FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

3 July 2025

347-25

Supporting document

Risk and technical assessment – Application A1315

Chitosan and (1,3)-β-glucans from white button mushrooms (*Agaricus bisporus*) as a food additive

Executive summary

Chinova Bioworks Inc. has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of a mixture of chitosan and (1,3)-β-D-glucans (mushroom chitosan) extracted from *Agaricus bisporus* as a preservative in food and beverage products. Chitosan from various sources is permitted as a processing aid in the Code. The applicant stated that mushroom chitosan is already permitted in the United States, Canada, Japan and Korea.

The use of mushroom chitosan at Good Manufacturing Process (GMP) levels as a food preservative is consistent with its typical function as an antimicrobial substance. It is, therefore, functioning as a food additive (preservative) for the purposes of the Code.

There are no relevant identity and purity specifications in the Code, therefore a new specification for mushroom chitosan will be included in Schedule 3 of the Code.

The production organism has a long history of safe human consumption. FSANZ's microbiological risk assessment has not identified any public health and safety concerns. *A. bisporus* has been determined to be neither pathogenic nor toxigenic.

The systemic absorption of chitosan in the stomach and small intestine varies depending on the molecular weight, viscosity and the degree of deacetylation. Following consumption in the diet chitosan is expected to remain intact in the upper gastrointestinal tract and be subject to fermentation by the microbiota in the large intestine.

No adverse effects of chitosan, chitosan oligomers or chitosan monomers were observed in acute, subchronic or chronic studies in rodents. No relevant reproductive or developmental toxicity studies were identified; however, systemic absorption of chitosan is negligible, suggesting that effects on reproduction or development are unlikely. No adverse effects of chitosan supplements were reported in human trials. There is no evidence of genotoxicity, and allergic reactions to oral exposure are very rare. Foods containing β-glucans have a long history of safe human use, and no experimental evidence or case reports of adverse effects of β-glucans have been identified.

Using a budget method approach and considering the worst-case scenario, the dietary exposure assessment calculated the theoretical maximum daily intake of food additives from foods and beverages to be 46.9 mg/kg bw/day. In the absence of an identified hazard for chitosan or β-glucans, an Acceptable Daily Intake (ADI) ‘not specified’ was established.

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1. Introduction

Chinova Bioworks Inc. (Chinova) is a Canadian manufacturer of chitosan from *Agaricus bisporus* for use as a preservative. The company was founded in 2016.

A. bisporus is one of the most common and widely cultivated species of edible mushrooms. It includes several varieties, with the most familiar being white button mushrooms (champignon, in its immature form), brown button mushrooms (in a more mature form), and portobello mushrooms (fully mature).

Chinova's application seeks to amend the Australia New Zealand Food Standards Code (the Code) to permit the use of a mixture of chitosan and (1,3)-β-D-glucans (i.e. mushroom chitosan) extracted from white button mushrooms (*Agaricus bisporus*) as a preservative in food and beverage products. In this document, this mixture is referred to as mushroom chitosan.

Mushroom chitosan extracted from *A. bisporus* would be used in a wide range of foods if approved. The usage level is at the minimum level required to achieve the desired effect, in accordance with the principles of Good Manufacturing Process (GMP). The applicant stated that the maximum levels range from 0.01 to 0.150 g/100 g (equivalent to 100 to 1,500 ppm).

Chitosan is derived from chitin, a carbohydrate polymer synthesised by various arthropods, molluscs, and fungi. The applicant has provided data showing that their chitosan is compositionally very similar to chitosan derived from crustaceans.

β-Glucans are polysaccharides of glucose molecules. Foods containing β–glucans, such as oats, barley, edible fungi, seaweeds and brewer's yeast (*Saccharomyces cerevisiae*) (Lante et al. 2023) have long histories of safe use. Over time, chitosan will naturally degrade to D-glucosamine and N-acetyl-D-glucosamine, while β-1,3-D-glucans gradually degrade to glucose.

1.1. Objectives of the assessment

The objectives of this risk and technical assessment were to:

- determine if mushroom chitosan extracted from *A. bisporus* performs the technological purpose of a preservative at GMP levels
- determine if mushroom chitosan achieves its technological function as an antimicrobial agent at GMP levels
- evaluate potential public health and safety concerns that may arise from the use of mushroom chitosan, chitosan, and (1,3)-β-glucans at GMP levels.

2. Food technology and antimicrobial activity assessment

2.1. Chemical names, identification and structure

2.1.1. Background

Mushroom chitosan is a mixture of chitosan and (1,3)-β-glucans. These two components form a natural co-polymer known as the *chitin-glucan complex* (CGC).

The starting material for preparing the CGC is *A. bisporus* only. The applicant uses the stem of *A. bisporus* that remains after removing the fruiting body grown for human consumption.

2.1.2. Chitosan

Chitosan is a linear chain of randomly distributed β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine (Figure 1). It is produced by deacetylating chitin (Aranaz *et al.* 2021, Chen *et al.* 2022, Velásquez 2023). Chitin is the second most abundant polysaccharide in nature (after cellulose) and is found in the exoskeletons of crustaceans, insects, and fungi. The main characteristics of chitosan are listed in Table 1.

Data included in the application demonstrate that the mushroom-derived chitosan moiety is chemically equivalent to crustacean-derived chitosan and to a chitosan reference standard described in the United States Pharmacopoeia (2024) monograph of chitosan.

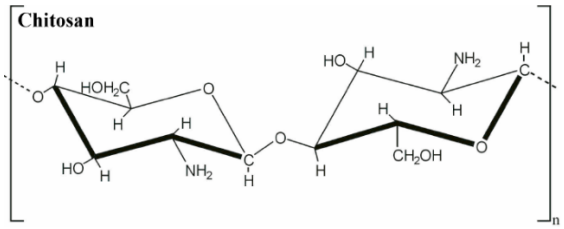

Figure 1. Structural formula of Chitosan (https://www.researchgate.net/figure/Chemical-structure-of-chitosan_fig4_355787682)

Table 1. Characteristics of Chitosan

Parameter	
Systematic name:	2-Amino-2-deoxy-β-D-glucopyranosyl-(1->4)-2-amino-2-deoxy-β-D-glucopyranosyl-(1->4)-2-amino-2-deoxy-β-D-glucopyranosyl-(1->4)-2-amino-2-deoxy-β-D-glucopyranosyl-(1->4)-2-amino-2-deoxy-β -D-glucopyranosyl-(1->4)-2-amino-2-deoxy-β-D-glucopyranosyl-(1->4)-2-deoxy-2-[(methoxycarbonyl)amino]-β-D-glucopyranosyl-(1->4)-2-amino-2-deoxy-β-D-glucopyranosyl-(1->4)-2-amino-2-deoxy-β- D-glucopyranose
Other names/common names	Poliglusam; Deacetylchitin; Poly-(D)glucosamine; BC; Chitopearl; Chitopharm; Flonac; Kytex, Quitosano
Molecular formula	$C_{56}H_{103}N_9O_{39}$
[1]Molecular weight*	1526.5 g/moβl
CAS number	9012-76-4

Sources: CAS Common Chemistry, Chitosan https://commonchemistry.cas.org/detail?cas_rn=9012-76-4, Luckachan and Pillai 2006, da Trindade Neto, et al. 2005, https://pubchem.ncbi.nlm.nih.gov/compound/71853

*Computed by PubChem 2.2 (PubChem release 2021.10.14)

2.1.3. (1,3)-β-glucans

(1,3)-β-Glucans are a polysaccharide of glucose molecules linked primarily by β-1,3-glycosidic bonds (Figure 2). In fungi and yeast (1,3)-β-Glucan forms a crucial part of the cell

wall, providing structural integrity and rigidity. It is found in smaller amounts in the bran of oats and barley. Some marine algae produce β-glucans with varying branching patterns (Gidley and Nishinari 2009, Barsanti *et al.* 2011).

The main chain consists of glucose units connected by β-1,3-glycosidic bonds. In some cases, there may be additional β-1,6-glycosidic linkages branching off from the β-1,3 backbone, especially in fungi (Ruiz-Herrera and Ortiz-Castellanos 2019). The main characteristics of (1,3)-β-glucans are listed in Table 2.

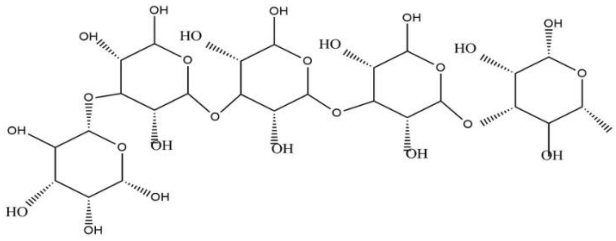

Figure 2. Structural formula of (1,3)-β-Glucans

Table 2. Characteristics (1,3)-β-glucans

Parameter	
Systematic name:	(2*S*,3*R*,4*S*,5*S*,6*R*)-2-[(2*R*,4*R*,5*R*,6*S*)-4,5-dihydroxy-2-(hydroxymethyl)-6-[(2*R*,4*R*,5*R*,6*S*)-4,5,6-trihydroxy-2-(hydroxymethyl)oxan-3-yl]oxyoxan-3-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol
Other names/common names	β-1,3-Glucan, β-D-Glucan, (1→3),(1→3)-β-D-Glucan, Polysaccharide 13140, TAK
Molecular formula	$C_{18}H_{32}O_{16}$
[1]Molecular weight*	504.4 g/mol
CAS number	9051-97-2

Source: PubChem: Beta-Glucan | C18H32O16 | CID 439262 - PubChem

*Computed by PubChem 2.2 (PubChem release 2024.11.20)

2.1.4. Mushroom chitosan

Mushroom chitosan is the chitin-glucan polysaccharide polymer complex found in the cell wall of most fungi and yeasts, including, *A. bisporus*, *Aspergillus niger* and *Saccharomyces cerevisiae* (Feofilova et al. 2006, Skorik et al. 2010, Beran, Holan, & Baldrián, 1972, Chakravarty 2011, Singh 2020).

While the composition and characteristics of chitosan extracted from different mushroom species can vary due to factors such as species type, growth conditions, and extraction method, the composition of mushroom chitosan is similar across fungal sources (Synowiecki, & Al-Khateeb.2003, Younes& Rinaudo, 2015, Pochanavanich & Suntornsuk, 2002, RekhaR & Sharma, 2009).

However, we note that this Application is concerned with mushroom chitosan extracted from *A. bisporus*. The species *A. bisporus*, the source of mushroom chitosan in this application, includes several varieties, with the most familiar being white button mushrooms

[1] PubChem typically calculates the molecular weight of a polymer by multiplying the molecular weight of the monomer unit by the degree of polymerization (chain length). If there is a distribution of polymer chain lengths, PubChem may use more advanced techniques to calculate the number average and weight average molecular weights.

(champignon, in its immature form), brown button mushrooms (in a more mature form), and portobello mushrooms (fully mature).

Mushroom chitosan is a natural co-polymer and comprises a chitin moiety (N-acetyl-d-glucosamine units) covalently linked to a β-glucans moiety (glucose units).

Mushroom chitosan combines the properties of chitin and β-glucans, both possessing antibacterial properties (Jayakumar et al., 2011; Lehtovaara & Gu, 2011).

Mushroom chitosan is used for various applications, such as a food additive (Agostoni *et al.*2010) or for wine clarification (Bornet and Teissedre 2008, Cosme and Vilela 2021).

