APPLICATION FOR THE APPROVAL OF CHINOVA'S FIBRE EXTRACTED FROM WHITE BUTTON MUSHROOMS (*AGARICUS BISPORUS*) UNDER THE *AUSTRALIA NEW ZEALAND FOOD STANDARDS CODE* – STANDARD 1.3.1 – FOOD ADDITIVES

CONFIDENTIAL

SUBMITTED BY:

[redacted]

DATE:
05 September 2024

Application for the Approval of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) Under the *Australia New Zealand Food Standards Code* – Standard 1.3.1 – Food Additives

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INTRODUCTION

Chinova Bioworks Inc. (hereinafter "Chinova") proposes to introduce fibre extracted from white button mushrooms (*Agaricus bisporus*) for use as a preservative in food and beverage products in Australia and New Zealand. The ingredient is a mixture of chitosan and *beta*-1,3-D-glucans. Chitosan is the main component, representing approximately 95% of the total volume and is a soluble polymer derived from the cell walls of non-genetically modified *A. bisporus* (white button mushroom) biomass. Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is not currently included in the *Australia New Zealand Food Standards Code* ("the Code").

This application has been prepared to gain authorisation of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) as a food additive in Australia and New Zealand. This food additive is intended for use as a preservative in food and beverage products at the minimum levels required to achieve the desired technical effect in accordance with current Good Manufacturing Practice (cGMP), with maximum levels ranging from 0.01 to 0.150 g/100 g (equivalent to 100 to 1,500 ppm).

Therefore, this dossier was prepared in accordance with the relevant sections of the following Guidelines, as presented in the Food Standards Australia New Zealand (FSANZ) *Application Handbook*:

- Guideline 3.1.1 – General Requirements (all sections)
- Guideline 3.3.1 – Food Additives (all sections)
- Guideline 3.3.2 – Processing Aids (parts of Section C and all of Section D)

A. GENERAL REQUIREMENTS

In accordance with Guideline 3.1.1 – General Requirements, of the Food Standards Australia New Zealand *Application Handbook* (FSANZ, 2019a), the following general information has been provided:

1. Form 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
12. Checklist

Each point is addressed in the following subsections.

A.1 Form of the Application

A.1.1 Information Related to Changes to Standard 1.3.1 – Food Additives

This application for an amendment to Standard 1.3.1 and related Schedules is prepared pursuant to Guideline 3.3.1 – Food Additives, of the FSANZ *Application Handbook* (FSANZ, 2019a), which requires the following structured format to assess an application for a new food additive:

A. General information on the application
B. Technical information on the food additive
C. Information on the safety of the food additive
D. Information on dietary exposure to the food additive

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

A.2 Applicant Details [CONFIDENTIAL]

Contact Information

[redacted]

Attention of:

[redacted]

Tel: [redacted]
Email: [redacted]

Nature of Applicant's Business

Chinova Bioworks Inc. is a Canadian manufacturer of the ingredient of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) for use as a preservative. The company was founded in 2016.

Details of Other Parties Involved with the Application

The following parties are involved in the preparation, submission, and stewardship of this application:

[redacted]

Tel: [redacted]
Email: [redacted]

A.3 Purpose of the Application

This application is being submitted to Food Standards Australia New Zealand (FSANZ) to seek approval for the use of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) as a food additive with the technical purpose of for use as a preservative. 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™. 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.

Chitosan, the main component of Chinova's fibre extracted from white button mushrooms (*A. bisporus*), is not currently listed in *Schedule 15 – Substances that may be used as food additives* or *Schedule 16 – Types of substances that may be used as food additives* of the Code. Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is proposed for use in Australia and New Zealand at levels consistent with cGMP. This food additive is intended for use as a preservative in food and beverage products at the minimum levels required to achieve the desired technical effect in accordance with cGMP, with maximum levels ranging from 0.01 to 0.150 g/100 g (equivalent to 100 to 1,500 ppm). The proposed food uses of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) are similar to those that have been recently approved in Canada and are Generally Recognized as Safe (GRAS) in the United States (U.S.), as well as those currently under evaluation by the relevant authorities in the European Union (EU) and the United Kingdom (UK). As such, the purpose of this application is to amend *Schedule 16 – Types of substances that may be used as food additives*, "Additives permitted at GMP" (S16—2) to include Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) (FSANZ, 2019b).

Additionally, in accordance with Standard 1.1.1—15(2), food additives must comply with any relevant specifications set out in *Schedule 3 – Identity and purity*, which can include those published in primary sources (such as *Food Chemicals Codex* [FCC] monographs) (S3—2), those published in secondary sources (S3—3), or specific provisions listed in the table to subsection (2) of (S3—2) (FSANZ, 2023a,b). Chinova has established product specifications for the fibre extracted from white button mushrooms (*A. bisporus*) consistent with the FCC monograph for chitosan derived from crustacean sources.

A.4 Justification of the Application

A.4.1 Need for the Proposed Change

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is proposed for use as a preservative. While other preservatives are already available for use in Australia and New Zealand, this ingredient will provide a sustainable option that is derived from a natural and non-genetically modified organism for consumers who are seeking such an option.

A.4.2 Costs and Benefits Associated with Use of the Food Additive

Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) is proposed for use in various food and beverage products for its antimicrobial properties at levels in accordance with current Good Manufacturing Practice. Thus, Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is intended for use as a preservative agent in the food and beverage products to which it is added. The availability of this food additive will benefit the food industry in Australia and New Zealand and globally, *via* an expanded catalogue of permissible food additives. Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) is suitably effective as a preservative agent, while being derived from a sustainable source that is a common food and non-genetically modified. This option may be more desirable to some consumers.

It is noted that this application for the use of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) in Australia and New Zealand is part of a global regulatory strategy; a recent approval was issued in Canada (30 May 2024; Reference Number: M-FAA-24-05[1]) and a "no questions" letter was issued by the FDA following notification of GRAS status (28 February 2022; GRN 997). Equivalent parallel submissions have been submitted and are currently under review by the EU (EFSA-Q-2023-00904) and the UK (reference: 8881-2987-6506-2235). Inclusion of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) as a permissible food additive in the Code will promote consistency between Australia and New Zealand food standards and those that have been established internationally.

Therefore, there are no additional costs (to consumers, industry, or government) or consumer health benefits expected to result from a decision to include provisions in the Code to permit the use of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) as a food additive in Australia and New Zealand.

[1] Chitosan from white button mushrooms (*Agaricus bisporus*) https://www.canada.ca/en/health-canada/services/food-nutrition/public-involvement-partnerships/modification-list-permitted-preservatives-enable-use-chitosan.html (Health Canada, 2024).

A.5 Information to Support the Application

Detailed technical information and data regarding Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) are provided to enable the objectives specified in Section 18 of the *Food Standards Australia New Zealand Act 1991*[2] ("the FSANZ Act") to be appropriately addressed (FSANZ, 2018).

To meet these objectives, technical information on the food additive has been provided in Section B of this application and detailed information related to the safety of the food additive has been presented in Section C. Moreover, exposure estimates for Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) have been recently conducted for evaluation by other regulatory authorities, based on the same proposed food uses presented herein. Details of the proposed uses in Australia and New Zealand, along with the details of these exposure estimates, are summarised in Section D.

This dossier was therefore prepared in accordance with the relevant sections of the following Guidelines, as presented in the FSANZ *Application Handbook* (FSANZ, 2019a):

- Guideline 3.1.1 – General Requirements (all sections)
- Guideline 3.3.1 – Food Additives (all sections)
- Guideline 3.3.2 – Processing Aids (parts of Section C and all of Sections D and E)

A.6 Assessment Procedure

The applicant considers that the most appropriate assessment procedure for the application herein is related to Standard 1.3.1 – Food Additives in the Code, which requires a substance to be included in Schedule 15 before it can be used as an ingredient in foods (FSANZ, 2019c). Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is comprised of 95% chitosan, and crustacean-derived chitosan has a long history of safe use in the global food supply (see Section C). The specifications for Chinova's fibre extracted from white button mushrooms (*A. bisporus*) are consistent with the FCC monograph for chitosan obtained from crustacean sources. Thus, it is expected that no changes to Schedule 3 of the FSANZ Act will be required due to the existence of established specifications in primary sources for chitosan. Moreover, an extensive safety database already exists for chitosan, and the ingredient has already been reviewed by international regulatory bodies (Health Canada and the U.S. FDA) at use levels that align with those discussed herein. As such, the requested modifications to the Code are expected to fall under the General Procedure (Subdivision D of the FSANZ Act), with an approximate Cost Category Level of 2.

[2] http://www8.austlii.edu.au/cgi-bin/viewdoc/au/legis/cth/consol_act/fsanza1991336/s18.html.

A.7 Confidential Commercial Information (CCI)

The applicant requests, and informs FSANZ in writing, that the following specific information related to Chinova's fibre extracted from white button mushrooms (*A. bisporus*) be considered confidential commercial information (CCI). Required proprietary data and information that are requested to remain confidential have been summarised in brief and provided within the application, as necessary. Data requested to be treated as CCI have been removed and provided in full in Appendix A. These data are described in Table A.7-1 below along with verifiable justification for them to be treated in a confidential manner.

Table A.7-1 Information Requested to be Considered as Confidential

Section(s)	Description	Justification
A.2	Applicant and contact person contact details.	Contact details for the persons responsible for this dossier are sensitive and should be treated as confidential. Public disclosure of this information is not required for the safety assessment of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*).
Appendix A	• Raw materials and processing aids used in the production process. • Detailed description of the production process.	Specific details related to the manufacturing process are considered confidential and proprietary, and therefore represent significant commercial value to the applicant. A non-confidential summary has been provided in Section B.5. Raw materials and processing aids are a crucial part of the confidential and proprietary manufacturing process. Publication of these data would provide a significant competitive disadvantage to the applicant.

A.8 Other Confidential Information

No other confidential information is contained within this application.

A.9 Exclusive Capturable Commercial Benefit (ECCB)

Currently, the applicant is not the only manufacturer of chitosan; however, due to the source being white button mushrooms (*A. bisporus*), it is assumed that, upon approval of this application, only the applicant will be able to commercially benefit from of the production of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) for use in Australia and New Zealand. Therefore, the application would confer exclusive capturable commercial benefit (ECCB) in accordance with Section 8 of the FSANZ Act.

A.10 International and Other National Standards

A.10.1 Australia

In Australia, Chitosan derived from *Aspergillus niger* (listed as poliglusam derived from *Aspergillus niger*) is included in the Therapeutic Good Administration's (TGA's) Permissible Ingredients Determination for use as an active ingredient in listed medicines at a maximum use level of 2,000 mg/day (TGA, 2016). As well, chitosan sourced from *A. niger* is permitted as a processing aid in the manufacture of wine, beer, cider, spirits, and food-grade ethanol, at a maximum permitted level of "*Good Manufacturing Practice (GMP)*."

A.10.2 Codex Alimentarius Commission

The FCC includes a monograph for chitosan derived from crustaceans, with functions listed as an antimicrobial, stabilizer, and acidity regulator (FCC, 2018).

A.10.3 The United States

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is GRAS in the U.S. for use in a variety of foods and beverages similar to those described herein. This GRAS status was notified to the U.S. Food and Drug Administration (FDA) as "*Chitosan and beta-1,3-glucans from white button mushrooms (Agaricus bisporus*)" (GRAS Notice [GRN] 997) and on 28 February 2022 the U.S. FDA issued a "no questions" letter in response to this Notice (U.S. FDA, 2022).

Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) previously received GRAS status by the Flavor and Extract Manufacturers Association of the United States (FEMA) for use as an ingredient with flavour-modifying properties (FEMA No. 4946). It should be noted that the intended use levels of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) as a food additive in Australia and New Zealand (150 to 1,500 mg/kg) are lower than the FEMA GRAS–approved use levels (1,500 to 2,000 ppm).

A.10.4 Canada

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is approved for use as a food additive in Canada for use as a preservative agent in a variety of foods and beverages similar to those described herein. This ingredient was added to Health Canada's List of Permitted Preservatives as an antibacterial (Class 2) and antifungal (Class 3) preservative, listed as "*Chitosan from Agaricus bisporus (average molecular weight 90 to 120 kDa and degree of deacetylation not less than 80%)*," on 30 May 2024 (reference: M-FAA-24-05; Health Canada, 2024).

It also is noted that 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).

A.10.5 The European Union/United Kingdom

The use of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) as a food additive in a variety of food and beverages is currently under review by the EU and the UK and is not yet approved. However, in the EU, chitosan extract from fungi (*Agaricus bisporus; Aspergillus niger*) are authorised for use in food supplements as defined in Directive 2002/46/EC[3] on the approximation of the laws of the Member States relating to food supplements at levels "*in line with normal use in food supplements of chitosan from crustacean sources*."[4] Chitosan from crustacean sources (as polyacetyl-glycosamine) is defined as not novel in food supplements within the EU Novel Food Catalogue, with no maximum levels listed (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 Regulation (EU) 2019/934.[5]

A.10.6 Japan and Korea

Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) is currently approved/permitted for use as a natural food additive for general food use in Japan and Korea (JFCRF, 2020; MFDS, 2020)

A.11 Statutory Declaration

A signed Statutory Declaration for Australia is provided in Appendix B.

A.12 Checklist

A completed checklist relating to the information required for submission with this application is provided in Appendix C.

[3] 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: https://eur-lex.europa.eu/eli/dir/2002/46/oj (current consolidated version: 06/02/2024).

[4] 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/eli/reg_impl/2017/2470/oj (current consolidated version: 27/06/2024).

[5] 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/eli/reg_del/2019/934/oj (current consolidated version: 08/02/2022).

B. TECHNICAL INFORMATION ON THE FOOD ADDITIVE

In accordance with Guideline 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 2019a) the following technical information must be provided:

1. Nature and technological purpose of the food additive
2. Information to enable identification of the additive
3. Information on the chemical and physical properties of the additive
4. Information on the impurity profile
5. Manufacturing process
6. Specifications for identity and purity
7. Information for food labelling
8. Analytical method for detection
9. Potential additional purposes of the food additive when added to food

Each point is addressed in the following subsections.

B.1 Nature and Technological Purpose of the Food Additive

Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) is intended for use as a preservative (antimicrobial preservative) as defined in *Schedule 14 – Technological purposes performed by substances used as food additives*, "Technical purposes" (S14—2) (FSANZ, 2023c). As a food additive, it is intended to achieve this technological purpose at use levels that are in accordance with cGMP in the food and beverage products to which it is added.

The antimicrobial properties of chitosan have been researched for several decades, and it has been reported to have bactericidal and/or bacteriostatic effects against a range of microbes, including yeast, bacteria, and fungi (Raafat *et al*., 2008; Goy *et al*., 2009). The mechanism of action by which chitosan exerts these properties has not yet been fully elucidated and likely varies between different microbes. The predominant theory on the mechanism of action against bacteria is *via* ionic interactions between the charged groups in the chitosan polymer backbone (protonated NH^{3+} groups) and negatively charged bacterial wall constituents (Goy *et al*., 2016). These interactions lead to hydrolysis effects on the peptidoglycans in the cell wall, resulting in the leakage of intracellular electrolytes and ultimately cell death. Other proposed mechanisms of action include coating the bacterial cells (film-forming) or interference with nutrient absorption/mineral displacement. Li *et al*. (2016) reported that the bactericidal properties of chitosan were increased against *Escherichia coli* and *Staphylococcus aureus* as the degree of deacetylation (DDA) increased. This finding was also reported by Omura *et al*. (2003), who found that samples with higher DDA (DDA = >70%) had higher bactericidal properties against *Bacillus subtilis*, *S. aureus*, *E. coli*, and *Pseudomonas aeruginosa versus* lower DDA samples (DDA = <70%). This is congruent with the efficacy of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus* as an antimicrobial agent given its high DDA, which is typically >90% (see Section B.6). At concentrations of 200 ppm and above, crustacean chitosan with molecular weights (MWs) of 55 to 155 kDa (DDA = ~80%) were generally equally effective against *E. coli* (Liu *et al*., 2006). At concentrations of 50 to 100 ppm, higher MW samples (96 to 155 kDa) had poorer antibacterial properties compared to lower MW samples (55 to 90 kDa) (Liu *et al*., 2006).

The technological function of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) (MW = 10 to 400 kDa) as an antimicrobial ingredient was evaluated in various beverage products, including carbonated soda, apple juice, and liquid-sugar syrup (see Table B.1.1.1-1), baked goods (see Table B.1.1.2-1), and dairy-based yoghurt and cream cheese (see Table B.1.1.3-1) and are discussed in the following sections.

B.1.1 Data Demonstrating the Technical Effect

B.1.1.1 Technological Function in Liquid Products

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was added at a concentration of 0, 50, 100, 200, 400, 600, or 800 ppm to apple juice. Control samples contained either no preservatives or a sorbate/benzoate preservative used at a total of 1,500 ppm. Samples of each mixture were aseptically inoculated with microorganisms associated with food spoilage (*i.e.*, *Lactobacillus brevis*, *A. niger*, *Zygosaccharomyces bailii*, *L. plantarum*, *Saccharomyces cerevisiae*) at an initial concentration of Log 3 colony-forming units (CFU)/mL, and then sealed and stored in a 30°C incubator for 42 days. Samples were assayed every 7 days by standard dilution and plate count.

Results of the microbiological analysis in beverage products, as presented in Table B.1.1.1-1, show that microbial counts decreased over time with the mushroom-derived fibre use by Day 35, whereas microbial growth increased over time in the control samples. A concentration of 400 ppm of the fibre extracted from white button mushrooms (*A. bisporus*) has comparable antimicrobial effects as the sorbate/benzoate control.

Table B.1.1.1-1 Summary of the Microbiological Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Apple Juice

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)[a]	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Zygosaccharomyces bailii	0 (Control)	3	3.1	3	3.2	3.4	4.1	4.7	5
	0 (Sorbate/ Benzoate Control)	3	2.1	1.9	2	1.7	1.4	0.5	0.1
	50	3	2.9	3.1	3.2	3.3	3.8	4.5	4.8
	100	3	3	3.1	2.9	3	3.4	3.9	4.1
	200	3	2.4	2.3	2.4	2.5	2.1	1.8	2.3
	400	3	2.3	2	1.9	1.3	0.9	0.4	0
	600	3	2	1.7	1.2	0.9	0.5	0.2	0
	800	3	1.3	1	0.8	0.8	0.4	0	0
Aspergillus niger	0 (Control)	3	2.2	2.4	2.3	2.9	4.2	4.8	5.2
	0 (Sorbate/ Benzoate Control)	3	1.9	0.2	0	0	0	0	0
	50	3	2.3	2.3	2.6	3	4.1	4.7	5.2
	100	3	1.8	1.9	2.1	2.8	4	4.6	5.1
	200	3	1.6	1.4	1	0.5	0.2	0	0
	400	3	0.9	0.4	0.1	0	0	0	0
	600	3	0.9	0.1	0	0	0	0	0
	800	3	0.8	0.1	0	0	0	0	0

Table B.1.1.1-1 Summary of the Microbiological Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Apple Juice

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)[a]	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Saccharomyces cerevisiae	0 (Control)	3	3.4	3.5	4.2	4.6	4.9	5.1	5.7
	0 (Sorbate/ Benzoate Control)	3	2.4	2.3	2.2	1.4	1	0.2	0
	50	3	3.4	3.5	3.3	4	3.7	4.1	4.5
	100	3	3	3.2	3	2.4	2.1	1.9	1.5
	200	3	2.7	2.5	2.6	2	1.8	1.4	1.2
	400	3	1.9	1.8	1.2	0.8	0.3	0	0
	600	3	1.3	1.3	0.8	0.3	0	0	0
	800	3	1.3	1.2	0.6	0	0	0	0
Lactobacillus plantarum	0 (Control)	3	2.7	2.3	2.5	2.4	2.9	3.4	4.3
	0 (Sorbate/ Benzoate Control)	3	2.1	0.4	0	0	0	0	0
	50	3	2.4	2.4	2.5	2.5	2.6	2.9	3.4
	100	3	2.4	2.5	2.4	2.7	2.9	2.1	2.4
	200	3	2.1	2.3	2	1.8	1.3	1.1	0.5
	400	3	1.4	1	0.3	0	0	0	0
	600	3	1	0.6	0	0	0	0	0
	800	3	1	0.5	0.1	0	0	0	0
Lactobacillus brevis	0 (Control)	3	2.9	3.4	3.4	3.5	3.6	4	5.2
	0 (Sorbate/ Benzoate Control)	3	0.8	0	0	0	0	0	0
	50	3	2.9	3.2	3.1	3.4	3.5	3.8	4.3
	100	3	2.4	2.3	2.2	2.8	3.1	3.6	4.3
	200	3	2.3	2.1	1.8	1.7	2	1.4	1.1
	400	3	2.1	1.4	1.3	0.3	0	0	0
	600	3	2.1	1.4	1	0	0	0	0
	800	3	1.8	0.7	0	0	0	0	0

CFU = colony-forming units; ppm = parts per million.

[a] Chinova's fibre from white button mushrooms (*A. bisporus*) was added at a concentration of 400 ppm to carbonated soda and apple juice products, and 1,000 ppm to flavoured sugar-syrup products. Control samples did not contain any preservatives.

