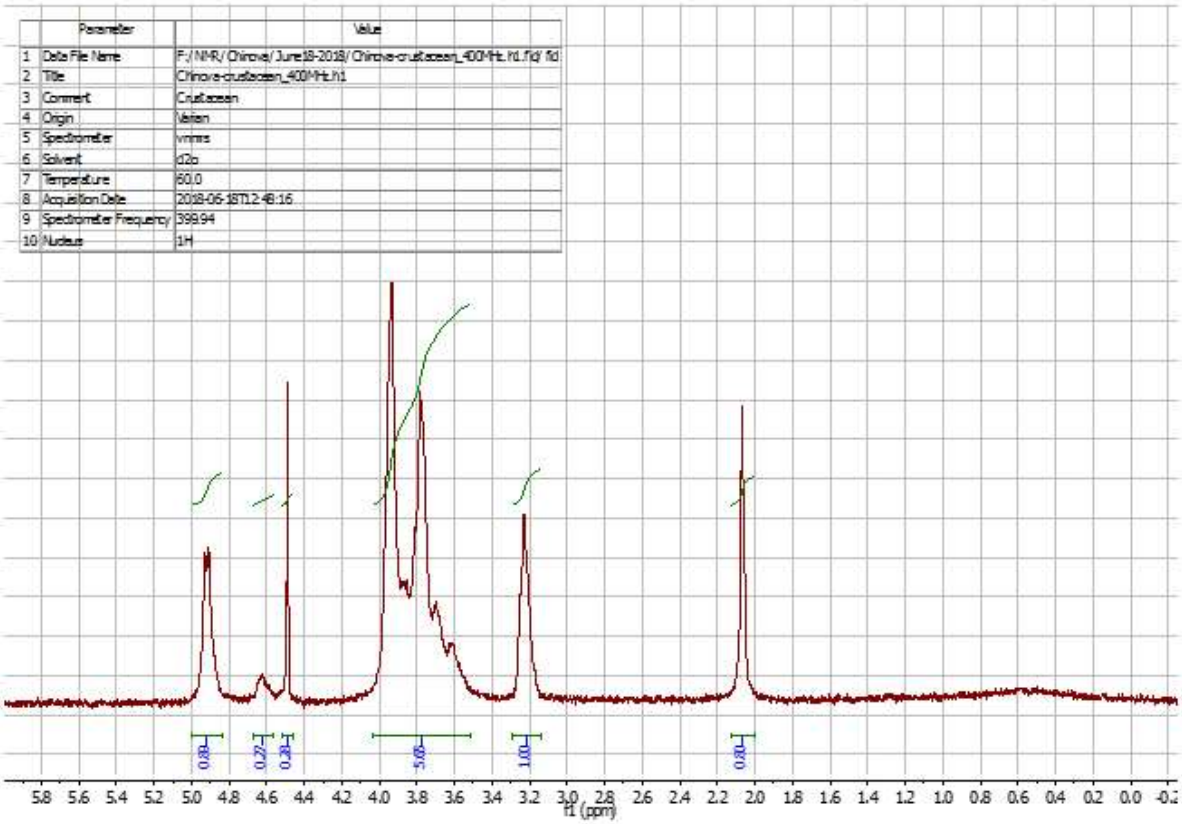
Sample 1) United States Pharmacopoeia Standard Crustacean Sourced Chitosan

	Parameter	Value
1	Data File Name	F:\NMR\Chinova\June18-2018\Chinova-USP_400MHz.h1.fid\fid
2	Title	Chinova-USP_400MHz.h1
3	Comment	USP
4	Origin	Varian
5	Spectrometer	vnmrs
6	Solvent	d2o
7	Temperature	60.0
8	Acquisition Date	2018-06-18T13:04:45
9	Spectrometer Frequency	399.94
10	Nucleus	1H



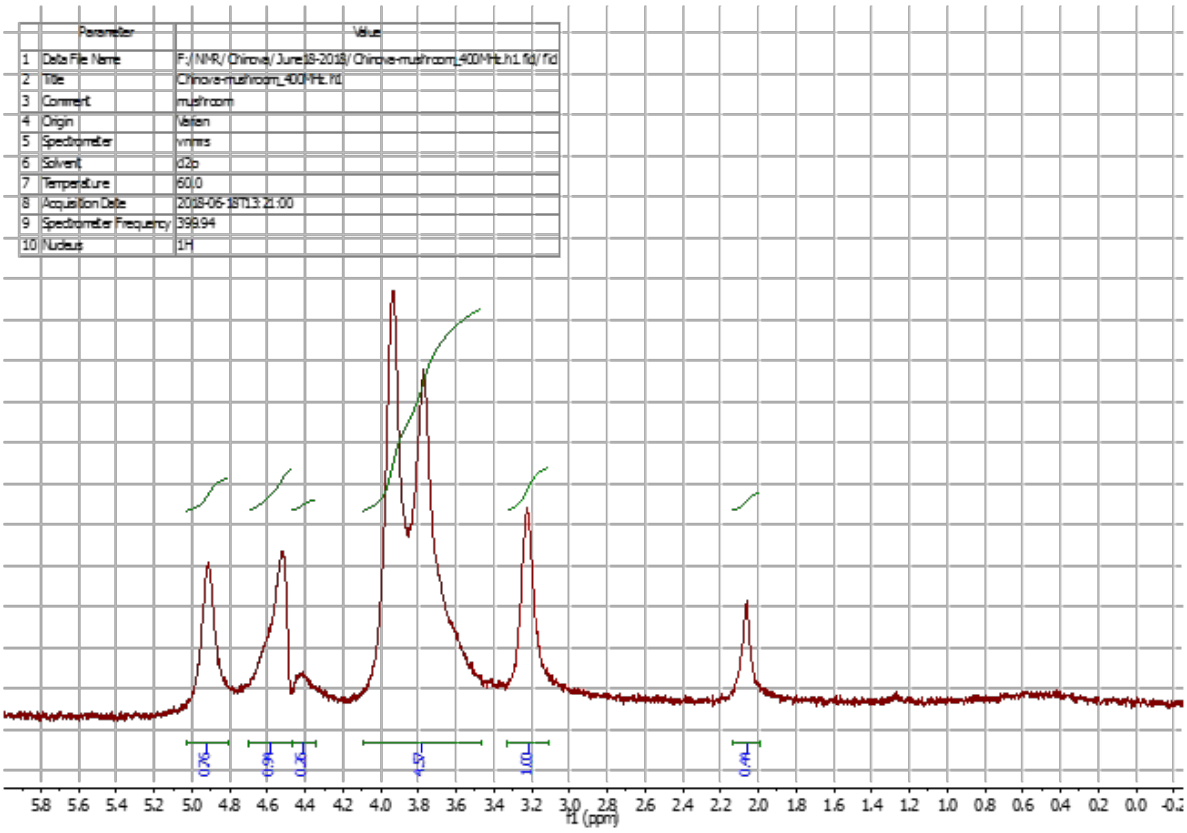
Figures 2.5.2-1 Nuclear Magnetic Resonance Spectra for Chitosan from *Agaricus bisporus* and Crustacean Sources

Sample 2) Crustacean Sourced Chitosan



Figures 2.5.2-1 Nuclear Magnetic Resonance Spectra for Chitosan from *Agaricus bisporus* and Crustacean Sources

Sample 3) A. bisporus Sourced Chitosan (Chiber)



2.6 Stability of Chinova's Fiber from White Button Mushrooms (*A. bisporus*)

The storage stability of Chinova's fiber extracted from white button mushrooms (*A. bisporus*) was tested under the recommended storage conditions (temperature: 25±2°C; relative humidity: 60±5%) and accelerated conditions (temperature: 40±2°C; relative humidity: 70±5%) using 3 non-consecutive lots of the fiber product. The results after 3, 6, and 9 months are within the specification limits, demonstrating stability of mushroom-derived fiber for at least 9 months when stored under ambient and accelerated conditions (Table 2.6-1), with an estimated shelf-life of 24 months based on the results of the accelerated stability testing.

Table 2.6-1 Stability of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) under Recommended and Accelerated Conditions

Parameter (*Specification Limit*)	Room Temperature Conditions (25±2°C; RH: 60±5%)				Accelerated Conditions (40±2°C; RH: 70±5%)			
	0 Months	3 Months	6 Months	9 Months	0 Months	3 Months	6 Months	9 Months
Lot #20170502-A								
Appearance	White	White	White	White	White	White	White	White
Moisture content (≤10%, w/w)	5.9	6.0	6.0	6.0	5.9	6.0	6.0	6.1
Solubility (%, w/w)	100	100	100	100	100	100	100	100
Molecular weight average (kDa)	100±5	100±5	100±5	100±5	100±5	100±5	100±5	100±5
Water activity (<0.5)	0.372	0.400	0.401	0.406	0.372	0.412	0.419	0.422
Total bacterial count (≤100 CFU/g)	1.5×10^1	<5.0	5	<5.0	1.5×10^1	<5.0	<5.0	<5.0
Yeast and mold count (≤100 CFU/g)	5	<5.0	<5.0	<5.0	5	<5.0	<5.0	<5.0
Escherichia coli (absent/10 g)	ND	ND	ND	ND	ND	ND	ND	ND
Salmonella spp. (absent/25 g)	ND	ND	ND	ND	ND	ND	ND	ND
Lot #20170502-B								
Appearance	White	White	White	White	White	White	White	White
Moisture content (≤10%, w/w)	6.0	6.0	6.0	6.0	6.0	6.2	6.2	6.2
Solubility (%, w/w)	100	100	100	100	100	101	102	103
Molecular weight average (kDa)	100±5	100±5	100±5	100±5	100±5	101±5	102±5	103±5
Water activity (*<0.5*)	0.372	0.380	0.381	0.388	0.372	0.399	0.404	0.419
Total bacterial count (≤100 CFU/g)	2.0×10^1	<5.0	1.0×10^1	<5.0	2.0×10^1	<5.0	<5.0	<5.0
Yeast and mold count (≤100 CFU/g)	1.5×10^1	<5.0	<5.0	<5.0	1.5×10^1	<5.0	<5.0	<5.0
Escherichia coli (absent/10 g)	ND	ND	ND	ND	ND	ND	ND	ND
Salmonella spp. (absent/25 g)	ND	ND	ND	ND	ND	ND	ND	ND
Lot #20170502-C								
Appearance	White	White	White	White	White	White	White	White
Moisture content (≤10%, w/w)	6.3	6.3	6.3	6.4	6.3	6.4	6.4	6.4
Solubility (%, w/w)	100	100	100	100	100	100	100	100
Molecular weight average (kDa)	100±5	100±5	100±5	100±5	100±5	100±5	100±5	100±5
Water activity (<0.5)	0.370	0.382	0.384	0.404	0.372	0.403	0.405	0.41
Total bacterial count (≤100 CFU/g)	1.0×10^1	<5.0	1.5×10^1	5	1.5×10^1	5.0	<5.0	<5.0

Table 2.6-1 Stability of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) under Recommended and Accelerated Conditions

Parameter (*Specification Limit*)	Room Temperature Conditions (25±2°C; RH: 60±5%)				Accelerated Conditions (40±2°C; RH: 70±5%)			
	0 Months	3 Months	6 Months	9 Months	0 Months	3 Months	6 Months	9 Months
Yeast and mold count (≤100 CFU/g)	<5.0	<5.0	<5.0	<5.0	5.0	<5.0	<5.0	<5.0
Escherichia coli (absent/10 g)	ND	ND	ND	ND	ND	ND	ND	ND
Salmonella spp. (absent/25 g)	ND	ND	ND	ND	ND	ND	ND	ND

CFU = colony-forming units; kDa = kilodaltons; ND = not detected; RH = relative humidity.

2.7 Technical Effect

The antimicrobial properties of chitosan have been researched for several decades, and it has been reported to have bactericidal and/or bacteriostatic effects against a range of microbes, including yeast, bacteria, and fungi (Raafat *et al*., 2008; Goy *et al*., 2009). The mechanism of action by which chitosan exerts these properties has not yet been fully elucidated and likely varies between different microbes. The predominant theory on the mechanism of action against bacteria is *via* ionic interactions between the charged groups in the chitosan polymer backbone (protonated NH^{3+} groups) and negatively charged bacterial wall constituents (Goy *et al*., 2016). These interactions lead to hydrolysis effects on the peptidoglycans in the cell wall, resulting in the leakage of intracellular electrolytes and ultimately cell death. Other proposed mechanisms of action include coating the bacterial cells (film-forming) or interference with nutrient absorption/mineral displacement. Li *et al*. (2016) reported that the bactericidal properties of chitosan were increased against *Escherichia coli* and *Staphylococcus aureus* as the DDA increased. This finding was also reported by Omura *et al*. (2003), who found that samples with higher DDA (>70% DDA) has higher bactericidal properties against *Bacillus subtilis*, *S. aureus*, *E. coli*, and *Pseudomonas aeruginosa versus* lower DDA samples (<70% DDA). This is congruent with the efficacy of the Chinova's mushroom-derived fiber as an antimicrobial agent given its high DDA, which is typically >90% (see Section 2.4). At concentrations of 200 ppm and above, crustacean chitosan with MWs of 55 to 155 kDa (~80% DDA) were generally equally effective against *E. coli* (Liu *et al*., 2006). At concentrations of 50 to 100 ppm, higher MW samples (96 to 155 kDa) had poorer antibacterial properties compared to lower MW samples (55 to 90 kDa) (Liu *et al*., 2006).

The technological function of Chinova's mushroom-derived fiber (10 to 400 kDa range) as an antimicrobial ingredient was evaluated in various beverage products, including carbonated soda, apple juice, and liquid sugar syrup (Table 2.7.1-1), baked goods (Table 2.7.2-1), and dairy based yogurt and cream cheese (Table 2.7.3-1).

2.7.1 Technological Function in Liquid Products

Chinova's mushroom-derived fiber was added at a concentration of 400 ppm to a cola-style carbonated beverage, 400 ppm to apple juice, and 1,000 ppm to a flavored sugar syrup containing no preservatives. Samples of each mixture were aseptically inoculated with microorganisms associated with food spoilage (*i.e.*, *Lactobacillus brevis*, *Aspergillus niger*, *Zygosaccharomyces bailii*, *Lactobacillus plantarum*, *Zygosaccharomyces rouxii*, *Saccharomyces cerevisiae diastaticus*, *Pichia anomala*, *Saccharomyces cerevisiae*) at an initial concentration of log 3 CFU/mL, and then sealed and stored in a 30°C incubator for 35 days. Samples were assayed every 7 days by standard dilution and plate count.

Results of the microbiological analysis in beverage products, as presented in Table 2.7.1-1, show that microbial counts decreased over time with the mushroom-derived fiber use by Day 35, whereas microbial growth increased over time in the control samples.

Table 2.7.1-1 Summary of the Microbiological Analysis of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) Liquid Products (Log CFU/mL)

Microorganism	Sample[a]	Timepoint (Days)						
		0	1	7	14	21	28	35
Carbonated Soda								
Zygosaccharomyces bailii	Control	3	1.2	1.1	1.3	1.3	1.5	2
	Chiber™	3	1.2	0	0	0	0	0
Aspergillus niger	Control	3	0.4	0.3	0.3	0.2	0.2	0.4
	Chiber™	3	0	0	0	0	0	0
Saccharomyces cerevisiae	Control	3	1.7	2.1	2.2	2.5	2.3	2.4
	Chiber™	3	0.2	0.4	0.4	0.2	0.1	0
Pichia anomala	Control	3	1.5	1.4	1.6	2.1	2.2	2.4
	Chiber™	3	1.1	0.5	0.2	0	0	0
Apple Juice								
Z. bailii	Control	3	3.1	3	3.2	3.4	4.1	4.7
	Chiber™	3	2.3	2	1.9	1.3	0.9	0.4
A. niger	Control	3	2.2	2.4	2.3	2.9	4.2	4.8
	Chiber™	3	0.9	0.4	0.1	0	0	0
S. cerevisiae	Control	3	3.4	3.5	4.2	4.6	4.9	5.1
	Chiber™	3	1.9	1.8	1.2	0.8	0.3	0
Lactobacillus plantarum	Control	3	2.7	2.3	2.5	2.4	2.9	3.4
	Chiber™	3	1.4	1	0.3	0	0	0
Lactobacillus brevis	Control	3	2.9	3.4	3.4	3.5	3.6	4
	Chiber™	3	2.1	1.4	1.3	0.3	0	0
Flavored Sugar Syrup								
Z. bailii	Control	3	2.8	3.1	3.3	3.5	4.2	4.8
	Chiber™	3	2.4	2.1	1.6	1.8	1.5	1.3
Zygosaccharomyces rouxii	Control	3	2.4	2.4	2.9	3.3	3.6	3.8
	Chiber™	3	2.3	2.2	2.1	1.8	1.5	1.6
S. cerevisiae	Control	3	3	3.2	3.5	3.9	4.1	4.3
	Chiber™	3	2.3	2.5	2.3	1.9	1.4	1.1
S. cerevisiae diastaticus	Control	3	3	3	3.2	3.4	3.9	4.3
	Chiber™	3	2.9	2.9	2.3	1.8	1.3	1.2
L. brevis	Control	3	2.7	2.4	2.4	2.8	2.9	3.2
	Chiber™	3	1.8	1.8	2.5	1.9	1.8	1.3

CFU = colony-forming units.

[a] Chinova's mushroom-derived fiber (Chiber™) was added at a concentration of 400 ppm to carbonated soda and apple juice products, and 1,000 ppm to flavored sugar syrup products. Control samples did not contain any preservatives.

2.7.2 Technological Function in Baked Good Products

The antimicrobial effect of Chinova's mushroom-derived fiber was also evaluated in baked good products. The test baked goods were prepared from raw ingredients containing no preservatives and 0 (control), and 1,000 ppm of mushroom-derived fiber. The baked good samples were incubated in a sealed plastic bag at 25°C for 35 days. The samples were inspected for visible mold growth and measured every 5 days, and the percent mold was calculated based on the total surface area. Results of the mold growth analysis in bread products demonstrate a delay in initial mold growth from 10 days in the control to 30 days with 1,000 ppm of mushroom-derived fiber (Table 2.7.2-1). The mold was also delayed from 10 days in the control to 25 days in the 1,000 ppm of mushroom-derived fiber English muffin sample. In the wheat tortilla the mold growth was delayed by 5 days from the control by the use of mushroom-derived fiber at 1,000 ppm.

Table 2.7.2-1 Summary of the Mold Growth Analysis of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) in Baked Good Products

Food Product	Sample[a]	Days to Mold Presence						
		0	5	10	15	20	25	30
Bread	Control	-	-	+	+	+++	+++	+++
	Chiber™	-	-	-	-	-	-	+
English Muffin	Control	-	-	+	++	+++	+++	+++
	Chiber™	-	-	-	-	-	++	++
Wheat Tortilla	Control	-	-	-	-	+	+++	+++
	Chiber™	-	-	-	-	-	+	+++

[a] Chinova's mushroom-derived fiber (Chiber™) was added at a concentration of 1,000 ppm to bread, English muffin, and wheat tortilla products. Control samples did not contain any preservatives.
-: No visual mold
+: 1 to 2 spots of visual mold
++: 10 to 25% coverage with mold
+++: >25% coverage with mold

2.7.3 Technological Function in Dairy Products

Technological function of mushroom-derived fiber was assessed in dairy products. A sample of plain yogurt and plain cream cheese (all preservative-free) with 0 (control), and 1 000 ppm mushroom-derived fiber each was placed in a small dish, sealed, and stored either in the refrigerator at 7°C. Samples of each dairy product were aseptically inoculated with microorganisms associated with food spoilage (*i.e.*, *Cladosporium cladosporiodes, Penicillium aurantiogriseum, Penicillium roqueforti, Geotrichum candidum, Yarrowia lipolytica, Aspergillus niger*) at an initial concentration of log 3 CFU/g, and then sealed and stored in a 7°C incubator for 35 days. Samples were assayed every 7 days by standard dilution and plate count. Results of the microbiological analysis in dairy products, as presented in Table 2.7.3-1, show that microbial counts decreased over time with the use of mushroom-derived fiber at 1,000 ppm, whereas microbial growth increased over time in the control samples.

Table 2.7.3-1 Summary of the Mold Growth Analysis of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) in Dairy Products (Log CFU/g)

Microorganism	Sample[a]	Timepoint (Days)					
		0	7	14	21	28	35
Plain Yogurt							
Cladosporium cladosporiodes	Control	3	2.4	2.9	3.3	3.9	4.7
	Chiber™	3	2.5	2.1	1.3	1	1
Penicillium aurantiogriseum	Control	3	1.5	1.6	2.3	4.3	4.4
	Chiber™	3	1.1	1.2	1.4	1.2	1
Penicillium roqueforti	Control	3	1.7	1.9	2.2	2.3	3.9
	Chiber™	3	0.9	0.3	0	0	0
Cream Cheese							
P. roqueforti	Control	3	3	2.9	3	3.2	4.1
	Chiber™	3	2.5	2.4	2	1.6	1.3
Geotrichum candidum	Control	3	2.9	2.9	3.4	3.5	4
	Chiber™	3	2.7	2.4	2.1	1.7	1.8
Yarrowia lipolytica	Control	3	2.4	2.5	2.5	2.9	3.4
	Chiber™	3	1	0.5	0.3	0.1	0.1
Aspergillus niger	Control	3	2.4	2.4	2.8	3.4	3.9
	Chiber™	3	1	0	0	0	0

CFU = colony-forming units.

[a] Chinova's mushroom-derived fiber (Chiber™) was added at a concentration of 1,000 ppm to plain yogurt and cream cheese products. Control samples did not contain any preservatives.

PART 3. § 170.235 DIETARY EXPOSURE

3.1 History of Use of Chitosan from Fungal and Crustacean Sources

Crustacean-derived chitosan has a long history of safe use in the global food supply. Crustacean-derived chitosan is currently approved/permitted for use as a natural food additive for general food use in Japan and Korea (JFCRF, 2014; MFDS, 2017) and has widespread use as a drug excipient, functional food ingredient, and dietary supplement product in the U.S., the European Union, and other regulatory jurisdictions throughout the world. Supplement products containing chitosan typically promote consumption of 1 to 5 g/person/day for use in weight control and/or maintenance of cardiovascular health (NIH, 2020).

Several GRAS Notices pertaining to chitosan derived from fungal and crustacean sources have been notified to the U.S. FDA to date (Table 3.1-1). In 2011, the use of an insoluble fungal-derived chitosan was concluded to be GRAS by KitoZyme as a secondary direct food ingredient in alcoholic beverage production at levels between 10 and 500 g/100 L. KitoZyme's GRAS conclusion was notified to the FDA on 08 August 2011 and filed by the Agency without objection under GRN 397 (U.S. FDA, 2011). In 2001, Primex Ingredients ASA (Primex) submitted a GRAS Notice to the FDA, regarding the GRAS conclusion of its shrimp-derived chitosan for use in foods for various applications, including as an antimicrobial, emulsifying, processing aid, antioxidant, dough strengthening, texturizing ingredient, amongst others (GRN 73 – U.S. FDA, 2002). At the notifier's request, the Agency ceased to evaluate the Notice. Primex resubmitted the GRAS Notice in 2005 with published clinical data demonstrating that chitosan does not adversely affect the absorption of fat-soluble vitamins; however, the Notice was subsequently withdrawn by the notifier. The Notice was re-submitted in 2012 and again withdrawn by the notifier (GRN 170 – U.S. FDA, 2005; GRN 443 – U.S. FDA, 2013a).

2-Amino-2-deoxy-poly-*D*-glucosamine [Chinova's fiber extracted from white button mushrooms (*A. bisporus*), as described herein] has the Flavor and Extract Manufacturers Association (FEMA) GRAS status for use as a flavoring ingredient with flavor modifying properties (FEMA No. 4946) at levels up to 2,000 ppm (see Appendix A). The FEMA-approved uses of Chinova's fiber extracted from white button mushrooms (*A. bisporus*) are the same as those proposed for use as an antimicrobial ingredient.

Table 3.1-1 Summary of GRAS Notifications for Chitosan

GRN No.	Substance	Intended Use	Outcome	Reference
73	Shrimp-derived chitosan	Use in foods in general for multiple technical effects in accordance with good manufacturing practice.	At notifier's request, FDA ceased to evaluate the Notice.	U.S. FDA (2002)
170	Shrimp-derived chitosan	Ingredient in food including meat and poultry products.	At notifier's request, FDA ceased to evaluate the Notice.	U.S. FDA (2005)
397	Chitosan from *Aspergillus niger*	As a secondary direct food ingredient in alcoholic beverage production at levels between 10 and 500 grams per hectoliter (100 liters).	No questions.	U.S. FDA (2011)
443	Shrimp-derived chitosan	Use in foods generally including meat and poultry, for multiple technical effects.	At notifier's request, FDA ceased to evaluate the Notice.	U.S. FDA (2013a)

FDA = United States Food and Drug Administration; GRAS = Generally Recognized as Safe; GRN = GRAS Notice.

3.2 Intended Condition of Use of Chinova's Fiber Extracted from White Button Mushrooms (*A. bisporus*)

Chinova's fiber extracted from white button mushrooms (*A. bisporus*) will be added to food and beverages products (see Table 1.3-1) as an antimicrobial ingredient, as defined under 21 CFR §170.3(o)(2), to control the growth of food-spoilage microbes (U.S. FDA, 2020a).

The use levels of Chinova's fiber extracted from white button mushrooms (*A. bisporus*) for its intended food uses as an antimicrobial ingredient will range from 0.01 to 0.150 g/100 g (equivalent to 100 to 1,500 ppm), which are much lower than the FEMA GRAS-approved use levels, which range from 1,500 to 2,000 ppm.

3.3 Estimated Dietary Consumption of Chinova's Fiber Extracted from White Button Mushrooms (*A. bisporus*)

3.3.1 Methodology

An assessment of the anticipated intake of Chinova's fiber extracted from white button mushrooms (*A. bisporus*) as an ingredient under proposed antimicrobial food uses (see Table 1.3-1) was conducted using consumption data available in the 2015-2016 cycle of the U.S. National Center for Health Statistics' National Health and Nutrition Examination Survey (NHANES) (CDC, 2018a,b; USDA, 2018). Chitosan is already permitted for use as a flavoring ingredient in foods where chitosan is proposed to be added as an antimicrobial ingredient. Exposure to chitosan from flavoring and antimicrobial uses would not be additive as the higher flavoring use levels would already be achieving the antimicrobial function. As a result, the maximum FEMA GRAS-approved use level of 2,000 ppm (0.20 g/100g) was applied to all proposed antimicrobial food uses of chitosan, to derive a worst-case estimate. A summary along with the pertinent results is presented herein.

The NHANES data are collected and released in 2-year cycles with the most recent cycle containing data collected in 2015-2016. Information on food consumption was collected from individuals *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2). Sample weights were incorporated with NHANES data to compensate for the potential under-representation of intakes from specific populations and allow the data to be considered nationally representative (CDC, 2018a,b; USDA, 2018). The NHANES data were employed to assess the mean and 90th percentile intake of Chinova's fiber extracted from white button mushrooms for each of the following population groups:

- Infants and toddlers, up to 2 years of age;
- Young children, ages 2 to 5;
- Children, ages 6 to 11;
- Female teenagers, ages 12 to 19;
- Male teenagers, ages 12 to 19;
- Female adults, ages 20 and up;
- Male adults, ages 20 and up; and
- Total population (ages 2 and older and both gender groups combined).

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of Chinova's fiber extracted from white button mushrooms by the U.S. population. Estimates for the daily intake of Chinova's fiber extracted from white button mushrooms represent projected 2-day averages for each individual from Day 1 and Day 2

of NHANES 2015-2016; these average amounts comprised the distribution from which mean and percentile intake estimates were determined. Mean and percentile estimates were generated incorporating survey weights in order to provide representative intakes for the entire U.S. population. "*Per capita*" intake refers to the estimated intake of Chinova's fiber extracted from white button mushrooms averaged over all individuals surveyed, regardless of whether they consumed food products in which the ingredient is proposed for use and, therefore, includes individuals with "zero" intakes (*i.e.*, those who reported no intake of food products containing Chinova's fiber extracted from white button mushrooms during the 2 survey days). "Consumer-only" intake refers to the estimated intake of Chinova's fiber extracted from white button mushrooms by those individuals who reported consuming food products in which the use of the ingredient is currently under consideration. Individuals were considered "consumers" if they reported consumption of 1 or more food products in which Chinova's fiber extracted from white button mushrooms (*A. bisporus*) is proposed for use on either Day 1 or Day 2 of the survey. The results of the assessment are presented in Section 3.3.2.

3.3.2 Intake Estimates for Chinova's Fiber Extracted from White Button Mushrooms (*A. bisporus*)

A summary of the estimated daily intake of Chinova's mushroom-derived fiber from proposed antimicrobial food uses in combination with the maximum FEMA GRAS-approved use level of 2,000 ppm is provided in Table 3.3.2-1 on an absolute basis (mg/person/day), and in Table 3.3.2-2 on a body weight basis (mg/kg body weight/day).

The percentage of consumers was high among all age groups evaluated in the current intake assessment; greater than 66.8% of the population groups consisted of consumers of food products in which Chinova's mushroom-derived fiber is currently proposed for use. Children 6 to 11 years of age had the greatest proportion of consumers at 100%. Among the total population (all ages), the mean and 90th percentile consumer-only intakes of Chinova's mushroom-derived fiber were determined to be 1.3 and 2.6 g/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest mean and 90th percentile consumer-only intakes of Chinova's mushroom-derived fiber on an absolute basis, at 1.6 and 3.2 g/person/day, respectively; while infants and toddlers had the lowest mean and 90th percentile consumer-only intakes of 0.5 and 1.0 g/person/day, respectively (Table 3.3.2-1).