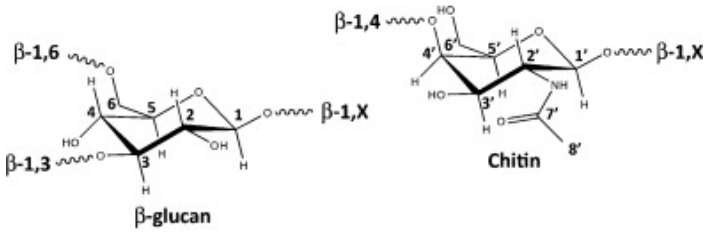

Figure 3. Mushroom chitosan: Chitin-glucan polysaccharide polymer complex

2.2. Manufacturing process

The applicant's information demonstrated that mushroom chitosan is produced from *A. bisporus* using an industrial process following current Good Manufacturing Practice (cGMP) for Food and the principles of Hazard Analysis and Critical Control Point (HACCP). The ingredient is extracted from the raw material using a deacetylation process followed by purification processes.

The applicant provided evidence that the materials used to manufacture mushroom chitosan are of food-grade[2] quality and meet predefined quality standards.

The application provided details on the manufacturing process, raw materials, and ingredients used in producing the applicant's mushroom chitosan preparation, some of which were Commercially Confidential information CCI.

2.3. Purity

The applicant is requesting permission for a new food additive.

Food additives in the Code must meet the identity and purity specifications set out in Schedule 3. Schedule 3 incorporates by reference the specifications listed in a variety of sources, including the Food Chemicals Codex (FCC) 13th edition (United States Pharmacopeial Convention 2022) and the International Oenological CODEX (2022a)[3].

Identity and purity specifications for chitosan (in the FCC) are incorporated by reference in Schedule 3 of the Code. However, the FCC monograph for chitosan is for chitosan obtained from crustacean and not fungal sources. There are no available references for fungal (1,3)-β-glucans from the FCC or other primary sources within Schedule 3.

[2] A "food grade" material is one that has been manufactured and tested to ensure it does not contain harmful chemicals or contaminants that could pose a risk to human health when it comes into contact with food.

[3] Federal Register of Legislation - Australia New Zealand Food Standards Code – Schedule 3 – Identity and purity

The International Oenological Codex (IOC) has a monograph applicable to chitosan sourced from *A. bisporus* (IOC 2022b) and on chitin-glucan from the cellular walls of *Aspergillus niger* (IOC 2022c).

Table 3. Specifications for Chinova's mushroom chitosan compared to FCC and FSC specifications for chitosan

Parameter	**Applicant's Specification**	**FCC (2023)**	**Code**	**Method of Analysis**
Identification	Positive	Conforms		[4]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 ([8]kDa)	10 to 400	-		[5]HPLC
Chitosan (%, w/w)	≥95	-		[1]H-NMR (Internal)
β-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	≤1	[7]ISO 11885 ([6]ICP-OES)
Lead (mg/kg)	≤0.2	≤0.5	≤2	ISO 11885
Cadmium (mg/kg)	≤0.2	≤0.2	≤1	ISO 11885
Mercury (mg/kg)	≤0.2	≤0.2	≤1	ISO 11885
Metals				
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 ([3]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	-		[2]AOAC 2013.01

[1]H-NMR = proton nuclear magnetic resonance; [2]AOAC = Association of Official Analytical Collaboration; [3]CFU = colony-forming units; [4]FTIR = Fourier-transform infrared spectroscopy; [5]HPLC = high-performance liquid chromatography; [6]ICP-OES = inductively coupled plasma–optical emission spectrometry; [7]ISO = International Organization for Standardization; [8]kDa = kilodaltons.

The applicant has established food-grade chemical and microbiological specifications for Chinova's mushroom chitosan that are generally consistent with the FCC specification (Table 3). However, the FCC monograph is only relevant to crustacean chitosan, not mushroom chitosan.

Preparations (such as mushroom chitosan) may be contaminated during production, even if they have antimicrobial properties. Microbiological specifications provide some evidence that GMPs that limit contamination are in place and provide helpful information to food manufacturers. However, because these specifications are not safety criteria, they would not be included in any identity and purity specifications set out in Schedule 3.

Specifications proposed for mushroom chitosan based on the identification and purity data reviewed are:

- chemical structure— a natural co-polymer and comprises a chitin moiety (N-acetyl-d-glucosamine units) covalently linked to a β-glucans moiety (glucose units)
- description—white to beige, odourless and flavourless powder, almost completely insoluble in aqueous or organic medium
- Molecular weight average—10 to 400 kDa
- degree of deacetylation —70.0 to 95.0 mol%
- water—less than 5.0%
- total chitosan content on a dry weight basis—no less than 95.0%
- total β-glucan content on a dry weight basis — no more than 5.0%.

In addition, Schedule 3 of the Code (Subsection S3—4) specifies that mushroom chitosan must not contain on a dry weight basis more than:

- 2 mg/kg of lead
- 1 mg/kg of arsenic
- 1 mg/kg of cadmium
- 1 mg/kg of mercury.

The levels of iron, chromium and nickel are not of toxicological concern and therefore have not been included in the proposed specifications

2.4. Technological purpose

Mushroom chitosan extracted from *A. bisporus* is intended for use as an antimicrobial preservative in food and beverages at levels consistent with GMP.

Chitosan has been shown to exhibit antimicrobial properties against various microbes (Raafat *et al*. 2008; Goy *et al*. 2009). Antibacterial action may occur through ionic interactions between the charged groups in the chitosan polymer backbone and the negatively charged components of bacterial cell walls (Goy *et al*. 2016). These interactions may lead to hydrolytic damage to the bacterial cell wall, resulting in leakage of intracellular electrolytes and ultimately leading to cell death. Other proposed mechanisms include forming a protective coating around bacterial cells and interference with nutrient absorption or mineral displacement.

The bactericidal effectiveness of chitosan improves with the degree of deacetylation above 70% (Li *et al.* 2016, Omura *et al.* 2003). The applicant reports that their mushroom chitosan has a degree of deacetylation of 90%. Liu *et al.* (2006) demonstrated the antibacterial properties of chitosan from crustacean sources. At 200 mg/kg concentrations, molecular weights (MWs) ranging from 55 to 155 kDa were effective. However, at 50 to 100 mg/kg concentrations, higher MW chitosan of 96 to 155 kDa had less antibacterial action. Mushroom chitosan MWs are 10 to 400 KDa (Table 3)

The applicant provided sufficient information (deemed as CCI) on the physical and chemical properties of mushroom chitosan from *A. bisporus* to demonstrate stability when used as a

preservative. The applicant also provided sufficient information demonstrating that the food additive can be incorporated homogeneously into different food matrices.

2.5. Methods of analysis

The IOC certified a range of tests in a monograph applicable to mushroom chitosan, including establishing identity, the degree of acetylation, the presence of various metals and microbiological control (IOC 2022a). IOC also has published methods on chitin-glucan from the cellular walls of *Aspergillus niger* (IOC 2022b) that include the determination of the chitin-glucan ratio, the presence of various metals and microbiological control.

The IOC method is relevant to Chinova's mushroom chitosan because fungal chitosan is similar across species. However, the applicant has also developed an assay to detect mushroom chitosan in foods and beverages. The assay follows GLP.

2.6. Antimicrobial activity

2.6.1. Mode of action and minimum inhibitory concentrations

The applicant seeks permission to use a mixture of chitosan (≥95%) and b-1,3-D-glucans (≤5%) derived from *A. bisporus* as a preservative in food and beverages. The antimicrobial activity of Chinova's mushroom chitosan was evaluated in various beverage products, including carbonated soda, apple juice, and liquid-sugar syrup, baked goods, and dairy-based yoghurt and cream cheese.

Mushroom chitosan is proposed for use in food and beverages to prevent the growth of microorganisms and subsequent spoilage. A range of microorganisms, notably yeasts and moulds, can cause spoilage. Spoilage yeasts include species from the Saccharomyces, Zygosaccharomyces, Candida and Brettanomyces genera. Typical spoilage moulds in include the genera Aspergillus, Cladosporium, and Penicillium. Bacteria, such as Bacillus cereus or certain lactic acid bacteria (such as Lactobacillus) are also known to cause spoilage

Chitosan is a versatile amino polysaccharide biopolymer that exhibits broad-spectrum antimicrobial activity against various spoilage and pathogenic microorganisms, including gram-negative and gram-positive bacteria, as well as yeast and mould (Aranaz et al. 2014; Goy et al. 2009; Goy et al. 2016; Nasaj et al. 2024; Raafat et al. 2008; Yan et al. 2021).

Though the exact mechanism of action has not been established, chitosan exhibits antimicrobial activity through at least four proposed mechanisms, which likely vary between different microbe targets. Firstly, the positively charged amino (NH_{3+}) groups in the chitosan polymer backbone interact with the negatively charged components of microbial cell membranes, leading to increased permeability, leakage of intracellular contents and eventual cell lysis (Goy et al. 2016; Raafat et al. 2008; Yan et al. 2021). Secondly, chitosan causes depolarisation of the microbial cell membrane, which disrupts essential cellular processes (Raafat et al. 2008). Thirdly, chitosan can penetrate microbial cells and bind to DNA, inhibiting RNA and protein synthesis, which hampers microbial growth (Goy et al., 2009; Yan et al. 2021). Finally, chitosan's chelating properties enable it to bind essential metal ions and nutrients, depriving microorganisms of necessary resources and inhibiting their growth (Raafat and Sahl 2009; Yan et al. 2021). These combined actions result in the inhibition of microbial growth and eventual cell death.

The antimicrobial efficacy of chitosan is influenced by factors such as its molecular weight, degree of deacetylation, temperature, salinity and pH of the environment, as well as the type of microorganism (Aranaz et al. 2014; Nasaj et al. 2024). For instance, lower molecular weight chitosan can more easily penetrate cell membranes, enhancing its antimicrobial effect (Ashrafizadeh et al. 2022; Liu et al. 2006). Additionally, Li et al. (2016) and Omuru et al. (2003) demonstrated increased degrees of deacetylation had higher bactericidal properties

against common pathogenic organisms *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa.*

2.6.2. Review of challenge studies

Data provided by the applicant concerned challenge studies for the *A. bisporus* chitosan fibre extract in commercially produced food and beverages. Challenge studies were performed using defined mixtures of yeasts (*Brettanomyces bruxellensis*, *Candida inconspicua*, *Pichia anomala*, *Saccharomyces cerevisiae, S. cerevisiae* subsp*. Diastaticus, Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, and *Yarrowia lipolytica*), yeast-like fungus (*Geotrichum candidum*), moulds (*Aspergilus niger*, *Cladosporium cladosporioides, Penicillium aurantiogriseum, Penicillium crustosum*, and *Penicillium roqueforti*) or bacteria (*Lactobacillus brevis* and *Lactobacillus plantarum*).

Chitosan fibre extract was added to non-preserved food and beverage samples at concentrations between 50 and 2,000 mg/kg. For each food and beverage tested, a negative control with no chitosan fibre extract and a positive control with an appropriate commonly used preservative were also included.

Liquid product test samples were spiked with an initial concentration of 3 Log colony-forming units (CFU)/mL of *S. cerevisiae, L. brevis* and *L. plantarum*, *Z. bailii*, or *A. niger* then sealed and stored in a 30°C incubator for 42 days. Chinova's mushroom chitosan was added at a concentration of 0, 50, 100, 200, 400, 600 or 800 mg/kg. Samples were then assayed every 7 days by standard dilution and plate count. For liquid products, a 3 Log reduction was seen and maintained with 400 mg/kg of mushroom chitosan, which was similar in effectiveness to the positive control (1,500 mg/kg sorbate/benzoate).

Baked good samples were not spiked but were incubated in a sealed plastic bag at 25°C for 30 days. Chinova's mushroom chitosan was added at a concentration of 0, 400, 600, 1,000, 1,200 or 1,600 mg/kg. 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. For baked good products, a similar effect compared to the preservative control, 0.3% calcium propionate of 25 days without mould presence was observed with 1,000 mg/kg mushroom chitosan.