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was added at a concentration of 400 ppm to flavoured water, wine spritzer, alcoholic tea and seltzer, soda, energy drinks, and sports drinks. Samples of each mixture were aseptically inoculated with microorganisms associated with food spoilage (*i.e.*, *L. brevis*, *A. niger*, *Z. bailii*, *L. plantarum*, *Zygosaccharomyces rouxii*, *Pichia anomala*, *S. cerevisiae*, *Brettanomyces bruxellensis*) at an initial concentration of Log 3 CFU/mL, and then sealed and stored in a 30°C incubator for 42 days. Samples were assayed every 7 days by standard dilution and plate count.

Results of the microbiological analysis in these representative beverage products, as presented in Table B.1.1.1-2, show that microbial counts decreased over time with the mushroom-derived fibre use by Day 42, whereas microbial growth increased over time in the control samples. The minimum concentration of the mushroom-derived fibre to have a comparable antimicrobial effect as sorbate/benzoate appears to be about 400 ppm.

Table B.1.1.1-2 Summary of the Microbiological Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) Liquid Products

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Flavoured Water									
Zygosaccharomyces bailii	0 (Control)	3	2.3	3.04	3.05	3.7	3.4	4.1	4.3
	0 (Sorbate/Benzoate Control)	3	0.5	0	0	0	0	0	0
	400	3	1.4	1	0	0	0	0	0
Aspergillus niger	0 (Control)	3	1.42	2	2.2	2.5	2.8	3.81	3.6
	0 (Sorbate/Benzoate Control)	3	1	0.1	0.1	0.2	0	0	0
	400	3	1.32	1.2	0.2	0	0	0	0
Saccharomyces cerevisiae	0 (Control)	3	3.06	4.38	5.71	6	5.8	5.6	5.4
	0 (Sorbate/Benzoate Control)	3	0.3	0.1	0.1	0	0	0	0
	400	3	2.3	0.7	0.5	0.1	0	0	0
Zygosaccharomyces rouxii	0 (Control)	3	0	0	0	0	0	0	0
	0 (Sorbate/Benzoate Control)	3	0	0	0	0	0	0	0
	400	3	0	0	0	0	0	0	0
Seltzer (5% Alcohol)									
Zygosaccharomyces bailii	0 (Control)	3	1.8	1.43	2	1.8	2.4	2.3	2.2
	0 (Sorbate/Benzoate Control)	3	0	0	0	0	0	0	0
	400	3	0	0	0	0	0	0	0
Aspergillus niger	0 (Control)	3	1.8	1.6	0	0	0	0	0
	0 (Sorbate/Benzoate Control)	3	0.3	0.1	0.1	0	0	0	0
	400	3	0.4	0	0	0	0	0	0

Table B.1.1.1-2 Summary of the Microbiological Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) Liquid Products

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Saccharomyces cerevisiae	0 (Control)	3	2.1	1.8	2.1	2.2	2.2	2.3	2.2
	0 (Sorbate/Benzoate Control)	3	1.3	0	0	0	0	0	0
	400	3	0.3	0	0	0	0	0	0
Zygosaccharomyces rouxii	0 (Control)	3	0	0	0	0	0	0	0
	0 (Sorbate/Benzoate Control)	3	0	0	0	0	0	0	0
	400	3	0	0	0	0	0	0	0
Tea (5% Alcohol)									
Zygosaccharomyces bailii	0 (Control)	3	2.44	5.4	6.1	6.6	6.8	6.5	6.6
	0 (Sorbate/Benzoate Control)	3	1.2	1.4	1.1	0.5	0	0	0
	400	3	2.3	1.3	0.2	0	0	0	0
Aspergillus niger	0 (Control)	3	1.8	1.6	0	0	0	0	0
	0 (Sorbate/Benzoate Control)	3	0.3	0.1	0.05	0.05	0	0	0
	400	3	0.4	0	0	0	0	0	0
Saccharomyces cerevisiae	0 (Control)	3	1.67	3.52	4.5	3.8	4	3.7	4.6
	0 (Sorbate/Benzoate Control)	3	0.2	0	0	0	0	0	0
	400	3	0.6	0.1	0	0	0	0	0
Zygosaccharomyces rouxii	0 (Control)	3	1.2	2.57	3.3	3.1	3.4	3.5	3.2
	0 (Sorbate/Benzoate Control)	3	1	0.2	0	0	0	0	0
	400	3	1.8	1.6	0.4	0	0	0	0
Wine Spritzer (3.5% Alcohol, Sulphate-free)									
Zygosaccharomyces bailii	0 (Control)	3	3.1	2.8	2.6	3	3.2	3.2	2.5
	0 (Sorbate/Benzoate Control)	3	0.4	0	0	0	0	0	0
	400	3	1	0	0	0	0	0	0
Aspergillus niger	0 (Control)	3	0.2	0.4	0.2	0	0	0	0
	0 (Sorbate/Benzoate Control)	3	0	0	0	0	0	0	0
	400	3	0	0	0	0	0	0	0

Table B.1.1.1-2 Summary of the Microbiological Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) Liquid Products

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Saccharomyces cerevisiae	0 (Control)	3	2.5	2.6	2.8	3.3	3.5	4.2	4.5
	0 (Sorbate/Benzoate Control)	3	0.2	0	0	0	0	0	0
	400	3	0.6	0.1	0	0	0	0	0
Brettanomyces bruxellensis	0 (Control)	3	3.3	3.5	3.6	4.2	4.7	5.8	6
	0 (Sorbate/Benzoate Control)	3	1	0.2	0	0	0	0	0
	400	3	1.8	1.6	0.4	0	0	0	0
Soda									
Zygosaccharomyces bailii	0 (Control)	3	1.2	1.1	1.3	1.3	1.5	2	2.4
	0 (Sorbate/Benzoate Control)	3	1.1	0.3	0	0	0	0	0
	400	3	1.2	0	0	0	0	0	0
Aspergillus niger	0 (Control)	3	0.4	0.3	0.3	0.2	0.2	0.4	0.6
	0 (Sorbate/Benzoate Control)	3	0	0	0	0	0	0	0
	400	3	0	0	0	0	0	0	0
Saccharomyces cerevisiae	0 (Control)	3	1.7	2.1	2.2	2.5	2.3	2.4	2
	0 (Sorbate/Benzoate Control)	3	0.3	0.1	0.1	0	0	0	0
	400	3	0.2	0.4	0.4	0.2	0.1	0	0
Pichia anomala	0 (Control)	3	1.5	1.4	1.6	2.1	2.2	2.4	2.4
	0 (Sorbate/Benzoate Control)	3	1.2	1	1.2	0.5	0	0	0
	400	3	1.1	0.5	0.2	0	0	0	0
Energy Drinks									
Zygosaccharomyces bailii	0 (Control)	3	3.2	3.4	3.5	3.3	3.2	3.4	3.8
	0 (Sorbate/Benzoate Control)	3	2.4	1.8	1.2	0.2	0	0	0
	400	3	2.9	2.3	1.8	0.4	0.1	0	0
Aspergillus niger	0 (Control)	3	1.1	1.2	1.1	1.5	1.9	2.4	4.3
	0 (Sorbate/Benzoate Control)	3	0.4	0.7	0.5	0.1	0	0	0
	400	3	1	0.9	0	0	0	0	0

Table B.1.1.1-2 Summary of the Microbiological Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) Liquid Products

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Saccharomyces cerevisiae	0 (Control)	3	3	3	2.9	3.1	3.4	3.5	3.9
	0 (Sorbate/Benzoate Control)	3	2.9	2.7	2.5	2.1	2	0.6	0.4
	400	3	3	2.5	2.6	2.5	2.3	1.5	1.1
Lactobacillus plantarum	0 (Control)	3	2.3	3.1	3	2.9	3.6	3.7	4
	0 (Sorbate/Benzoate Control)	3	2.2	2.1	2.2	0.9	0.5	0.2	0.4
	400	3	2	1.9	1.8	0	0	0	0
Lactobacillus brevis	0 (Control)	3	3	2.5	2.4	2.5	2.9	3.9	4.5
	0 (Sorbate/Benzoate Control)	3	2.6	2.5	2.2	1.9	1.4	0.5	0
	400	3	2.4	2	0.9	0.3	0	0	0
Sports Drinks									
Zygosaccharomyces bailii	0 (Control)	3	2.8	2.9	3.4	3.7	4.3	4.6	4.8
	0 (Sorbate/Benzoate Control)	3	2	2.2	2.1	2	1.7	1.1	0.7
	400	3	2.4	2.1	1.7	1.5	0.8	0.3	0
Aspergillus niger	0 (Control)	3	1.1	0.9	1	1.4	2	2.21	2.4
	0 (Sorbate/Benzoate Control)	3	0.4	0.4	0.1	0	0	0	0
	400	3	0.8	0.8	0.7	0.3	0	0	0
Saccharomyces cerevisiae	0 (Control)	3	2.5	2.9	3.1	3.2	3.9	4.3	5.3
	0 (Sorbate/Benzoate Control)	3	1.8	1.4	0	0	0	0	0
	400	3	1.4	1.5	1.3	0.8	0.3	0	0
Lactobacillus plantarum	0 (Control)	3	3	3.1	3	3.8	4	5.4	5.8
	0 (Sorbate/Benzoate Control)	3	3	1.8	1.57	1.4	0.3	0	0
	400	3	3	3.1	1.2	1.1	0.5	0.1	0
Lactobacillus brevis	0 (Control)	3	3.1	3.4	3.9	4.3	4.9	5.9	6.1
	0 (Sorbate/Benzoate Control)	3	2.3	2	1.4	1.1	0.5	0.1	0
	400	3	2.1	1.5	1.2	0.5	0.1	0	0

Table B.1.1.1-2 Summary of the Microbiological Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) Liquid Products

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Juice									
Zygosaccharomyces bailii	0 (Control)	3	3.1	3	3.2	3.4	4.1	4.7	5
	0 (Sorbate/Benzoate Control)	3	2.1	1.9	2	1.7	1.4	0.5	0.1
	400	3	2.3	2	1.9	1.3	0.9	0.4	0
Aspergillus niger	0 (Control)	3	2.2	2.4	2.3	2.9	4.2	4.8	5.2
	0 (Sorbate/Benzoate Control)	3	1.9	0.2	0	0	0	0	0
	400	3	0.9	0.4	0.1	0	0	0	0
Saccharomyces cerevisiae	0 (Control)	3	3.4	3.5	4.2	4.6	4.9	5.1	5.7
	0 (Sorbate/Benzoate Control)	3	2.4	2.3	2.2	1.4	1	0.2	0
	400	3	1.9	1.8	1.2	0.8	0.3	0	0
Lactobacillus plantarum	0 (Control)	3	2.7	2.3	2.5	2.4	2.9	3.4	4.3
	0 (Sorbate/Benzoate Control)	3	2.1	0.4	0	0	0	0	0
	400	3	1.4	1	0.3	0	0	0	0
Lactobacillus brevis	0 (Control)	3	2.9	3.4	3.4	3.5	3.6	4	5.2
	0 (Sorbate/Benzoate Control)	3	0.8	0	0	0	0	0	0
	400	3	2.1	1.4	1.3	0.3	0	0	0

CFU = colony-forming units; ppm = parts per million.

B.1.1.2 Technological Function in Baked Good Products

The antimicrobial effect of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was also evaluated in baked good products such as bread, bagels, English muffins, cakes, muffins, waffles, wheat tortillas and corn tortillas. The test baked goods were prepared from raw ingredients containing no preservatives (control) or 1,000 ppm of mushroom-derived fibre. Bread samples were prepared with 0 (control), 400, 600, 800, 1,000, 1,200, or 1,600 ppm of mushroom-derived fibre. Control samples included no preservatives or 0.3% calcium propionate. The baked good samples were incubated in a sealed plastic bag at 25°C for 30 days. The samples were inspected for visible mould growth and measured every 5 days, and the percent mould was calculated based on the total surface area.

Results of the mould growth analysis in baked good products demonstrate a delay in initial mould growth from 10 days in the control to at least 25 days with 1,000 ppm of mushroom-derived fibre (see Table B.1.1.2-1).

Table B.1.1.2-1 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Baked Good Products

Food Product	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)[a]	Days to Mould Presence						
		0	5	10	15	20	25	30
Bread	0 (Control)	-	-	+	+	+++	+++	+++
	0 (0.3% Calcium Propionate)	-	-	-	-	-	-	+
	400	-	-	-	+	++	++	+++
	600	-	-	-	-	+	++	+++
	800	-	-	-	-	+	+	+++
	1,000	-	-	-	-	-	-	+
	1,200	-	-	-	-	-	-	+
	1,600	-	-	-	-	-	-	+
Bagel	0 (Control)	-	-	+	+	+++	+++	+++
	1,000	-	-	-	-	+	++	++
English Muffin	0 (Control)	-	-	+	++	+++	+++	+++
	1,000	-	-	-	-	-	++	++
Cake	0 (Control)	-	-	+	++	+++	+++	+++
	1,000	-	-	-	-	+	++	++
Muffin	0 (Control)	-	-	++	+++	+++	+++	+++
	1,000	-	-	-	-	+	++	++
Waffle	0 (Control)	-	-	-	+	++	+++	+++
	1,000	-	-	-	-	-	+	++
Corn Tortilla	0 (Control)	-	-	-	+	+	+++	+++
	1,000	-	-	-	-	-	+	+++
Wheat Tortilla	Control	-	-	-	-	+	+++	+++
	Chinova's fibre	-	-	-	-	-	+	+++

- = no visual mould; + = <10% coverage of visual mould; ++ = 10 to 25% coverage with mould; +++ = >25% coverage with mould; ppm = parts per million.

[a] Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was added at a concentration of 1,000 ppm to bread, English muffin, and wheat tortilla products. Control samples did not contain any preservatives.

B.1.1.3 Technological Function in Dairy Products

Technological function of fibre extracted from white button mushrooms (*A. bisporus*) was assessed in various dairy products, including plain yoghurt, cream cheese, cottage cheese, and cheese-based sauce. The mushroom-derived fibre was added at concentrations of 400, 600, 800, 1,000, 1,200, and 1,600 ppm in plain yoghurt products, and 1,000 ppm in cream cheese, cottage cheese, and cheese-based sauce products. Control samples did not contain any preservatives. Samples were placed in a small dish, sealed, and stored either in the refrigerator at 7°C. Samples of each dairy product were aseptically inoculated with microorganisms associated with food spoilage (*i.e.*, *Cladosporium cladosporioides, Penicillium aurantiogriseum, Penicillium roqueforti, Geotrichum candidum, Yarrowia lipolytica, A. niger*) at an initial concentration of Log 3 CFU/g, and then sealed and stored in a 7°C incubator for 42 days. Samples were assayed every 7 days by standard dilution and plate count.

Results of the microbiological analysis in dairy products, as presented in Table B.1.1.3-1, show that microbial counts decreased over time with the use of mushroom-derived fibre at 1,000 ppm, whereas microbial growth increased over time in the control samples.

Table B.1.1.3-1 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Dairy Products

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)[a]	Results (log CFU/g)						
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Plain Yoghurt								
Cladosporium cladosporioides	0 (Control)	3	2.4	2.9	3.3	3.9	4.7	5.4
	400	3	2.4	3	3.2	3.6	4.1	4.8
	600	3	2.5	2.2	2.8	3.5	4	4.6
	800	3	2.5	2.4	2.5	2.7	2.9	3.3
	1,000	3	2.5	2.1	1.3	1	1	0.3
	1,200	3	2.0	2.1	1.1	0.9	0.5	0.2
	1,600	3	2.1	1.8	1.1	0.7	0.4	0
Penicillium aurantiogriseum	0 (Control)	3	1.5	1.6	2.3	4.3	4.4	4.8
	400	3	1.6	1.7	2.2	4.1	4.0	4.3
	600	3	1.4	1.5	2.1	2.8	3.3	3.5
	800	3	1.1	1.3	1.5	1.4	1.8	2.3
	1,000	3	1.1	1.2	1.4	1.2	1.0	0.5
	1,200	3	1.1	1.2	1.3	1.0	0.8	0.4
	1,600	3	0.9	0.8	0.5	0.5	0.2	0.3
Penicillium roqueforti	0 (Control)	3	1.7	1.9	2.2	2.3	3.9	4.6
	400	3	1.6	1.9	2.4	2.5	3.5	4.1
	600	3	1.2	1.3	1.8	2.1	2.7	3.4
	800	3	1.2	1.1	0.8	0.4	0	0
	1,000	3	0.9	0.3	0	0	0	0
	1,200	3	0.7	0	0	0	0	0
	1,600	3	0.9	0	0	0	0	0
Cream Cheese								
Penicillium roqueforti	Control	3	3	2.9	3	3.2	4.1	4.3
	Chinova's fibre	3	2.5	2.4	2	1.6	1.3	0.9
Geotrichum candidum	Control	3	2.9	2.9	3.4	3.5	4	4.3
	Chinova's fibre	3	2.7	2.4	2.1	1.7	1.8	1.2
Yarrowia lipolytica	Control	3	2.4	2.5	2.5	2.9	3.4	3.6
	Chinova's fibre	3	1	0.5	0.3	0.1	0.1	0
Aspergillus niger	Control	3	2.4	2.4	2.8	3.4	3.9	4.6
	Chinova's fibre	3	1	0	0	0	0	0
Cottage Cheese								
Penicillium roqueforti	Control	3	2.6	2.8	2.9	3.6	3.8	4.3
	Chinova's fibre	3	2.3	2.4	2.3	2.0	1.8	1.0
Geotrichum candidum	Control	3	3.5	3.9	4.1	4.3	4.5	5.4
	Chinova's fibre	3	3.0	3.1	2.8	2.3	2.0	1.2
Yarrowia lipolytica	Control	3	2.2	2.4	3.7	4.1	4.3	4.7
	Chinova's fibre	3	1.0	1.9	1.2	0.4	0.1	0.3

Table B.1.1.3-1 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Dairy Products

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)[a]	Results (log CFU/g)						
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Aspergillus niger	Control	3	3.0	3.2	2.9	3.4	3.6	5.3
	Chinova's fibre	3	2.3	2.3	2.1	1.7	1.2	1.1
Cheese-Based Sauce								
Penicillium roqueforti	Control	3	3.0	3.1	3.3	3.9	4.3	4.8
	Chinova's fibre	3	2.6	2.5	2.1	1.2	1.1	0.5
Geotrichum candidum	Control	3	2.9	2.3	2.5	2.4	2.5	2.8
	Chinova's fibre	3	2.4	2.5	2.3	2.1	1.8	1.4
Yarrowia lipolytica	Control	3	2.6	3.0	2.8	3.4	3.5	4.0
	Chinova's fibre	3	1.3	1.1	0.6	0.5	0	0
Aspergillus niger	Control	3	1.7	1.9	1.9	2.6	4.0	5.1
	Chinova's fibre	3	1.2	1.0	0.5	0.1	0	0

CFU = colony-forming units; ppm = parts per million.
[a] Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was added at a concentration of 1,000 ppm to plain yoghurt and cream cheese products. Control samples did not contain any preservatives.

The antimicrobial effect of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was also investigated in natural cheese (cheddar) and processed cheese (mozzarella). Samples were prepared containing 0 (control), 1,000, or 1,500 ppm of mushroom-derived fibre. Samples were stored in sealed plastic bags at 7°C for up to 42 days and inspected for visible mould growth every 7 days. The percent mould growth was calculated based on the total surface area. The results are summarised in Table B.1.1.3-2, and demonstrate that mould growth was delayed in samples containing Chinova's fibre extracted from white button mushrooms (*A. bisporus*).

Table B.1.1.3-2 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Natural and Processed Cheese Products

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Days to Mould Presence						
		0	7	14	21	28	35	42
Natural Cheese (Cheddar)								
Penicillium roqueforti	0 (Control)	-	-	-	-	+	++	+++
	1,000	-	-	-	-	+	++	++
	1,500	-	-	-	-	-	-	-
Geotrichum candidum	0 (Control)	-	-	-	+	+	++	++
	1,000	-	-	-	-	+	++	++
	1,500	-	-	-	-	-	-	+
Yarrowia lipolytica	0 (Control)	-	-	-	-	-	-	+
	1,000	-	-	-	-	-	-	+
	1,500	-	-	-	-	-	-	-

Table B.1.1.3-2 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Natural and Processed Cheese Products

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Days to Mould Presence						
		0	7	14	21	28	35	42
Aspergillus niger	0 (Control)	-	-	-	-	-	++	++
	1,000	-	-	-	-	-	+	++
	1,500	-	-	-	-	-	-	-
Processed Cheese (Mozzarella)								
Penicillium roqueforti	0 (Control)	-	-	-	+	+	++	+++
	1,000	-	-	-	-	-	+	+++
	1,500	-	-	-	-	-	-	+
Geotrichum candidum	0 (Control)	-	-	+	+	+	++	+++
	1,000	-	-	-	+	+	++	++
	1,500	-	-	-	-	+	+	+
Yarrowia lipolytica	0 (Control)	-	-	-	-	+	+++	+++
	1,000	-	-	-	-	+	+	+++
	1,500	-	-	-	-	-	+	+
Aspergillus niger	0 (Control)	-	-	-	-	++	++	++
	1,000	-	-	-	-	+	++	++
	1,500	-	-	-	-	-	+	+

- = no visual mould; + = <10% coverage of visual mould; ++: 10 to 25% coverage with mould; +++ = >25% coverage with mould; ppm = parts per million.