Table 3.3.2-1 Summary of the Estimated Daily Intake of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) from Proposed Antimicrobial Food Uses at the Maximum FEMA GRAS-Approved Use Level in the U.S. by Population Group (2015-2016 NHANES Data)

Population Group	Age Group (Years)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)	
		Mean	90th Percentile	Mean	90th Percentile
Infants and Toddlers	0 to <2	0.3	0.8	0.5	1.0
Young Children	2 to 5	0.8	1.5	0.8	1.5
Children	6 to 11	1.1	1.9	1.1	1.9
Female Teenagers	12 to 19	1.0	1.9	1.0	1.9
Male Teenagers	12 to 19	1.3	2.6	1.3	2.6
Female Adults	20 and older	1.1	2.2	1.1	2.2
Male Adults	20 and older	1.6	3.2	1.6	3.2
Total Population	2 and older	1.3	2.5	1.3	2.6

Table 3.3.2-1 Summary of the Estimated Daily Intake of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) from Proposed Antimicrobial Food Uses at the Maximum FEMA GRAS-Approved Use Level in the U.S. by Population Group (2015-2016 NHANES Data)

Population Group	Age Group (Years)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)	
		Mean	90th Percentile	Mean	90th Percentile

n = sample size; FEMA; Flavor and Extract Manufacturers Association of the United States; GRAS = Generally recognized as Safe; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

On a body weight basis, the total population (all ages) mean and 90th percentile consumer-only intakes of Chinova's fiber from mushrooms were determined to be 19.8 and 40.2 mg/kg body weight/day, respectively. Among the individual population groups, young children were identified as having the highest mean consumer-only intakes of 48.7 mg/kg body weight/day, while infants and toddlers had the highest 90th percentile consumer-only intakes of 93.0 mg/kg body weight/day. Female adults had the lowest mean and 90th percentile consumer-only intakes of 15.2 and 30.0 mg/kg body weight/day, respectively (Table 3.3.2-2).

Table 3.3.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) from Proposed Antimicrobial Food Uses at the Maximum FEMA GRAS-Approved Use Level in the U.S. by Population Group (2015-2016 NHANES Data)

Population Group	Age Group (Years)	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)	
		Mean	90th Percentile	Mean	90th Percentile
Infants and Toddlers	0 to <2	29.8	76.7	44.7	93.0
Young Children	2 to 5	48.5	89.3	48.7	89.3
Children	6 to 11	34.1	62.2	34.1	62.2
Female Teenagers	12 to 19	17.3	34.2	17.3	34.2
Male Teenagers	12 to 19	19.6	36.5	19.7	36.5
Female Adults	20 and older	15.2	30.0	15.2	30.0
Male Adults	20 and older	17.8	36.4	17.9	36.9
Total Population	2 and older	19.7	40.2	19.8	40.2

bw = body weight; FEMA; Flavor and Extract Manufacturers Association of the United States; GRAS = Generally recognized as Safe; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

PART 4. § 170.240 SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with Chinova's fiber extracted from white button mushrooms (*A. bisporus*).

PART 5. §170.245 EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

Not applicable.

PART 6. § 170.250 NARRATIVE AND SAFETY INFORMATION

6.1 Safety Narrative

Chitosan derived from crustaceans have a long history of safe use in the food supply. The white button mushroom chitosan fiber manufactured by Chinova has been demonstrated to be compositionally similar to chitosan derived from shellfish (see Section 2.5 for further details). Chinova's fiber derived from white button mushrooms is manufactured to an average MW in the range of 10-400 kDa and a DDA greater than 80%. Chitosan oligosaccharides are a mixture containing glucosamine, dimers, trimers, tetramers, pentamers, and hexamers, and typically have an average MW less than 1 kDa and a DDA of 100%, and are not considered to be chemically representative of Chinova's fiber extracted from white button mushrooms (*A. bisporus*). Absorption and distribution resulting in systemic exposure to chitosan following consumption from the diet is influenced by the MW of the compound (Chae *et al*., 2005). Chitosan was not detected in the plasma of rats administered chitosan with a MW of 230 kDa, suggesting low bioavailability following exposure to high MW chitosan, while increased plasma chitosan concentrations were reported after administration of 3.8 to 22 kDa chitosan. As MW is expected to impact the bioavailability of the material, studies on chitosan oligosaccharides are not considered to be of toxicological relevance in the safety assessment of Chinova's fiber extracted from white button mushrooms (*A. bisporus*) as these compounds would be readily available and absorbed into the systemic circulation. Nevertheless, studies on chitosan oligosaccharides were included in the sections that follow for the sake of completeness.

The safety of various chitosan preparations, derived from crustacean or fungal sources or chitosan oligosaccharides, was investigated in a number of animal, human, and *in vitro* studies and discussed in previous GRAS Notifications (*e.g*., GRN 73, 170, 397, 443). Published studies on the metabolic fate of chitosan and toxicological studies on chitosan derived from crustacean sources were previously discussed in GRN 397, and is incorporated by reference to support the safety of Chinova's fiber derived from white button mushrooms (*A. bisporus*). The studies discussed in GRN 397 are briefly discussed in the sections that follow. An updated search of the scientific literature was conducted to identify studies related to chitosan that have been published since 2011. According to GRN 170, the U.S. FDA stated:

> "*Chitosan was non-toxic to humans and other test animals, but questioned whether or not chitosan would interfere with fat-soluble vitamin and mineral status in humans, when the substance was consumed on a chronic basis as part of a general diet*" (U.S. FDA, 2005).

It was noted that these concerns were raised based on the results of a publication (Deuchi *et al*., 1995), in which rats consuming a high-fat diet containing 5% chitosan (source and MW not reported; DDA 90%) experienced significant reductions in fat digestibility, and as a result, reduced levels of vitamins A, D, and E, and certain minerals (calcium, magnesium, iron) (U.S. FDA, 2005). The National Toxicology Program (NTP) conducted a long-term toxicity study of USP-grade crustacean-derived chitosan in rats, and in 2017 published the entirety of the study report (NTP, 2017). In this study, the chitosan test article had an average purity of 94%, average MW of 81.6 kDa and a DDA of 86.5%, and was mixed in with rat feed with 4% fat content. The NTP study reported statistically significant changes in fat-soluble vitamins and reductions in liver and thymus weights in animals consuming 3% or 9% chitosan, equivalent to approximately 1,500 or 1,800 mg/kg body weight/day for males and females, respectively, or 5,200 or 6,000 mg/kg body weight/day for males and females, respectively, for 6 months. These findings are discussed in further detail in Section 6.3.3. Based on the reported effects of chitosan on serum vitamin E levels, the authors concluded the "*lowest-observed-effect level for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats*". The crustacean-derived chitosan

used in the NTP study is chemically and compositionally similar to Chinova's fiber derived from white button mushrooms, and was considered to be pivotal in the safety assessment of Chinova's chitosan. Similar nutritional findings were not reported in human clinical studies at doses up to 6.75 g/day, and therefore, the changes in fat-soluble vitamins were not considered to be toxicologically significant at clinically relevant doses.

6.2 Metabolic Fate of Chitosan

Chitosan is a soluble biopolymer derived from the deacetylation of chitin, a naturally occurring carbohydrate polymer that is widely distributed in nature (*e.g.*, crustacean shells, fungal cell walls). As discussed in Section 2.5, Chinova's fiber derived from white button mushrooms is compositionally similar to chitosan derived from crustacean sources, and therefore, it is expected that Chinova's chitosan will follow the same metabolic fate as other crustacean-derived chitosans.

The metabolic fate of chitosan was previously discussed in Section C of GRN 397 and is incorporated by reference (U.S. FDA, 2011). Chitosan is not subject to digestion *via* human digestive enzymes; absorption and systemic exposure to intact chitosan molecules consumed in the diet will not occur. Following consumption in the diet, chitosan is expected to dissolve in water and travel intact throughout the upper gastrointestinal tract to the colon where the material is subject to fermentation by the microbiota in the large intestine (Lattimer and Haub, 2010). Enzymatic digestion of chitosan is dependent on the DDA of chitosan (Yang *et al*., 2007). The rate of degradation increased with the DDA of chitosan; chitosan with a DDA of 7.7% had a reported degradable percentage of 2.9%, while chitosan with a DDA of 82.5% had a degradable percentage of 60.2% (Yang *et al*., 2007). Chitosan with a DDA of 93.4% was completely degradable. Microbial fermentation of chitosan yields normal metabolites of fermentation, similar to other dietary fibers, such as short-chain fatty acids, and hydrogen, carbon dioxide, and methane gases. Although enzymatic degradation of chitosan during digestion is not likely, possible hydrolysis products generated during gastric transit would consist of compounds, such as chitosan oligomers, glucosamine, *N*-acetylglucosamine and glucose, which are known to be non-toxic even when consumed at high dietary concentrations in animals and humans (Lee *et al*., 2004; Anderson *et al.*, 2005; Takahashi *et al*., 2009).

Considering that Chinova's fiber derived from white button mushrooms has an average MW of 60±5 kDa and DDA greater than 90%, it is not expected that the ingredient will be absorbed following consumption from the diet and would not be enzymatically digested. Thus, systemic exposure to Chinova's fiber derived from white button mushrooms is not expected to occur, and the ingredient will pass intact through the gastrointestinal tract.

6.3 Toxicological Studies

6.3.1 Acute Toxicity

The acute oral toxicity of chitosan from fungal sources (*i.e*., *A. bisporus*) or a chitosan oligosaccharide preparation was discussed in GRN 397 (U.S. FDA, 2011). The median lethal dose (LD_{50}) for mushroom-derived (*A. bisporus*) chitosan was reported to be >2,000 mg/kg body weight in female Sprague-Dawley rats, while maximum acute tolerated oral dose of a chitosan oligosaccharide preparation (MW of 1.86 kDa) was reported to be greater than 10,000 mg/kg in Kunming mice. Two acute oral toxicity studies on lobster-derived chitosan and chitosan oligosaccharides were identified in the scientific literature since GRN 397. These studies are described briefly as follows.

Female Wistar rats (6/group) were administered lobster-derived chitosan (MW of 309 kDa and a DDA of 83%) *via* gavage at doses of 0 or 2,000 mg/kg body weight (Lagarto *et al*., 2015). Mortality, clinical signs, body weight, and organ abnormalities were monitored; however, no signs of toxicity or mortality were observed. The authors concluded that the acute LD_{50} was >2,000 mg/kg (Lagarto *et al.*, 2015). The lobster-derived chitosan test article used in the study by Lagarto *et al.* (2015) had a reported MW of 309 kDa and a DDA of 83%, and is considered to be compositionally similar to Chinova's fiber derived from white button mushrooms. The results of the study by Lagarto *et al.* (2015) suggest that Chinova's chitosan is of low acute toxicity.

In another acute toxicity study, chitosan oligosaccharides (90% purity; not further specified) were orally administered at doses of 0, 1,150, 1,400, 1,700, and 1,900 mg/kg body weight to Wistar female rats (5/group) (Eisa *et al*., 2018). The acute oral LD_{50} of 1,500 mg/kg body weight in female rats was determined by plotting lethality results against a linear regression line and probit analysis. Reduced locomotion was reported in all treated animals. These results are inconsistent with the results reported in Sprague-Dawley and Wistar rats administered acute doses of chitosan derived from crustaceans, wherein signs of toxicity were not reported in the animals at the highest dose of chitosan tested (*i.e.*, 2,000 mg/kg body weight/day). The reason for this difference is unclear; however, it could relate to the specific nature of the chitosan test article, which was not characterized by Eisa *et al*. (2018) and therefore, its compositional equivalence to Chinova's fiber extracted from white button mushrooms (*A. bisporus*) is unknown. As well, in oral repeat-dose studies evaluating the safety of chitosan at doses of 2,000 mg/kg body weight/day or higher (see Section 6.3.2), chitosan did not elicit increased mortality. Therefore, the results of the study reported by Eisa *et al*. (2018) are not considered relevant to the safety assessment of Chinova's mushroom-derived fiber.

6.3.2 Repeated-Dose Oral Toxicity

6.3.2.1 Studies on Chitosan

The repeated-dose oral toxicity of chitosan derived from crustacean sources was investigated in mice, rats, and guinea pigs. The test articles investigated in these studies were reported as low molecular weight chitosan (LMWC) or high molecular weight chitosan (HMWC), chitin-chitosan (containing 80% chitosan), or water-soluble chitosan.

A number of studies reported statistically significant changes in liver weight and liver enzymes [*e.g.*, aspartate transaminase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT)] that suggest hepatic effects in mice, rats, and guinea pigs. In a subchronic oral toxicity study in female Kunming mice, dietary administration of high-MW and water-soluble chitosan preparations of varying molecular weights and solubility (MW ranging 32.7 to 760 kDa; DDA ~85%) for 90 days was without significant adverse effects in any study parameter, and in particular liver and kidney weights and histopathology (Zeng *et al*., 2008). The authors noted that consumption of medium molecular weight chitosan (MW = 32.7 kDa; DDA = 85.2%) resulted in increased concentrations of minerals in the liver, spleen, and heart. These findings were attributed to the accumulation of HMWC in these organs and corresponding chelation of endogenous minerals (Zeng *et al*., 2008). In rats, no significant changes in liver weight were reported in male Wistar rats consuming chitosan (MW = 250 kDa; DDA = 94%) in the diet at levels of 5%, equivalent to 5,000 mg/kg body weight/day, for 21 days (Fukada *et al*., 1991) or in male and female Wistar rats administered chitosan derived from lobster chitin (MW = 309 kDa; DDA = 83%) by gavage at doses up to 1,000 mg/kg body weight/day for 28 days (Lagarto *et al*., 2015). In the study by Lagarto *et al.* (2015) and no signs of toxicity, mortality, or statistically significant changes in biochemistry parameters were reported following chitosan treatment. A statistically significant increase in erythrocyte count was reported in females in the 300 and

1,000 mg/kg body weight/day groups and in males in the 1,000 mg/kg body weight/day group compared to controls. No statistically significant variations in relative organ weight (as a percentage of total body weight) were reported in chitosan-dosed animals compared to controls. No treatment-related increase in organ lesions were reported based on histopathology examination (Lagarto *et al*., 2015). Lagarto *et al.* reported the short-term no-observed-adverse-effect level (NOAEL) to be 1,000 mg/kg body weight/day, the highest dose tested, for "*effects other than transient variation in erythrocyte count for chitosan under the conditions of this investigation*". The increase in erythrocyte count was considered to be unreliable due to the short duration of this study (*i.e.*, 28 days) and on the basis that no corroborative findings were reported in the long-term study in Sprague-Dawley rats by NTP (2017) (see Section 6.3.3 for further details). Conversely, Chiang *et al.* (2000) and Chiu *et al.* (2020) reported significant decreases in liver weight following consumption of chitosan (MW ranging from 80 to 740 kDa; DDA = 84 to 91%) in the diet at concentrations up to 5%, equivalent to 5,000 mg/kg body weight/day, for up to 8 weeks. The decrease in liver weight reported by Chiang *et al.* (2000) was associated with a decrease in liver total lipids, resulting in a decrease in liver fat accumulation.

Several other studies reported statistically significant changes in liver weights and liver enzyme activities following chitosan exposure; however, these studies did not report the source of chitosan, purity, average molecular weight, or DDA (Landes and Bough, 1976; Sugano *et al*., 1988; Han *et al*., 1999; Kimura *et al*., 2004; Sumiyoshi and Kimura, 2006; Moon *et al*., 2007; Neyrinck *et al*., 2009; Yao *et al*., 2010; Omara *et al*., 2012; Do *et al*., 2018; Ali *et al*., 2019; Chiu *et al*., 2020). Thus, it was difficult to evaluate their compositional similarity to Chinova's fiber derived from white button mushrooms and assess the suitability of these studies in the safety evaluation of Chinova's fiber derived from white button mushrooms. Furthermore, it is noted that the majority of these studies were designed to evaluate an efficacious effect of chitosan (*e.g*., amelioration of consumption of a high fat diet or non-alcoholic fatty liver disease, measurement of lipid profiles, serum antioxidant concentration, and biomarkers of lipid peroxidation and inflammation) and were not specifically designed to evaluate the toxicity of chitosan; the identified studies reporting a liver-related finding were not conducted according to an internationally recognized test protocol [*e.g*., Organisation for Economic Co-operation and Development (OECD) Test Guideline 408]. Nevertheless, the findings suggest that chitosan may impact liver function and elicit hepatomodulatory effects. In the 6-month study by NTP (2017), the absolute and relative liver weights of Sprague-Dawley rats were significantly decreased following consumption of 9% chitosan in the diet and a significant reduction in relative liver weight in animals consuming 3% chitosan in the diet (NTP, 2017). The decrease in liver weights was accompanied by decreases in liver fat accumulation and increases in ALT. The fatty change was characterized by hepatocytes with clear vacuoles within the periportal region, and was considered to be a biological adaptive response to fat-soluble vitamin and mineral depletion, and may not be a toxicological effect (NTP, 2017). Diets containing 3% and 9% chitosan provided a daily dose of approximately 450 and 6,000 mg/kg body weight, respectively. The available data suggest a possible liver effect of chitosan exposure at doses of 450 mg/kg body weight/day, which is approximately 11-fold higher than the highest intake of Chinova's fiber from white button mushrooms, based on its proposed food uses and FEMA-approved uses (*i.e.*, 40.2 mg/kg body weight/day, see Table 3.3.2-2). No decreases in serum fat-soluble vitamins (vitamin A, D, E), α-carotene, or β-carotene were reported in mildly hypercholesterolemic male and female subjects consuming 6.75 g/day of chitosan for 8 weeks (Tapola *et al*., 2008) or changes in clinically relevant serum parameters (see Section 6.4 for further details), and therefore a similar hepatotoxic effect is not expected in humans.

In a 35-day oral toxicity study, Omara *et al.* (2012) administered chitosan (test material not further characterized) *via* gavage at doses of 0 (distilled water), 150, or 300 mg/kg body weight/day to Swiss albino mice (7/sex/group). A consistent, dose-dependent increase in hypercellularity and degenerated glomeruli and tubules in the kidney of both sexes at 150 and 300 mg/kg body weight/day was reported. In addition, severe degeneration and hypercellularity of glomeruli and tubules in kidneys of females compared to males were reported in the high-dose group. Serum creatinine and urea were significantly increased in a dose-dependent manner in males and females. Quantitative analysis demonstrated a statistically significant, dose-dependent decrease in glycogen and total protein content (mean percent of grey area) in renal tubules and glomeruli of the kidneys *versus* controls, and this decrease was statistically significantly greater in females compared to males in the low and high chitosan groups. Similar histopathological findings were not reported in NTP (2017), and with the exception of a statistically significant increase in absolute right kidney weight in males of the high-dose group (9%; 450 mg/kg body weight/day), no adverse renal effects were reported. The authors reported increases in urinary creatinine concentration that corresponded with decreases in urine volume, indicating "proper kidney function" (NTP, 2017). Furthermore, it should be noted that the study by Omara *et al.* (2012) was not conducted in accordance with Good Laboratory Practice (GLP) or internationally-accepted standards for toxicity testing of chemicals and the test article was not adequately described by the authors (*i.e.*, molecular weight, DDA, purity), as such, its relevance to Chinova's fiber derived from white button mushrooms could not be determined.

The repeated-dose oral toxicity studies on various chitosan preparations are summarized in Table 6.3.2.1-1.

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Studies in Mice						
LMWC and HMWC Source: NR DDA: 80% Size: MW of 20,000 (LMWC) and 50,000 (HMWC)	Mice (CF_1) F Approximately 12/group	Diet 42 d	Group 1: 0 (control) Group 2: 2% LMWC (3,000) Group 3: 2% HMWC (3,000)	bw, frequency of aberrant crypt foci	• Chitosan groups had lowered bw, but HMWC was not statistically significant. • NSD in mice; HMWC decreased the number of aberrant crypt foci in azoxymethane-treated mice.	Torzsas *et al.* (1996)[c]
Chitosan Source: Crab shell DDA: 80% Size: 3.6 µm in diameter	Mice (BALB/c) M, F	Diet 28 d	Group 1: 0 (control) Group 2: 0.5% (750) Group 3: 5.0% (7,500)	bw, food consumption, fecal bacteria	• After 4 wks of feeding, Group 3 had a statistically significant reduction in bw. • Average food consumption in Week 4 was statistically lower in Group 3 than control group. • Facultative anaerobes and lactobacillus concentrations were statistically lower in Group 3 than control. • Anaerobe colonies were higher in Group 3 than controls. • NSD in *Bifidobacterium* and *Enterobacteriaceae*. NSD between Group 2 and controls.	Tanaka *et al.* (1997)[c]
Chitin-chitosan (80% chitosan) Source: NR DDA: NR Size: NR	Mice (ICR) F 13/group	Diet 63 d	Group 1: 0 (control) Group 2: 3% (4,500) Group 3: 7% (10,500) Group 4: 15% (22,500)	bw, liver weight, serum lipids, cholesterol	• Groups 2, 3, 4 significantly reduced the increase in bw following HFD. • Reduced liver weight in Groups 3, 4 following a HFD. • Serum triacylglycerol significantly reduced in Groups 2, 3, 4.	Han *et al.* (1999)[c]
Chitosan Source: NR DDA: NR Size: NR	Mice (Swiss Webster) M, F 29 to 30/group	Diet 70 d	Group 1: 0 (control) Group 2: 10% (15,000)	bw, small intestine length, liver weight, retinol concentration	• Chitosan group had reduction in weight gain at 10 wks. • Increased small intestine length in chitosan group. • Absolute and relative liver mass increased in chitosan group. • NSD in whole-blood, tissue accumulation, and fecal and urinary excretion during 2-wk retinol exposure period	Kimura *et al.* (2004)[c]

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Water-soluble chitosan Source: NR DDA: NR Size: 46 kDa	Mice (C57Bl/6J) M 4/group	Oral (gavage) 140 d (20 wks)	Group 1: 0 (control) Group 2: 200 Group 3: 600	bw and food consumption, plasma triglycerides, total cholesterol, liver weight and lipids, liver and kidney damage markers	• NSD in weight gain until Week 17: Group 3 had reduced bw gain when fed a HFD. • NSD in plasma triglycerides; Group 3 inhibited the increase of total cholesterol when fed a HFD. • Group 3 had significantly lower liver weight and hepatic triglyceride and total cholesterol. • NSD in glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, and blood nitrogen urea.	Sumiyoshi and Kimura, 2006[c]
Chitosan, high-molecular weight Source: NR DDA: 85.5% Size: 760 kDa Chitosan, middle molecular weight Source: NR DDA: 85.2% Size: 32.7kDa Chitosan, water-soluble Source: NR DDA: 52.6% Size: 39.1 kDa	Mice (Kunming) F 10/group	Diet 90 d	Group 1: 0 (control) Group 2: 1.05% HCS (1,575) Group 3: 1.05% MCS (1,575) Group 4: 1.05% WSC (1,575)	General condition, bw, food intake, absolute and relative organ weights, histopathology, trace iron, trace zinc, trace copper	• NSD in appearance and behavior. • NSD in bw in chitosan groups compared to control. • NSD in food intake. • In Group 4: statistically significant increase in relative thymus weight. • Other groups: NSD in relative heart, liver, spleen, thymus, kidney, and lung weights. • NSD in histopathology in chitosan groups compared to control. • Iron levels in liver, heart, spleen, kidney not different in Groups 2 and 4 when compared to control; iron level in liver and spleen elevated in Group 3. • Zinc levels in liver, heart, spleen, kidney not different in Groups 2 and 4 when compared to control; zinc level in liver, spleen, heart significant elevated in Group 3. • Copper levels in liver, heart, spleen, kidney not different in Groups 2 and 4 when compared to control; copper level in liver, spleen significant elevated in Group 3.	Zeng *et al.* (2008)[c]
Chitosan Source: exoskeleton fungi DDA: NR Size: NR	Mice (C57bl6/J) M 8/group	Diet 10 wks	Group 1: 0 (HFD) Group 2: 5% (7,500; in HFD)	bw gain, feed efficiency, fat mass development, liver weight, epididymal, visceral, and subcutaneous white adipose tissue weight, oral glucose tolerance test, plasma insulin,	• Decreased bw gain compared to non-supplemented HFD; feed efficiency was significantly lower compared to control. • NSD in liver weight; white adipose tissue weight was systematically lower compared to controls. • NSD in glucose tolerance. • NSD in insulin resistance index; decreased serum triglycerides, cholesterol; NSD in serum non-	Neyrinck *et al.* (2009)[c]

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
				glucose, triglycerides, cholesterol, non-esterified fatty acids, and β-hydroxybuterate, lipid analysis in cecal content, liver and muscle	esterified fatty acids. • Fat staining of the tissue demonstrate that lipid accumulation was reduced in liver and muscle compared to controls.	
Chitosan (Sedico Pharmaceutical Co., Cairo) NFS	Mouse (Swiss albino) M, F 7/sex/group	Oral (gavage) 35 d	0 (distilled water), 150, or 300	ALT, AST, ALP, LDH, GPI, HK, PFK in liver homogenate; glycogen and protein levels in liver and kidney homogenate; TC, HDL-C, LDL-C, TG, and total lipid; glucose, creatinine, and urea in serum; histopathology of liver and kidney	<u>Dose-dependent significant effects</u> • ↑ ALT, AST, urea, creatinine (M, F) [150, 300]. • ↑ ALP in F [150, 300]. • ↓ total lipids, TG in M [150, 300]. • ↓ TC, HDL-C, LDL-C (M, F) [150, 300]. • ↓ protein, glycogen in kidney and liver homogenate (M, F) [150, 300]. • ↑ LDH, GPI, HK (M, F) [150, 300]. • ↑ PFK in F [150, 300]. <u>Significant effects</u> • ↑ ALP in M [0 *vs.* 300]. • ↓ total lipids, TG in F [0 *vs.* 150, 300]. • ↓ serum glucose in F [0 *vs.* 300]. • ↑ PFK in M [0 *vs.* 150, 300]. <u>Kidney</u> • Dose-dependent hyper-cellularity and degenerated glomeruli and tubules were consistently observed (M, F) [150, 300]. • Severe degeneration and hyper cellularity of glomeruli and tubules in F *vs.* M [300]. <u>Liver</u> • M: Degeneration, necrosis, and eosinophilic substances in hepatic lobules, vacuolated cytoplasm, and presence of intracellular hemorrhage between hepatocytes [300]. • F: Dilated central veins, destructed red blood cells	Omara *et al.* (2012)[c]

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
					[150]. • F: Cytoplasmic vacuolation in hepatocytes, fatty degeneration, and leukocytic infiltration [300]. • Severe pathological changes (especially the degree of degeneration, necrosis, and mononuclear cell infiltration in portal tracts) in F *vs.* M [300]. <u>Quantitative analysis</u> • Significant, dose dependent ↓ in glycogen and total protein content (mean percent of grey area) in renal tubules and glomeruli of the kidneys and hepatocytes *vs.* control; significantly lower in F *vs.* M [150, 300].	

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
LC chitosan (390 kDa) and SC chitosan (210 kDa)	Mouse (C57BL/6J)[d] Sex NR 10/group	Diet 12 wks	0 or 1% (0 or 1,500)	bw, food consumption, plasma adipokine level (leptin, adiponectin, resistin, PAI-1), serum and hepatic lipid profile (TC, TG, HDL-C, apolipoprotein A-I, apolipoprotein B	• ↓ bw in LC and SC groups compared to HFD control. • NSD food consumption in LC and SC groups compared to HFD control. • ↓ total white adipose tissue, TC in SC group compared to HFD control; NSD in LC group. • NSD in serum leptin, adiponectin in LC and SC groups compared to HFD control. • ↓ serum resistin, PAI-1 levels, TG, free fatty acid, apolipoprotein B in LC and SC groups compared to HFD control. • ↑ leptin, resistin, PAI-1, TG, TC, free fatty acid, HDL-C, apolipoprotein B in HFD control compared to normal diet control. • ↓ adiponectin, apolipoprotein A-I in HFD control compared to normal diet control. • NSD in HDL-C in LC and SC groups compared to HFD control. • ↓ hepatic TG and TC in LC and SC groups compared to HFD; NSD in hepatic free fatty acids. • ↑ hepatic TG, TC, free fatty acids in HFD control compared to normal diet control.	Do *et al.* (2018)
LMWC NFS	Mice (C57BL/6J) M 12/group	Diet 4 wks	0 or 5% (0 or 7,500)	Blood glucose, OGTT, serum leptin, insulin, total cholesterol, triglycerides, LDL-C, HDL-C, epi-WAT cell area	• ↑ bw in high-fat controls *vs.* basal diet controls and chitosan. • ↓ bw, weight gain, and food consumption in high-fat chitosan *vs.* high-fat controls. • ↓ food consumption in low-fat chitosan *vs.* low-fat controls. • ↑ serum leptin levels of high-fat chitosan *vs.* high-fat controls. • ↑ fat/bw ratio and epi-WAT cell area in high-fat controls *vs.* low-fat chitosan and low-fat controls. • ↓ fat/bw ratio and epi-WAT cell area in high-fat chitosan *vs.* high-fat controls. • NSD in blood glucose, OGTT, serum insulin or lipids levels in any group.	Tang *et al.* (2020)

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: NR MW 21.7 x 10^4 Da DDA: NR	Mouse (Kunming)[e] M 10/group	Oral (gavage) 15 d	0, 150, 250 mg/kg/d	bw, colon histopathology	• ↓ bw in treatment groups compared to normal control; attenuation of bw ↓ compared to DSS control. • ↓ colon length in DSS control compared to normal control; attenuation of colon length ↓ in treatment groups compared to DSS control. • Loss of colonic epithelial cells, distortion of crypt structure, and massive inflammatory cell infiltration in DSS control compared to normal control; effects were ameliorated in treatment groups with significant reduction in histology injury caused by DSS.	Wang *et al.* (2019a)
Studies in Rats						
Chitosan Source: NR DDA: NR Size: NR	Rat (Sprague-Dawley) Male 10/group	Diet 58 d	Group 1: 0 (control) Group 2: 1% (1,000) Group 3: 2.5% (2,500) Group 4: 5% (5,000) Group 5: 10% (10,000) Group 6: 15% (15,000)	bw, food intake, hematology, absolute and relative organ weights	• Weight gain reductions occurred in Groups 5 and 6. • Efficiency of food utilization was decreased in Groups 5 and 6. • Hemoglobin and packed cell volume decreased in Groups 5 and 6; total serum protein decreased in Group 6. • Relative liver and kidney weights were reduced in Group 6.	Landes and Bough (1976)[c]
Chitosan Source: crab shell DDA:81 to 99% Size: NR	Rat (Sprague-Dawley) • 6 to 7/group • 6/group	Diet • 22 d • 28 d	Group 1: 0 (control) Group 2: 2% (2,000) Group 3: 5% (5,000)	Food intake, growth, organ weights, serum cholesterol levels, serum and liver lipids	• NSD in bw, food intake. • Relative liver weight was lower in chitosan groups. • Chitosan prevented the rise of serum cholesterol due to feeding cholesterol. • Liver cholesterol concentrations decreased in chitosan groups.	Sugano *et al.* (1988)[c]

