APPLICATION TO AMEND STANDARD 1.3.3 OF THE AUSTRALIA AND NEW ZEALAND FOOD STANDARDS CODE TO INCLUDE *AGARICUS BISPORUS* AS A SOURCE ORGANISM FOR CHITOSAN

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Application to Amend Standard 1.3.3 of the Australia and New Zealand Food Standards Code to Include *Agaricus bisporus* as a Source Organism for Chitosan.

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A. GENERAL REQUIREMENTS

In accordance with Section 3.1.1 – General Requirements of the Food Standards Australia New Zealand (FSANZ) *Application Handbook* (FSANZ, 2019), the following general information must be provided:

1. Format of the application;

2. Applicant details;

3. Purpose of the application;

4. Justification for the application;

5. Information to support the application;

6. Assessment procedure;

7. Confidential commercial information;

8. Other Confidential information;

9. Exclusive capturable commercial benefit;

10. International and other national standards;

11. Statutory declaration; and

12. Checklist.

Each point is addressed in the following subsections.

A.1 Format of the Application

A.1.1. Information Related to Changes to Standard 1.3.3 – Processing Aids

This application for an amendment to Standard 1.3.3 and related Schedules is prepared pursuant to Section 3.3.2 – Processing Aids of the FSANZ *Application Handbook* (FSANZ, 2019), which requires the following structured format to assess an application for a new processing aid:

A. General information on the application;

B. Technical information on the processing aid;

C. Information related to the safety of a chitosan processing aid;

D. Additional information related to the safety of a chitosan processing aid derived from a microorganism;

and

E. Information related to the dietary exposure to the processing aid.

The application is presented in this format. At the start of each section (A to E) the information that
must be addressed therein is specified in more detail. Additionally, an executive summary for the
application is provided as a separate electronic document to this application. The application has been prepared in English and submitted electronically, as required by the FSANZ *Application handbook* (FSANZ, 2019).

A.1.2 Executive Summary

Chitin is the main component of the cell walls of fungi, plants, and insects. Chitosan is a natural occurring polysaccharide, obtained by the de-acetylation of chitin. Chitosan and chitin-glucan are permissible products that can be used for the decrease of undesirable micro-organisms, settling aids, antioxidants, decrease of copper and iron concentrations and removal of contaminants. Chitosan can also control the growth of undesirable yeast like *Brettanomyces*, lactic acid bacteria like *Lactobacillus*, *Oenococcus* and *Pediococcus* and acetic acid bacteria like *Acetobacter.* Chitosan's mechanism of action against microorganisms comes down to its strong cationic charge when in acidic solution, and that charge binds to anionic components of the microorganism's cell wall and physically shears open the cell wall. This ionic interaction kills the microorganisms.

The degree of acetylation (DA) of chitin is a significant parameter influencing the biological, physicochemical, and mechanical properties and an important parameter that determines its classification whether it is chitin or chitosan. Chitosan is emerging as a very important raw material for the synthesis of a wide range of products used for food, medical, pharmaceutical,

health care, agriculture, industry, and environmental pollution protection. Chitosan is used as a processing aid in the manufacturing of wine, beer, cider, and spirits.
Regardless of the technological purpose, the sediments that contain the chitosan are removed from the wine, must or spirits at the end of the treatment by physical separation processes such as racking, centrifugation and/ or filtration. Since chitosan is insoluble at slightly acidic to neutral pH levels, as well as in aqueous and ethanol solutions, it is unlikely that any residual chitosan will remain in the treated products. High-performance liquid chromatography analyses have confirmed that the final product is free from chitosan. Therefore, the estimated intake of chitosan from a wine source can be considered as negligible.
Resolutions permitting the use of fungal chitosan from *Aspergillus niger* and *Agaricus bisporus* as a fining agent and contaminant treatment have been granted by the International Organisation of Vine and Wine (OIV/OENO 336A/2009; 337A/2009; 338A/2009; 339A/2009) (OIV, 2011; Appendix 3 - 6). A monograph for fungal chitosan has also been added to the International Oenological Codex by decision of the OIV general assembly dated July 2009 considering the works of the group of experts "Specifications of Oenological Products" (OIV/OENO 368/2009, Appendix 7), but currently FSANZ only permit the use of chitosan from shellfish and from the fungi *A. niger* as a processing aid. As part of the OIV approval process they do evaluate toxicity of processing aids and the safety risk for wine consumers.

A number of animal, human, and *in vitro* studies on the safety of shellfish chitosan (and other sources) have been published and summarised in this application. In FSANZ application A1077 the applicants demonstrated how similar chitosan from *Aspergillus niger* are to shellfish chitosan and in the FSANZ review of all the data they accepted the safety information, and that the data are applicable to *A. niger* chitosan due to its similarity to shellfish chitosan and approved chitosan from *A. niger* as safe to use as a processing aid in wine. Likewise, in this application Chinova Bioworks demonstrated how similar chitosan from *Agaricus bisporus* is to chitosan from shellfish and from *A. niger*. Also, that their product Pinnacle Mycrobrio received GRAS status for use as a processing-aid in the manufacturing of alcoholic beverages. Both the Australian Grape and Wine and the New Zealand Winegrowers support this application.

A.2 Applicant Details

[redacted]

Global Wine Market Director

Commercial Director – Asia-Pacific and South Africa

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A.3 Purpose of the Application

AB Mauri is submitting this application to FSANZ to request an amendment to Standard 1.3.3 of the Food Standards Code ("the Code") to include chitosan derived from *Agaricus bisporus* (button mushroom) as a processing aid as a finning agent for microbial stabilisation in wine, beer, and cider. The trade name for the chitosan preparation as described herein is "Pinnacle Mycobrio™", it is produced by a Canadian company Chinova Bioworks.

A.4 Justification for the Application

Chitosan derived from *Aspergillus niger* are currently permitted for use under schedule 18 of the Code for use as a processing aid in (FSANZ, 2002, 2020a). Chitosan from *Agaricus bisporus* as a processing aid has been approved for use in some of Australia's major competitor countries. In addition, under the Australia – European wine agreement, wine from Europe made using this fungal chitosan can be sold in Australia, but Australian producers cannot yet use chitosan from this source for domestic production.
Therefore, as Schedule 18 of the Code currently only permits chitosan from *Aspergillus niger,* this application is submitted to amend the Code to include a different source organism, *A. bisporus*, as a source of chitosan. Both the Australian National Association of Winegrape and Wine Producers (appendix 9) and New Zealand Wine (appendix 10) strongly support this application.

A.4.1 Technological Function of the Processing Aid

Chitosan is a natural polysaccharide, obtained by the de-acetylation of chitin. Chitin is the main component of the cell walls of fungi, plants, and insects. Chitosan and chitin-glucan of fungal origin (*Aspergillus niger*) are permissible products that can be used for the decrease of undesirable micro-organisms, settling aid, antioxidants, decrease of copper and iron concentrations and removal of contaminants. Chitosan is important in the food and wine industries mainly because of its anti-microbial characteristics. It can control the growth of undesirable yeast like *Brettanomyces*, lactic acid bacteria like *Lactobacillus*, *Oenococcus* and *Pediococcus* and acetic acid bacteria like *Acetobacter.*

A.4.2 Costs and Benefits for Industry, Consumers, and Government Associated with Use of the Processing Aid

The inclusion of chitosan derived from *Agaricus bisporus* as a processing aid will provide wine and cider producers with an alternative source of chitosan that is derived from, a non-pathogenic and non-toxigenic source organism, the white button mushroom. Because this chitosan is made from a sustainably farmed biomass it will be a much more environmentally friendly product. This will also make Australia consistent with the standards from other major producers. The inclusion of *A. bisporus* as a source organism will not result in any additional cost to the regulator as chitosan is already approved for use as a processing aid by the OIV.

A.5 Information to Support the Application

FSANZ previously reviewed applications to include chitosan from *A. niger*, (Application A1077), for use as a processing aid in the production of wine, cider, and beer. As part of their evaluation, FSANZ reviewed safety information on chitosan, including pre-clinical and human

safety data, and raised no safety concerns with the use of chitosan as a processing aid. Technical information specific to Chinova Bioworks chitosan derived from A*. bisporus*, including product-specific safety data, are presented in the sections that follow. The information is presented to support the safety of chitosan derived from A*. bisporus* in accordance with the requirements listed in Section 3.3.2 (Processing Aids) of the FSANZ *Application Handbook* (FSANZ, 2019). The information presented herein pertains to the commercial product, Pinnacle Mycobrio, containing chitosan from A*. bisporus* for which approval is being sought.

A.6 Assessment Procedure

AB Mauri considers the most appropriate assessment procedure for assessing the application to include A*. bisporus* as a source organism for chitosan to be the General Procedure. It is anticipated that this application will involve amending Standard 1.3.3 of the Code to include chitosan derived from A. *bisporus* as a processing aid. As noted, chitosan derived from *A. niger* is already permitted as a processing aid as listed in Schedule 18 of the Code.

A.7 Confidential Commercial Information (CCI)

None.

A.8 Other Confidential Information

None.

A.9 Exclusive Capturable Commercial Benefit (ECCB)

It is not anticipated that this application would confer Exclusive Capturable Commercial Benefit (ECCB) in accordance with Section 8 of the FSANZ Act based on the following:

"*An exclusive, capturable commercial benefit is conferred upon a person who applies for the development of a food regulatory measure or the variation of food regulatory measure under Section 22 if:*

(a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard to draft variation of the standard that would be prepared in relation to the application; and

b) any other unrelated persons or bodies, including unrelated commercial entities, would require the agreement of the applicant to benefit financially from the approval of the application".

A.10 International and Other National Standards

This application is for a processing aid and therefore the Codex General Standard for Food Additives is not applicable.
Resolutions permitting the use of fungal chitosan from *Aspergillus niger* and *Agaricus bisporus* as a fining agent and contaminant treatment have been granted by the International Organisation of Vine and Wine (OIV/OENO 336A/2009; 337A/2009; 338A/2009; 339A/2009) (OIV, 2011; Appendix 3 - 6). A monograph for fungal chitosan has been added to the International Oenological Codex by decision of the OIV general assembly dated July 2009 considering the works of the group of experts "Specifications of Oenological Products" (OIV/OENO 368/2009, Appendix 7).

A.11 Statutory Declaration

Signed Statutory Declarations for Australia and New Zealand are provided in Appendix B.

A.12 Checklist

Completed checklists relating to the information required for submission with this application based on the relevant guidelines in the FSANZ *Application Handbook* are provided in Appendix C.

B. TECHNICAL INFORMATION ON THE PROCESSING AID

In accordance with Section 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 2019),
the following technical information must be provided:
1. Information on the type of processing aid;

2. Information on the identity of the processing aid;

3. Information on the chemical and physical properties of the processing aid;

4. Manufacturing process;

5. Specification for identity and purity; and

6. Analytical method for detection.

Each point is addressed in the following subsections.

B.1 Information on the Type of Processing Aid

Chitosan is a linear polysaccharide of glucosamine and N-acetylglucosamine that is derived from chitin, a naturally occurring carbohydrate polymer that is widely distributed in nature. Chitin is found in cell walls of crustaceans, fungi, insects and in some algae, microorganisms, and some invertebrate animals. The traditional extraction process involves various steps, namely, demineralization, deproteination, bleaching/discoloration and finally deacetylation to form chitosan. Fungal extraction does not require the demineralization step but requires

deproteinization using bases and strong acids at high temperatures and further neutralization. Deacetylation converts chitin into chitosan. The process involves the removal of the acetyl groups attached to amino group to expose the -NH_2 groups. The degree of acetylation (DA) of chitin is a significant parameter influencing the biological, physicochemical, and mechanical properties and an important parameter that determines its classification whether it is chitin or chitosan. Chitosan is emerging as a very important raw material for the synthesis of a wide range of products used for food, medical, pharmaceutical, health care, agriculture, industry, and environmental pollution protection.

Fungal chitosan is obtained by the deacetylation of chitin. Fungal chitosan is used as a processing aid in the manufacturing of wine, beer, cider, and spirits. Chitosan is used in stabilizing wine, cider, and beer through their antimicrobial effect on several economically important microbial contaminants such as *Brettanomyces* as well for flotation, clarification to reduce cloudiness and the content of unstable colloids, for use as a fining agent in the treatment of wine, for stabilizing of the colour, for riddling of sparkling wine, for reducing organic and mineral contaminants. Chitosan can be used at different stages throughout the winemaking process to achieve different outcomes.

Regardless of the technological purpose, the sediments that contain the chitosan are removed from the wine, must or spirits at the end of the treatment by physical separation processes such as racking, centrifugation and/ or filtration. Since chitosan is insoluble at slightly acidic to neutral pH levels, as well as in aqueous and ethanol solutions, it is unlikely that any residual chitosan will remain in the treated products.

Therefore, the estimated intake of chitosan from all proposed technological uses can be considered as negligible. Chitosan derived from *A. bisporus*, was shown to be chemically and structurally equivalent to *A. niger* and Crustacean derived chitosan (Appendix 1). Through chemical analysis by FTIR and NMR we can see the compositional equivalency of all 3 sources of chitosan to one another. The fungal sourced White Button mushroom (*A. bisporus*) and *Aspergillus niger* are completely equivalent to the United State Pharmacopeia standard crustacean chitosan. In addition, the specifications of the chitosan set by the suppliers of each source of chitosan agree with one another for key characteristics such as ash content, heavy metals, and microbiology. See specifications in Table 1 below.

Table 1. Chitosan specifications from different sources.

	Chitosan Source		
	Crustacean	**Aspergillus niger**	**Agaricus bisporus**
Degree of deacetylation (mol %)	>80	>70	>80
Loss on drying (%)	≤10	≤10	≤10
Ash (%, w/w)	≤5	≤3	≤3
Mercury (mg/kg)	≤0.1	≤0.1	≤0.1
Arsenic (mg/kg)	≤1	≤1	≤1
Cadmium (mg/kg)	≤1	≤1	≤1
Copper (mg/kg)	≤10	≤10	≤10

Therefore, data established the safety of shellfish-derived and *A. niger* derived chitosan are considered relevant to the safety evaluation of fungal chitosan for the proposed food used described herein.