B.1.1.4 Technological Function in Plant-Based Dairy Products

The technological function of fibre extracted from white button mushrooms (*A. bisporus*) was assessed in various plant-based dairy products (yoghurt and cream cheese). The mushroom-derived fibre was added at concentrations of 500, 1,000, 1,500, or 2,000 ppm in plant-based yoghurt and cream cheese products. Control samples did not contain any preservatives or 1.5% cultured dextrose. Samples were placed in a small dish, sealed, and stored in the refrigerator at 7°C. Samples of each product were aseptically inoculated with microorganisms associated with food spoilage (*i.e.*, *C. cladosporioides, Penicillium crustosum, Candida inconspicua, P. roqueforti, A. niger*) at an initial concentration of Log 2 CFU/g and/or Log 3 CFU/g in the plant-based cream cheese samples, and then sealed and stored in a 7°C incubator for 42 days. Samples were assayed every 7 days by standard dilution and plate count.

Results of the microbiological analysis in these plant-based dairy products, as presented in Tables B.1.1.4-1 and B.1.1.4-2, show that microbial counts decreased over time with the use of fibre extracted from white button mushrooms (*A. bisporus*), whereas microbial growth increased over time in the control samples.

Table B.1.1.4-1 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Almond-based Yoghurt

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)						
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Candida inconspicua	0 (Control)	2	1.4	1.3	1.5	1.76	1.6	1.8
	Cultured dextrose 1.5%	2	1.3	1.3	1.8	1.7	1.3	1.2
	500	2	1.5	1.3	1.9	2	1.9	2.1
	1,000	2	1.3	1.4	1.8	1.9	2	1.7
	1,500	2	1	1	1	1.1	1	0
	2,000	2	0.8	0.7	0.4	0.1	0	0
Cladosporium cladosporioides	0 (Control)	2	1.6	1.7	2.4	2.8	2.7	3.4
	Cultured dextrose 1.5%	2	1.1	1.2	0.8	0.9	1	1.5
	500	2	1.7	1.7	2.2	2.7	2.9	3.2
	1,000	2	1.5	1.4	1.5	1.9	2.2	2.4
	1,500	2	1.2	1.4	1.1	1	1.2	0.7
	2,000	2	0.8	0.9	0.4	0.1	0	0
Aspergillus niger	0 (Control)	2	1.6	1	0	0	0	0
	Cultured dextrose 1.5%	2	1.2	0.8	0.4	0	0	0
	500	2	1.7	0.8	0.3	0	0	0
	1,000	2	1.4	0.8	0.3	0	0	0
	1,500	2	1	0	0	0	0	0
	2,000	2	0.2	0	0	0	0	0
Penicillium crustosum	0 (Control)	2	2.1	2.44	3.85	4.5	5.6	8.3
	Cultured dextrose 1.5%	2	2.1	2.2	2.3	2.1	1.9	2.6
	500	2	2	2.3	3.1	3.8	4.6	5.3
	1,000	2	1.8	1.9	2.4	2.5	2.9	3.2
	1,500	2	1.4	1.5	1.6	1.2	1.3	1.6
	2,000	2	1.2	1.4	1.3	0.9	1	0.8
Penicillium roqueforti	0 (Control)	2	2.4	3.4	4.3	5.1	5.9	7.4
	Cultured dextrose 1.5%	2	1.4	1.9	2.2	2.1	2.4	2.6
	500	2	2.1	2.2	2.8	3.4	3.8	4.8
	1,000	2	1.5	1.9	2.2	2.4	2.9	3.8
	1,500	2	1.2	1.3	1.8	2	1.9	1.8
	2,000	2	1	1	0.9	1.2	1.4	1.5

CFU = colony-forming units; N/A = not available; ppm = parts per million.

Table B.1.1.4-2 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Plant-based Cream Cheese

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)					
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28
Geotricum candidum	0 (Control)	2	1.7	3.6	5.3	5.4	5.6
	1,500	2	1.3	1.3	1.6	1.5	1.2
	0 (Control)	3	2.1	3.8	5.4	5.6	6.1
	1,500	3	1.6	2.1	2.6	2.5	2.3
Penicillium aurantiogriseum	0 (Control)	2	1.6	2.2	3.2	3.4	3.8
	1,500	2	1.4	1.2	1.6	1.4	1.2
	0 (Control)	3	2.6	2.8	3.5	4.1	4.8
	1,500	3	2.1	1.4	1.8	2.0	2.1
Cladosporium cladosporioides	0 (Control)	2	2.5	2.7	3.0	4.3	5.1
	1,500	2	1.4	1.2	1.3	1.5	1.9
	0 (Control)	3	3.1	3.3	2.8	4.2	5.3
	1,500	3	1.9	1.9	1.7	1.8	2.0

CFU = colony-forming units; N/A = not available; ppm = parts per million.

B.1.1.5 Technological Effect in Sauces, Spreads, Syrups, and Condiments

The technological function of fibre extracted from white button mushrooms (*A. bisporus*) was assessed in a high-sugar jam and high-fat coconut cream sauce. The fibre extracted from white button mushrooms (*A. bisporus*) was added at concentrations of 400, 600, 800, 1,000, 1,200, or 1,600 ppm. Control samples did not contain any preservatives or a mixture of sorbate and benzoate preservative at a total of 1,500 ppm. Samples of each product were aseptically inoculated with microorganisms associated with food spoilage (*i.e.*, *L. brevis*, *A. niger*, *Z. bailii*, *Z. rouxii*, *S. cerevisiae, S. cerevisiae diastaticus, P. aurantiogriseum*) at an initial concentration of Log 3 CFU/g, and then sealed and stored in a 25°C incubator for 42 days. Samples were assayed every 7 days by standard dilution and plate count.

Results of the microbiological analysis in these products, as presented in Table B.1.1.5-1, show that microbial counts decreased over time with the use of mushroom-derived fibre at concentrations of 1,000 ppm that was comparable to the sorbate/benzoate preservative control, whereas microbial growth increased over time in the control samples containing no preservatives.

Table B.1.1.5-1 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in High-sugar Jam and High-fat Coconut Cream

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
High-sugar Jam									
Zygosaccharomyces bailii	0 (Control)	3	2.8	2.8	2.9	3.4	3.5	3.6	3.9
	0 (Sorbate/ Benzoate Control)	3	1.8	1.7	1.3	1.3	1.2	1	0.5
	400	3	2.8	2.7	2.9	3.3	3.4	3.4	3.8
	600	3	2.5	2.8	2.8	3.2	3.3	3.4	3.7
	800	3	2.3	2.2	2.6	2.4	2.3	2.5	2.6
	1,000	3	2.2	2.3	2.4	1.8	1.5	1.3	0.8
	1,200	3	1.4	1.1	0.5	0.1	0	0	0
	1,600	3	1.2	0.3	0.1	0	0	0	0
Zygosaccharomyces rouxii	0 (Control)	3	3	3.3	3.7	4	4.3	4.8	5.2
	0 (Sorbate/ Benzoate Control)	3	2.9	2.2	2.2	2.1	1.8	1.4	1.1
	400	3	3.1	3.2	3.8	4.1	4.2	4.7	5.1
	600	3	3.2	3.3	3.5	3.8	4	4.3	4.5
	800	3	3	2.8	2.5	2.5	2.2	2.1	1.8
	1,000	3	2.9	2.7	2.4	2.3	1.6	1.1	1
	1,200	3	2.5	2.3	1.9	1.7	1.3	0.6	0.3
	1,600	3	2.4	2.1	2	1.5	1.1	0.3	0.1
Saccharomyces cerevisiae	0 (Control)	3	3	3.5	4.2	4.8	5.2	5.4	6.3
	0 (Sorbate/ Benzoate Control)	3	2.4	2.3	2.4	2.2	1.7	1.2	1
	400	3	3.1	3.2	4.1	4.6	4.8	5.2	5.8
	600	3	2.8	2.9	3.4	4.1	4.3	4.5	5.4
	800	3	2.6	2.8	2.5	2.8	2.9	3	3.7
	1,000	3	2.6	2.6	2.3	2.2	2.1	1.7	1.4
	1,200	3	2.3	2.4	2.1	2	1.8	1	1
	1,600	3	2.6	2.5	2.2	2.1	2.3	1.1	0.9
Saccharomyces cerevisiae diastaticus	0 (Control)	3	3.4	3.7	4.4	4.4	5.2	5.4	5.8
	0 (Sorbate/ Benzoate Control)	3	2.3	2.1	2	2	1.7	1.4	1.1
	400	3	3.4	3.5	3.7	4.1	4.3	4.8	5.2
	600	3	3.1	3.2	2.8	2.7	2.4	2.1	1.9
	800	3	2.8	2.8	2.4	2.1	2	1.8	1.4
	1,000	3	2.8	2.7	2.3	1.8	1.9	1.2	0.7
	1,200	3	2.4	2.1	1.8	1.5	1.3	0.5	0.2
	1,600	3	2.1	2	1.9	1.4	1	0.4	0

Table B.1.1.5-1 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in High-sugar Jam and High-fat Coconut Cream

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Lactobacillus brevis	0 (Control)	3	2.2	1.9	2.4	2.7	2.3	3	3.2
	0 (Sorbate/ Benzoate Control)	3	1.7	1.8	1.3	0.4	0	0	0
	400	3	2.2	2	2.3	2.5	1.9	1.8	1.4
	600	3	2.3	2.2	1.7	0.9	0.4	0.2	0
	800	3	1.9	1.7	0.7	0.5	0	0	0
	1,000	3	1.4	1.4	0	0	0	0	0
	1,200	3	0.9	0.4	0.1	0	0	0	0
	1,600	3	1	0.1	0	0	0	0	0
Aspergillus niger	0 (Control)	3	2.7	2.3	2.8	3.4	3.5	3.9	4.3
	0 (Sorbate/ Benzoate Control)	3	2.3	2.1	1.2	0.8	0	0	0
	400	3	2.8	3	2.8	3.1	3.3	3.8	4.1
	600	3	2.7	2.3	2.4	1.9	2.6	3.3	3.4
	800	3	2.7	2.5	2.3	2.4	1.8	0.7	0.4
	1,000	3	2.7	2.4	1.2	1.3	0.4	0	0
	1,200	3	2.3	1.9	1.1	0.9	0.1	0	0
	1,600	3	2.4	2	1.7	0.7	0.2	0	0
High-fat Coconut Cream Sauce									
Penicillium aurantiogriseum	0 (Control)	3	2.9	2.8	2.9	3.3	3.4	4.3	5.1
	0 (Sorbate/ Benzoate Control)	3	2.4	2.2	1.9	1.8	1.1	1.4	1.9
	600	3	2.5	2.6	3	3.4	3.7	4.2	4.8
	800	3	2.5	2.4	3.1	3	3.2	3.7	4.3
	1,000	3	2.3	2.5	2.8	2.1	1.8	1.7	1.4
	1,200	3	2.1	2	2.2	1.7	1.3	1.1	0.8
	1,600	3	2	1.8	1.4	1.3	1	0.7	0.4
Zygosaccharomyces rouxii	0 (Control)	3	2.3	2.1	2.4	2.3	2.9	3.1	3.6
	0 (Sorbate/ Benzoate Control)	3	1.8	1.7	1.3	1.2	0.4	0.1	0
	600	3	2.3	2.2	2.3	2.4	2.7	2.8	3.1
	800	3	2.1	2.2	1.8	1.9	1.7	2.3	2.8
	1,000	3	1.9	2.1	1.3	0.5	0.2	0.2	0.1
	1,200	3	1.9	1.4	1	0.3	0.1	0	0
	1,600	3	1.7	1.3	1.2	0.8	0.3	0	0

Table B.1.1.5-1 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in High-sugar Jam and High-fat Coconut Cream

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Saccharomyces cerevisiae	0 (Control)	3	2	1.7	1.3	1.2	1.4	1.1	1
	0 (Sorbate/ Benzoate Control)	3	1	0	0	0	0	0	0
	600	3	2	1.5	1.3	1.2	0.6	0.4	0.2
	800	3	1.7	1.5	1.2	0.4	0.1	0	0
	1,000	3	1	0.2	0.1	0	0	0	0
	1,200	3	0.4	0	0	0	0	0	0
	1,600	3	0.2	0	0	0	0	0	0
Lactobacillus brevis	0 (Control)	3	2.1	2.1	2.2	2.9	3.5	3.2	3.4
	0 (Sorbate/ Benzoate Control)	3	1.7	1.4	1.2	0.4	0.4	0.1	0
	600	3	2.4	2.1	2.4	2.5	2.8	3.4	3.6
	800	3	2.3	2	2.1	1.8	1.5	1.2	0.9
	1,000	3	1.9	2	1.4	0.5	0.5	0.3	0.2
	1,200	3	1.4	1.2	1.1	0.4	0	0	0
	1,600	3	1.2	1.2	1	0.2	0.1	0	0
Aspergillus niger	0 (Control)	3	1.2	1.3	1.2	0.5	0	0	0
	0 (Sorbate/ Benzoate Control)	3	0.3	0.1	0	0	0	0	0
	600	3	1.2	1.4	1.1	0.4	0.1	0	0
	800	3	1.2	1.2	1	0.1	0	0	0
	1,000	3	0.6	0.4	0.3	0	0	0	0
	1,200	3	0	0	0	0	0	0	0
	1,600	3	0.1	0	0	0	0	0	0

CFU = colony-forming units; ppm = parts per million.

The technological function of fibre extracted from white button mushrooms (*A. bisporus*) was assessed in coffee syrup (vanilla), cocoa syrup, custard, jam, frosting, relish, coconut cream sauce, margarine, reduced fat mayonnaise, salad dressing (ranch). The mushroom-derived fibre was added at concentrations of 800 or 1,000 ppm. Control samples did not contain any preservatives or a mixture of sorbate and benzoate preservatives used at a total of 1,500 ppm. Samples of each product were aseptically inoculated with microorganisms associated with food spoilage (*i.e.*, *L. brevis*, *A. niger*, *Z. bailii*, *Z. rouxii*, *S. cerevisiae, S. cerevisiae diastaticus, P. aurantiogriseum*) at an initial concentration of Log 3 CFU/g, and then sealed and stored in a 25°C incubator for 42 days. Samples were assayed every 7 days by standard dilution and plate count.

Results of the microbiological analysis in these products, as presented in Table B.1.1.5-2, show that microbial counts decreased over time with the use of fibre extracted from white button mushrooms (*A. bisporus*) at concentrations of 800 or 1,000 ppm that was comparable to the sorbate/benzoate preservative control, whereas microbial growth increased over time in the control samples containing no preservatives.

Table B.1.1.5-2 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Various Sauces, Spreads, Syrups, and Condiments

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Coffee Syrup (Vanilla)									
Zygosaccharomyces bailii	0 (Control)	3	2.8	3.1	3.3	3.5	4.2	4.8	5.8
	0 (Sorbate/ Benzoate Control)	3	2.1	1.9	1.4	1.5	1.4	1.2	1
	1,000	3	2.4	2.1	1.6	1.8	1.5	1.3	1.3
Zygosaccharomyces rouxii	0 (Control)	3	2.4	2.4	2.9	3.3	3.6	3.8	4.3
	0 (Sorbate/ Benzoate Control)	3	2.3	1.4	1.5	1.7	1.4	1.2	0.4
	1,000	3	2.3	2.2	2.1	1.8	1.5	1.6	1.1
Saccharomyces cerevisiae	0 (Control)	3	3	3.2	3.5	3.9	4.1	4.3	4.5
	0 (Sorbate/ Benzoate Control)	3	2.4	2.3	2.5	2.3	2	1.5	1.3
	1,000	3	2.3	2.5	2.3	1.9	1.4	1.1	1.1
Saccharomyces cerevisiae diastaticus	0 (Control)	3	3	3	3.2	3.4	3.9	4.3	5.4
	0 (Sorbate/ Benzoate Control)	3	3	2.9	2.8	2	1.8	1.5	1.2
	1,000	3	2.9	2.9	2.3	1.8	1.3	1.2	0.5
Lactobacillus brevis	0 (Control)	3	2.7	2.4	2.4	2.8	2.9	3.2	3.4
	0 (Sorbate/ Benzoate Control)	3	2.1	2	2.9	2.3	1.7	1.4	1.5
	1,000	3	1.8	1.8	2.5	1.9	1.8	1.3	1.1
Aspergillus niger	0 (Control)	3	2	2.1	2.4	2.8	3.4	3.8	4.5
	0 (Sorbate/ Benzoate Control)	3	1.9	1.8	1.8	1.3	1.2	1.1	0.4
	1,000	3	1.9	2	1.9	1.5	1.8	1.2	0.9
Cocoa Syrup									
Zygosaccharomyces bailii	0 (Control)	3	2.8	3.1	3.3	3.4	4	4.9	5.5
	0 (Sorbate/ Benzoate Control)	3	2.2	1.9	1.4	1.1	1.1	1.1	0.5
	1,000	3	2.3	2.1	1.4	1.4	1.2	1.3	1.3
Zygosaccharomyces rouxii	0 (Control)	3	2.6	2.6	3	3.3	3.6	3.7	4.2
	0 (Sorbate/ Benzoate Control)	3	2.1	1.8	1.7	1.6	1.4	1	0.4
	1,000	3	2.1	2	2	1.7	1.3	1.3	0.7
Saccharomyces cerevisiae	0 (Control)	3	3.1	3	3.2	3.7	3.9	4.1	4.4
	0 (Sorbate/ Benzoate Control)	3	2.5	2.2	2.1	2	1.8	0.9	0.6
	1,000	3	2.1	2.4	2.2	2	1.9	1.2	0.9
Saccharomyces cerevisiae diastaticus	0 (Control)	3	2.8	2.9	2.9	3.1	3.4	4	4.7
	0 (Sorbate/ Benzoate Control)	3	2.1	2	1.5	1.8	1.2	1.1	0.8
	1,000	3	2.5	2.2	2.1	1.8	1.3	1.2	0.4

Table B.1.1.5-2 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Various Sauces, Spreads, Syrups, and Condiments

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Lactobacillus brevis	0 (Control)	3	3	2.9	3.1	3	2.3	2.4	3.8
	0 (Sorbate/ Benzoate Control)	3	1.9	0.8	0.4	0	0	0	0
	1,000	3	2.3	1	0.3	0.1	0	0	0
Aspergillus niger	0 (Control)	3	2	2.1	2.4	2.8	3	3.8	4.6
	0 (Sorbate/ Benzoate Control)	3	1.9	1.8	1.8	1.3	1	0.9	0.4
	1,000	3	1.9	2.1	2	1.4	1.3	1	0.7
Custard									
Zygosaccharomyces bailii	0 (Control)	3	3.1	3.3	3.5	4.1	4.7	4.9	5.7
	0 (Sorbate/ Benzoate Control)	3	2.8	2.7	2.7	2.2	1.6	1.2	0.8
	800	3	2.9	2.9	2.4	2.1	1.8	1.1	1
Zygosaccharomyces rouxii	0 (Control)	3	3.1	3.7	4.1	4.3	4.4	4.8	5.1
	0 (Sorbate/ Benzoate Control)	3	2.8	2.3	2.3	2	1.7	1.4	1.3
	800	3	3	2.8	2.4	2.2	1.8	1.3	1.3
Saccharomyces cerevisiae	0 (Control)	3	3.2	3.7	4.5	4.9	5.2	5.4	5.9
	0 (Sorbate/ Benzoate Control)	3	2	2.1	2.4	2.5	2.1	2.8	2.5
	800	3	2.4	2.1	1.9	2.4	2.5	3	2.8
Saccharomyces cerevisiae diastaticus	0 (Control)	3	3.4	3.7	4.4	4.9	5.3	5.7	6.7
	0 (Sorbate/ Benzoate Control)	3	2.3	2.1	2.4	2.8	2.1	1.8	1.9
	800	3	2.8	2.7	2.5	2.1	1.9	1.4	1.5
Lactobacillus brevis	0 (Control)	3	2.3	2.2	2.8	3.2	3.4	3.8	4.1
	0 (Sorbate/ Benzoate Control)	3	1.8	1.2	0.9	0.4	0.1	0	0
	800	3	1.9	2	1.8	1.7	1.2	0.5	0.2
Aspergillus niger	0 (Control)	3	1.3	1.4	2.4	2.8	3	3.2	4.1
	0 (Sorbate/ Benzoate Control)	3	0.8	0.3	0	0	0	0	0
	800	3	1	0	0	0	0	0	0
Jam									
Zygosaccharomyces bailii	0 (Control)	3	2.8	2.8	2.9	3.4	3.5	3.6	3.9
	0 (Sorbate/ Benzoate Control)	3	1.8	1.7	1.3	1.3	1.2	1	0.5
	1,000	3	2.2	2.3	2.4	1.8	1.5	1.3	0.8
Zygosaccharomyces rouxii	0 (Control)	3	3	3.3	3.7	4	4.3	4.8	5.2
	0 (Sorbate/ Benzoate Control)	3	2.9	2.2	2.2	2.1	1.8	1.4	1.1
	1,000	3	2.9	2.7	2.4	2.3	1.6	1.1	1

