Application to amend the Australia New Zealand Food Standard Code to permit the use of long-chain glycolipids from *Dacryopinax spathularia* ("Natural Glycolipids") as a preservative in non-alcoholic beverages

Date 13/05 2019

Contents

Part 1 General Information about the application

1.1 Applicant details

The application **must** contain the following contact details:

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(f)	nature of applicant's business	The core business of LANXESS is the development, manufacturing and marketing of chemical intermediates, additives, specialty chemicals and plastics.
(g)	details of other individuals, companies or organisations associated with the application	This application was prepared in consultation with: Dr Simon Brooke-Taylor Brooke-Taylor & Co Pty/Ltd Consultants PO Box 1404 Milawa Victoria 3678 Please refer all correspondence regarding this application to Dr Brooke-Taylor email: simon@brooketaylor.com.au pH: +61 411 156 773

1.2 Purpose of the application

This application seeks an amendment Australia New Zealand Food Standard Code to permit the use of long-chain glycolipids from *Dacryopinax spathularia* ("Natural Glycolipids") as a preservative in non-alcoholic beverages at use levels ranging from 2 to 100 ppm. "Natural Glycolipids" may also be described by its product development name "AM-1", the descriptive term "Natural Glycolipids" or its Trade Name "Nagardo™".

1.3 Justification for the application

Natural Glycolipids is a natural glycolipid mixture obtained via fermentation of glucose by the edible jelly fungus *Dacryopinax spathularia*, also known as sweet osmanthus ear, *Cantharellus spathularius or Guepinia spathularia*. It has prominent antifungal effects against common yeasts, moulds and spoilage bacteria and can be used to prolong shelf life and guarantee the microbiological quality of beverages. Natural Glycolipids is a versatile alternative or complement to other techniques such as heat treatment, which may lead to loss of nutritional value, vitamin content or taste. It may also substitute for established chemical preservatives, such as sorbic acid, benzoic acid or sulphites, in beverages, at lower concentrations in use. In addition, it may also be used to control yeast and mould strains which have adapted to current chemical preservatives and / or are tolerant to heat treatment. Natural Glycolipids is consequently particularly useful as a naturally derived preserving agent to prevent spoilage of beverages.

The application seeks its approval as a food additive under Standard 1.3.1.

Natural Glycolipids, under the descriptive name "Jelly Mushroom Glycolipids", is recognised as GRAS in the USA for the purpose proposed (GRN 740) (Attachment 6).

Letters of support from the Australian beverage industry are included in Appendix 3.3.

Details of similar applications submitted or proposed to be submitted in other countries is provided in section 2.3

1.4 Regulatory impact information

1.4.1 Costs and benefits of the application

- The application offers consumer the benefit of consuming beverages preserved with a safe naturally derived product rather than synthetic preservatives or preserving systems like heat treatment that reduce the nutritional value and taste of the beverage. Consumers will be informed about the use of Natural Glycolipids as a preservative through ingredient labelling in accordance with Standard 1.2.4.
- The application will benefit beverage manufacturers choosing a naturally derived preservative. There are no costs imposed on food manufacturers from the approval of Natural Glycolipids as preservative for beverages.
- There are no anticipated costs for government from the approval of Natural Glycolipids as a preservative for beverages.

1.4.2 Impact on international trade

Natural Glycolipids is permitted to be used as preservative in beverages in the USA. Approval in Australia and New Zealand is anticipated to facilitate trade in beverage products with the USA. Other major countries of the world are expected to follow.

Part 2 Information about Natural Glycolipids

[based on Application Handbook section 3.3.1 Food Additives]

2.1 Technical information about Natural Glycolipids

2.1.1 Nature and technological purpose of Natural Glycolipids

Natural Glycolipids (in the following also referred to as "AM-1") may be used as preservative to prevent microbial spoilage in beverages. They may be added to beverages immediately prior to packaging, in a similar manner to existing chemical preservatives

Natural Glycolipids (AM-1) has prominent antifungal effect against common yeasts, moulds and bacteria making it a particularly useful naturally derived product to prevent microbiological spoilage of beverages (Table 1). They prolong shelf life and guarantee the microbiological quality of beverages, making them a versatile alternative or complement to other techniques such as heat treatment, which is associated with loss of nutritional value, vitamin content and taste. Natural Glycolipids may also substitute current chemical preservatives in beverages, such as sorbic acid, benzoic acid or sulphites while being applied at considerably lower concentrations in use (Table 2). Since Natural Glycolipids is also active against yeast and mould strains which have adapted to current chemical preservatives, it represents an attractive technical solution to overcome technical challenges which cannot be handled with currently approved preservative options.

Natural Glycolipids (AM-1) has good activity against Gram-positive bacteria (including spoilage organisms like *Bacillus cereus* or *Listeria spp.*) and selected Gram-negative bacteria (e.g. acetic acid bacteria) but weak or no activity against many other Gram-negative bacteria (the mechanism of action of Natural Glycolipids is discussed below in this section) (Attachment 1). Under identical test conditions (method DIN 58940-8), Minimum Inhibitory Concentrations (MICs) of 3.1 and 7.8 mg/L were determined for Natural Glycolipids against *Aspergillus niger* in SDB (Sabouraud Dextrose Broth) medium (pH 5.6) or clear apple juice medium (pH 3.3), respectively, compared to MICs of 250 mg/L (in SDB) or ≥1000 mg/L (in apple juice medium) for sorbic acid and benzoic acid, respectively (Table 2). The dose level used in these studies was 10^5 cfu/ml and all tests were performed in duplicate.

Table 1 - MIC values of Natural Glycolipids (AM-1) against common spoilage bacteria, yeasts, and molds*:

Category	Microbial strain	MIC [mg/l]
Bacteria	*Bacillus cereus* (ATCC11778)	12.5
	Bacillus subtilis (ATCC6633)	1.6
	Propionibacterium acnes (ATCC6919)	60
	Clostridium perfringens (ATCC13124)	60
	Clostridium sporogenes (ATCC3584)	50
	Enterococcus faecalis (ATCC19433)	50
	Listeria welshimeri (DSM15452)	25
	Listeria monocytogenes (ATCC19111)	50
	Lactobacillus plantarum (DSM12028)	25
	Leuconostoc mesenteroides (ATCC 8293)	6.3
	Staphylococcus aureus (ATCC6538)	25
Molds	*Aspergillus fumigatus* (ATCC1028)	20
	Aspergillus niger (ATCC16404)	6.3
	Byssochlamys fulva (DSM62097)	3.1
	Mucor plumbeus (MUCL49355)	6.3

	Talaromyces luteus (CBS348.51)	<3.9
	Dekkera bruxellensis (DSM70726)	6.3
	Dekkera naardenensis (DSM70743)	12.5
Yeasts	*Saccharomyces cerevisiae* (MUCL 53497)	12.5
	Zygosaccharomyces bailii (DSM70492)	3.1
	Zygosaccharomyces bailii (ATCC 60484)	25
	Zygosaccharomyces bisporus (ATCC 52407)	3.1
	Zygosaccharomyces bisporus (DSM70415)	12.5
	Zygosaccharomyces florentinus (DSM70506)	6.3
	Zygosaccharomyces rouxii (NCYC381)	6.3
	Candida albicans (ATCC10231)	12.5

* Standard media: Orange Serum Agar (OSA) and malt extract glucose media

Table 2 - MIC values of Natural Glycolipids (AM-1) compared to sorbis and benzoic acid

	Minimum Inhibitory Concentrations (MICs) in [mg/l]			
Organism	**AM-1**	**Sorbic acid**	**Benzoic acid**	**Conditions**
Saccharomyces cerevisiae	3.1	250	250	SDB medium, pH5.6, 28°C, 72 h
Aspergillus niger	3.1	250	250	
Zygosaccharomyces bailii	<3.9	>1000	>1000	Clear apple juice medium, pH 3.3, 28°C, 4 weeks
Dekkera bruxeliensis	<3.9	1000	250	
Aspergillus niger	7.8	1000	>1000	
Byssochlamys fulva	<3.9	500	500	

SDB = Sabouraud Dextrose Broth

Application data for AM-1 in beverages

Application data for Natural Glycolipids (AM-1) were successfully determined in more than 150 commercially available non-alcoholic beverages, proving the applicability of AM-1 in different beverage matrices and types.