B.2 Information on the Identity of the processing aid

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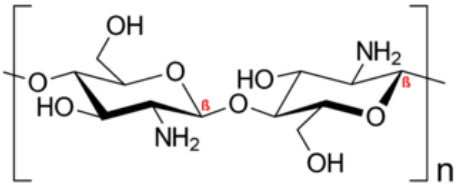
B.2.1 Identity of the processing aid

Source (strain): *Agaricus bisporus*
Common/Accepted Name: white button mushroom
Chemical Abstracts Name: 2-amino-2-deoxy-poly-D-glucosamine
International Union for Pure and Applied Chemistry name: (2R, 3R, 4R, 5S, 6R) 3-amino-6-hydroxymethyl oxane-2, 4, 5-triol
Structural formula:

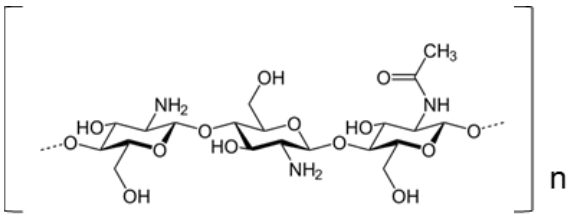


Common name: Fungal Chitosan
Chemical Abstract Service (CAS) number: 9012-76-4
Marketing names: Various marketing names currently exists and are marketed under distributors tradenames.
Molecular formula: $(C_8H_{11}NO_4)n$
Viscosity Mv of 10-15K (Mv = viscosimetric molecular weight
Appearance and odour: Odourless, off white to slightly brownish fine free-flowing powder or liquid.
It has FDA GRAS status (Appendix 2).

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is a mixture of chitosan and *beta*-1,3-D-glucans and is sold under the trade name Chiber™ to AB Biotek and rebranded to Pinnacle Mycobrio™. Chitosan is the main component, representing approximately 95% of the total volume; chitosan is a soluble polymer derived from the cell walls of a non–genetically modified white button mushroom (*A. bisporus*) biomass with a molecular weight (MW) of 10 to 400 kDa.[1] Chitosan [(1,4)-2-amino-2-desoxy-*beta*-D-glucan; poly-β-(1,4)-2-amino-2-deoxy-D-glucose; CAS 9012-76-4] is a linear polycationic polysaccharide composed of D-glucosamine and *N*-acetyl-D-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.*, in crustacean shells and fungal cell walls), where more than 60% of the acetyl groups are removed (*i.e.*, >60% deacetylation). The chemical structure of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is shown in Figure 1.

[1] Chitosan in this MW range is considered low molecular weight chitosan.

Figure 1. **Chemical Structure of Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*).**



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 (*e.g.*, vegetable and *Aspergillus niger* sources) or branched (*e.g.*, Baker's yeast) or both (*e.g.*, mushrooms). Chinova's mushroom-derived fibre may contain up to 5% *beta*-1,3-D-glucans.

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is specified to contain an average MW of 10 to 400 kDa, with a degree of acetylation (DDA) greater than 80%. Analysis of different production batches of the ingredient demonstrates the average MW to be approximately 100 kDa and the DDA to be 90 to 94%.

B.3 Information on the Chemical and Physical Properties of the processing aid

Chitin was first discovered by French scientist Prof. Henri Braconnot in mushrooms in 1811. In 1823 the same compound was also found by Odier in the insect's cuticles and named as "Chitin," which in Greek means tunic, covering, or envelope. In 1859 C. Rouget's research publication indicated "modified chitin" could be synthesized by treating chitin with boiling aqueous solution of concentrated potassium hydroxide. He also observed that chitin was influenced by chemical and temperature-dependent treatment.

Chitosan molecules possess almost similar structure of cellulose and chitin except at C-2 position in the functional group. Cellulose and chitin possess hydroxyl (–OH) group and *N*-acetylamine ($–NHCOCH_3$) group, respectively. However, chitosan consists of amino ($–NH_2$) group at C-2 position. Chitosan is a linear-chain polysaccharide that consists of *N*-acetyl-2-amino-2-deoxy-d-glucopyranose (acetylated unit) and 2-amino 2-deoxyd-glucopyranose (deacetylated unit), where the repeating units are linked by *β*-(1→4)-glycosidic bonds.

The physicochemical properties of chitosan in solution are related to the degree of deacetylation and molecular weight. After the deacetylation process, the resulting chitosan has diverse functional groups: some capable of being ionized, the amino moieties, and the remaining acetamide groups that are prone to form hydrophobic associations. This chemical characteristic of chitosan has influence in many functional properties of this molecule (i.e., at acid pH values, the amino groups become cationic, promoting the dissolution of chitosan). Also, the polycationic character of chitosan allows it to interact with diverse types of molecules. This, together with its structural capacities, biocompatibility, and other properties, make chitosan attractive for producing functional materials applicable in several fields.

The proportion of acetylated and deacetylated groups, or degree of acetylation (DA), determines the distinction among chitin and chitosan. Chitin deacetylation reaction progresses by exposing amino groups along the molecule. The extent and distribution of this

modification causes several changes in the main properties of the molecule. One of the most notorious changes is that, as the amino groups are ionizable, chitosan becomes polycationic in acidic media. This unusual quality for a biopolymer allows chitosan to be capable of forming solutions and actively interacting with diverse molecules. Thus, the DA determines most of the properties of chitosan, including solubility, extent of swelling in water, susceptibility to biodegradation, bioactivity, and biocompatibility among others. Practically, the DA has influence in all the functional properties of chitosan.

Adsorption is a process involving ionic, hydrophobic, hydrogen bonding, or Van der Waals forces. Chitosan has functional groups that could generate these interactions with molecules of several types. Adsorption of proteins, lipids, metals, and other substances, such as aromatic compounds and dyes, over chitosan have been studied. The adsorption capacity of chitosan is dependent on its physicochemical characteristics, mainly DA and molecular weight, the conditions of interaction (e.g., pH, ionic strength, etc.), and type and characteristics of the adsorbate.

The interactions of chitosan with proteins are mainly influenced by the pH that determines the degree of ionization of both compounds. To generate electrostatic interactions the isoelectric point of the protein should be lower that the pK_a of chitosan. Generally, chitosan–protein interactions are not strong; thus, proteins can be desorbed on appropriate conditions. Protein adsorption on chitosan is displayed in chitosan functional properties as muco-adhesion or cell and microbial flocculation. Currently, the adsorption capacity of chitosan is fundamental on its main commercial uses, such as flocculating agent in wastewater or food-related applications.

B.4 Manufacturing Process

Chitin is found in association with other biopolymers in different organisms. For example, in fungi, chitin is covalently bonded directly or indirectly via peptide bridges to glucans in cell walls while in insects and other invertebrates, it is either covalently or non-covalently associated with certain proteins. This variation implies that different extraction techniques maybe necessary.

The traditional extraction process involves various steps, namely, demineralization, deproteination, bleaching/discoloration and finally deacetylation to form chitosan. Dissimilar to fungi and insect, the presence of minerals in crustaceans makes demineralization a crucial step. Demineralization is achieved through acid treatment using sulfuric, hydrochloric, nitric, acetic, oxalic and formic acids. Acid treatment breaks down calcium carbonate into calcium chloride and carbon dioxide. While hydrochloric acid is the most preferred reagent for the demineralization of both insect and crustacean shells, attempts are being made to replace with more environmentally friendly organic acids. Fungal extraction does not require the demineralization step but requires deproteinization using bases and strong acids at high temperatures and further neutralization. A typical extraction process of chitin from fungi requires first treatment with alkali, usually 1 M NaOH at 60–120 ◦C for 0.5–12 h), to remove proteins, lipids, and other alkali-soluble carbohydrates. The remaining alkali insoluble material containing mainly chitin is further treated with acids such as 2–10% acetic acid at 50–95 ◦C, to remove acid soluble material. The obtained acid soluble material, rich in chitosan, is then treated with alkali up to 2 N NaOH followed by centrifugation and washing with acetone and ethanol, followed by centrifugation, and washing with acetone and ethanol.

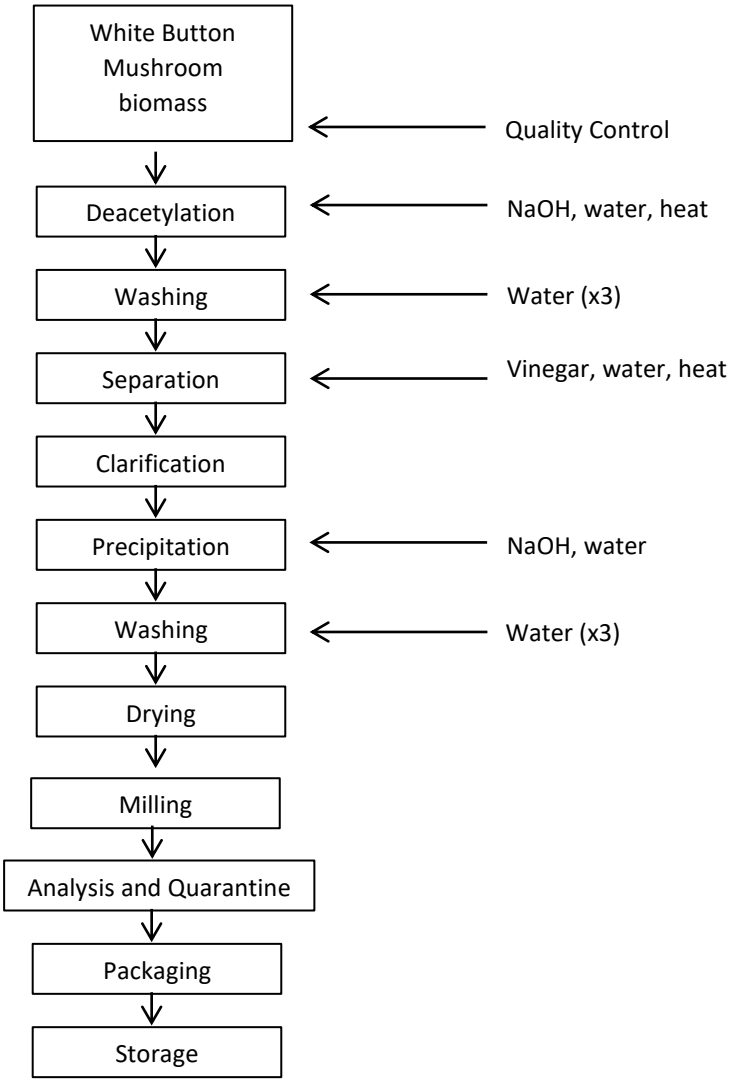
Alkaline conditions degrade cell wall material resulting in insoluble proteins and chitin which is then further treated with an acid such as hydrochloric, lactic, or acetic acid. Acetic acid is preferred for effectively removing phosphates and insoluble materials. It should be noted that high alkali concentration can cause chitosan oxidation, extensive chain degradation especially at high temperatures and long deproteinization incubation time. Similarly, acid treatment can also affect the final yield of chitosan during the extraction process. Lactic acid produced higher yield of chitosan than hot sulfuric acid even under lower temperature, formic acid (6% v/v) gave a higher yield of chitin compared with acetic acid. Although hydrochloric acid causes a greater extent of hydrolysis of the acetyl moieties, it produces chitosan with higher DDA compared with acetic and formic acid.
Generally increasing the concentration of acids results in increased DDA and darker coloured chitosan. This procedure does not include the deproteinization nor the demineralization processes required in the extraction of chitosan from crustacean sources.
The fungal chitin extraction process has been shown to result in chitin free of proteins that could cause allergic reaction, making them suitable for biomedical applications.
Discoloration is easily achieved using organic solvents for example, acetone while bleaching is achieved using sodium hypochlorite or hydrogen peroxide.
In summary, it is important to note the harsher the chemical extraction techniques employed during demineralization, deproteination and discoloration treatments with regards to chemicals used, pH, temperature, and incubation time, the higher the degree of hydrolysis and may affect the quality of obtained chitin. Despite the advances in developing new environmentally friendly, efficient chitin extraction techniques, chemical extraction techniques remain, to date, the preferred routes due to the availability of chemicals and the possibilities of scalability.

Deacetylation converts chitin into chitosan. The process involves the removal of the acetyl groups attached to amino group to expose the -NH2 groups. The degree of acetylation (DA) of chitin is a significant parameter influencing the biological, physicochemical, and mechanical properties and an important parameter that determines its classification whether it is chitin or chitosan. The deacetylation process results in a polymer containing both N-acetyl-glucosamines and glucosamines units. If the deacetylation produces a polymer with >50% N-acetyl-glucosamine units, it is still referred to as chitin if it is lower, than it is termed chitosan. Thus, deacetylation does not only affect acid-base behaviour, electrostatic characteristics, biodegradability, self-aggregation, solubility, sorption properties, ability to chelate metal ions among many other properties but also determine its classification and affect its suitability for specific applications. The percentage of N-acetyl-glucosamine units is termed the degree of acetylation (DA) and can vary from 50% to 100%. During the deacetylation process, random depolymerization also occurs due to the extreme process conditions (for example, strong base, high temperatures and pressures) leading to the production of chitosan with varying chain length and water-solubility properties. Although chitin can be deacetylated using either acids or alkalis, glycosidic bonds are very susceptible to acid hydrolysis therefore alkali-deacetylation using NaOH at high temperature is increasingly being used more frequently to avoid unwanted chain termination. Satisfactory deacetylation is achieved with concentrated NaOH or KOH (40–50%) at temperatures above 100 °C. This industrial approach hydrolyses the amide bonds makes it possible to produce several chitosan products in the form of flakes, fine powder, beads, or fibers.

B.4.1 Chinova Bioworks Manufacturing Process

The chitosan extracted from white button mushrooms (*Agaricus bisporus*) is manufactured in accordance with current Good Manufacturing Practice. 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 the figure 2 below. Chinova Bioworks procures white button mushroom from Canadian mushroom farmers. Each mushroom farm is inspected and accredited by the Canadian Food Inspection Agency and Agriculture and Agri-Food Canada. Prior to processing the mushroom biomass is inspected and samples analyses according to the company's specifications for white button mushroom material. The company ensures only *A. bisporus* is utilized by inspection of the lot's received, and by working with mushroom farms which only grow *A. bisporus* mushrooms.

Figure 2 The *Agaricus bisporus* chitosan production process.



The white button mushroom (*A. bisporus*) biomass is initially inspected for conformance with the internal raw material standards (heavy metal content, moisture content, microbiology [total aerobic plate count, yeast, and moulds] 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 fibre and simultaneously hydrolyses proteins and saponifies lipids from the biomass and will also destroy any mycotoxins present. The deacetylated biomass is removed from the alkali solution and is 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 fibre out of the solution. The precipitated fibre is collected by centrifugation and is repeatedly washed with water. The collected dewatered fibre is dried using a drum dryer. The dried fibre is milled into a fine powder and held for quality control analysis. Subject to approval from the quality control analysis, the fibre is then packaged and stored.