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: NR DDA: 94% Size: 250 kDa	Rat (Wistar) M	Diet 21 d	Group 1: 0 (control) Group 2: 2% (2,000) Group 3: 5% (5,000)	bw, food intake, liver weight, fecal weight, serum cholesterol, fecal neutral sterol excretion, fecal bile acid excretion	• NSD in growth, food intake, liver weight, dried fecal weight. • NSD in fecal excretion of neutral sterols and bile acids. • Composition of bile acids and neutral sterols in cecum was statistically different in 5% chitosan group; chitosan expanded the neutral sterol pool and cholesterol, and decreased coprostanol. • Statistically significant decrease in serum cholesterol in 5% chitosan group.	Fukada *et al.* (1991)[c]
Chitosan Source: NR DDA: 90% Size: NR	Rat (Sprague-Dawley) 10/group	Diet 14 d	Group 1: 0 (cellulose control) Group 2: 5% (5,000)	bw, food efficiency, apparent fat digestibility, vitamin and mineral status	• bw gain reduced in chitosan group. • Food efficiency ratio decreased in chitosan group. • Apparent fat digestibility decreased in chitosan group. • Chitosan group had lower Ca, Mg, Fe absorption, and lower bone mineral content. • Liver retinol and retinyl palmitate lower in chitosan groups. • Lower serum and liver vitamin E observed in chitosan group. • Lower serum triglyceride. • higher plasma vitamin K concentration.	Deuchi *et al.* (1995)[c]
Chitosan (high viscosity) Chitosan (low viscosity) Source: shrimp shell DDA: 90% Size: 480 kDa (high viscosity) 340 kDa (low viscosity)	Rat (Sprague-Dawley) 6/group	Diet 28 d	Group 1: 0 (control) Group 2: 5% high viscosity chitosan (5,000) Group 3: 5% low viscosity chitosan (5,000)	Liver weight, plasma lipid, transaminase, lactic acid, frutosamine, *beta*-hydroxybutyric acid, free fatty acid levels, plasma and liver lipid peroxides, liver and fecal lipids, liver glucose-6-phosphate dehydrogenenase	• NSD in bw. • Decreased relative liver weight. • Higher liver lipid peroxide in chitosan (high viscosity) group. • NSD plasma lipid peroxide values. • NSD found in other tissue weights. • Chitosan decreased plasma total cholesterol, VLDL-C, LDL-C, HDL-C. • Decreased liver total lipids, but no significant difference in liver triacylglycerol content.	Chiang *et al.* (2000)[c]

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: NR DDA: NR Size: NR	Rat (Sprague-Dawley) M 8 to 9/group	Diet 18 d	Group 1: 0 (control) Group 2: Week 1: 10% (10,000) Week 2+: 7.5% (7,500)	bw, food intake, liver lipids, fecal fat, cholesterol absorption	• Chitosan group had a slower rate of growth. • Reduced food intake with 10% and 7.5% supplementation. • Lower liver cholesterol contents in chitosan group. • Higher fat excretion. • No changes in intestinal contents supernatant viscosity.	Gallaher *et al.* (2000)[c]
Chitosan, dietary Source: shrimp shells DDA: 85 to 98% Size: 350 kDa	Rat (Long Evans) F 5/group	Diet 56 d	Group 1: 0 Group 2: 2% (2,000)	bw, food consumption, plasma cholesterol, liver lipids, plasma fatty acid profile	• NSD in weight and food consumption. • Plasma total cholesterol decreased by 16%. • NSD in liver lipids. • NSD in plasma palmitic and steric acid levels, increases in oleic, linoleic, and docosapentaenoic acid; decreased arachidonic acid.	Hossain *et al.* (2007)[c]
Chitosan Source: crab shell DDA: NR Size: NR	Rat (Sprague-Dawley) M 8/group	Diet 28 d	Group 1: 0 (control) Group 2: 2% (2,000) Group 3: 5% (5,000)	Food intake, bw gain, plasma lipids, microsomal CYP7A1 activity	• NSD bw gain, food intake, food efficiency ratio. • Chitosan-treated rats had significantly lower plasma TC and LDL-C concentration. • Consumption of chitosan resulted in elevated activity of CYP7A1 by 123% in Group 2, and 165% in Group 3.	Moon *et al.* (2007)[c]
Chitosan Source: shrimp shell DDA: 83% Size: 625 kDa	Rat (Sprague-Dawley) M 7/group	Diet 28 d	Group 1: 0 (control) Group 2: 5% (5,000)	bw, liver weight, liver metabolizing enzymes	• Significantly lower final bw in chitosan group. • Significantly lower absolute and relative liver weight. • Lower levels of CYP 3A, CYP 1A1 in chitosan group, decrease in glutathione S-transferase.	Yao *et al.* (2010)[c]

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: Lobster chitin MW: 309 kDa DDA: 83%	Rat (Wistar) M, F 7/sex/group	Oral (gavage) 28 d	0, 100, 300, or 1,000	Mortality, clinical signs, bw, food consumption, serum biochemistry, hematology, organ weights (liver, kidney, adrenals, testis, epididymides, ovaries, thymus, spleen, heart, and brain), and histopathology of organs	• No signs of toxicity, mortality, or changes in biochemical parameters compared to controls. • Significant ↑ erythrocyte count in F [300, 1,000] and in M [1,000]. • NSD in relative organ weight (%/total bw) in any of the groups. • No treatment-related organ lesions.	Lagarto *et al.* (2015)
High-MW Chitosan Source: NR MW: 310 to 375 kDa DDA: NR	Rat (Sprague-Dawley)[f] M 5/group	Diet 10 wks	0, 400, 800 mg/kg diet	bw, serum biochemistry (lipid, total protein, ALT, AST, ALP, CK, creatinine, urea, calcium, vitamin A and E), lipid peroxidation biomarkers (MDA, LPO, GSH, SOD), organ weight and histology	• ↓ bw gain, food consumption, relative-to-body heart and liver weight, TC, TG, LDL-C, VLDL-C, ALT, AST, ALP, CK, creatinine, urea, calcium, vitamin A, vitamin E, MDA in treatment groups compared to control. • ↑ relative-to-body kidney weight, HDL-C, total protein, albumin, globulin, albumin/globulin ratio, GSH, SOD in treatment groups compared to control. • No histological lesions reported in heart or renal tissues. • Liver steatosis was reported in the HFD control and 400 mg/kg group and not in the 800 mg/kg group.	Ali *et al.* (2019)
Chitosan (MW 2.5 x 10^5 Da, CS1; 3.8 x 10^4, CS2) and Chitosan quaternary ammonium salt (MW 2.4 x 10^5, HACC1; 3.5 x 10^4, HACC2)	Rat (Sprague-Dawley) M 8/group	Oral (gavage) 30 d	0 or 4.5% wt% suspensions (1 mL/100 g)	bw, food consumption, serum and liver lipid profile (TG, TC, LDL-C, HDL-C, lipoprotein lipase), serum free fatty acids, lipid peroxide, SOD	• ↓ bw in CS2, HACC1, HACC2 compared to HFD control; NSD in CS1. • NSD in food consumption. • ↓ serum TG, LDL-C in CS2, HACC1, HACC2 compared to HFD control; NSD in CS1. • ↑ serum TG, TC, LDL-C and ↓ HDL-C in HFD control compared to normal diet control. • ↑ hepatic TG and TC in HFD control compared to normal diet control. • ↓ hepatic TG in CS2, HACC1, HACC2 compared to HFD control; NSD in CS1. • ↓ hepatic TC in CS and HACC2 compared to HFD control; NSD in CS1 and HACC1. • ↑ serum lipoprotein lipase activity in CS1, CS2,	Wang *et al.* (2019b)

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
					HACC1, HACC2 compared to HFD control. • ↑ serum lipoprotein lipase activity HACC1 and HACC2 compared to HFD control; NSD in CS1 and CS2. • ↓ serum free fatty acids, lipid peroxide and ↑ SOD in HACC1 and HACC2 compared to HFD control. • ↓ lipid peroxide in CS1 compared to HFD control; NSD in CS2. • ↑ SOD in CS2 compared to HFD control; NSD in CS1.	
LMWC Source: Crustacean shells MW: 80 kDa DDA: 83.9% HMWC Source: Crustacean shells MW: 740 kDa, DDA: 91%	Rat (Sprague-Dawley) M 6/group	Diet 8 wks	0 or 5% (0 or 2,500)	AST, ALT, serum total cholesterol, HDL-C, LDL-C, VLDL-C, TNF-α, liver and intestinal weight	• ↓ bw in low-MW *vs.* high-fat controls. • NSD in food consumption or intestinal weight in any group. • ↑ liver weight in high-fat controls. • ↓ liver weight in LMWC and HMWC *vs.* high-fat controls • ↑ serum total cholesterol, HDL-C, LDL-C, VLDL-C in high-fat controls; ↓ in same parameters in low-MW, high-MW groups. • NSD in ALT, AST, and TNF-α in low-MW or high-MW chitosan groups.	Chiu *et al.* (2020)
LMWC and HMWC NFS	Rat (Sprague-Dawley) M 6/group	Diet 8 wks	0 (standard diet), 0 (HFD), HFD with 5% HMWC (2,500), or HFD with 5% LMWC (2,500)	bw, indicators of liver function and hypercholesterolemia, liver and intestinal analysis (weight and histopathology)	• NSD in food consumption between groups. • ↓ liver weight in HMWC and LMWC groups. • NSD in intestinal weight between groups. • ↓ total cholesterol, LDL-C, VLDL-C, HDL-cholesterol. • ↓ plasma AST and ALT in HMWC and LMWC groups.	Chiu *et al.* (2020)
Studies in Guinea Pigs						
Chitosan Source: NR DDA: NR Size: NR	Guinea pigs (Hartley) 6/group	Diet 35 d	Group 1: 0 (control) Group 2: 5% (2,000)	bw, food intake, food efficiency ratio, relative organ weight and fat pad, fecal excretion, plasma cholesterol, lipid peroxide and GSH levels	• NSD in bw, food intake, food efficiency ratio compared to controls. • NSD in relative organ weights. • NSD in fat pads except percentage of epididymal fat pad in chitosan group was significantly lower than control. • Chitosan increased fecal weight, fecal fat excretion,	Jun *et al.* (2010)[c]

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
					fecal water excretion, fecal water content. • Total cholesterol, LDL-C, triacylglycerol decreased in chitosan group. • GSH level in liver of chitosan group was higher compared to control.	

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Studies in Pigs						
LMWC Source: NR MW: 20 to 30 kDa DDA: NR	Pig (Duroc x Landrace x Yorkshire) Sex NR 20/group	Diet 28 d	0 or 50 mg/kg/d	bw, food consumption, diarrhea rate, serum CAT, GSH-Px, T-SOD, MDA, T-AOC, intestinal morphology and cytokines	• NSD in bw, diarrhea rate, serum activity of T-AOC, CAT, GSH-Px, T-SOD, MDA. • ↑ food consumption. • NSD in villus height, crypt depth, or ratio of villus height and crypt depth. • ↓ expression of intestinal IL-1β and TNF-α in jejunal mucosa. • NSD in expression of intestinal IL-10 or TGF-β.	Hu *et al.* (2018)
Chitosan Source: NR MW: 232 kDa DDA: NR	Pig (Duroc x Yorkshire x Landrace) M, F 12/group	Diet 14 d	0, 500 mg/kg	bw, food consumption, diarrhea rate, serum cytokines (IL-1, IL-2, IL-6, TNF-α), IgA, IgG, IgM, ACTH, cortisol	• ↑ growth performance (bw, daily weight gain, feed conversion ratio). • NSD daily food consumption, IL-1, IL-6, TNF-α, IgM, IgA, ACTH. • Improvement in fecal score. • ↑ IL-2 and IgG. • ↓ cortisol.	Xu *et al.* (2018)
LMWC Source: NR MW: 20 to 30 kDa DDA: >85%	Pig (Duroc x Landrace x Yorkshire)[i] Sex NR 8/group	Diet 2 wks	0, 100 mg/kg	bw, food consumption, intestinal cytokines, serum D-lactic acid, LPS, DAO, ALP, cortisol	• NSD in growth performance (average daily gain, feed intake, gain to feed ratio). • ↑ serum D-lactic acid, LPS, DAO in ETEC control compared to non-ETEC control; effects were reversed in treatment group. • NSD serum ALP activity and cortisol concentration. • Attenuation of jejunal and ileal occludin protein abundance caused by ETEC infection. • NSD duodenal, jejunal, and ileal IL-1, IL-10, IFN-γ in all groups. • ↑ jejunal and ileal IL-6, TNF-α in ETEC control compared to non-ETEC control; NSD in treatment group compared to non-ETEC control. • ↓ jejunal and ileal TGF-β in ETEC control compared to non-ETEC control; NSD in treatment group compared to ETEC and non-ETEC control.	Wan *et al.* (2019)
LMWC NFS	Pigs (Duroc x Landrace x Yorkshire) 15 d	Diet ETEC challenge at	0, 50, or 100 mg/kg	Average daily gain, average daily feed intake, gain-to-feed	• NSD in average daily gain, average daily feed intake, gain-to-feed ratio on Days 1 to 11 in any group. • ↑ average daily gain [100].	Zhang *et al.* (2020)

Table 6.3.2.1-1 Summary of Repeated-Dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
	Sex NR 8/group		Day 11	ratio, serum IL-1, IL-6, IL-10, TNF-α, IgA, IgG, and IgM, and intestinal morphology	• ↑ gain-to-feed ratio [50, 100]. • ↓ serum TNF-α, IgG, and IgM [50, 100]. • NSD in IL-1, IL-6, IL-10, or IgA [50, 100]. • ↑ villus height and villus height-to-crypt ratio in jejunum and ileum [50, 100]. • NSD in duodenal morphology.	

↓ = decrease; ↑ = increase; ACTH = adrenocorticotropic hormone; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate transaminase; bw = body weight; Ca = calcium; CAT = catalase; CK = creatine kinase; CYP = cytochrome P450; d = day(s); DAO = diamine oxidase; DDA = degree of deacetylation; DSS = dextran sodium sulphate; ETEC = enterotoxigenic *Escherichia coli*; F = females; Fe = iron; GPI = glucose phosphate isomerase; GSH = glutathione; GSH-Px = glutathione peroxidase; HCS = high molecular weight chitosan with molecular weight of 7.60×10^5 and DDA of 85.5%; HDL-C = high-density lipoprotein cholesterol; HFD = high-fat diet; HK = hexokinase; HMWC = high molecular weight chitosan; IFN = interferon; Ig = immunoglobulin; IL = interleukin; kDa = kilodaltons; LC = long-chain; LDH = lactate dehydrogenase; LDL-C = low-density lipoprotein cholesterol; LMWC = low molecular weight chitosan; LPO = lactoperoxidase; LPS = lipopolysaccharides; M = males; MCS = middle molecular weight chitosan with molecular weight of 3.27×10^4 and DDA of 85.2% ; MDA = malondialdehyde; Mg = magnesium; MW = molecular weight; NFS = not further specified; NR = not reported; NSD = no statistical difference; OGTT = oral glucose tolerance test; PAI-1 = plasminogen activator inhibitor-1; PFK = phosphofructokinase; SC = short-chain; SOD = superoxide dismutase; T-AOC = total antioxidant capacity; T-SOD = total superoxide dismutase; TC = total cholesterol; TG = triglycerides; TGF-β = transforming growth factor beta; TNF-α = tumor necrosis factor-alpha; VLDL-C = very-low-density lipoprotein cholesterol; wk(s) = week(s); WSC = water-soluble chitosan with molecular weight of 3.91×10^4 and DDA of 52.6%;

[a] Doses were estimated using default values of U.S. FDA (1993) unless reported otherwise by the study authors.

[b] The reported findings are statistically significant compared to the control unless otherwise stated.

[c] The details on test substance, assay, test system, concentration/dose, and results are presented as reviewed in GRN 397 (U.S. FDA, 2011).

[d] Animals were provided a HFD.

[e] Animals were administered 3% dextran sulfate sodium to induce ulcerative colitis.

[f] Animals were provided a HFD in addition to supplementation of 10 g/kg diet calcium, 11 mg/kg diet vitamin A, and 350 mg/kg diet vitamin E.

[g] Animals were infected with ETEC.

6.3.2.2 Studies on Chitosan Oligomers/Oligosaccharides

Several repeated-dose studies were identified on chitosan oligomers/oligosaccharides (Table 6.3.2.2-1). Consistent with the studies on chitosan, studies on chitosan oligomers/oligosaccharides also reported statistically significant changes in liver weights and liver enzyme activities (Kim *et al*., 2001; Qin *et al*., 2006; Sumiyoshi and Kimura, 2006; Yao *et al*., 2012; Teodoro *et al*., 2016; Lan *et al*., 2019; Qian *et al*., 2019; Chiu *et al*., 2020). As previously discussed, chitosan oligosaccharides typically have an average molecular weight less than 1 kDa and a DDA of 100%, and are not chemically representative of Chinova's fiber extracted from white button mushrooms (*A. bisporus*). These compounds are readily bioavailable and would be absorbed into the systemic circulation which would not occur with Chinova's fiber.

Table 6.3.2.2-1 Summary of Repeated-Dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Studies in Mice						
Chito-oligomer Source: NR DDA: 85.7% Size: 0.99 kDa	Mice (Kunming) F 10/group	Diet 90 d	0 (control), 1.05% (1,575)	General condition, bw, food intake, absolute and relative organ weights, histopathology, trace iron, trace zinc, trace copper	• NSD in appearance and behavior. • NSD in bw in chitosan groups compared to control. • NSD in food intake. • NSD in relative heart, liver, spleen, thymus, kidney, and lung weights. • NSD in histopathology in chitosan groups compared to control. • Iron levels in liver, heart, spleen, kidney not different compared to control. • Zinc levels in liver, heart, spleen, kidney not different compared to control. • Copper levels in liver, heart, spleen, kidney not different compared to control.	Zeng *et al.* (2008)[c]
CO NFS	Mouse (C57BL/6J)[d] M 5/group	Oral (drinking water) 5 months	0 or 1 mg/mL (200)	bw, fasting glucose, liver parameters	• ↓ bw and fasting glucose compared to HFD control. • Treatment alleviated glucose intolerance due to HFD. • ↓ mRNA expression of IL-6, MCP-1 TNF-α, and glucolipid metabolism regulators (SCD-1, ACC1, PCK1-α), and translation of PPARγ in liver tissue.	Bai *et al.* (2018)
CO NFS	Mouse (C57BL/6J)[d] M 10/group	Oral (drinking water) 10 wks	0, 4% (0, 10,000)	bw, food consumption, plasma and liver AST, ALT, ALP, TG, glucose tolerance test, lipid profile	• NSD in bw, food consumption, glucose tolerance test, relative liver weight, adipose fat weight, brown adipose fat, white adipose fat. • ↓ plasma ALT, AST, ALP, absolute liver weight. • Amelioration of hepatic steatosis.	Qian *et al.* (2019)
CO Source: NR DDA: NR MW: 5 kDa	Mouse (C57BL/6) M 8/group	Oral (gavage) 7 wks	0, 200, 400 mg/kg bw/d	Serum TC, TG, HDL-C, LDL-C, AST, ALT, liver TC, TG, MPO, T-AOC, GSH-Px, SOD, MDA, CAT, liver weight and histology	• NSD serum TG in all groups. • ↑ serum TC, HDL-C, LDL-C, LDL/HDL ratio, AST, ALT in HFD group compared to normal diet group. • ↑ serum TC, HDL-C, LDL-C in CO groups compared to normal diet control. • ↓ serum TC in 400 CO group compared to HFD	Tao *et al.* (2019)

Table 6.3.2.2-1 Summary of Repeated-Dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
					control. • ↓ serum LDL-C, LDL/HDL ratio, AST, ALT in CO groups compared to HFD control. • NSD in serum AST or ALT in CO groups compared to normal diet control. • Amelioration of hepatic steatosis. • ↓ liver IL-1β, IL-6, MPO in CO groups compared to HFD control. • ↓ liver TNF-α in 400 CO group and NSD in 200 CO group compared to HFD control. • ↑ liver TNF-α, IL-1β, IL-6, MPO in HFD control compared to normal diet control. • NSD T-AOC, CAT in all CO groups compared to HFD control or normal diet control. • ↓ GSH-Px and ↑ NDA and NO in HFD control compared to normal diet control. • ↑ GSH-Px in CO groups compared to HFD control. • ↑ SOD in 400 CO group and NSD in 200 CO group compared to HFD control. • ↓ MDA and NO in CO groups compared to HFD control.	

Table 6.3.2.2-1 Summary of Repeated-Dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Studies in Rats						
CO Source: NR DDA: NR Size: <1 kDa	Rat (Sprague-Dawley) 9/sex/group	Oral (gavage) 28 d	Group 1: 0 (control) Group 2: 500 Group 3: 1,000 Group 4: 2,000	Clinical signs, bw, hematological and biochemical parameters, histopathological examinations	• NSD in behavior or external appearance. • Normal bw, food consumption. • Normal urinalysis, hematology, blood chemistry, relative organ weights. • Normal histopathological findings. • NOAEL >2,000 mg/kg bw/d.	Kim *et al.* (2001)[c]
Chito-oligosaccharides NFS	Rat (Sprague-Dawley) F, ovariectomized 8/group	Diet 42 d	Group 1: 0 (control) Group 2: 2% (2,000)	bw, food consumption, urinary and fecal calcium, serum calcium, bone mineral density	• NSD in weight gain, food intake, total calcium intake. • Rate of calcium loss into feces significantly lower in ovariectomized rats in CO group (retain calcium better). • NSD in serum calcium in treatment group. • CO increased the bone marrow density in distal region of femur.	Jung *et al.* (2006)[c]
Chitosan oligomer Source: shrimp DDA: NR Size: 1.86 kDa	Rat (Sprague-Dawley) 10/sex/group	Diet 30 d	Group 1: 0% (control) Group 2: 0.75% (750) Group 3: 1.5% (1,500) Group 4: 3.0% (3,000)	Daily food intake, weekly bw, hematology test, clinical chemistry tests, organ weights, histopathological examination	• NSD food intake, feces, hair, behavior, bw. • NSD in absolute or relative bw. • NSD in hematology and clinical chemistry parameters.	Qin *et al.* (2006)[c]

Table 6.3.2.2-1 Summary of Repeated-Dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan oligosaccharides Source: shrimp DDA: 95% MW: NR	Rat (Sprague-Dawley) M 8/group	Diet 5 wks	0, 1, or 3% (0, 500, or 1,500)	bw, liver and kidney weight, AST, ALT, creatinine, blood urine nitrogen, Phase I and Phase II enzyme activities of the liver and kidneys	• NSD in bw, liver and kidney weight, AST, ALT, creatinine, blood urine nitrogen. • Some statistically significant, but not dose dependent effects on metabolizing enzymes and glutathione of the liver and kidneys. <u>Liver</u> • ↓ CYP450, CYP3A, CYP2C, CYP4A *vs.* controls [1, 3%] • ↑ NADPH: quinone oxidoreductase 1 *vs.* controls [1, 3%] <u>Kidney</u> • ↑ CYP2C [3% *vs.* control]	Yao *et al.* (2012)
Chitosan oligosaccharides NFS	Healthy rats (Wistar Han) M Diabetic rats (Goto Kakizaki) M	Oral (drinking water) 6 wks	0 or 0.5% (0 or 500)	bw, ALP, ALT, AST, GGT, glucose, cholesterol, triglycerides, bilirubin, liver weight, hepatic and skeletal muscle mitochondrial toxicity (altered activities of complexes)	• ↓ bw in healthy-chitosan, diabetic controls, and diabetic-chitosan *vs.* healthy-controls. • ↓ cholesterol in healthy-chitosan *vs.* healthy-controls; in healthy-chitosan *vs.* diabetic-chitosan; and in healthy-chitosan *vs.* diabetic-controls. • ↑ cholesterol in diabetic controls *vs.* healthy-controls. • ↑ glucose in diabetic-controls *vs.* healthy-controls; in diabetic-chitosan *vs.* healthy-controls; and in diabetic-chitosan *vs.* healthy-chitosan. • ↑ AST in diabetic-chitosan *vs.* diabetic-controls; and in diabetic-chitosan *vs.* healthy-controls. • NSD in ALP, ALT, or liver weight in any group	Teodoro *et al.* (2016)

Table 6.3.2.2-1 Summary of Repeated-Dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
CO NFS	Rat (Sprague-Dawley) M 10/group	Oral (gavage) 8 wks	0 (HFD), 150, 300, 600 mg/kg/d	bw, food consumption, serum lipid (TC, TG, HDL-C, LDL-C) AST, ALT, fat pad, fat-body ratio, visceral index, liver histology	• NSD bw or food consumption. • ↓ TC, TG, LDL-C, leptin and ↑ HDL-C in all CO groups compared to HFD control. • NSD TC, TG, LDL-C, HDL-C, leptin in all CO groups compared to normal diet control. • ↓ liver weight, index, TC, TG, AST, ALT in 300 and 600 CO group compared to HFD control. • NSD in liver weight, index, TC, TG, AST, ALT in 150 CO group compared to HFD control. • NSD in liver weight, index, TC, TG, AST, ALT in CO groups compared to normal control. • Amelioration of hepatic steatosis, epididymal and perirenal white adipose tissue weight in all CO groups compared to HFD control.	Pan *et al.* (2018)
CO Source: NR MW: 2.3 x 10^3 Da DDA: 91%	Rat (Sprague-Dawley)[e] M 9/group	Oral (gavage) 4 wks	0, 200 mg/kg	Blood parameters (albumin, BUN, CR, LDH, LA, TC, TG, HDL-C, LDL-C)	• ↓ BUN, TC, LDL-C in treatment groups compared to sedentary and exercise controls. • ↑ RBC, hematocrit, MCV in treatment groups compared to sedentary and exercise controls.	Xiong *et al.* (2018)
CO NFS	Rat (Wistar)[f] M 10/group	Oral (gavage) 3 d	0, 500 mg/kg	Markers of lung damage (protein, LDH activity) and inflammation (IL-1β, IL-8, TNF-α)	• Treatment inhibitor PM2.5-induced lung damage and lung inflammatory response (IL-1β, IL-8, TNF-α).	Zhao *et al.* (2018)
CO (95% purity; >95% DDA; average MW <32 kDa)	Rat (Sprague-Dawley)[g] M 10/group	Diet 7 d	0 or 200 mg/kg/d	bw, food and water consumption, organ weight (liver, kidney, spleen), inflammatory and antioxidant parameters (MDA, SOD, CAT, GSH-Px, GSH, T-AOC, IL-1β, IL-6, IL-10, TNF-α)	• NSD in bw, food consumption, liver weight between groups. • ↑ spleen and kidney weight in CO group compared to heat-stress control group; NSD compared to normal control group. • ↓ spleen and kidney weight in heat-stress control group compared to normal control group. • NSD in liver MDA, SOD, CAT, GSH, or T-AOC, or IL-6 and TNF-α in CO group compared to heat-stress control group.	Lan *et al.* (2019)

Table 6.3.2.2-1 Summary of Repeated-Dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
					• ↑ liver IL-1β in CO group compared to heat-stress control group. • ↑ liver MDA, IL-1β and ↓ CAT, GSH-Px, T-AOC, IL-10 in heat-stress control group compared to normal control group. • NSD in spleen MDA, SOD, CAT, T-AOC, or IL-1β, IL-6, TNF-α in CO group compared to heat-stress control group. • NSD in spleen IL-1β between groups. • ↑ spleen MDA, IL-6, TNF-α and ↓ SOD, GSH-Px, GSH, IL-10 in heat-stress control group compared to normal control group. • ↓ spleen IL-10 in heat-stress control group compared to normal control group. • NSD kidney MDA, SOD, CAT, GSH, T-AOC, IL-1β, IL-6, IL-10, TNF-α in CO group compared to heat-stress control group. • ↑ kidney MDA, IL-6, TNF-α and ↓ SOD, GSH-Px, T-AOC, IL-10 in heat-stress control group compared to normal control group.	
Chito-oligosaccharides (CO) Source: Crustacean shells MW: 719 Da DDA: 100%	Rat (Sprague-Dawley) M 6/group	Diet 8 wks	0 or 5% (0 or 2,500)	AST, ALT, serum total cholesterol, HDL-C, LDL-C, VLDL-C, TNF-α, liver and intestinal weight	• ↓ bw in CO *vs.* high-fat controls. • NSD in food consumption or intestinal weight in any group. • ↑ liver weight in high-fat controls. • NSD in liver weight CO group *vs.* high-fat controls. • ↑ serum total cholesterol, HDL-C, LDL-C, VLDL-C in high-fat controls; ↓ in same parameters in CO group. • ↑ ALT, AST, and TNF-α in CO group.	Chiu *et al.* (2020)
CO NFS	Rat (Sprague-Dawley) M 6/group	Diet 8 wks	0 (standard diet), 0 (HFD), HFD with 5% CO (2,500)	bw, indicators of liver function and hypercholesterolemia, liver and intestinal analysis (weight and histopathology)	• NSD in food consumption between groups. • NSD in liver weight in CO group. • NSD in intestinal weight between groups • ↓ total cholesterol, LDL-C, VLDL-C, HDL-cholesterol. • ↑ plasma AST, ALT, TNF-α in CO group.	Chiu *et al.* (2020)

Table 6.3.2.2-1 Summary of Repeated-Dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Studies in Pigs						
Chitosan Source: NR MW: ≤1,000 Da DDA: NR	Pig (Yorkshire) F 12/group	Diet 108 d	0, 100 mg/kg	bw, serum cytokines (IL-1, IL-6, TNF-α), immunoglobulins (IgA, IgG, IgM), antioxidants (MDA, SOD, CAT, GSH-Px, T-AOC)	• ↑ average daily bw gain, average piglet weaning weight. • NSD in SOD, GSH-Px, IL-1, IL-6, TNF-α on Lactation Day 1. • ↑ CAT, T-AOC, IL-10, IgA, IgG, IgM and ↓ MDA on Lactation Day 1. • NSD in SOD, CAT, GSH-Px, MDA, IL-1, IL-6, IL-10, TNF-α, IgA, IgG, IgM on Lactation Day 21. • ↑ T-AOC on Lactation Day 21.	Wan *et al.* (2018)

ACC1 = acetyl-CoA carboxylase; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate transaminase; BUN = blood urea nitrogen; bw = body weight; CAT = catalase; CO = chitosan oligosaccharides; CR = creatinine; CYP = cytochrome P450; d = day(s); DDA = degree of deacetylation; F = females; GGT = gamma-glutamyl transferase; GSH = glutathione; GSH-Px = glutathione peroxidase; HDL-C = high-density lipoprotein cholesterol; HFD = high-fat diet; Ig = immunoglobulin; IL = interleukin; kDa = kilodaltons; LA = lactic acid; LDH = lactate dehydrogenase; LDL-C = low-density lipoprotein cholesterol; M = males; MCP-1 = monocyte chemoattractant protein 1; MCV = mean corpuscular volume; MDA = malondialdehyde; MPO = myeloperoxidase; mRNA = messenger ribonucleic acid; MW = molecular weight; NADPH = nicotinamide adenine dinucleotide phosphate; NFS = not further specified; NO = nitric oxide; NOAEL = no-observed-adverse-effect level; NR = not reported; NSD = no statistical difference; PCK1-α = phosphoenolpyruvate carboxykinase 1; PPARγ = peroxisome proliferator-activated receptor; RBC = red blood cells; SCD = stearoyl-CoA desaturase; SOD = superoxide dismutase; T-AOC = total antioxidant capacity; TC = total cholesterol; TG = triglycerides; TNF-α = tumor necrosis factor-alpha; VLDL-C = very-low-density lipoprotein cholesterol; wk(s) = week(s).