Table B.1.1.5-2 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Various Sauces, Spreads, Syrups, and Condiments

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Saccharomyces cerevisiae	0 (Control)	3	3	3.5	4.2	4.8	5.2	5.4	6.3
	0 (Sorbate/ Benzoate Control)	3	2.4	2.3	2.4	2.2	1.7	1.2	1
	1,000	3	2.6	2.6	2.3	2.2	2.1	1.7	1.4
Saccharomyces cerevisiae diastaticus	0 (Control)	3	3.4	3.7	4.4	4.4	5.2	5.4	5.8
	0 (Sorbate/ Benzoate Control)	3	2.3	2.1	2	2	1.7	1.4	1.1
	1,000	3	2.8	2.7	2.3	1.8	1.9	1.2	0.7
Lactobacillus brevis	0 (Control)	3	2.2	1.9	2.4	2.7	2.3	3	3.2
	0 (Sorbate/ Benzoate Control)	3	1.7	1.8	1.3	0.4	0	0	0
	1,000	3	1.4	1.4	0	0	0	0	0
Aspergillus niger	0 (Control)	3	2.7	2.3	2.8	3.4	3.5	3.9	4.3
	0 (Sorbate/ Benzoate Control)	3	2.3	2.1	1.2	0.8	0	0	0
	1,000	3	2.7	2.4	1.2	1.3	0.4	0	0
Frosting									
Zygosaccharomyces bailii	0 (Control)	3	3	3.1	3.4	3.6	4.5	5.4	6.1
	0 (Sorbate/ Benzoate Control)	3	2.7	2.4	2.3	1.9	2	2.3	2
	1,000	3	2.8	2.7	2.5	2.5	2.3	2.2	1.9
Zygosaccharomyces rouxii	0 (Control)	3	2.9	3.1	3.3	3.2	3.6	4.1	5.3
	0 (Sorbate/ Benzoate Control)	3	2.3	2.3	2.4	2	1.9	1.3	0.8
	1,000	3	2.5	2.7	2.2	2.2	1.9	1.1	1
Saccharomyces cerevisiae	0 (Control)	3	2.5	2.4	2.9	3.3	3.5	3.2	4.1
	0 (Sorbate/ Benzoate Control)	3	1.8	1.7	1.4	1.5	1.3	1	0.8
	1,000	3	2.3	2.2	2	1.7	1.7	0.9	0.9
Saccharomyces cerevisiae diastaticus	0 (Control)	3	2.8	2.6	2.7	2.9	3.9	4.3	4.8
	0 (Sorbate/ Benzoate Control)	3	2.2	2	1.9	1.4	1.5	1.2	0.5
	1,000	3	2.4	2.4	2.5	1.8	1	1.2	0.4
Lactobacillus brevis	0 (Control)	3	3.2	3.4	3.2	3.9	4.4	4.3	4.3
	0 (Sorbate/ Benzoate Control)	3	1.4	1.4	1.2	0.5	0.5	0.2	0
	1,000	3	1.9	1.7	1.3	0.8	0.3	0.4	0.4
Aspergillus niger	0 (Control)	3	2.7	2.3	2.9	3.2	3.6	4.3	4.4
	0 (Sorbate/ Benzoate Control)	3	2.5	2	1.4	0.9	0.5	0.4	0.1
	1,000	3	2.9	2.3	1.4	1.1	0.6	0.5	0.3

Table B.1.1.5-2 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Various Sauces, Spreads, Syrups, and Condiments

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Relish									
Zygosaccharomyces bailii	0 (Control)	3	3	3.4	3.9	4.1	4.8	5.6	6.4
	0 (Sorbate/ Benzoate Control)	3	2.7	2.4	2.3	2.3	2	2	0.8
	800	3	3	2.8	2.6	2.4	2	2.2	1
Zygosaccharomyces rouxii	0 (Control)	3	2.9	3.4	3.5	3.7	3.9	4.2	5.3
	0 (Sorbate/ Benzoate Control)	3	2.3	2.2	2.3	2.1	1.8	0.6	0.4
	800	3	2.5	2.3	2	2.4	2.3	1.7	1.3
Saccharomyces cerevisiae	0 (Control)	3	2.4	2.3	2.8	3.4	3.8	3.9	4.4
	0 (Sorbate/ Benzoate Control)	3	1.4	1.4	1.3	1.2	1	0.6	0.8
	800	3	2.4	2.1	1.8	1.4	1.4	0.5	0.8
Saccharomyces cerevisiae diastaticus	0 (Control)	3	2.8	2.6	2.9	3.3	3.4	4	4.5
	0 (Sorbate/ Benzoate Control)	3	2.2	2	1.2	1.2	1	0.9	0.7
	800	3	2.4	2.1	2.4	2.3	2.3	2.1	1.4
Lactobacillus brevis	0 (Control)	3	3	2.3	2.3	2.4	2.8	3.5	3.8
	0 (Sorbate/ Benzoate Control)	3	1	0.9	0.3	0	0	0	0
	800	3	2.2	2.1	0.9	0.4	0	0	0
Aspergillus niger	0 (Control)	3	2.1	1.9	1.9	2.3	2.4	2.5	2.4
	0 (Sorbate/ Benzoate Control)	3	0	0	0	0	0	0	0
	800	3	1.4	1.2	0	0	0	0	0
Coconut Cream Sauce									
Penicillium aurantiogriseum	0 (Control)	3	2.9	2.8	2.9	3.3	3.4	4.3	5.1
	0 (Sorbate/ Benzoate Control)	3	2.4	2.2	1.9	1.8	1.1	1.4	1.9
	1,000	3	2.3	2.5	2.8	2.1	1.8	1.7	1.4
Zygosaccharomyces rouxii	0 (Control)	3	2.3	2.1	2.4	2.3	2.9	3.1	3.6
	0 (Sorbate/ Benzoate Control)	3	1.8	1.7	1.3	1.2	0.4	0.1	0
	1,000	3	1.9	2.1	1.3	0.5	0.2	0.2	0.1
Saccharomyces cerevisiae	0 (Control)	3	2	1.7	1.3	1.2	1.4	1.1	1
	0 (Sorbate/ Benzoate Control)	3	1	0	0	0	0	0	0
	1,000	3	1	0.2	0.1	0	0	0	0
Lactobacillus brevis	0 (Control)	3	2.1	2.1	2.2	2.9	3.5	3.2	3.4
	0 (Sorbate/ Benzoate Control)	3	1.7	1.4	1.2	0.4	0.4	0.1	0
	1,000	3	1.9	2	1.4	0.5	0.5	0.3	0.2

Table B.1.1.5-2 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Various Sauces, Spreads, Syrups, and Condiments

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Aspergillus niger	0 (Control)	3	1.2	1.3	1.2	0.5	0	0	0
	0 (Sorbate/ Benzoate Control)	3	0.3	0.1	0	0	0	0	0
	1,000	3	0.6	0.4	0.3	0	0	0	0
Margarine									
Penicillium aurantiogriseum	0 (Control)	3	1.8	1.3	1.4	1.2	1.4	1.5	1.6
	0 (Sorbate/ Benzoate Control)	3	1.2	1.2	1	0.8	0.2	0.1	0.3
	1,000	3	1.4	1	0.4	0.5	0.6	0.3	0.2
Zygosaccharomyces rouxii	0 (Control)	3	0.9	0.5	0.3	0.2	0.5	0.8	1.2
	0 (Sorbate/ Benzoate Control)	3	1	0.3	0.1	0	0	0	0
	1,000	3	0.8	0.5	0	0	0	0	0
Saccharomyces cerevisiae	0 (Control)	3	1	0.4	0.2	0.1	0	0	0
	0 (Sorbate/ Benzoate Control)	3	0	0	0	0	0	0	0
	1,000	3	0.3	0.1	0	0	0	0	0
Lactobacillus brevis	0 (Control)	3	0.9	0.3	0.4	0.4	0.5	0.6	0.8
	0 (Sorbate/ Benzoate Control)	3	0.3	0.1	0	0	0	0	0
	1,000	3	0.4	0.2	0.1	0	0	0	0
Aspergillus niger	0 (Control)	3	0.9	0.7	0.5	0.9	1.2	1.4	1.5
	0 (Sorbate/ Benzoate Control)	3	0.5	0.1	0.1	0	0	0	0
	1,000	3	0.3	0.2	0.1	0	0	0	0
Mayonnaise (Reduced Fat)									
Penicillium aurantiogriseum	0 (Control)	3	2.2	2.3	2.1	2.4	2.5	2.9	3.5
	0 (Sorbate/ Benzoate Control)	3	1.9	1.4	1.5	1.2	0.8	0.7	0.6
	1,000	3	2.1	1.9	1.6	1.5	1.4	1.2	0.9
Zygosaccharomyces rouxii	0 (Control)	3	2.9	2.9	3.3	3.4	3.2	3.6	4.3
	0 (Sorbate/ Benzoate Control)	3	2.5	2.4	2.4	2.2	0.9	1.2	1.1
	1,000	3	2.8	2.5	2.3	1.9	1.5	1.3	1.5
Saccharomyces cerevisiae	0 (Control)	3	1.4	1.5	1.9	2.2	2.4	2.2	2.1
	0 (Sorbate/ Benzoate Control)	3	1.2	0.4	0.5	0.3	0.1	0	0
	1,000	3	1.1	1.1	1.2	1	0.6	0.4	0
Lactobacillus brevis	0 (Control)	3	1.4	1.2	1.6	1.6	1.9	2.2	2.4
	0 (Sorbate/ Benzoate Control)	3	0.9	0.5	0.2	0	0	0	0
	1,000	3	1.2	1.2	0.8	0.5	0.3	0	0

Table B.1.1.5-2 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Various Sauces, Spreads, Syrups, and Condiments

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)							
		Day 0	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Aspergillus niger	0 (Control)	3	2.3	2.4	2.1	2.7	2.4	2.9	3.7
	0 (Sorbate/ Benzoate Control)	3	2	1.5	1.2	1.2	0.5	0.2	0
	1,000	3	2.1	1.8	1.5	1.2	1.1	0.7	0.4
Salad Dressing (Ranch)									
Penicillium aurantiogriseum	0 (Control)	3	2.3	2.5	2.4	2.4	2.8	3.3	3.6
	0 (Sorbate/ Benzoate Control)	3	2	1.5	1.6	1.4	1.2	0.9	1.1
	1,000	3	2.2	2	1.1	1.2	0.8	1.1	1.2
Zygosaccharomyces rouxii	0 (Control)	3	2.9	3	3.2	3.6	3.5	3.8	4.4
	0 (Sorbate/ Benzoate Control)	3	2.3	2.3	2.8	2.5	1.3	1.5	0.9
	1,000	3	2.4	2.3	2.3	1.9	1.2	0.8	1.1
Saccharomyces cerevisiae	0 (Control)	3	2	2.2	1.9	2.2	2.4	2.2	2.1
	0 (Sorbate/ Benzoate Control)	3	1.6	0.4	0.5	0.4	0.6	0.2	0.4
	1,000	3	1.9	1.2	0.9	0.7	0.2	0.2	0
Lactobacillus brevis	0 (Control)	3	1.4	1.2	1.6	1.6	1.9	2.2	2.4
	0 (Sorbate/ Benzoate Control)	3	0.9	0.5	0.2	0	0	0	0
	1,000	3	1.2	1.2	0.8	0.5	0.3	0	0
Aspergillus niger	0 (Control)	3	2.1	2.2	1.9	1.7	2.2	2.4	3.3
	0 (Sorbate/ Benzoate Control)	3	1.9	1.5	1.2	1	0.7	0.3	0.3
	1,000	3	2.3	2	1.3	1	0.9	0.5	0.2

CFU = colony-forming units; ppm = parts per million.

B.1.1.6 Technological Effect in Plant-based Meat Analogues

The technological function of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was assessed in plant-based meat analogues (*i.e.*, soy-based ground beef). The fibre extracted from white button mushrooms (*A. bisporus*) was added at concentrations of 500, 1,000, 1,500, 2,000, or 2,500 ppm. Control samples did not contain any preservatives or 2% cultured dextrose preservative. Samples of each product were aseptically inoculated with *L. brevis* that is associated with food spoilage at an initial concentration of Log 5 CFU/g, and then sealed and stored in a 25°C incubator for 42 days. Additional samples were aseptically inoculated with *A. niger* and *C. cladosporioides* with mushroom-derived fibre concentrations of 0 or 1,500 ppm. Samples were assayed every 7 days by standard dilution and plate count.

Results of the microbiological analysis, as presented in Tables B.1.1.6-1, show that microbial counts decreased over time with the use of mushroom-derived fibre at concentrations of 1,500 ppm that was comparable with the cultured dextrose control samples, whereas microbial growth increased over time in the control samples containing no preservatives.

Table B.1.1.6-1 Summary of the Mould Growth Analysis of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Soy-based Ground Beef

Microorganism	Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) Concentration (ppm)	Results (log CFU/g)						
		Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Lactobacillus brevis	0 (Control)	5	3	2.1	2.2	2.4	2.5	2.6
	0 (Cultured Dextrose 2%)	5	2.1	2.1	2.1	1.7	1.5	1.6
	500	5	2	2.1	1.8	2.3	2.5	2.5
	1,000	5	1.4	1.4	1.2	1.5	1.8	2.1
	1,500	5	1.5	1.2	1	1.1	1	0.5
	2,000	5	1.5	1.2	0.8	0.5	0.3	0.2
	2,500	5	1.2	0.8	0.4	0.2	0.1	0
Cladosporium cladosporioides	0 (Control)	5	1.2	1.4	1.9	2.5	4.3	5.5
	0 (Cultured Dextrose 2%)	5	1	1.3	1.4	1.8	2.4	2.9
	1,500	5	0.8	0.4	0.2	0	0	0
Aspergillus niger	0 (Control)	5	2.6	2.5	2.3	2.8	3.6	4.8
	0 (Cultured Dextrose 2%)	5	2.4	2.2	2.7	2.4	3.1	3.3
	1,500	5	2.1	1.7	1.5	1.8	1.9	2.4

CFU = colony-forming units; ppm = parts per million.

B.1.2 Stability

B.1.2.1 Stability of Fibre Extracted from White Button Mushrooms (Agaricus bisporus)

Chinova recommends storing the fibre extracted from white button mushrooms (*Agaricus bisporus*) at 25 ± 2°C and 60 ± 5% humidity in original intact packaging. When stored at ambient temperature (25 ± 2°C), the shelf life is 1 year.

The storage stability of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) was tested under the recommended storage conditions (temperature: 25 ± 2°C; relative humidity: 60 ± 5%) and accelerated conditions (temperature: 40 ± 2°C; relative humidity: 70 ± 5%) using 3 non-consecutive lots of the fibre product. The results after 3, 6, and 9 months were within the specification limits, demonstrating stability of mushroom-derived fibre for at least 9 months when stored under ambient and accelerated conditions (see Table B.1.2.1-1), with an estimated shelf life of 24 months based on the results of the accelerated stability testing.

Table B.1.2.1-1 Stability of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) Under Recommended and Accelerated Conditions

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

Table B.1.2.1-1 Stability of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) Under Recommended and Accelerated Conditions

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

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

B.1.2.2 Stability and Fate in Foods to Which the Additive is Added

As presented in Section B.1.2.1, studies have been conducted to determine the shelf life of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) at normal and accelerated conditions in order to demonstrate the stability of the fibre over time to ensure proper antimicrobial function will be demonstrated up to 12 months after the date of manufacture. The results of the storage stability study under both ambient/normal storage conditions and at accelerated conditions (*i.e.*, increased temperature and relative humidity conditions) demonstrated no significant changes in any of the observed parameters, indicating that the mushroom-derived fibre is stable under the recommended storage conditions. Furthermore, once the ingredient is added to a food or beverage, the stability of the ingredient would not be a concern, as it would be bound to other molecules present in the food or beverage, such as proteins or metal ions; therefore, it is not technologically feasible to separate chitosan for chemical analysis. Considering the ingredient is a fibre, it would not degrade into any harmful substance. It should also be noted that results of the stability studies on the mushroom-derived fibre demonstrate that the ingredient maintains its antimicrobial and antifungal activity as displayed by the absence or reduction of microbial growth in the product.

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is a mixture of chitosan and *beta*-glucan fibres extracted from the mushrooms. The chitosan and *beta*-glucan fibres will naturally slowly degrade into shorter fibre lengths over time and eventually down to their individual monomers. Chitosan is known to naturally degrade into its glucosamine and *N*-acetyl-glucosamine monomers (Muzzarelli, 1997). *beta*-Glucan is known to naturally degrade into glucose monomers over time (GRN 397 – U.S. FDA, 2011). Chinova has tested the antimicrobial effect of fibre extracted from white button mushrooms (*A. bisporus*) in a wide variety of foods and beverages (see Section B.1.1) that have a wide range of pHs, have been exposed to high shear in the processing steps, and have been exposed to both high and low temperatures. The antimicrobial effect of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is primarily, but not solely, and depending on the type of food or beverage product, intended to be relevant only from the period the food or beverage is produced to the point the packaging is opened by the consumer. As such, the ingredient does not need to remain active beyond a relatively short time period. While chitosan and *beta*-glucan fibres are known to be stable and not prone to rapid degradation, their ability to remain stable in various food and beverage products is unknown. It is noted that the *beta*-glycosidic bonds of chitosan are resistant to the hydrochloric acid of the human stomach, which indicates the ingredient's tolerance to highly acidic, low-pH environments (U.S. FDA, 2011). Other publications have indicated that chitosan is heat-tolerant and can withstand temperatures up to 200 to 220°C before mild degradation occurs (Grząbka-Zasadzińska *et al*., 2017). Chinova has evidence that the antimicrobial effect of fibre extracted from white button mushrooms (*A. bisporus*) is unaffected by commercial pasteurisation processes used by beverage producers when tested in a study comparing a pasteurised beverage to a control that was not pasteurised or heat-treated in any way. Chinova also has determined that high shear mixing does not impact the antimicrobial effect of the ingredient. Given this, along with the published literature, the stability of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) has been demonstrated to be suitable to have the intended effect in various food and beverages.

B.2 Information to Enable Identification of the Food Additive

Information to enable the identification of Chinova's fibre extracted from white button mushrooms (*A. bisporus*), including the identity, chemical composition, chemical structure, the chemical name, the molecular weight and formula, and the common name, is presented below.

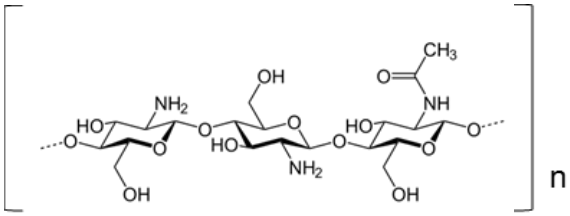
B.2.1 Identity of Substance

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™. 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 MW of 10 to 400 kDa.[6] Chitosan [(1,4)-2-amino-2-desoxy-*beta*-D-glucan; poly-β-(1,4)-2-amino-2-deoxy-D-glucose; Chemical Abstracts Service (CAS) number 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 B.2.1-1.

[6] Chitosan in this MW range is considered low-MW chitosan.

beta-1,3-D-Glucans are a major constituent of the cell walls of fungi; 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 MW averages and can be linear (*e.g.*, vegetable and *A. 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.

Figure B.2.1-1 Chemical Structure of Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*)



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 3 production batches of the ingredient demonstrates the average MW to be approximately 100 kDa and the DDA to be 90 to 94% (see Section B.6.2). Chinova has established product specifications for the fibre extracted from white button mushrooms (*A. bisporus*) consistent with the FCC monograph for chitosan derived from crustacean sources (see Section B.6.1). As discussed in Section B.2.2 below, Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is compositionally equivalent to crustacean-derived chitosan as confirmed by Fourier-transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy.

B.2.2 Chemical Composition and Identity

B.2.2.1 Compositional Equivalent of Chinova's Fibre Extracted from White Button Mushrooms (Agaricus bisporus) to Crustacean-derived Chitosan

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.[7] 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.2.2.2, 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.2.2.3). 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.