Dairy samples were spiked with an initial concentration of 3 Log CFU/mL *C. cladosporioides*, *P. aurantiogriseum*, *P. crustosum, P. roqueforti, G. candidum, Y. lipolytica or A. niger* then sealed and stored in a 7°C incubator for 42 days. Chinova's mushroom chitosan was added at a concentration of 400, 600, 800, 200, 1,000, 1,200 or 1,600 mg/kg. Samples were then assayed every 7 days by standard dilution and plate count. For dairy products the use of mushroom chitosan at 1,000 mg/kg caused a 0.5-2 Log reduction after 7 days and a 3 Log reduction over 42 days. Whereas microbial growth increased over time in the negative control samples. No preservative control was used with the dairy products.

Natural and processed cheese samples were incubated with *P. roqueforti*, *G. candidum, Y. lipolytica* or *A. niger* and stored in sealed plastic bags at 7°C for up to 42 days. Chinova's mushroom chitosan was added at a concentration of 1,000 or 1,500 mg/kg. Samples were inspected for visible mould growth every 7 days. The percent mould growth was calculated based on the total surface area. No preservative positive control was used with these cheese products. Compared to the negative control, the effective concentration of mushroom chitosan to limit each of the tested microbes' growth was 1,500 mg/kg. For natural cheese, 1,500 mg/kg of mushroom chitosan prevented growth for each microbe for 42 days, while the negative control saw growth of 10-25% coverage after 35 days for *P. roqueforti, G. candidum,* and *A. niger*. *Y. lipolytica* grew to a coverage level of <10% of the natural cheese after 42 days for the negative control.

For processed cheese 1,500 mg/kg of mushroom chitosan prevented growth of *P. roqueforti, Y. lipolytica,* and *A. niger* for at least 28 days with <10% coverage at 42 days. 1,500 mg/kg of mushroom chitosan prevented *G. candidum* growth for 21 days with <10% coverage at 42 days. The negative control for the processed cheese saw microbe growth at 14 days for *G. candidum,* 21 days for *P. roqueforti*, and 28 days for *Y. lipolytica,* and *A. niger*. The negative controls had growth of at least 25% coverage at 42 days.

Table 4. Minimal inhibitory concentration and effective concentration for Chinova's chitosan extract from A. bisporus in various food and beverages.

Type	**Food/Beverage**	**Minimal Inhibitory Concentration** mg/kg	**Effective Concentration** mg/kg
Liquid Products	Apple Juice	200	400
	Flavoured Water	200	400
	Seltzer (5% Alcohol)	200	400
	Tea (5% Alcohol	200	400
	Wine Spritzer (3.5% Alcohol, Sulphate-free)	200	400
	Soda	200	400
	Energy Drinks	200	400
	Sports Drinks	200	400
	Other Juice	200	400
Baked Good Products	Bread	600	1,000
	Bagel	600	1,000
	English Muffin	600	1,000
	Cake	600	1,000
	Muffin	600	1,000
	Waffle	600	1,000
	Corn Tortilla	600	1,000
	Wheat Tortilla	600	1,000
Dairy	Plain Yoghurt	800	1,000
	Cream Cheese	800	1,000
	Cottage Cheese	800	1,000
	Cheese-Based Sauce	800	1,000
Natural Cheese	Cheddar	1,500	1,500
Processed Cheese	Mozzarella	1,500	1,500
Plant-Based Dairy	Almond-Based Yoghurt	1,000	1,500
	Plant-Based Cream Cheese	1,000	1,500
Sauces, Spreads, Syrups and Condiments	High-Sugar Jam	800	1,000
	High-Fat Coconut Cream Sauce	800	1,000
	Coffee Syrup	800	1,000
	Cocoa Syrup	800	1,000
	Custard	800	800
	Frosting	800	1,000
	Relish	800	800
	Margarine	800	1,000
	Mayonnaise	800	1,000
	Salad Dressing	800	1,000
Plant-Based Meat Analogues	Soy-Based Ground Beef	500	1,500

Plant-based yoghurt samples were spiked with *C. cladosporioides, P. crustosum, C. inconspicua, P. roqueforti,* or *A. niger* at an initial concentration of 2 Log CFU/mL and/or 3 Log CFU/mL and then sealed and stored in a 7°C incubator for 42 days. Chinova's mushroom chitosan was added at concentrations of 500, 1,000, 1,500 or 2,000 mg/kg. Samples were assayed every 7 days by standard dilution and plate count. For plant-based yoghurt, a 3 Log reduction in microbe concentration was seen with 1,500 mg/kg of mushroom chitosan, which was more than similar to the positive preservative control (1.5% cultured dextrose).

Plant-based cream cheese samples were spiked with *G. candidum, P. aurantiogriseum,* or *C. cladosporioides* at an initial concentration of 2 Log CFU/mL or 3 Log CFU/mL and then sealed and stored in a 7°C incubator for 42 days. Chinova's mushroom chitosan was added at a concentration of 1,500 mg/kg. Samples were assayed every 7 days by standard dilution and plate count. Compared to the negative control, the effective concentration of mushroom chitosan to limit each of the tested microbes' growth was 1,500 mg/kg. No preservative positive control was used with these products.

Sauces, spreads, syrups, and condiments samples were spiked with *Z. bailii, Z. rouxii, S. cerevisiae, S. cerevisiae diastaticus, L. brevis, A. niger,* or *P. aurantiogriseum* at an initial concentration of 3 Log CFU/mL, and then sealed and stored in a 25°C incubator for 42 days. Chinova's mushroom chitosan was added at concentrations of 400, 600, 800, 1,000, 1,200 or 1,600 mg/kg. Samples were assayed every 7 days by standard dilution and plate count. For these products, at least a 2 Log reduction in microbe concentration was seen with 1,000 mg/kg of mushroom chitosan, which was similar in effectiveness to the positive control (1,500 mg/kg sorbate/benzoate).

Plant-based meat analogues samples were spiked with *L. brevis, C. cladosporioides,* or *A. niger* at an initial concentration 5 Log CFU/mL, and then sealed and stored in a 25°C incubator for 42 days. Chinova's mushroom chitosan was added at concentrations of 500, 1,000, 1,500, 2,000 or 2,500 mg/kg. Samples were assayed every 7 days by standard dilution and plate count. A 3 Log reduction in microbe concentration was seen with 1,500 mg/kg of mushroom chitosan, similar in effectiveness to the positive control (2% cultured dextrose).

The challenge study results showed that the minimum inhibitory concentration (MIC) and/or effective concentration of Chinova's mushroom chitosan depends on the nature of the food or beverage (Table 4). The lowest effective concentration was 400 mg/kg for liquid products, with the highest effective concentration being 1,500 mg/kg for natural and processed cheese as well as plant-based dairy and meat products.

2.7. Food technology and antimicrobial activity assessment conclusion

FSANZ undertook a food technology and antimicrobial activity assessment to determine if mushroom chitosan achieves its technological purpose in the quantity and form proposed.

FSANZ concluded that:

- Mushroom chitosan is the chitin-glucan complex found in the cell wall of most fungi and yeasts.
- The composition of mushroom chitosan is similar across fungal sources.
- The information on the safety and efficacy of mushroom chitosan as a food preservative in various foods is consistent with its typical function of exhibiting antimicrobial properties against various microbes. Therefore, it functions as a food additive (preservative) for the purposes of the Code.
- Mushroom chitosan may be used as an alternative to existing permitted preservatives and to reduce spoilage from microbes.

- There are no relevant identity and purity specifications in the Code. Therefore, FSANZ proposes to include a specification for mushroom chitosan, i.e. chitosan and (1,3)-β-D-glucans extracted and purified from mushrooms.
    - The submitted data and information from other sources are suitable for assessing the antimicrobial efficacy of mushroom chitosan extracted from *A. bisporus*.
    - Efficacy data demonstrate that mushroom chitosan extracted from *A. bisporus* has broad-spectrum antibacterial and antifungal preservative effects in various representative food matrices against microbes commonly associated with spoilage in these food matrices.
    - The challenge data using defined mixtures of yeasts, moulds, and bacteria highlighted differences in minimum inhibitory concentration and/or effective concentration between food and beverage types.

3. Safety assessment

3.1. Production organism history of use

The most commonly consumed mushrooms worldwide belong to the *A. bisporus* species, which includes button (or white) mushrooms, portobello and cremini mushrooms (Blumfield et al., 2020). In total, *A. bisporus* accounts for 35–45% of total worldwide edible mushroom production (Ramos et al. 2019). The consumption of *A. bisporus* is second only to the baker's yeast *Saccharomyces cerevisiae* as the most commonly ingested fungal species (Ramos et al. 2019).

The *A. bisporus* from which the applicant's mushroom chitosan is extracted is obtained from Canadian mushroom growers and must meet the Canadian guidelines for mushroom cultivation. Therefore, the raw material used is a common edible food and is considered fit for human consumption in Canada. The applicant also performs raw material inspection of *A. bisporus* biomass prior to manufacturing in order to qualify its use in the production of mushroom chitosan.

There is recent evidence for the possibility of mycotoxin accumulation within *A. bisporus* grown on contaminated compost (Varga et al. 2022). However, Canadian guidelines set out that mycotoxins, namely ochratoxin A, cannot be present at levels more than 15 ppb in mushrooms destined for human consumption. Additionally, during the mushroom chitosan's manufacturing process the mushroom biomass is exposed to sodium hydroxide at concentrations high enough to destroy any potential mycotoxin (Moerck et al. 1980). The applicant also provided data for one test confirming mycotoxins were below the limit of detection. Additional testing was not deemed necessary because of the manufacturing process followed and the trusted source of mushroom biomass.

The microbiological risk assessment undertaken by FSANZ has not identified any public health and safety concerns associated with the use of *A. bisporus* in the production of mushroom chitosan to be used as a food additive. *A. bisporus* has been determined to be neither pathogenic nor toxigenic.

3.2. Antimicrobial resistance

Microorganisms developing resistance and cross-resistance to antimicrobials is of increasing concern. Within their GRAS submission, Chinova reported that developing acquired resistance to chitosan by microorganisms is unlikely (GRN 997; U.S. FDA 2023). The basis for this was that the polymer is thought to kill bacteria by interacting with bacterial cell wall proteins through cationic-mediated processes, and acquired resistance was unlikely. There is one reported investigation into the development of chitosan resistance and associated cross-resistance to other antimicrobials in bacteria. Raafat et al. (2017) conducted a serial passage experiment with S. aureus, exposing it to increasing chitosan concentrations over 15 passages (0 - 2,000 mg/kg). A chitosan-resistant variant emerged after six passages, marked by a sharp increase in minimum inhibitory concentration (MIC). The rapid development of stable resistance to chitosan was unexpected and the authors identified differences in bacterial surface properties, metabolic pathways, and regulatory mechanisms of the resistant mutant compared to the non-resistant isolate. While slight increases in the MIC values for antibiotics important for human health were reported for the chitosan resistant mutant, these were not above critical limits (epidemiological cut offs) that would suggest the bacteria had acquired resistance to these antibiotics (EUCAST 2025). Other authors investigating chitosan for various applications have noted that due to its broad-spectrum action, its mode of action being not solely reliant on targeting specific bacterial functions, and absence of widely reported known resistance mechanisms, the development of bacterial resistance is unlikely (Ma et al. 2016; Saito et al. 2019; Yan et al. 2021).

Chinova also demonstrated the stability of its mushroom chitosan to ensure proper antimicrobial function will be maintained up to 12 months after the date of manufacture. Additionally, the mushroom chitosan maintains its antimicrobial and antifungal activity during a product's shelf life when added to a food or beverage.

The antimicrobial action of Chinova's mushroom chitosan was evaluated in various food and beverage products. These included liquid products (e.g. juices, carbonated beverages, sports drinks); baked goods; dairy-based yoghurt and cheeses; sauces, spreads, syrups and condiments; and plant-based analogues. A MIC was determined from the data provided and represents the lowest concentration of mushroom chitosan where an antimicrobial effect was observed. Additionally, an effective concentration was determined, representing where the antimicrobial effect was more than similar to a commonly used preservative control (Table 4). The preservative controls used included 1,500 mg/kg sorbate/benzoate (liquid products and sauces, spreads, syrups and condiments), 0.3% calcium propionate (baked goods), 1.5% cultured dextrose (plant-based dairy) and 2% cultured dextrose (plant-based meat).