Tests were designed as antimicrobial challenge tests, using the following test organisms:

Yeasts

Saccharomyces cerevisiae

Zygosaccharomyces rouxii

Zygosaccharomyces bailii

Molds

Aspergillus brasiliensis

Byssochlamys nivea

Penicillium roqueforti

AM-1 was added to the non-preserved beverages, usually as certain volume of a 10 mg/l stock solution in water. The beverage was mixed thoroughly to assure homogenous dissolution of AM-1. Then, the test organisms depicted above were added either as yeast mixture or mold mixture. In both cases, the initial inoculum was ca. 100 cfu/ml as proven by viable cell count at test start. Incubation was done at ambient temperature for three months and included benchmark controls (preserved with either benzoic or sorbic acid) and growth controls (i.e. the non-preserved beverage).

In case of flasks, tests were carried out in the original container without protection from light. In case of cans, Tetra-Pak® or other non-closable containers, the beverages were filled into sterile glass bottles before start of

the test and protected against light during the test, as would have been the case in the original container. Readout was done visually on a regular basis and – after three months – by determination of the viable cell count (cfu/ml) using the spread or pour plate method with SDB or OSA agar.

Results indicate that the minimum effective concentration of AM-1 depends on the nature of the beverage, as summarized in Table 3.

Table 3 - **Typical AM-1 use levels in different beverages**

Beverage category	**Typical AM-1 use level [mg/l]**
Carbonated soft drinks (CSD)	Cola or citrus type, incl. concentrates: 3 – 10
	10% juice, turbid: 10 – 25
Fruit drinks	Clear fruit drinks: 3 – 5
	10% juice, turbid: 5 – 25
	Spritzer, nectar, other turbid fruit drinks: 25 – 80
Teas RTD	Clear 3 – 10
	with juice content: 10 – 50
Enhanced waters	2 – 25
Energy drinks	10 – 50
Sport drinks	3 – 50
Juice	Clear juices: 5 – 10
	turbid, apple: 25
	turbid, other: 50 – 100

Comparison of antimicrobial efficacy of AM-1 with sorbate and benzoate

Efficacy against benzoate- and/or sorbate-tolerant spoilage yeasts:

Test of specific microorganisms (isolates from the beverage industry) with resistance/adaptation against sorbate and benzoate showed that, most likely due to a unique mode of action, no cross resistance to existing preservative solutions exists for AM-1.

Example: *Saccharomyces cerevisiae* strain FU74037 was isolated as a sorbate- and benzoate-tolerant strain from a commercial carbonated soft drink. The MIC value at pH 3.6 against AM-1 was found to be **3.1 mg/l**, while 200 mg/l benzoate or benzoate could not completely inhibit growth of this organism.

Comparison of 4-day MIC data in selected beverages:

Four selected commercial beverages (bought from hot fill in Germany, (i.e. without chemical preservative) were subjected to an antimicrobial challenge test as outlined in Section 5, but – deviating from the described protocol – with incubation at 28 °C for four days. Then, viable cell counts were determined using the streaking plate method on SDB agar plates (Table 4, Figure 1).

Mechanism of action

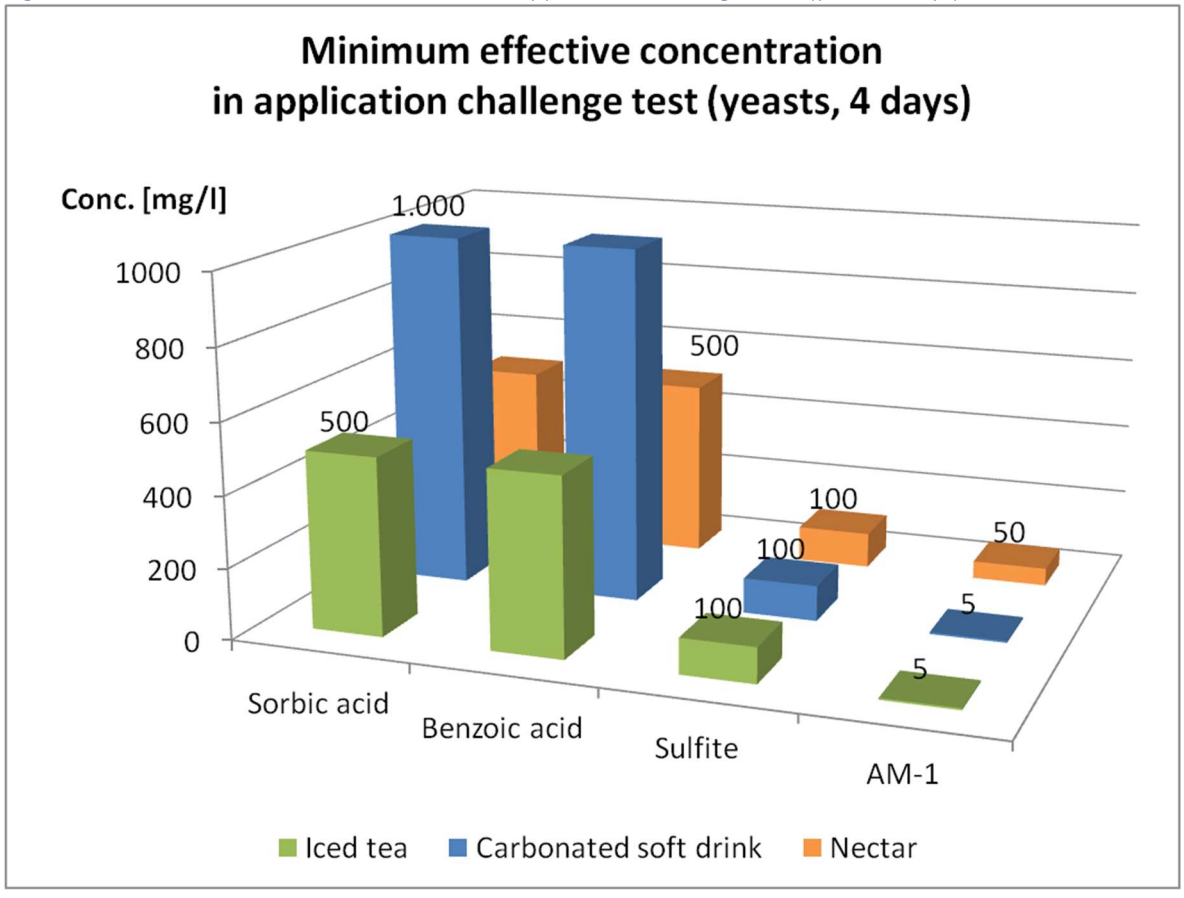
Tests of specific microorganisms (isolates from the beverages) with resistance/adaptation against sorbate and benzoate showed that the mechanism of action of Natural Glycolipids is most likely due to a unique mode of action, as there is no cross-resistance to existing preservative solutions. The mechanism is not fully understood at the present time. Literature regarding the effects of glycolipids indicate the following mode of action: Biosurfactant glycolipids alter the cell membrane, suggesting a mainly physical effect, due to changes in the permeability of the cell membranes. Consequently, metabolism is affected and the growth of microorganism is

inhibited by the glycolipids. Effects such as leakage of intracellular potassium, ATP, Cl-, Na+ and inhibition of acidification of the medium by plasma membrane ATPases have been reported (Cortés-Sánchez *et al.,* 2013; Kulakovskaya *et al.*, 2003; Mimee *et al.,* 2009; Pereda-Mirandaa *et al*., 2009; Sotirova *et al*., 2008).