[a] Doses were estimated using default values of U.S. FDA (1993) unless otherwise reported by the study authors.

[b] The reported findings are statistically significant compared to the control unless otherwise stated.

[c] The details on test substance, assay, test system, concentration/dose, and results are presented as reviewed in GRN 397 (U.S. FDA, 2011).

[d] Animals were provided a high-fat diet.

[e] Animals were exercised (swimming) for 30 minutes in Week 1, 1 hour in Week 2, and 2 hours in Weeks 3 and 4. Exercise was performed 6 times per week.

[f] Animals were exposed to 1.2 mg/kg PM2.5 by intratracheal injection approximately 2 hours before administration of chitosan oligosaccharides.

[g] Animals were heat-stressed by exposure to cyclical heat stress conditions (35°C from 08:00 to 12:00 and 24° from 12:00 or 08:00).

6.3.3 Chronic Toxicity

The NTP conducted a 6-month feeding study to investigate the safety of chitosan[3] in Sprague-Dawley rats (NTP, 2017). Male and female Sprague-Dawley rats (10 animals/sex/group/dose[4]) were fed *ad libitum* feed containing 0, 1, 3, or 9% chitosan (approximately 450, 1,500, or 5,200 mg/kg body weight/day in males and 650, 1,800, or 6,000 mg/kg body weight/day in females). The test material had an average purity of 94%, and was mixed with a rat feed with 4% fat content[5]. The test material had an average percent deacetylation of 86.5% and an average molecular weight of 81.6 kDa (ranged from 62,755 to 87,343 Da; considered LMWC). The study was conducted according to U.S. FDA GLP.

The following endpoints were measured over the course of the study: feed consumption (recorded weekly), body weights, serum vitamin A, D, E, and K_1, levels (at Weeks 7, 13, 19, and 26), hepatic vitamin E and K levels (at Week 26), bone histomorphometry, bone calcium, ash, and moisture, clinical chemistry (Week 7 and/or Weeks 13, 19, and 25 with a single measurement for ALT and sorbitol dehydrogenase taken at Week 25), and hematology (at Week 25), along with a sperm morphology and vaginal cytology examination, urinalysis (at all 4 time points), feed and fecal analysis, and gross histopathology of major organs (liver, pancreas, stomach, forestomach, heart, blood vessel, adrenal cortex, parathyroid, pituitary, and thyroid glands, prostate, testes, preputial, mammary, and clitoral glands, brain, lymph node, spleen, thymus, skin, skeletal muscle, lung, nose, eye, Harderian gland, kidney, and urinary bladder).

Three male rats (1 in the control group and 2 in the 9% group) and 2 female rats (1 in the 1% group and 1 in the 3% group) died before the end of the study (cause of death was indeterminant). Body weight of animals remained comparable across all dosed groups at the end of the study compared to controls and there were no clinical signs reported in the 9% group compared to the controls at all time points. Statistically significant decreases of toxicity were sporadically reported. Statistically significantly decreased serum levels of cholesterol (26 to 48%) were reported for triglyceride serum levels in the 9% male (47 to 57%) and female (30%) rats. Serum phosphorus levels were significantly decreased in the 9% male rats (12 to 18%) and in the 3% males (14%). Similarly, phosphorus levels were significantly decreased in the 3% and 9% females (9 to 20%). ALT was slightly but statistically significantly elevated at Week 25 in the 9% male rats (104%) and in the 3% and 9% female rats (28% and 88%, respectively). However, sorbitol dehydrogenase (another marker of hepatocellular injury) was not significantly increased relative to the controls, and hepatocellular changes associated with increases in ALT were not reported microscopically. The authors reported that the toxicologic significance of the ALT increases was uncertain. A slight, but statistically significant increase in urea nitrogen was reported in the 9% males (23%) and females (15%) at Week 25 (only timepoint measured).

Mild but statistically significant increases (4 to 6%) in automated hematocrit, hemoglobin concentration, mean cell volume, and mean cell hemoglobin reported in 9% males compared to controls. These changes were considered by investigators to be due to biological variability and were likely not toxicologically relevant (NTP, 2017). All other differences from control values in hematology data were mild or sporadic and not considered toxicologically significant.

[3] The chitosan test article was analytically demonstrated to be absent of organochlorine and organophosphorus pesticides, nitrosamines, aflatoxins.

[4] Animals were split into 3 groups (A, B, and C) and different parameters were measured in each group (10 animals/sex/group/dose level): Group A (feed consumption, body weight, clinical findings, gross lesions/histopathology, bone analysis, and sperm morphology and vaginal cytology examinations), Group B (vitamin A, E, D and bone analysis) and C (fat digestion, hematology, clinical chemistry, urinalysis, and fecal analysis).

[5] It was noted in the study report that the rat feed AIN-93M was used instead of the typical feed (NTP-2000), as the latter feed typically has double the amount of fat soluble vitamins and double the fat content compared to AIN-93M.

Statistically significant, dose-dependent decreases (15 to 29%) were reported in serum vitamin A concentrations starting at Week 13 in males of the 3% and 9% group. Females were less affected with significant decreases (18 to 21%) observed in the 9% group. Significant, concentration-dependent decreases (17 to 82%) were also reported in serum vitamin E concentrations in male rats at all doses and all time points. Females were less affected with significant decreases (~60%) in serum vitamin E levels reported in the 9% group only at all time points. Hepatic vitamin E concentrations of exposed rats were significantly lower than those in control rats, which were significantly reduced (48 to 87%) in 3% males and the 9% group.

Serum concentrations of vitamin D were statistically significantly increased in 9% males (105 to 142%) and females (100 to 180%) at Weeks 7, 19, and 26 compared to the control groups. Calcium absorption was significantly increased (55 to 154%) in 9% females at Weeks 19 and 25. However, serum levels of calcium were mildly but statistically decreased (4%) in 9% males at Weeks 19 and 25. Total osteocalcin and parathyroid hormone levels were occasionally elevated (38% and 56 to 96%, respectively) in the 9% group throughout the study. Bone moisture was significantly increased by 7% in 9% females compared to controls. Results for vitamin K were not presented as many samples were below the level of detection.

At the completion of the study, urine volume was significantly decreased in males (all doses) and females of the 9% group. Increases in urine creatinine concentration paralleled the decreases in urine volume suggestive of proper kidney function.

No changes in testis or epididymis weights or sperm parameters were reported. The absolute and relative liver and thymus weights were significantly lower than controls in the 9% dosed animals (both sexes) and 3% dosed males (thymus only). The relative liver weights of 3% males were also significantly lower than controls.

Exposure to chitosan was reported to elicit various digestive effects, including decreases in percent fat digested and increases in fecal weight and moisture. Compared to the control groups, percent fat digested was statistically significantly decreased from 8 to 33% in all treated animals. A statistically significant decrease in the incidence of hepatic periportal fatty change in females of the 9% group was reported compared to the control group, while non-significant reductions in number of incidences were also seen in 1% and 3% females. Fatty change was characterized by hepatocytes with large, well-defined, clear vacuoles (lipid) within the cell, displacing the nuclei and cytoplasm to the cell periphery. Fecal weight was significantly increased up to 170% in the 3% and 9% group and up to 29% in 1% females. Fecal moisture was statistically significantly increased in both males and females in the 9% group compared to controls.

Based on a review of the data, the only statistically significant effects reported in the 1% chitosan dosed animals at the completion of the study were: decreased serum vitamin E levels at Week 13 (males only); decreased urine volume at Weeks 13, 19, and 25 (males only); decreased fat digested at Weeks 24 to 25 (males and females); decreased deoxypyridinoline/creatinine levels at Weeks 13 and 19 (females only); and increased fecal weight at Weeks 12 to 13, 18 to 19, and 24 to 25 (females only). None of the other parameters evaluated at the 1% dose level reached statistical significance. These effects were likely a consequence of increased intakes of a fiber-like substance, impacting fat and water absorption/digestion and not a direct toxic effect of chitosan. As well, these effects were not consistently reported in both sexes, with the exception of decreased vitamin E levels and fat digestion. These findings were considered indirect consequences of the recognized fat binding properties of chitosan[6] resulting in excretion of dietary fat and reduced absorption of fat-soluble vitamins, and as such were not direct toxic effects of chitosan on organ systems. It was noted that the study was conducted using AIN-93M diet instead of the NTP-2000 diet because of the high levels of fat-soluble vitamins and higher total fat content found in the NTP-2000 diet. The NTP-2000 feed contains almost double the amount of required fat-soluble vitamins and has a higher fat content (7% to 8%) than the AIN-93M feed (4%); therefore, the study would have been particularly sensitive to effects on fat soluble vitamin absorption (NTP, 2017). The effects on fat-soluble vitamins were considered relevant to the safety of Chinova's fiber derived from white button mushrooms. However, the sensitive nature of the study design and the differences in the dietary requirements and in the metabolism of fats between rodents and humans suggest that small changes in the absorption of nutrients reported in the study may not necessarily be of nutritional significance to humans consuming Chinova's chitosan. The generalized effects of resistant dietary fibers on nutrient absorption have been long known, are well characterized, and are not considered of nutritional relevance at levels that are commonly consumed in the diet (Dahl and Stewart, 2015). Similar effects on fat-soluble vitamins were not reported in mildly hypercholesterolemic male and female subjects consuming 6.75 g/day of chitosan for 8 weeks (Tapola *et al*., 2008) or in overweight subjects consuming β-chitosan (MW not reported; 75.5% DDA) or "rapidly-soluble chitosan" (MW = >100 kDa; >78% DDA) at doses of 3 g/day for up to 24 weeks (Schiller *et al*., 2001; Mhurchu *et al*., 2004).

The authors of NTP study concluded that dietary exposure to chitosan for 6 months resulted in decreased fat digestion and depletion of some fat-soluble vitamins in male and female rats. Based on the above results, "*The lowest observed effect level (LOEL) for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats*" (NTP, 2017). On a body weight-basis, the 1% dose is equivalent to a human consuming approximately 31.5 g of chitosan per day (for a 70-kg individual).

Chronic toxicity studies on chitosan are summarized in Table 6.3.3-1 below.

[6] Chitosan is marketed as a dietary supplement for weight loss, and the USP monograph for chitosan includes fat binding capacity as a qualitative specification parameter for the ingredient.

Table 6.3.3-1 Summary of Chronic Oral Toxicity Studies of Chitosan

Test Substance	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Dose in mg/kg bw/d (concentration)[a]	Parameters Evaluated	LO(A)EL[b]	Significant Findings[c,d]	Reference
Chitosan Source: prawn shells DDA: 78% Size: NR	Mice (transgenic homozygous apo E-deficient), mixed gender 10 /control 13/experimental	Diet 182 days (26 weeks)	Group 1: 0 Group 2: 5% (7,500)	bw, general condition, select organ weights, food consumption	N/A	• Chitosan-fed mice had significantly higher body weight on Day 126 and 154 of study (improved growth). • NSD in general condition. • NSD in liver, epididymal, uterine horn fat pad weights. • Food intake of all chitosan mice was marginally more than that of controls.	Ormrod *et al.* (1998)[e]
Low molecular weight chitosan powder Average MW: 82 kDa DDA: 86.5% Purity: 94%	Rat (Sprague-Dawley) M, F 10/sex/group	Oral (diet) 25 to 26 weeks	M: 0, 450, 1,500, or 5,200 F: 0, 650, 1,800, or 6,000 (0, 1, 3, 9%)	Feed consumption, bw, vitamin A, D, K_1, and E levels in serum and/or liver, bone histomorphometry, clinical chemistry, hematology, sperm morphology, vaginal cytology examination, urinalysis, feed and fecal analysis, and gross histopathology of major organs	1%	• No significant effect on body weights in any dosed group *vs.* control. • 3 M, 2 F died before study end (cause of death unknown). • ↑ (4 to 6%) in automated hematocrit, hemoglobin concentration, mean cell volume, and mean cell hemoglobin in M [9%]. • ↓ in cholesterol (26 to 48%) in both sexes [9%]. • NSD in triglycerides at end of study. • ALT ↑ 104% in M and ↑ 88% in F at Week 25 [9%]; ALT ↑ 28% in F [3%]. • No changes in testis or epididymis weights or sperm parameters. • Absolute and relative liver and thymus weights ↓ in both sexes [9%] and in M [3%, thymus]. • ↓ incidence of hepatic periportal fatty change in F [9%]. • Dose-dependent ↓ (15 to 29%) in serum vitamin A in M [3%, 9%] and ↓(18 to 21%) in F [9%]. • ↓ (17 to 82%) in serum vitamin E in M [3, 9%] and ↓ (~60%) in F [9%]. • Hepatic vitamin E levels ↓ (48 to 87%) in M [3%, 9%] and F [9%]. • Serum vitamin D ↑ (142%) in M and (180%) in F [9%]. • Calcium absorption ↑(154%) in F [9%]. • Serum calcium ↓ (4%) in M [9%]. • Percent fat digested ↓ (8 to 33%) in all dosed	NTP (2017)

Table 6.3.3-1 Summary of Chronic Oral Toxicity Studies of Chitosan

Test Substance	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Dose in mg/kg bw/d (concentration)[a]	Parameters Evaluated	LO(A)EL[b]	Significant Findings[c,d]	Reference
						groups [1%, 3%, 9%]. • ↑ Fecal weight in M [3%, 9%] and F [1%, 3%, 9%]. • Fecal moisture ↑ 4 to 10% in both sexes [9%].	

ALT = alanine aminotransferase; bw = body weight; d = day(s); DDA = degree of deacetylation; F = females; LO(A)EL = lowest-observed-(adverse)-effect level; M = males; MW = molecular weight; NR = not reported; NSD = no statistical difference.

[a] Doses were estimated using default values of U.S. FDA (1993) unless otherwise reported by the study authors.

[b] The effect level is designated in parenthesis as either being "reported" (the publication had defined an effect level for the study) or "assumed" (in the event that an effect level was not reported and was estimated based on the available information).

[c] The reported findings are statistically significant compared to the control unless otherwise stated.

[d] Information in [ ] corresponds to the dose in which the reported effects were observed.

[e] The details on test substance, assay, test system, concentration/dose, and results are presented as reviewed in GRN 397 (U.S. FDA, 2011).

6.3.4 Developmental and Reproductive Toxicity Studies

Three studies evaluating the developmental and reproductive effects of water-soluble chitosan and chitosan oligosaccharides were identified in the scientific literature and previously discussed in GRN 397 (Choi *et al.*, 2002; Yoon *et al.*, 2005; Qin *et al.*, 2006). These studies are briefly discussed below. B6C3F1 female mice (15/group) induced to ovulate were orally administered water-soluble chitosan (approximately 300 kDa; >90% deacetylation), at daily doses of 480 mg/kg body weight/day for 4 days (Choi *et al.*, 2002). Chitosan treatment did not have any effects on the oocyte and fertilization rates in animals fed a standard control diet. In contrast, chitosan treatment increased the number of ovulated oocytes and normal oocytes, as well as the *in vivo* and *in vitro* fertilization rates, compared to controls in animals fed a high-fat diet. The authors suggested that chitosan "*might improve the functions of the ovary and the oviduct in obese mice*". In a study by Yoon *et al.* (2005), 4 generations of ICR mice ingested approximately 10 mg/kg body weight/day of chitosan oligosaccharide *via* drinking water for up to 180 days. Though developmental and reproductive toxicity endpoints were not specifically examined in the study, no adverse effects were reported in any of the generations. Male and female ICR mice of the parental generation were provided with drinking water containing 0.1% chitosan oligosaccharide (equivalent to approximately 1 mg chitosan oligosaccharide/kg body weight/day) for 30 days. It was not indicated whether a control group was included in the parental generation. Subsequent generations (referred to as F1, F2, and F3 generations) were provided drinking water containing 0, 0.01, 0.1, or 1% chitosan oligosaccharide (equivalent to approximately 0, 0.1, 1, or 10 mg chitosan oligosaccharide/kg body weight/day) for up to 180 days. Timing and conditions of mating and euthanizing animals were not specified (age of parental generation at mating was not specified, although animals were purchased at 8 to 10 weeks of age). Following the experimental periods, bone marrow was taken from the femur of each mouse and used to assess the formation of chromosomal aberrations. The authors reported no significant differences in chromosomal aberrations between any of the treated groups compared to the control group. Other adverse effects or safety parameters were not assessed. Chitosan oligomers did not induce morphologic sperm abnormalities in male mice following oral gavage daily for 5 days with up to 5,000 mg/kg (Qin *et al.*, 2006).

Subsequent to GRN 397, 1 developmental toxicity study on chitosan oligosaccharides was identified in the scientific literature (Eisa *et al*., 2018). In this study, chitosan oligosaccharides (90% purity, agricultural grade, not further specified) were administered by gavage to groups of 3 pregnant female Wistar rats at doses of 0 (distilled water), 50, or 150 mg/kg body weight/day from Gestation Day (GD) 6 to 15. Body weights, placenta and uterus weights, number of fetuses, implantation sites, and resorbed fetuses, fetal weights and lengths, and physical and skeletal examination of fetuses were measured. The following statistically significant effects were reported at 50 and 150 mg/kg body weight/day doses of chitosan: decreased maternal body weight on GD 15 and 20, decreased absolute placenta and uterus weight, decreased fetal weight and length, and increased incidences of cleft palate, heart hypoplasia, atrophy of liver and kidneys, absence of skull cranial bone, caudal vertebrae, sternbrae, and limbs, and ribs shortage. There were no significant effects in behavior or clinical signs in treated and control groups, and no significant difference in relative organ weight and in number of fetuses, implantation sites, and resorbed fetuses at 50 and 150 mg chitosan. It should be noted that this study was not conducted according GLP or current testing guidelines for teratogenicity and used a very small maternal population (3/group) and only 2 dose groups compared to OECD testing guidelines, which recommend at least 10 animals per group and at least 3 dose groups. These deficiencies limit the value of this study in the safety assessment of Chinova's fiber derived from white button mushrooms.

Based on the 6-month dietary feeding study in which male and female Wistar rats were administered chitosan at intake levels of up to 6,000 mg/kg body weight/day (Section 6.3.3), no adverse effects were reported on testes or epididymis weights or sperm parameters or on uterus weights, indicating that chitosan did not elicit any effects that would suggest chitosan to be a reproductive toxin.

6.3.5 Short-Term Tests for Genotoxicity

The genotoxic potential of chitosan (derived from *Aspergillus bisporus*) and chitosan oligosaccharides was investigated in *in vitro* and *in vivo* studies and reviewed in GRN 397 (U.S. FDA, 2011). These studies are summarized in Table 6.3.5-1. Chitosan derived from *A. bisporus* (KiOmedine-CsU) did not increase the number of revertant colonies in an Ames test conducted according to OECD Test Guideline 471 at doses up to 1,000 µg/plate with and without S9 metabolic activation (Kitozyme, 2008 – reviewed in Kitozyme sa, 2011 – GRN 397). The incidence of micronuclei formation and chromosomal aberrations in male ICR mice following administration of chitosan oligosaccharides (MW <10 kDa, 90% DDA) at concentrations up to 1% w/v of the drinking water, equivalent to 10 mg/kg body weight/day, for up to 180 days (Yoon *et al*., 2005). No increases in micronuclei formation or chromosomal aberrations (in F1, F2, and F3 generations) were reported in any treatment group. Negative findings were also reported in an *in vivo* micronucleus test in Kunming mice administered chitosan oligomer (MW 1.86 kDa, 85% DDA) at doses of 5,000 mg/kg (Qin *et al*., 2006).

The cytotoxic effect of chitosan oligosaccharides (MW 1.4 kDa, 78% degree of acetylation) at concentrations up to 0.5% was investigated in human spermatozoa (Schimpf *et al*., 2019). Human sperm kinetic parameters, morphology, plasma membrane integrity, reactive oxygen species production, and DNA damage were measured. Sperm samples were collected from human volunteers aged 18 to 45 years. The authors reported no significant changes in any study parameter at concentrations of 0.1 to 0.5%, with the exception of a significant decrease in velocity at chitosan oligosaccharide concentrations of 0.25 and 0.5%. Based on the results of this study, the authors concluded that chitosan oligosaccharides do not show any sign of toxicity to sperm function (Schimpf *et al*., 2019).

No other mutagenic or genotoxic findings were reported in non-standard assays (*e.g*., mutagenicity in *Euglena gracilis*, chromosome damage and cytogenetic damage in *Allium cepa*, sister chromatid exchange in Chinese hamster lung cells, and aberrant crypts and proliferative indices in female CF1 mice) (Ohe, 1996; Torzsas *et al*., 1996; Kogan *et al*., 2004; de Lima *et al*., 2010).

The available evidence indicate that chitosan and chitosan oligosaccharides do not have genotoxic potential.

Table 6.3.5-1 Summary of Genotoxicity Studies of Chitosan and Chitosan Oligosaccharides

Test Substance	Test	Test System	Concentration/Dose	Result	Reference
In Vitro					
Chitosan derived from *Aspergillus bisporus* (KiOmedine-CsU)	Ames test[a]	*Salmonella typhimurium* strain TA98, TA100, TA1535, and TA1537, *Escherichia coli* WP2 strain pKM101	Up to 1,000 µg/plate (±S9)	• Negative.	Kitozyme (2008)[b]
Chitosan oligomer Source: shrimp DDA: 85% MW: 1.86 kDa	Ames test	*S. typhimurium* strain TA97, TA98, TA100, and TA102	0.5, 5, 50, 500, 5,000 µg/plate (±S9)	• Negative.	Qin *et al.* (2006)[b]
N-carboxyethyl derivatives of chitosan Source: NR DDA: NR MW: 150 kDa	*Euglena gracilis* mutagenicity assay	*E. gracilis*	10, 50, 100, 200 µg/mL	• N-carboxyethyl chitosan did not cause formation of mutant colonies at any concentration tested. • No change in cell viability observed. • Co-treatment of carboxyethyl chitosan protected against acridine orange genotoxicity.	Kogan *et al.* (2004)[b]
Chitosan polymerized with poly(methacrylic acid) nanoparticles Source: NR DDA: 94% MW: 71.3 kDa	*Allium cepa* assay for chromosome damage	*A. cepa*	1.8, 19, 180 mg/L	• No differences in mean mitotic index values in *A. cepa* test.	de Lima *et al.* (2010)[b]
Chitosan polymerized with poly(methacrylic acid) nanoparticles Source: NR DDA: 94% MW: 71.3 kDa	Cytogenetic assay	Human lymphocyte cell cultures	1.8, 19, 180 mg/L	• No numerical or structural changes in chromosomes.	de Lima *et al.* (2010)[b]
Chitosan Oligosaccharides Source: NR DDA: 78% MW: 1.4 kDa	Cytotoxicity	Human spermatozoa	0.1 to 0.5%	• Significant decrease in sperm velocity at 0.25% and 0.5%. • No sign of toxicity to sperm function.	Schimpf *et al.* (2019)

Table 6.3.5-1 Summary of Genotoxicity Studies of Chitosan and Chitosan Oligosaccharides

Test Substance	Test	Test System	Concentration/Dose	Result	Reference
In Vivo					
Chitosan oligomer Source: NR DDA: 90% MW: <10 kDa	Bone marrow micronuclei test	Male ICR mice (20/group)	0, 0.01%, 0.1%, 1% dietary chitosan oligosaccharide administered for up to 180 days	• No differences in formation of micronuclei in bone marrow cells.	Yoon *et al.* (2005)[b]
Chitosan oligomer Source: NR DDA: 90% MW: <10 kDa	Chromosome aberration test (4 generations)	Male ICR mice (20/group)	0, 0.01%, 0.1%, 1% dietary chitosan oligosaccharide administered for up to 180 days	• No differences in chromosome aberrations in parents and F1-3.	Yoon *et al.* (2005)[b]
Chitosan oligomer (single dose) Source: shrimp DDA: 85% MW: 1.86 kDa	Micronucleus test	Kunming mice M, F 5/sex/group	5,000 mg/kg	• Negative.	Qin *et al.* (2006)[b]
Chitosan oligomer (single dose) Source: shrimp DDA: 85% MW: 1.86 kDa	Sperm abnormality test	Kunming mice M 5/group	5,000 mg/kg	• Negative.	Qin *et al.* (2006)[b]
Anti-genotoxicity					
Chitin and chitosan	Sister chromatid exchange	Chinese hamster lung cells (CHL)	20 mg/mL	• Chitin and chitosan were anti-genotoxic when co-treated with 4-nitroquinoline N-oxide, dinitropyrene, mitomycin C, or Adriamycin.	Ohe (1996)[b]

Table 6.3.5-1 Summary of Genotoxicity Studies of Chitosan and Chitosan Oligosaccharides

Test Substance	Test	Test System	Concentration/Dose	Result	Reference
LMWC Source: NR DDA: 80% MW: 20 kDa HMWC Source: NR DDA: 80% MW: 20 kDa	Determination of aberrant crypts and proliferative indices in colon	Female CF1 mice (12 to 13/group)	Pretreatment with azoxymethane (known colon-specific carcinogen) for 2 weeks (i.p.), followed by diets supplemented with 2% LMWC or HMWC for 6 weeks	• 2% HMWC significantly decreased number of aberrant crypt foci, and decreased crypt height and circumference, in mice exposed to azoxymethane. • 2% LMWC decreased (not significant) number of aberrant crypt foci in mice exposed to azoxymethane. • 2% LMWC and HMWC significantly decreased number of mitotic figures per crypt in azoxymethane treated mice.	Torzsas *et al.* (1996)[b]

DDA = degree of deacetylation; F = females; HMWC = high molecular weight chitosan; i.p. = intraperitoneal; kDa = kilodaltons; LMWC = low molecular weight chitosan; M = males; MW = molecular weight; NR = not reported.

[a] Conducted according to Organisation for Economic Co-operation and Development Test Guideline 471.

[b] The details on test substance, assay, test system, concentration/dose, and results are presented as reviewed in GRN 397 (U.S. FDA, 2011).

6.3.6 Studies on Related Compounds (*N*-Acetylglucosamine)

As previously discussed, according to the USP monograph, "*chitosan in an unbranched binary polysaccharide consisting of N-acetyl-D-glucosamine and D-glucosamine units linked in a β(1-4) manner*". Although it is unlikely that chitosan would be digested by gastric enzymes, *N*-acetylglucosamine is a potential hydrolysis byproduct generated during gastric transit (see Section 6.2 for further details). The chronic toxicity and carcinogenicity of *N*-acetyl-D-glucosamine, the monomeric constituent of chitosan, was evaluated in F344 rats in 2 separate studies conducted by Takahashi *et al.* (2009). This study was previously reviewed in GRN 397 (U.S. FDA, 2011). In the first study, F344 rats (10 animals/sex/group) were provided *N*-acetyl-D-glucosamine in the diet at concentrations of 1.25, 2.5, or 5% for 52 weeks. In the second study, F344 rats (50 animals/sex/group) were provided *N*-acetyl-D-glucosamine in the diet at concentrations of 0, 2.5, or 5% for 104 weeks. No treatment-related mortality or effects related to clinical signs of toxicity, food consumption, hematology, serum biochemistry and histopathological evaluations were reported compared to control in either study. Body weights were slightly but statistically significantly decreased in high-dose (5%) males in both studies and in females (2.5% and 5%) in the carcinogenicity study. No statistically significant increase in tumors was reported in any of the dose groups of animals compared to controls. The slight suppression of body weights was considered by the authors to relate to reductions in caloric intake due to the high levels of intake of the test article and not direct toxic effect. Based on the results of this study, the NOAEL was concluded to be 5% in the diet in both studies, equivalent to 2,323 and 2,545 mg/kg body weight/day in males and females, respectively.

6.4 Clinical Studies

Chitosan has an apparent history of safe use in food supplement products, and several human clinical studies in which healthy, hypercholesterolemic, smokers, and/or obese subjects have been administered chitosan or chitosan oligosaccharides in the diet are published in the literature [see Section G of GRN 397 and Section D of GRN 443] (Kitozyme sa, 2011; U.S. FDA, 2011, 2013a; GRAS Associates, LLC, 2012). These studies demonstrated that chitosan consumption was well-tolerated at levels ranging from 1 to 6 g per day, for periods up to 24 weeks (Table 6.4-1). According to GRN 170, the U.S. FDA has raised concerns on potential effects on fat-soluble vitamins and mineral status in humans following consumption of chitosan (Lee B. Dexter and Assoc., 2005 – GRN 170). These concerns were raised due to a rat study reporting significant reductions in levels of vitamins A, D, and E, and calcium, magnesium, and iron (Deuchi *et al*., 1995), and a more recent long-term toxicity study reported similar findings (NTP, 2017). These findings have not been substantiated in human clinical studies conducted with clinically relevant dosages (Tapola *et al*., 2008). As such, the altered absorption of dietary nutrients reported in animals is not relevant to the safety of chitosan, given that the doses used in animal studies were much larger on a grams/kilogram body weight basis, therefore, were not considered representative of human intake levels.

A summary of the human clinical studies discussed in GRN 397 is provided in Table 6.4-1. Clinical studies published since GRN 397 that were identified in an update literature search are summarized below. The results of the new clinical studies support the previous conclusions regarding the safety of chitosan in humans.

In a multi-center, single-blind, placebo controlled, and randomized clinical study, 96 adult patients in India (36 males, 60 females, mean age: 35.5±11.2 years) took five 500 mg chitosan capsules (KiOnutrime-CsG® chitosan derived from *A. niger*) per day for a total dose of 2,500 mg chitosan daily for 90 days (n=64) or a placebo (n=32; microcrystalline cellulose powder) (Trivedi *et al*., 2016). Study participants were generally free from disease; however, 15 subjects in the chitosan group and 6 from the placebo group had hypertension, diabetes mellitus, and/or dyslipidemia. The following parameters were measured or tracked during the study: safety, quality of life (*via* questionnaire), adverse events and effects, biochemical parameters (urea, serum creatinine, ALT, AST), mean body weight changes, body mass index (BMI), body fat, visceral fat, muscle mass, upper abdominal circumference, hip, and waist, waist to hip ratio, lipid profile [triglycerides (TG), high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL)], and glycated hemoglobin levels.