The processing aid has been tested for the presence of food allergens including sulphites, milk, eggs, peanuts, nuts, almonds, pistachio, gluten, soy, celery, mustard, sesame seeds, lupin, molluscs, crustaceans, and fish. Results are negative (or at concentration below the detection limit) which confirms that the manufacturing process does not carry-over any allergens.

The *A. bisporus* chitosan is also manufactured in compliance with the principles of HACCP and Chinova Bioworks has established appropriate quality control procedures to ensure production of a high-purity liquid concentrate that is free of contaminants, including the use of an established safe production strain (See Chinova Bioworks HACCP plan in Appendix 15). Quality Chinova Bioworks is certified under the Global Food Safety Institute through the Safe Quality Foods (SQF) program in Canada. control steps for these critical control points have been included as part of this plan to ensure adherence with the established manufacturing process and to produce a high quality and consistent product. Furthermore, each manufactured batch is analysed for conformity to the following specifications listed in table 2.

B.5 Specification for Identity and Purity

B.5.1 Product Specifications

The specifications for chitosan from white button mushrooms (*Agaricus bisporus*) are presented in Table 2. Internal methods of analysis are provided below as well (Some internal methods are listed as appendix 11-13). Parameters for physical and compositional characteristics of the ingredient, as well as appropriate limits for heavy metal impurities, have been established.

Table 2 **Product Specifications for Chitosan Extracted from White Button Mushrooms (*Agaricus bisporus*)**

Specification Parameter	Specification Limit	FCC (2023)	Method of Analysis
Identification	Positive	Conforms	FTIR, ^{1}H-NMR
Colour of powder	White to beige	White to light yellow	Validated Internal (visual)
Degree of deacetylation (mol%)	≥80	70 to 95	Validated Internal
Molecular weight average (kDa)	10 to 400	-	HPLC
Moisture (%, w/w)	≤5	≤5	Validated Internal
Heavy Metals			
Arsenic (mg/kg)	≤0.2	≤0.5	ISO 11885 (ICP-OES)
Lead (mg/kg)	≤0.2	≤0.5	ISO 11885
Cadmium (mg/kg)	≤0.2	≤0.2	ISO 11885
Mercury (mg/kg)	≤0.2	≤0.2	ISO 11885
Minerals			
Iron (mg/kg)	≤10	≤10	ISO 11885
Chromium (mg/kg)	≤1.0	≤1.0	ISO 11885
Nickel (mg/kg)	≤1.0	≤1.0	ISO 11885
Microbiological Parameters			
Aerobic microbial count (CFU/g)	≤100	-	ISO 4833 Part 2 2013
Yeast and mould count (CFU/g)	≤100	-	ISO 21527-2
Escherichia coli (CFU/g)	Absent	-	ISO 7251
Salmonella (absent/present)	Absent	-	AOAC 2013.01

^{1}H-NMR = proton nuclear magnetic resonance; AOAC = Association of Official Analytical Collaboration; CFU = colony-forming units; FTIR = Fourier-transform infrared spectroscopy; = HPLC = high-performance liquid chromatography; ICP-OES = inductively coupled plasma-optical emission spectrometry; ISO = International Organization for Standardization; kDa = kilodaltons.

To characterise the particle size distribution of the chitosan from white button mushrooms (*A. bisporus*), particle size analysis of 3 lots was carried out on a Malvern Mastersizer 3000 equipped with HydroEV wet volume dispersion cell. Results of the analysis, presented below in Table 3, demonstrate that less than 10% of the particles are smaller than 500 nm, the practical threshold upper limit for the range of "small particles" in accordance with Section 3.3 of the European Food Safety Authority *Guidance on risk assessment of nanomaterials to be applied in the food and feed chain: human and animal health* (EFSA Scientific Committee, 2021). Therefore, the chitosan from white button mushrooms (*A. bisporus*) does not meet the definition of engineered nanomaterial as set out in Regulation (EU) 2015/2283[2] on novel foods.

[2] Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. OJ L 327, 11.12.2015, p. 1–22. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32015R2283 (current consolidated version: 27/03/2021).

Table 3 **Particle Size Analysis for 3 Lots of Chitosan Extracted from White Button Mushrooms (*Agaricus bisporus*)**

Lot	Particle Size (microns)		
	D10	D50	D90
Lot 20200922-1	0.592	0.868	1.87
Lot 20201015-1	0.586	0.871	1.83
Lot 20201118-1	0.596	0.876	1.89

D10 = size below which 10% of the particles are found; D50 = size below which 50% of the particles are found; D90 = size below which 90% of the particles are found.

Analysis of 5 production lots of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) demonstrates that the manufacturing process, as described in Section B4.1 above, produces a consistent product that meets the established product specifications. A summary of the chemical and microbiological analyses is provided in Table 4. Certificates of analysis are provided in Appendix 11-13.

Table 4 **Analysis of 5 Independent Representative Batches of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*)**

Specification Parameter	Specification Limit	Manufacturing Lot No.				
		20201118-1	20201015-1	20200922-1	20210212-1	20210220-1
Identification	Positive	Positive	Positive	Positive	Positive	Positive
Colour of powder	White to beige	Beige	Beige	Beige	Beige	Beige
Degree of deacetylation (mol%)	≥80	94	90	92	90	92
Molecular weight average (kDa)	10 to 400	102	100	105	107	105
Moisture (%, w/w)	≤5	4.5	4.8	4.6	4.8	4.7
Heavy Metals						
Arsenic (mg/kg)	≤0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Lead (mg/kg)	≤0.2	<0.02	<0.02	<0.02	<0.02	<0.02
Cadmium (mg/kg)	≤0.2	<0.002	<0.002	<0.002	<0.002	<0.002
Mercury (mg/kg)	≤0.2	<0.01	<0.01	<0.01	<0.01	<0.01
Minerals						
Iron (mg/kg)	≤10	9	8	9	8	9
Chromium (mg/kg)	≤1.0	0.6	0.6	0.8	0.8	0.8
Nickel (mg/kg)	≤1.0	0.2	0.2	0.2	0.2	0.2
Microbiological Parameters						
Aerobic microbial count (CFU/g)	≤100	<1	<1	<1	<1	<1
Yeast and mould count (CFU/g)	≤100	<1	<1	<1	<1	<1
Escherichia coli (CFU/g)	≤1	<0.5	<0.5	<0.5	<0.5	<0.5
Salmonella (absent/present)	Absent	Absent	Absent	Absent	Absent	Absent

CFU = colony-forming units; kDa = kilodaltons.

B.5.2 Analytical methods

Test methods are briefly described below.

B.5.2.1 Degree of acetylation

Titration methods are the most common and affordable methods of measuring DA of chitosan. Several titration methods have been used by several researchers including acid–base titration, potentiometric titration, colloidal titration, and conductometric titration. In alkali titration, chitosan is dissolved in an organic acid and then titrated with dilute alkali solution with continuous measurement of the pH of the solution. Plotting pH of the solution against the volume of added alkali will yield two points of inflection, which are indicators of the amount of alkali consumed for the conversion of the amine groups into ammonium salts. In acid titration DA is calculated from the amount of acid consumed to neutralize free amino groups of chitosan dissolved in an alkali. The ninhydrin (triketohydrinedene hydrate) method is based on the determination of the free amine group of glucosamine units. In the picric acid method, the acid is used to adsorb free amino groups of chitin/chitosan with a ratio of 1:1.

Acid hydrolysis/ high-performance liquid chromatography can also be used. Acid hydrolysis of chitosan is performed first by digestion of the chitin/chitosan sample by acid to release acetic acid and later analysing the acetic acid by HPLC. Analysis with HPLC involves the measurement of the peak area and its conversion into molar concentration using a standard curve. The standard curve should be constructed using glucosamine or *N*-acetylglucosamine as the standard material. The method is relatively valid over a wide range of DA. It is affordable and easy to perform. It can also be used to analyse insoluble chitin. However, a calibration curve is usually required, and the measurement is sensitive to the presence of water.

Infrared is probably the most used method in the determination of DA of chitosan. It involves recording the infrared spectrum of the chitosan sample and calculating the DA or DD from the absorbance of some specific peaks using specific equations that have been developed over the years. In most cases baselines and internal reference bands are used to correct the spectra before they are used to calculate DA. The absorption ratios of A_{7669}/A_{7474} and A_{6039}/A_{5342} were chosen as the best ratios for chitin and chitosan model compounds, respectively.
In appendix 11 Chinova Bioworks describes their method for calculation of the percent degree of deacetylation.

B.5.2.2 Residual glucans

The determination of residual beta-glucan is performed by UV spectrophotometry. Beta-glucan is thermally decomposed by the addition of hot sulphuric acid R, leading to hydroxymethylfurfural moieties that react with phenol to form a yellowish to brown coloured product which absorbs at a wavelength of 420 nm. The absorption intensity of the solution is then compared to an external calibration curve established with a reference oat beta-glucan by using this method.

B.5.2.3 Viscosity

The apparent viscosity of the chitosan solution is measured using a calibrated rotational viscometer at controlled temperature, using an appropriate spindle, spindle rotation speed and a temperature-controlled water bath.

B.5.2.4 Loss on drying

The loss on drying is determined thermogravimetrically. A known quantity is heated at 105°C, and the sample weight loss is continuously measured using a calibrated moisture analyser until reaching a value less than 1 mg per 90s. When this value is reached, the weight of dry matter is calculated by removing the loss on drying value from the total weight.

B.5.2.5 Ash

The total ash content is determined by first weighing an empty porcelain crucible and then place a known quantity of chitosan in the crucible and heat it for 10 hrs at 600°C in a calibrated muffle oven. After the combustion the crucible containing the chitosan is weighed again.

B.5.2.6 Soluble residues

A known quantity of chitosan is washed with water and filtered. The residual matter is then filtered and weighed.

B.5.2.7 Heavy metals

The Heavy metals is determined by ICP-MS (as per the ISO 11885 standard).

B.5.2.8 Total viable aerobic microbial count

Is determined according to the ISO 4833:2003 standard, a method for enumeration of microorganisms by counting the colonies growing on a solid media after aerobic incubation at 30°C.

B.5.2.9 Total yeast and moulds count

Is determined according to the ISO 7954 standard, a method for enumeration of microorganisms by counting the colonies growing on a solid media after aerobic incubation.

B.5.2.10 Coliforms

The amount of *Escherichia coli* colonies is determined according to the ISO 7251 standard, a method for the enumeration of beta-glucuronidase-positive *Escherichia coli*.

B.5.2.11 Enterobacteriaceae

The amount of Enterobacteriaceae colonies is determined according to the ISO 215-28-2 standard.

B.5.2.12 Salmonella

The amount of *Salmonella* colonies (including *Salmonella typhi* and *S. paratyphi*) is determined according to the ISO 6579 standard.

B.6 Analytical methods for chitosan identification

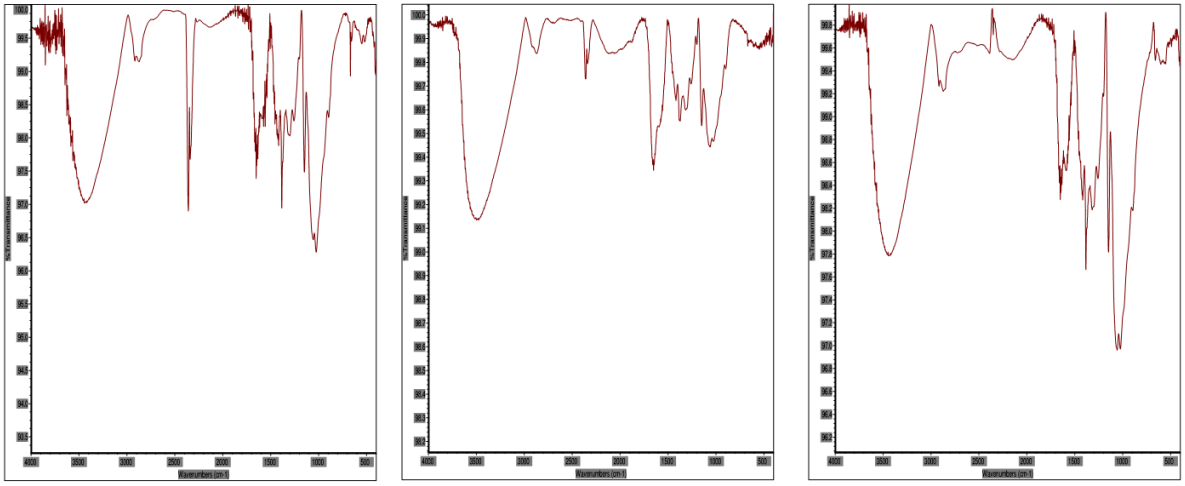
A compositional analysis of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was conducted to demonstrate that the mushroom-derived chitosan is equivalent to crustacean-derived chitosan, as well as to a chitosan reference standard described in the *United States Pharmacopoeia* (USP) monograph of chitosan. 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 B.6.1 below. In addition, chitosan derived from

A. bisporus, crustacean-derived chitosan, and USP monograph reference chitosan were analysed by ^{1}H-NMR spectroscopy (see Section B.6.1). The results of the infrared and ^{1}H-NMR spectroscopy demonstrate that chitosan derived from *A. bisporus* is compositionally identical to chitosan derived from crustacean sources.