Table 4: MIC values after 4 days in [mg/l] in selected beverages

	Carbonated soft drink		Nectar		Orange juice		Iced tea	
Preservative	yeasts	molds	yeasts	molds	yeasts	molds	yeasts	molds
Sorbic acid	1000	250	500	250	500	250	500	250
Benzoic acid	1000	100	500	100	2000	100	500	500
Metabisulfite	100	100	100	100	500	100	100	100
AM-1	5	5	50	25	100	50	5	3

Figure 1 – Minimum effective concentrations in application challenge tests (yeast, 4 days)



(Detailed efficacy data, including references, for Natural Glycolipids in beverages is provided in Attachment 1 (GRN 740 Appendix 1-E pages 79-86).

2.1.2 Information to enable identification of the additive

The subject of this application is the material identified as long-chain glycolipids from *Dacryopinax spathularia*, also referred to as "Natural Glycolipids" or "AM-1".

CAS Registry Number: 2205009-17-0

Molecular and Structural Formula

The major components of Natural Glycolipids are three structurally-related glycolipid congeners. Representative structure diagrams for these main components (i, ii, iii) are illustrated in Figure 2. The remaining components are congeners of the major components, sharing the saturated C26 fatty acid and the glucopyranosyl-(1→2)-xylopyranosyl-(1→2)-xylopyranosyl trisaccharide moiety but differing in the acylation pattern, i.e. the number and position of acyl groups attached to the sugar units.

2.1.3 Information on the chemical and physical properties of Natural Glycolipids

Physical Description

Natural Glycolipids is a white to off-white / ivory solid with weak, characteristic odour.

Specifications and Composition

The specifications for Natural Glycolipids are summarized below in Part 2.1.6, including a total glycolipids content of ≥ 93% (dry weight basis, calculated as sodium salt). The remaining ≤ 7% of dry weight is comprised of protein, fat, and sodium chloride.

Analytical Characterization

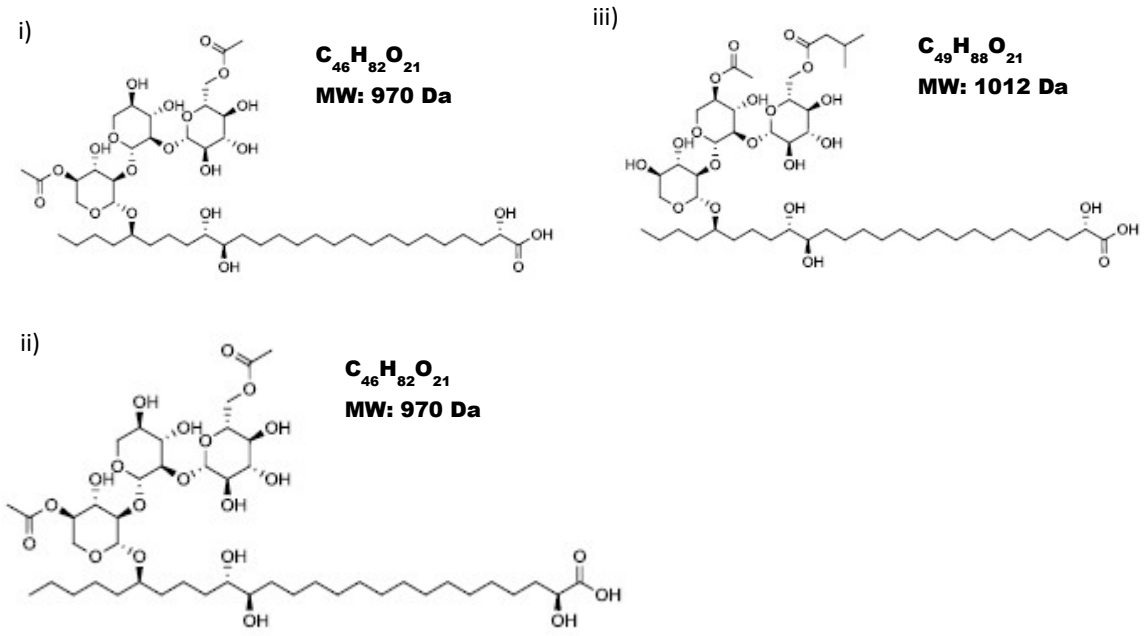
Analytical results for non-consecutive lots/batches of commercially representative Natural Glycolipids are provided in Attachment 2 and demonstrate conformance to specifications (section 2.1.6) and consistency of manufacturing.

Identity and consistency of the product is measured by an HPLC-MS method analysing the glycolipid composition. The glycolipids are clustered into eight groups by their molecular weight. The relative ratio of each glycolipid group is analysed and compared with the internal specification which fixes certain ranges for each component.

Total glycolipid content is obtained based on quantification of glucose and xylose. Glycolipids are quantitatively hydrolysed under acidic conditions and the resulting monosaccharides (glucose, xylose) are derivatized and quantified by gas chromatography using established text book standard methods. Then, total glycolipid content is recalculated based on the average molecular weight obtained from above mentioned HPLC-MS analysis.

Details of both validated analytical methods can be provided on request.

Figure 2. Representative structures for Natural Glycolipids (AM-1)



Stability

When stored as a dry powder in a closed container at temperatures of 40°C and below, Natural Glycolipids was shown to be stable for at least three years without any detectable degradation or modification of its composition (Attachment 2).

In aqueous solution, Natural Glycolipids is stable at room temperature for at least six months, with minor hydrolysis occurring for some of the glycolipid acyl moieties (<5%). Hydrolysis products are low concentrations of acetate and isovalerate. Antimicrobial efficacy is not influenced significantly. Aqueous solutions may be stored under refrigerated (4-8°C) conditions for up to 1 year. Detailed stability data for Natural Glycolipids in aqueous solution beverages is provided in Attachment 3.

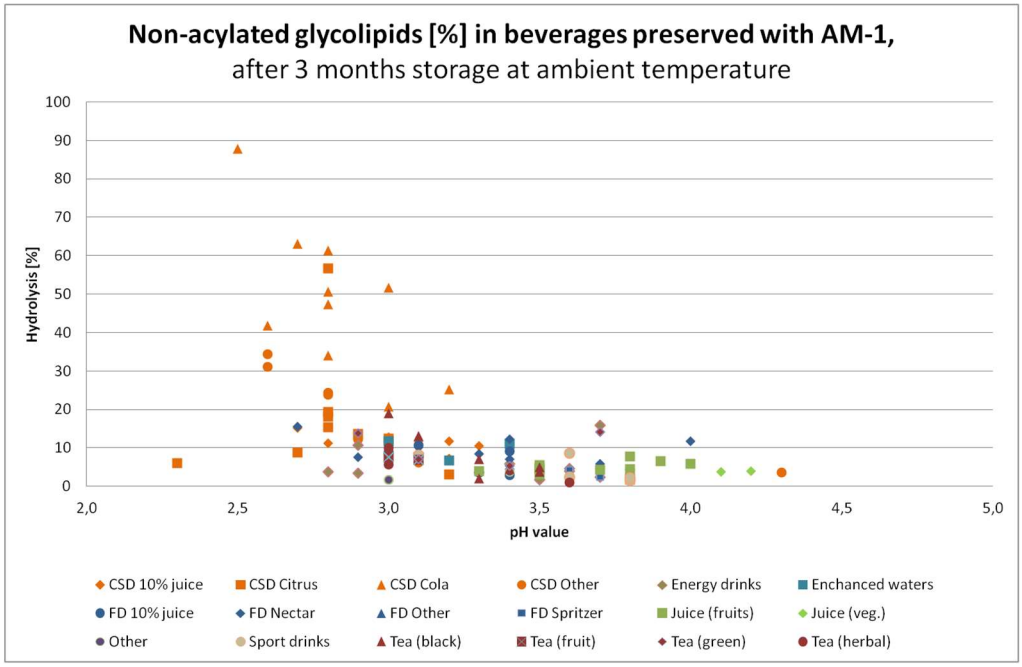
Stability of AM-1 in beverages was tested in 110 commercially obtained beverages (Attachment 3). The beverages were chosen from different categories, including carbonated soft drinks (CSD), fruit drinks (FD), Enhanced waters, sport drinks, energy drinks, syrups and various ready-to-drink teas. Storage conditions were 3 months at ambient temperature. Analysis was done by high performance liquid chromatography coupled with mass spectrometry (LCMS), being capable of differentiating the individual glycolipid congeners of the jelly mushroom glycolipid mixture.