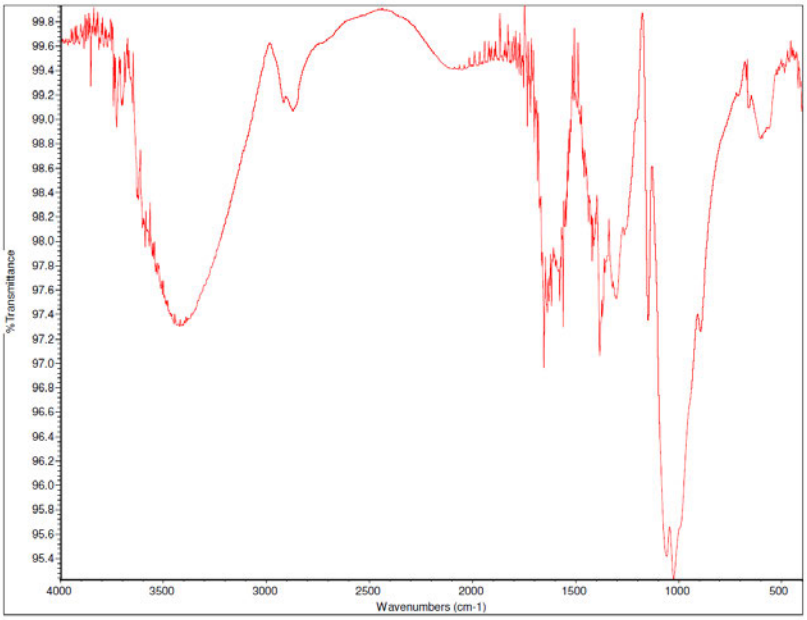
[7] "*Chitosan in an unbranched binary polysaccharide consisting of N-acetyl-D-glucosamine and D-glucosamine units linked in a β(1-4) manner. Chitosan is obtained by partial deacetylation of chitin, which is extracted from the shells of edible shrimps and crabs suitable for human use. Its degree of deacetylation is NLT* [not less than] *70.0% and NMT* [not more than] *95%*" (USP, 2020).

B.2.2.2 Infrared Spectroscopy Analysis

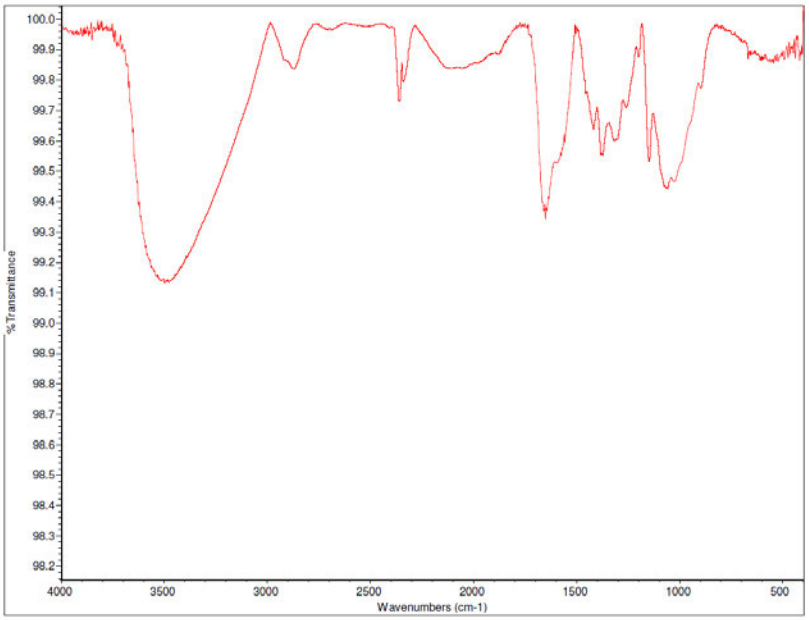
FTIR is the most commonly used method for the identification of chitosan (Kumirska *et al*., 2010). Samples prepared for chitosan from *A. bisporus*, crustacean-derived chitosan, and USP monograph–reference chitosan were analysed by FTIR. The FTIR spectra demonstrates that Chinova's fibre derived from white button mushrooms (*A. bisporus*) is chemically identical to crustacean-derived chitosan products, including the USP monograph–reference chitosan (see Figure B.2.2.2-1). 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.

Figure B.2.2.2-1 Fourier-Transform Infrared Spectra for Chitosan from *Agaricus bisporus* and Crustacean Sources

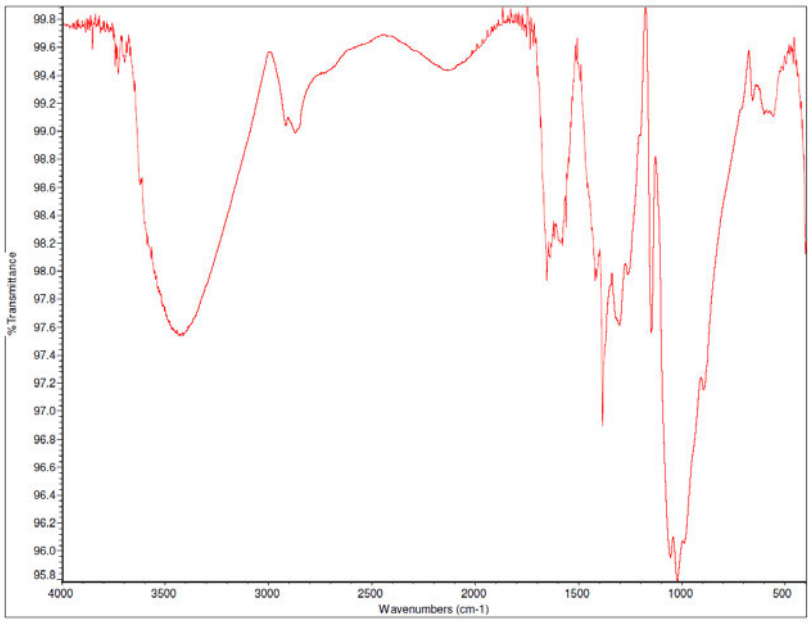
USP – Crustacean Chitosan



Commercial – Crustacean Chitosan



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



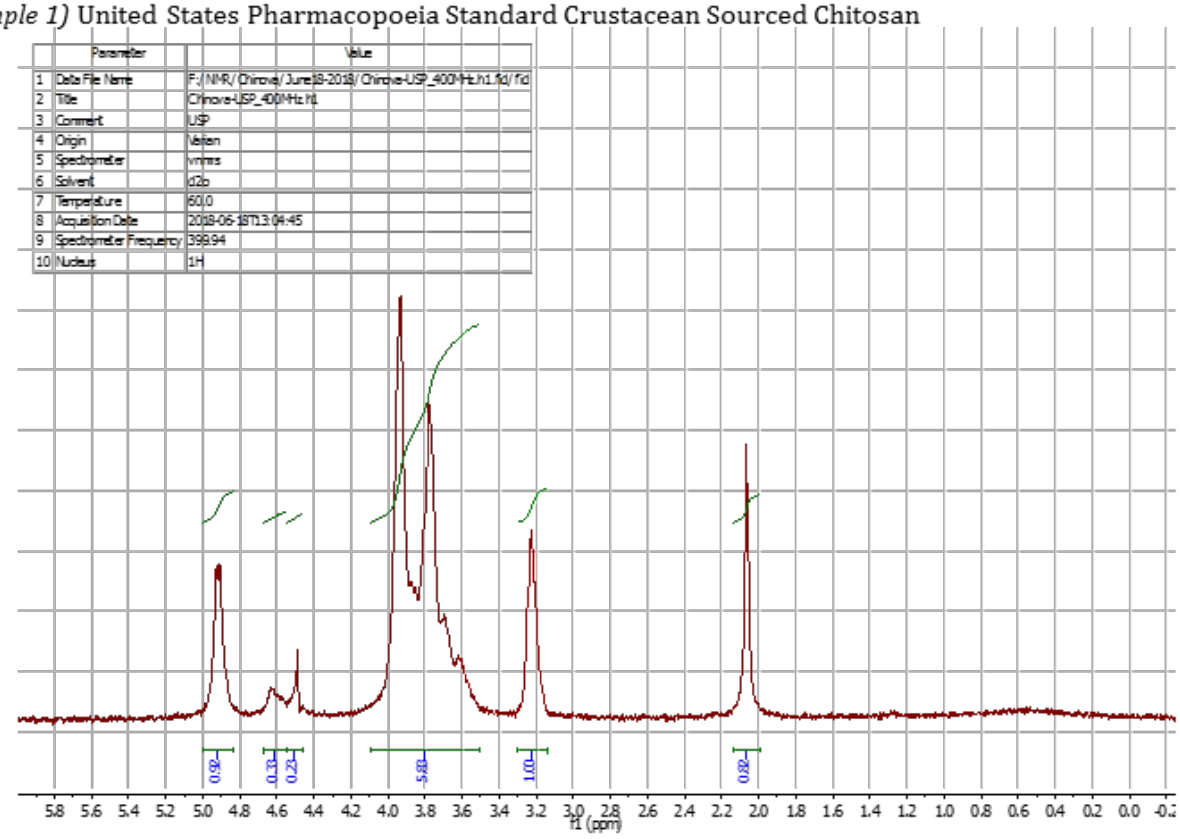
USP = *United States Pharmacopeia*.

B.2.2.3 Nuclear Magnetic Resonance Spectroscopy

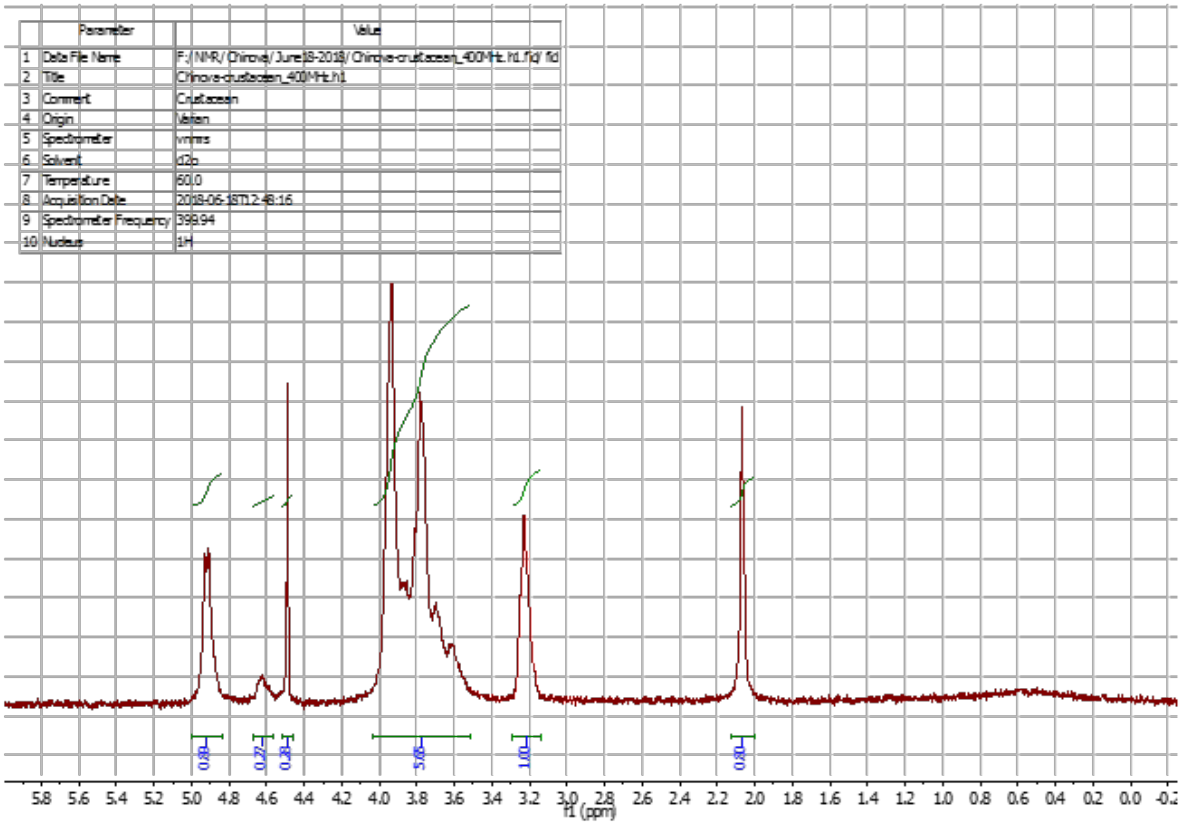
Samples of Chinova's fibre from white button mushrooms (*A. bisporus*), crustacean-derived chitosan, and USP monograph–reference chitosan were analysed by ^{1}H-NMR spectroscopy to provide information regarding the DDA of the compound and the compositional equivalency. The spectra shown in Figure B.2.2.3-1 demonstrates that chitosan derived from white button mushrooms (*A. bisporus*) is compositionally equivalent to crustacean-derived chitosan products, including the USP monograph–reference chitosan.

Figure B.2.2.3-1 Nuclear Magnetic Resonance Spectra for Chitosan from *Agaricus bisporus* and Crustacean Sources

Sample 1) United States Pharmacopoeia Standard Crustacean Sourced Chitosan

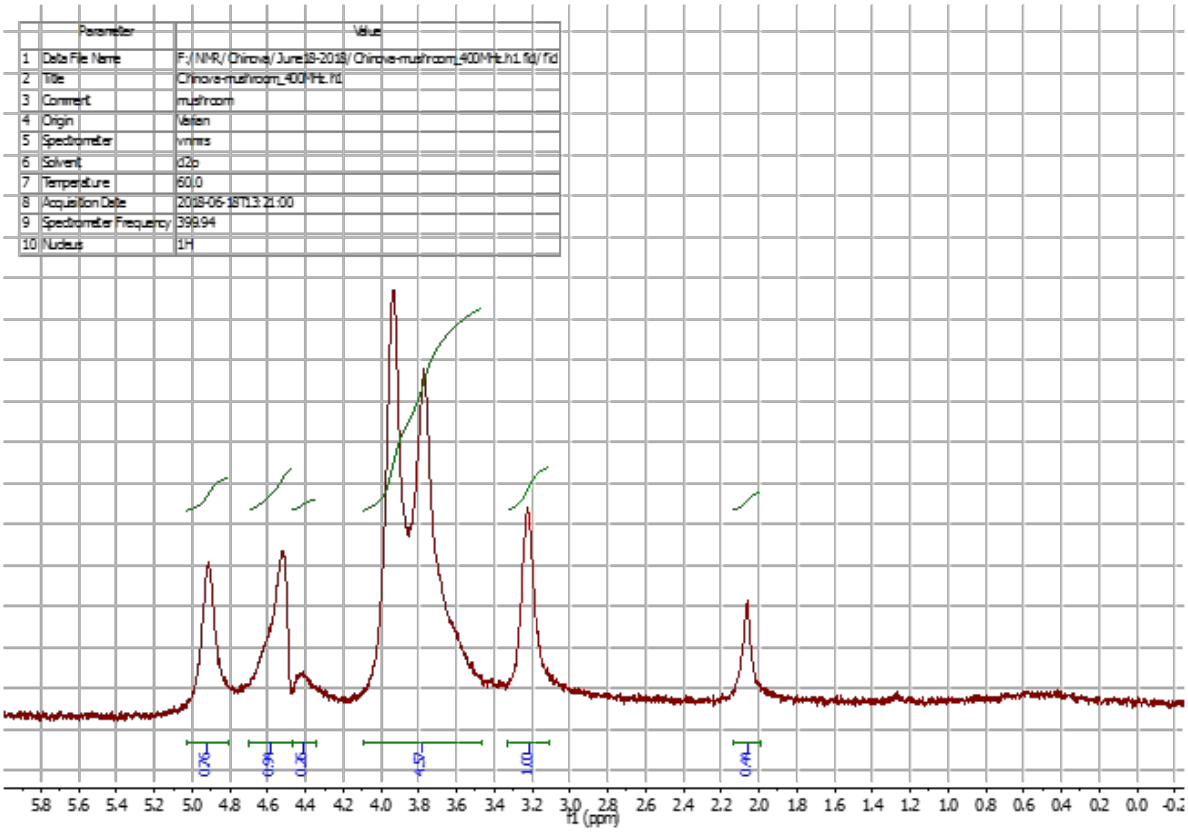


Sample 2) Crustacean Sourced Chitosan



	Parameter	Value
1	Data File Name	F:\NMR\Chinova\June18-2018\Chinova-crustacean_400MHz.h1.fid\fid
2	Title	Chinova-crustacean_400MHz.h1
3	Comment	Crustacean
4	Origin	Varian
5	Spectrometer	vnmrs
6	Solvent	d2o
7	Temperature	60.0
8	Acquisition Date	2018-06-18T12:48:16
9	Spectrometer Frequency	399.94
10	Nucleus	1H

Sample 3) A. bisporus Sourced Chitosan (Chiber)



	Parameter	Value
1	Data File Name	F:\NMR\Chinova\June18-2018\Chinova-mushroom_400MHz.h1.fid\fid
2	Title	Chinova-mushroom_400MHz.h1
3	Comment	mushroom
4	Origin	Varian
5	Spectrometer	vnmrs
6	Solvent	d2o
7	Temperature	60.0
8	Acquisition Date	2018-06-18T13:21:00
9	Spectrometer Frequency	399.94
10	Nucleus	1H

B.3 Information on the Chemical and Physical Properties of the Food Additive

Chinova's fibre extracted from white button mushrooms (A. bisporus) is a white to beige powder. As discussed in Section B.2.1, is a mixture of chitosan and *beta*-1,3-D-glucans, with chitosan being the main component, representing approximately 95% of the total volume. The solubility is 100% (w/w) when tested in a solution of 1% acetic acid solution (the ingredient is not soluble in water).

B.4 Information on the Impurity Profile of the Food Additive

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is compositionally equivalent to crustacean-derived chitosan. Potential contaminants (heavy metals or microbials) were either not detected or were well below specification limits (see Section B.6.2). Since Chinova's fibre from white button mushrooms (*A. bisporus*) is intended as a preservative, risk of microbial contamination is low.

B.5 Manufacturing Process of the Food Additive

B.5.1 Raw Materials

All processing aids and materials used in the manufacture of the ingredient are of high quality and are acceptable for use in food production. All processing aids and raw materials conform to FCC (2023) quality standards, where available. A detailed description of the raw materials is included in Appendix A (CCI). Certificates of Analysis for the raw materials are provided in Appendix D.

B.5.2 Production Method

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is manufactured in accordance with cGMP with a Hazard Analysis and Critical Control Points (HACCP) plan in place. The manufacturing process includes controls to ensure the quality of the final product prior to its release.

The ingredient is produced by extraction of chitosan from white button mushrooms (*A. bisporus*) using a deacetylation process. Purification processes results in the final ingredient. A detailed description of the production process is included in Appendix A (CCI).

B.6 Specification for Identity and Purity of the Food Additive

B.6.1 Proposed Specifications for the Food Additive

Food-grade chemical and microbiological specifications have been established for Chinova's fibre extracted from white button mushrooms (*A. bisporus*) that are generally consistent with the FCC monograph for chitosan obtained from crustacean sources (Table B.6.1-1). All methods of analysis are internationally recognised (*e.g.*, International Organization for Standardization [ISO]) or equivalent, or have been developed internally and validated by Chinova (internal methods of analysis are provided in Appendix E).

Table B.6.1-1 Product Specifications for Chinova's Fibre 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
Chitosan (%, w/w)	≥95	-	^{1}H-NMR (Internal)
beta-glucan (%, w/w)	≤5	-	Enzymatic Assay (Megazyme K-EBHLG; Internal)
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.

B.6.2 Batch Analysis

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 B.5 above, produces a consistent product that meets the established product specifications. A summary of the chemical and microbiological analyses is provided in Table B.6.2-1. Certificates of Analysis are provided in Appendix F.

Table B.6.2-1 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
Chitosan (%, w/w)	≥95	98	96	97	98	98
beta-glucan (%, w/w)	≤5	2	4	3	2	2
Moisture (%, w/w)	≤5	4.5	4.8	4.6	4.8	4.7

Table B.6.2-1 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
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.6.3 Other Potential Contaminants

The white button mushrooms (*A. bisporus*), from which the fibre is extracted, must meet guidelines for crops grown in Canada. This implies that the general maximum residue level (MRL) of pesticides is 0.1 ppm.[8] It also implies that mycotoxins, namely ochratoxin A, cannot be present at levels more than 15 ppb. Cultivated mushrooms are not known to have significant contamination from polycyclic aromatic hydrocarbons. Cultivated mushrooms grown in Canada are not at risk of contamination from pesticides, mycotoxins, and polycyclic aromatic hydrocarbons. As such, the raw material mushroom, which is a common edible food itself, is considered fit for human consumption in Canada. During the extraction process of the fibre, the mushroom biomass is exposed to sodium hydroxide caustic soda at concentrations high enough to destroy any potential mycotoxin.

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) also was analysed for potential toxicants, including pesticides and mycotoxins. All individual pesticides and mycotoxins were below the limit of detection. Certificates of Analysis are available in Appendix F.

B.7 Information for Food Labelling

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is a mixture of chitosan (≥95%) and *beta*-1,3-D-glucans (≤5%), intended for use as a preservative in food and beverages. This ingredient would be labelled in accordance with the relevant standards established for such ingredients when used in the food products proposed for use in Australia and New Zealand, as defined in Section D. No additional labelling statements or claims would be used for food products containing Chinova's fibre extracted from white button mushrooms (*A. bisporus*).

[8] https://pest-control.canada.ca/pesticide-registry/en/mrl-search.html.

B.8 Analytical Method for Detection

Detecting Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) (chitosan and *beta*-glucan fibres) 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 DDA 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 FTIR. 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 1 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.