There were 6 adverse events (common cold, hypertriglyceridemia, body ache, hypertension, and 2 counts of constipation) in the chitosan group and 4 adverse events (2 counts of mild headache, hypertriglyceridemia, and fracture) in the placebo group. The authors reported that all adverse events were mild and unrelated to study treatment. There was no statistically significant difference in ALT, AST, serum creatinine, or urea from Day 0 to 90 in either group. The authors reported no study withdrawals due to adverse effects and stated that overall, chitosan was safe and well tolerated. Compared to placebo, a statistically significant reduction in mean body weight change, BMI, body fat percentage, and upper abdominal, hip, and waist circumference at Day 45 and Day 90 were reported.

Compared to baseline measures, a statistically significant decrease in body weight, BMI, body fat percentage, visceral fat percentage, muscle mass, upper abdominal, hip, and waist circumference were reported at Day 45 and Day 90. Percent glycated hemoglobin was significantly decreased in the chitosan group at Day 45 and 90 as well as in the placebo group at Day 45, though returning to baseline at Day 90 in the latter group. A statistically significant increase in LDL was reported in the chitosan group at Day 45 and in the placebo at Day 90, an effect attributable to only 1 subject/group, and was therefore considered transient and clinically non-significant by the authors. No significant differences were reported by the authors for all other lipid parameters compared to baseline (Trivedi *et al*., 2016).

In a 12-week randomized, double-blind, placebo-controlled study conducted with 60 pre-diabetic adult patients (characterized by impaired fasting glucose and impaired glucose tolerance), a low-molecular weight chitosan oligosaccharide capsule (100% purity, not further specified) or a placebo capsule (roasted barley meal powder) was administered 6 times/day for a total daily dose of 1,500 mg (Kim *et al*., 2014). Adverse effects, serum levels of glucose and C-peptide, cholesterol and immune markers, triglycerides, insulin, adiponectin, and glycated hemoglobin were measured throughout the study period. No adverse effects were reported by any of the subjects. Statistically significantly increased lean body mass was reported in the chitosan group compared to placebo. Significantly decreased glycated hemoglobin, glucose at 30 and 60 minutes, and interleukin-6 (IL-6) and significantly increased adiponectin were reported compared to baseline. There were no significant differences in insulin, C-peptide, and area under the curve of glucose and C-peptide compared to baseline. Significant changes from baseline to after 12 weeks of chitosan use *versus* changes in the placebo group were reported as a decrease in body fat percentage, waist circumference, blood glucose at 60 minutes, and glycated hemoglobin. There was no significant difference in changes in total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, insulin, adiponectin, IL-6, and tumor necrosis factor-alpha (TNF-α) between treatment and placebo groups (Kim *et al.*, 2014).

In a randomized, double-blind, controlled crossover study conducted with 37 healthy adults (age 20 to 75 years), chitosan oligosaccharide capsules were provided to subjects at a daily dose of 250 mg (Jeong *et al*., 2019). The treatment was provided in addition to 75 g of sucrose within 15 minutes. After 7 days, subjects were provided a placebo. Blood samples were collected after a 12-hour overnight fast. Serum glucose concentrations were measured at 0, 30, 60, 90, and 120 minutes. Total energy expenditure was calculated for each subject. No side effects were reported in any study subjects. No significance changes in white blood cells, red blood cells, hemoglobin, hematocrit, platelets, or parameters of daily food intake and total energy expenditure (basal metabolic rate) in any study subject. Blood glucose levels peaked at 30 minutes and returned to baseline after 2 hours. No significant differences in blood glucose levels were reported between treatment and placebo groups (Jeong *et al*., 2019).

A meta-analysis of randomized, double-blind, placebo-controlled trials was conducted to evaluate the effects of chitosan administration on systolic blood pressure and diastolic blood pressure (Huang *et al*., 2018a). Chitosan was administered at doses ranging from 1 to 4.5 g/day for up to 24 weeks in 617 subjects that were overweight, obese, hypercholesterolemic, or prehypertensive from 8 trials with 10 arms and chitosan did not result in any significant decreases in systolic or diastolic blood pressure. However, analyses of subgroups indicated that diastolic blood pressure was decreased in the short-term (<12 weeks) and at high doses (>2.4 g/day). The reported forms of chitosan were “chitosan” or microcrystalline chitosan. No further information on the molecular weight or DDA was reported. Based on the results of this meta-analysis, the authors concluded that chitosan consumption significantly decreased diastolic blood pressure at high doses (>2.4 g/day) and in short-term interventions (Huang *et al*., 2018a).

In another meta-analysis of randomized controlled trials conducted to investigate the effects of chitosan consumption on serum lipids, 1,108 subjects that were overweight, obese, hypercholesterolemic, or prediabetic from 14 trials with 21 treatment arms were evaluated (Huang *et al*., 2018b). Chitosan administration at doses ranging from 0.312 to 6.75 g/day for up to 24 weeks significantly increased the total cholesterol and LDL-cholesterol in all subjects. No significant changes in HDL-cholesterol or triglycerides and no serious adverse events were reported (Huang *et al*., 2018b).

The effects of chitosan on body weight and body composition were investigated in a meta-analysis of 15 trials with 18 treatment arms that included 1,130 subjects (Huang *et al*., 2019). The studies included subjects that were overweight or obese with hypercholesterolemia or overweight or obese but otherwise healthy consuming chitosan at doses ranging from 0.312 to 4.5 g/day for 4 to 24 weeks. The reported treatments included chitosan capsules, microcrystalline chitosan capsules, water-soluble chitosan capsules, or beta-glucan-chitin-chitosan fraction. No details on the molecular weight or DDA were reported. Chitosan consumption was associated with a significant decrease in body weight. Analysis of subgroups indicated that consuming high doses of chitosan (>2.4 g/day) for short-term (<12 weeks) was associated with a decrease in body weight. In addition, consumption of chitosan was well tolerated and was not associated with any serious adverse events (Huang *et al*., 2019).

Table 6.4-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
Healthy Subjects						
10 subjects, healthy volunteers, not taking antioxidants (such as vitamin E or C) during the 3 months before inclusion in the study	Oral preparation 4 wks Open-label, placebo-controlled, cross-over study	Water-soluble chitosan Source: NR DDA: 95% Size: average MW of 20 kDa	Group 1: 0 Group 2: 0.54	• Blood pressure, BMI • HDL and LDL cholesterol, triglycerides • Atherogenic index • Calcium and phosphorous levels • Plasma antioxidant capacity	• NSD in blood pressure, BMI, levels of total cholesterol, phosphorous, or calcium. • Decrease in levels of plasma glucose, and atherogenic index after 2 wks and persisted until the end of study. • Concentration of HDL-cholesterol increased during treatment period; no significant difference in LDL-cholesterol. • Lowered the ratio of oxidized to reduced albumin, and increased total plasma antioxidant activity.	Anraku *et al.* (2009)
24 subjects, healthy males and females	Oral capsule 12 d Double-blind, placebo-controlled, cross-over study	Chitosan Source: NR DDA: NR Size: NR	Group 1: 0 Group 2: 2.5	• Food intake • Weight • Fecal fat content	• NSD in weight or food intake. • Very small increase in fecal fat content in men, but NSD in women. • No adverse effects reported.	Gades and Stern (2005)
8 subjects, healthy male volunteers	Oral biscuits 14 d	Chitosan Source: sea crab shells DDA: NR Size: NR	Week 1: 0 Week 2: 3 Week 3: 6 Week 4: 0	• Mean energy and nutrient intake • Fecal microbiota, bacterial metabolites, fecal weight, moisture content, pH value	• Decrease in lecithinase-negative clostridia ("may lead to improvement in intestinal environment"). • Decrease in fecal ammonia. • Chitosan inhibits putrefactive activity of intestinal microbiota and may contribute to reduction of factors that lead to disease states.	Terada *et al.* (1995)

Table 6.4-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
8 subjects, healthy males	Biscuits 14 d Random, placebo-controlled cross-over study	Chitosan Source: NR DDA: 90.5% Size: 500 kDa	Group 1: 0 Group 2: Week 1: 3 Week 2: 6	• Body weight • Nutrition survey • Serum lipid • Bile acid and neutral cholesterol in feces	• Intake of energy, protein, fat, and cholesterol did not change. • Average total serum cholesterol level decreased, serum HDL-cholesterol increased, NSD in serum triglyceride and phospholipid. • NSD in bile acid excretion, amount of secondary bile acid excreted as lithocholic acid significantly decreased. • Excreted amount of metabolite of cholesterol, coprostanol, was significantly lower.	Maezaki *et al.* (1993)
Hypercholesterolemic Subjects						
56 subjects, mild hypercholesterolemia	Oral tablets 55 d Parallel, placebo-controlled, single-blind trial	Chitosan (commercial food grade, shellfish-derived) Source: NR DDA: >95% Viscosity: <500 mPa·s	Group 1: 0 (placebo) Group 2: 4.5 Group 3: 6.75	• Hematology: blood count, plasma creatinine, urate, γ-glutamyl transferase, calcium, serum ferritin • Serum: *alpha*- and *beta*-carotene, vitamin A, vitamin E, 25-hydroxyvitamin D • Plasma total and HDL-cholesterol, total triglyceride concentrations • Body weight, blood pressure • RAND 36-item Health Survey • Incidence and severity of gastrointestinal, skin and other symptoms	• NSD in hematology, serum biochemistry, plasma lipids, body weight. • Association in incidence of constipation, heartburn, nausea in first 4-week period in chitosan groups (not significant between groups after performing pair-wise comparisons). • Three subjects in chitosan group and 1 subject in placebo group reported skin symptoms.	Tapola *et al.* (2008)

Table 6.4-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
95 subjects, mild or moderate hypercholesterolemia	Oral tablet 12 wks Multicenter, placebo-controlled, randomized study	HEP-40, low-molecular weight chitosan Source: NR DDA: 93% Size: 40 kDa	Group 1: 0 (placebo) Group 2: 1.2 Group 3: 1.6 Group 4: 2.4	• Blood cholesterol levels • Incidence of adverse events • Serum parameters	• NSD in non-serious adverse events. • No serious adverse events reported. • No clinically important changes in any laboratory safety parameters. • NSD in serum 25(OH)D. • HEP-40 reduced serum LDL-cholesterol and total cholesterol at Weeks 4, 8. • At 12 wks, NSD in lipid profile parameters.	Jaffer and Sampalis (2007)
90 women, Mild to moderate hypercholesterolemia	Oral capsules 8 wks Double-blind, placebo-controlled, randomized study	Chitosan Source: NR DDA: 89.5% Viscosity: 160 mPa·s	Group 1: 0 (placebo) Group 2: 1.2	• Serum chemistry profiles • Complete blood counts • Changes in physical findings and signs • Blood pressure	• NSD in body weight, BMI, blood pressure, food consumption. • Chitosan therapy produced statistically significant reduction in total cholesterol at 8 wks. • NSD in HDL cholesterol, triglyceride levels.	Bokura and Kobayashi (2003)
Overweight Subjects						
12 subjects, obese, without diabetes mellitus	Oral tablet 3 months Placebo-controlled, randomized, double-blind trial	Chitosan (Vitamin World, 750 mg chitosan) Source: NR DDA: NR Size: NR	Group 1: 0 (placebo) Group 2: 2.25	• Serum glucose, total cholesterol, HDL cholesterol, triglycerides	• NSD serum glucose levels, lipid profile. • Significant decrease in triglycerides. • No adverse events with interventions. • Insulin sensitivity increased significantly.	Hernández-González *et al.* (2010)
30 subjects, overweight, hyperlipemic, under physical training	Oral tablet 4 months Double-blind, placebo-controlled	Low molecular weight chitosan, polyglucosamine	Group 1: 0 (placebo) Group 2: 2	• Anthropometric measures • Blood pressure • LDL and HDL-cholesterol, blood glucose and triacylglycerol	• More significant reduction in body weight, waist circumference, LDL-cholesterol, triacylglycerol than placebo control. • HDL increase was higher than placebo control. • Metabolic syndrome was reduced in 12 cases in the supplement group.	Cornelli *et al.* (2008)

Table 6.4-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
134 subjects, Overweight adults, 83% women	Oral capsules 60 d Double-blind, placebo-controlled study	Chitosan Source: NR DDA: NR Size: NR	Group 1: 0 (placebo) Group 2: 3	• Body composition • Blood chemistries • Tracking forms (daily caloric intake, activity levels)	• Significant reduction in mean scale weight, fat mass. • NSD in total cholesterol, HDL, LDL, or bone mineral density.	Kaats *et al.* (2006)
250 subjects, Overweight adults, 82% women	Oral capsule 24 wks Randomized, double-blind, placebo-controlled trial	β-Chitosan Source: squid pens DDA: 75.5% Size: NR	Group 1: 0 (placebo) Group 2: 3	• Body weight • Blood pressure • Waist circumference • Serum lipids • Plasma glucose • Fat-soluble vitamins in serum • Fecal fat losses • Health-related quality of life questionnaire	• NSD in BMI, waist circumference, body fat, blood pressure, fat-soluble vitamins, fecal fat loss. • Statistically significant decrease in total cholesterol levels, LDL-cholesterol, but not clinically significant. • NSD in HDL-cholesterol. • NSD in health-related quality of life questionnaire answers.	Mhurchu *et al.* (2004)
68 subjects, Normoglycemic obese individuals	Oral tablet 12 wks Randomized, double-blind, placebo controlled	Absorbitol, a salt of chitosan Source: shellfish DDA: NR Size: NR	Group 1: 0 (placebo) Group 2: 3	• Body weight • Waist/hip ratio • Blood pressure • Bioelectric impedance analysis • Serum total cholesterol, triglyceride, HDL cholesterol, glucose	• NSD in adverse effects reporting. • NSD in weight, body composition, blood composition, blood pressure, lipid profile, fasting insulin levels.	Ho *et al.* (2001)

Table 6.4-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
59 subjects, overweight, mildly obese, females	Oral capsule 8 wks Randomized, double-blind, placebo-controlled	Rapidly-soluble chitosan, LipoSan Ultra™ Source: NR DDA: > 78% Size: > 100 kDa	Group 1: 0 (placebo) Group 2: 3	• Body weight • Waist/hip ratio • Symptom Observational Survey questionnaire • Routine calorie and dietary fat intake; exercise diary • Fasting serum lipid levels • Fecal fat	• NSD in calorie and dietary fat intake. • NSD in total Symptom Observational Survey results, though chitosan group reported more incidences of gastrointestinal discomfort, mild nausea, and heartburn; were alleviated by increasing water consumption. • In placebo group, mean weight increased significantly by 1.5 kg while treatment group decreased mean weight by 1.0 kg. • BMI was lower in chitosan group. • Chitosan group exhibited an increasing trend in fecal fat excretion, but no statistical conclusion (sample size too small).	Schiller *et al.* (2001)
30 subjects, overweight volunteers	Oral capsules 28 d Randomized, double-blind, placebo-controlled	Chitosan Source: NR DDA: NR Size: NR	Group 1: 0 (placebo) Group 2: 2	• Body mass index • Blood pressure • Quality of life • Serum cholesterol • Serum triglycerides • Vitamin A, D, E, *beta*-carotene	• NSD in body mass index, serum cholesterol, serum triglycerides, vitamin A, D, E, *beta*-carotene. • Small increase in vitamin K after 4 wks in chitosan group compared with placebo. • Minor adverse events reported in 9 subjects in chitosan group to be constipation.	Pittler *et al.* (1999)
Diabetic (Type 2) Subjects						
18 subjects, dyslipidemic type 2 diabetic subjects	Dietary supplementation 12 wks Random, placebo-controlled	Chitosan Source: NR DDA: 90% Size: 1,000 kDa	Group 1: 0 Group 2: 1.8	• Body weight • Plasma cholesterol • HDL-cholesterol, LDL-cholesterol, triglyceride • Adverse events	• NSD in cholesterol, triglyceride concentration. • Increase in HDL-cholesterol, concomitant reduction in LDL-cholesterol. • Mild digestive discomfort.	Ausar *et al.* (2003)

BMI = body mass index; d = day(s); DDA = degree of deacetylation; HDL = high-density lipoprotein; kDA = kilodaltons; LDL = low-density lipoprotein; MW = molecular weight; NR = not reported; NSD = no significant difference; wk(s) = week(s).

[a] Study details were taken as reported in GRN 397 (U.S. FDA, 2011).

6.5 Information Pertaining to the Safety of *Beta*-1,3-Glucans

Chinova's fiber derived from white button mushrooms contains *beta*-1,3-glucans at concentrations of up to 5% on a w/w% basis, and as crustacean-derived chitosan preparations do not contain *beta* glucans, ancillary safety data on the toxicity of *beta*-1,3-glucans are necessary. As described in GRN 397 (U.S. FDA, 2011), several studies have been conducted which evaluated the safety of beta-glucan. In 1 study, groups of male and female Wistar rats (20/sex/group) [Crl:WI(WU)] were administered chitin-glucan as a dietary admixture at concentrations of 0 (control), 1, 5, or 10% (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) for a period of 13 weeks. Food intake in high-dose rats was statistically significantly increased with no changes in body weight, in comparison to control rats. The author considered this finding to be toxicologically irrelevant due to the lower energy content of the high-dose diet compared to the control diet. A statistically 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. Cecal enlargement occurs in rodents administered large dietary quantities of non-digestible polysaccharides/polyols and is an effect that is not considered relevant to humans (WHO, 1987). The authors concluded that under the conditions of the study, the NOAEL was 10% in the diet, the highest concentration tested, which was equivalent to an overall estimated daily intake of 6,589 mg/kg body weight/day for males and 7,002 mg/kg body weight/day for females.

Similar findings were reported in studies evaluating the effect of orally administered insoluble fungal derived *beta*-glucan preparations in rodents (Feletti *et al.*, 1992; Babícek *et al*., 2007). In a GLP- and OECD No. 408-compliant subchronic toxicity study (OECD, 1998a,b), a NOAEL of 100 mg/kg body weight (the maximum deliverable gavage dose) was derived for Fisher-344 rats administered a *Saccharomyces cerevisiae* derived *beta*-1,3-glucan preparation on a repeated basis over a period of 91 days (Babícek *et al*., 2007). The chronic (52 weeks) toxicity of a *Candida albicans* derived *beta*-1,3-D-glucan insoluble isolate was evaluated by Feletti *et al*. (1992). Groups of Sprague-Dawley rats (20/sex/group) were randomized to treatment groups receiving gavage doses of *beta*-glucan at 0 (saline), 50, 100, or 200 mg/kg body weight/day. Similar to findings reported by Jonker *et al*. (2010), high-dose male and female treatment groups (200 mg/kg body weight/day) experienced soft stools, diarrhea, and cecal enlargement with variable hyperplasia of the colon mucosa. A NOAEL of 200 mg/kg body weight per day, the highest dose tested, can be determined from this study.

The safety of soluble beta-glucans derived from oat bran, barley, baker's yeast, and fungi has been reviewed in numerous GRAS Notifications to the U.S. FDA (*e.g.*, GRN 239 – U.S. FDA, 2008a; GRN 309 – U.S. FDA, 2010; GRN 437 – U.S. FDA, 2013b; GRN 544 – U.S. FDA, 2015). Based on the intended uses of beta-glucan, the estimated intake in consumers was calculated to be as high as 16.5 g beta-glucan/person/day in 90th percentile (GRN 437 – U.S. FDA, 2013b). The Agency did not raise any objections any of the GRAS determinations.

The safety of beta-glucans in the diet is also supported by the fact that the U.S. FDA has approved several health claims for soluble fibers derived from oats containing beta-glucan and providing at least 0.75 g beta-glucan soluble fiber per serving (U.S. FDA, 2008b). The European Food Safety Authority also approved health claims related to the maintenance of normal blood cholesterol concentrations and intake of oat beta-glucan of at least 3 g/day (EFSA, 2010). The safety of Baker's yeast-derived beta-glucan was also concluded to be safe for use in foods at levels providing 600 mg/day (EFSA, 2011).

Based on the intended uses of chitosan derived from white button mushrooms, the highest intake under the intended conditions of use is estimated to result in intakes of 1.53 g/day. This would amount to approximately 76.5 mg of beta-glucan, which is well-below intakes that are anticipated to be consumed from the current GRAS uses of beta-glucans in the US. Therefore, no safety concerns are anticipated due to the presence of up to 5% beta-glucan in Chinova's fiber derived from white button mushrooms.

6.6 Discussion of the Available Safety Information on Chitosan

The safety of chitosan was discussed in numerous GRAS notifications that were notified to the U.S. FDA (*i.e.*, GRN 73, 170, 397, 443). Based on the information provided in GRN 170, the main concern raised by the reviewers at the Center for Food Safety and Applied Nutrition (CFSAN) were related to the "*nutritional effects of consuming shrimp-derived chitosan on a chronic basis as part of a normal diet*" (Lee B. Dexter and Assoc., 2005 – GRN 170). According to the Notifier, the FDA noted that "*chitosan was non-toxic to humans and other test animals*"; however, the Agency "*questioned whether or not chitosan would interfere with fat-soluble vitamin and mineral status in humans, when the substance was consumed on a chronic basis as part of a general diet*". The nutritional effects discussed in GRN 170 were based on a study by Deuchi *et al.* (1995), in which rats consuming a high-fat diet containing 5% chitosan experienced significant reductions in fat digestibility, and reduced reserves of vitamins A, D, and E, and minerals, such as calcium, magnesium, and iron. The findings in Deuchi *et al.* (1995) are not considered of clinical significance, given the differences in the digestions of dietary fiber-like substances (*i.e.*, chitosan) and fat between rats and humans. Rats have no gallbladder, and therefore, cannot emulsify high fat content meals for complete digestion and the shorter transit time in rats impacts their ability to digest dietary fiber-like substances such as chitosan (Bach Knudsen et al., 1994; Wisker *et al*., 1997). These species differences limit the direct applicability of the rat as a model for evaluating nutritional effects of fat sequestering compounds, such as chitosan. In addition, considering that the effects on vitamin absorption are secondary to effects on fat absorption, an understanding of threshold effects of chitosan on fat absorption in a clinical setting is more relevant for use in risk assessment.

The nutritional effects of chitosan were further assessed in a 6-month feeding study conducted by the NTP, wherein Sprague-Dawley rats were provided low-MW chitosan powder (purity: 94%; average MW: 82 kDa; DDA: 86.5%; compositionally equivalent to chitosan from white button mushrooms) in the diet at levels of 0, 1, 3, or 9% for 6 months (NTP, 2017). Further details of this study, which was not published at the time GRN 443 was filed, are provided in Section 6.3.3. Dietary concentrations of chitosan up to 9% in the diet were well-tolerated by rats. However, statistically significant reductions in serum concentrations of fat-soluble vitamins and reduced relative liver and thymus weights were reported at dietary concentrations of 3% and 9% in males, and 9% in females. No histopathological changes attributable to chitosan were reported in any of the groups. A statistically significant decrease in fat soluble vitamins at the 1% level in male rats was only reported at Week 13 for serum vitamin E. The reduction of serum vitamin E in male rats was not consistent throughout the study. Dietary exposure to chitosan for 6 months resulted in decreased fat digestion and depletion of some fat-soluble vitamins in male and female rats. There were no histological findings associated with the observed decreases in vitamin levels. Based on the effects of chitosan on serum vitamin E levels, the authors concluded the "*lowest-observed-effect level for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats*". These effects are considered to be indirect consequences of the recognized fat binding properties of chitosan[7] resulting in excretion of dietary fat and reduced absorption of fat-soluble vitamins. In addition, generalized effects of resistant dietary fibers, such as chitosan on nutrient absorption have been

[7] Chitosan is marketed as a dietary supplement for weight loss, and the USP monograph for chitosan includes fat binding capacity as a qualitative specification parameter for the ingredient.

long known, are well characterized, and are not considered nutritionay relevant at levels that are commonly consumed in the diet (Dahl and Stewart, 2015). As such, these effects are not considered to be a direct toxic effect of chitosan on organ systems or a finding of toxicological or nutritional significance and the reported fatty change is considered to be a biological adaptive response to depletion of fat-soluble vitamins and minerals and contingent upon consumption of supraphysiological intakes that would affect lipid absorption.

Concerns regarding chitosan reducing the absorption of lipid and other nutrients, such as, fat soluble vitamins and minerals were mainly reported in studies with rats (Deuchi *et al*., 1995; NTP, 2017). This is further collaborated by the results of several clinical studies, wherein no significant decreases in fat-soluble vitamins were reported in human studies as follows:

- Vitamins A, E, D, α-carotene, and β-carotene in mild hypercholesterolemic subjects (n=56) consuming chitosan derived from shellfish at levels of 6.75 g/day for 55 days (Tapola *et al*., 2008);

- Vitamin D in mild or moderate hypercholesterolemic subjects (n=96) consuming LMWC at doses up to 2.4 g/day for 12 weeks (Jaffer and Sampalis, 2007);

- Vitamin A (retinol), D, E (α-tocopherol), β-carotene, and prothrombin time (surrogate for vitamin K) in overweight adults (n=250) consuming 3 g/day of β-chitosan for 24 weeks (Mhurchu *et al*., 2004); and

- Vitamin A, D, E, and β-carotene in overweight subjects (n=30) consuming 2 g/day of chitosan (not further characterized) for 28 days (Pittler *et al*., 1999).

A number of repeated-dose studies were identified in mice, rats, guinea pigs, and pigs, which reported an effect of chitosan administration (see Section 6.3.2). The weight of the available evidence indicates that typical chitosan preparations, when ingested are non-toxic. Some evidence of toxicity (*e.g*., increased or decreased relative organ weights, accumulation of iron, zinc in copper in organs, decrease fat soluble vitamins) has been reported in rodent studies following administration of LMWC oligomers and/or fully deacetylated oligomers at high dietary concentrations (>1%). Evidence of toxicity in these studies is typically dose limiting (only observed at dietary levels >1%) and in some cases were confounded by application of non-validated study designs.

Fifteen clinical studies were discussed in GRN 397 in which chitosan was consumed at doses of 0.54 to 6.75 g/day for 2 to 24 weeks without significant treatment-related adverse effects reported (U.S. FDA, 2011). An updated search of the scientific literature identified studies published since GRN 397 that were conducted with chitosan doses of 1.5 to 2.5 g/day for up to 90 days (see Section 6.4). No treatment-related adverse events were reported throughout the studies, but a statistically significant decrease in body weight, body mass index, body fat percentage, visceral fat percentage, muscle mass, and upper abdominal, hip, and waist circumference were reported (Kim *et al.*, 2014; Trivedi *et al*., 2016). These findings are considered to be an expected effect of chitosan, as the substance is commonly used in food supplements products for its fat binding ability.

The reported LOEL from NTP (2017) was 1% in male rats, equivalent to 450 mg/kg body weight/day, based on the reported nutritionally-related findings. On a body weight basis, this dose is equivalent to a human consuming approximately 31.5 g of chitosan per day (for a 70-kg individual). In the parallel, placebo-controlled study by Tapola *et al.* (2008), no effects on fat absorption were reported at clinically relevant doses (*i.e.*, 6.75 g/day). Based on the proposed antimicrobial food uses of the chitosan derived from white button mushrooms, considering maximum FEMA-GRAS approved use levels, the estimated daily intake of chitosan derived from white button mushrooms was determined to be highest in male adults, at 3.2 g/day at the 90th percentile, approximately 10-fold less than the reported LOEL of chitosan by NTP (2017), and an order of magnitude below levels that have been demonstrated to not affect vitamin absorption in human studies. Therefore, the proposed uses of fiber derived from white button mushrooms is not expected to be associated with any adverse outcomes, including vitamin or mineral deficiencies.

REFERENCES

Ali AAM, Mansour A, Hassanine EA, Abdel-Razek AH, Mahmoud HAH (2019). Ameliorative role of a combination of chitosan, calcium, vitamins A and E against high fat diets-induced adverse effects in rats. J Basic Appl Zool 80:11 [12pp]. DOI:10.1186/s41936-019-0081-7.

Anderson JW, Nicolosi RJ, Borzelleca JF (2005). Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. Food Chem Toxicol 43(2):187-201. DOI:10.1016/j.fct.2004.11.006.

Anraku M, Fujii T, Furutani N, Kadowaki D, Maruyama T, Otagiri M, et al. (2009). Antioxidant effects of a dietary supplement: reduction of indices of oxidative stress in normal subjects by water-soluble chitosan. Food Chem Toxicol 47(1):104-109. DOI:10.1016/j.fct.2008.10.015.

Ausar SF, Morcillo M, León AE, Ribotta PD, Masih R, Vilaro Mainero M, et al. (2003). Improvement of HDL- and LDL-cholesterol levels in diabetic subjects by feeding bread containing chitosan. J Med Food 6(4):397-399. DOI:10.1089/109662003772519985.

Babícek K, Cechová K, Simon RR, Harwood M, Cox DJ (2007). Toxicological assessment of a particulate yeast (1,3/1,6)-β-D-glucan in rats. Food Chem Toxicol 45(9):1719-1730. DOI:10.1016/j.fct.2007.03.013.

Bach Knudsen KE, Wisker E, Daniel F, Feldheim W, Eggum BO (1994). Digestibility of energy, protein, fat and non-starch polysaccharides in mixed diets: comparative studies between man and rat. Br J Nutr 71(4):471-487. DOI:10.1079/bjn19940156.

Bai Y, Zheng J, Yuan X, Jiao S, Feng C, Du Y, et al. (2018). Chitosan oligosaccharides improve glucolipid metabolism disorder in liver by suppression of obesity-related inflammation and restoration of peroxisome proliferator-activated receptor gamma (PPARγ). Mar Drugs 16(11):455 [15pp]. DOI:10.3390/md16110455.

Bokura H, Kobayashi S (2003). Chitosan decreases total cholesterol in women: a randomized, double-blind, placebo-controlled trial. Eur J Clin Nutr 57(5):721-725. DOI:10.1038/sj.ejcn.1601603.

CDC (2018a). *National Health and Nutrition Examination Survey (NHANES): 2015-2016*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/ContinuousNhanes/Default.aspx?BeginYear=2015 [NHANES Home Page last reviewed: October 30, 2018].

CDC (2018b). *National Health and Nutrition Examination Survey (NHANES): 2015-2016 – Dietary Data*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/search/datapage.aspx?Component=Dietary&CycleBeginYear=2015 [Last updated: July 2018].

Chae SY, Jang M-K, Nah J-W (2005). Influence of molecular weight on oral absorption of water soluble chitosans. J Control Release 102(2):383-394. DOI:10.1016/j.jconrel.2004.10.012.

Chiang MT, Yao HT, Chen HC (2000). Effect of dietary chitosans with different viscosity on plasma lipids and lipid peroxidation in rats fed on a diet enriched with cholesterol. Biosci Biotechnol Biochem 64(5):965-971. DOI:10.1271/bbb.64.965.