Degradation of the glycolipids to its principal components glucose, xylose and long-chain fatty acids (LCFA) did not occur in any beverage.

Hydrolysis of ester moieties of the glycolipids was found, to a degree dependent on the pH value and cloudiness of the individual beverage (**Figure 3**). For instance, carbonated soft drinks (CSD) with low pH generally showed a higher degree of ester hydrolysis compared to cloudy fruit juices. Further, beverages with pH >3.5 showed only low degrees of ester hydrolysis.

The result of this hydrolysis process is a higher ratio of deacylated glycolipids being present in the glycolipid mixture than initially. The preserving properties of this partially deacylated glycolipid mixture are still very good, although slightly reduced compared to the initial material as shown by antimicrobial tests. Hydrolysis products are acetate and isovalerate.

Figure 3 – Percent of non-acylated glycolipids versus pH value for AM-1 in various beverages preserved with AM-1, after 3 months storage at ambient temperature.



[CSD = carbonated soft drinks, FD = fruit drinks].

There are no degradation products of safety concern associated with Natural Glycolipids, as a bulk material ingredient or when formulated in low-pH beverage applications, under typical storage and use conditions. Consistent with the hydrolysis pathway of Natural Glycolipids in the gastrointestinal tract determined in experimental studies (Bitzer *et al*., 2017a), initial hydrolysis products are acetate and isovalerate which are normal constituents of the human diet and, if absorbed, are rapidly eliminated and may be ultimately metabolized to CO_2.

2.1.4 Information on the impurity profile

Analytical results for three non-consecutive lots/batches of commercially representative Natural Glycolipids are presented below and are also provided in Attachment 2.

2.1.5 Manufacturing process

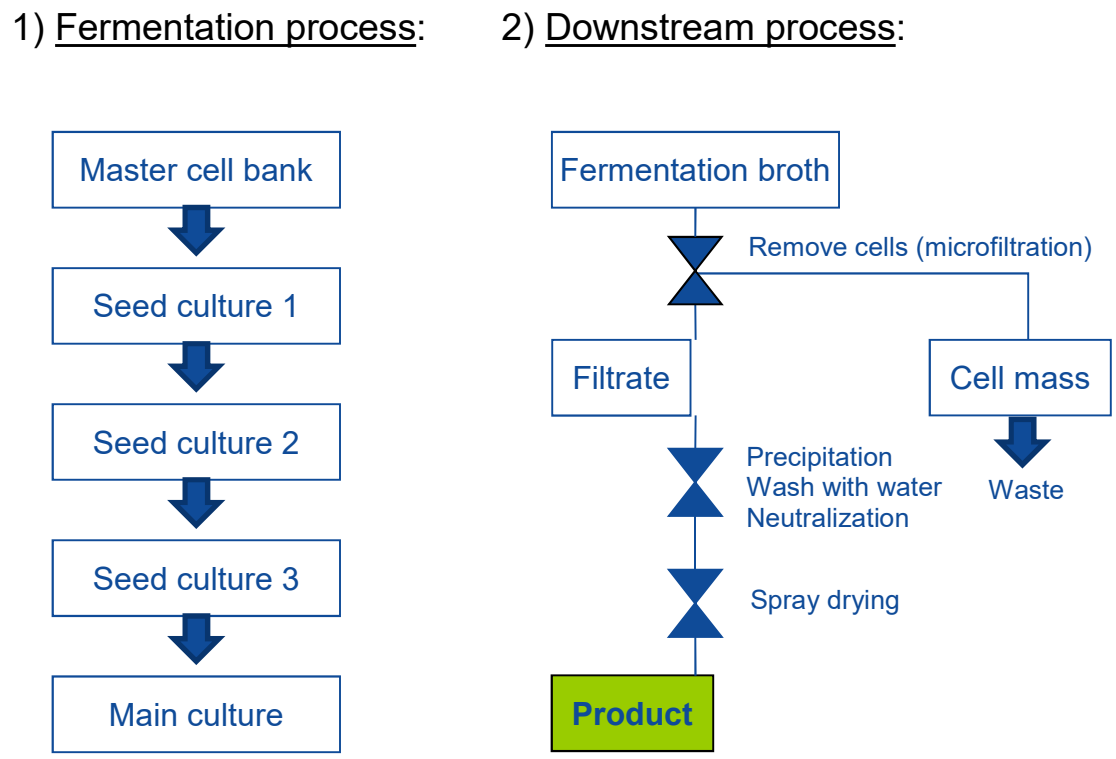
Natural Glycolipids represents a natural glycolipid mixture obtained via fermentation of glucose by the edible jelly fungus *Dacryopinax spathularia* (Schwein.). This mushroom species is also known as sweet osmanthus ear or under the scientific synonym *Cantharellus spathularius* and *Guepinia spathularia (Schwein.) Fr.*. It belongs to the phylum Basidiomycota and builds edible, orange-coloured, spatula-shaped fruiting bodies. The safety of *Dacryopinax spathularia* strain MUCL 53181 is summarised in section 2.2 (below). The producer organism is a wild-type strain without any genetic modification (non-GMO).

Fermentation of *Dacryopinax spathularia* strain MUCL 53181 is conducted using glucose as carbon source in aerobic submerged culture. Starting from a cryogenic Working Cell Bank (WCB), a series of seed cultures is conducted in shake flasks and then in bioreactors with increasing volumes in order to obtain sufficient living fungal cells to inoculate the main culture. The main culture is conducted in fed-batch mode, with start medium and feed medium both consisting of glucose and smaller amounts of yeast extract (commercial extract of autolysed Baker's yeast *Saccharomyces cerevisiae*). The culture is maintained at 30 °C for several days until maximum titre of glycolipids is achieved. The feed is stopped and the cells are further cultivated until no free glucose is found in the culture medium.

Then, the fungal cells are quantitatively removed by microfiltration, followed by acidic precipitation of the glycolipids. The precipitate is washed with water and neutralized using sodium hydroxide solution. Spray or freeze drying leads to the final product as an off-white, water soluble powder. The absence of any remaining intact fungal cells from the source organism *Dacryopinax spathularia* is technically excluded by the design of the microfiltration step and has been confirmed by viable fungal cell count (using preferred growth conditions for *Dacryopinax spathularia* strain MUCL 53181) as well as microscopic control of representative batches.

Nutrient media used in the fermentation process contain glucose and yeast extract. Hydrochloric acid and sodium hydroxide solution are used for pH adjustment during downstream processing. All nutrient components and solutions for pH adjustment are food-grade. The simple production process consists only of typical food-grade processing steps. No organic solvents are used. No chemical reaction or modification of the glycolipids is done. The production process follows good manufacturing practice and a certified quality management compliant to FSSC22000, including a Hazard Analysis and Risk-based Preventive Controls (HARPC) plan as demanded by the US FDA Food Safety Modernization Act (FSMA). A Manufacturing Process Flow Diagram for Natural Glycolipids (AM-1) is provided below in Figure 4.