B.6.1 Infrared Spectroscopy

Infrared (IR) spectroscopy is one of the most common methods used for chemical characterization of chitosan (Kumirska et al., 2010). Chinova Bioworks had samples of the white button mushroom chitosan, compared to a sample of *Aspergillus niger* chitosan origin and a sample of the USP chitosan reference standard which is crustacean source. The FTIR was run by the Department of Chemistry at the University of New Brunswick, Canada. The resulting spectra show that the white button mushroom and *A. niger*, sourced chitosan samples are indistinguishable from the crustacean sourced chitosan. The spectra are shown in figure 3 below:

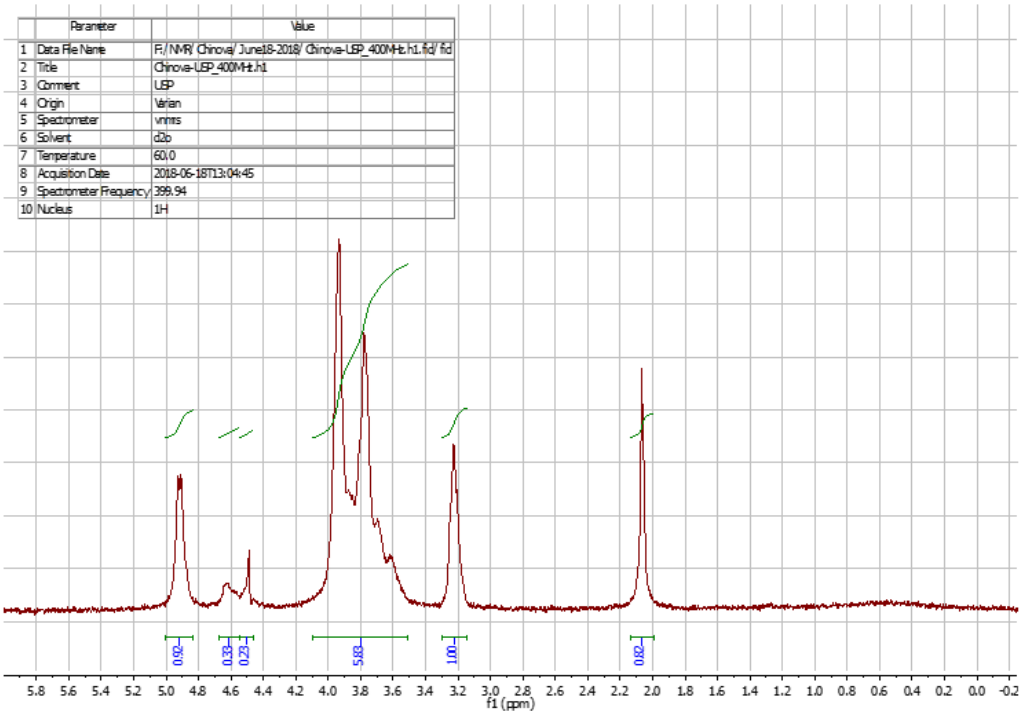
Figure 3. FTIR spectra of different sources of chitosan.



As can be seen in this figure all three sources of chitosan produced comparable FTIR spectral profiles, supporting chemical equivalence of *A. bisporus* derived chitosan sources. The peak shown in each spectrum at an approximate wavelength of 2,300 cm^{-1} is associated with carbon dioxide from the environment and is not associated with the chitosan sample.

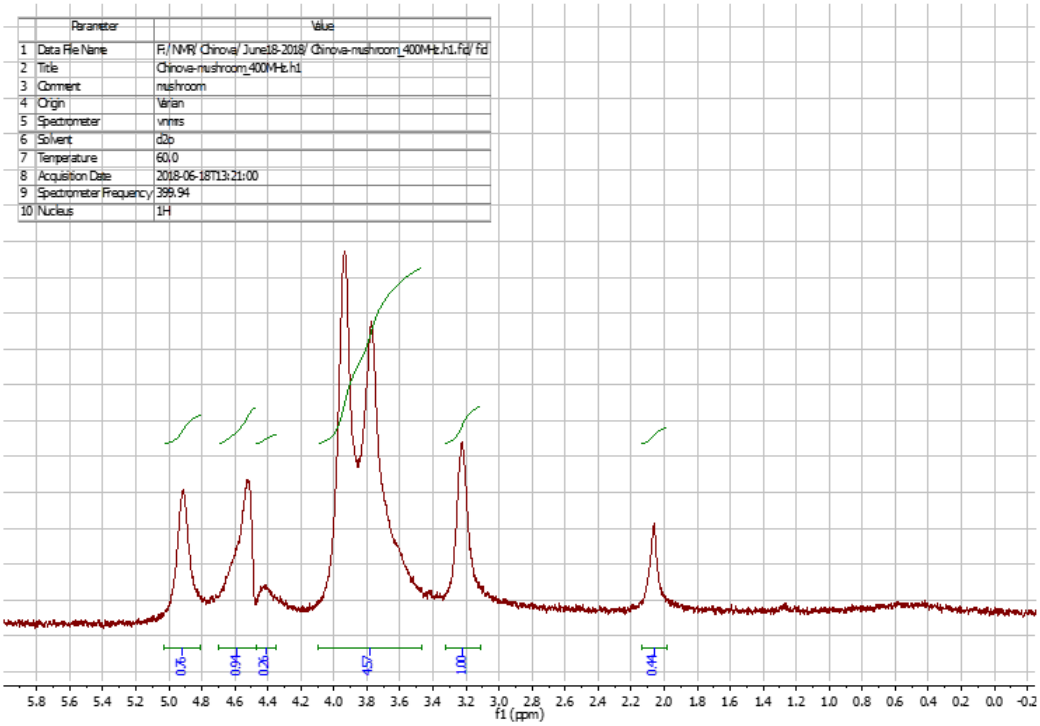
Another commonly used method of characterizing chitosan is Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy. This technique is well suited for chitosan since it can more readily give information about the degree of deacetylation of the chitosan compared to FTIR. Chinova had samples analyzed using ^{1}H-NMR by the Department of Chemistry at the University of New Brunswick, Canada. The samples included the white button mushroom (*A. bisporus*) chitosan, the *A. niger* fungal chitosan, and the USP monograph reference crustacean chitosan. The spectra are shown in the following figures 4a-c below:

Figure 4a. Proton Nuclear Magnetic Resonance spectroscopy profile of crustacean chitosan.



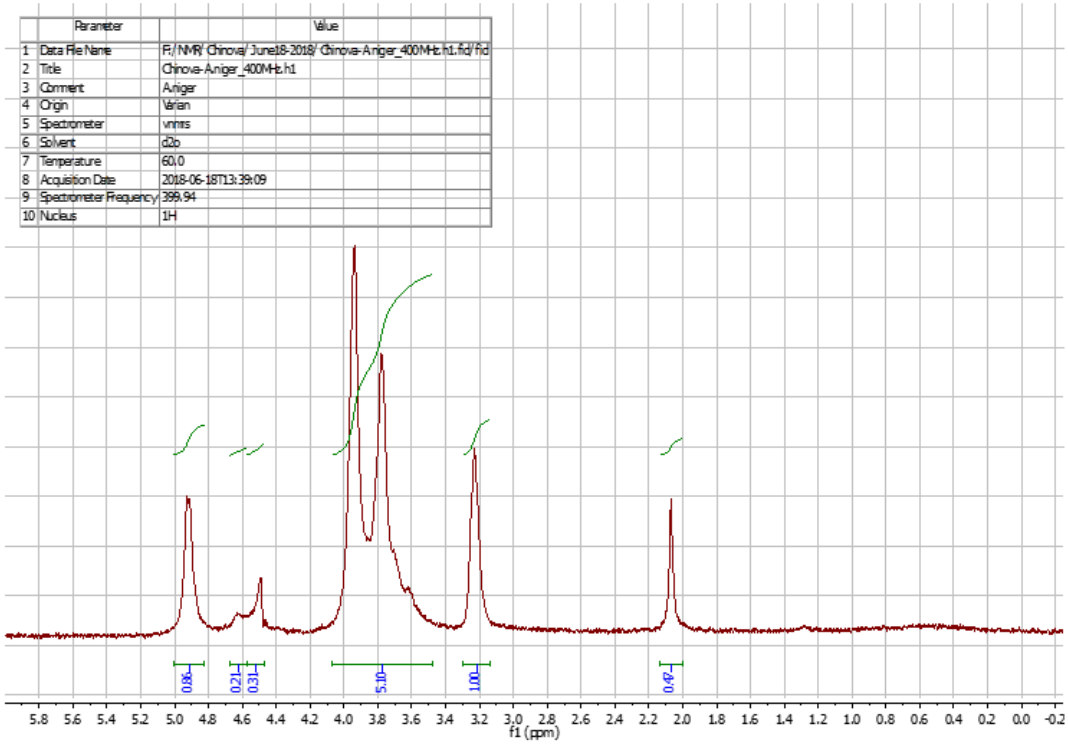
USP Standard – Crustacean Chitosan

Figure 4b. Proton Nuclear Magnetic Resonance spectroscopy profile of *A. bisporus* chitosan.



White Button Mushroom (*A. bisporus*) Chitosan

Figure 4c. Proton Nuclear Magnetic Resonance spectroscopy profile of *A. niger* chitosan.



A. *niger* Chitosan

Through chemical analysis by FTIR and NMR we can see the compositional equivalency of all 3 sources of chitosan to one another. The fungal sourced White Button mushroom (*A. bisporus*) and *Aspergillus niger* are completely equivalent to the United State Pharmacopeia standard crustacean chitosan. In addition, the specifications of the chitosan set by the suppliers of each source of chitosan agree with one another for key characteristics such as ash content, heavy metals, and microbiology.

C. INFORMATION RELATED TO THE SAFETY OF THE PROCESSING AID

C.1 General information on non-food industrial use of chitosan

Due to its physical and chemical properties, chitosan is being used in a wide variety of product and applications going from pharmaceutical, medical and cosmetics products to water treatment and plant production. In pharmaceutical applications, chitosan is used as an excipient for drug delivery systems. In medical applications it is used for its haemostatic, wound healing, and antibacterial properties in different forms (sponges, granules, bandages, fibers, etc). In cosmetics, chitosan is used for hair care (in shampoos), skin care (in body washes, creams, lotions, etc) and oral care (in toothpastes, mouthwashes, chewing gum, etc) to prevent the formation of plaque. Other industrial applications of chitosan include water treatment (flocculating agent, chelating agent, and heavy metal removing), textile and paper industry and agriculture. Considerable amount of chitosan is used in wastewater treatment.

Several animals, human, and *in vitro* studies relevant to the safety of shellfish chitosan, which has a long history of safe use in food supply, have been published. Chitosan from *A. bisporus*, was shown to be chemically and structurally equivalent to shellfish derived chitosan.

Therefore, data establishing the safety of shellfish-derived chitosan are considered relevant to the safety evaluation of fungal chitosan for the proposed food uses described in this application.

Published studies examining the metabolism and kinetics; acute, sub chronic, and chronic toxicity; reproductive toxicity in animals; and safety in human of shellfish-derived chitosan or chitosan oligosaccharides are summarized below. Generally available studies conducted in adult subjects have evaluated the safety and tolerability of repeated consumption of chitosan, and multiple studies investigating the effects of consuming shellfish-derived chitosan on various biological parameters (*e.g.*, plasma lipid levels, mineral and vitamin absorption, weight gain, sugar metabolism) have been reported.
Shellfish derived chitosan has a long history of safe use in the food industry. It is currently approved for use as a natural food additive for general food use in Japan and Korea (Japan Food Chemical Research Foundation, 2011; KFDA, 2011), and has widespread use as a dietary supplement product in the United States, the European Union, and other regulatory jurisdictions throughout the world. Supplement products typically promote consumption of 1 to 2 g/person/day for use in weight control and/or maintenance of cardiovascular health.
Also, fungal chitosan (derived from *A. bisporus* and *A. niger* sources) has been granted Novel Food approval by the European Commission, for use in supplement products in the European Union based on its substantial equivalence to existing shellfish derived chitosan products that are currently in the market.

Detecting chitosan in food products is very difficult. The issue lies in the separation of chitosan from the rest of the ingredients in the matrix. Chitosan is soluble in food products until approximately pH 6.5, at which point it will precipitate out, forming an insoluble precipitate. Several colourimetric assays, using ninhydrin, O-phthalaldehyde, or Cibacron Brilliant Red 3B-A, have been used to quantify chitosan. However, the response during these reactions depends strictly upon the degree of deacetylation of chitosan and cannot be used when chitosan is mixed with other proteins in a food and/or beverage system, which limits the application of these colourimetric assays (Yan and Evenocheck, 2012).

To characterise and quantify chitosan on its own, various other direct analytical methods can be employed, including capillary electrophoresis, size exclusion chromatography, high-performance liquid chromatography, and Fourier-transform infrared spectroscopy. Most of these quantification methods entail a total hydrolysis of chitosan into glucosamine (GlcN) followed by the subsequent characterisation of the monomer. Acid hydrolysis with hydrochloric acid is the most widely used because of its effectiveness in both the hydrolysis of the glycosidic linkage (depolymerisation) and the *N*-acetyl linkage (deacetylation) of chitosan. However, the recovery rate of GlcN can vary significantly from one analytical method to another, which can lead to high variability and improper quantification of chitosan (El-Saharty and Bary, 2002). Moreover, these analytical methods are not specific enough for routine analysis of chitosan in complex matrices such as foods and beverages. Chitosan readily interacts with various ingredients (carbohydrates, proteins, polyphenols, *etc*.) present in a food or beverage matrix to form complexes, which, from a technical perspective, makes isolating and separating chitosan extremely challenging (Li *et al*., 2013). This leads to

interference from other compounds during quantification and makes it nearly impossible to quantify chitosan or GlcN in complex food or beverage matrices.

C.1.2 Metabolic Fate

A limited number of studies have examined the metabolic fate of chitosan. Like dietary fibers, chitosan is poorly absorbed, is not subject to digestive processes within the gastrointestinal tract, and therefore travels intact throughout the small intestine to the colon where it is subject to microbial fermentation and excretion in the faeces. These findings are briefly summarized below.

C.1.2.1 Absorption and Distribution

Chitosan is poorly absorbed due to its highly insoluble physico-chemical properties. Upon ingestion, chitosan is solubilized by hydrochloric acid in the stomach and converted into a viscous liquid that emulsifies dietary fat droplets. As this viscous chitosan gel enters the duodenum, it starts to precipitate due to the gradual increase in pH and is excreted in the faeces (Furda, 2000). *In vitro* cellular models have also provided additional evidence that chitosan is poorly absorbed. Chitosan with molecular weight of 30 kDa or higher was not taken up by intestinal epithelial Caco-2 cells (Schipper *et al*., 1997). A subsequent study also demonstrated water-soluble chitosans of 230 kDa did not penetrate through the Caco-2 cell layer (Chae *et al*., 2005). It was also reported that the extent of chitosan absorption was inversely related to its molecular weight and occurred in the following order: H-chitosan<M-chitosan<chitosan oligosaccharides. Water-soluble chitosan had the greatest amount absorbed compared to all the compounds tested despite its moderate molecular weight, which may be attributed to its greater water solubility.

C.1.2.2 Metabolism

The human digestive tract can efficiently hydrolyse glucose polymers linked by *alpha*-glycosidic linkages, such as those found in starch and glycogen, and the *beta*-glycosidic bond in lactose can be hydrolysed by beta-galactosidase (Wisker *et al*., 1985). The *beta*-glycosidic bonds in chitosan are resistant to hydrolysation by hydrochloric acid present in the stomach; however, these bonds can be hydrolysed by chitosanases to produce a mixture of chitosan oligomers ranging from between 2 and 8 degrees of polymerization, which can be subsequently degraded further to glucosamine (Deshpande, 1993; Muzzarelli *et al*., 1997).