B.9 Potential Additional Purposes of the Food Additive When Added to Food

Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) is intended for use as food additive in a range of food and beverage products to perform its technological purpose as a preservative. No additional purposes are expected related to the use of this food additive at the proposed use levels described in Section D.

C. INFORMATION RELATED TO THE SAFETY OF THE FOOD ADDITIVE

In accordance with Guideline 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 2019a), the following safety information must be provided for new food additives:

1. Information on the toxicokinetics and metabolism of the food additive and, if necessary, its degradation products and/or major metabolites;

2. Information on the toxicity of the food additive and, if necessary, its degradation products and major metabolites; and

3. Safety assessment reports prepared by international agencies or other national government agencies, if available.

Each point is addressed in the section that follows.

C.1 Introduction

Chitosan derived from crustaceans has a long history of safe use in the food supply. Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*) has been demonstrated to be compositionally similar to chitosan derived from shellfish (see Section B.2.2.1 for further details). Chinova's fibre derived from white button mushrooms (*A. bisporus*) is manufactured to an average 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 (HMWC), 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.

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 U.S. GRAS 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, U.S. FDA (2005) 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 USP-grade crustacean-derived chitosan in rats, and in 2017, published the entirety of the study report (NTP, 2017). In this study, the chitosan test article had an average purity of 94%, 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. These findings are discussed in further detail in Section C.2.2.3 Based on the reported effects of chitosan on serum vitamin E levels, the authors concluded the "*lowest-observed-effect level for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats*." The crustacean-derived chitosan used in the NTP study is chemically and compositionally similar to Chinova's 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.

It is noted that this data package was submitted, by Chinova, to the Food Directorate of Health Canada and Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus)* was recently (*i.e*., 2024) approval for use as a preservative in a variety of foods and beverages in Canada (Health Canada, 2023).

C.2 Safety Data of the Food Additive

C.2.1 Metabolic Fate of the Food Additive

Chitosan is a soluble biopolymer derived from the deacetylation of chitin, a naturally occurring carbohydrate polymer that is widely distributed in nature (*e.g.*, in crustacean shells and fungal cell walls). As discussed in Section B.2, Chinova's fibre derived from white button mushrooms (*A. bisporus*) is compositionally similar to chitosan derived from crustacean sources, and therefore, it is expected that Chinova's fibre derived from white button mushrooms (*A. bisporus*) will follow the same metabolic fate as other crustacean-derived chitosan.

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

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

C.2.2 Toxicological Studies

C.2.2.1 Acute Toxicity

The acute oral toxicity of chitosan from fungal sources (*i.e*., *A. bisporus*) or a chitosan oligosaccharide preparation was discussed in GRN 397 (U.S. FDA, 2011). The median lethal dose (LD_{50}) for 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 repeated-dose oral studies evaluating the safety of chitosan at doses of 2,000 mg/kg body weight/day or higher (see Section C.2.2.2), chitosan did not elicit increased mortality. Therefore, the results of the study reported by Eisa *et al*. (2018) are not considered relevant to the safety assessment of Chinova's mushroom-derived fibre.

C.2.2.2 Subchronic Toxicity

C.2.2.2.1 Studies on Chitosan

The repeated-dose oral toxicity of chitosan derived from crustacean sources was investigated in mice, rats, and guinea pigs. The test articles investigated in these studies were reported as low-molecular-weight chitosan (LMWC) or 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 MWs and solubility (MW = 32.7 to 760 kDa; DDA = ~85%) for 90 days was without significant adverse effects in any study parameter, and in particular liver and kidney weights and histopathology (Zeng *et al*., 2008). The authors noted that consumption of medium-molecular-weight chitosan (MW = 32.7 kDa; DDA = 85.2%) resulted in increased concentrations of minerals in the liver, spleen, and heart. These findings were attributed to the accumulation of HMWC in these organs and corresponding chelation of endogenous minerals (Zeng *et al*., 2008). 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 (NOAEL) to be 1,000 mg/kg body weight/day, the highest dose tested, for "*effects other than transient variation in erythrocyte count for chitosan under the conditions of this investigation*." The increase in erythrocyte count was considered to be unreliable due to the short duration of this study (*i.e.*, 28 days) and on the basis that no corroborative findings were reported in the long-term study in Sprague-Dawley rats by NTP (2017) (see Section C.2.2.3 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 [OECD] Test Guideline 408 – *Repeated dose 90-day oral toxicity study in rodents*). Nevertheless, the findings suggest that chitosan may impact liver function and elicit hepatomodulator 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 D.1). 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 C.2.3 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 (GLP) 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 C.2.2.2.1-1.

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Studies in Mice						
LMWC and HMWC Source: NR DDA: 80% Size: MW of 20,000 (LMWC) and 50,000 (HMWC)	Mice (CF_1) F Approximately 12/group	Diet 42 d	Group 1: 0 (control) Group 2: 2% LMWC (3,000) Group 3: 2% HMWC (3,000)	bw, frequency of aberrant crypt foci	• Chitosan groups had lowered bw, but HMWC was not statistically significant. • NSD in mice; HMWC ↓ 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% (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]

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: NR DDA: NR Size: NR	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 transaminase, and blood urea nitrogen.	Sumiyoshi and Kimura, 2006[c]

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan, 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 C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: 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 caecal 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].	Omara *et al.* (2012)[c]

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

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

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
LC chitosan 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)

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
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. • NSD in blood glucose, OGTT, serum insulin, or lipid levels in any group.	Tang *et al.* (2020)
Chitosan Source: NR DDA: NR Size: 21.7 × 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)

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Studies in 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 caecum 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 C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: NR DDA: 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]

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: NR DDA: NR Size: NR	Rat (Sprague-Dawley) M 8 to 9/group	Diet 18 d	Group 1: 0 (Control) Group 2: Week 1: 10% (10,000) Week 2+: 7.5% (7,500)	bw, food intake, liver lipids, faecal fat, cholesterol absorption	• Chitosan group had a slower rate of growth. • Reduced food intake with 10 and 7.5% supplementation. • Lower liver cholesterol contents in chitosan group. • Higher fat excretion. • No changes in intestinal contents supernatant viscosity.	Gallaher *et al.* (2000)[c]
Chitosan, dietary Source: Shrimp 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 C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: Lobster chitin 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)
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)

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Chitosan Source: NR DDA: NR Size: 2.5×10^5 Da, CS1; 3.8×10^4, CS2 NFS Chitosan quaternary ammonium salt Source: NR DDA: NR Size: 2.4×10^5, HACC1; 3.5×10^4, 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 C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
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 C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
Studies in Pigs						
LMWC Source: NR 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)

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
LMWC Source: NR DDA: >85% Size: 20 to 30 kDa	Pig (Duroc x Landrace x Yorkshire)[g] 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)

Table C.2.2.2.1-1 Summary of Repeated-dose Oral Toxicity Studies of Various Chitosan Preparations

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference

↓ = 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); DAO = diamine oxidase; DDA = degree of deacetylation; DSS = dextran sodium sulphate; ETEC = enterotoxigenic *Escherichia coli*; F = females; Fe = iron; GPI = glucose phosphate isomerase; GSH = glutathione; GSH-Px = glutathione peroxidase; HCS = high-molecular-weight chitosan with molecular weight of 7.60×10^5 and DDA of 85.5%; HDL-C = high-density lipoprotein cholesterol; HFD = high-fat diet; HK = hexokinase; HMWC = high-molecular-weight chitosan; IFN = interferon; Ig = immunoglobulin; IL = interleukin; kDa = kilodaltons; LC = long-chain; LDH = lactate dehydrogenase; LDL-C = low-density lipoprotein cholesterol; LMWC = low-molecular-weight chitosan; LPO = lactoperoxidase; LPS = lipopolysaccharides; M = males; MCS = middle-molecular-weight chitosan with molecular weight of 3.27×10^4 and DDA of 85.2%; MDA = malondialdehyde; Mg = magnesium; MW = molecular weight; NFS = not further specified; NR = not reported; NSD = no statistical difference; OGTT = oral glucose tolerance test; PAI-1 = plasminogen activator inhibitor-1; PFK = phosphofructokinase; SC = short-chain; SOD = superoxide dismutase; T-AOC = total antioxidant capacity; 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 3.91×10^4 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.2.2.2.2 Studies on Chitosan Oligomers/Oligosaccharides

Several repeated-dose studies were identified on chitosan oligomers/oligosaccharides (see Table C.2.2.2.2-1). Consistent with the studies on chitosan, studies on chitosan oligomers/oligosaccharides also reported statistically significant changes in liver weights and liver enzyme activities (Kim *et al*., 2001; Qin *et al*., 2006; Sumiyoshi and Kimura, 2006; Yao *et al*., 2012; Teodoro *et al*., 2016; Lan *et al*., 2019; Qian *et al*., 2019; Chiu *et al*., 2020; Kamal *et al*., 2023a). As previously discussed, chitosan oligosaccharides typically have an average molecular weight of less than 1 kDa and a DDA of 100% and are not chemically representative of Chinova's fibre extracted from white button mushrooms (*Agaricus bisporus*). These compounds are readily bioavailable and would be absorbed into the systemic circulation, which would not occur with Chinova's fibre.

Table C.2.2.2.2-1 Summary of Repeated-dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

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

Table C.2.2.2.2-1 Summary of Repeated-dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
CO Source: NR DDA: NR Size: 5 kDa	Mouse (C57BL/6) M 8/group	Oral (Gavage) 7 wks	0, 200, 400 mg/kg bw/d	Serum TC, TG, HDL-C, LDL-C, AST, ALT, liver TC, TG, MPO, T-AOC, GSH-Px, SOD, MDA, CAT, liver weight and histology	• NSD serum TG in all groups. • ↑ serum TC, HDL-C, LDL-C, LDL/HDL ratio, AST, and ALT in HFD group compared to normal diet group. • ↑ serum TC, HDL-C, and LDL-C in CO groups compared to normal diet control. • ↓ serum TC in 400 CO group compared to HFD control. • ↓ serum LDL-C, LDL/HDL ratio, AST, and ALT in CO groups compared to HFD control. • NSD in serum AST or ALT in CO groups compared to normal diet control. • Amelioration of hepatic steatosis. • ↓ liver IL-1β, IL-6, and MPO in CO groups compared to HFD control. • ↓ liver TNF-α in 400 CO group and NSD in 200 CO group compared to HFD control. • ↑ liver TNF-α, IL-1β, IL-6, and MPO in HFD control compared to normal diet control. • NSD T-AOC and CAT in all CO groups compared to HFD control or normal diet control. • ↓ GSH-Px and ↑ NDA and NO in HFD control compared to normal diet control. • ↑ GSH-Px in CO groups compared to HFD control. • ↑ SOD in 400 CO group and NSD in 200 CO group compared to HFD control. • ↓ MDA and NO in CO groups compared to HFD control.	Tao *et al.* (2019)

Table C.2.2.2.2-1 Summary of Repeated-dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

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

Table C.2.2.2.2-1 Summary of Repeated-dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

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

Table C.2.2.2.2-1 Summary of Repeated-dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
CO NFS	Rat (Sprague-Dawley) M 10/group	Oral (Gavage) 8 wks	0 (HFD), 150, 300, 600 mg/kg/d	bw, food consumption, serum lipid (TC, TG, HDL-C, LDL-C) AST, ALT, fat pad, fat-body ratio, visceral index, liver histology	• NSD bw or food consumption. • ↓ TC, TG, LDL-C, and leptin and ↑ HDL-C in all CO groups compared to HFD control. • NSD in TC, TG, LDL-C, HDL-C, or leptin in all CO groups compared to normal diet control. • ↓ liver weight, index, TC, TG, AST, and ALT in 300 and 600 CO group compared to HFD control. • NSD in liver weight, index, TC, TG, AST, or ALT in 150 CO group compared to HFD control. • NSD in liver weight, index, TC, TG, AST, or ALT in CO groups compared to normal control. • Amelioration of hepatic steatosis, epididymal and perirenal white adipose tissue weight in all CO groups compared to HFD control.	Pan *et al.* (2018)
CO Source: NR DDA: 91% Size: 2.3 × 10^3 Da	Rat (Sprague-Dawley)[e] M 9/group	Oral (Gavage) 4 wks	0, 200 mg/kg	Blood parameters (albumin, BUN, creatinine, LDH, LA, TC, TG, HDL-C, LDL-C)	• ↓ BUN, TC, and LDL-C in treatment groups compared to sedentary and exercise controls. • ↑ RBC, haematocrit, and MCV in treatment groups compared to sedentary and exercise controls.	Xiong *et al.* (2018)
CO NFS	Rat (Wistar)[f] M 10/group	Oral (Gavage) 3 d	0, 500 mg/kg	Markers of lung damage (protein, LDH activity) and inflammation (IL-1β, IL-8, TNF-α)	• Treatment inhibitor PM2.5-induced lung damage and lung inflammatory response (IL-1β, IL-8, and TNF-α).	Zhao *et al.* (2018)

Table C.2.2.2.2-1 Summary of Repeated-dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
CO Purity: 95% DDA: >95% Size: average MW <32 kDa	Rat (Sprague-Dawley)[g] M 10/group	Diet 7 d	0 or 200 mg/kg/d	bw, food and water consumption, organ weight (liver, kidney, spleen), inflammatory and antioxidant parameters (MDA, SOD, CAT, GSH-Px, GSH, T-AOC, IL-1β, IL-6, IL-10, TNF-α)	• NSD in bw, food consumption, or liver weight between groups. • ↑ spleen and kidney weight in CO group compared to heat-stress control group; NSD compared to normal control group. • ↓ spleen and kidney weight in heat-stress control group compared to normal control group. • NSD in liver MDA, SOD, CAT, GSH, T-AOC, IL-6, or TNF-α in CO group compared to heat-stress control group. • ↑ liver IL-1β in CO group compared to heat-stress control group. • ↑ liver MDA and IL-1β and ↓ CAT, GSH-Px, T-AOC, and IL-10 in heat-stress control group compared to normal control group. • NSD in spleen MDA, SOD, CAT, T-AOC, IL-1β, IL-6, or TNF-α in CO group compared to heat-stress control group. • NSD in spleen IL-1β between groups. • ↑ spleen MDA, IL-6, and TNF-α and ↓ SOD, GSH-Px, GSH, and IL-10 in heat-stress control group compared to normal control group. • ↓ spleen IL-10 in heat-stress control group compared to normal control group. • NSD in kidney MDA, SOD, CAT, GSH, T-AOC, IL-1β, IL-6, IL-10, or TNF-α in CO group compared to heat-stress control group. • ↑ kidney MDA, IL-6, and TNF-α and ↓ SOD, GSH-Px, T-AOC, and IL-10 in heat-stress control group compared to normal control group.	Lan *et al.* (2019)

Table C.2.2.2.2-1 Summary of Repeated-dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

Test Substance(s)	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Concentration (Dose in mg/kg bw/d)[a]	Parameters Evaluated	Significant Findings[b]	Reference
CO Source: Crustacean shells DDA: 100% Size: 719 Da	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 CO *vs.* high-fat controls. • NSD in food consumption or intestinal weight in any group. • ↑ liver weight in high-fat controls. • NSD in liver weight CO group *vs.* high-fat controls. • ↑ serum TC, HDL-C, LDL-C, and VLDL-C in high-fat controls; ↓ in same parameters in CO group. • ↑ ALT, AST, and TNF-α in CO group.	Chiu *et al.* (2020)
CO NFS	Rat (Sprague-Dawley) M 6/group	Diet 8 wks	0 (Standard Diet), 0 (HFD), HFD with 5% CO (2,500)	bw, indicators of liver function and hypercholesterolemia, liver and intestinal analysis (weight and histopathology)	• NSD in food consumption between groups. • NSD in liver weight in CO group. • NSD in intestinal weight between groups • ↓ TC, LDL-C, VLDL-C, and HDL-C. • ↑ plasma AST, ALT, and TNF-α in CO group.	Chiu *et al.* (2020)
Studies in Rabbits						
CO NFS	Rabbits (New Zealand White) M and F 6 M and 10 F/group	Diet 8 wks	0, 0.2, 0.4, or 0.6 g/kg diet	Growth Performance, Carcass traits, haematological and biochemical parameters (total protein, glucose, TC, TG, LDL, HDL, ALT, AST), histology of ileum, economic efficiency	• Significantly improved final bw, bw gain, and feed conversion ratio in CO groups. • NSD in mortality rate. • NSD in carcass traits except levels of abdominal fat. ↑ abdominal fat levels in CO groups • ↑ serum total protein, glucose, TG, and HDL levels. • NSD in TC, LDL, ALT, or AST. • Significantly improved final bw, bw gain, feed conversion ratio, economic efficiency, and histological measurements of ileum in CO groups.	Kamal *et al.* (2023a)

Table C.2.2.2.2-1 Summary of Repeated-dose Oral Toxicity Studies of Chitosan Oligomers/Oligosaccharides

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

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

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

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

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

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

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

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

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

C.2.2.3 Chronic Toxicity and Carcinogenicity

The NTP conducted a 6-month feeding study to investigate the safety of chitosan[9] in Sprague-Dawley rats (NTP, 2017). Male and female Sprague-Dawley rats (n=10 animals/sex/group/dose[10]) 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.[11] The test material had an average percent deacetylation of 86.5% and an average MW of 81.6 kDa (ranging from 62,755 to 87,343 Da; considered LMWC). The study was conducted according to U.S. FDA GLP.

The following endpoints were measured over the course of the study: feed consumption (recorded weekly); body weights; serum vitamin A, D, E, and K_1, levels (at Weeks 7, 13, 19, and 26); hepatic vitamin E and K levels (at Week 26); bone histomorphometry; bone calcium; ash and moisture; clinical chemistry (Week 7 and/or Weeks 13, 19, and 25 with a single measurement for 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 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.

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

[10] 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).

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

Statistically significant, dose-dependent decreases (15 to 29%) were reported in serum vitamin A concentrations starting at Week 13 in males of the 3% and 9% 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,[12] 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 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; 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 C.2.2.3-1 below.

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

Table C.2.2.3-1 Summary of Chronic Oral Toxicity Studies of Chitosan

Test Substance	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Dose in mg/kg bw/d (concentration)[a]	Parameters Evaluated	LO(A)EL[b]	Significant Findings[c,d]	Reference
Chitosan Source: Prawn shells DDA: 78% Size: NR	Mice (transgenic homozygous apo E-deficient), mixed gender 10 /control 13/experimental	Diet 182 days (26 weeks)	Group 1: 0 Group 2: 5% (7,500)	bw, general condition, select organ weights, food consumption	N/A	• Chitosan-fed mice had significantly higher 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 histopathology of major organs	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 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%].	NTP (2017)

Table C.2.2.3-1 Summary of Chronic Oral Toxicity Studies of Chitosan

Test Substance	Species (Strain), Sex, and Number of Animals	Route of Administration and Study Duration	Dose in mg/kg bw/d (concentration)[a]	Parameters Evaluated	LO(A)EL[b]	Significant Findings[c,d]	Reference
						• ↓ (17 to 82%) in serum vitamin E in M [3, 9%] and ↓ (~60%) in F [9%]. • Hepatic vitamin E levels ↓ (48 to 87%) in M [3%, 9%] and F [9%]. • Serum vitamin D ↑ (142%) in M and (180%) in F [9%]. • Calcium absorption ↑(154%) in F [9%]. • Serum calcium ↓ (4%) in M [9%]. • Percent fat digested ↓ (8 to 33%) in all dosed 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).

C.2.2.4 Reproductive and Developmental Toxicity

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 (MW = approximately 300 kDa; DDA = >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 GLP or current testing guidelines for teratogenicity and used a very small maternal population (n=3/group) and only 2 dose groups compared to OECD testing guidelines, which recommend at least 10 animals per group and at least 3 dose groups. These deficiencies limit the value of this study in the safety assessment of Chinova's fibre derived from white button mushrooms (*A. 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 C.2.2.3), 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.