Figure 4. Manufacturing Process Flow Diagram for Natural Glycolipids (AM-1)



2.1.6 Specification for identity and purity

Analytical Parameter	Acceptable Target/Range	Methods of Analysis
Appearance	Off-white to ivory powder	In-house method, based on RAL classic colour scheme
Odour	Weak, characteristic	Olfactory assessment
Aqueous solubility	> 20 g/l	Shake-flask
Turbidity (0.1% in water)	< 8 NTU	Turbidity meter
pH value (1% in water)	5.0 – 7.0	pH meter
Water content	< 5.0%	Karl Fischer (USP 921)
Total protein	< 3.0%	Kjeldahl (USP 461, N × 6.25)
Total fat	< 2.0%	Gravimetric (AOAC, 2000)
Sodium	1.7 – 3.3%	AAS (USP 852)
Total glycolipids (dry weight basis, calc. as sodium salt)	≥ 93.0%	GC, in-house method
Identity	Conforms to standard chromatogram and mass spectra	HPLC-MS, in-house method
Heavy metals	< 1 ppm (As, Cd, Hg) < 2 ppm (Ni, Pb)	ICP-MS (USP 233)

TAMC (total aerial microbial count)	≤100 CFU/g	USP 61
TYMC (total yeast/mould count)	≤10 CFU/g	USP 61
Coliforms	≤ 3MPN/g	AOAC 966.24
Escherichia coli	≤ 3MPN/g	AOAC 966.24
Salmonella spec.	absent in 25 g	AOAC 967.26, AOAC 967.27
Known allergens of concern (as listed in Standard 1.2.3-4)	None	

AAS = atomic absorption spectroscopy; AOAC = Association of Official Analytical Chemists; CFU = colony forming units; GC = gas chromatography; HPLC-MS = high-performance liquid chromatography-mass spectrometry; ICP-MS = inductively coupled plasma-mass spectrometry; MPN = Most Probable Number; NTU =Nephelometric Turbidity Unit; USP = United States Pharmacopeia

2.1.7 Information for food labelling

Natural Glycolipids is intended to be used as a preservative and would be identified on food labels as such. There is currently no agreed food additive code number for Natural Glycolipids. The applicant proposes the use of the Food Additive Number 246 (the same number will also be requested in the EU approval dossier currently in preparation) and the prescribed name "natural glycolipids" (the name to be used in the USA).

2.1.8 Analytical method for detection

Natural Glycolipids is a mixture of glycolipids from the edible mushroom *Dacryopinax spathularia*. The glycolipids share the same core structure (hydroxylated fatty acid attached to a trisaccharide) and differ in the acylation pattern of the trisaccharide having acetyl and/or isovaleryl groups at different positions.

Quantification is done by alkaline saponification (in order to simplify the mixture) followed by LCMS-based quantification of the main product. Accuracy of the method is typically estimated as ± 15%. It is suitable for a Natural Glycolipids content of 2 - 30 ppm. For higher concentrations, samples have to be diluted accordingly. For more details please refer to Attachment 4.

Natural Glycolipids can be detected and quantified in application (i.e. in beverages) by a robust HPLC-MS method (Attachment 5). While being a very sensitive detection method, mass spectrometry also assures high selectivity for Natural Glycolipids independent from the beverage matrix by focusing on the relevant molecular weights only.

2.1.9 Potential additional purposes of the food additive when added to food

There are currently no other proposed purposes for Natural Glycolipids.

2.2 Information related to the safety of the food additive

The safety of Natural Glycolipids is summarised in detail in Attachment 6 Section 6.

Historical Consumption

Glycolipids are part of the normal human diet with the most abundant sources of glycolipids identified as eggs and dairy products, cereals, and soybeans[1].

The fruiting bodies of the Natural Glycolipids production organism *Dacryopinax spathularia* (syn.: *Cantharellus spathularius*) are edible and evidence for their traditional use as food in many countries in Asia and Africa is

[1] Leray, C. 2015. Lipids: Nutrition and Health. CRC Press, Taylor & Francis Group. Boca Raton,FL, pp.154-155. ISBN 978-1-4822-4231-7.

documented. *Dacryopinax spathularia* is listed in the Food and Agriculture Organization of the United Nations (FAO) compendium on edible mushrooms[2] with proven food use documented in China, Japan, and Cameroon. Additional species of the Dacryomycetaceae family (e.g. *Dacrymyces palmatus* and *Ditiola peziziformis*) are also known to be edible and confirmed in the FAO compendium. Culinary use of the fruiting bodies is further described for Malaysia and India. The presence of Natural Glycolipids (AM-1) as constituents in collected fruiting bodies of *Dacryopinax spathularia* has been confirmed by HPLC-MS analyses; however, concentrations have not been determined.

Safety Assessment of Natural Glycolipids Production Organism

The safety of the Natural Glycolipids (AM-1) production organism *Dacryopinax spathularia* MUCL 53181 was assessed utilizing scientific procedures as outlined by Pariza and Johnson (2001)[3] for safety evaluation of microbial enzyme preparations used in food processing (Attachment 7). Using this paradigm, the jelly mushroom glycolipid ingredient was considered the "test article" that is produced by a mushroom fermentation culture, which is comparable to an enzyme preparation produced by a microbial culture. Based on the outcome of the decision tree (Attachment 7) including strain characterization, screening for undesirable attributes and metabolites, and experimental evidence of safety for the produced jelly mushroom glycolipid ingredient, it was concluded that *D. spathularia* MUCL 53181 is safe for use in the production of Natural Glycolipids as an ingredient for human consumption.

The production organism *D. spathularia* MUCL 53181 is a wild-type, not genetically modified strain (non-GMO). No genetic engineering or gene manipulation has been performed on this microorganism.

2.2.1 Overview of toxicokinetic and Toxicology Database for Natural Glycolipids

Summary

Natural Glycolipids are poorly absorbed by the oral route and are primarily eliminated in the faeces without absorption. There are no identified target tissues for residence or accumulation. Systemic exposure to Natural Glycolipids or its metabolites would be very limited following oral ingestion. A suite of in vivo and in vitro toxicity studies indicates no evidence of acute toxicity or mutagenicity. In 90 day studies in rats and dogs and in reproductive and multi-generational development studies in rats, no treatment related adverse effects were observed at the highest doses tested (1000mg/kg bodyweight/day and higher) (Bitzer et al., 2017a,b,c, Bitzer et al., 2018a, b). Furthermore, the highest doses were self-limiting due to the surfactant properties of strong aqueous solutions of Natural Glycolipids. The applicant therefore proposes that an ADI of "not specified" is appropriate for Natural Glycolipids. Alternatively, if FSANZ prefers to establish a numerical ADI, a value of 10mg/kg bodyweight/day, based on the application of a 100 fold safety factor to 1000mg/kg bodyweight/day, the maximum dose tested in the 90 day oral study in dogs, would be appropriate.

2.2.1.1 Toxicokinetics and metabolism

The pharmacokinetics, excretion balance (i.e. mass balance as a measurement of test-article equivalents in excreta samples), and tissue distribution of [14C]-Natural Glycolipids and [14C]-Long Chain Fatty Acid (LCFA) equivalents following single or repeated administration to Sprague Dawley rats, aged 8-10 weeks, were evaluated (Appendix 1-1, Bitzer *et al.,* 2017a). The study was performed in compliance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations (21 CFR Part 58) (FDA, 1987) and the study protocol was designed in general accordance with U.S. FDA Redbook II Guidelines (FDA, 1993) and OECD Testing Guideline No. 417. For all study phases, rats received equimolar doses of either [14C]- Natural Glycolipids or [14C]-LCFA via oral or intravenous (IV) administration followed by collection of biological samples (blood, urine, faeces, and expired air) at specified intervals. [14C]-LCFA constitutes the ultimate hydrolysis product of [14C]-Natural Glycolipids (after acidic and/or enzymatic saponification and deglycosylation) and was chosen as additional study item in order to follow specifically the radioactive equivalents of the lipid part of the glycolipid molecules and differentiate its metabolic fate from that of the monosaccharides (glucose, xylose) and small organic acids (acetate, isovalerate). The carcasses of select animals were also retained for processing by quantitative whole body autoradiography (QWBA).