In vitro studies have reported that other digestive enzymes, including pepsin, amylase, and lipase, can hydrolyse chitosan at rates comparable to chitosanase (Xia *et al*., 2008). However, it is unknown whether these enzymes can effectively hydrolyse chitosan in the human gastrointestinal tract. Lysozyme, in addition to hydrolysing the glycosidic linkages of bacterial cell wall peptidoglycans, can also hydrolyse chitosan and chitin (Aam *et al*., 2010). Varum *et al*. (1997) incubated three different chitosan with different degrees of deacetylation (42, 51, and 60%) in human serum, and measured degradation rates by changes in viscosity as a function of time. Addition of lysozyme increased degradation rates, while addition of allosamidin, a chitinase inhibitor, had no effect. The authors concluded that the degradation of chitosan in human serum is mediated primarily by lysozyme and no other enzymes or depolymerizing mechanisms.

C.1.2.3 Fermentation by Intestinal Microflora

Bacteria are known to express chitosanases (Gooday, 1989), and it is plausible that the intestinal microflora may also possess the ability to hydrolyse chitosan. Although it is unknown whether the intestinal microflora in humans expresses chitosanases or other enzymes that can degrade chitosan, rat bacterial enzymes isolated from colon were able to degrade chitosan *in vitro* (Zhang and Neau, 2002). Although it is unknown whether bacterial strain with chitosanase activity exists in the human intestines, *Clostridium paraputrificum* was isolated from human faeces containing endochitinase and *beta*-N-acetylglucosaminidase activity. In vitro cultivation of bacteria with colloidal chitin resulted in the production of hydrogen, carbon dioxide, acetate, and lactate, as well as minute amounts of propionate and butyrate (Simunek *et al*., 2002).

C.1.2.4 Elimination

Onishi *et al*., (1999) administered FITC-labelled chitosan (50% deacetylated) intraperitoneally (29 mg/kg body weight)- to 3 male ddy mice. Urine was collected at 1-, 14-, and 24-hours following chitosan administration; approximately 25% of the dose was excreted in the urine within 1 hour, and nearly the entire dose was accounted for in the urine by 14 hours. In addition, Richardson *et al*. (1999) administered [^{125}I]-labelled chitosan of various molecular weights (<5 kDa, between 5 to 10 kDa, and >10 kDa) intravenously to male Wistar rats. Chitosan with molecular weights >5 kDa was rapidly cleared from the plasma. At 60 minutes following the injection, <10% of the administered dose recovered in the plasma while more than 50% of the administered dose was recovered in the liver. In contrast, approximately 30% of chitosan with smaller molecular weights (<5 kDa) was recovered in the plasma, and approximately 30% was found in the liver, at 60 minutes post-injection.

As a conclusion, chitosan is not absorbed from the gastrointestinal tract, thus systemic exposure does not occur. Although there is no evidence presented in literature to suggest that chitosan would be digested / hydrolysed during gastrointestinal transit, putative hydrolysis products generated during transit would consist of compounds (chitosan oligomers, glucosamine, and glucose) that are known to be poorly bioavailable, and 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). Therefor it can be concluded that chitosan would not be chemically altered in the human gastrointestinal tract.

C.2 General information on the use of chitosan as a food processing aid in other countries:

C.2.1. OIV Resolutions and Monograph

Resolutions permitting the use of fungal chitosan (both from *A. bisporus* and *A. niger*) in winemaking as a fining agent and contaminant treatment have been granted by the International Organisation of Vine and Wine (OIV/OENO 336A/2009; 337A/2009; 338A/2009) (OIV, 2011; Appendix 3 - 6).

A monograph for fungal chitosan has been added to the International Oenological Codex by decision of the OIV general assembly in July 2009 considering the works of the group of experts “Specifications of Oenological Products (OIV/OENO OIV/OENO 368/2009) (Appendix 7).

C.2.2. Approval in European Union

The corresponding approval for use of fungal chitosan (both sources) in wine products marketed within the European Union has been issued by the European Commission *EU 2019/934/2022-02-08.*

Since 2011, chitosan from Fungal origin (first from *A. niger* and then later from *A bisporus* was added) was approved for the oenological practices of clarification according to ANNEX 1 of REGULATION (EU) No 53/2011 and for treatment of wines under the conditions set up in Appendix 8.

C.3 Information on the toxicity of the chemical processing aid and if necessary, its major metabolites.

Crustacean-derived chitosan has a long history of safe use in the global food supply. It has widespread use as a drug excipient, functional food ingredient, and food supplement product in the EU, the United States (U.S.), and other regulatory jurisdictions and is currently approved/permitted for use as a natural food additive for general food use in Japan and Korea (JFCRF, 2020; MFDS, 2020). 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, 2023). In Canada, chitosan obtained from crustacean sources are used in licensed natural health products (NHPs), and a monograph for the use of crustacean-derived chitosan in NHPs indicates dose levels of 0.5 to 3 g of chitosan, 2 times/day, for a total of 6 g/day (Health Canada, 2018).

In the U.S., Chinova's fibre extracted from white button mushrooms (*A. bisporus*) has Flavour and Extract Manufacturers Association of the United States (FEMA) Generally Recognized as Safe (GRAS) status for use as a flavouring ingredient with flavour modifying properties (FEMA No. 4946 – Cohen *et al.*, 2022) at levels up to 2,000 parts per million (ppm). It should be noted that the intended use levels of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) as an antimicrobial ingredient in the EU (150 to 1,500 mg/kg) are lower than the FEMA GRAS–approved use levels (1,500 to 2,000 ppm). Several GRAS Notices pertaining to chitosan derived from fungal and crustacean sources have been notified to the U.S. Food and Drug Administration to date, including Chinova's fibre derived from white button mushrooms (GRN 997 – U.S. FDA, 2022), for which a letter of "no questions" was received on 28 February 2022 for the ingredient's use as an antimicrobial in a variety of foods at levels ranging from 0.015 to 0.15 g per 100 g. In Canada, Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is currently authorised for use under the same proposed food uses and maximum use levels as described herein; thus, it is included on Health Canada's List of Permitted Food additives as an anti-bacterial (Class 2) and anti-fungal (Class 3) preservative (Health Canada, 2023).

C.3.1 Potential Risk of Mycotoxins from source material

There is no potential risk of mycotoxins to white button mushroom chitosan as the processing used to extract it is intense enough to destroy any possible mycotoxin / aflatoxin

contamination in the white button mushrooms. Research (Moerck, 1980) has shown that even at concentrations as low as 2.0%, sodium hydroxide can destroy aflatoxin to below FDA guideline limits. The extraction processing uses higher concentrations of sodium hydroxide, paired with increased temperature, which would have an even stronger result then the cited research.

Chinova Bioworks uses a certified external labrotory to perform ELISA test on chitosan batches, which always confirm that no mycotoxin / aflatoxin is detected. Example can be seen at appendix 14.

C.3.2 Safety Evaluation Strategy and Corresponding Testing Strategy

Chitosan derived from crustaceans has a long history of safe use in the food supply, and its use in the European Union (EU) is defined as not novel in food supplements (European Commission, 2023). Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) has been demonstrated to be compositionally similar to chitosan derived from shellfish (see Section B5 and B6 for further details). As well, it is important to note that the authorisation of chitosan extract from fungi such as *Agaricus bisporus* or *Aspergillus niger* in the EU for use in food supplements was based on it being substantially equivalent to crustacean-derived chitosan, which is not novel[3]. Chinova's fibre derived from white button mushrooms (*A. bisporus*) is manufactured to an average molecular weight (MW) of 10 to 400 kDa and a degree of deacetylation (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 fibre 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 molecular weight 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 fibre 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.

[3] Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, p. 72–201. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1533914206967&uri=CELEX:32017R2470 (current consolidated version: 31/05/2023).

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 United States (U.S.) Generally Recognized as Safe Notices (*e.g*., GRN 73 – U.S.FDA, 2002; GRN 170 – U.S. FDA, 2005; GRN 397 – U.S. FDA, 2011; GRN 443 – U.S. FDA, 2013a), which are publicly available. Published studies on the metabolic fate of chitosan and toxicological studies on chitosan derived from crustacean sources were included in GRN 397 (U.S. FDA, 2011) and are discussed herein to support the safety of Chinova's fibre derived from white button mushrooms (*A. bisporus*). 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. Food and Drug Administration 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.* (GRN 170 – U.S. FDA, 2005)

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) (GRN 170 – U.S. FDA, 2005). The National Toxicology Program (NTP) conducted a long-term toxicity study of *United States Pharmacopeia*–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%, an average MW of 81.6 kDa, 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. 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 fibre derived from white button mushrooms (*A. bisporus*) and was considered to be pivotal in the safety assessment of Chinova's fibre extracted from white button mushrooms (*A. bisporus*). Similar nutritional findings were not reported in human clinical studies at doses up to 6.75 g/day; therefore, the changes in fat-soluble vitamins were not considered to be toxicologically significant at clinically relevant doses.

No product-specific toxicological studies have been conducted for Chinova's fibre derived from white button mushrooms (*A. bisporus*). As stated in Section 4 of the European Food Safety Authority (EFSA) *Guidance for submission of food additive evaluations*:

> *Applicants are reminded that Directive 2010/63/EU, on the protection of animals used for experimental and other scientific purposes, requires that care is taken to avoid unnecessary use of animals.*

Since it is evident that Chinova's fibre derived from white button mushrooms (*A. bisporus*) is not systemically absorbed, and it is structurally and compositionally equivalent to the crustacean-derived chitosan products used in the numerous toxicological studies that are available, conducting *in vivo* toxicological studies specifically with Chinova's fibre derived from white button mushrooms (*A. bisporus*) would be an unnecessary use of animals. Moreover, according to the tiered approach described in the EFSA *Guidance for submission for food additive evaluations* (EFSA ANS Panel, 2021), only Tier 1 would be required for the safety assessment of Chinova's fibre derived from white button mushrooms; however, for completeness, all Tier 2 and Tier 3 toxicological studies that are already available for the various chitosan preparations have been included in this application dossier.

List of Abbreviations

DDA	degree of deacetylation
EFSA	European Food Safety Authority
EU	European Union
MW	molecular weight
NTP	National Toxicology Program
U.S.	United States

Most information below has been collected from literature on shellfish chitosan unless the source is specified.

C.3.3 Acute Toxicity Studies

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 white button 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 = 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 herein.

Female Wistar rats (n=6/group) were administered lobster-derived chitosan (MW = 309 kDa; DDA = 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 fibre derived from white button mushrooms (*A. bisporus*). 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 (n=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 characterised by Eisa *et al*. (2018) and therefore, its compositional equivalence to Chinova's fibre 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 3.11), 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 fibre.

C.3.4 Subacute Toxicity Studies

Kim *et al*. (2001) evaluated the subacute toxicity of chitosan oligosaccharide in rats. The study was not conducted using GLP, and study methodologies were not reported to be consistent with recognized U.S., or international guidelines for toxicity testing of chemicals. Five-week-old male and female SPF Sprague Dawley rats (n=9/sex/group) were administered 0, 1000 or 2000 mg chitosan oligosaccharide/kg body weight/day by gavage for 28 consecutive days. Body weight and feed consumption were monitored weekly during the administration period. After the administration period, rats were killed by exsanguination under phenobarbital anaesthesia. No significant differences between groups were observed at any point with respect to feed intake, body weight, clinical signs, or mortality. All urinalysis parameters measured at necropsy (*i.e*., colour, pH, and concentrations of glucose, ketone bodies, nitrites, protein, occult blood, urobilinogen, and bilirubin) were within normal ranges except for increased mean leukocyte concentrations in the urine of male rats administered 500 mg chitosan oligosaccharide/kg body weight/day. However, this finding was not considered to be toxicologically relevant as it was observed only in males, and its occurrence was not dose related.

C.3.5 Sub chronic Toxicity Studies

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, alkaline phosphatase, 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 HMWC and water-soluble chitosan preparations of varying molecular weights (MWs) and solubility (MW = 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). 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 increase in organ lesions were reported based on histopathology examination (Lagarto *et al*., 2015). Lagarto *et al.* (2015) reported the short-term no-observed-adverse-effect level 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 = 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 MW, 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 fibre derived from white button mushrooms (*A. bisporus*) and assess the suitability of these studies in the safety evaluation of Chinova's fibre derived from white button mushrooms (*A. bisporus*). Furthermore, 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 recognised test protocol (*e.g.*, Organisation for Economic Co-operation and Development Test Guideline 408 – *Repeated Dose 90-Day Oral Toxicity Study in Rodents*). 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 there was 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 characterised 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 highest intake of Chinova's fibre from white button mushrooms (*A. bisporus*), based on its proposed food uses (*i.e.*, 1.2 g/person/day or 21 mg/kg body weight/day; see Section 3.7). No decreases in serum fat-soluble vitamins (vitamin A, D, E), *alpha*-carotene, or *beta*-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); 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 characterised) *via* gavage at doses of 0 (distilled water), 150, or 300 mg/kg body weight/day to Swiss albino mice (n=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 or internationally accepted standards for toxicity testing of chemicals and the test article was not adequately described by the authors (*i.e*., MW, DDA, purity). As such, its relevance to Chinova's fibre derived from white button mushrooms (*A. bisporus*) could not be determined.

The repeated-dose oral toxicity studies on various chitosan preparations are summarised in Table 5.