C.2.2.5 Genotoxicity

The genotoxic potential of chitosan (derived from *Aspergillus bisporus*) and chitosan oligosaccharides was investigated in *in vitro* and *in vivo* studies and reviewed in GRN 397 (U.S. FDA, 2011). These studies are summarised in Table C.2.2.5-1. Chitosan derived from *A. bisporus* (KiOmedine-CsU) did not increase the number of revertant colonies in an Ames test conducted according to OECD Test Guideline 471 (*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 (MW = <10 kDa; 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 C.2.2.5-1 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)

Table C.2.2.5-1 Summary of Genotoxicity Studies of Chitosan and Chitosan Oligosaccharides

Test Substance(s)	Test	Test System	Concentration/Dose	Result(s)	Reference
In Vivo					
Chitosan oligomer Source: NR DDA: 90% MW: <10 kDa	Bone marrow micronuclei test	ICR mice M 20/group	0, 0.01, 0.1, 1% dietary chitosan oligosaccharide administered for up to 180 days	• No differences in formation of micronuclei in bone marrow cells.	Yoon *et al.* (2005)[b]
Chitosan oligomer Source: NR DDA: 90% MW: <10 kDa	Chromosome aberration test (4 generations)	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]

Table C.2.2.5-1 Summary of Genotoxicity Studies of Chitosan and Chitosan Oligosaccharides

Test Substance(s)	Test	Test System	Concentration/Dose	Result(s)	Reference

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

C.2.2.6 Studies on Related Compounds (N-Acetylglucosamine)

As previously discussed, according to the USP monograph, "*chitosan in an unbranched binary polysaccharide consisting of N-acetyl-D-glucosamine and D-glucosamine units linked in a β(1-4) manner*." Although it is unlikely that chitosan would be digested by gastric enzymes, *N*-acetylglucosamine is a potential hydrolysis by-product generated during gastric transit (see Section C.2.1 for further details). The chronic toxicity and carcinogenicity of *N*-acetyl-D-glucosamine, the monomeric constituent of chitosan, was evaluated in F344 rats in 2 separate studies conducted by Takahashi *et al.* (2009). This study was previously reviewed in GRN 397 (U.S. FDA, 2011). In the first study, F344 rats (n=10 animals/sex/group) were provided *N*-acetyl-D-glucosamine in the diet at concentrations of 1.25, 2.5, or 5% for 52 weeks. In the second study, F344 rats (n=50 animals/sex/group) were provided *N*-acetyl-D-glucosamine in the diet at concentrations of 0, 2.5, or 5% for 104 weeks. No treatment-related mortality or effects related to clinical signs of toxicity, food consumption, haematology, serum biochemistry, and histopathological evaluations were reported compared to control in either study. Body weights were slightly but statistically significantly decreased in high-dose (5%) males in both studies and in females (2.5 and 5%) in the carcinogenicity study. No statistically significant increase in tumours was reported in any of the dose groups of animals compared to controls. The slight suppression of body weights was considered by the authors to relate to reductions in caloric intake due to the high levels of intake of the test article and not direct toxic effect. Based on the results of this study, the NOAEL was concluded to be 5% in the diet in both studies, equivalent to 2,323 and 2,545 mg/kg body weight/day in males and females, respectively.

C.2.3 Human 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 C.2.3-1). According to GRN 170, the U.S FDA has raised concerns on potential effects on fat-soluble vitamins and mineral status in humans following consumption of chitosan (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 C.2.3-1. 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 multicentre, 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 prediabetic adult patients (characterised by impaired fasting glucose and impaired glucose tolerance), a LMWC 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 haemoglobins, 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 haemoglobins. 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 (ages 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 MW or DDA was reported. Based on the results of this meta-analysis, the authors concluded that chitosan consumption significantly decreased diastolic blood pressure at high doses (>2.4 g/day) and in short-term interventions (Huang *et al*., 2018a).

In another meta-analysis of 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 C.2.3-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
Healthy Subjects						
10 subjects Healthy volunteers, not taking antioxidants (such as vitamin E or C) during the 3 months before inclusion in the study	Oral preparation 4 wks Open-label, placebo-controlled, cross-over study	Water-soluble chitosan Source: NR DDA: 95% Size: average MW of 20 kDa	Group 1: 0 Group 2: 0.54	• Blood pressure, BMI • HDL-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)
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)

Table C.2.3-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
8 subjects Healthy males	Biscuits 14 d Random, placebo-controlled cross-over study	Chitosan Source: NR DDA: 90.5% Size: 500 kDa	Group 1: 0 Group 2: Week 1: 3 Week 2: 6	• 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						
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 Multicentre, 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)

Table C.2.3-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
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						
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)

Table C.2.3-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
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)
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 C.2.3-1 Summary of Human Studies of Chitosan and Chitosan Oligosaccharides[a]

Number and Characteristics of Subjects	Route of Administration, Study Duration, and Study Design	Test Article and Properties	Dose (g/d)	Parameters Measured Related to Safety	Reported Effects	Reference
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; GRN = Generally Recognized as Safe Notice; 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.2.4 Information Pertaining to the Safety of *beta*-1,3-Glucans

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 caecum of mid- and high-dose males and high-dose females, and a significant increase in the full and empty caecum weights relative to body weight in the high-dose males and females were reported compared to controls. Caecal enlargement occurs in rodents administered large dietary quantities of non-digestible polysaccharides/polyols and is an effect that is not considered relevant to humans (WHO, 1987). The authors concluded that under the conditions of the study, the NOAEL was 10% in the diet, the highest concentration tested, which was equivalent to an overall estimated daily intake of 6,589 mg/kg body weight/day for males and 7,002 mg/kg body weight/day for females.

Similar findings were reported in studies evaluating the effect of orally administered insoluble fungal derived *beta*-glucan preparations in rodents (Feletti *et al.*, 1992; Babíček *et al*., 2007). In a GLP- and OECD 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 (Babíček *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 caecal enlargement with variable hyperplasia of the colon mucosa. A NOAEL of 200 mg/kg body weight per day, the highest dose tested, can be determined from this study.

The safety of soluble *beta*-glucans derived from oat bran, barley, Baker's yeast, and fungi has been reviewed in numerous GRAS notices to the U.S. FDA (*e.g.*, GRN 239 – U.S. FDA, 2008a; GRN 309 – U.S. FDA, 2010; GRN 437 – U.S. FDA, 2013b; GRN 544 – U.S. FDA, 2015). Based on the intended uses of *beta*-glucan, the estimated intake in consumers was calculated to be as high as 16.5 g *beta*-glucan/person/day in 90th percentile (GRN 437 – U.S. FDA, 2013b). The Agency did not raise any objections to 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, 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, 2011a).

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*).

C.3 Summary of Safety Opinions of the Food Additive

C.3.1 Canada

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is approved for use as a food additive in Canada for use as a preservative agent in a variety of foods and beverages similar to those described herein. This ingredient was added to Health Canada's List of Permitted Preservatives as an antibacterial (Class 2) and antifungal (Class 3) preservative, listed as "*Chitosan from Agaricus bisporus (average molecular weight 90 to 120 kDa and degree of deacetylation not less than 80%),"* on 30 May 2024 (reference: M-FAA-24-05; Health Canada, 2024). Within the summary of their assessment, Health Canada States:

> *The Food Directorate concluded that information related to the safety and efficacy of chitosan from white button mushrooms (A. bisporus) supports its use as an antibacterial agent and an antifungal agent in the foods of interest.*

Therefore, based on the premarket assessment of Chinova's fibre extracted from white button mushrooms (Agaricus bisporus), Health Canada authorised the intended uses (Health Canada, 2023, 2024).

C.4 History of Use of Chitosan from Fungal and Crustacean Sources

Crustacean-derived chitosan has a long history of safe use in the global food supply. Chitosan obtained from crustacean sources are permitted for use in NHPs in Canada (Health Canada, 2018). Health Canada has prepared a monograph for the use of crustacean-derived chitosan in NHPs as part of a weight management program, lower blood total and low-density lipoprotein cholesterol, and to help maintain health cholesterol levels which indicates dose levels of 0.5 to 3 g of chitosan, 2 times/day, for a total of 6 g/day (Health Canada, 2018). Crustacean-derived chitosan is currently approved/permitted for use as a natural food additive for general food use in Japan and Korea (JFCRF, 2020; MFDS, 2020) and has widespread use as a drug excipient, functional food ingredient, and dietary supplement product in the U.S., the EU, and other regulatory jurisdictions throughout the world. Supplement products containing chitosan typically promote consumption of 1 to 5 g/person/day for use in weight control and/or maintenance of cardiovascular health (NIH, 2023). In the EU, chitosan extract from fungi such as *A. bisporus* or *A. niger* are authorised for use in food supplements as defined in Directive 2002/46/EC[13] on the approximation of the laws of the Member States relating to food supplements at levels consistent with chitosan from crustacean sources.

[13] 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: https://eur-lex.europa.eu/eli/dir/2002/46/oj (current consolidated version: 06/02/2024).

In the EU, chitosan extract from fungi (*Agaricus bisporus; Aspergillus niger*) are authorised for use in food supplements as defined in Directive 2002/46/EC[14] on the approximation of the laws of the Member States relating to food supplements at levels "*in line with normal use in food supplements of chitosan from crustacean sources.*"[15] Chitosan from crustacean sources (as polyacetyl-glycosamine) is defined as not novel in food supplements within the EU Novel Food Catalogue, with no maximum levels listed (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 Regulation (EU) 2019/934.[16]

In 2011, the use of an insoluble fungal-derived chitosan was concluded to be GRAS in the U.S. by KitoZyme as a secondary direct food ingredient in alcoholic beverage production at levels between 10 and 500 g/100 L. KitoZyme's GRAS conclusion was notified to the U.S. Food and Drug Administration on 08 August 2011 and filed by the Agency without objection under GRN 397 (U.S. FDA, 2011). 2-Amino-2-deoxy-poly-*D*-glucosamine (Chinova's fibre extracted from white button mushrooms [*A. bisporus*], as described herein) has FEMA 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 ppm. Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is GRAS in the U.S. for use in a variety of foods and beverages similar to those described herein. This GRAS status was notified to the U.S. Food and Drug Administration (FDA) as "*Chitosan and beta-1,3-glucans from white button mushrooms (Agaricus bisporus*)" (GRAS Notice [GRN] 997) and on 28 February 2022 the U.S. FDA issued a "no questions" letter in response to this Notice (U.S. FDA, 2022). In Canada, Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is 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 antibacterial (Class 2) and antifungal (Class 3) preservative (Health Canada, 2024).

[14] 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: https://eur-lex.europa.eu/eli/dir/2002/46/oj (current consolidated version: 06/02/2024).

[15] 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/eli/reg_impl/2017/2470/oj (current consolidated version: 27/06/2024).

[16] 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/eli/reg_del/2019/934/oj (current consolidated version: 08/02/2022).

C.5 Discussion of the Available Safety Information on Chitosan

The safety of chitosan was discussed in numerous GRAS notices that were notified to the U.S. 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 LMWC powder (purity = 94%; average MW = 82 kDa; DDA = 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 C.2.2.3. Dietary concentrations of chitosan up to 9% in the diet were well tolerated by rats. However, statistically significant reductions in serum concentrations of fat-soluble vitamins and reduced relative liver and thymus weights were reported at dietary concentrations of 3 and 9% in males, and 9% in females. No histopathological changes attributable to chitosan were reported in any of the groups. A statistically significant decrease in fat soluble vitamins at the 1% level in male rats was only reported at Week 13 for serum vitamin E. The reduction of serum vitamin E in male rats was not consistent throughout the study. Dietary exposure to chitosan for 6 months resulted in decreased fat digestion and depletion of some fat-soluble vitamins in male and female rats. There were no histological findings associated with the observed decreases in vitamin levels. Based on the effects of chitosan on serum vitamin E levels, the authors concluded the "*lowest-observed-effect level for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats*." These effects are considered to be indirect consequences of the recognised fat-binding properties of chitosan,[17] resulting in excretion of dietary fat and reduced 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.

[17] 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.

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 C.2.2.2). The weight of the available evidence indicates that typical chitosan preparations, when ingested are non-toxic. Some evidence of toxicity (*e.g*., increased or decreased relative organ weights, accumulation of iron, zinc in copper in organs, decrease fat soluble vitamins) has been reported in rodent studies following administration of LMWC oligomers and/or fully deacetylated oligomers at high-dietary concentrations (>1%). Evidence of toxicity in these studies is typically dose-limiting (only observed at dietary levels >1%) and in some cases were confounded by application of non-validated study designs.

Fifteen clinical studies were discussed in GRN 397 (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 C.2.3). 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. Chitosan is approved for use in supplements in the EU, therefore, a conservative high-level cumulative estimate of exposure is 5.7 g/day, which is 5-fold less than the LOEL and below the aforementioned clinically relevant dose. 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.

D. INFORMATION RELATED TO THE DIETARY EXPOSURE TO THE FOOD ADDITIVE

In accordance with Guideline 3.3.1 – Food Additives of the FSANZ *Application Handbook* (FSANZ, 2019a), the following dietary exposure information must be provided:

1. A list of the foods or food groups proposed to contain the food additive;

2. The maximum proposed level and/or concentration range of the food additive for each food group or food; and

3. For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs), information on the likely level of consumption (not applicable).

Each point is addressed in the following subsections.

D.1 Proposed Food Uses and Use Levels of the Food Additive

Chinova's fibre derived from white button mushrooms (*A. bisporus*) is intended for use in the Australia and New Zealand marketplace as a preservative (antimicrobial preservative) as defined in Schedule 14 at the minimum levels required to achieve the desired technical effect in accordance with cGMP, with maximum levels ranging from 0.01 to 0.150 g/100 g (equivalent to 100 to 1,500 ppm). A summary of the proposed food categories and use levels for Chinova's fibre derived from white button mushrooms (*A. bisporus*) is provided in Table D.1-1 below, which is organised according to the food standards as listed in Chapter 2 of the Code (FSANZ, 2024). Notably, these food uses and use levels are considered reflective of those that have been recently approved in Canada and are GRAS in the U.S., as well as those currently under evaluation by the relevant authorities in the EU and the UK.

Table D.1-1 Summary of the Individual Proposed Food Uses and Use Levels for Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Australia/New Zealand

Food Category[a]	Food Uses	Maximum Use Level (mg/kg)
Cereal and cereal products (Referencing Standard 2.1.1)	Pasta products Examples: pasta, noodles	200
	Cereal bars	200
	Baked goods Examples: Biscuits, Cakes, Pastries, Pies	1,000
	Bread and rolls Examples: Bread stuffing, Crisp bread, Croutons, Extruded, pressed or puffed bread, Gluten free bread, Leavened breads, Unleavened or flat breads	1,000
Meat and meat products (Referencing Standard 2.1.1)	Meat and poultry analogue products Examples: Tofu, Meat substitutes such as patties, sausages, grounded meat, fillets, *etc.*	1,500
Fruit and vegetables (Referencing Standard 2.3.1)	Fruit and vegetable purees Examples: Tomato puree, Potato puree, Banana puree	400
Jam (Referencing Standard 2.3.2)	Fruit and vegetable spreads, Fruit sauces Examples: Jams, marmalades, compotes, strawberry sauce	1,000
Edible oil spreads (Referencing Standard 2.4.2)	Margarine and other edible oil spreads	1,000
Dairy alternatives and dairy products (Referencing Standard 2.5.3 to 2.5.4)	Yoghurt and non-dairy yoghurt alternatives (including yoghurt drinks)	1,000
	Cheese Examples: firm-ripened cheeses, soft-ripened cheese veined with blue mould (blue Bavarian, blue de graven type), soft-ripened cheese with white and blue mould (cambozola type), unripened cheese (cottage cheese, mascarpone, mozzarella, quark, burrata, chevre frais, clotted cream, crescenza, juustoleipa, cream cheese, cheese curd, feta, mizithra, urda, ricotta	1,000
	Processed cheese and spreads	1,500
	Imitation cheese	1,500
Fruit juice and vegetable juice (Referencing Standard 2.6.1)	Vegetable juices and nectars, Mixed fruit and vegetable juices or nectars, Coconut water, Aloe vera Juice	400
	Fruit juices and nectars	600
Non-alcoholic beverages and brewed soft drinks (Referencing Standard 2.6.2)	Carbonated and non-carbonated beverages Examples: Regular and diet soft drinks, vitamin water, flavoured water	400
Formulated caffeinated beverages (Referencing Standard 2.6.4)	Energy drinks	400
Alcoholic beverages (Referencing Standard 2.7)	Cocktail drinks	400
Sugar and sugar products (Referencing Standard 2.8.1)	Icing sugar	400
Formulated meal replacements and formulated supplementary foods (Referencing Standard 2.9.3)	Single meal replacement products	1,000
Formulated supplementary sports foods (Referencing Standard 2.9.4)	Isotonic and sports drinks	400
Miscellaneous standards for other foods (Referencing Standard 2.10.4)	Coffee and coffee imitate beverages	150
	Tea beverages	400
Mustard	Mustard	400

Table D.1-1 Summary of the Individual Proposed Food Uses and Use Levels for Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Australia/New Zealand

Food Category[a]	Food Uses	Maximum Use Level (mg/kg)
Savory sauces	Tomato-based cooked sauces, Barbecue or steak sauces, Continental European brown cooked sauce.	200
	Tomato ketchup	400
	Relishes	800
	All other savoury sauces, mayonnaise, salad dressing Examples: Herbs/spices sauces, Mayonnaise, hollandaise sauce, soy sauce, Teriyaki sauce, Vegetables-based cooked sauces, White sauces, salad dressing	1,000
Soups	All varieties of soup Examples: chicken, tomato, minestrone, vegetable *etc.*	400
Sweet sauces and syrups	All varieties of sweet sauces and syrups Examples: Chocolate sauce, Fudge sauce, Alcoholic sweet sauce	1,000
Table-top sweeteners	Table-top sweeteners in all formats (liquid, powder, tablet)	1,000

[a] Food Standards where listed are based on Chapter 2: Food Standards https://www.foodstandards.gov.au/code/Pages/default.aspx.

D.1.1 Canada

The food uses and use levels for Chinova's fibre derived from white button mushrooms (*A. bisporus*) that were authorized for use in Canada on 30 May 2024 (Reference Number: M-FAA-24-05; Health Canada, 2024) are presented below in Table D.1.1-1. These uses and use levels are similar to those proposed for use in Australia and New Zealand.

Table D.1.1-1 Summary of the Individual Authorised Food Uses and Use Levels for Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Canada

Food Uses	Proposed Use Levels
Apricot nectar; Concentrated (naming the fruit) juice; Concentrates for unstandardized beverages containing fruit juice; Fruit-based or dairy-based smoothie beverages; Fruit-flavoured drinks; (naming the fruit) Juice; (naming the fruits) Juice; Peach nectar; Pear nectar; Unstandardized beverages containing fruit juice	600 ppm
Bread; Unstandardized bakery products	1,000 ppm
Caffeinated energy drinks; Unstandardized alcoholic beverages; Unstandardized fermented tea beverages; Unstandardized non-alcoholic water-based beverages; Unstandardized tea beverages; Unstandardized vegetable juices; Water-based beverages with vitamin and mineral nutrients added, except beverages with vitamins added in accordance with Part D of the Food and Drug Regulations	400 ppm
Cheddar cheese; (naming the variety) Cheese; Cold-pack (naming the variety) cheese; Cold-pack cheese food; Cold-pack (naming the variety) cheese with (naming the added ingredients); Cold-pack cheese food with (naming the added ingredients); Cottage cheese; Cream cheese; Cream cheese with (naming the added ingredients); Cream cheese spread; Cream cheese spread with (naming the added ingredients); Processed cheese food; Processed cheese food with (naming the added ingredients); Processed cheese spread; Processed cheese spread with (naming the added ingredients); Processed (naming the variety) cheese; Processed (naming the variety) cheese with (naming the added ingredients); Unstandardized cheese-based sauces; Unstandardized processed cheese products; Unstandardized shredded cheese products	1,500 ppm
Egg-based desserts; Fruit-based desserts	800 ppm
Fillings; Toppings; Unstandardized table syrups	1,000 ppm
Fresh pasta; Fresh noodles	200 ppm
Frostings; Icings	400 ppm

Table D.1.1-1 Summary of the Individual Authorised Food Uses and Use Levels for Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in Canada

Food Uses	Proposed Use Levels
Liquid plant protein isolate-based products that resemble egg products	1,500 ppm
Liquid soup bases; Liquid soup mixes; Soups	400 ppm
Liquid table-top sweeteners	1,000 ppm
Meal replacement bars; Nutritional supplement bars	200 ppm
Plant-based products that resemble cheese	1,500 ppm
Relishes	800 ppm
Simulated meat products; Simulated poultry products	1,500 ppm
Unstandardized coffee beverages	150 ppm
Unstandardized confectionery coatings	1,000 ppm
Unstandardized fruit spreads	1,000 ppm
Unstandardized salad dressings	1,000 ppm
Unstandardized sauces	1,000 ppm
Unstandardized snack bars	200 ppm
Unstandardized vegetable purées	400 ppm
Yoghurt	1,000 ppm

ppm = parts per million.

D.1.2 The United States

In the U.S., chitosan has FEMA GRAS status for use as a flavouring ingredient (FEMA No. 4946) at levels up to 2,000 ppm. Chinova's fibre extracted from white button mushrooms (*A. bisporus*) also is GRAS in the U.S. for use in a variety of foods and beverages similar to those proposed for use in Australia and New Zealand. This GRAS status was notified to the U.S. FDA for which a "no questions" letter was issued by the U.S. FDA (GRN 997; U.S. FDA 2022). The individual food uses and use levels are presented below in Table D.1.2-1.