Approximately 88% to 101% of the administered dose was recovered in expired air, urine, faeces, and carcass

[2] http://www.fao.org/docrep/018/y5489e/y5489e.pdf

[3] Pariza, M.W. and Johnson, E.A. 2001. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul. Toxicol. Pharmacol. 33, 173-186.

following single or repeated oral administration of [14C]-Natural Glycolipids at 100 mg/kg or equimolar doses of [14C]-LCFA at 46 mg/kg (Appendix 1-1, Bitzer *et al.,* 2017a). There appeared to be no difference in the excretion of Natural Glycolipids or LCFA equivalents based on sex (males versus females) or single versus repeated exposures. C_{max} and AUC_{last} for [14C]-Natural Glycolipids and [14C]-LCFA-equivalents-derived radioactivity detected by quantitative whole body autoradiography was highest in the tissues of the GI tract, as expected following oral administration. The remaining tissues had low concentrations of test article equivalents relative to the administered dose and **no target tissues for residence or accumulation were identified**. Oral bioavailability of both Natural Glycolipids and LCFA including their metabolites was low at approximately 11% (overall average for males and females combined). The pharmacokinetic, tissue distribution, and excretion balance data derived in this study (Bitzer *et al.*, 2017a) are consistent with an interpretation that following ingestion, Natural Glycolipids is partially hydrolysed to its components, glucose, xylose, acetate, isovalerate and LCFA. The expected small primary metabolites glucose, xylose, acetate, and isovalerate are expected to have a fast and high bioavailability but rapid clearance and thus to contribute marginally to the observed test article equivalents in blood and tissues after oral administration of Natural Glycolipids. **In conclusion, Natural Glycolipids and LCFA are poorly absorbed by the oral route and are primarily eliminated in the faeces without absorption. These results support an interpretation that systemic exposure to Natural Glycolipids or its metabolites would be very limited following oral ingestion. In 90 oral toxicity studies in rats and dogs, and in reproductive and multi-generational** (Appendix 1-1, Bitzer *et al.,* 2017a).

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

2.2.1.2.1 Acute toxicity

Natural Glycolipids has low acute toxicity, via the oral and dermal routes of exposure, non-irritating to the skin and eyes and non-sensitizing as outlined below. The studies are also summarised in Bitzer *et al*. (2017b).

Test	Test model details	Max dose	Conclusions	Reference
Acute oral toxicity and LD50	female rats (WISTAR Crl: WI(Han))(OECD Testing Guideline No. 423) n=3	2,000 mg/kg bw	No adverse effects	Appendix 1-2
Acute Dermal Toxicity and Irritation	*in vitro* 3D-tissue cultures of Human Reconstructed Epidermis (RHE 42.BIS-OECD Testing Guideline No. 439)	Dissolved in 100% DMSO . Applied as 2% DMSO solution in saline (16 ± 0.5 µL)	non-irritant	Appendix 1-3
In vitro Skin Corrosion Test	*in vitro* Human Skin Model Test with EpiDerm™ tissues models (OECD Testing Guideline No. 431)	0.5% (w/w) solution or 5.0% (w/w) suspension	non-corrosive	Appendix 1-4
Dermal Sensitization Study in Guinea Pigs (Buehler Method)	dermal sensitization test with Hartley albino guinea pigs - 3 groups (preliminary irritation group 4; test group 20; naive control group 10)	60% w/w mixture	not a contact sensitizer.	Appendix 1-5
Dermal Sensitization	repeat insult patch test in human (HRIPT) n=50	0.5% in distilled water	non-primary irritant and non-primary sensitizer to skin	Appendix 1-6
Acute Eye Irritation	3D-tissue cultures of Human Corneal Epithelium (the HCE model)	test item was dissolved in 100% DMSO and subsequently applied as a 2% DMSO solution in saline (30 µL)	not an eye irritant	Appendix 1-7

Phototoxicity	Balb/c 3T3 cells in monolayer. (OECD Testing Guideline No. 432)		Mean Photo Effect (MPE) calculated as 0.03 - not phototoxic.	Appendix 1-8

2.2.1.2.2 Assessment of Mutagenicity and Carcinogenicity Potential for Natural Glycolipids

Based on the chemical structural characteristics of Natural Glycolipids, i.e. a glycolipid mixture with no chemically reactive groups, there is low potential for carcinogenicity or mutagenicity from dietary intake of this material. In addition, as discussed above, the metabolism profile for Natural Glycolipids is well understood (Bitzer *et al.*, 2017a) and allows one to draw the conclusion that there are no carcinogenic or mutagenic metabolites of Natural Glycolipids formed *in vivo*.

As summarised in Bitzer *et al*. (2018a), a series of three *in vitro* studies was performed to confirm the expectation that Natural Glycolipids is non-genotoxic and have low potential for carcinogenicity. The results of these studies, described below, corroborate the above conclusions based on the structure and metabolism profile for Natural Glycolipids, which are generally available (Bitzer *et al*., 2017a).

Genotoxicity Studies with Natural Glycolipids

A series of three studies was conducted on Natural Glycolipids to assess the genotoxic potential of this material. In the *in vitro* bacterial mutation assay (Ames test; OECD Testing Guideline No. 471) with NATURAL GLYCOLIPIDS, there was no evidence of mutagenic activity in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, or in the *Escherichia coli* strain WP2 uvrA, in both the absence and the presence of metabolic activation (S9-mix), at any non-cytotoxic dose level (Appendix 1-9. Three bacterial reverse mutation tests were performed. In the first and second test the test substance was dissolved at 50 mg/mL and in the third test at 200 mg/mL based on a purity of 99% (five concentrations were tested for all strains: 62, 185, 556, 1667 and 5000 µg/plate). Certain concentrations of the test material were toxic to strain TA 1537 at 185 µg/plate in ±S9 mix in the first test and to strain TA 100 at 5000 µg/plate in –S9 mix, in the second test as well to strain TA 1537 at 300 µg/plate in ±S9 mix. It was concluded that Natural Glycolipids is not mutagenic under the conditions employed in this study.

In the *in vitro* human lymphocyte study (micronucleus test; OECD Testing Guideline No. 487), the Natural Glycolipids was examined for their potential to induce micronuclei in cultured binucleated human lymphocytes, in both the absence and presence of an S9 metabolic activation system. The highest concentration tested was 5000 µg/mL (Appendix 1-10). There was no statistically significant increase in the number of binucleated cells containing micronuclei when compared to concurrent control cultures, in the presence and absence of S9, at all time points and at any of the concentrations analysed. It was concluded that, under the conditions used in this study, the test substance, Natural Glycolipids is not clastogenic and/or aneugenic to cultured human lymphocytes.

Natural Glycolipids was tested for mutagenic potential in the *in vitro* mouse lymphoma thymidine kinase assay (MLA) according to OECD Testing Guideline No. 490 and EC No. 440/2008 (Appendix 1-11). A maximum dose of 4000 µg/mL was selected as the upper dose limit for the preliminary toxicity test. A suspension of L5178Y mouse lymphoma cells was incubated with and without S9 metabolic activation at test material concentrations ranging from 52 – 4000 µg/mL. In the presence or absence of S9, Natural Glycolipids did not induce a significant increase in the mutation frequency. It was concluded that Natural Glycolipids is not mutagenic in the TK mutation test system under the experimental conditions of the study.

2.2.1.2.3 Subchronic (Repeated Dose) Toxicity Studies with Natural Glycolipids

90-Day Oral (Drinking Water Administration) Toxicity Study in Rats

The subchronic toxicity of Natural Glycolipids was evaluated in a 90-day oral study in male and female CD® Crl:CD(SD) rats, approximately 5-6 weeks of age (Appendix 1-12, Bitzer *et al*., 2017b). The study was performed in compliance with the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice Regulations (OECD, 1998). The study protocol was designed in general accordance with OECD Testing Guideline No. 408 “Repeated Dose 90-day Oral Toxicity Study in Rodents”. Main study groups were comprised of 20 animals/sex/group. An additional 10 animals/sex for Groups 1 and 4, designated as Recovery animals, were maintained on study for a 28-day observation period following cessation of test article

administration. The test article was diluted with tap water, at target concentrations of 0.15%, 0.5%, and 1.5% (1.5, 5.0, and 15 mg/mL, respectively) and provided to the test animals *ad libitum* for 90 days. The control animals received fresh tap water on the same schedule/regimen as the treatment group animals. Dose levels were selected based on the results of a dose range-finding toxicity study in which no test article-related signs of systemic toxicity were noted in rats treated with dose levels of 0.1%, 0.5%, or 1.0% in drinking water for 14 days.