Table 5. 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 ↓ 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, faecal 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, and 4 significantly reduced the ↑ in bw following HFD. • Reduced liver weight in Groups 3, 4 following an HFD. • Serum triacylglycerol significantly reduced in Groups 2, 3, and 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. • ↑ small intestine length in chitosan group. • Absolute and relative liver mass ↑ in chitosan group. • NSD in whole-blood, tissue accumulation, and faecal and urinary excretion during 2-wk retinol exposure period.	Kimura *et al.* (2004)[c]
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 TG, TC, liver weight and lipids, liver and kidney damage markers	• NSD in weight gain until Week 17: Group 3 had reduced bw gain when fed an HFD. • NSD in plasma TG; Group 3 inhibited the ↑ of TC when fed an HFD. • Group 3 had significantly lower liver weight and hepatic TG and TC. • NSD in glutamic oxaloacetic transaminase, glutamic pyruvic	Sumiyoshi and Kimura, 2006[c]

Table 5. 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
					transaminase, and blood urea nitrogen.	
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 Fe, trace zinc, trace copper	• NSD in appearance and behaviour. • NSD in bw in chitosan groups compared to control. • NSD in food intake. • Group 4: statistically significant ↑ in relative thymus weight. • Other groups: NSD in relative heart, liver, spleen, thymus, kidney, or lung weights. • NSD in histopathology in chitosan groups compared to control. • Fe levels in liver, heart, spleen, and kidney not different in Groups 2 and 4 when compared to control; Fe level in liver and spleen elevated in Group 3. • Zinc levels in liver, heart, spleen, and kidney not different in Groups 2 and 4 when compared to control; zinc level in liver, spleen, and heart significantly elevated in Group 3. • Copper levels in liver, heart, spleen, and kidney not different in Groups 2 and 4 when compared to control; copper level in liver and spleen significantly elevated in Group 3.	Zeng *et al.* (2008)[c]

Table 5. 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: 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, OGTT, plasma insulin, glucose, TG, cholesterol, non-esterified fatty acids, and β-hydroxybutyrate, lipid analysis in cecal content, liver, and muscle	• ↓ 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; ↓ serum TG, cholesterol; NSD in serum non-esterified fatty acids. • Fat staining of the tissue demonstrate that lipid accumulation was reduced in liver and muscle compared to controls.	Neyrinck *et al.* (2009)[c]
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, and creatinine (M, F) [150, 300]. • ↑ ALP in F [150, 300]. • ↓ total lipids and TG in M [150, 300]. • ↓ TC, HDL-C, and LDL-C (M, F) [150, 300]. • ↓ protein and glycogen in kidney and liver homogenate (M, F) [150, 300]. • ↑ LDH, GPI, and HK (M, F) [150, 300]. • ↑ PFK in F [150, 300]. <u>Significant effects</u> • ↑ ALP in M [0 *vs.* 300]. • ↓ total lipids and 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 haemorrhage between hepatocytes [300]. • F: Dilated central veins and destructed red blood cells [150]. • F: Cytoplasmic vacuolation in hepatocytes, fatty degeneration, and leukocytic infiltration [300].	Omara *et al.* (2012)[c]

Table 5. 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
					• 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].	
LC chitosan Source: NR DDA: NR Size: 390 kDa SC chitosan Source: NR DDA: NR Size: 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, and apolipoprotein B in LC and SC groups compared to HFD control. • ↑ leptin, resistin, PAI-1, TG, TC, free fatty acid, HDL-C, and apolipoprotein B in HFD control compared to normal diet control. • ↓ adiponectin and 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, and 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, TC, TG, 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.	Tang *et al.* (2020)

Table 5. 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
					• NSD in blood glucose, OGTT, serum insulin, or lipid levels in any group.	
Chitosan Source: NR DDA: NR Size: 21.7 x 10^4 Da	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) M 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, haematology, absolute and relative organ weights	• Weight gain reductions occurred in Groups 5 and 6. • Efficiency of food utilisation ↓ in Groups 5 and 6. • Haemoglobin and packed cell volume ↓ in Groups 5 and 6; total serum protein ↓ 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 or food intake. • Relative liver weight was lower in chitosan groups. • Chitosan prevented the rise of serum cholesterol due to feeding cholesterol. • Liver cholesterol concentrations ↓ in chitosan groups.	Sugano *et al.* (1988)[c]
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, faecal weight, serum cholesterol, faecal neutral sterol excretion, faecal bile acid excretion	• NSD in growth, food intake, liver weight, or dried faecal weight. • NSD in faecal 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 ↓ coprostanol. • Statistically significant ↓ in serum cholesterol in 5% chitosan group.	Fukada *et al.* (1991)[c]

Table 5. 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: 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 ↓ in chitosan group. • Apparent fat digestibility ↓ in chitosan group. • Lower Ca, Mg, and Fe absorption, and lower bone mineral content, in chitosan group. • Liver retinol and retinyl palmitate lower in chitosan group. • Lower serum and liver vitamin E observed in chitosan group. • Lower serum TG. • 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, fructosamine, *beta*-hydroxybutyric acid, free fatty acid levels, plasma and liver lipid peroxides, liver and faecal lipids, liver glucose-6-phosphate dehydrogenase	• NSD in bw. • ↓ relative liver weight. • Higher liver lipid peroxide in chitosan (high viscosity) group. • NSD in plasma lipid peroxide values. • NSD found in other tissue weights. • Chitosan ↓ plasma TC, VLDL-C, LDL-C, and HDL-C. • ↓ liver total lipids, but NSD in liver triacylglycerol content.	Chiang *et al.* (2000)[c]
					•	
Chitosan, dietary Source: Shrimp shell 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 TC ↓ by 16%. • NSD in liver lipids. • NSD in plasma palmitic and steric acid levels; ↑ oleic, linoleic, and docosapentaenoic acid; ↓ 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 in bw gain, food intake, or 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 and CYP 1A1 in chitosan group; ↓ in GSH S-transferase.	Yao *et al.* (2010)[c]

Table 5. 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 DDA: 83% Size: 309 kDa	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, haematology, 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)

Table 5. 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
HMWC Source: NR DDA: NR Size: 310 to 375 kDa	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, Ca, 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, Ca, vitamin A, vitamin E, and MDA in treatment groups compared to control. • ↑ relative-to-body kidney weight, HDL-C, total protein, albumin, globulin, albumin/globulin ratio, GSH, and 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 Source: NR DDA: NR Size: 2.5 x 10^5 Da, CS1; 3.8 x 10^4, CS2 NFS Chitosan quaternary ammonium salt Source: NR DDA: NR Size: 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, and HACC2 compared to HFD control; NSD in CS1. • NSD in food consumption. • ↓ serum TG, LDL-C in CS2, HACC1, and HACC2 compared to HFD control; NSD in CS1. • ↑ serum TG, TC, and 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, and HACC2 compared to HFD control; NSD in CS1. • ↓ hepatic TC in CS and HACC2 compared to HFD control; NSD in CS1 or HACC1. • ↑ serum lipoprotein lipase activity in CS1, CS2, HACC1, and HACC2 compared to HFD control. • ↑ serum lipoprotein lipase activity HACC1 and HACC2 compared to HFD control; NSD in CS1 or CS2. • ↓ serum free fatty acids and 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.	Wang *et al.* (2019b)

Table 5. 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
LMWC Source: Crustacean shells DDA: 83.9% Size: 80 kDa HMWC Source: Crustacean shells DDA: 91% Size: 740 kDa	Rat (Sprague-Dawley) M 6/group	Diet 8 wks	0 or 5% (0 or 2,500)	AST, ALT, serum TC, HDL-C, LDL-C, VLDL-C, TNF-α, liver and intestinal weight	• ↓ bw in LMWC group *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 TC, HDL-C, LDL-C, and VLDL-C in high-fat controls; ↓ in same parameters in LMWC and HMWC groups. • NSD in ALT, AST, and TNF-α in LMWC or HMWC 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. • ↓ TC, LDL-C, VLDL-C, and HDL-C. • ↓ plasma AST and ALT in HMWC and LMWC groups.	Chiu *et al.* (2020)
Studies in Guinea Pigs						
Chitosan NFS	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, faecal excretion, plasma cholesterol, lipid peroxide and GSH levels	• NSD in bw, food intake, or 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 ↑ faecal weight, faecal fat excretion, faecal water excretion, and faecal water content. • ↓ TC, LDL-C, and triacylglycerol in chitosan group. • GSH level in liver of chitosan group was higher compared to control.	Jun *et al.* (2010)[c]

Table 5. 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 DDA: NR Size: 20 to 30 kDa	Pig (Duroc x Landrace x Yorkshire) Sex NR 20/group	Diet 28 d	0 or 50 mg/kg/d	bw, food consumption, diarrhoea rate, serum CAT, GSH-Px, T-SOD, MDA, T-AOC, intestinal morphology and cytokines	• NSD in bw, diarrhoea rate, serum activity of T-AOC, CAT, GSH-Px, T-SOD, and 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 DDA: NR Size: 232 kDa	Pig (Duroc x Yorkshire x Landrace) M, F 12/group	Diet 14 d	0, 500 mg/kg	bw, food consumption, diarrhoea rate, serum cytokines (IL-1, IL-2, IL-6, TNF-α), IgA, IgG, IgM, ACTH, cortisol	• ↑ growth performance (bw, daily weight gain, and feed conversion ratio). • NSD daily food consumption, IL-1, IL-6, TNF-α, IgM, IgA, or ACTH. • Improvement in faecal score. • ↑ IL-2 and IgG. • ↓ cortisol.	Xu *et al.* (2018)
LMWC Source: NR DDA: >85% Size: 20 to 30 kDa	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, and 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 in duodenal, jejunal, and ileal IL-1, IL-10, or IF*N*-γ in all groups. • ↑ jejunal and ileal IL-6 and 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) Sex NR 8/group	Diet 15 d	0, 50, or 100 mg/kg ETEC challenge at Day 11	Average daily gain, average daily feed intake, gain-to-feed ratio, serum IL-1, IL-6, IL-10, TNF-α, IgA, IgG, and IgM, and intestinal morphology	• NSD in average daily gain, average daily feed intake, or gain-to-feed ratio on Days 1 to 11 in any group. • ↑ average daily gain [100]. • ↑ 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.	Zhang *et al.* (2020)

↓ = decrease(d); ↑ = increase(d); 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);

Table 5. 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

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 x 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 x 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; = TC = total cholesterol; TG = triglycerides; TGF-β = transforming growth factor-*beta*; TNF-α = tumour necrosis factor-*alpha*; T-SOD = total superoxide dismutase; VLDL-C = very-low-density lipoprotein cholesterol; wk(s) = week(s); WSC = water-soluble chitosan with molecular weight of 3.91 x 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 an HFD.

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

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

[g] Animals were infected with ETEC.

C.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 (n=15/group) induced to ovulate were orally administered water-soluble chitosan (molecular weight = approximately 300 kDa; degree of deacetylation = >90%), 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 fertilisation 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* fertilisation 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 euthanising 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, a 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 foetuses, implantation sites and resorbed foetuses, foetal weights and lengths, and physical and skeletal examination of foetuses 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 foetal weight and length; and increased incidences of cleft palate, heart hypoplasia, atrophy of liver and kidneys, absence of skull cranial bone, caudal vertebrae, sternebrae, and limbs, and ribs shortage. There were no significant effects in behaviour or clinical signs in treated and control groups, and no significant difference in relative organ weight and in number of foetuses, implantation sites, and resorbed foetuses at 50 and 150 mg chitosan. It should be noted that this study was not conducted according to Good Laboratory Practice or current testing guidelines for teratogenicity and used a very small maternal population (n=3/group) and only 2 dose groups compared to Organisation for Economic Co-operation and Development 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 fibre derived from white button mushrooms (*Agaricus bisporus*).

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 (see 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.

An additional study on chitosan oligosaccharides was identified in the scientific literature; this study was designed to assess positive effects on the ovarian development and reproduction of New Zealand White rabbits. While this study was designed to assess beneficial effects, it does include relevant safety endpoints. Healthy weaned female rabbits were randomly distributed into 4 experimental groups (n=10 females/group) and fed *ad libitum* for 6 months (Kamal *et al*., 2023b). A basal diet without chitosan oligosaccharides was used as a control. The other 3 experimental groups were fed a basal diet plus 0.2, 0.4, or 0.6 g chitosan/kg diet. After 8 weeks, 3 females/group were sacrificed for morphological observation of ovarian

tissues, and the remaining animals were used for reproductive evaluation including sexual receptivity, conception rate, gestation period, reproductive performance, and mortality rates. Specific details of the administration through mating and gestation were not reported. The results demonstrate that the significant effects were related to improvement of reproductive performance. No significant differences were reported in weight at birth, weight at weaning, offspring weight at birth, offspring weight at weaning, milk yield during the lactating period, and offspring and dam mortality. As such, no safety concerns were identified during the study.

List of Abbreviations

GD Gestation Day

C.5 Chronic Toxicity Studies

The National Toxicology Program (NTP) conducted a 6-month feeding study to investigate the safety of chitosan[4] in Sprague-Dawley rats (NTP, 2017). Male and female Sprague-Dawley rats (n=10 animals/sex/group/dose[5]) were fed *ad libitum* feed containing 0, 1, 3, or 9% chitosan (approximately 0, 450, 1,500, or 5,200 mg/kg body weight/day in males and 0, 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.[6] The test material had an average percent deacetylation of 86.5% and an average molecular weight (MW) of 81.6 kDa (ranging from 62,755 to 87,343 Da; considered low molecular weight chitosan). The study was conducted according to United States Food and Drug Administration Good Laboratory Practice.

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 alanine aminotransferase (ALT) and sorbitol dehydrogenase taken at Week 25); haematology (at Week 25); along with a sperm morphology and vaginal cytology examination; urinalysis (at all 4 time points); feed and faecal 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). The body weights of the animals remained comparable across all dosed

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

[5] Animals were split into 3 groups (A, B, and C) and different parameters were measured in each group (n=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, haematology, clinical chemistry, urinalysis, and faecal analysis).

[6] 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.

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. 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% group male (47 to 57%) and female (30%) rats. Serum phosphorus levels were significantly decreased in the 9% group male rats (12 to 18%) and in the 3% group males (14%). Similarly, phosphorus levels were significantly decreased in the 3% and 9% group females (9 to 20%). ALT was slightly but statistically significantly elevated at Week 25 in the 9% group male rats (104%) and in the 3% and 9% group 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% group males (23%) and females (15%) at Week 25 (only time point measured).

Mild but statistically significant increases (4 to 6%) in automated haematocrit, haemoglobin concentration, mean cell volume, and mean cell haemoglobin were reported in the 9% group 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 haematology data were mild or sporadic and not considered toxicologically significant.

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% groups. 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 the 3% group males and the 9% group.

Serum concentrations of vitamin D were statistically significantly increased in the 9% group 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 the 9% group females at Weeks 19 and 25. However, serum levels of calcium were mildly but statistically decreased (4%) in the 9% group 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 the 9% group 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% group animals (both sexes) and 3% dosed males (thymus only). The relative liver weights of the 3% group 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 faecal 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 the 1% and 3% group females. Fatty change was characterised by hepatocytes with large, well-defined, clear vacuoles (lipid) within the cell, displacing the nuclei and cytoplasm to the cell periphery. Faecal weight was significantly increased up to 170% in the 3% and 9% group and up to 29% in the 1% group females. Faecal 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 faecal 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 fibre-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 recognised fat binding properties of chitosan,[7] 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 twice 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 fibre derived from white button mushrooms (*A. bisporus*). 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 generalised effects of resistant dietary fibres on nutrient absorption have been long known, are well characterised, and are not considered of

[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.

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 *beta*-chitosan (MW = not reported; degree of deacetylation [DDA] = 75.5%) or "*rapidly-soluble chitosan*" (MW = >100 kDa; DDA = >78%) 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 summarised in Table 6 below.