3.3. Kinetics and Metabolism

In vertebrates, chitosan is generally degraded by lysozymes and by bacterial enzymes in the colon. Three active chitinases have been identified in humans, of which acidic mammalian chitinase is found in the gastrointestinal tract but has not been investigated regarding its activity in the degradation of chitosan (Kean and Thanou 2010).

The systemic absorption of chitosan varies depending on the molecular weight (Kean and Thanou 2010), viscosity, and the degree of deacetylation (Baldrick 2009). Chitosan with a mean MW of 230 kDa (degree of deacetylation [DDA] 84.9%) had negligible absorption from the gastrointestinal tract of Sprague Dawley rats (Chae et al. 2005). Kean and Thanou (2010) concluded that absorption from the gastrointestinal tract is negligible for chitosan polymers whereas chitosan oligosaccharides may be absorbed to some extent. *In vitro* degradation of chitosan decreases with increasing DDA (Baldrick 2009). However, Wijesekara and Xu (2024) suggest that higher DDA may increase the bioavailability of chitosan *in vivo* by increasing its interaction with the intestinal epithelium. Chitosan is thought to be more soluble in the small intestine than in the acid conditions of the stomach (Wijesekara and Xu 2024). It has been suggested that chitosan forms a gel with dietary fats in the stomach, preventing the absorption of fats from the intestine (Baldrick 2009).

It is noteworthy that the possible hydrolysis products generated by any enzymatic breakdown of chitosan would be chitosan oligomers, glucosamine, N-acetylglucosamine and glucose, all of which are practically nontoxic.

Intact or minimally degraded chitosan that reaches the large intestine may be expected to be fermented by the resident microbiota.

3.4. Gut microbiome effects

As described in 3.3, chitosan is not digested via human digestive enzymes. Following consumption in the diet, chitosan is expected to travel intact throughout the upper gastrointestinal tract to the colon, where the material is fermented by the microbiota in the large intestine (Lattimer and Haub 2010).

Like 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. Vernazza et al. (2005) investigated *in vitro* fermentation of chitosan derivatives by mixed cultures of human gut flora. This work showed that despite the ability of some bifidobacteria and lactobacilli to grow on chitosan oligomers, bifidobacteria were not increased in a mixed culture model system. They also demonstrated that low, medium and high molecular weight chitosans were less able to support growth of bacteria, with a trend for the longer molecules to be less well metabolised (Vernazza et al. 2005).

Recent reviews looking at the role of chitosan in antimicrobial therapy have not detected the development of AMR within bacterial populations exposed to chitosan (Egorov et al. 2023, Hemmingsen et al. 2021; Ma et al. 2016; Saito et al. 2019; Yan et al. 2021).

3.5. Safety of chitosan

3.5.1. Safety of chitosan in animals

3.5.1.1. Acute studies in animals

No toxicity was observed in an unpublished acute toxicity study in female Sprague Dawley rats, cited in GRAS Notice 397, up to a maximum dose of 2000 mg chitosan/kg bw. The chitosan used in the study was derived from *Agaricus bisporus*, but the MW and DDA are not stated.

An acute oral toxicity study of chitosan derived from lobster (*Panurilus argus*) shell was conducted in female Wistar rats and found no toxicity at a maximum dose of 2000 mg/kg bw. The chitosan used in the study had a mean MW of 309 kDa and a DDA of 83% (Lagarto et al. 2015). The authors of the study also cited a 1968 study that reported an acute oral LD_{50} of 16 g chitosan/kg bw in mice.

3.5.1.2. Subchronic studies in animals

Zeng et al. (2008) conducted a 90-day study in Kunming mice with chitosan with a range of different molecular weights, at 1.05% of the diet. These included a high molecular weight chitosan (760 kDa; DDA 85.5%), a low molecular weight chitosan (32.7 kDa; DDA 85.0%), a chito-oligomer (0.9 kDa; DDA 85.7%) and a water-soluble chitosan (39.1 KDa; DDA 52.6%). None of the test articles had any adverse effects. Using JECFA conversion guidelines, 1.05% in the diet would be approximately 1.05 g/kg bw/day, or 1050 mg/kg bw/day.

Twenty-eight-day oral gavage studies of lobster shell chitosan (309 kDa; DDA 83%) and its lactate and acetate salts were conducted in rats by Largato et al. (2015). Chitosan and lactate chitosan were administered daily at doses up to a maximum of 1000 mg/kg bw. The physical properties of the acetate chitosan restricted the highest dose to 700 mg/kg bw/day. Test article-related effects were limited to statistically significant but biologically minimal changes in haematology and clinical chemistry, and no changes were considered to be adverse.

Yao et al. (2010) conducted a four-week dietary study in rats. The chitosan used in the study had a mean MW of 625 kDa and a DDA of 83%. Chitosan was added to low-fat and high-fat diets at 5% (equivalent to approximately 5000 mg/kg bw/day). Supplementation with chitosan was associated with a negative effect on the group mean value for weight gain only in rats fed the high-fat diet but was associated with decreased group mean values for terminal liver weight and liver: bodyweight ratio both in rats on the low-fat diet and rats on the high-fat diet. Plasma concentrations of liver enzymes were not measured, and histological examination of the liver was not performed, so there was insufficient information to determine if the effect on the liver was adverse.

Do et al. (2018) compared the effects of long-chain and short-chain chitosan in a 12-week dietary study in mice that were made obese by a high fat diet. The test articles were added to the diet at a concentration of 1%, equivalent to approximately 1000 mg/kg bw/day. The long chain chitosan had a mean MW of 390 kDa, whereas the short chain chitosan had a mean MW of 210 kDa. The long chain chitosan had a DDA of 82.8%. The DDA of the short chain chitosan was not specified, but it was derived from the long chain chitosan. The chitosan supplements were associated with lower group mean body weights than those of control mice on the high fat diet, but this was regarded as a beneficial rather than an adverse effect since the mice were obese. No adverse effects were reported.

Chiang et al. (2020) conducted a four-week dietary study of chitosan in male rats to study its effects on plasma lipids and markers of lipid peroxidation. Two different test articles were

prepared from shrimp shells. Both had a DDA of approximately 90%, but one had a MW of 480 kDa, and the other had a MW of 340 kDa. The test articles comprised 5% of the diet, equivalent to approximately 5000 mg/kg bw/day. This study was not designed as a toxicity study, but no adverse effects on survival or terminal body weight were observed. Dietary chitosan was associated with significantly lower plasma total cholesterol, VDL-cholesterol and VLDL-cholesterol, and lower hepatic concentrations of total lipid and of cholesterol, compared to those of controls.

An eight-week dietary study was conducted in male rats, including groups fed high MW chitosan (740 kDa; DDA 91%), low MW chitosan (80 kDa; DDA 83.9%) and chitosan oligosaccharides (0.7 kDa; 100%), all at 5% of the diet (approximately 5000 mg/kg bw/day) (Chiu et al. 2020). The study was not designed as a toxicity study and did not measure the usual toxicity endpoints. Still, no adverse effects of feeding high MW or low MW chitosan were reported.

Further details of these subchronic studies are provided in Appendix 1.

3.5.1.3. Chronic studies in animals

The National Toxicology Program (NTP) conducted a six-month dietary study of chitosan in Sprague Dawley rats. Dietary concentrations of chitosan (MW 94 kDa; DDA 86.5%) ranged up to a maximum of 9%, which was equivalent to a group mean intake of 5200 mg/kg bw/day in male rats and 6000 mg/kg bw/day in female rats. The study was conducted to determine if chitosan consumption is a cause of osteoporosis. No evidence of effects related to osteoporosis was found, but the study may have been of inadequate duration to detect such effects. All rats survived to the end of the study and there were no adverse clinical observations in the core study group (NTP 2017).

Further details of this study are summarized in Appendix 1.

3.5.1.4. Reproductive and developmental studies in animals

No reproductive or developmental studies of chitosan conducted according to OECD Guidelines were submitted or located, except for a publication by Eisa et al. (2018). That study is not considered to be useful for risk assessment of chitosan because the identity of the test article is unclear. It is alternatively called chitosan or chitosan oligosaccharide, and a chemical description that might elucidate its identity is not provided.

3.5.1.5. Safety of potential metabolites or breakdown products of chitosan in animals

Oligomers

Kim et al (2001) conducted a four-week gavage study of chitosan oligosaccharide, MW less than 1000 Da, in rats. Doses up to 2000 mg/kg bw/day were administered. No effects were observed.

Chitosan oligomers with a mean MW of 1860 Da were prepared by Qin et al. (2006) and used as the test article in an acute oral gavage study in mice, a sperm abnormality study in mice, and a 30-day dietary study in rats. No mortalities or adverse effects were observed in the acute study in mice, in which the highest dose was 10 g/kg bw. There were no increases in sperm abnormalities in mice following administration of chitosan oligomers by oral gavage for 5 days at doses up to 5000 mg/kg bw/day. All rats survived the 30-day study, in which the concentration of the test article in the diet was up to 3%, and there were no significant effects on clinical observations, body weights, body weight changes, clinical pathology parameters, necropsy findings or microscopic findings.

A low MW chitosan (46 kDa) produced by enzymatic digestion of chitosan was used for ten weeks (100 or 300 mg/kg bw twice daily by oral gavage) in a 20-week dietary study by Sumiyoshi and Kimura (2006) to investigate its effects on mice consuming a high-fat diet. There were no adverse effects associated with this intervention.

Yao et al (2012) conducted a five-week dietary study in rats to investigate the effects of chitosan oligomers at up to 3% in the diet (approximately 3000 mg/kg bw/day) on drug-metabolizing enzymes in liver and kidneys. The test article was a diverse mix of chitosan oligomers of different molecular weights. No adverse effects were found on body weights, organ weights, or clinical chemistry parameters examined.

Teodoro et al. (2016) administered up to 0.5% chitosan oligosaccharides (equivalent to 0.1 mg/kg bw/day) of unspecified MW range, in the water of normal and spontaneously hypertensive rats for six weeks. There were no biologically relevant adverse effects of this intervention.

No adverse effects were observed in association with the addition of chitosan oligosaccharides (average MW 32 kDa, DDA over 95%) to the diet of rats at 200 mg/kg feed (equivalent to 20 mg/kg bw/day) for seven days (Lan et al. 2019).

The eight-week dietary study by Chiu et al. (2020) in male rats on a high fat diet, which included groups fed high MW chitosan, low MW chitosan, and chitosan oligosaccharides (0.7 kDa; 100%) at approximately 5000 mg/kg bw/day has previously been mentioned in Section 4.3.1.1.1.2. The study was not designed as a toxicity study and did not measure the usual toxicity endpoints. Administration of dietary chitosan oligosaccharides, but not chitosan, was associated with significant increases in circulating AST, ALT, markers of liver damage, as well as TNF-α, which may reflect inflammation.

Further details of these studies are provided in Appendix 1.

Monomers

Lee et al. (2004) conducted a 13-week dietary study of N-acetylglucosamine (>98% purity) in F344 rats, using dietary concentrations up to 5% and complying with OECD Test Guideline 408. No adverse effects were found, and the no observed adverse effect level (NOAEL) was identified as 5% dietary concentration, equivalent to 2476 and 2834 mg/kg/day for male and female rats, respectively.

The same research team conducted a 52-week chronic dietary toxicity study and a 104-week dietary carcinogenicity study of N-acetylglucosamine (>98% purity), in F344 rats, in compliance with MHLW (1996) guidelines for food additives. There was no evidence of toxicity or carcinogenicity at dietary concentrations up to the maximum concentration used, 5%, which was equivalent to 2323 and 2545 mg/kg/day in males and females, respectively (Takahashi et al 2009).