All main study and recovery animals were randomly assigned to groups for neurological screening evaluation during pre-treatment and in study week 12. Recovery phase animals were also evaluated during study week 16. Body weights and food consumption were recorded weekly and water consumption was recorded daily. Blood samples for haematology, coagulation, and serum chemistry parameters were collected during study weeks 2 (Day 14), 6 (Day 42), and 13 (Day 91) for all main study and recovery animals, and during study week 17 (Day 119) for all recovery animals (Groups 1 and 4). Urine samples were collected over a 16-hour interval during study weeks 10 (Day 65) and 13 (Day 85) from all main study and recovery animals, and during study week 17 (Day 115) from all recovery animals. A standard listing of clinical chemistry and urinalysis parameters were analysed in accordance with the applicable testing guidelines of OECD and FDA Redbook. On Day 91 (main study, one day after the last administration of the test article) or Day 119 (recovery phase, 28 days following cessation of test article administration), a complete gross necropsy was performed on all animals euthanized under CO2 anaesthesia and exsanguinated. The organs and tissues of all main study animals of the control and high-dose (1.5% Natural Glycolipids) treatment groups and any prematurely deceased animals were examined microscopically after preparation of paraffin sections and hematoxylin-eosin (H-E) staining. The stomach of all main study animals in the low- and mid-dose (0.15% and 0.5%) treatment groups as well as of all recovery animals was examined histologically due to observations in the stomach/forestomach of the main study animals in the high-dose group. Additional microscopic examinations were performed on the heart, liver, and one kidney (males and females using Oil Red O staining) and on one testis and one epididymis (males only using periodic acid-Schiff staining) from the main study animals in the control and high-dose treatment groups.

A detailed discussion of the study results, along with supporting data tables and figures, is also provided in Bitzer *et al.* (2017b). There were no test article-related deaths or changes in behaviour or external appearance of the study animals. The decreased drinking water consumption of male and female rats in the high dose group, particular during study weeks 1 and 2, and a few later intervals, was attributed to higher viscosity, surfactant qualities and/or a decreased palatability of the drinking water containing high concentrations of Natural Glycolipids. Further, the food consumption of male and female rats in the high dose group was decreased in study week 1 and the body weight of the high-dose male animals was marginally below the body weight of the control animals from study week 1 throughout the treatment period (statistically significant during the first 6 weeks of the study only); however, bodyweights of females high dose group were unaffected. It is well established in the scientific literature that under normal circumstances rats maintain a fairly constant ratio of food intake to water intake. Thus, reduced water intake is associated with reduced food intake and a reduction in body weight gain. The differences observed were considered to be an artefact of the route of dose administration related to high viscosity, surfactancy, and reduced palatability, and not an indication of systemic toxicity of the test article.

The statistically significant differences noted for select clinical pathology parameters in the 90-day rat study (Bitzer *et al*., 2017b; Tables 3 and 4) were also regarded as secondary stress-related effects due to decreased drinking water intake and the resulting slight dehydration of the animals and not due to a direct systemic toxic effect of the test article. Consistent patterns that would suggest biological significance were not observed for several statistically significant parameters, since the results were within the laboratory's historical control data ranges, were not present in a dose-related manner, and/or lacked correlation with histopathological findings.

No adverse test article-related effects were noted on haematological and serum chemistry parameters, urinalysis, eyes or optic region, or relative (to body weight at necropsy) and absolute organ weights at any dose levels at the end of the treatment period in the present study. Macroscopic inspection at necropsy did not reveal any test article-related changes in the organs or tissues of treated male and female rats. Microscopic examination of all scheduled organs and tissues of the high-dose (1.5%) group male and female animals, gross lesions of the high dose (1.5%) group animals, and the stomach of the low- and intermediate-dose (0.15% and 0.5%) rats did not reveal morphological changes that are considered to be related to the administration of the test article.

At the end of the 4-week recovery period (limited to the high-dose and control groups) mean body weights among the high-dose (1.5%) group males and females were within the range of the control group, supporting the conclusion that the slightly decreased body weights of the high-dose group male animals during the treatment period were associated with reduced intake of the treated drinking water. Additionally, clinical pathology parameters in the high-dose group animals were comparable to control group values at the end of the 4-week recovery period with the exception of select parameters in males (RBC, LUC, MCV, MCH, albumin, and Ca) or females (LUC and TPT), which appeared to be spurious statistical variation and without biological relevance due to only slight differences from controls (some of which were at the high or low end of the historical control range).

In conclusion, minor variations in some parameters (i.e. body weight, select clinical chemistry) evaluated were considered incidental and secondary to reduced drinking water consumption / mild dehydration due to reduced palatability of the test article-treated drinking water. **The no-observed-adverse-effect level (NOAEL) for systemic toxicity was considered to be 1.5% Natural Glycolipids in the drinking water, equivalent to 1201 and 1423 mg/kg bw/day for male and female rats, respectively**. This NOAEL is corroborated by the results of a 90-day subchronic oral (capsule administration) toxicity study of AM-1 in Beagle dogs in which no adverse effects were observed at doses up to 1000 mg/kg bw/day (Bitzer *et al.*, 2017c).

90-Day Oral (Capsule Administration) Study in Dogs

The subchronic toxicity of Natural Glycolipids was evaluated in a 90-day oral capsule study in male and female Beagle dogs, approximately 4-5 months of age (Appendix 1-13, Bitzer *et al*., 2017c). The study protocol was designed in general accordance with OECD Testing Guideline No. 409 "Repeated Dose 90-day Oral Toxicity Study in Non-Rodents". Study groups were comprised of 4 animals/sex/group. The test article was administered by oral capsule at doses of 150, 500, or 1000 mg/kg bw/day for 90 days. The control animals received the same number of empty capsules equivalent to that used for the same sex in the high-dose group. Each animal received an approximate 5 to 15 mL flush of water using a graduated syringe following the daily administration of capsules.

The selected route of administration for this study was oral (capsule) because the oral route is the intended route of exposure. Oral capsule administration was selected for the present study to mitigate the palatability issues observed in the rat drinking water administration study (Bitzer *et al.*, 2017b). All animals were randomly assigned to groups for neurological screening evaluation during pre-treatment and in study weeks 11 and/or 12. Body weights were recorded weekly and food consumption was recorded daily. Blood and urine samples for clinical pathology evaluations (haematology, coagulation, serum chemistry, and urinalysis) were collected during acclimation (study week -1), during study weeks 2 and 6, and at the scheduled necropsy (study week 13). A standard listing of clinical chemistry and urinalysis parameters were analysed in accordance with the applicable OECD testing guidelines. A complete gross necropsy was performed on all animals. The organs and tissues of all animals of the control and high-dose treatment groups were examined microscopically after preparation of paraffin sections and hematoxylin-eosin (H-E) staining. Gross lesions were examined microscopically from all animals. A detailed discussion of the study results, along with supporting data tables and figures, is provided in Bitzer *et al*. (2017c). There were no test article-related deaths or changes in behaviour or external appearance of the study animals. Clinical observations attributed to the capsule administration method included emesis, clear or frothy (white) material around the mouth and salivation. Additionally, abnormal (soft or mucoid) faeces and diarrhea were noted occasionally and occurred in similar incidence in the control groups; therefore, these observations were not considered test article-related. A minimal test article-related reduction in body weight compared to the control group (not statistically significant at any weekly interval) was observed in the high-dose (1000 mg/kg bw/day) treatment group females (approximately 6% by study week 12). Cumulative body weight gains in the 1000 mg/kg bw/day group females were statistically significantly lower than the control group from Study Weeks 0 to 1, 0 to 12 (approximately 27%), and 0 to 13 (approximately 30%). A minimal reduction in food consumption was also observed for most weeks of dosing for the 1000 mg/kg bw/day group females compared to the control group, although not statistically significant. It may be hypothesised that the higher doses of Natural Glycolipids, at levels which have surfactant properties at high aqueous concentrations, may have made the dogs feel unwell for some time after dosing, resulting in slightly lessened appetite.