Chiu C-Y, Yen T-E, Liu S-H, Chiang M-T (2020). Comparative effects and mechanisms of chitosan and its derivatives on hypercholesterolemia in high-fat diet-fed rats. Int J Mol Sci 21(1):92 [11pp]. DOI:10.3390/ijms21010092.

Choi HG, Kim JK, Kwak DH, Cho JR, Kim JY, Kim BJ, et al. (2002). Effects of high molecular weight water-soluble chitosan on *in vitro* fertilization and ovulation in mice fed a high-fat diet. Arch Pharm Res 25(2):178-183. DOI:10.1007/BF02976560.

Cornelli U, Belcaro G, Cesarone MR, Cornelli M (2008). Use of polyglucosamine and physical activity to reduce body weight and dyslipidemia in moderately overweight subjects. Minerva Cardioangiol 56(5, Suppl.):71-78.

Dahl WJ, Stewart ML (2015). Position of the Academy of Nutrition and Dietetics: health implications of dietary fiber. J Acad Nutr Diet 115(11):1861-1870. DOI:10.1016/j.jand.2015.09.003.

de Lima R, Feitosa L, do Espírito Santo Pereira A, de Moura MR, Ahmad Aouada F, Capparelli Mattoso LH, et al. (2010). Evaluation of the genotoxicity of chitosan nanoparticles for use in food packaging films. J Food Sci 75(6):N89-N96. DOI:10.1111/j.1750-3841.2010.01682.x.

Deuchi K, Kanauchi O, Shizukuishi M, Kobayashi E (1995). Continuous and massive intake of chitosan affects mineral and fat-soluble vitamin status in rats fed on a high-fat diet. Biosci Biotechnol Biochem 59(7):1211-1216. DOI:10.1271/bbb.59.1211.

Do AR, Cho S-J, Cho Y-Y, Kwon E-Y, Choi J-Y, Lee JH, et al. (2018). Antiobesity effects of short-chain chitosan in diet-induced obese mice. J Med Food 21(9):927-934. DOI:10.1089/jmf.2017.4115.

EFSA (2010). Scientific Opinion on the substantiation of a health claim related to oat beta-glucan and lowering blood cholesterol and reduced risk of (coronary) heart disease pursuant to Article 14 of Regulation (EC) No 1924/2006 (EFSA Panel on Dietetic Products, Nutrition and Allergies/NDA) (Question no EFSA-Q-2008-681, adopted: 12 November 2010 by European Food Safety Authority). EFSA J 8(12):1885. [15 pp.] DOI:10.2903/j.efsa.2010.1885. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/1885.

EFSA (2011). Scientific Opinion on the safety of `Yeast *beta*-glucans' as a Novel Food ingredient (EFSA Panel on Dietetic Products, Nutrition and Allergies/NDA) (Question no EFSA-Q-2010-00952, adopted: 08 April 2011 by the European Food Safety Authority). EFSA J 9(5):2137 [22 pp]. DOI:10.2903/j.efsa.2011.2137. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/2137.

Eisa AAA, Aboelghar GES, Ammar IM, Metwally HG, Arafa SS (2018). Teratogenic effects induced by chitosan oligosaccharide in Wistar female rat *Rattus norvegicus*. Environ Sci Pollut Res 25(10):9371-9379. DOI:10.1007/s11356-018-1199-8.

Feletti F, De Bernardi di Valserra M, Contos S, Mattaboni P, Germogli R (1992). Chronic toxicity study on a new glucan extracted from Candida albicans in rats. Arzneimittelforschung 42(11):1363-1367.

Fukada Y, Kimura K, Ayaki Y (1991). Effect of chitosan feeding on intestinal bile acid metabolism in rats. Lipids 26(5):395-399. DOI:10.1007/BF02537206.

Gades MD, Stern JS (2005). Chitosan supplementation and fat absorption in men and women. J Am Diet Assoc 105(1):72-77. DOI:10.1016/j.jada.2004.10.004.

Gallaher CM, Munion J, Hesslink R, Wise J, Gallaher DD (2000). Cholesterol reduction by glucomannan and chitosan is mediated by changes in cholesterol absorption and bile acid and fat excretion in rats. J Nutr 130(11):2753-2759. DOI:10.1093/jn/130.11.2753.

Goy RC, de Britto D, Assis OBG (2009). A review of the antimicrobial activity of chitosan. Polímeros: Ciência e Tecnologia 19(3):241-247. DOI:10.1590/S0104-14282009000300013.

Goy RC, Morais STB, Assis OBG (2016). Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on *E. coli* and *S. aureus* growth. Rev Bras Farmacogn 26(1):122-127. DOI:10.1016/j.bjp.2015.09.010.

GRAS Associates, LLC (2012). *GRAS Assessment: ChitoClear® Shrimp-derived Chitosan. Food Usage Conditions for General Recognition of Safety*. (GRAS Notice GRN No. 443). Submitted for Siglufjordur, Iceland: Primex, ehf by Bend (OR): GRAS Associates, LLC to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=443 [Resubmission GRN No. 443; Feb. 11, 2013 – At notifier's request, FDA ceased to evaluate the notice].

Han LK, Kimura Y, Okuda H (1999). Reduction in fat storage during chitin-chitosan treatment in mice fed a high-fat diet. Int J Obes Relat Metab Disord 23(2):174-179. DOI:10.1038/sj.ijo.0800806.

Hernández-González SO, González-Ortiz M, Martínez-Abundis E, Robles-Cervantes JA (2010). Chitosan improves insulin sensitivity as determined by the euglycemic-hyperinsulinemic clamp technique in obese subjects. Nutr Res 30(6):392-395. DOI:10.1016/j.nutres.2010.06.005.

Ho SC, Tai ES, Eng PH, Tan CE, Fok AC (2001). In the absence of dietary surveillance, chitosan does not reduce plasma lipids or obesity in hypercholesterolaemic obese Asian subjects. Singapore Med J 42(1):6-10.

Hossain S, Rahman A, Kabir Y, Shams AA, Afros F, Hashimoto M (2007). Effects of shrimp (*Macrobracium rosenbergii*)-derived chitosan on plasma lipid profile and liver lipid peroxide levels in normo-and hypercholesterolaemic rats. Clin Exp Pharmacol Physiol 34(3):170-176. DOI:10.1111/j.1440-1681.2007.04568.x.

Hu S, Zheng C, Wang L, Wen X, Wang Y, Jiang Z (2018). Effects of low-molecular-weight chitosan on the growth performance, intestinal morphology, barrier function, cytokine expression and antioxidant system of weaned piglets. BMC Vet Res 14(1):215 [7pp]. DOI:10.1186/s12917-018-1543-8.

Huang H, Zou Y, Chi H (2018a). Quantitative assessment of the effects of chitosan intervention on blood pressure control. Drug Des Devel Ther 12:67-75. DOI:10.2147/DDDT.S148064.

Huang H, Zou Y, Chi H, Liao D (2018b). Lipid-modifying effects of chitosan supplementation in humans: a pooled analysis with trial sequential analysis. Mol Nutr Food Res 62(8):e1700842 [10pp, plus supplementary data]. DOI:10.1002/mnfr.201700842.

Huang H, Liao D, Zou Y, Chi H (2019). The effects of chitosan supplementation on body weight and body composition: a systematic review and meta-analysis of randomized controlled trials. Crit Rev Food Sci Nutr [online ahead of print - Apr. 16, 2019]. DOI:10.1080/10408398.2019.1602822.

Jaffer S, Sampalis JS (2007). Efficacy and safety of chitosan HEP-40 in the management of hypercholesterolemia: a randomized, multicenter, placebo-controlled trial. Altern Med Rev 12(3):265-273.

Jeong S, Min Cho J, Kwon Y-I, Kim S-C, Yeob Shin D, Ho Lee J (2019). Chitosan oligosaccharide (GO2KA1) improves postprandial glycemic response in subjects with impaired glucose tolerance and impaired fasting glucose and in healthy subjects: a crossover, randomized controlled trial. Nutr Diabetes 9(1):31 [9pp]. DOI:10.1038/s41387-019-0099-4.

JFCRF (2014). Chitosan. In: *List of Existing Food Additives* [Complied and published by the Ministry of Health and Welfare on April 16, 1996]. Tokyo, Japan: Ministry of Health, Labour and Welfare, Japan (MHLW) and Japan Food Chemical Research Foundation (JFCRF). Available at: http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/list-exst.add [Effective from January 30, 2014, Last update: 04/10/2014].

Jonker D, Kuper CF, Maquet V, Nollevaux G, Gautier S (2010). Subchronic (13-week) oral toxicity study in rats with fungal chitin-glucan from *Aspergillus niger*. Food Chem Toxicol 48(10):2695-2701. DOI:10.1016/j.fct.2010.06.042.

Jun SC, Jung EY, Kang DH, Kim JM, Chang UJ, Suh HJ (2010). Vitamin C increases the fecal fat excretion by chitosan in guinea-pigs, thereby reducing body weight gain. Phytother Res 24(8):1234-1241. DOI:10.1002/ptr.2970.

Jung W-K, Moon S-H, Kim S-K (2006). Effect of chitooligosaccharides on calcium bioavailability and bone strength in ovariectomized rats. Life Sci 78(9):970-976. DOI:10.1016/j.lfs.2005.06.006.

Kaats GR, Michalek JE, Preuss HG (2006). Evaluating efficacy of a chitosan product using a double-blinded, placebo-controlled protocol. J Am Coll Nutr 25(5):389-394. DOI:10.1080/07315724.2006.10719550.

Kim S-K, Park P-J, Yang H-P, Han S-S (2001). Subacute toxicity of chitosan oligosaccharide in Sprague-Dawley rats. Arzneimittelforschung 51(9):769-774. DOI:10.1055/s-0031-1300113.

Kim HJ, Ahn HY, Kwak JH, Shin DY, Kwon YI, Oh CG, et al. (2014). The effects of chitosan oligosaccharide (GO2KA1) supplementation on glucose control in subjects with prediabetes. Food Funct 5(10):2662-2669. DOI:10.1039/c4fo00469h.

Kimura Y, Nagata Y, Buddington RK (2004). Some dietary fibers increase elimination of orally administered polychlorinated biphenyls but not that of retinol in mice. J Nutr 134(1):135-142. DOI:10.1093/jn/134.1.135.

Kitozyme (2008) [unpublished]. *Final Report Ames Test B-00561: Chitosan (Cs). Confidential.* Prepared by Madrid, Spain: Vivotecnia Research S.L. for Cedex, France: Dermscan for submission to Herstal, Belgium: Kitozyme sa. Cited In: Kitozyme sa, 2011.

Kitozyme sa (2011). *Chitosan GRAS Notice*. (GRAS Notice GRN No. 397). Submitted by Herstal, Belguim: Kitozyme sa to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=397 [Dec. 19, 2011 – FDA has no questions].

Ko YT, Lin YL (2004). 1,3-*β*-glucan quantification by a fluorescence microassay and analysis of its distribution in foods. J Agric Food Chem 52(11):3313-3318. DOI:10.1021/jf0354085.

Kogan G, Skorik YA, Žitňanová I, Križková L, Ďuračková Z, Gomes CA, et al. (2004). Antioxidant and antimutagenic activity of N-(2-carboxyethyl)chitosan. Toxicol Appl Pharmacol 201(3):303-310. DOI:10.1016/j.taap.2004.05.009.

Kumirska J, Czerwicka M, Kaczynski Z, Bychowska A, Brzozowski K, Thöming J, et al. (2010). Application of spectroscopic methods for structural analysis of chitin and chitosan. Mar Drugs 8(5):1567-1636. DOI:10.3390/md8051567.

Lagarto A, Merino N, Valdes O, Dominguez J, Spencer E, De La Paz N, et al. (2015). Safety evaluation of chitosan and chitosan acid salts from {*Panurilus argu*} lobster. Int J Biol Macromol 72:1343-1350. DOI:10.1016/j.ijbiomac.2014.10.030.

Lan R, Li S, Chang Q, Zhao Z (2019). Chitosan oligosaccharides protect Sprague Dawley rats from cyclic heat stress by attenuation of oxidative and inflammation stress. Animals 9(12):1074 [11pp]. DOI:10.3390/ani9121074.

Landes DR, Bough WA (1976). Effects of chitosan—a coagulating agent for food processing wastes—in the diets of rats on growth and liver and blood composition. Bull Environ Contam Toxicol 15(5):555-563. DOI:10.1007/BF01685704.

Lattimer JM, Haub MD (2010). Effects of dietary fiber and its components on metabolic health. Nutrients 2(12):1266-1289. DOI:10.3390/nu2121266.

Lee B. Dexter and Assoc. (2005). *Shrimp-derived Chitosan GRAS Notification*. (GRAS Notice GRN No. 170). Prepared by Austin (TX): Lee B. Dexter and Assoc. and submitted by Siglufjordun, Iceland: Primex ehf to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=170 [resubmitted as GRN No. 443 Oct. 31, 2005 - At notifier's request, FDA ceased to evaluate the notice].

Lee KY, Shibutani M, Takagi H, Arimura T, Takigami S, Uneyama C, et al. (2004). Subchronic toxicity study of dietary *N*-acetylglucosamine in F344 rats. Food Chem Toxicol 42(4):687-695. DOI:10.1016/j.fct.2003.12.005.

Li J, Wu Y, Zhao L (2016). Antibacterial activity and mechanism of chitosan with ultra high molecular weight. Carbohydr Polym 148:200-205. DOI:10.1016/j.carbpol.2016.04.025.

Liu N, Chen X-G, Park H-J, Liu C-G, Liu C-S, Meng X-H, et al. (2006). Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli.* Carbohydr Polym 64(1):60-65. DOI:10.1016/j.carbpol.2005.10.028.

Maezaki Y, Tsuji K, Nakagawa Y, Kawai Y, Akimoto Y, Akimoto M, et al. (1993). Hypocholesterolemic effect of chitosan in adult males. Biosci Biotechnol Biochem 57(9):1439-1444. DOI:10.1271/bbb.57.1439.

MFDS (2017). 70. Chitosan. In: *Korea Food Additives Code*. (All designated chemicals and some natural additives are currently regulated by Food Additives Code. It includes specifications, standards and general test methods for each additives). Chungcheongbuk-do, South Korea.: Ministry of Food and Drug Safety (MFDS), pp. 1238-1239.

Mhurchu CN, Poppitt SD, Mcgill A-T, Leahy FE, Bennett DA, Lin RB, et al. (2004). The effect of the dietary supplement, chitosan, on body weight: a randomised controlled trial in 250 overweight and obese adults. Int J Obes Relat Metab Disord 28(9):1149-1156. DOI:10.1038/sj.ijo.0802693.

Moon MS, Lee MS, Kim CT, Kim Y (2007). Dietary chitosan enhances hepatic CYP7A1 activity and reduces plasma and liver cholesterol concentrations in diet-induced hypercholesterolemia in rats. Nutr Res Pract 1(3):175-179. DOI:10.4162/nrp.2007.1.3.175.

Neyrinck AM, Bindels LB, De Backer F, Pachikian BD, Cani PD, Delzenne NM (2009). Dietary supplementation with chitosan derived from mushrooms changes adipocytokine profile in diet-induced obese mice, a phenomenon linked to its lipid-lowering action. Int Immunopharmacol 9(6):767-773. DOI:10.1016/j.intimp.2009.02.015.

NIH (2020). Search results for "Chitosan". In: *Dietary Supplements Labels Database (DSLD)*. Bethesda (MD): National Library of Medicine (NLM) / National Institutes of Health (NIH), Office of Dietary Supplements. Available at: https://dsld.nlm.nih.gov/dsld/ [Version 7.0.12 – August 2020, 19e5a30; Last accessed: January 4, 2020].

NTP (2017). *NTP Technical Report on the Toxicity Study of Chitosan (CAS No. 9012-76-4) Administered in Feed to Sprague Dawley [Crl:CD(SD)] Rats*. (NTP Toxicity Report Series, no 93). Research Triangle Park (NC): National Toxicology Program (NTP). Available at: https://ntp.niehs.nih.gov/ntp/htdocs/st_rpts/tox093_508.pdf.

OECD (1998a). *OECD Principles of Good Laboratory Practice*. (Series on Principles of Good Laboratory Practice and Compliance Monitoring, no. 1 [ENV/MC/CHEM(98)17]). Paris, France: Organisation for Economic Co-Operation & Development (OECD), Environment Directorate, Chemicals Group and Management Committee, OECD Environmental Health and Safety Publications. Available at: http://www.oecd-ilibrary.org/environment/oecd-principles-on-good-laboratory-practice_9789264078536-en [As revised in 1997].

OECD (1998b). Repeated dose 90-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline no 408) [Updated & Adopted: 21 September 1998]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en [New Version Updated & Adopted 25 June 2018].

Ohe T (1996). Antigenotoxic activities of chitin and chitosan as assayed by sister chromatid exchange. Sci Total Environ 181(1):1-5. DOI:10.1016/0048-9697(95)04949-5.

Omara EA, Aly HF, Nada SA (2012). Chitosan induced hepato-nephrotoxicity in mice with special reference to gender effect in glycolytic enzymes activities. Regul Toxicol Pharmacol 62(1):29-40. DOI:10.1016/j.yrtph.2011.11.010.

Omura Y, Shigemoto M, Akiyama T, Saimoto H, Shigemasa Y, Nakamura I, et al. (2003). Antimicrobial activity of chitosan with different degrees of acetylation and molecular weights. Biocontrol Sci 8(1):25-30. DOI:10.4265/bio.8.25.

Ormrod DJ, Holmes CC, Miller TE (1998). Dietary chitosan inhibits hypercholesterolaemia and atherogenesis in the apolipoprotein E-deficient mouse model of atherosclerosis. Atherosclerosis 138(2):329-334. DOI:10.1016/S0021-9150(98)00045-8.

Pan H, Fu C, Huang L, Jiang Y, Deng X, Guo J, et al. (2018). Anti-obesity effect of chitosan oligosaccharide capsules (COSCs) in obese rats by ameliorating leptin resistance and adipogenesis. Mar Drugs 16(6):198 [16pp]. DOI:10.3390/md16060198.

Pittler MH, Abbot NC, Harkness EF, Ernst E (1999). Randomized, double-blind trial of chitosan for body weight reduction. Eur J Clin Nutr 53(5):379-381. DOI:10.1038/sj.ejcn.1600733.

Qian M, Lyu Q, Liu Y, Hu H, Wang S, Pan C, et al. (2019). Chitosan oligosaccharide ameliorates nonalcoholic fatty liver disease (NAFLD) in diet-induced obese mice. Mar Drugs 17(7):391 [14pp]. DOI:10.3390/md17070391.

Qin C, Gao J, Wang L, Zeng L, Liu Y (2006). Safety evaluation of short-term exposure to chitooligomers from enzymic preparation. Food Chem Toxicol 44(6):855-861. DOI:10.1016/j.fct.2005.11.009.

Raafat D, von Bargen K, Haas A, Sahl HG (2008). Insights into the mode of action of chitosan as an antibacterial compound. Appl Environ Microbiol 74(12):3764-3773. DOI:10.1128/AEM.00453-08.

Schiller RN, Barrager E, Schauss AG, Nichols EJ (2001). A randomized, double-blind, placebo-controlled study examining the effects of a rapidly soluble chitosan dietary supplement on weight loss and body composition in overweight and mildly obese individuals. J Am Nutraceut Assoc 4(1):42-49.

Schimpf U, Nachmann G, Trombotto S, Houska P, Yan H, Björndahl L, et al. (2019). Assessment of oligo-chitosan biocompatibility toward human spermatozoa. ACS Appl Mater Interfaces 11(50):46572-46584. DOI:10.1021/acsami.9b17605.

Sugano M, Watanabe S, Kishi A, Izume M, Ohtakara A (1988). Hypocholesterolemic action of chitosans with different viscosity in rats. Lipids 23(3):187-191. DOI:10.1007/BF02535456.

Sumiyoshi M, Kimura Y (2006). Low molecular weight chitosan inhibits obesity induced by feeding a high-fat diet long-term in mice. J Pharm Pharmacol 58(2):201-207. DOI:10.1211/jpp.58.2.0007.

Takahashi M, Inoue K, Yoshida M, Morikawa T, Shibutani M, Nishikawa A (2009). Lack of chronic toxicity or carcinogenicity of dietary *N*-acetylglucosamine in F344 rats. Food Chem Toxicol 47(2):462-471. DOI:10.1016/j.fct.2008.12.002.

Tanaka Y, Tanioka S, Tanaka M, Tanigawa T, Kitamura Y, Minami S, et al. (1997). Effects of chitin and chitosan particles on BALB/c mice by oral and parenteral administration. Biomaterials 18(8):591-595. DOI:10.1016/s0142-9612(96)00182-2.

Tang D, Wang Y, Kang W, Zhou J, Dong R, Feng Q (2020). Chitosan attenuates obesity by modifying the intestinal microbiota and increasing serum leptin levels in mice. J Funct Foods 64:103659 [10pp, plus supplementary data]. DOI:10.1016/j.jff.2019.103659.

Tao W, Sun W, Liu L, Wang G, Xiao Z, Pei X, et al. (2019). Chitosan oligosaccharide attenuates nonalcoholic fatty liver disease induced by high fat diet through reducing lipid accumulation, inflammation and oxidative stress in C57BL/6 mice. Mar Drugs 17(11):645 [15pp]. DOI:10.3390/md17110645.

Tapola NS, Lyyra ML, Kolehmainen RM, Sarkkinen ES, Schauss AG (2008). Safety aspects and cholesterol-lowering efficacy of chitosan tablets. J Am Coll Nutr 27(1):22-30. DOI:10.1080/07315724.2008.10719671.

Teodoro JS, Gomes AP, Varela AT, Duarte FV, Rolo AP, Palmeira CM (2016). Hepatic and skeletal muscle mitochondrial toxicity of chitosan oligosaccharides of normal and diabetic rats. Toxicol Mech Methods 26(9):650-657. DOI:10.1080/15376516.2016.1222643.

Terada A, Hara H, Sato D, Higashi T, Nakayama S, Tsuji K, et al. (1995). Effect of dietary chitosan on faecal microbiota and faecal metabolites of humans. Microb Ecol Health Dis 8(1):15-21. DOI:10.3109/08910609509141377.

Torzsas TL, Kendall CW, Sugano M, Iwamoto Y, Rao AV (1996). The influence of high and low molecular weight chitosan on colonic cell proliferation and aberrant crypt foci development in CF1 mice. Food Chem Toxicol 34(1):73-77. DOI:10.1016/0278-6915(95)00083-6.

Trivedi VR, Satia MC, Deschamps A, Maquet V, Shah RB, Zinzuwadia PH, et al. (2016). Single-blind, placebo controlled randomised clinical study of chitosan for body weight reduction. Nutr J 15:3 [12pp]. DOI:10.1186/s12937-016-0122-8.

U.S. FDA (1993). Appendix I. Table 14. Conversion table for test chemical treatment doses used in PAFA. In: . *Priority Based Assessment of Food Additives (PAFA) Database*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: https://web.archive.org/web/20170215164333/http://www.fda.gov/ohrms/DOCKETS/DOCKETS/95s0316/95s-0316-Rpt0272-36-Appendix-D-Reference-F-FDA-vol205.pdf.

U.S. FDA (2002). *Agency Response Letter GRAS Notice No. GRN 000073 [Shrimp-derived chitosan, Avaldsnes, Norway: Primex Ingredients ASA]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=73 [Jan 31, 2002 - At notifier's request, FDA ceased to evaluate the notice].

U.S. FDA (2005). *Agency Response Letter GRAS Notice No. GRN 000170 [Shrimp-derived Chitosan], Siglufjordur, Iceland: Primex ehf]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=170 [resubmitted as GRN No. 443; Oct. 31, 2005 - At notifier's request, FDA ceased to evaluate the notice].

U.S. FDA (2008a). *Agency Response Letter GRAS Notice No. GRN 000239 [Bakers yeast beta-glucan, Eagan (MN): Biothera, Inc.]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=239 [July 22, 2008 – FDA has no questions].

U.S. FDA (2008b). Food labeling: health claims; soluble fiber from certain foods and risk of coronary heart disease. Interim final rule. Fed Regist (US) 73(37):9938-9947.

U.S. FDA (2010). *Agency Response Letter GRAS Notice No. GRN 000309 [Beta-glucan derived from Aureobasidium pullulan, South Korea: Glucan Corporation, Limited Marine Bio Center 221, Silla University]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=309 [June 14, 2010 – FDA has no questions].

U.S. FDA (2011). *Agency Response Letter GRAS Notice No. GRN 000397 [Chitosan from Aspergillus niger, Herstal, Belgium: KitoZyme sa]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=397 [Dec. 19, 2011 – FDA has no questions].

U.S. FDA (2013a). *Agency Response Letter GRAS Notice No. GRN 000443 [Shrimp-derived chitosan, Siglufjordur, Iceland: Primex ehf]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=443 [Resubmission of GRN No. 170; Feb. 11, 2013 - At notifier's request, FDA ceased to evaluate the notice].

U.S. FDA (2013b). *Agency Response Letter GRAS Notice No. GRN 000437 [Beta-glucans from oat bran, Exeter (CA): Garuda International, Inc.]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=437 [Jan. 27, 2013 – FDA has no questions].

U.S. FDA (2015). *Agency Response Letter GRAS Notice No. GRN 000544 [β-glucans from oat bran, Hoffman Estates (IL): Tate and Lyle]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=544 [May 5, 2015 – FDA has no questions].

U.S. FDA (2020a). Part 170—Food additives. Section §170.3—Definitions. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs* (Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/2019/title21 [current to 4-1-20, last updated 4-1-19].

U.S. FDA (2020b). Part 170—Food additives. Section §170.30—Eligibility for classification as generally recognized as safe (GRAS). In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs* (Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/2019/title21 [current to 4-1-20, last updated 4-1-19].

USDA (2018). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2015-2016.* Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release [Last Modified: July 31, 2018].

USP (2020). Chitosan. In: *The United States Pharmacopeia (USP 43) / The National Formulary (NF 38).* Rockville (MD): United States Pharmacopeial Convention, Inc. [online subscription].

Wan J, Xu Q, He J (2018). Maternal chitosan oligosaccharide supplementation during late gestation and lactation affects offspring growth. Ital J Anim Sci 17(4):994-1000. DOI:10.1080/1828051X.2018.1435313.

Wan J, Zhang J, Wu G, Chen D, Yu B, Huang Z, et al. (2019). Amelioration of enterotoxigenic *Escherichia coli*-induced intestinal barrier disruption by low-molecular-weight chitosan in weaned pigs is related to suppressed intestinal inflammation and apoptosis. Int J Mol Sci 20(14):3485 [19pp]. DOI:10.3390/ijms20143485.

Wang J, Zhang C, Guo C, Li X (2019a). Chitosan ameliorates DSS-induced ulcerative colitis mice by enhancing intestinal barrier function and improving microflora. Int J Mol Sci 20(22):5751 [12pp]. DOI:10.3390/ijms20225751.

Wang Z, Zhang F, Yan Y, Zhang Z, Wang L, Qin C (2019b). Lipid-lowering activities of chitosan and its quaternary ammonium salt for the hyperlipidemia rats induced by high-fat diets. Int J Biol Macromol 132:922-928. DOI:10.1016/j.ijbiomac.2019.03.214.

WHO (1987). Test procedures and evaluations. 5.5.3. Toxicological versus physiological responses. In: *Principles for the Safety Assessment of Food Additives and Contaminants in Food*. (Environmental health criteria, no 70). Geneva, Switz.: World Health Organization (WHO), International Programme on Chemical Safety (IPCS), p. 82. Available at: http://www.inchem.org/documents/ehc/ehc/ehc70.htm.

Wisker E, Bach Knudsen KE, Daniel M, Eggum BO, Feldheim W (1997). Energy values of non-starch polysaccharides: comparative studies in humans and rats. J Nutr 127(1):108-116. DOI:10.1093/jn/127.1.108.

Xiong Y, Li X, Xiong M, Vikash S, Liu P, Wang M, et al. (2018). Chitosan combined with swimming promotes health in rats. Int J Biol Macromol 118:2092-2097. DOI:10.1016/j.ijbiomac.2018.07.067.

Xu Y, Wang Z, Wang Y, Yan S, Shi B (2018). Effects of chitosan as growth promoter on diarrhea, nutrient apparent digestibility, fecal microbiota and immune response in weaned piglets. J Appl Anim Res 46(1):1437-1442. DOI:10.1080/09712119.2018.1531763.

Yang Y, Hu W, Wang X, Gu X (2007). The controlling biodegradation of chitosan fibers by N-acetylation in vitro and in vivo. J Mater Sci Mater Med. 18(11):2117-2121. DOI:10.1007/s10856-007-3013-x.

Yao HT, Lii CK, Chou RH, Lin JH, Yang HT, Chiang MT (2010). Effect of chitosan on hepatic drug-metabolizing enzymes and oxidative stress in rats fed low- and high-fat diets. J Agric Food Chem 58(8):5187-5193. DOI:10.1021/jf903857m.

Yao HT, Luo MN, Hung LB, Chiang MT, Lin JH, Lii CK, et al. (2012). Effects of chitosan oligosaccharides on drug-metabolizing enzymes in rat liver and kidneys. Food Chem Toxicol 50(5):1171-1177. DOI:10.1016/j.fct.2012.02.022.

Yoon HJ, Park HS, Bom HS, Roh YB, Kim JS, Kim YH (2005). Chitosan oligosaccharide inhibits $^{203}HgCl_2$-induced genotoxicity in mice: micronuclei occurrence and chromosomal aberration. Arch Pharm Res 28(9):1079-1085 [erratum, 28(10):1203]. DOI:10.1007/BF02977405.

Zeng L, Qin C, He G, Wang W, Li W, Xu D (2008). Effect of dietary chitosans on trace iron, copper and zinc in mice. Carbohydr Polym 74(2):279-282. DOI10.1016/j.carbpol.2008.02.022.

Zhang J, Wan J, Wu G, Chen D, Yu B, Huang Z, et al. (2020). Low-molecular-weight chitosan relieves enterotoxigenic Escherichia coli-induced growth retardation in weaned pigs. Int Immunopharmacol 78:105798. DOI:10.1016/j.intimp.2019.105798.

Zhao Y, Xu G, Wang S, Yi X, Wu W (2018). Chitosan oligosaccharides alleviate PM2.5-induced lung inflammation in rats. Environ Sci Pollut Res 25(34):34221-34227. DOI:10.1007/s11356-018-3365-4.