Table 6. 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 bw on Day 126 and 154 of study (improved growth). • NSD in general condition. • NSD in liver, epididymal, or uterine horn fat pad weights. • Food intake of all chitosan mice was marginally more than that of controls.	Ormrod *et al.* (1998)[e]
LMWC powder Source: NR DDA: 86.5% Size: average MW 82 kDa 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, haematology, sperm morphology, vaginal cytology examination, urinalysis, feed and faecal analysis, and gross	1%	• No significant effect on bw in any dosed group *vs.* control. • 3 M and 2 F died before study end (cause of death unknown). • ↑ (4 to 6%) in automated haematocrit, haemoglobin concentration, mean cell	NTP (2017)

Table 6. 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
				histopathology of major organs		volume, and mean cell haemoglobin in M [9%]. • ↓ in cholesterol (26 to 48%) in both sexes [9%]. • NSD in TG 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	

Table 6. 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
						dosed groups [1%, 3%, 9%]. • ↑ Faecal weight in M [3%, 9%] and F [1%, 3%, 9%]. • Faecal moisture ↑ 4 to 10% in both sexes [9%].	

↓ = decrease(d); ↑ = increase(d); 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; N/A = not applicable; NR = not reported; NSD = no statistical difference; TG = triglycerides.

[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).

List of Abbreviations

ALT	alanine aminotransferase
DDA	degree of deacetylation
MW	molecular weight
NTP	National Toxicology Program

C.6 Genotoxicity / Mutagenicity Studies

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 summarised in Table 6. Chitosan derived from *A. bisporus* (KiOmedine-CsU) did not increase the number of revertant colonies in an Ames test conducted according to Organisation for Economic Co-operation and Development Test Guideline 471 (*Bacterial Reverse Mutation Test*) at doses up to 1,000 µg/plate with and without S9 metabolic activation (OECD, 1997; Kitozyme, 2008 [unpublished] – reviewed in Kitozyme sa, 2011 – GRN 397). The incidence of micronuclei formation and chromosomal aberrations in male ICR mice following administration of chitosan oligosaccharides (molecular weight [MW] = <10 kDa; degree of deacetylation [DDA] = 90%) 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; DDA = 85%) at doses of 5,000 mg/kg (Qin *et al*., 2006).

The cytotoxic effect of chitosan oligosaccharides (MW = 1.4 kDa; DDA = 78%) 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 indicates that chitosan and chitosan oligosaccharides do not have genotoxic potential.

Table 7. Summary of Genotoxicity Studies of Chitosan and Chitosan Oligosaccharides.

Test Substance(s)	Test	Test System	Concentration/Dose	Result(s)	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 [unpublished])[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 polymerised 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 polymerised 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)
In Vivo					
Chitosan oligomer Source: NR	Bone marrow micronuclei test	ICR mice M	0, 0.01, 0.1, 1% dietary chitosan oligosaccharide	• No differences in formation of	Yoon *et al.* (2005)[b]

Table 7. Summary of Genotoxicity Studies of Chitosan and Chitosan Oligosaccharides.

Test Substance(s)	Test	Test System	Concentration/Dose	Result(s)	Reference
DDA: 90% MW: <10 kDa		20/group	administered for up to 180 days	micronuclei in bone marrow cells.	
Chitosan oligomer Source: NR DDA: 90% MW: <10 kDa	Chromosome aberration test (4 generations)	ICR mice M 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 to F3.	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]
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	CF1 mice F 12 to 13/group	Pre-treatment 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 (*Bacterial Reverse Mutation Test* – OECD, 1997).

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

C.7 Other relevant Animal Studies

Examples of studies on selective biological endpoints of orally administered chitosan or chitosan oligomers to animals are listed in literature. In most of these studies did chitosan treatment does not alter body or organ weights, and no changes in urinalysis, blood biochemistry and haematological parameters were observed. Deuchi *et al*. (1995) reported significant reduction in serum levels of minerals (Ca, Fe, and Mg) and lipid soluble vitamins (A, D, E, and K) in Sprague-Dawley rats fed diets supplemented with 5% chitosan. However, these findings have not been replicated in other animal studies (Gordon and Beach-Williford, 1984; Kimura *et al*., 2004; Jung *et al*., 2006). In several randomized, double-blind, placebo-controlled human clinical trials, supplementation of the diet with grams of chitosan did not affect the absorption of fat-soluble vitamins or minerals (Pittler *at al*., 1999; Mhurchu *et al*., 2004; Tapola *et al*., 2008). Overall, no observations were identified in these studies.

C.8 Human 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 were 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; Primex ehf, 2012). These studies demonstrate that chitosan consumption is well tolerated at levels ranging from 1 to 6 g per day, for periods up to 24 weeks (see Table 3.14.1-1). According to GRN 170, the United States Food and Drug Administration has raised concerns on potential effects on fat-soluble vitamins and mineral status in humans following consumption of chitosan (Primex ehf, 2005 – GRN 170). These concerns were raised due to a rat study that reported significant reductions in levels of vitamins A, D, and E, as well as 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; these values were not considered representative of human intake levels.

A summary of the human clinical studies discussed in GRN 397 is provided in Table 3.14.1-1. Clinical studies published since GRN 397, identified through an update literature search, are summarised below. The results of the new clinical studies support the previous conclusions regarding the safety of chitosan in humans.

In a multi-centre, single-blind, placebo-controlled, randomised 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 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 dyslipidaemia. 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, alanine aminotransferase [ALT], aspartate transaminase [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, high-density lipoproteins [HDL], low-density lipoproteins [LDL], and very low-density lipoproteins), and glycated haemoglobin 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 haemoglobin was significantly decreased in the chitosan group at Day 45 and 90, as well as in the placebo group at Day 45, though it returned 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; this effect was 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 randomised, double-blind, placebo-controlled study conducted with 60 pre-diabetic adult patients (characterised 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 haemoglobin 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 hemoglobins, 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 hemoglobins. There was no significant difference in changes in total cholesterol, HDL-cholesterol, LDL-cholesterol,

triglycerides, insulin, adiponectin, IL-6, and tumour necrosis factor-*alpha* between treatment and placebo groups (Kim *et al.*, 2014).

In a randomised, 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, haemoglobin, haematocrit, 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 randomised, 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 (MW) or degree of deacetylation (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 randomised 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*., 2020). The studies included subjects who 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 MW 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*., 2020).

Table 8. Summary of Human Studies of Chitosan and Chitosan Oligosaccharides.

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-C and LDL-C, TG • Atherogenic index • Calcium and phosphorous levels • Plasma antioxidant capacity	• NSD in blood pressure, BMI, or levels of TC, 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-C increased during treatment period; no significant difference in LDL-C. • Lowered the ratio of oxidised 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 • Faecal fat content	• NSD in weight or food intake. • Very small increase in faecal fat content in men, but NSD in women. • No adverse effects reported.	Gades and Stern (2005)

Table 8. Summary of Human Studies of Chitosan and Chitosan Oligosaccharides.

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 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 • Faecal microbiota, bacterial metabolites, faecal weight, moisture content, pH value	• Decrease in lecithinase-negative clostridia ("*may lead to improvement in intestinal environment*"). • Decrease in faecal ammonia. • Chitosan inhibits putrefactive activity of intestinal microbiota and may contribute to reduction of factors that lead to disease states.	Terada *et al.* (1995)
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	• bw • Nutrition survey • Serum lipid • Bile acid and neutral cholesterol in faeces	• Intake of energy, protein, fat, and cholesterol did not change. • Average total serum cholesterol level decreased, serum HDL-C increased, NSD in serum TG 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						

Table 8. Summary of Human Studies of Chitosan and Chitosan Oligosaccharides.

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
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	• Haematology: blood count, plasma creatinine, urate, γ-glutamyl transferase, calcium, serum ferritin • Serum: α- and β-carotene, vitamin A, vitamin E, 25-hydroxyvitamin D • Plasma total and HDL-C, total TG concentrations • bw, blood pressure • RAND 36-item Health Survey • Incidence and severity of gastrointestinal, skin and other symptoms	• NSD in haematology, serum biochemistry, plasma lipids, or body weight. • Association in incidence of constipation, heartburn, nausea in first 4-wk period in chitosan groups (not significant between groups after performing pair-wise comparisons). • 3 subjects in chitosan group and 1 subject in placebo group reported skin symptoms.	Tapola *et al.* (2008)
95 subjects Mild or moderate hypercholesterolemia	Oral tablet 12 wks Multi-centre, placebo-controlled, randomised 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-C and TC at Weeks 4 and 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, randomised 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 bw, BMI, blood pressure, food consumption. • Chitosan therapy produced statistically significant reduction in TC at 8 wks. • NSD in HDL-C or TG levels.	Bokura and Kobayashi (2003)
Overweight Subjects						

Table 8. Summary of Human Studies of Chitosan and Chitosan Oligosaccharides.

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
12 subjects Obese, without diabetes mellitus	Oral tablet 3 mo Placebo-controlled, randomised, 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, TC, HDL-C, TG	• NSD in serum glucose levels or lipid profile. • Significant decrease in TG. • No adverse events with interventions. • Insulin sensitivity increased significantly.	Hernández-González *et al.* (2010)
30 subjects Overweight, hyperlipemic, under physical training	Oral tablet 4 mo Double-blind, placebo-controlled	Low molecular weight chitosan, polyglucosamine	Group 1: 0 (placebo) Group 2: 2	• Anthropometric measures • Blood pressure • LDL-C and HDL-C, blood glucose and triacylglycerol	• More significant reduction in bw, waist circumference, LDL-C, and triacylglycerol than placebo control. • HDL-C increase was higher than placebo control. • Metabolic syndrome was reduced in 12 cases in the supplement group.	Cornelli *et al.* (2008)
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 TC, HDL, LDL, or bone mineral density.	Kaats *et al.* (2006)
250 subjects Overweight adults, 82% women	Oral capsule 24 wks Randomised, double-blind, placebo-controlled trial	β-Chitosan Source: Squid pens DDA: 75.5% Size: NR	Group 1: 0 (placebo) Group 2: 3	• bw • Blood pressure • Waist circumference • Serum lipids • Plasma glucose • Fat-soluble vitamins in serum • Faecal fat losses • Health-related quality of life questionnaire	• NSD in BMI, waist circumference, body fat, blood pressure, fat-soluble vitamins, or faecal fat loss. • Statistically significant decrease in TC levels, LDL-C, but not clinically significant. • NSD in HDL-C. • NSD in health-related quality of life questionnaire answers.	Mhurchu *et al.* (2004)

Table 8. Summary of Human Studies of Chitosan and Chitosan Oligosaccharides.

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
68 subjects Normoglycemic obese individuals	Oral tablet 12 wks Randomised, double-blind, placebo controlled	Absorbitol, a salt of chitosan Source: Shellfish DDA: NR Size: NR	Group 1: 0 (placebo) Group 2: 3	• bw • Waist/hip ratio • Blood pressure • Bioelectric impedance analysis • Serum TC, TG, HDL-C, glucose	• NSD in adverse effects reporting. • NSD in weight, body composition, blood composition, blood pressure, lipid profile, or fasting insulin levels.	Ho *et al.* (2001)
59 subjects Overweight, mildly obese, females	Oral capsule 8 wks Randomised, double-blind, placebo-controlled	Rapidly-soluble chitosan, LipoSan Ultra™ Source: NR DDA: > 78% Size: > 100 kDa	Group 1: 0 (placebo) Group 2: 3	• bw • Waist/hip ratio • Symptom Observational Survey questionnaire • Routine calorie and dietary fat intake; exercise diary • Fasting serum lipid levels • Faecal fat	• NSD in calorie or dietary fat intake. • NSD in total Symptom Observational Survey results, though chitosan group reported more incidences of gastrointestinal discomfort, mild nausea, and heartburn; 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 faecal fat excretion, but no statistical conclusion (sample size too small).	Schiller *et al.* (2001)

Table 8. Summary of Human Studies of Chitosan and Chitosan Oligosaccharides.

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
30 subjects Overweight volunteers	Oral capsules 28 d Randomised, double-blind, placebo-controlled	Chitosan Source: NR DDA: NR Size: NR	Group 1: 0 (placebo) Group 2: 2	• BMI • Blood pressure • Quality of life • Serum cholesterol • Serum TG • Vitamin A, D, E, *beta*-carotene	• NSD in body mass index, serum cholesterol, serum TG, vitamin A, D, E, or *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	• bw • Plasma cholesterol • HDL-C, LDL-C, TG • Adverse events	• NSD in cholesterol or TG concentration. • Increase in HDL-C and concomitant reduction in LDL-C. • Mild digestive discomfort.	Ausar *et al.* (2003)

BMI = body mass index; bw = body weight; d = day(s); DDA = degree of deacetylation; HDL = high-density lipoprotein; kDA = kilodaltons; LDL-C = low-density lipoprotein cholesterol; mo = month(s); MW = molecular weight; NR = not reported; NSD = no significant difference; TC = total cholesterol; TG = triglycerides; wk(s) = week(s).
[a] Study details were taken as reported in GRN 397 (U.S. FDA, 2011).

C. 9 Information Pertaining to the Safety of Residual beta-1,3-glucans in the processing aid.

Chinova's fibre from white button mushrooms (*Agaricus bisporus*) 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 (n=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 no-observed-adverse-effect level (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 Good Laboratory Practice– and Organisation for Economic Co-operation and Development Test Guideline 408 (*Repeated Dose 90-Day Oral Toxicity Study in Rodents*)–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 (n=20/sex/group) were randomised 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, diarrhoea, 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 Generally Recognized as Safe (GRAS) Notices to the United States (U.S.) Food and Drug Administration (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 fibres derived from oats containing *beta*-glucan and providing at least 0.75 g *beta*-glucan soluble fibre 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 NDA Panel, 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 NDA Panel, 2011).

Based on the intended uses of Chinova's fibre from white button mushrooms (*A. bisporus*), the highest intake under the intended conditions of use is estimated to result in intakes of 1.2 g/day. This would amount to approximately 60 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 U.S. Therefore, no safety concerns are anticipated due to the presence of up to 5% *beta*-glucan in Chinova's fibre derived from white button mushrooms (*A. bisporus*).