Further details of these studies are provided in Appendix 1.

Anderson et al. (2005) reviewed numerous animal studies, ranging in duration from acute to 12 months, none of which showed any adverse effects of oral glucosamine supplementation. They concluded that glucosamine is well tolerated by mice, rats, rabbits, dogs and horses.

3.5.1.6. Other animal studies

Choi et al. (2002) conducted a study designed to investigate if chitosan affected ovulation and *in vitro* fertilisation in mice that had been fed a high-fat diet. Female mice were fed a diet high in fat and protein for four weeks. On the last four days of this period, a treatment group of the mice were gavaged daily with water-soluble chitosan (MW 300 kDa; DDA >90%) at 480 mg/kg bw/day while a control group was gavaged with an equivalent volume of physiological saline. No adverse effects of chitosan on reproductive performance, which included a number of oocytes ovulated in response to superovulation treatment (PMSG followed by HCG), in vitro fertilisation rate and number of zygotes progressing to the blastocyst stage in vitro, were observed. The relevance of this study to reproductive and developmental safety is limited because the endpoints measured are not standard endpoints in reproductive or developmental toxicity assays.

Fourteen other animal studies submitted by the applicant were not considered valuable for assessment because insufficient characterisation of the chitosan used as the test article made it impossible to determine their relevance. Only two of these studies reported adverse effects of chitosan supplementation. Since most studies contradict these findings, their validity must be questioned.

3.5.2. Safety of chitosan in humans

3.5.2.1. History of safe human use of chitosan

Chitosan from *Aspergillus niger* has been approved as a processing aid in the Code since 2014.

3.5.2.2. Studies of chitosan in humans

Tapola et al. (2008) conducted an eight-week parallel placebo-controlled single-blind study of two different doses of chitosan (4.5 or 6.75 g per day) compared to placebo (cellulose/lactose tablets) or supplementation with 6.75 g per day glucomannan. The chitosan used in the study had a MW of 152 kDa and DDA > 95%. Between 12 and 15 healthy adults completed each intervention. There were no significant differences between groups in the incidence of common gastrointestinal symptoms after pairwise comparison. However, since subjects consuming 6.75 g chitosan/day were most likely to stop participating in the study, the authors concluded that higher doses cannot be recommended.

This study is described in more detail in Appendix 1.

Huang *et al*., (2018a) conducted a meta-analysis of eight clinical trials investigating the effects of chitosan on blood pressure control. A drawback of this meta-analysis was that the chemical characteristics of the chitosans were not described, so the study may have included low molecular weight chitosans and chitosan oligosaccharides. Overall, they concluded that chitosan supplementation may reduce diastolic blood pressure but not systolic blood pressure. They did not report any adverse effects. When the same team pooled the results of fourteen studies of the effects of chitosan on blood lipids (Huang et al 2018b) they found no adverse effects, but did not report the chemical characteristics of the chitosans used in the studies. The risk of adverse events did not differ between participants consuming chitosan and those consuming the placebo. Finally, the same team conducted a systematic review and meta-analysis of fifteen randomised controlled trials of the effects of chitosan on body weight and body composition. No serious adverse effects were reported, but the chemical properties of the chitosans were not described. Minor adverse events were not more frequent in participants consuming chitosan than in those consuming placebo.

3.5.2.3. Other studies of chitosan and chitosan oligosaccharides in humans

The relevance of these studies to the current application is uncertain, because the test article was either of different molecular weight range, or insufficiently characterised. Relevant aspects are briefly described here.

Kim et al. (2014) conducted a randomised, double-blind, placebo-controlled clinical trial of the effects of supplementations with chitosan oligosaccharide on people with prediabetes. The test article was of low molecular weight. No adverse events were reported.

A randomised, multicentre, single-blind, placebo-controlled, clinical study reported by Trivedi et al. (2016) investigated the potential of chitosan, 250 mg/day, to act as a weight loss agent. There were no adverse events related to chitosan supplementation. The chemical properties (MW, DDA) of the test article were not specified.

Jeong et al. (2019) conducted a crossover randomised controlled trial to investigate the effects of chitosan oligosaccharide on postprandial glycaemic response in healthy subjects and in subjects with impaired glucose tolerance and impaired fasting glucose. No adverse events were reported.

The cytotoxic effect of chitosan oligosaccharides (MW = 1.4 kDa; DDA = 78%) at concentrations up to 0.5% was investigated in *ex vivo* human spermatozoa from human volunteers aged 18 to 45 years. Kinetic parameters, morphology, plasma membrane integrity, reactive oxygen species production, and DNA damage were measured. The authors concluded that chitosan oligosaccharides do not show any sign of toxicity to sperm function (Schimpf et al., 2019).

3.5.3. Genotoxicity

An unpublished bacterial reverse mutation assay (Ames test) is described in GRAS Notice 397. No increase in revertant colonies, compared with negative controls, was observed at concentrations of fungal chitosan up to 1000 µg/plate. Qin et al. (2006) similarly found no increase in revertant colonies using shrimp chitosan oligomer (1.86 kDa, DDA 85%) at concentrations up to 5000 µg/plate, although only four test strains of bacteria were used in the assay. Qin et al. (2006) also reported that the results of a mouse bone marrow cell micronucleus test and a mouse sperm abnormality test showed no mutagenic potential. The studies conducted by Qin et al. are described in more detail in Appendix 1.

Ohe (1996) reported negative results with chitin and chitosan in a sister chromatid exchange assay conducted using Chinese hamster lung cells. The MW and DDA of the chitosan were not reported, and therefore the relevance of these assays to the current application is uncertain.

3.6. Safety of β-1,3-glucans

Babíček et al. (2007) conducted acute and subchronic oral gavage studies of β-1,3-glucans in F344 rats. In the acute study, no toxicity was observed over 14 days following an acute dose of 2000 mg/kg bw. The subchronic study was conducted for 91 days, using doses of β-1,3-glucan up to 100 mg/kg bw/day. No treatment-related adverse effects were observed, and a NOAEL of 100 mg/kg bw/day was identified.

A subchronic dietary study using a test article with a ratio of 30:70 chitin:glucan was conducted in Wistar rats by Jonker et al. (2010). No adverse effects were observed and the highest dietary concentration, 10%, was identified as the NOAEL. This concentration was equivalent to 6.6 and 7.0 g chitin-glucan/kg bw/day in male and female rats, respectively.

These studies are described in more detail in Appendix 1.

The European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies reviewed the safety of both soluble and insoluble yeast β-glucans. The Panel concluded that systemic absorption of these β-glucans is minimal and that toxicity data from animals and humans do not give cause for concern. The Panel concluded that yeast β-glucans are safe for use in foods at consumption levels up to 600 mg/day (EFSA 2011a).

FSANZ notes that foods containing β-glucans, such as oats, barley, edible fungi, seaweeds and brewer's yeast (*Saccharomyces cerevisiae*) (Lante et al. 2023) have long histories of safe use.

3.7. Potential for allergenicity

In a literature review, Peng et al. (2022) found only one case of allergic reaction to chitosan via the oral route. On this basis, FSANZ concludes that allergy to chitosan is very rare.

He *et al*. (2024) found that β-1,3-glucan potentiated the development of food allergy in mice, while β-1,3/1,6-glucan did not have the same effect. FSANZ notes that the dietary level of β-glucan in He *et al*. (2024) was higher than expected human exposure. In addition, spontaneous sensitisation murine models poorly replicate human food allergy (Kanagaratham et al. 2018).

FSANZ did not find any cases of human food allergy due to β-glucan on literature search of PubMed.

Overall, FSANZ concludes that allergy to mushroom chitosan is very rare.

3.8. Assessments by other regulatory agencies

The chitosan that is the subject of this application is approved for use in Canada. It 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

3.9. Dietary exposure assessment

3.9.1. Introduction and purpose

The applicant requested permission to use Chinova's mushroom chitosan as a food additive (preservative) for a variety of general purpose foods and a small number of special purpose foods such as formulated supplementary sports foods (sports foods), formulated meal replacements (FMR) and formulated supplementary foods (FSF), with proposed maximum use levels ranging from 150 mg/kg to 1500 mg/kg in final foods (see Table D.1-1 in the application for details).

The purpose of the dietary exposure assessment was to estimate the level of chronic dietary exposure to the food additive for the Australian and New Zealand populations. Chronic dietary exposure estimates are used to represent the long term, usually life-long, dietary exposure for the population from the range of foods containing the chemical of interest.

3.9.2. Approach to estimating dietary exposures and results

Dietary exposure assessments at FSANZ are conducted using a tiered approach. The first assessment is conducted using the most conservative assumptions and the least amount of resources, with refinements made following this assessment if needed. A detailed discussion of the FSANZ methodology and approach to conducting dietary exposure assessments is set out in *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (FSANZ, 2024).

The safety assessment did not identify any population sub-groups or at-risk groups for which there were specific safety considerations or where separate chronic dietary exposure estimates were needed. Hence, the budget method calculation was used as a 'worse-case scenario' approach to estimating likely levels of dietary exposure to the food additive assuming it is added to all processed general-purpose foods, including sports foods, FMR and FSF given they are a supplement to a normal diet and not a total diet replacement.

3.9.2.1. Budget method

The budget method is a valid screening tool for estimating the theoretical maximum daily intake (TMDI) of a food additive (Douglass et al., 1997) and used by international regulatory bodies and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 2020). The calculation is based on physiological food and liquid requirements, the food additive concentration in foods and beverages, and the proportion of foods and beverages that may contain the food additive.

In this budget method calculation, FSANZ made the following assumptions that are conservative and reflective of a first tier in estimating dietary exposure (FAO/WHO, 2009):

- the maximum physiological requirement of solid foods (including milk) is 50 g/kg body weight/day. This is the standard level used in a budget method calculation where there is a potential for the food additive to be present in baby foods or general-purpose foods that would be consumed by infants (Hansen 1966).
- the maximum physiological requirement for liquids is 100 mL/kg body weight/day. This is the standard level used in a budget method calculation.
- 12.5% of solid foods and 25% of non-milk beverages contain the food additive. These

are commonly used default proportions noted in FAO/WHO, 2009.
- all solid foods and non-milk beverages contain the food additive at the maximum proposed use level of 1500 mg/kg in the final food (see Table D.1-1 in the application).

Based on these assumptions, FSANZ calculated the TMDI of the food additive to be 46.9 mg/kg bw/day. The calculated TMDI will be an overestimate of the dietary exposure to the food additive given the conservatisms in the budget method. This includes that it was assumed that 12.5% of solid foods and 25% of non-milk beverages contain the food additive at the highest maximum proposed use level.

3.9.2.2. Dietary supplements

Chitosan is also approved for use in dietary supplements. Some specific chitosan dietary supplements are included on the Australian Register of Therapeutic Goods (ARTG) (TGA, 2024). Of the products listed, and based on product dosage information available online, doses can provide up to around 29 mg/kg bw/day chitosan (often as noted as poliglusam). Information sheets on the ARTG for some of the products note the presence of crustaceans which are alternate sources of chitosan. The applicant noted similarities between this source and the mushroom source.

3.10. Discussion and risk characterisation

The production organism has a long history of safe human consumption. The microbiological risk assessment undertaken by FSANZ has not identified any public health and safety concerns associated with the use of *A. bisporus* in the production of chitosan to be used as a food additive. *A. bisporus* has been determined to be neither pathogenic nor toxigenic.

The systemic absorption of chitosan in the stomach and small intestine varies depending on the molecular weight viscosity, and the degree of deacetylation. Following consumption in the diet, chitosan is expected remain intact in the upper gastrointestinal tract, and be subject to fermentation by the microbiota in the large intestine.