APPENDIX A

FEMA-GRAS Approved Uses

The Expert Panel of the Flavor and Extract Manufacturers Association of the United States

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June 10, 2019

Ernst Wagner, Ph.D.
Group Lead
Regulatory Affairs & Quality Management
Personal Care & Aroma Chemicals
Global Product Registration & Quality Management
DSM Nutritional Products Ltd.
Wurmisweg 576
4303 Kaiseraugst
Switzerland

Dear Dr. Wagner:

Your application and supporting information regarding the proposed new uses of 2-amino-2-deoxy-poly-*D*-glucosamine (FEMA 4946; CAS 9012-76-4) was reviewed by the FEMA Expert Panel during its May 2019 meeting. It was the decision of the Panel to recognize the uses of the substance as a flavor ingredient with modifying properties as GRAS in the food categories and at the use levels specified in the attached table. The additional food categories for 2-amino-2-deoxy-poly-*D*-glucosamine are scheduled to be published in GRAS 30.

It is only the use of 2-amino-2-deoxy-poly-*D*-glucosamine for the technical effect of flavoring that is considered GRAS by the FEMA Expert Panel. The technical effect of flavoring includes the ability to impart or modify the perception of flavor. Technical effects other than that of flavoring achieved in the finished food must have separate determinations of regulatory authority to use.

Significant changes in use levels within an approved category, or use in new food categories, require a re-evaluation of this material by the Expert Panel. Re-evaluation may also be required if there is a significant change in the composition or production method of the product in commerce.

Any new data, either collected by your company or that your company becomes aware of, that is relevant to the safety evaluation of the material should be provided to the FEMA Expert Panel. The Expert Panel reserves the right to re-evaluate the GRAS status of this substance if new relevant data becomes available or if there is a significant increase in the annual volume of use of this substance.

The regulations regarding the proper labeling of flavoring substances in the United States and foods containing them can be found in the Food and Drug Administration's regulations at 21 CFR 101.22. Please contact John Hallagan, the Expert Panel's Legal Advisor, for additional information on labeling matters (Hondobear@aol.com; 202.331.2333).

If you have any questions or comments, please feel free to contact me.

Sincerely,

Sean V. Taylor, Ph.D.
Scientific Secretary to the FEMA Expert Panel

Use Levels and Food Categories

Name 2-Amino-2-deoxy-poly-D-glucosamine

FEMA No: 4946

Food Category	Average Usual Use Level (ppm)	Average Maximum Use Level (ppm)
Baked Goods	1500	2000
Beverages Type I, Non-alcoholic	1500	2000
Beverages Type II, Alcoholic	1500	2000
Breakfast Cereals	0	0
Cheese	1500	2000
Chewing Gum	1500	2000
Condiments and Relishes	1500	2000
Confectionery and Frostings	1500	2000
Egg Products	0	0
Fats and Oils	1500	2000
Fish Products	1500	2000
Frozen Dairy	0	0
Fruit Ices	0	0
Gelatins and Puddings	1500	2000
Granulated Sugar	0	0
Gravies	1500	2000
Hard Candy	0	0
Imitation Dairy Products	1500	2000
Instant Coffee and Tea	1500	2000
Jams and Jellies	1500	2000
Meat Products	1500	2000
Milk Products	1500	2000
Nut Products	0	0
Other grains	1500	2000
Poultry	0	0
Processed Fruits	1500	2000
Processed Vegetables	1500	2000
Reconstituted Vegetable Protein	1500	2000
Seasonings and Flavors	1500	2000
Snack Foods	0	0
Soft Candy	1500	2000
Soups	1500	2000
Sugar Substitutes	1500	2000
Sweet Sauce	1500	2000

APPENDIX B

GRAS Panel Consensus Statement

GRAS Panel Statement Concerning the Generally Recognized as Safe (GRAS) Status of the Proposed Uses of Fiber Extracted from White Button Mushrooms for Use as an Antimicrobial Ingredient

11 January 2021

INTRODUCTION

At the request of Chinova Bioworks (Chinova), a panel of independent scientists (the "GRAS Panel"), qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information related to Chinova's fiber extracted from white button mushrooms (*Agaricus bisporus*) (Chiber™) to determine whether the intended use as an antimicrobial ingredient in various food and beverage products would be Generally Recognized as Safe (GRAS) based on scientific procedures. For purposes of the GRAS Panel's evaluation, "safe" or "safety" indicates that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2020).

The GRAS Panel consisted of the below-signed qualified scientific experts: Professor Emeritus Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine); Professor Emeritus George C. Fahey (University of Illinois), and Professor Eric A. Johnson (University of Wisconsin-Madison). The GRAS Panel was selected and convened in accordance with the United States (U.S.) Food and Drug Administration's (FDA's) guidance for industry on *Best Practices for Convening a GRAS Panel* (U.S. FDA, 2017). Chinova ensured that all reasonable efforts were made to identify and select a balanced GRAS Panel with expertise in food safety, toxicology, and microbiology. Efforts were placed on identifying conflicts of interest or relevant "appearance issues" that could potentially bias the outcome of the deliberations of the GRAS Panel; no such conflicts of interest or appearance issues were identified. The GRAS Panel received an honorarium as compensation for their time; the honorarium provided to the GRAS Panel was not contingent upon the outcome of their deliberations.

The GRAS Panel, independently and collectively, critically evaluated a comprehensive package of all publicly available scientific data and information compiled from a comprehensive search of the scientific literature conducted by Chinova, which included all available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food uses of Chinova's fiber extracted from white button mushrooms. The data evaluated by the GRAS Panel included the method of manufacture and product specifications, analytical data, intended use and use levels in specified food products, consumption estimates, and generally available safety information on chitosan obtained from a comprehensive search of the literature using several online databases. The GRAS Panel also evaluated other publicly available information, as considered appropriate.

Following its independent and collaborative critical evaluation of the data and information, the GRAS Panel concluded that under the conditions of intended use described herein, Chinova's fiber derived from white button mushrooms, meeting appropriate food-grade specifications, and manufactured consistent with current Good Manufacturing Practice (cGMP), is GRAS based on scientific procedures. A summary of the basis for the GRAS Panel's conclusion is provided below.

IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECTS

Chitosan is a biopolymer derived from the deacetylation of chitin, a naturally occurring carbohydrate polymer that is widely distributed in nature (*e.g.*, crustacean shells, fungal cell walls). Chitosan is characterized as a linear polycationic polysaccharide composed of glucosamine and N-acetyl glucosamine molecules linked *via* β-1,4-linkages. The level of deacetylation of chitin during the manufacture of chitosan will vary depending on the manufacturing conditions; however, typical chitosan products [*e.g.*, United States Pharmacopeia (USP)-grade chitosan] generally display a deacetylation level of greater than 60%. Fiber manufactured by Chinova is a mixture of chitosan and *beta*-1,3-D-glucans. Chitosan is the main component, representing 95% of the total volume, and is a soluble polymer derived from the cell walls of non-genetically modified *A. bisporus* (white button mushroom) biomass with a molecular weight (MW) range of 10 to 400 kDa[1].

Chinova has stated:

> "*Chiber™ is manufactured consistent with cGMP and that all raw materials, processing aids, and food contact articles used in the manufacture of Chiber™ are food-grade and permitted for their respective uses in accordance with appropriate federal regulations, have been previously determined to be GRAS, or have been the subject of an effective food contact notification*".

The mushroom or fungal biomass is initially inspected for conformity to the company's raw material standards [heavy metal content, moisture content, microbiology (total aerobic plate count, yeast, and molds), and visual appearance], and upon approval, it undergoes a thermal alkali hydrolysis process resulting in the removal of acetyl groups from chitin and simultaneous hydrolysis of proteins and saponification of lipids. The other downstream processes include pH adjustment, washing, centrifugation, heating, and drying. The final dried chitosan is milled into a fine powder and held for quality control analysis. Subject to approval from the analysis, the chitosan is then packaged and stored. The GRAS Panel noted that the manufacturing procedures applied during the production of Chinova's fiber derived from white button mushrooms use processing aids and food processing conditions that are common to the food industry (*e.g.*, acid/base treatment, heating, pH balancing, and drying).

The GRAS Panel noted that contamination of soil with *Clostridium* and *Bacillus* spores is common and, therefore, the presence of microbial spores on mushrooms is expected from typical production methods (Notermans *et al*., 1989). Chinova stated that the production conditions for Chinova's fiber derived from white button mushrooms involve heat treatment of the fungal biomass sources in the presence of caustic soda. As these chemical reaction processes are sufficient to fully degrade the cell wall components of the biomass, microbial spores would not be expected to survive intact in this environment. Residual metabolites or other small molecule impurities also would be removed during the wash steps.

[1] Chitosan in this molecular weight range is considered low molecular weight chitosan (LMWC).

Chemical analysis of 5 lots of Chinova's fiber derived from white button mushrooms demonstrates that the manufacturing process produces a consistent product that meets the ingredient chemical and microbiological specifications, regardless of the source of chitin. The degree of deacetylation was approximately 95%, while the molecular weight average was approximately 60 kDa. Microbiological analysis of the 5 lots of Chinova's fiber derived from white button mushrooms also demonstrates that the product consistently meets the microbiological specifications.

The stability of Chinova's fiber derived from white button mushrooms was tested under Chinova's recommended storage conditions (temperature: 25±2°C; relative humidity: 60±5%) and accelerated conditions (temperature: 40±2°C; relative humidity: 70±5%) using 3 non-consecutive lots of the chitosan product. The results after 3, 6, and 9 months are within the specification limits, demonstrating the suitable stability of Chinova's fiber derived from white button mushrooms for at least 9 months.

Chinova presented results of analytical data on Chinova's fiber derived from white button mushrooms demonstrating that the ingredients are qualitatively and quantitatively equivalent to traditional crustacean-derived chitosan used in food and pharmaceutical preparations, and to a chitosan standard meeting USP specifications (also from a crustacean source). This data included analyses conducted using Fourier-transform infrared spectroscopy (FT-IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy by the Department of Chemistry at the University of New Brunswick, Canada. The GRAS Panel agreed that this information demonstrated that Chinova's fiber derived from white button mushrooms is chemically comparable to chitosan samples produced from crustacean sources and Chinova's fiber derived from white button mushrooms can be concluded to be food-grade quality on the basis of the qualitative analyses and conformance to the USP specifications for shellfish chitosan.

INTENDED USE AND ESTIMATED EXPOSURE

Chinova's fiber extracted from white button mushrooms (*Agaricus bisporus*), comprised of chitosan and beta-glucan, is intended for use as an antimicrobial ingredient, as defined under 21 CFR §170.3(o)(2), in select food and beverage products in the U.S. A summary of the food categories and use levels in which the mushroom-derived fiber is intended for use is provided in Table 1 below. The proposed food uses for Chinova's fiber extracted from white button mushrooms (*A. bisporus*) are similar to those that have previously received GRAS status by the Flavor and Extract Manufacturers Association (FEMA) for use as an ingredient with flavor modifying properties (FEMA No. 4946) at levels up to 2,000 ppm. The use levels of Chinova's fiber derived from white button mushrooms for use as an antimicrobial ingredient range from 0.01 to 0.150 g/100 g (equivalent to 100 to 1,500 ppm), which are much lower than the FEMA GRAS-approved use levels, which range from 1,500 to 2,000 ppm.

Table 1 Summary of the Individual Proposed Food Uses and Use Levels for Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020)	Food Uses[a]	Proposed Use Levels (g/100 g)
Baked Goods and Baking Mixes	Bagels and English muffins	0.100
	Bread (excluding sweet type breads and rolls)	0.100
	Cakes	0.100
	Light weight cakes	0.100
	Medium weight cakes	0.100
	Heavy weight cakes	0.100

Table 1 Summary of the Individual Proposed Food Uses and Use Levels for Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020)	Food Uses[a]	Proposed Use Levels (g/100 g)
	Cornbread, corn muffins, or tortillas	0.100
	Croissants	0.100
	Doughnuts (Donuts)	0.100
	French toast, pancakes, and waffles	0.100
	Muffins	0.100
	Pastries	0.100
	Pies	0.100
Beverages, Alcoholic	Cocktail drinks	0.040
Beverages and Beverage Bases	Energy drinks	0.040
	Enhanced or fortified waters	0.040
	Flavored or carbonated waters	0.040
	Soft drinks (regular and diet)	0.040
	Sport or electrolyte drinks, fluid replacement drinks	0.040
Cheeses	Cheese-based sauces	0.100
	Cottage cheese	0.100
	Cream cheese and cheese-based spreads	0.100
	Natural cheese	0.150
	Processed cheese or cheese mixtures	0.150
Coffee and Tea	Ready-to-drink coffees	0.015
	Ready-to-drink tea beverages	0.040
Condiments and Relishes	Ketchup	0.040
	Mustard	0.040
	Relish	0.080
Confections and Frostings	Coatings	0.100
	Frostings and icings	0.040
Dairy Product Analogs	Imitation cheese	0.150
Fats and Oils	Fat-based sauces	0.100
	Margarine and margarine-like spreads	0.100
	Mayonnaise and mayonnaise-type dressings	0.100
	Salad dressings	0.100
Gelatins, Puddings, and Fillings	Flans, custards, and other egg-based desserts	0.080
Grain Products and Pastas	Cereal and granola bars	0.020
	Energy bars or protein bars or meal replacement bars	0.020
	Macaroni and noodle products	0.020
Gravies and Sauces	Gravies	0.020
	Tomato-based sauces	0.020
	White sauces	0.100
Jams and Jellies	Jams, jellies, preserves, and marmalades	0.100
Milk Products	Plain or flavored yogurt	0.100
Processed Fruits and Fruit Juices	Fruit drinks and ades and smoothies	0.060
	Fruit juices	0.060

Table 1 Summary of the Individual Proposed Food Uses and Use Levels for Chinova's Fiber Extracted from White Button Mushrooms (*Agaricus bisporus*) in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2020)	Food Uses[a]	Proposed Use Levels (g/100 g)
	Fruit nectars	0.060
	Fruit-based desserts	0.080
Plant Protein Products	Meat analogs	0.150
Processed Vegetables and Vegetable Juices	Vegetable juices	0.040
	Vegetable purees[b]	0.040
Soups and Soup Mixes	Prepared and canned soups	0.040
Sugar Substitutes	Sugar substitutes	0.100
Sweet sauces, toppings, and syrups	Sweet sauces, syrups, and toppings (including fruit-based)	0.100
	Cocoa syrups	0.100

CFR = *Code of Federal Regulations*; U.S. = United States.
[a] Chinova's mushroom-derived fiber is intended for use in unstandardized products when standards of identity, as established under 21 CFR §130 to 169, do not permit its addition.
[b] Food codes for vegetable mixtures and vegetable combinations (which are likely to be used to make purees) were included as a surrogate for 'vegetable purees'.

The technological function of Chinova's fiber derived from white button mushrooms as a preservative was evaluated by Chinova in carbonated soda, ready-to-drink tea, liquid sweetener, bread, and yogurt. Chinova's fiber derived from white button mushrooms at 50 ppm was shown to decrease microbial counts in inoculated products and prevent mold growth in products with no preservatives. The antimicrobial properties of chitosan have been researched for several decades and it has been reported to have bactericidal and/or bacteriostatic effects against a range of microbes, including yeast, bacteria, and fungi (Raafat *et al*., 2008; Goy *et al*., 2009).

The estimated daily intake of mushroom-derived chitosan from proposed antimicrobial food uses in combination with the maximum FEMA GRAS-approved use level of 2,000 ppm was evaluated on an absolute basis (mg/person/day), and on a body weight basis (mg/kg body weight/day). Among the total population (all ages), the mean and 90th percentile consumer-only intakes of mushroom-derived chitosan were determined to be 1.3 and 2.6 g/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest mean and 90th percentile consumer-only intakes of mushroom-derived chitosan on an absolute basis, at 1.6 and 3.2 g/person/day, respectively; while infants and toddlers had the lowest mean and 90th percentile consumer-only intakes of 0.5 and 1.0 g/person/day, respectively. On a body weight basis, the total population (all ages) mean and 90th percentile consumer-only intakes of mushroom-derived chitosan were determined to be 19.8 and 40.2 mg/kg body weight/day, respectively. Among the individual population groups, young children were identified as having the highest mean consumer-only intakes of 48.7 mg/kg body weight/day, while infants and toddlers had the highest 90th percentile consumer-only intakes of 93.0 mg/kg body weight/day. Female adults had the lowest mean and 90th percentile consumer-only intakes of 15.2 and 30.0 mg/kg body weight/day, respectively.

DATA PERTAINING TO ASSESSMENT OF SAFETY

History of Use

Crustacean-derived chitosan has a long history of safe use in the food supply. It is currently approved/permitted for use as a natural food additive for general food use in Japan and Korea (JFCRF, 2014; MFDS, 2017) and has widespread use as a drug excipient, functional food ingredient, and dietary supplement product in the U.S., the European Union, and other countries. Supplement products containing chitosan typically promote consumption of 1 to 5 g/person/day for use in weight control and/or maintenance of cardiovascular health (NIH, 2020).

Primex Ingredients ASA (Primex) submitted a GRAS Notice to the U.S. FDA in 2001 regarding the GRAS conclusion of its shrimp-derived chitosan for use in foods for various purposes, including as an antimicrobial, emulsifier, processing aid, antioxidant, dough strengthening-aid, and texturizing ingredient (GRN 73 – U.S. FDA, 2002). At the notifier's request, the Agency ceased to evaluate the notice. Primex resubmitted the GRAS Notice in 2005 and then again in 2012, each time requesting the Agency to cease evaluating the notice (GRN 170 – U.S. FDA, 2005; GRN 443 – U.S. FDA, 2013). The reasons for the retractions were not published.

KitoZyme submitted a GRAS Notice to the U.S. FDA in 2011 regarding the GRAS conclusion of its insoluble fungal-derived chitosan for use as a secondary direct food ingredient in alcoholic beverage production at levels between 10 and 500 g/100 L. KitoZyme's ingredient was filed by the Agency without objection as GRAS Notice 397 (U.S. FDA, 2011).

Absorption, Distribution, Metabolism, Elimination

Chitosan and *beta*-1,3-D-glucans are not subject to hydrolytic digestion and are not absorbed. Therefore, systemic exposure to chitosan following ingestion is highly unlikely. Chitosan may be subject to microbial fermentation in the gastrointestinal tract, resulting in normal non-toxic fermentation products associated with the digestion of common non-digestible dietary fibers.

Toxicological Studies

Acute toxicity studies in rats demonstrated the low acute oral toxicity of chitosan (GRN 397 – U.S. FDA, 2011; Lagarto *et al.*, 2015). The approximate lethal dose of chitosan (approximately 309 kDa, and a degree of deacetylation of 83%; compositionally equivalent to Chinova's fiber derived from white button mushrooms) in rats was reported to be >2,000 mg/kg (Lagarto *et al.*, 2015).

The GRAS Panel also reviewed the results of several repeated-dose oral toxicity studies conducted with chitosan derived from crustacean sources that were investigated in mice, rats, and guinea pigs. The test articles investigated in these studies were reported as low molecular weight chitosan (LMWC) or high molecular weight chitosan (HMWC), chitin-chitosan (containing 80% chitosan), or water-soluble chitosan. A number of studies reported statistically significant changes in liver weight and liver enzymes [*e.g.*, aspartate transaminase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT)] that may suggest hepatic effects in mice, rats, and guinea pigs. In a subchronic oral toxicity study in female Kunming mice, dietary administration of high-MW and water-soluble chitosan preparations of varying molecular weights and solubility [MW ranging from 32.7 to 760 kDa; degree of deacetylation (DDA) ~85%] for 90 days was without significant adverse effects in any study parameter, and in particular liver and kidney weights and

histopathology (Zeng *et al*., 2008). The authors noted that consumption of medium molecular weight chitosan (MW = 32.7 kDa; DDA = 85.2%) resulted in increased concentrations of minerals in the liver, spleen, and heart. These findings were attributed to the accumulation of HMWC in these organs and corresponding chelation of endogenous minerals (Zeng *et al*., 2008). In rats, no significant changes in liver weight were reported in male Wistar rats consuming chitosan (MW = 250 kDa; DDA = 94%) in the diet at levels of 5%, equivalent to 5,000 mg/kg body weight/day, for 21 days (Fukada *et al*., 1991) or in male and female Wistar rats administered chitosan derived from lobster chitin (MW = 309 kDa; DDA = 83%) by gavage at doses up to 1,000 mg/kg body weight/day for 28 days (Lagarto *et al*., 2015). In the study by Lagarto *et al.* (2015), no signs of toxicity, mortality, or statistically significant changes in biochemistry parameters were reported following chitosan treatment. A statistically significant increase in erythrocyte count was reported in females in the 300 and 1,000 mg/kg body weight/day groups and in males in the 1,000 mg/kg body weight/day group compared to controls. No statistically significant variations in relative organ weight (as a percentage of total body weight) were reported in chitosan-dosed animals compared to controls. No treatment-related increases in organ lesions were reported based on histopathology examination (Lagarto *et al*., 2015). Lagarto *et al.* reported the short-term no-observed-adverse-effect level (NOAEL) to be 1,000 mg/kg body weight/day, the highest dose tested, for "*effects other than transient variation in erythrocyte count for chitosan under the conditions of this investigation*". The increase in erythrocyte count was considered to be unreliable due to the short duration of this study (*i.e.*, 28 days) and on the basis that no corroborative findings were reported in a long-term study in Sprague-Dawley rats by the National Toxicology Program (NTP) (NTP, 2017). Conversely, Chiang *et al.* (2000) and Chiu *et al.* (2020) reported significant decreases in liver weight following consumption of chitosan (MW ranging from 80 to 740 kDa; DDA = 84 to 91%) in the diet at concentrations up to 5%, equivalent to 5,000 mg/kg body weight/day, for up to 8 weeks. The decrease in liver weight reported by Chiang *et al.* (2000) was associated with a decrease in liver total lipids, resulting in a decrease in liver fat accumulation.

Several other studies reported statistically significant changes in liver weights and liver enzyme activities following chitosan exposure; however, these studies did not report the source of chitosan, purity, average molecular weight, or DDA (Landes and Bough, 1976; Sugano *et al*., 1988; Han *et al*., 1999; Kimura *et al*., 2004; Sumiyoshi and Kimura, 2006; Moon *et al*., 2007; Neyrinck *et al*., 2009; Yao *et al*., 2010; Omara *et al*., 2012; Do *et al*., 2018; Ali *et al*., 2019; Chiu *et al*., 2020). Thus, it was difficult to evaluate their compositional similarity to the Chinova's fiber derived from white button mushrooms and assess the suitability of these studies in the safety evaluation of chitosan derived from white button mushrooms. Furthermore, it is noted that the majority of these studies were designed to evaluate an efficacious effect of chitosan (*e.g.*, amelioration of consumption of a high fat diet or non-alcoholic fatty liver disease, measurement of lipid profiles, serum antioxidant concentration, and biomarkers of lipid peroxidation and inflammation) and were not specifically designed to evaluate the toxicity of chitosan; the identified studies reporting a liver-related finding were not conducted according to an internationally recognized test protocol [*e.g.*, Organisation for Economic Co-operation and Development (OECD) Test Guideline 408]. Nevertheless, the findings suggest that chitosan may impact liver function and elicit hepatomodulatory effects. In the 6-month study by NTP (2017), the absolute and relative liver weights of Sprague-Dawley rats were significantly decreased following consumption of 9% chitosan in the diet and a significant reduction in relative liver weight in animals consuming 3% chitosan in the diet. The decrease in liver weights were accompanied by decreases in liver fat accumulation and increases in ALT. The fatty change was characterized by hepatocytes with clear vacuoles within the periportal region, and was considered to be a biological adaptive response to fat-soluble vitamin and mineral depletion, and may not be a toxicological effect (NTP, 2017). Diets containing 3% and 9% chitosan provided a daily dose of approximately 450 and 6,000 mg/kg body weight, respectively. The available data suggest a possible liver effect of chitosan exposure at doses of 450 mg/kg body weight/day, which is approximately 21-fold higher than the cumulative intake of Chinova's fiber from white button mushrooms based on its proposed food uses (*i.e*., 21.9 mg/kg body weight/day). No decreases in serum fat-

soluble vitamins (vitamin A, D, E), α-carotene, or β-carotene were reported in mildly hypercholesterolemic male and female subjects consuming 6.75 g/day of chitosan for 8 weeks (Tapola *et al*., 2008) or changes in clinically relevant serum parameters (see Section 6.4 for further details) and; therefore, a similar hepatotoxic effect is not expected in humans.

In a 35-day oral toxicity study, Omara *et al.* (2012) administered chitosan (test material not further characterized) *via* gavage at doses of 0 (distilled water), 150, or 300 mg/kg body weight/day to Swiss albino mice (7/sex/group). A consistent, dose-dependent increase in hypercellularity and degenerated glomeruli and tubules in the kidney of both sexes at 150 and 300 mg/kg body weight/day was reported. In addition, severe degeneration and hypercellularity of glomeruli and tubules in kidneys of females compared to males were reported in the high-dose group. Serum creatinine and urea were significantly increased in a dose-dependent manner in males and females. Quantitative analysis demonstrated a statistically significant, dose-dependent decrease in glycogen and total protein content (mean percent of grey area) in renal tubules and glomeruli of the kidneys *versus* controls, and this decrease was statistically significantly greater in females compared to males in the low and high chitosan groups. Similar histopathological findings were not reported in NTP (2017), and with the exception of a statistically significant increase in absolute right kidney weight in males of the high-dose group (9%; 450 mg/kg body weight/day), no adverse renal effects were reported. The authors reported increases in urinary creatinine concentration that corresponded with decreases in urine volume, indicating "proper kidney function" (NTP, 2017). Furthermore, it should be noted that the study by Omara *et al.* (2012) was not conducted in accordance with Good Laboratory Practice (GLP) or internationally-accepted standards for toxicity testing of chemicals and the test article was not adequately described by the authors (*i.e*., molecular weight, DDA, purity), as such, its relevance to Chinova's fiber derived from white button mushrooms could not be determined.

The GRAS Panel reviewed results of a long-term toxicity study of USP-grade crustacean derived chitosan conducted by the NTP (NTP, 2017). The chitosan used in this study had an average purity of 94% and was mixed with a rat feed with 4% fat content. The chitosan had an average percent deacetylation of 86.5% and an average molecular weight of 81.6 kDa (ranged from 62,755 to 87,343 Da; considered low molecular weight chitosan; compositionally equivalent to Chinova's fiber derived from white button mushrooms). The crustacean-derived chitosan was administrated to rats at concentrations of 0%, 1%, 3%, and 9% in the diet for 6 months (NTP, 2017). Dietary concentrations of chitosan up to 9% in the diet were well-tolerated by the rodents. However, statistically significant reductions in serum concentrations of fat-soluble vitamins and reduced relative liver and thymus weights were reported at dietary concentrations of 3% and 9% in males, and 9% in females. No histopathological changes attributable to chitosan were reported in any of the groups. A statistically significant decrease in fat soluble vitamins at the 1% level in male rats was only reported at Week 13 for serum vitamin E. The reduction of serum vitamin E in male rats was not consistent throughout the study. Dietary exposure to chitosan for 6 months resulted in decreased fat digestion and depletion of some fat-soluble vitamins in male and female rats. There were no histological findings associated with the reported decreases in vitamin levels. Based on the effects of chitosan on serum vitamin E levels, the authors concluded the "*lowest-observed-effect level for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats*". The GRAS Panel noted that these effects were indirect consequences of the recognized fat binding properties of chitosan[2] resulting in excretion of dietary fat and reduced absorption of fat-soluble vitamins, and as such were not direct toxic effects of chitosan on organ systems. The GRAS Panel noted that the study was conducted using AIN-93M diet instead of the NTP-2000 diet because of the high levels of fat-soluble vitamins and higher total fat content found in the NTP-2000 diet. The NTP-2000 feed contains almost double the amount of required fat-soluble vitamins and has a higher fat content (7 to 8%) than the

[2] Chitosan is marketed as a dietary supplement for weight loss, and the USP monograph for chitosan includes fat binding capacity as a qualitative specification parameter for the ingredient.

AIN-93M diet (4%); therefore, the study would have been particularly sensitive to effects on fat soluble vitamin absorption (NTP, 2017). The GRAS Panel concluded that the effects of chitosan on fat absorption were an expected finding and are not of nutritional or toxicological significance. The GRAS Panel considered the effects of chitosan on fat soluble vitamin absorption to be relevant to the safety of Chinova's fiber derived from white button mushrooms; however, the sensitive nature of the study design and the differences in the dietary requirements and metabolism of fats between rodents and humans suggest that small changes in the absorption of nutrients reported in the study may not necessarily be of nutritional significance to humans consuming Chinova's fiber derived from white button mushrooms. The GRAS Panel agreed that the NTP study demonstrated the potential of chitosan to sequester nutrients; however, the nutritional significance of chitosan on fat soluble vitamin absorption should be determined by well-designed clinical trials. The GRAS Panel also noted that generalized effects of resistant dietary fibers on nutrient absorption have been long known, are well characterized, and are not considered of nutritional relevance at levels that are commonly consumed in the diet (Dahl and Stewart, 2015). As such, these effects are not considered to be a direct toxic effect of chitosan on organ systems or a finding of toxicological or nutritional significance and the reported fatty change is considered to be a biological adaptive response to depletion of fat-soluble vitamins and minerals and contingent upon consumption of supraphysiological intakes that would affect lipid absorption.

Reproductive and developmental toxicity studies on Chinova's fiber derived from white button mushrooms demonstrated that chitosan did not have any toxic developmental and reproductive effects (GRN 397 – U.S. FDA, 2011). B6C3F1 female mice (induced to ovulate) gavaged with water-soluble chitosan (approximately 300 kDa; >90% deacetylation; compositionally equivalent to Chinova's fiber derived from white button mushrooms) at a dose of 480 mg/kg body weight/day for 4 days did not demonstrate any effects on the oocyte and fertilization rates (Choi *et al.*, 2002).

Genotoxicity tests including the bacterial reverse mutation assay, Ames reverse mutation test, and the bone marrow micronucleus test demonstrated that chitosan is not mutagenic or genotoxic (Qin *et al.*, 2006; GRN 397 – U.S. FDA, 2011).

Additional toxicity studies were identified during the literature search and were evaluated by the GRAS Panel; however, they were not considered relevant to the safety of Chinova's fiber derived from white button mushrooms based on the identity of the test item and/or the quality of the study. Several studies did not characterize the identity or composition of chitosan used; several studies used chitosan oligosaccharides preparations, which are not representative of USP chitosan such as Chinova's fiber derived from white button mushrooms; and several studies were not conducted according to GLP or current internationally-accepted testing guidelines for the toxicity of chemicals (Kim *et al*., 2001; Yoon *et al*., 2005; Qin *et al.*, 2006; Naito *et al*., 2007; Zeng *et al*., 2008; Omara *et al*., 2012; Yao *et al*., 2012; Lagarto *et al*., 2015; Eisa *et al*., 2018).