List of Abbreviations

FDA	Food and Drug Administration
GRAS	Generally Recognized as Safe

NOAEL	no-observed-adverse-effect level
U.S.	United States

C.10 Discussion of the Available Safety Information on Chitosan

The safety of chitosan was discussed in numerous Generally Recognized as Safe Notices that were notified to the United States Food and Drug Administration (FDA) (*i.e*., GRN 73 – U.S. FDA, 2002; GRN 170 – U.S. FDA, 2005; GRN 397 – U.S. FDA, 2011; GRN 443 – U.S. FDA, 2013a). Based on the information provided in GRN 170, the main concern raised by the reviewers at the Center for Food Safety and Applied Nutrition were related to the "*nutritional effects of consuming shrimp-derived chitosan on a chronic basis as part of a normal diet*" (Primex ehf, 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, including 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 fibre-like substances (*i.e.*, chitosan) and fat between rats and humans. As rats do not have a gallbladder, they cannot emulsify high-fat meals for complete digestion, and the shorter transit time in rats impacts their ability to digest dietary fibre-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 like 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 National Toxicology Program, wherein Sprague-Dawley rats were provided low molecular weight chitosan (LMWC) powder (purity = 94%; average molecular weight = 82 kDa; degree of deacetylation = 86.5%; compositionally equivalent to Chinova's fibre from white button mushrooms [*A. bisporus*]) 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 3.12. 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 recognised fat-binding properties of chitosan,[8] resulting in excretion of dietary fat and reduced

[8] 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.

absorption of fat-soluble vitamins. In addition, generalised effects of resistant dietary fibres like chitosan on nutrient absorption have been long known, are well characterised, and are not considered nutritionally 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 *beta*-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), *beta*-carotene, and prothrombin time (surrogate for vitamin K) in overweight adults (n=250) consuming 3 g/day of *beta*-chitosan for 24 weeks (Mhurchu *et al*., 2004); and

- Vitamin A, D, E, and *beta*-carotene in overweight subjects (n=30) consuming 2 g/day of chitosan (not further characterised) 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 3.11). 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 (U.S. FDA, 2011) 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. 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 3.14.1). 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 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 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 (*A. bisporus*), the estimated daily intake of chitosan derived from white button mushrooms was determined to be highest in adults, at 1.2 g/day at the highest 95th percentile intakes chitosan derived from white button mushrooms (*A. bisporus*), approximately 26-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 fibre derived from white button mushrooms (*A. bisporus*) is not expected to be associated with any adverse outcomes, including vitamin or mineral deficiencies.

List of Abbreviations

FDA	Food and Drug Administration
LMWC	low molecular weight chitosan
LOEL	lowest-observed-effect level

C.11 Overall Conclusion on the Safety of the Food Additive

Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*), comprised of chitosan and *beta*-glucan, is proposed for use as an antimicrobial ingredient in multiple food and beverage products in the European Union (EU) at levels in accordance with current Good Manufacturing Practice (cGMP). In the EU, chitosan extract from fungi such as *Agaricus bisporus* or *Aspergillus niger* are authorised for use in food supplements as defined in Directive 2002/46/EC[9] on the approximation of the laws of the Member States relating to food supplements at levels consistent with chitosan from crustacean sources. Chitosan from crustacean sources is defined as not novel in food supplements within the EU Novel Food catalogue (as polyacetyl-glycosamine) (European Commission, 2023). As well, chitosan derived from *A. niger* is authorised for use in the EU as a processing aid: clarifying agent in several wine products, and as a processing aid for the correction of defects in the above product categories and also in new wine still in fermentation as defined in Commission Delegated Regulation (EU) 2019/934[10] supplementing Regulation (EU) No 1308/2013. In Canada, Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is currently authorised for use under the same proposed food uses and maximum use levels as described herein; thus, it is included on Health Canada's List of Permitted Food additives as an anti-bacterial (Class 2) and anti-fungal (Class 3) preservative (Health Canada, 2023).

Chitosan [(1,4)-2-amino-2-deoxy-*beta*-D-glucan; poly-β-(1,4)-2-amino-2-deoxy-D-glucose; CAS9012-76-4] is the main component of Chinova's fibre extracted from white button mushrooms (*A. bisporus*). The ingredient has a degree of deacetylation (DDA) of 90 to 94%, and an average molecular weight (MW) of 100 kDa. Chinova's fibre extracted from white button mushrooms (*A. bisporus*) has been demonstrated analytically to be compositionally similar to crustacean-derived

[9] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. OJ L 183, 12.7.2002, p. 51–57. Available online: http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32002L0046&qid=1442597959590 (current consolidated version: 30/09/2022).

[10] Commission Delegated Regulation (EU) 2019/934 of 12 March 2019 supplementing Regulation (EU) No 1308/2013 of the European Parliament and of the Council as regards wine-growing areas where the alcoholic strength may be increased, authorised oenological practices and restrictions applicable to the production and conservation of grapevine products, the minimum percentage of alcohol for by-products and their disposal, and publication of OIV files. OJ L 149, 7.6.2019, p. 1–52. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32019R0934 (current consolidated version: 08/02/2022).

chitosan as confirmed by Fourier-transform infrared spectroscopy and proton nuclear magnetic resonance spectroscopy.

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is manufactured in accordance with cGMP. The final product, fibre extracted from white button mushrooms (*A. bisporus*), is specified to contain less than 5% moisture, and an average MW of 10 to 400 kDa with a DDA greater than 80%. The specifications for Chinova's fibre extracted from white button mushrooms (*A. bisporus*) are consistent with the *Food Chemicals Codex* monograph for chitosan obtained from crustacean sources, and analysis of 3 production lots demonstrate the product consistently meets the established food-grade specifications. Particle size analysis demonstrates that Chinova's fibre extracted from white button mushrooms (*A. bisporus*) does not meet the definition of engineered nanomaterial as set out in the Regulation (EU) 2015/2283[11] on novel foods. Bulk stability data demonstrates that Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is stable for at least 9 months when stored under ambient and accelerated conditions, with an estimated shelf-life of 24 months based on the results of the accelerated stability testing. Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is expected to be stable and have the intended antimicrobial effect in the final foods. The chitosan and *beta*-glucan fibres will naturally slowly degrade into shorter fibre lengths over time, and eventually down to their individual monomers.

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is proposed for use in various food and beverage products for its antimicrobial properties at levels in accordance with cGMP, at maximum use levels ranging from 150 to 1,500 mg/kg in food. The dietary intakes of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was estimated using 2 different modelling approaches. The intakes estimated using the DietEx tool were subtly lower than those estimated using the Food Additives Intake Model 2.1 tool, with larger differences observed from children through to the elderly. The refined results using the DietEx tool are considered a more realistic estimation of intakes in the EU population and were therefore determined to be the appropriate results to be used for the risk assessment. Based on the proposed food uses of Chinova's fibre extracted from white button mushrooms (*A. bisporus*), on a body weight basis, intakes were highest in infants and toddlers, as expected based on their relatively smaller body weights, with up to 19 and 34 mg/kg body weight/day at the mean and up to 74 and 59 mg/kg body weight/day at the high level, respectively. The highest intakes on an absolute basis were calculated for adults, at up to 696 mg/person/day at the mean and up to 1,197 mg/person/day at the high level.

The safety assessment of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was conducted through an evaluation of the metabolic profile of the substance and the available animal toxicology and human studies on chitosan. Chitosan is a soluble biopolymer derived from the deacetylation of chitin, which is widely distributed in nature. As Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is compositionally similar to chitosan derived from crustacean sources, the metabolic fate of the fibre extracted from white button mushrooms (*A. bisporus*) will be similar as crustacean-derived chitosan. It also is important to note that the authorisation of chitosan extract from fungi such as *A. bisporus* or *A. niger* in the EU for use in food supplements was based on it being substantially equivalent to crustacean derived chitosan, which is not novel[12]. Chitosan is not

[11] Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. OJ L 327, 11.12.2015, p. 1–22. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32015R2283 (current consolidated version: 27/03/2021).

[12] Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351,

subject to digestion by gastric enzymes, and travels intact through the gastrointestinal tract. The safety of chitosan was discussed in numerous Generally Recognized as Safe Notices notified to the United States Food and Drug Administration, and the main concern related to nutritional effects of chitosan on fat-soluble vitamin and mineral status in humans when consumed on a consistent basis. The nutritional effects of chitosan were investigated in a 6-month feeding study wherein Sprague-Dawley rats consumed a low molecular weight chitosan powder that is compositionally equivalent to Chinova's fibre extracted from white button mushrooms (*A. bisporus*) at levels of 0, 1, 3, or 9% of the diet (NTP, 2017). Dietary concentrations of chitosan up to 9% in the diet were well tolerated by rats. Significant reductions in fat-soluble vitamins and reduced relative liver and thymus weights were reported at concentrations of 9% in males and females, and 3% in males only. 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. Based on the findings on serum vitamin E levels, the lowest-observed-adverse-effect level (LOAEL) was concluded to be 1%, equivalent to 450 mg/kg in males, and 9%, equivalent to 6,000 mg/kg in females. The effects on fat-soluble vitamins were considered to be indirect consequences of the recognised fat-binding properties of chitosan and therefore, these findings 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. Similar findings on fat-soluble vitamins were not observed in human studies on chitosan at doses up to 6.75 g/day for up to 24 weeks (Pittler *et al*., 1999; Mhurchu *et al*., 2004; Jaffer and Sampalis, 2007; Tapola *et al*., 2008). The reported LOAEL from the NTP (2017) study was equivalent to 450 mg/kg body weight/day, or about 31.5 g/day for a 70 kg individual. In comparison, no effects on fat absorption were reported in a parallel, placebo-controlled human study consuming 6.75 g/day (Tapola *et al*., 2008). Based on the proposed food uses of Chinova's fibre extracted from white button mushrooms (*A. bisporus*), the estimated daily intake of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was determined to be highest in adults, at 1.2 g/day at the highest 95th percentile intakes, approximately 26-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 fibre extracted from white button mushrooms (*A. bisporus*) is not expected to be associated with any adverse outcomes, including vitamin or mineral deficiencies.

Together, the totality of the available scientific data and information on Chinova's fibre extracted from white button mushrooms (*A. bisporus*) indicates no safety concerns with its proposed use in food and beverage products as an antimicrobial agent at levels in accordance with cGMP.

List of Abbreviations

cGMP	current Good Manufacturing Practice
DDA	degree of deacetylation
EU	European Union
LOAEL	lowest-observed-adverse-effect level
MW	molecular weight

30.12.2017, p. 72–201. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1533914206967&uri=CELEX:32017R2470 (current consolidated version: 31/05/2023).

D. INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE PROCESSING AID.

D.1 Information on the toxicity of chemical processing aid and if necessary, its major metabolites:

Information on the toxicity is available in section C.3 above.

D.2 Safety assessment reports by international agencies or other national government agencies:

The only one available is reported in section C.1 and C.2 above.

D.3 Information related to the safety of an enzyme processing aid.

Not applicable. Fungal chitosan is not an enzyme processing aid.

D.4 Additional information related to the safety of an enzyme processing aid derived from a microorganism.

Not applicable. Fungal chitosan is not an enzyme processing aid.

D.5 Information related to the dietary exposure of the processing aid.

D5.1 A list of food groups likely to contain the processing aid or its metabolites.

Fungal chitosan from *Agaricus bisporus* is proposed for use as a processing-aid in the manufacturing of wine, beer, cider, seltzers, and alcoholic cocktails.
Chitosan derived from *A. bisporus* and *A. niger* was shown to be chemically and structurally equivalent to shellfish derived chitosan. Therefore, data establishing the safety of shellfish-derived chitosan are considered relevant to the safety evaluation of fungal chitosan for the proposed food uses described in this document.
Shellfish derived chitosan is widely available in foods and is used in dietary supplement products, industrial, pharmaceutical, agricultural, and cosmetic applications, and background exposures to chitosan are therefore expected to exceed those occurring from the food uses of fungal chitosan. Thus, based on the absence exposure to chitosan under the proposed food uses, calculations of the estimated intake were not deemed necessary in the assessment of the safety of the material under the proposed food uses in wine / alcoholic beverages processing for the GRAS determination.

A number of animal, human, and *in vitro* studies relevant to the safety of shellfish chitosan, which has a long history of safe use in the food supply, have been published. Published studies examining the metabolism and kinetics; acute, sub chronic, and chronic toxicity; reproductive toxicity in animals; and safety in human of shellfish-derived chitosan or oligosaccharides are present in the dossier. Shellfish derived chitosan has a long history of safe use in the food industry. It is currently approved for use as a natural food additive for general food use in Japan and Korea (Japan Food Chemical Research Foundation, 2011; KFDA, 2011), and has widespread use as a dietary supplement product in the United States, the European Union, and other regulatory jurisdictions throughout the world. Finally, fungal chitosan (derived from *Agaricus bisporus* and *Aspergillus niger* sources) has been granted Novel Food approval by the European Commission, for use in supplement products in the European Union based

on its substantial equivalence to existing shellfish derived chitosan products that are currently in the market.

D5.2 The levels of residues of the processing aids or its metabolites for each food or food group

Regardless of the technology purpose, the sediments that contain the chitosan are removed from the wine, must, or spirits at the end of the treatment by physical separation processes such as racking, centrifugation and/ or filtration. Since chitosan is insoluble at slightly acidic to neutral pH levels, as well as in aqueous and ethanol solutions, it is unlikely that any residual chitosan will remain in the treated products. High-performance liquid chromatography (HPLC) analyses for residual chitosan in wine processed with chitosan indicate that the final product is free from chitosan carry-over products up to the limit of detection of the analysis method (10 mg/L). Therefore, the estimated intake of chitosan from all proposed technological uses can be considered as negligible.

D.6 Information on likely levels of consumption

. Chitosan extracted from white button mushrooms is intended for use as a secondary direct food ingredient (processing aid) in the production of alcoholic beverages at use levels ranging from 10 to 50 g/hL. A summary of the beverage categories and use levels in which the mushroom-derived fiber is intended for use is provided in Table 9. The conditions of use for this ingredient are similar to those outlined in US GRAS Notice (GRN) 397, wherein chitosan derived from *Aspergillus niger* is GRAS for use as a processing aid in the production of wine, spirits, cider, and beer for the purpose of microbial stabilization, removal of organic and mineral contaminants and clarification.

Table 9. Summary of beverage categories and use levels for chitosan products.

Technological Use	Proposed Products	Use-Level
Microbial stabilization	Wine	10-50 g/hL
	Cider	
	Beer	
	Seltzer	
	Alcoholic cocktails	

D.7 Percentage of food groups to use processing aid

There is no information on the expected use of this processing aid in Australian wine or imported products currently being sold in Australia.

D.8 Information on residues in foods in other countries

There is no information on residues in wines where it is approved as a processing aid in other countries.

D.9 Where consumption has changed, information on likely consumption

Not applicable.

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