No adverse effects of chitosan, chitosan oligomers or chitosan monomers were observed in acute, subchronic or chronic studies in rodents. No relevant reproductive or developmental toxicity studies were identified, but systemic absorption of chitosan is negligible and therefore effects on reproduction or development are improbable. No adverse effects of chitosan supplements were reported in human trials. There is no evidence of genotoxicity and allergic reactions to oral exposure are very uncommon.

Foods containing β-glucans have a long history of safe human use, and no experimental evidence or case reports of adverse effects of β-glucans were located.

Due to mushroom chitosan's broad-spectrum action, its mode of action is not solely reliant on targeting specific bacterial functions, and the absence of widely reported known resistance mechanisms makes the development of bacterial resistance unlikely. Moreover, the risk of developing resistance against mushroom chitosan decreases when used at its effective concentration.

3.11. Safety Assessment Conclusion

Considering the worst-case scenario, the TMDI of the food additive from use in foods and beverages was calculated to be 46.9 mg/kg bw/day. In the absence of an identifiable hazard for chitosan and β-glucans, an Acceptable Daily Intake (ADI) "not specified" was established.

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Appendix 1 Review of toxicological studies

I Studies in animals

I.I Acute studies

Acute oral toxicity studies of chitosan and its acetate and lactate salts (Lagarto et al 2015). Regulatory status: None.

The test item was chitosan derived from lobster (*Panuliris argus*) chitin. The chitosan had a MW of 309 kDa and a DDA of 83%. The acetate and lactate salts had a DDA of 48.9% and 57.1% respectively. The test system comprised female Wistar rats, 7-8 weeks old, 6 rats/group, group-housed under standard laboratory conditions. The rats were assigned to four groups: 2000 mg/kg bw chitosan, 2000 mg/kg bw lactate chitosan, 2000 mg/kg bw acetate chitosan, and control (vehicle only). Test articles were suspended in 0.5% carboxymethylcellulose and administered on Day 1 by oral gavage. Rats were examined for survival and clinical signs for 8 h after dosing, and twice daily from Day 2 to Day 14. Bodyweights were recorded prior to dosing on Day 1, and on Days 7 and 14. On day 14, all rats were killed by anaesthesic overdose, and gross necropsies were conducted. All rats survived to scheduled termination and there were no clinical signs or necropsy findings indicative of toxicity.

I.II Subchronic studies

Mice

Ninety-day dietary study of chitosans in mice (Zeng et al. 2008) Regulatory status: None.

The purpose of this study was to investigate the effects of dietary chitosan on iron, zinc and copper in liver, spleen, heart and kidney. The test articles were supplied at 1.05% of the diet. They included a high molecular weight chitosan (760 kDa; DDA 85.5%), a low molecular weight chitosan (32.7 kDa; DDA 85.0%), a chito-oligomer (0.9 kDa; DDA 85.7%) and a water-soluble chitosan (39.1 KDa; DDA 52.6%). A control group of mice did not receive any chitosan supplement. The test system comprised female Kunming mice, 4 weeks old at receipt. They were group-housed at 5/cage, and 10 mice were assigned to each dose group. Feed and water were provided *ad libitum*. Feed intake was monitored. Mice were observed daily and weighed weekly. Mice were decapitated on Day 90 and gross necropsies conducted. Weights of fresh heart, liver, kidneys, spleen, thymus and lung were recorded. All mice survived to the end of the study and no clinical signs of toxicity were observed. There were no gross or microscopic lesions indicative of toxicity. Significantly higher group mean values for hepatic iron, zinc and copper were found in the group fed low molecular weight chitosan, when compared to the control group, but no similar effects were found in the mice fed high molecular weight chitosan, chito-oligomer or water-soluble chitosan.

Twelve-week dietary study of chitosan in diet-induced obese mice (Do et al 2018). Regulatory status: None.

The purpose of this study was to compare the anti-obesity effects of long-chain chitosan (LC) and short-chain chitosan (SC) in C57BL/6J mice which had been made obese by feeding a high-fat diet. The chitosan was from crab shells. The LC had a MW of 390 kDa and a DDA of 82.8%. The SC was made from the LC by hydrolytic treatment with hydrogen peroxide and had a MW of 210 kDa. Each chitosan was added to the diet at 1%. The authors did not provide a conversion to a dose in mg/kg bw. It is apparent from the numbers used that all the mice were the same sex, but the paper does not state if they were male or female. They were maintained under standard laboratory environmental conditions. Groups of 10 mice/group were fed a control diet (ND), high-fat diet with no chitosan (HFD), high-fat diet

with LC, or high-fat diet with SC. After 12 weeks the mice were killed by anaesthetic overdose after blood sampling, and the liver and adipose tissues were removed.

All mice survived to scheduled termination and no adverse effects of chitosan supplementation were reported. Group mean values for terminal body weight and calculated bodyweight gain were significantly lower in the LC and SC groups compared to the mice fed the high-fat diet without chitosan, although calculated feed intake and energy intake were not significantly different. Mice in the SC group had significantly lower group mean values for mass of epididymal, perirenal, and mesenteric white adipose tissue, and particularly for visceral fat and total white adipose tissue, when compared to the mice in the HFD and LC groups. Supplementation with SC was also associated with beneficial effects on plasma and hepatic lipids, and on hepatic histopathology.

Rats

Twenty-eight day studies of chitosan and chitosan salts in rats (Lagarto et al. 2015)
Regulatory status: None

The test articles for these studies were the same as those used in the acute study reviewed in section 6.1.1.

For the chitosan repeat-dose study, Wistar rats, 5 weeks old, were assigned to groups of 14/sex/group. The vehicle and control article was 0.5% carboxymethylcellulose. Chitosan was administered daily by oral gavage at a dose of 0, 100, 300 or 1000 mg/kg bw/day. Lactate chitosan was administered daily to 14 rats/sex at a dose of 1000 mg/kg bw/day, and acetate chitosan was administered daily to 14 rats/sex at a dose of 700 mg/kg bw/day. There were also 14 vehicle control rats/sex. For each treatment group, seven/sex were killed on Day 28 and seven/sex maintained as recovery cohorts for 14 days.

Rats were group-housed by dose. All rats were examined daily for mortality and clinical observations. Feed intake was recorded daily and rats were weighed weekly. Blood samples were collected on Day 28 prior to killing by anaesthetic overdose, and gross necropsies were performed. Fresh organ weights were recorded for selected organs, and a selected range of major organs were preserved for histopathology. Recovery cohorts were killed after a 14 day recovery period.

All rats survived to scheduled termination of the in-life phase and no abnormal clinical signs were observed in association with any of the test articles. The only treatment related effect observed in rats dosed with chitosan was a statistically significant increase in group mean erythrocyte count in females dosed with ≥300 mg/kg bw/day chitosan, and males dosed with 1000 mg/kg bw/day chitosan. Similar modest but significant increases in group mean erythrocyte counts were observed in both sexes dosed with 1000 mg/kg bw lactate chitosan, and both sexes dosed with 700 mg/kg bw/day acetate chitosan. An increase in group mean leukocyte count was observed in female rats dosed with lactate chitosan, and both sexes dosed with acetate chitosan. Other group mean values that were significantly different to control values in rats treated with acid salts of chitosan were increased glucose and decreased AST in both sexes treated with acetate chitosan, and an increase in urea in males treated with lactate chitosan. Treatment-related changes in group mean values for organ weights were limited to decreased relative kidney weight in males in the lactate chitosan group. No gross or microscopic lesions were found in any rats.

Clinical chemistry results from recovery groups showed that the increased erythrocyte count was not reversible in males treated with 1000 mg/kg bw/day chitosan, but apparently reversible in rats treated with the acid salts. Changes in leukocyte count and plasma glucose observed in association with acid salts of chitosan were still present 14 days after the end of treatment.

None of the observations were interpreted as adverse. The increases in erythrocyte counts and blood glucose levels were small in absolute terms. The increases in leukocyte counts were not associated with increases in thymus or spleen weights, or lesions in those organs. The authors concluded that chitosan and the tested acid salts are of low toxicity in rats.

Four-week dietary study of chitosans of different viscosity in rats (Chiang et al 2020). Regulatory status: None

The two test articles for this study originated from shrimp shells. Both had a DDA of approximately 90%. One was prepared by alkali deacetylation using NaOH and was identified as CCS. This chitosan had a MW of 480 kDa and intrinsic viscosity of 6.29 dl/g. The other chitosan was prepared by microbial fermentation and was identified as FCS. This chitosan had MW of 340 kDa and intrinsic viscosity of 5.32 dl/g. The test system comprised male Sprague Dawley rats, six weeks old at study start. Rats were individually housed, with *ad libitum* access to feed and water. Rats were assigned to one of three groups, with 6 rats/group. Groups were the Cellulose (CE) group, the group fed and the group fed FCS. Inclusion of either chitosan in the diet at 5% resulted in significantly lower group mean values for plasma total cholesterol, VDL-cholesterol and VLDL-cholesterol, and significantly lower group mean values for concentrations of total lipid and total cholesterol in the liver, when compared to the CE group. Conversion to a dose expressed in mg/kg bw was not provided. No adverse effects on survival or terminal bodyweight were observed.

Eight-week study of the effects of chitosan and derivatives on hypercholesterolaemia in rats fed a high-fat diet (Chiu et al. 2020). Regulatory status: None

The test system for this study comprised male Sprague Dawley rats, seven weeks old at study start. Rats were individually housed under standard laboratory environmental conditions and assigned to groups of 6 rats/group. Groups were rats fed a standard rat feed (NC), rats fed a high fat diet (HF group), rats fed a high-fat diet and a 5% supplement with high MW chitosan (HF+HC), rats fed a high-fat diet and a 5% supplement with low MW chitosan (HF+LC), and rats fed a high-fat diet and a 5% supplement with chitosan oligosaccharides (HF+CO). The characteristics of the supplements were as follows: High MW chitosan 740 kDa; DDA 91%; low MW chitosan 80 kDa; DDA 83.9%; chitosan oligosaccharides 0.7 kDa; DDA 100%. In normal and high-fat diets, 5% cellulose was added in place of chitosan derivatives.

Bodyweights were recorded weekly during the in-life phase. After 8 weeks the rats were killed under anaesthesia. Plasma samples were collected for tests on liver function and indicators of hypercholesterolemia, and liver and intestines were collected. The study was not designed as a toxicity study and did not measure the usual toxicity endpoints, but no adverse effects of feeding high MW or low MW chitosan were reported. Unexpectedly, the CO group exhibited significantly elevated group mean values for aspartate aminotransferase, alanine aminotransferase and tumour necrosis factor-α when compared to all the other groups, indicating hepatocellular damage.

I.III Chronic studies

Six-month dietary toxicity study of chitosan in rats (NTP 2017). Regulatory status: GLP; non-guideline

The test article for this study had a MW of 94 kDa and DDA 86.5% and was estimated to be 94% pure. The study was conducted to determine if chitosan consumption is a cause of Vitamin E depletion and osteoporosis.

Sprague Dawley rats were housed individually under standard laboratory environmental conditions. There were three cohorts of rats; core study rats for feed consumption, bodyweights, clinical observations, gross and microscopic postmortem findings, bone

histomorphometry, and sperm morphology/vaginal cytology evaluations (group A); rats for measurements of vitamins A, E and D, and for bone calcium measurement (Group B); and rats for haematology, clinical chemistry, vitamin K measurement, feed and faecal analysis, and urinalysis.

Group A rats, 10/sex/group, were fed diets containing chitosan at concentrations of 0%, 1%, 3%, or 9%, for 25 weeks. Rats in Groups B and C, 10/sex/group, were fed the same dietary concentrations for up to 26 weeks. All male and female Group A rats survived to the end of the study. Mean body weights and feed consumption of exposed Group A groups were similar to those of the control groups. Dietary concentrations of 1%, 3%, and 9% resulted in average daily doses of approximately 450, 1,500, and 5,200 mg chitosan/kg body weight/day in males and 650, 1,800, and 6,000 mg/kg/day in females.