Clinical Studies

Chitosan has an apparent history of safe use in food supplement products, and several human clinical studies in which healthy, hypercholesterolemic, smokers, and/or obese subjects have been administered chitosan or chitosan oligosaccharides in the diet are published in the literature (see Section G of GRN 397 and Section D of GRN 443) (Kitozyme sa, 2011; U.S. FDA, 2011, 2013; GRAS Associates, LLC, 2012). These studies demonstrated that chitosan consumption was well-tolerated at levels ranging from 1 to 6 g per day, for periods up to 24 weeks. According to GRN 170, the U.S. FDA has raised concerns on potential effects on fat-soluble vitamins and mineral status in humans following consumption of chitosan (Lee B. Dexter and Assoc., 2005 – GRN 170). These concerns were raised due to a rat study reporting significant reductions in

levels of vitamins A, D, and E, and calcium, magnesium, and iron (Deuchi *et al*., 1995), and a more recent long-term toxicity study reported similar findings (NTP, 2017). These findings have not been substantiated in human clinical studies conducted with clinically relevant dosages (Tapola *et al*., 2008). As such, the altered absorption of dietary nutrients reported in animals is not relevant to the safety of chitosan, given that the doses used in animal studies were much larger on a grams/kilogram body weight basis; therefore, they were not considered representative of human intake levels.

In a multi-center, single-blind, placebo controlled, and randomized clinical study, 96 adult patients in India (36 males, 60 females, mean age: 35.5±11.2 years) took five 500 mg chitosan capsules (KiOnutrime-CsG® chitosan derived from *Aspergillus niger*) per day for a total dose of 2,500 mg chitosan for 90 days (n=64) or a placebo (n=32; microcrystalline cellulose powder) (Trivedi *et al*., 2016). Study participants were generally free from disease; however, 15 subjects in the chitosan group and 6 from the placebo group had hypertension, diabetes mellitus, and/or dyslipidemia. The following parameters were measured or tracked during the study: safety, quality of life (*via* questionnaire), adverse events and effects, biochemical parameters (urea, serum creatinine, ALT, AST), reduction in mean body weight, body mass index (BMI), body fat, visceral fat, muscle mass, circumference of the upper abdominal, hip, and waist, waist-to-hip ratio, lipid profile [triglycerides (TG), high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL)], and glycated hemoglobin levels.

There were 6 adverse events (common cold, hypertriglyceridemia, body ache, hypertension, and 2 counts of constipation) in the chitosan group and 4 adverse events (2 counts of mild headache, hypertriglyceridemia, and fracture) in the placebo group. The authors reported that all adverse events were mild and unrelated to study treatment. There was no statistically significant difference in ALT, AST, serum creatinine, or urea from Day 0 to 90 in either group. The authors reported no study withdrawals due to adverse effects and stated that overall, chitosan was safe and well tolerated. Compared to placebo, a statistically significant reduction in mean body weight change, BMI, body fat percentage, and upper abdominal, hip, and waist circumference at Day 45 and Day 90 were reported.

Compared to baseline measures, a statistically significant decrease in body weight, BMI, body fat percentage, visceral fat percentage, muscle mass, upper abdominal, hip, and waist circumference were reported at Day 45 and Day 90. Percent glycated hemoglobin was significantly decreased in the chitosan group at Day 45 and 90 as well as in the placebo group at Day 45, though returning to baseline at Day 90 in the latter group. A statistically significant increase in LDL was reported in the chitosan group at Day 45 and in the placebo at Day 90, an effect attributable to only 1 subject/group, and was; therefore, considered transient and clinically non-significant by the authors. No significant differences were reported for all other lipid parameters compared to baseline (Trivedi *et al*., 2016).

In a 12-week randomized, double-blind, placebo-controlled study conducted with 60 pre-diabetic adult patients (characterized by impaired fasting glucose and impaired glucose tolerance), a low-molecular weight chitosan oligosaccharide capsule (100% purity, not further specified) or a placebo capsule (roasted barley meal powder) was administered 6 times/day for a total daily dose of 1,500 mg (Kim *et al*., 2014). Adverse effects, serum levels of glucose and C-peptide, cholesterol and immune markers, triglycerides, insulin, adiponectin, and glycated hemoglobin were measured throughout the study period. No adverse effects were reported by any of the subjects. Statistically significant increased lean body mass was reported in the chitosan group compared to placebo. Significantly decreased glycated hemoglobin, glucose at 30 and 60 minutes, and IL-6 and significantly increased adiponectin were seen compared to baseline. There were no significant differences in insulin, C-peptide, and area under the curve for glucose and C-peptide compared to baseline. Significant changes from baseline to after 12 weeks of chitosan use *versus* changes in the placebo group were reported as a decrease in body fat percentage, waist circumference, blood

glucose at 60 minutes, and glycated hemoglobin. There was no significant difference in changes in total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, insulin, adiponectin, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) between treatment and placebo groups (Kim *et al.*, 2014).

In a randomized, double-blind, controlled crossover study conducted with 37 healthy adults (age 20 to 75 years), chitosan oligosaccharide capsules were provided to subjects at a dose of 250 mg (Jeong *et al*., 2019). The treatment was provided in addition to 75 g of sucrose within 15 minutes. After 7 days, subjects were provided a placebo. Blood samples were collected after a 12-hour overnight fast. Serum glucose concentrations were measured at 0, 30, 60, 90, and 120 minutes. Total energy expenditure was calculated for each subject. No side effects were reported in any study subjects. No significant changes in white blood cells, red blood cells, hemoglobin, hematocrit, platelets, or parameters of daily food intake and total energy expenditure (basal metabolic rate) were reported in any study subject. Blood glucose levels peaked at 30 minutes and returned to baseline after 2 hours. No significant differences in blood glucose levels were reported between treatment and placebo groups (Jeong *et al*., 2019).

A meta-analysis of randomized, double-blind, placebo-controlled trials was conducted to evaluate the effects of chitosan administration on systolic blood pressure and diastolic blood pressure (Huang *et al*., 2018a). Consumption of chitosan at doses ranging from 1 to 4.5 g/day for up to 24 weeks in 617 subjects that were overweight, obese, hypercholesterolemic, or prehypertensive from 8 trials with 10 arms did not result in any significant decreases in systolic or diastolic blood pressure. However, analyses of subgroups indicated that diastolic blood pressure was decreased in the short-term (<12 weeks) and at high doses (>2.4 g/day). The reported forms of chitosan were “chitosan” or microcrystalline chitosan. No further information on the molecular weight or DDA were reported. Based on the results of this meta-analysis, the authors concluded that chitosan consumption significantly decreased diastolic blood pressure at high doses (>2.4 g/day) and in short-term interventions (Huang *et al*., 2018a).

In another meta-analysis of randomized controlled trials conducted to investigate the effects of chitosan consumption on serum lipids, 1,108 subjects that were overweight, obese, hypercholesterolemic, or prediabetic from 14 trials with 21 treatment arms were evaluated (Huang *et al.*, 2018b). Chitosan administration at doses ranging from 0.312 to 6.75 g/day for up to 24 weeks significantly increased total cholesterol and LDL-cholesterol in all subjects. No significant changes in HDL-cholesterol or triglycerides and no serious adverse events were reported (Huang *et al.*, 2018b).

The effects of chitosan on body weight and body composition were investigated in a meta-analysis of 15 trials with 18 treatment arms that included 1,130 subjects (Huang *et al*., 2019). The studies included subjects that were overweight or obese with hypercholesterolemia or overweight or obese but otherwise healthy consuming chitosan at doses ranging from 0.312 to 4.5 g/day for 4 to 24 weeks. The reported treatments included chitosan capsules, microcrystalline chitosan capsules, water-soluble chitosan capsules, or beta-glucan-chitin-chitosan fraction. No details on the molecular weight or DDA were reported. Chitosan consumption was associated with a significant decrease in body weight. Analysis of subgroups indicated that consuming high doses of chitosan (>2.4 g/day) short-term (<12 weeks) was associated with a decrease in body weight. In addition, consumption of chitosan was well tolerated and was not associated with any serious adverse events (Huang *et al*., 2019).

Other Considerations

Chitosan is intended for use as an antimicrobial in a large number of food and beverage categories and, therefore, widespread consumption in the diet could occur throughout the day on a continual basis. The GRAS Panel noted that increasing concerns over the use of antimicrobial substances in the environment

have been reported in the literature (Halden *et al*., 2017; Hartmann *et al*., 2017; Tun *et al*., 2018). The mechanism of action of chitosan is unknown; however, the polymer is believed to kill bacteria through a process mediated *via* cationic interactions with bacterial cell wall proteins. Development of acquired resistance to chitosan by microorganisms is unlikely. Foods to which Chinova may be added are expected to require preservatives to maintain the shelf-life of the food and, therefore, they are necessary for consumer safety of perishable foods. Chinova's fiber derived from white button mushrooms will serve as an alternative to existing chemical preservatives such as benzoates and sorbates. The health risk of introducing Chinova's fiber derived from white button mushrooms as a preservative to the food supply will not be any greater than that currently attributed to existing preservatives that are used in food.

Information Pertaining to the Safety of B*eta*-1,3-Glucans

Chinova's fiber derived from white button mushrooms contains *beta*-1,3-glucans at concentrations of up to 5% on a w/w% basis.

Wistar rats administered chitin-glucan as a dietary admixture at concentrations of 0% (control), 1%, 5%, or 10% for 13 weeks did not demonstrate any treatment-related significant adverse effects (Jonker *et al*., 2010). The authors concluded that, under the conditions of the study, the NOAEL was 10% in the diet, the highest concentration tested, which was equivalent to an overall estimated daily intake of 6,589 mg/kg body weight/day for males and 7,002 mg/kg body weight/day for females. A NOAEL of 100 mg/kg body weight (the maximum deliverable gavage dose) was derived for Fisher-344 rats administered a *Saccharomyces cerevisiae*-derived *beta*-1,3-glucan preparation at doses of 0, 2, 33.3, or 100 mg/kg body weight/day for 91 days (Babícek *et al*., 2007). In another study, Sprague-Dawley rats received, by gavage, *Candida albicans*-derived *beta*-1,3-D-glucan insoluble isolate at doses of 0 (saline), 50, 100, or 200 mg/kg body weight/day for 52 weeks, with no treatment-related adverse effects reported and a NOAEL of 200 mg/kg body weight per day (Feletti *et al*., 1992). No safety concerns are anticipated from the presence of *beta*-1,3-glucans in Chinova's fiber derived from white button mushrooms.

Safety Summary

Overall, the concerns regarding chitosan reducing the absorption of lipids and other nutrients, such as fat-soluble vitamins and minerals were mainly reported in studies with rats (Deuchi *et al*., 1995; NTP, 2017). This is further corroborated by the results of several clinical studies wherein no significant decreases in fat-soluble vitamins were reported in human studies as follows:

- Vitamins A, E, D, α-carotene, and β-carotene in mild hypercholesterolemic subjects (n=56) consuming chitosan derived from shellfish at levels of 6.75 g/day for 55 days (Tapola *et al.*, 2008);
- Vitamin D in mild or moderate hypercholesterolemic subjects (n=96) consuming LMWC at doses up to 2.4 g/day for 12 weeks (Jaffer and Sampalis, 2007);
- Vitamin A (retinol), D, E (α-tocopherol), β-carotene, and prothrombin time (surrogate for vitamin K) in overweight adults (n=250) consuming 3 g/day of β-chitosan for 24 weeks (Mhurchu *et al*., 2004); and
- Vitamin A, D, E, and β-carotene in overweight subjects (n=30) consuming 2 g/day of chitosan (not further characterized) for 28 days (Pittler *et al*., 1999).

A number of repeated-dose studies were identified in mice, rats, guinea pigs, and pigs, which reported an effect of chitosan administration. The weight of the available evidence indicates that typical chitosan preparations, when ingested, are non-toxic. Some evidence of toxicity (*e.g.*, increased or decreased relative organ weights, accumulation of iron, zinc and copper in organs, decreased fat soluble vitamins) has been reported in rodent studies following administration of LMWC oligomers and/or fully deacetylated oligomers at high dietary concentrations (>1%). Evidence of toxicity in these studies is typically dose limiting (only observed at dietary levels >1%) and, in some cases, were confounded by application of non-validated study designs.

Fifteen clinical studies were discussed in GRN 397 in which chitosan was consumed at doses of 0.54 to 6.75 g/day for 2 to 24 weeks without significant treatment-related adverse effects reported (U.S. FDA, 2011). An updated search of the scientific literature identified studies published since GRN 397was submitted that were conducted with chitosan doses of 1.5 to 2.5 g/day for up to 90 days (see Section 6.4). No treatment-related adverse events were reported throughout the studies, but a statistically significant decrease in body weight, body mass index, body fat percentage, visceral fat percentage, muscle mass, and upper abdominal, hip, and waist circumference were reported (Kim *et al.*, 2014; Trivedi *et al*., 2016) was reported. These findings are considered to be an expected effect of chitosan, as the substance is commonly used in food supplements for its fat binding ability.

The reported lowest-observed-effect level (LOEL) from NTP (2017) was 1% in male rats, equivalent to 450 mg/kg body weight/day, based on the reported nutritional-related findings. On a body weight basis, this dosechino is equivalent to a human consuming approximately 31.5 g of chitosan per day (for a 70-kg individual). In the parallel, placebo-controlled study by Tapola *et al.* (2008), no effects on fat absorption were reported at clinically relevant doses (*i.e.*, 6.75 g/day). Based on the proposed antimicrobial food uses of Chinova's fiber derived from white button mushrooms, considering maximum FEMA-GRAS approved use levels, the estimated daily intake of Chinova's fiber derived from white button mushrooms was determined to be highest in male adults at 3.2 g/day at the 90th percentile, approximately 10-fold less than the reported LOEL of chitosan by NTP (2017), and an order of magnitude below levels that have been demonstrated to not affect vitamin absorption in human studies. Therefore, the proposed uses of Chinova's fiber derived from white button mushrooms is not expected to be associated with any adverse outcomes, including vitamin or mineral deficiencies.

CONCLUSION

We, the members of the GRAS Panel, have independently and collectively critically evaluated the data and information summarized above and unanimously conclude that the intended use of Chinova's fiber extracted from white button mushrooms as a preservative ingredient in food and beverages, meeting appropriate food-grade specifications and produced consistent with current Good Manufacturing Practice (cGMP), is Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

[redacted]

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REFERENCES

Ali AAM, Mansour A, Hassanine EA, Abdel-Razek AH, Mahmoud HAH (2019). Ameliorative role of a combination of chitosan, calcium, vitamins A and E against high fat diets-induced adverse effects in rats. J Basic Appl Zool 80:11 [12pp]. DOI:10.1186/s41936-019-0081-7.

Babícek K, Cechová K, Simon RR, Harwood M, Cox DJ (2007). Toxicological assessment of a particulate yeast (1,3/1,6)-β-D-glucan in rats. Food Chem Toxicol 45(9):1719-1730. DOI:10.1016/j.fct.2007.03.013.

Chiang MT, Yao HT, Chen HC (2000). Effect of dietary chitosans with different viscosity on plasma lipids and lipid peroxidation in rats fed on a diet enriched with cholesterol. Biosci Biotechnol Biochem 64(5):965-971. DOI:10.1271/bbb.64.965.

Chiu C-Y, Yen T-E, Liu S-H, Chiang M-T (2020). Comparative effects and mechanisms of chitosan and its derivatives on hypercholesterolemia in high-fat diet-fed rats. Int J Mol Sci 21(1):92 [11pp]. DOI:10.3390/ijms21010092.

Choi HG, Kim JK, Kwak DH, Cho JR, Kim JY, Kim BJ, et al. (2002). Effects of high molecular weight water-soluble chitosan on *in vitro* fertilization and ovulation in mice fed a high-fat diet. Arch Pharm Res 25(2):178-183. DOI:10.1007/BF02976560.

Dahl WJ, Stewart ML (2015). Position of the Academy of Nutrition and Dietetics: health implications of dietary fiber. J Acad Nutr Diet 115(11):1861-1870. DOI:10.1016/j.jand.2015.09.003.

Deuchi K, Kanauchi O, Shizukuishi M, Kobayashi E (1995). Continuous and massive intake of chitosan affects mineral and fat-soluble vitamin status in rats fed on a high-fat diet. Biosci Biotechnol Biochem 59(7):1211-1216. DOI:10.1271/bbb.59.1211.

Do AR, Cho S-J, Cho Y-Y, Kwon E-Y, Choi J-Y, Lee JH, et al. (2018). Antiobesity effects of short-chain chitosan in diet-induced obese mice. J Med Food 21(9):927-934. DOI:10.1089/jmf.2017.4115.

Eisa AAA, Aboelghar GE, Ammar IM, Metwally HG, Arafa SS (2018). Teratogenic effects induced by chitosan oligosaccharide in Wistar female rat Rattus norvegicus. Environ Sci Pollut Res Int 25(10):9371-9379. DOI:10.1007/s11356-018-1199-8.

Feletti F, De Bernardi di Valserra M, Contos S, Mattaboni P, Germogli R (1992). Chronic toxicity study on a new glucan extracted from Candida albicans in rats. Arzneimittelforschung 42(11):1363-1367.

Fukada Y, Kimura K, Ayaki Y (1991). Effect of chitosan feeding on intestinal bile acid metabolism in rats. Lipids 26(5):395-399. DOI:10.1007/BF02537206.

Goy RC, de Britto D, Assis OBG (2009). A review of the antimicrobial activity of chitosan. Polímeros: Ciência e Tecnologia 19(3):241-247. DOI:10.1590/S0104-14282009000300013.

GRAS Associates, LLC (2012). *GRAS Assessment: ChitoClear® Shrimp-derived Chitosan. Food Usage Conditions for General Recognition of Safety*. (GRAS Notice GRN No. 443). Submitted for Siglufjordur, Iceland: Primex, ehf by Bend (OR): GRAS Associates, LLC to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=443 [Resubmission GRN No. 443; Feb. 11, 2013 – At notifier's request, FDA ceased to evaluate the notice].

Halden RU, Lindeman AE, Aiello AE, Andrews D, Arnold WA, et al. (2017). The Florence Statement on Triclosan and Triclocarban. Environ Health Perspect 125(6):064501 [13pp]. DOI:10.1289/EHP1788

Han LK, Kimura Y, Okuda H (1999). Reduction in fat storage during chitin-chitosan treatment in mice fed a high-fat diet. Int J Obes Relat Metab Disord 23(2):174-179. DOI:10.1038/sj.ijo.0800806.

Hartmann EM, Hickey R, Hsu T, Betancourt Román CM, Chen J, et al. (2017). Antimicrobial chemicals are associated with elevated antibiotic resistance genes in the indoor dust microbiome. Environ Sci Technol 50(18):9807-9815. DOI:10.1021/acs.est.6b00262.

Huang H, Zou Y, Chi H (2018a). Quantitative assessment of the effects of chitosan intervention on blood pressure control. Drug Des Devel Ther 12:67-75. DOI:10.2147/DDDT.S148064.

Huang H, Zou Y, Chi H, Liao D (2018b). Lipid-modifying effects of chitosan supplementation in humans: a pooled analysis with trial sequential analysis. Mol Nutr Food Res 62(8):e1700842 [10pp, plus supplementary data]. DOI:10.1002/mnfr.201700842.

Huang H, Liao D, Zou Y, Chi H (2019). The effects of chitosan supplementation on body weight and body composition: a systematic review and meta-analysis of randomized controlled trials. Crit Rev Food Sci Nutr [online ahead of print - Apr. 16, 2019]. DOI:10.1080/10408398.2019.1602822.

Jaffer S, Sampalis JS (2007). Efficacy and safety of chitosan HEP-40 in the management of hypercholesterolemia: a randomized, multicenter, placebo-controlled trial. Altern Med Rev 12(3):265-273.

Jeong S, Min Cho J, Kwon Y-I, Kim S-C, Yeob Shin D, Ho Lee J (2019). Chitosan oligosaccharide (GO2KA1) improves postprandial glycemic response in subjects with impaired glucose tolerance and impaired fasting glucose and in healthy subjects: a crossover, randomized controlled trial. Nutr Diabetes 9(1):31 [9pp]. DOI:10.1038/s41387-019-0099-4.

JFCRF (2014). *List of Existing Food Additives* [Compiled and published by the Ministry of Health and Welfare on April 16, 1996]. Tokyo, Japan: Ministry of Health, Labour and Welfare, Japan (MHLW) and Japan Food Chemical Research Foundation (JFCRF). Available at: http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf/pages/list-exst.add [Effective from January 30, 2014, Last update: 04/10/2014].

Jonker D, Kuper CF, Maquet V, Nollevaux G, Gautier S (2010). Subchronic (13-week) oral toxicity study in rats with fungal chitin-glucan from *Aspergillus niger*. Food Chem Toxicol 48(10):2695-2701. DOI:10.1016/j.fct.2010.06.042.

Kim S-K, Park P-J, Yang H-P, Han S-S (2001). Subacute toxicity of chitosan oligosaccharide in Sprague-Dawley rats. Arzneimittelforschung 51(9):769-774. DOI:10.1055/s-0031-1300113.

Kim HJ, Ahn HY, Kwak JH, Shin DY, Kwon YI, Oh CG, et al. (2014). The effects of chitosan oligosaccharide (GO2KA1) supplementation on glucose control in subjects with prediabetes. Food Funct 5(10):2662-2669. DOI:10.1039/c4fo00469h.

Kimura Y, Nagata Y, Buddington RK (2004). Some dietary fibers increase elimination of orally administered polychlorinated biphenyls but not that of retinol in mice. J Nutr 134(1):135-142. DOI:10.1093/jn/134.1.135.

Kitozyme sa (2011). *Chitosan GRAS Notice*. (GRAS Notice GRN No. 397). Submitted by Herstal, Belguim: Kitozyme sa to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=397 [Dec. 19, 2011 – FDA has no questions].

Lagarto A, Merino N, Valdes O, Dominguez J, Spencer E, De La Paz N, et al. (2015). Safety evaluation of chitosan and chitosan acid salts from {*Panurilus argu*} lobster. Int J Biol Macromol 72:1343-1350. DOI:10.1016/j.ijbiomac.2014.10.030.

Landes DR, Bough WA (1976). Effects of chitosan—a coagulating agent for food processing wastes—in the diets of rats on growth and liver and blood composition. Bull Environ Contam Toxicol 15(5):555-563. DOI:10.1007/BF01685704.

Lee B. Dexter and Assoc. (2005). *Shrimp-derived Chitosan GRAS Notification*. (GRAS Notice GRN No. 170). Prepared by Austin (TX): Lee B. Dexter and Assoc. and submitted by Siglufjordun, Iceland: Primex ehf to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=170 [resubmitted as GRN No. 443 Oct. 31, 2005 - At notifier's request, FDA ceased to evaluate the notice].

MFDS (2017). 70. Chitosan. In: *Korea Food Additives Code*. (All designated chemicals and some natural additives are currently regulated by Food Additives Code. It includes specifications, standards and general test methods for each additives). Chungcheongbuk-do, South Korea.: Ministry of Food and Drug Safety (MFDS), pp. 1238-1239.

Mhurchu CN, Poppitt SD, Mcgill A-T, Leahy FE, Bennett DA, Lin RB, et al. (2004). The effect of the dietary supplement, chitosan, on body weight: a randomised controlled trial in 250 overweight and obese adults. Int J Obes Relat Metab Disord 28(9):1149-1156. DOI:10.1038/sj.ijo.0802693.

Moon MS, Lee MS, Kim CT, Kim Y (2007). Dietary chitosan enhances hepatic CYP7A1 activity and reduces plasma and liver cholesterol concentrations in diet-induced hypercholesterolemia in rats. Nutr Res Pract 1(3):175-179. DOI:10.4162/nrp.2007.1.3.175.

Naito Y, Tago K, Nagata T, Furuya M, Seki T, Kato H, et al. (2007). A 90-day *ad libitum* administration toxicity study of oligoglucosamine in F344 rats. Food Chem Toxicol 45(9):1575-1587. DOI:10.1016/j.fct.2007.02.018.

Neyrinck AM, Bindels LB, De Backer F, Pachikian BD, Cani PD, Delzenne NM (2009). Dietary supplementation with chitosan derived from mushrooms changes adipocytokine profile in diet-induced obese mice, a phenomenon linked to its lipid-lowering action. Int Immunopharmacol 9(6):767-773. DOI:10.1016/j.intimp.2009.02.015.

NIH (2020). Search results for "Chitosan". In: *Dietary Supplements Labels Database (DSLD)*. Bethesda (MD): National Library of Medicine (NLM) / National Institutes of Health (NIH), Office of Dietary Supplements. Available at: https://dsld.nlm.nih.gov/dsld/ [Version 7.0.12 – August 2020, 19e5a30; Last accessed: January 4, 2020].

Notermans S, Dufrenne J, Gerrits JPG (1989). Natural occurrence of *Clostridium botulinum* on fresh mushrooms (*Agaricus, Bisporus*). J Food Prot 52(10):733-736. DOI:10.4315/0362-028X-52.10.733.

NTP (2017). *NTP Technical Report on the Toxicity Study of Chitosan (CAS No. 9012-76-4) Administered in Feed to Sprague Dawley [Crl:CD(SD)] Rats*. (NTP Toxicity Report Series, no 93). Research Triangle Park (NC): National Toxicology Program (NTP). Available at: https://ntp.niehs.nih.gov/ntp/htdocs/st_rpts/tox093_508.pdf.

Omara EA, Aly HF, Nada SA (2012). Chitosan induced hepato-nephrotoxicity in mice with special reference to gender effect in glycolytic enzymes activities. Regul Toxicol Pharmacol 62(1):29-40. DOI:10.1016/j.yrtph.2011.11.010.

Pittler MH, Abbot NC, Harkness EF, Ernst E (1999). Randomized, double-blind trial of chitosan for body weight reduction. Eur J Clin Nutr 53(5):379-381. DOI:10.1038/sj.ejcn.1600733.

Qin C, Gao J, Wang L, Zeng L, Liu Y (2006). Safety evaluation of short-term exposure to chitooligomers from enzymic preparation. Food Chem Toxicol 44(6):855-861. DOI:10.1016/j.fct.2005.11.009.

Raafat D, von Bargen K, Haas A, Sahl HG (2008). Insights into the mode of action of chitosan as an antibacterial compound. Appl Environ Microbiol 74(12):3764-3773. DOI:10.1128/AEM.00453-08.

Sugano M, Watanabe S, Kishi A, Izume M, Ohtakara A (1988). Hypocholesterolemic action of chitosans with different viscosity in rats. Lipids 23(3):187-191. DOI:10.1007/BF02535456.

Sumiyoshi M, Kimura Y (2006). Low molecular weight chitosan inhibits obesity induced by feeding a high-fat diet long-term in mice. J Pharm Pharmacol 58(2):201-207. DOI:10.1211/jpp.58.2.0007.

Tapola NS, Lyyra ML, Kolehmainen RM, Sarkkinen ES, Schauss AG (2008). Safety aspects and cholesterol-lowering efficacy of chitosan tablets. J Am Coll Nutr 27(1):22-30. DOI:10.1080/07315724.2008.10719671.

Trivedi VR, Satia MC, Deschamps A, Maquet V, Shah RB, Zinzuwadia PH, et al. (2016). Single-blind, placebo controlled randomised clinical study of chitosan for body weight reduction. Nutr J 15:3 [12pp]. DOI:10.1186/s12937-016-0122-8.

Tun MH, Tun HM, Mahoney JJ, Konya TB, Guttman DS, Becker AB, et al. (2018). Postnatal exposure to household disinfectants, infant gut microbiota and subsequent risk of overweight in children (CHILD Study Investigators). CMAJ. 190(37):E1097-E1107. DOI:10.1503/cmaj.170809.

U.S. FDA (2002). *Agency Response Letter GRAS Notice No. GRN 000073 [Shrimp-derived chitosan, Avaldsnes, Norway: Primex Ingredients ASA]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=73 [Jan 31, 2002 - At notifier's request, FDA ceased to evaluate the notice].

U.S. FDA (2005). *Agency Response Letter GRAS Notice No. GRN 000170 [Shrimp-derived Chitosan], Siglufjordur, Iceland: Primex ehf]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=170 [resubmitted as GRN No. 443; Oct. 31, 2005 - At notifier's request, FDA ceased to evaluate the notice].

U.S. FDA (2011). *Agency Response Letter GRAS Notice No. GRN 000397 [Chitosan from Aspergillus niger, Herstal, Belgium: KitoZyme sa]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=397 [Dec. 19, 2011 – FDA has no questions].

U.S. FDA (2013). *Agency Response Letter GRAS Notice No. GRN 000443 [Shrimp-derived chitosan, Siglufjordur, Iceland: Primex ehf]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=443 [Resubmission of GRN No. 170; Feb. 11, 2013 - At notifier's request, FDA ceased to evaluate the notice].

U.S. FDA (2017). *Best Practices for Convening a GRAS Panel: Guidance for Industry [Draft].* (Docket No: FDA-2017-D-0085). Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN) / Center for Veterinary Medicine (CVM). Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/draft-guidance-industry-best-practices-convening-gras-panel [Current as of: 11/16/2017].

U.S. FDA (2020). Part 170—Food additives. Section §170.3—Definitions. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs* (Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/2019/title21 [current to 4-1-20, last updated 4-1-19].

Yao HT, Lii CK, Chou RH, Lin JH, Yang HT, Chiang MT (2010). Effect of chitosan on hepatic drug-metabolizing enzymes and oxidative stress in rats fed low- and high-fat diets. J Agric Food Chem 58(8):5187-5193. DOI:10.1021/jf903857m.

Yao H-T, Luo M-N, Hung L-B, Chiang M-T, Lin J-H, Lii C-K, et al. (2012). Effects of chitosan oligosaccharides on drug-metabolizing enzymes in rat liver and kidneys. Food Chem Toxicol 50(5):1171-1177. DOI:10.1016/j.fct.2012.02.022.

Yoon HJ, Park HS, Bom HS, Roh YB, Kim JS, Kim YH (2005). Chitosan oligosaccharide inhibits $^{203}HgCl_2$-induced genotoxicity in mice: micronuclei occurrence and chromosomal aberration. Arch Pharm Res 28(9):1079-1085 [erratum, 28(10):1203]. DOI:10.1007/BF02977405.

Zeng L, Qin C, He G, Wang W, Li W, Xu D (2008). Effect of dietary chitosans on trace iron, copper and zinc in mice. Carbohydr Polym 74(2):279-282. DOI10.1016/j.carbpol.2008.02.022.