Table D.1.2-1 Summary of the Individual Food Uses and Use Levels Notified as Generally Recognized as Safe in the United States for Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*)

Food Category (21 CFR §170.3) (U.S. FDA, 2021a)	Food Uses[a]	Maximum Proposed Use Levels (g/100 g)
Baked Goods and Baking Mixes	Bagels and English muffins	0.060
	Bread (excluding sweet type breads and rolls)	0.060
	Cakes	
	Light-weight cakes	0.060
	Medium-weight cakes	0.060
	Heavy-weight cakes	0.060
	Cornbread, corn muffins, or tortillas	0.060
	Muffins	0.040
	Pastries	0.050
Beverages, Alcoholic	Beer	0.010
	Cider	0.040
	Cocktail drinks	0.040
	Seltzer	0.040
	Wine	0.040
Beverages and Beverage Bases	Energy drinks	0.010
	Flavored or carbonated waters	0.010
	Soft drinks (regular and diet)	0.010
	Sport or electrolyte drinks, fluid-replacement drinks	0.010
Cheeses	Cheese-based sauces	0.100
	Cottage cheese	0.050
	Cream cheese and cheese-based spreads	0.100
	Processed cheese or cheese mixtures	0.060
Coffee and Tea	Ready-to-drink tea beverages	0.010
Condiments and Relishes	Relish	0.080
Confections and frostings	Coatings	0.100
	Frostings and icings	0.100
Dairy Product Analogs	Imitation cheese	0.150
Fats and Oils	Fat-based sauces	0.050
	Margarine and margarine-like spreads	0.050
	Mayonnaise and mayonnaise-type dressings	0.060
	Salad dressings	0.080
Gelatins, Puddings, and Fillings	Flans, custards, and other egg-based desserts	0.100
Grain Products and Pastas	Energy bars or protein bars or meal-replacement bars	0.020
Gravies and Sauces	Gravies	0.020
	White sauces	0.100
Jams and Jellies	Jams, jellies, preserves, and marmalades	0.100
Milk Products	Plain or flavored yogurt	0.060
Processed Fruits and Fruit Juices	Fruit drinks and ades and smoothies	0.020
	Fruit-based desserts	0.100
Plant Protein Products	Meat	0.150
	Egg substitutes	0.080
Processed Vegetables and Vegetable Juices	Vegetable purees[b]	0.040

Table D.1.2-1 Summary of the Individual Food Uses and Use Levels Notified as Generally Recognized as Safe in the United States for Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*)

Food Category (21 CFR §170.3) (U.S. FDA, 2021a)	Food Uses[a]	Maximum Proposed Use Levels (g/100 g)
Sugar Substitutes	Sugar substitutes	0.100
Sweet Sauces, Toppings, and Syrups	Sweet sauces, syrups, and toppings (including fruit-based)	0.060
	Cocoa syrups	0.100

CFR = *Code of Federal Regulations*.
[a] Chinova's mushroom-derived fibre is intended for use in unstandardized products when standards of identity, as established under 21 CFR §130 to 169, do not permit its addition (U.S. FDA, 2021b).
[b] Food codes for vegetable mixtures and vegetable combinations (which are likely to be used to make purees) were included as a surrogate for "vegetable purees."

D.1.3 The European Union/United Kingdom

The proposed food uses and maximum use levels of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) that are currently under review in the EU and the UK are presented in Table D.1.3-1.

Table D.1.3-1 Proposed Food Uses and Maximum Use Levels of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in the European Union and the United Kingdom

Specified Food Category[a,b]	Maximum Level of Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) (mg/kg or mg/L), as Marketed [Intended Food Use]
1.2 – Unflavoured fermented milk products, including natural unflavoured buttermilk (excluding sterilised buttermilk) non heat-treated after fermentation	1,000 [Yoghurt]
1.4 – Flavoured fermented milk products including heat-treated products	1,000 [Yoghurt drinks, including sweetened and/or flavoured variants]
1.7.1 – Unripened cheese excluding products falling in category 16	1,000 [Cottage cheese]
	1,000 [Mascarpone]
	1,000 [Mozzarella]
	1,000 [Quark]
	1,000 [Cheese, burrata]
	1,000 [Cheese, chevre frais]
	1,000 [Clotted cream]
	1,000 [Cheese, crescenza]
	1,000 [Cheese, juustoleipa]
	1,000 [Cream cheese]
	1,000 [Cheese curd]
	1,000 [Soft brined cheese (feta type)]
1.7.2 – Ripened cheese	1,000 [Soft-ripened cheese veined with blue mould (blue Bavarian, blue de graven type)]
	1,000 [Soft-ripened cheese with white and blue mould (cambozola type)]
	1,000 [Firm-ripened cheeses]

Table D.1.3-1 Proposed Food Uses and Maximum Use Levels of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in the European Union and the United Kingdom

Specified Food Category[a,b]	Maximum Level of Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) (mg/kg or mg/L), as Marketed [Intended Food Use]
1.7.4 – Whey cheese	1,000 [Ricotta]
	1,000 [Cheese, mizithra]
	1,000 [Cheese, urda]
	1,000 [Firm brined cheese (ricotta salata type)]
1.7.5 – Processed cheese	1,500 [Processed cheese and spreads]
1.8 – Dairy analogues, including beverage whiteners	1,500 [Imitation cheese]
	1,000 [Soya yoghurt]
	1,000 [Imitation yoghurt, non-soy]
2.2 – Fat and oil emulsions mainly of type water-in-oil	1,000 [Margarines and similar]
4.2 – Processed fruits and vegetables	400 [Fruit or fruit-vegetable puree]
	400 [Potato puree]
	1,000 [Fruit/vegetable spreads and similar]
	1,000 [Fruit sauce]
5.4 – Decorations, coatings and fillings, except fruit based fillings covered by category 4.2.4	1,000 [Biscuit with inclusions, filling or coating]
	1,000 [Chocolate sauce]
	1,000 [Fudge sauce]
	1,000 [Alcoholic sweet sauce]
	1,000 [Syrups (molasses and other syrups)]
	400 [Sugar, icing – powder]
6.4 – Pasta	200 [Fresh pasta]
	200 [Pasta wholemeal]
	200 [Fresh stuffed pasta]
	200 [Pasta, gluten free]
6.5 – Noodles	200 [Glass noodle]
	200 [Noodle, rice]
7.1 – Bread and rolls	1,000 [Unleavened or flat bread and similar]
	1,000 [Leavened bread and similar]
	1,000 [Crisp bread]
	1,000 [Extruded, pressed or puffed bread]
	1,000 [Bread stuffing]
	1,000 [Gluten free bread]
	1,000 [Croutons]
7.2 – Fine bakery wares	1,000 [Choux pastry]
	1,000 [Cakes]
	1,000 [Yeast leavened pastry]
	1,000 [Shortcrust (pies-tarts)]
	1,000 [Pastry based on laminated dough]
	1,000 [Various pastry]
	200 [Cereal bars]
11.4.1 – Table-top sweeteners in liquid form	1,000 [Table-top sweeteners in liquid form]
11.4.2 – Table-top sweeteners in powder form	1,000 [Table-top sweeteners in powder form]
11.4.3 – Table-top sweeteners in tablets	1,000 [Table-top sweeteners in tablets]
12.4 – Mustard	400 [Mustard and related sauces]

Table D.1.3-1 Proposed Food Uses and Maximum Use Levels of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) in the European Union and the United Kingdom

Specified Food Category[a,b]	Maximum Level of Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) (mg/kg or mg/L), as Marketed [Intended Food Use]
12.5 – Soups and broth	400 [Soups (Ready-to-eat)]
12.6 – Sauces	200 [Continental European brown cooked sauce, gravy]
	200 [Tomato-containing cooked sauces]
	400 [Tomato ketchup and related sauces]
	200 [Barbecue or steak sauces]
	1,000 [Mayonnaise, hollandaise and related sauces]
	1,000 [White sauces]
	1,000 [Salad dressing]
	800 [Relishes]
	1,000 [Vegetables-based cooked sauce]
	1,000 [Herbs/spices sauces]
	1,000 [Sauces from fermented/hydrolised sources and similar]
12.9 – Protein products	1,500 [Meat imitates]
	1,500 [Tofu]
13.3 – Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)	1,000 [Single meal replacement for weight reduction]
14.1.2 – Fruit and vegetable juices	400 [Vegetable juices]
	400 [Other (mixed) fruit and vegetable juices or nectars]
	600 [Fruit juices (100% from named source)]
	400 [Liquid or gel separated from plant RPCs]
14.1.3 – Fruit nectars as defined by Directive 2001/112/EC[c] and vegetable nectars and similar products	600 [Fruit nectars (min. 25-50% fruit as defined in EU legislation)]
14.1.4 – Flavoured drinks	400 [Functional drinks]
	400 [Fortified bottled water]
	400 [Flavoured bottled water]
	400 [Soft drinks]
14.1.5.1 – Coffee, coffee extracts	150 [Coffee beverages]
	400 [Tea beverages]
14.1.5.2 – Other	150 [Coffee imitate beverages]
14.2.8 – Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol	400 [Cocktail drink]

EFSA = European Food Safety Authority.

[a] Food Categories as per European Commission Guidance document describing the food categories in Part E of Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16-33. Available online: http://data.europa.eu/eli/reg/2008/1333/2024-04-23 (current consolidated version: 23/04/2024).

[b] Food categories from Regulation (EC) No 1333/2008 have been matched as closely as possible to FoodEx2 categories, the standardised food classification system included in the EFSA Comprehensive European Food Consumption Database.

[c] Council Directive 2001/112/EC of 20 December 2001 relating to fruit juices and certain similar products intended for human consumption. OJ L 10, 12.1.2002, p. 58-66. Available online: http://data.europa.eu/eli/dir/2001/112/2014-10-05 (current consolidated version: 05/10/2014).

D.2 Exposure Data

Chinova's fibre extracted from white button mushrooms (*A. bisporus*) is intended for use in Australia and New Zealand as a preservative under the same conditions of use as those presently authorised for Chinova's fibre extracted from white button mushrooms (*A. bisporus*) in the U.S. and Canada, as well as those currently under review in the EU and the UK.

Recent exposure estimates have been conducted for the U.S., and the EU/UK populations to support the use of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) as a food additive in those jurisdictions. At the time of submission to Canada, exposure estimates were permitted to be based on the U.S. population and so the exposure assessment calculated for the U.S. GRAS procedure was adapted for the submission of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) to Health Canada. Despite minor differences in food category naming conventions used for each jurisdiction, the proposed food uses and use levels remain consistent. Estimated intakes of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) from its use as a food additive in the U.S., the EU, and the UK are therefore considered representative of the anticipated exposure to Chinova's fibre extracted from white button mushrooms (*A. bisporus*) in Australia and New Zealand, and as such, a separate intake assessment for Chinova's fibre extracted from white button mushrooms (*A. bisporus*) in Australia and New Zealand was not performed for the purposes of this application. In place of a separate exposure estimate for Australia and New Zealand, the recent intake estimates conducted for the U.S., the EU, and the UK are presented in the sections below.

D.2.1 The United States

An assessment of the anticipated intake of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) as an ingredient under the intended conditions of use described in D.1.2 was conducted using data available in the 2017-2018 cycle of the U.S. National Center for Health Statistics National Health and Nutrition Examination Survey (NHANES) (CDC, 2021a,b; USDA, 2021).

A summary of the estimated daily intake of Chinova's fibre extracted from white button mushrooms from all proposed food uses in the U.S. population groups is provided in Table D.2.1-1 on an absolute basis (g/person/day) and on a body weight basis (mg/kg body weight/day). The percentage of consumers was high among all age groups evaluated in the current intake assessment; more than 96.7% of the population groups consisted of consumers of food products in which Chinova's fibre extracted from white button mushrooms is currently proposed for use. Children ages 6 to 11 years had the greatest proportion of consumers, at 99.3%. The consumer-only estimates are more relevant to risk assessments, as they represent exposures in the target population; consequently, only the consumer-only intake results are discussed in detail herein.

Among the total population (ages 2 years and older), the mean and 90th percentile consumer-only intakes of Chinova's fibre extracted from white button mushrooms were determined to be 0.12 and 0.25 g/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest mean and 90th percentile consumer-only intakes of Chinova's fibre extracted from white button mushrooms on an absolute basis, at 0.15 and 0.30 g/person/day, respectively, while children (ages 2 to 5 years) had the lowest mean and 90th percentile consumer-only intakes, at 0.07 and 0.13 g/person/day, respectively.

On a body weight basis, the total population (ages 2 years and older) mean and 90th percentile consumer-only intakes of Chinova's fibre extracted from white button mushrooms were determined to be 1.9 and 3.8 mg/kg body weight/day, respectively. Among the individual population groups, children (ages 2 to 5 years) were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, at 4.1 and 7.3 mg/kg body weight/day, respectively. Female teenagers had the lowest mean and 90th percentile consumer-only intakes, at 1.5 and 2.6 mg/kg body weight/day, respectively.

Table D.2.1-1 Summary of the Estimated Daily Intake of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) from Proposed Food Uses at the Maximum Use Level in the United States by Population Group (2017-2018 NHANES Data)

Population Group	Age Group (Years)	Consumer-only Intake					
		Percentage of Population (%)	n	Absolute Basis (g/day)		Body Weight Basis (mg/kg bw/day)	
				Mean	90th Percentile	Mean	90th Percentile
Children	2 to 5	96.7	446	0.07	0.13	4.1	7.3
Children	6 to 11	99.3	675	0.09	0.16	2.7	5.3
Female teenagers	12 to 19	98.4	439	0.09	0.17	1.5	2.6
Male teenagers	12 to 19	96.8	426	0.11	0.21	1.7	3.4
Female adults	20 and older	98.5	2,114	0.12	0.23	1.6	3.2
Male adults	20 and older	98.6	1,943	0.15	0.30	1.7	3.5
Total population	2 and older	98.4	6,043	0.12	0.25	1.9	3.8

bw = body weight; n = sample size; NHANES = National Health and Nutrition Examination Survey.

D.2.2 The European Union/United Kingdom

Estimates for the intake of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) in the EU and the UK were assessed using a tiered approach, based on the proposed and authorised food uses and use levels in combination with food consumption data from the European Food Safety Authority (EFSA) Comprehensive European Food Consumption Database (hereafter referred to as the "EFSA Comprehensive Database") using the following resources (EFSA, 2022a):

- The Food Additives Intake Model (FAIM) 2.1 tool, which estimates chronic dietary exposure to additives using the food categories in Part E of Annex II to Regulation (EC) No 1333/2008[18] on food additives (EFSA, 2022b); and
- The DietEx tool, which estimates chronic dietary exposure using the more refined FoodEx food classification (EFSA, 2022c).

D.2.2.1 FAIM 2.1 Tool

Table D.2.2.1-1 summarises the estimated exposure to Chinova's fibre extracted from white button mushrooms (*A. bisporus*) from the proposed food uses in all population groups.

[18] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16–33. Available online: https://eur-lex.europa.eu/eli/reg/2008/1333/oj (current consolidated version: 23/04/2024).

On a body weight basis, toddlers had the highest mean intakes, ranging from 8 to 27 mg/kg body weight/day, while infants had the highest 95th percentile intakes of the ingredient, ranging from 3 to 75 mg/kg body weight/day. On an absolute basis (using default body weights), elderly had the highest mean intakes of the ingredient, ranging from 242 to 627 mg/person/day, while adolescents had the highest 95th percentile intakes, ranging from 348 to 1,598 mg/person/day.

Table D.2.2.1-1 Estimated Daily Intakes of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) from the Proposed Food Uses Based on the FAIM 2.1 Tool for Different Population Groups in the European Union

Population Group	Age Group	Intakes of Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) (mg/kg bw/day)		Intakes of Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) (mg/person/day)[a]	
		Mean Range	P95 Range	Mean Range	P95 Range
Infants	≤11 months	1 to 21	3 to 75	3 to 107	13 to 373
Toddlers	12 to 35 months	8 to 27	18 to 74	92 to 321	220 to 891
Children	3 to 9 years	8 to 22	15 to 60	180 to 517	352 to 1,376
Adolescents	10 to 17 years	3 to 11	7 to 31	156 to 572	348 to 1,598
Adults	18 to 64 years	4 to 8	8 to 21	269 to 593	533 to 1,496
Elderly and very elderly	≥65 years	3 to 9	6 to 16	242 to 627	439 to 1,214

bw = body weight; EFSA = European Food Safety Authority; FAIM = Food Additives Intake Model; P95 = 95th percentile.
[a] Absolute intakes calculated by multiplying body weight intakes by EFSA default (or mean) body weights for respective age categories (EFSA, 2012); *i.e.*, infants = 5 kg; toddlers = 12 kg; other children = 23.1 kg; adolescents = 52.35 kg; adults = 70 kg; elderly and very elderly = 73.6 kg.

D.2.2.2 DietEx Tool

Estimated mean and high-level intakes of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) in EU Member States from its proposed conditions of use, determined using the DietEx tool, are presented in Table D.2.2.2-1. Results are provided as a range based on the estimates determined from individual surveys, for each population group, on both an absolute (mg/person/day) and per kilogram body weight basis (mg/kg body weight/day). As mentioned, it is not possible to directly compare the intake results across countries due to the different methodologies used in separate surveys; however, some overall observations can be made on the range of estimated intakes.

When expressed on a body weight basis, the highest mean intakes of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) were observed in toddlers, with intakes of up to 34 mg/kg body weight/day. The highest 95th percentile intakes were observed in infants, with intakes ranging from 4 to 74 mg/kg body weight/day. Higher intakes by the younger age groups are expected based on the relatively higher intake of foods and beverages on a body weight basis when compared to the rest of the population (EFSA, 2011b). In the remaining population groups (from other children to the very elderly and infants), mean intakes were up to 25 mg/kg body weight/day, while high-level intakes were up to 44 mg/kg body weight/day.

The highest intakes of Chinova's fibre extracted from white button mushrooms (*A. bisporus*) on an absolute basis were calculated in adults, with mean intakes ranging from 256 to 696 mg/person/day and high-level intakes ranging from 482 to 1,197 mg/person/day. The lowest mean and high-level intakes were calculated in infants at up to 157 and 569 mg/person/day, respectively.

Table D.2.2.2-1 Estimated Daily Intake of Chinova's Fibre Extracted from White Button Mushrooms (*Agaricus bisporus*) from the Proposed Uses Based on the EFSA Comprehensive Database (DietEx Tool) in Different Population Groups

Population Group	No. of Surveys[a]	Intakes of Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) (mg/kg bw/day)		Intakes of Chinova's Fibre Extracted from White Button Mushrooms (*A. bisporus*) (mg/person/day)	
		Mean Range	High-level Range[b]	Mean Range	High-level Range[b]
Infants (≤11 months)	12 (11)	1 to 19	4 to 74	7 to 157	31 to 569
Toddlers (12 to 35 months)	15 (13)	7 to 34	20 to 59	69 to 458	212 to 666
Other children (3 to 9 years)	19 (19)	9 to 25	18 to 44	210 to 443	406 to 835
Adolescents (10 to 17 years)	21 (20)	5 to 11	10 to 21	270 to 592	520 to 1,194
Adults (18 to 64 years)	22 (22)	4 to 9	7 to 16	256 to 696	482 to 1,197
Pregnant and lactating women	8 (8)	4 to 7	7 to 14	258 to 463	491 to 863
Elderly (65 to 74 years)	19 (19)	3 to 8	6 to 14	216 to 615	412 to 1,051
Very elderly (≥75 years)	14 (10)	3 to 8	6 to 14	213 to 601	416 to 988

bw = body weight; EFSA = European Food Safety Authority; No. = number.

[a] Number of surveys in which individuals within the population group were identified to be consumers of the food groups of interest. Number of surveys with a statistically reliable number of consumers (used to calculate high-level intakes) are presented in parentheses.

[b] Results that were not statistically reliable are not presented (n<60).

The intakes estimated using the DietEx tool were subtly lower than those estimated using the FAIM 2.1 tool, with larger differences observed from children through to the elderly. For example, in the FAIM 2.1 tool, the estimated high-level intakes in infants ranged from 3 to 75 mg/kg body weight/day, compared to 4 to 74 mg/kg body weight/day using the DietEx tool; however, the estimated high-level intakes in adolescents ranged from 348 to 1,598 mg/person/day using the FAIM 2.1 tool, compared to 520 to 1,194 mg/person/day using the DietEx tool. This is due to the broader food categories in the FAIM 2.1 tool compared to the more refined FoodEx classification in the DietEx tool. The rationale for the observed differences in infants' intakes between both models can be reasoned since FAIM Category 1.2 "Unflavoured fermented milk products, including natural unflavoured buttermilk (excluding sterilised buttermilk) non-heat-treated after fermentation" was a significant contributor to infant intakes within the FAIM 2.1 model. It can be assumed that all the FoodEx2 codes that would be applicable to FAIM Category 1.2 would heavily contribute towards intakes within the DietEx tool. For infants, the highest contributor in the DietEx tool was "Yoghurt," which is likely to have significant coverage for all food consumption data that relate to the FAIM Category 1.2; therefore, subtle differences between intakes models would be expected.

As the proposed uses for Chinova's fibre extracted from white button mushrooms (*A. bisporus*) are specific and align better with the FoodEx2 classifications, the refined results using the DietEx tool are considered a more realistic estimation of intakes in the EU population and should therefore be used for the risk assessment. These refined intakes are still considered conservative, as they assume that each food category contains the maximum proposed use levels, whereas in reality the levels added to foods and beverage will vary depending on the manufacturing process. Furthermore, it is unlikely that Chinova's fibre extracted from white button mushrooms (*A. bisporus*) will have 100% market penetration in all categories. Therefore, in accordance with DietEx tool, on a body weight basis, intakes were highest in infants and toddlers, 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 95th percentile, 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 95th percentile.

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