There were no treatment-related clinical findings. Treatment-related decreases in percentage fat digestion of 20% to 33% in males and 5% to 14% in females, relative to controls, were consistently observed in the 9% group with effects also noted in males in the 3% group, in which decreases were 2% to 8%. However, overall, there were no effects of chitosan on group mean values for bodyweight. The dietary chitosan level of 9% was also associated with decreased group mean cholesterol, and with decreased levels of vitamins A and E. Although there was no clinical evidence of deficiency of either of these vitamins, these decreases were interpreted as adverse. In contrast, vitamin D levels were elevated, whereas bone calcium levels remained stable. Chitosan supplementation was associated with decreased incidence of periportal lipid accumulation in the liver.

The NTP concluded that the lowest observed effect level (LOEL) was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats, although the basis for their reasoning is not clearly explained.

I.III.I Oligomers

Mice

Acute toxicity study of chitosan oligomers in mice (Qin et al 2006) Regulatory status: None

The test article for this study was a mixture of chitosan oligomers prepared by enzymatic depolymerization of chitosan. The average MW was 1860 Da.

Kunming mice of both sexes, weighing 18 to 22 g, were housed under conditions of controlled temperature. Mice were assigned to four groups of 5 mice/sex/group. Mice were fasted overnight prior to a single dose by oral gavage of 1000, 2150, 4640 or 10,000 mg chitosan oligomer/kg body weight. Mice were then maintained for 7 days with daily observation. All mice survived until the end of the observation period, and no clinical abnormalities were observed. The authors of the study concluded that the LD50 for this preparation of chitosan oligomers was greater than 10,000 mg/kg bw.

Sperm abnormality study in mice (Qin et al 2006). Regulatory status: compliant with Ministry of Health, PR China, 2003

Twenty-five male Kunming mice, weighing 27 to 35 g, were randomly assigned to five groups of 5 mice/group. The test article was chitosan oligomers with a mean MW of 1860, and the vehicle and control article was distilled water. Four groups were gavaged daily for five days with 0, 1250, 2500, 5000 mg chitosan oligomer/kg bw. A fifth positive control group was administered 40 mg/kg cyclophosphamide in physiological saline by intraperitoneal injection. Thirty-five days after the first dose, the mice were killed by cervical dislocation and sperm were collected from the epididymides. One hundred sperm from each animal were examined microscopically for morphological abnormalities.

The group mean values for frequency of sperm abnormalities for the groups dosed with chitosan oligomers were not significantly different from that of the negative control group.

The group mean value for sperm abnormalities of the positive control group was significantly greater than that of the negative control group, confirming the validity of the assay.

Study of low molecular weight chitosan in mice (Sumiyoshi and Kimura 2006) Regulatory status: None

The test article for this study was a low molecular weight chitosan (46 kDa) obtained by enzymatic hydrolysis of a high molecular weight chitosan. It was of low viscosity and was water-soluble. The purpose of the experiment was to investigate its effects on male C57BL/6J mice fed a high-fat diet. The mice were kept under standard laboratory conditions and started on the study when they were 5 weeks old. Mice were assigned to groups of 7 or 8 mice/group and fed either a low fat diet (negative controls), a high fat diet with no chitosan intervention (positive control), a high fat diet plus twice-daily gavage with chitosan at 100 mg/kg bw, or a high fat diet plus twice-daily gavage with chitosan at 300 mg/kg bw. The in-life phase lasted 20 weeks. Group mean bodyweight values for the chitosan-treated mice were intermediate between those of the negative and positive controls, with the greatest effect occurring at ≥17 weeks. The group mean values for liver weight, hepatic triglyceride, hepatic cholesterol, subcutaneous adipose tissue mass, mesenteric adipose tissue mass, epididymal adipose tissue mass, and mean white adipose cell diameter of the 300 mg/kg bw group were significantly lower than those of the positive control group. No adverse effects of the chitosan supplementation were observed, and serum biomarkers of liver and kidney function remained normal. The authors concluded that water-soluble 46 kDa chitosan is a safe functional food.

Rats

Twenty-eight-day oral gavage study of chitosan oligosaccharide in rats (Kim et al. 2001). Regulatory status: None

The test article for this study was chitosan oligosaccharide with MW less than 1000 Da. Doses up to 2000 mg/kg bw/day were administered. No effects were observed. Four-week-old Sprague Dawley rats were individually housed and acclimatized to standard laboratory conditions for one week prior to study start. Rats were assigned to groups of 9/sex/group, and gavaged once daily with 0, 500, 1000 or 2000 mg/kg bw/day chitosan oligosaccharide at a constant volume of 10 mL/kg bw. Rats were observed, and feed intake measured, daily throughout the in-life phase. Bodyweight was recorded weekly. At the end of the in-life phase, urine and blood were collected and rats were killed by exsanguination while under anaesthesia. A detailed necropsy was performed on each rat, and fresh weights of kidney, spleen, liver, lung and gonads were recorded.

All rats survived to the end of the study and there were no treatment-related clinical observations or effects on group mean values for bodyweight or bodyweight gain. There were no treatment-related effects on urinalysis, plasma biochemistry, gross necropsy, group mean values for absolute or relative organ weights, or microscopic findings. The group mean value for total leukocyte count in the 2000 mg/kg bw/day males was elevated to a statistically significant extent compared to that of control males but remained within normal historical control range.

Thirty-day dietary study of chitosan oligomers in rats (Qin et al. 2006). Regulatory status: None

Young Sprague Dawley rats with bodyweights ranging from 60 to 80 g were individually housed. They were assigned to four groups of 10/sex/group and fed diets containing 0, 0.75%, 1.5% or 3.0% chitosan oligomers with a mean MW of 1860 Da. Feed and water were provided *ad libitum*. Clinical observations and feed intake were recorded daily, and bodyweights were recorded weekly. At the end of the in-life phase, rats were anaesthetised, and blood was collected for haematology and clinical chemistry, and rats were then killed.

Necropsies were performed and fresh weights of selected major organs were recorded. Livers, kidneys and small intestines were processed for microscopic examination.

All rats survived to the end of the study and no abnormal clinical signs were observed. Addition of chitosan oligomers to the diet had no effect on bodyweights, bodyweight gains, feed consumption, haematology, clinical chemistry, absolute or relative organ weights, or gross or microscopic necropsy findings.

Five-week dietary study of chitosan oligomers in male rats (Yao et al. 2012) Regulatory status: None

The test article for this study was a diverse mix of chitosan oligomers of different molecular weights. The average composition of each gram of the test article was glucosamine, 4.0 mg; chitosan dimer, 66.5 mg; chitosan trimer,137.1 mg; chitosan tetramer, 148.2 mg; pentamer, 178.8 mg and hexamer,53.1 mg; others: 412.3 mg. The test system comprised male Sprague Dawley rats, 6 weeks old at time of receipt. The rats were acclimatised to standard laboratory conditions for a week before being assigned to three groups of 8 rats/group. They were fed diets containing 0, 1% or 3% chitosan oligomers. Feed and drinking water were provided *ad libitum*. At the end of the in-life phase, rats were killed by exsanguination while under anaesthesia. Plasma AST, ALT, creatinine and BUN were measured. Liver and kidneys were weighed and stored at -80°C for microsome preparations and enzyme assays.

Dietary supplementation with up to 3% of the chitosan oligomer mix had no effect on group mean values for bodyweight, liver weight, kidney weight, AST, ALT, creatinine or BUN.

Six-week drinking water study of chitosan oligosaccharides in normal and diabetic male rats (Teodoro et al 2016). Regulatory status: None

The test article for this study is not clearly characterised but is described as water-soluble and of low molecular weight. Normal rats were Han Wistars, whereas the diabetic rats were a spontaneously diabetic strain designated GK/Slc. All rats were male. Rats were kept under standard laboratory conditions, with free access to feed and to acidified water, and were four weeks old at the start of the study. The four study groups, each made up of four rats, were Wistar Han rats; Wistar Han rats given 0.5% chitosan oligosaccharides in their water; GK/Slc rats; or GK/Slc rats given 0.5% chitosan oligosaccharides in their water. Rats were maintained on the study for six weeks. At the end of the study, rats were killed by decapitation and blood was collected immediately for haematology and clinical chemistry. Liver and skeletal muscle were processed for isolation of mitochondria.

Group mean values for bodyweight of GK/Slc rats were lower than those of Han Wistar rats throughout the study. Han Wistar rats receiving chitosan oligosaccharide supplementation had a significantly lower group mean bodyweight than control Han Wistars at the end of the study, but the group mean bodyweight of treated GK/Slc rats was not significantly different to that of untreated GK/Slc rats. The group mean value for plasma cholesterol of treated Han Wistar rats was significantly lower than that of control Han Wistars, but other clinical chemistry parameters were not affected. Untreated GK/Slc rats had significantly higher group mean values for plasma glucose and cholesterol when compared to control Han Wistars. GK/Slc rats treated with chitosan oligosaccharides had significantly higher values for direct and total bilirubin, aspartate aminotransferase activity, and glucose than untreated rats of both strains. Treated rats had significantly lower group mean cholesterol levels than untreated rats of the same strain. Treatment with chitosan oligosaccharides did not affect hepatic mitochondrial function but had an inhibitory effect on mitochondrial respiration in skeletal muscle but this does not appear to have any clinical significance.

Seven-day study of the effects of chitosan oligosaccharides in heat-stressed (Lan et al. 2019) Regulatory status: None

The aim of this study was to evaluate the effects of chitosan oligosaccharides on heat stressed male Sprague Dawley rats. The chitosan oligosaccharides had an average MW 32 kDa and DDA over 95%. Rats, 6 to 8 weeks at time of receipt, were acclimatised to standard laboratory conditions before being assigned to one of three groups, 10 rats/group. The control group was provided with basal feed and maintained at 24°C. The second group was also provided with basal feed, but heat-stressed by maintenance of an ambient temperature of 35°C for four hours/day. The third group was heat-stressed by the same conditions, but also supplemented with chitosan oligosaccharides at 200 mg/kg feed. Feed and water were provided *ad libitum* to all rats. The duration of the study was seven days. Bodyweight, water intake and feed intake were recorded daily.

Heat-stressed rats exhibited lower group mean bodyweight gain and lower mean feed consumption than control rats, but these effects were partially ameliorated by chitosan oligosaccharide supplementation. Group mean values for relative weights of spleen and kidneys were also higher in the heat-stressed rats than the controls, but not in heat-stressed rats supplemented with chitosan oligosaccharides. Chitosan oligosaccharides also had an ameliorative effect on tissue markers of oxidative damage and inflammatory response.

I.III.II Monomers

Thirteen-week toxicity study of n-*acetylglucosamine in rats (Lee et al 2004) Regulatory status: Compliant with OECD Test Guideline 408. GLP status unspecified.*

N-acetylglucosamine, > 98% purity, was blended into the diet at concentrations of 0, 0.625, 1.25, 2.5 and 5%, after which the diet was pelleted. F344 rats, five weeks old at receipt, were acclimatized to standard laboratory conditions for one week prior to study start. Rats were housed in groups of three or four and assigned to five groups comprising 10/sex/group. Feed and tap water were provided *ad libitum*. Rats were observed daily. Feed intake and bodyweight were recorded weekly. At the end of the in-life phase, rats were bled and then exsanguinated while under anaesthesia. Haematology, clinical chemistry, fresh organ weights and tissues preserved for histopathology were compliant with OECD Test Guideline 408 at the time. Histopathology was limited to the control and 5% group in the first instance.

Based on group mean feed consumption data, the concentrations of *n*-acetylglucosamine of 0, 0.625, 1.24, 2.5 and 5.0% were equivalent to group mean doses of 0, 302, 588, 1218 and 2476 mg/kg bw/day in males, and 0, 351, 695, 1412 and 2834 mg/kg bw/day in females.

There were no treatment-related effects on survival, clinical observations, bodyweight, bodyweight gain, feed consumption, gross necropsy findings or histopathological lesions. Statistically significant increases in mean corpuscular volume in females fed ≥ 1.25% *n*-acetylglucosamine were biologically negligible in magnitude. Slight decreases in plasma sodium and chloride in females fed ≥ 2.5% *n*-acetylglucosamine were of no biological relevance. Other minimal but statistically significant differences in haematology and clinical chemistry parameters between treated and control rats did not show consistent dose-dependence. Group mean values for absolute and relative organ weights were comparable to those of control females for all weighed organs, but statistically significant decreases in relative weights of lungs, heart, spleen, liver and kidneys were observed at all doses in males, compared to control males, but absolute weights of those organs were comparable to those of control males.

The authors of the study identified 5% *n*-acetylglucosamine in the diet as the NOAEL in both sexes, the highest concentration tested. This dietary concentration is equivalent to 2476 and 2834 mg/kg/day for male and female rats, respectively.

Chronic dietary toxicity and carcinogenicity studies of n-*acetylglucosamine in rats (Takahashi et al. 2009). Regulatory status: Compliant with MHLW (1996) guidelines for food additives. GLP status unspecified.*

The two studies reported in this paper were conducted by the same research team as the Lee et al. (2004) study and used the same test article. The chronic toxicity study was of 52 weeks' duration and the carcinogenicity study was of 104 weeks duration. In both studies, F344 rats were group-housed by sex under standard laboratory conditions with *ad libitum* access to feed and water and started on the study at 6 weeks of age. Dietary concentrations of n-acetylglucosamine were 0%, 1.25%, 2.5% or 5% in the chronic toxicity study and 0%, 2.5% or 5% in the carcinogenicity study.

Rats in the chronic toxicity study were assigned to groups of 10/sex. Rats were observed daily. Body weights and feed consumption were recorded weekly through to Week 8 and monthly thereafter. At scheduled termination of the in-life phase, blood was collected from anaesthetised rats for haematology and clinical chemistry, and rats were exsanguinated. Fresh organ weights were recorded for brain, heart, lungs, liver, spleen, adrenals, kidneys and testes were weighed. A comprehensive list of organs and tissues was preserved for histopathology. Histopathological assessment was performed on tissues from the control and 5% rats in the first instance. Since there were no treatment-related lesions at the 5% dietary concentration, further histopathological examination was no performed.

The same general methods were used for the 104-week carcinogenicity study, but with 50 rats/sex/group. Rats found dead or in moribund condition were subject to detailed necropsy.

All rats survived to the end of the 52-week study. Although a small number of deaths or humane euthanasias occurred on the 104-week study, unscheduled deaths were not more frequent in treated rats than in controls.

In the chronic toxicity study, statistically significant suppression of bodyweight gain was observed in males at 5% from week 32 to the end of the experiment, but no dose-related change in body weights was found in females. Episodes of suppression of bodyweight gain were also found in males of the 5% group in the 104-week study, while in females, body weights were slightly but significantly depressed at more than 2.5% in a dose-dependent manner from week 1. Effects on bodyweight and bodyweight gain were not considered to be of sufficient magnitude to represent adverse effects. No intergroup differences in feed consumption were observed in either sex in either study. The mean values for food consumption/kg bodyweight were slightly higher in males of all treatment groups than in controls in the 52-week study but not in either sex in the 104-week study.

No treatment-related effects on haematological parameters were observed in either sex in either study. In the 52-week study, there was a slight but statistically significant dose-related decrease in total cholesterol in males fed ≥2.5% *n*-acetylglucosamine, but no corresponding change was observed in females and the effect was not of toxicological relevance. ALT was slightly increased in females in the 5% group, but there was no histological correlate. There were no treatment-related lesions found during gross necropsy or microscopic examination in the 52-week study, and there were no biologically relevant differences in fresh organ weights. There were no treatment-related differences in incidences of neoplastic or non-neoplastic lesions in the 104-week study. It was concluded that the highest dietary concentration tested of 5% *n*-acetylglucosamine, equivalent to 2323 and 2545 mg/kg/day in males and females, respectively, did not have any toxic or carcinogenic effects in F344 rats.

II Studies in humans

Study of the effects of chitosan tablets in human volunteers (Tapola et al 2008). Regulatory status: None

The study was a parallel, single-blind, placebo-controlled trial. Chitosan, placebo (negative control) and glucomannan (positive control) were supplied as tablets. Sixty-five healthy adults were eligible for the study after initial screening, and 56 completed the study. The subjects were assigned randomly to one of the four groups. Men and women were randomized separately. Prior to commencement of the intervention, body mass index and

plasma level of total cholesterol, LDL-cholesterol, HDL-cholesterol and total triglyceride were recorded for each subject. The subjects were given 0, 4.5 or 6.75 g of chitosan per day or 6.75 g of glucomannan per day in the form of tablets with main meals for eight weeks. The mean duration of the intervention period was 55 days (minimum 50, maximum of 58) in the chitosan 4.5 group, 55 days (53 - 56) in the chitosan 6.75 group, 54 days (50 - 58) in the glucomannan group and 55 days (48- 62) in the placebo group. Tablets were taken with a glass of water 15 minutes before breakfast, lunch and dinner. To blind the participants to what they were taking, placebo tablets were used to ensure that all participants took the same number of tablets. Participants kept a daily diary of health, medication and lifestyle during the intervention period. Dietary composition was measured pre-study and during the intervention, by keeping a four-day food diary.

Compliance during the intervention period was 95 to 96%. Chitosan supplementation had no adverse effects on serum levels of vitamin A, vitamin E, 25-hydroxyvitamin D, carotenes (α- and β-carotene), haematological parameters or clinical chemistry parameters. There were no statistically significant differences between study groups in total or LDL-cholesterol levels. There were no significant differences between groups in frequency of adverse gastrointestinal symptoms after performing pairwise comparison. Because discontinuation of participation was greatest in the 6.75 g chitosan group, the authors concluded that doses ≥ 6.75 g chitosan cannot be recommended.

III Genotoxicity assays

Bacterial reverse mutation assay (Ames test) of chitooligomers (Qin et al. 2006). Regulatory status: Compliant with the guidelines of the National Standards of the Peoples' Republic of China, Ministry of Health, Peoples' Republic of China 2003.

The test article for this assay comprised chitosan oligomers with a mean MW of 1860 Da. The assay was conducted using the treat and plate method. The test strains were *Salmonella enteriditis* var. Typhimurium TA97, TA98, TA100 and TA102. Distilled water was used as the vehicle. Bacteria were exposed to the test article at concentrations of 0, 0.5, 5, 50, 500 and 5000 µg/plate, with and without addition of S9 mix for metabolic activation. All tests were conducted in duplicate. In the preliminary test, there was no evidence of toxicity or growth inhibition at 5000 µg/plate. Negative control articles run in parallel were distilled water and DMSO. For TA97, positive controls were fenaminosulf in the absence of S9 and 2-aminofluorene in the presence of S9. For TA98, positive controls were 2-aminofluorene in the absence of S9 and fenaminosulf in the presence of S9. For TA100, positive controls were sodium azide in the absence of S9 and 2-aminofluorene in the presence of S9. For TA102, positive controls were fenaminosulf in the absence of S9 and 1,8-dihydroxyanthraquinone in the presence of S9. No increase in revertant colonies was observed at any concentration of test article in any of the test strains, when compared to negative control plates. Significant increases in the numbers of revertant colonies were seen in response to exposure to all of the positive control articles, confirming the validity of the assay. It was concluded that the chitosan oligomers had no mutagenic effect on the test strains at any of the concentrations applied.

IV Studies of beta glucans

Acute and sub-chronic oral gavage studies of β-1,3-glucans in rats (Babíček et al. 2007). Regulatory status: Compliant with OECD Guidelines 420 and 408.

The test article for these studies is described as an extract of *Saccharomyces cerevisiae* comprising "highly pure" (1,3/1,6)-β-D-glucan.

The acute study was conducted using male and female F344 rats maintained under standard laboratory conditions. Rats were 6 weeks old at dosing and assigned to two groups of 5/sex/group. The control rats were gavaged with water. The rats in the treatment group were

gavaged with a suspension of test article in water, at a dose of 2000 mg/kg bw. Rats were then observed for 14 days, with bodyweights recorded pre-study, on Day 7 and on Day 14. All rats survived to the end of the observation period and no abnormal clinical signs were observed. There were no significant differences in group mean bodyweights between treated rats and controls of the same sex. It was concluded that the LD_{50} for the β-1,3-glucans was >2000 mg/kg bw.

Dose levels in the subchronic study were 0, 2, 33.3 or 100 mg/kg bw/day, administered by gavage once daily for 91 days. Rats were 5 to 6 weeks old when dosing commenced. Ten rats/sex/group were assigned to the main study and additional recovery cohorts were included for the control and highest dose groups, for a recovery period of 14 days. Rats were observed twice daily and subjected to detailed examination and bodyweight measurement weekly. Ophthalmic examinations were conducted pre-study and close to scheduled necropsies. A functional observational battery for behavioural/neurological changes was conducted close to the end of the dosing period. Rats were bled prior to kill, and haematological and clinical chemistry parameters were measured. At scheduled kill, rats were anaesthetised and exsanguinated. Necropsies were performed on all rats, fresh organ weights were recorded for major organs, and a comprehensive range of organs and tissues preserved for histopathology.

All rats survived to scheduled termination, and there were no treatment-related effects on clinical observations, ophthalmic findings, bodyweights, bodyweight gains, feed consumption, results of the functional observational battery, haematological values, clinical chemistry values, gross necropsy findings, absolute or relative organ weights, or histopathological findings. The authors concluded that the NOAEL in this study was 100 mg/kg bw/day, the highest dose tested.

Thirteen-week dietary study of 30:70 chitin:glucan in rats (Jonker et al. 2010). Regulatory status: GLP; conducted in accordance with OECD, EC and FDA guidelines at the time.

The chitin:glucan content of the test article was 94%. The material was derived from the cell wall of the mycelium of *Aspergillus niger.* The test article was mixed into standard rodent diet at levels of 1%, 5% and 10%. Wistar rats were approximately six weeks old at the start of the study. They were allocated to four groups, including a control group, comprising 20/sex/group. Feed and water were provided *ad libitum*. Rats were observed daily, and subject to detailed examination once weekly. A functional observational battery was conducted in Week 13. Ophthalmic examinations were conducted pre-study and in Week 13. Bodyweights were recorded pre-study and weekly thereafter. Feed and water consumption were recorded for each rat. Haematology and clinical chemistry parameters were measured pre-study, mid-study and prior to scheduled termination. Urine was collected overnight prior to termination. At the end of the in-life phase all rats were killed under anaesthesia and subjected to detailed necropsy. Fresh weights of organs were recorded according to guidelines, and a comprehensive list of organs and tissues was preserved for histopathology.

All rats survived to scheduled termination of the in-life phase, and there were no treatment-related effects on clinical signs or performance on the functional observational battery. Dietary exposure to chitin:glucan had no effect on group mean values for bodyweight, water consumption, ophthalmology, haematology, clinical chemistry, urinalysis, gross necropsy findings or microscopic findings. Group mean feed consumption was slightly higher in the 10% chitin:glucan group than in sex-matched controls. The group mean weights of full and empty caecum were significantly increased over those of sex-matched controls in males consuming ≥5% chitin:glucan, and females consuming 10% chitin:glucan. Increase in caecal size and volume is a well-recognised effect of increased fibre consumption in rats. The authors identified the highest dietary concentration, equivalent to 6.6 and 7.0 g chitin-glucan/kg bw/day in male and female rats, respectively, as the NOAEL.