There were no other test article-related effects on food consumption or body weights noted. Statistically significant changes were noted infrequently for some haematological, serum chemistry, or coagulation parameters; however, these differences were not considered test article-related because they were minimal and/or lacked a dose response, or values were similar to those measured during acclimation or were within

the laboratories historical control ranges. No test article-related effects were noted on the urinalysis parameters

The microscopic examination of all scheduled organs and tissues of the high-dose group male and female animals and gross lesions from all animals showed no signs of test article related histologic alterations. Histologic changes were considered to be incidental findings or related to some aspect of experimental manipulation other than administration of the test article.

In conclusion, oral administration of Natural Glycolipids to sexually mature Beagle dogs at dose levels of 150, 500, and 1000 mg/kg bw/day for a minimum of 91 days was well tolerated at all dosages.

Test substance-related changes were limited to minimal effects on food consumption and body weights in the 1000 mg/kg bw/day group females. **Therefore, the no-observed-adverse-effect level (NOAEL) for this study was considered to be 1000 mg/kg bw/day, the highest dosage level tested. This NOAEL is comparable with the NOAEL from the 90-day oral (drinking water administration) toxicity study in rats of. 15 mg/mL, equivalent to 1201 and 1423 mg/kg bw/day for male and female rats, respectively** (Bitzer *et al*., 2017b).

2.2.1.2.4 Assessment of Reproductive and Developmental Toxicity Potential for Natural Glycolipids

Natural Glycolipids is poorly absorbed via the oral route (~11% oral bioavailability) and there was no accumulation in the reproductive organs of rats given single or repeated oral doses via gavage (Bitzer *et al*., 2017a). In addition, as discussed above, repeated oral administration had no effect on reproductive organ weights of rats or dogs, and there were no test-article related macroscopic or microscopic findings in reproductive organs (Bitzer *et al*., 2017b/c). Therefore, it can be concluded that Natural Glycolipids has low potential for reproductive and developmental toxicity.

Developmental and reproductive toxicity study studies were performed to confirm the expectation that Natural Glycolipids is not a reproductive or developmental toxicant (Bitzer *et al*., 2018b). The results of these studies corroborate the above conclusion (Bitzer *et al*., 2017a/b/c).

Rat Developmental Toxicity Study with Natural Glycolipids

Natural Glycolipids was evaluated in an oral gavage developmental toxicity study in female Crl:CD(SD) rats approximately 11-14 weeks of age (Charles River Laboratories Ashland Study No. WIL-294507; Appendix 1-14). The study protocol was designed in general accordance with the OECD Testing Guideline No. 414 “Prenatal Developmental Toxicity Study”. Study groups were comprised of 24 successfully mated female rats/group. The test material was diluted with the vehicle (reverse osmosis-treated water) to achieve concentrations of 15, 50, and 100 mg/mL (adjusted based on the glycolipid content of 95%). Doses were administered at a dose volume of 10 mL/kg by oral gavage to female rats from Gestation Days 6-19 resulting in final dose levels of 150, 500, and 1000 mg/kg bw/day. Control animals received vehicle only on the same schedule/regimen as the treatment group animals. The test article formulations were confirmed to be stable under the conditions of storage during the study and met the laboratory defined acceptance criteria for concentration and homogeneity of suspensions. The test article was not detected in the analysed vehicle formulation that was administered to the control group. The selected route of administration for the definitive developmental toxicity study was oral (gavage) because this is a potential route of exposure for humans and the typical route employed for this study design.

Individual clinical observations were recorded daily during Gestation Days 0–20 (prior to dose administration during the treatment period). Animals were also observed for signs of toxicity 1–2 hours following dose administration. Body weights and food consumption were recorded on Gestations Days 0 and 6-20 (daily). All rats were euthanized on Gestation Day 20 by carbon dioxide inhalation and a laparohysterectomy and macroscopic examination was conducted according to the above-referenced testing guidelines and standard laboratory procedures for developmental and reproductive toxicity studies. Foetuses were examined for external, visceral, and skeletal malformations and developmental variations.

All females in the 150, 500, and 1000 mg/kg bw/day groups survived to the scheduled necropsy on Gestation Day 20. Rales were noted in a dose-dependent manner 1–2 hours after dose administration in the 150, 500, and 1000 mg/kg bw/day groups sporadically throughout the treatment period (Gestation Days 6–19). Clear and/or red material around the nose and/or mouth were noted in the 500 and 1000 mg/kg bw/day groups 1–2 hours after dose administration sporadically during Gestation Days 7–16. The rales and clear and/or red material findings were considered test article-related but not adverse because they generally did not persist to the daily examination the following day and there were no corresponding signs of systemic toxicity at any dosage level. Thus, these observations were likely an artefact of the oral gavage route of administration. There

were no test article-related effects on body weights, body weight gains, gravid uterine weights, net body weights (the Gestation Day 20 body weight minus gravid uterine weight), or net body weight gains (the Gestation Day 0–20 body weight change exclusive of the gravid uterine weight) in the 150, 500, and 1000 mg/kg bw/day groups or food consumption in the 150 mg/kg bw/day group. Lower mean food consumption was noted during Gestation Days 6–9 and 15–20 for the 1000 mg/kg bw/day group and Gestation Days 15–20 for the 500 mg/kg bw/day group compared to the control group. These changes at 500 and 1000 mg/kg bw/day were considered test article-related but not adverse because the effects were transient and not of sufficient magnitude to impact mean absolute body weights. At the scheduled necropsy on Gestation Day 20, no test article-related macroscopic findings were noted for females at any dosage level. Intrauterine growth and survival and foetal morphology in all test article-treated groups were unaffected by maternal test article administration.

In conclusion, given the lack of any adverse test article-related effects on survival, clinical observations, body weights or food consumption, necropsy, intrauterine growth and survival, or foetal morphology, **a dosage level of 1000 mg/kg bw/day (the highest dosage level evaluated) was considered to be the no-observed-adverse-effect level (NOAEL) for maternal toxicity and embryo/fetal developmental toxicity** when Natural Glycolipids was administered orally by gavage to bred female Crl:CD(SD) rats (Appendix 1-14).

Rat Two-Generation Reproduction Toxicity Study with Natural Glycolipids

AM-1 was evaluated in an oral gavage two-generation reproductive toxicity study in male and female Crl:CD(SD) rats (Charles River Laboratories Ashland Study No. WIL-294508, Appendix 1-15). The study protocol was designed in general accordance with the OECD Testing Guideline No. 416 “Two-Generation Reproduction Toxicity Study”.

The experimental design for this study consisted of 3 test article-treated groups and 1 control group, composed of 25 rats/sex/group. The selected animals were approximately 6 weeks old at the initiation of test article administration. During cohabitation, the rats were paired (1 female to 1 male). Following positive evidence of mating, or at the end of the 14-day mating period, the F0 and F1 females were individually housed until weaning on Lactation Day 21. The weaned F1 pups selected as parents for the next generation were housed 2–3 per cage by sex until pairing.

The test article (Natural Glycolipids) was diluted with the vehicle (reverse osmosis-treated water) to prepare dose formulation concentrations of 15, 50, and 100 mg/mL (adjusted based on the glycolipid content of 95%). Doses were administered at a dose volume of 10 mL/kg by oral gavage resulting in final dose levels of 150, 500, and 1000 mg/kg bw/day.

F0 and F1 males and females were dosed once daily for a minimum of 70 consecutive days prior to mating. Dose administration for the F0 and F1 males continued throughout mating and through the day prior to euthanasia. The F0 and F1 females continued to be dosed throughout mating, gestation, and lactation, through the day prior to euthanasia. F0 males and females were dosed for 128–133 consecutive days and F1 males and females were directly dosed for 138–148 consecutive days. The offspring of the F0 and F1 generations (F1 and F2 litters, respectively) were potentially exposed to the test article in utero, as well as via the milk while nursing. The F1 pups selected for mating (25 sex/group) were directly administered the test article following weaning (beginning on PND 21) and dosing continued following a similar regimen as the F0 parental generation. Control animals received vehicle on the same schedule/regimen as the test article treatment group animals. The selected route of administration for the definitive study was oral gavage because this is a potential route of human exposure and allowed for precise dosing of the test article.

Individual F0 and F1 male body weights were recorded weekly throughout the study and prior to the scheduled necropsy. Individual F0 and F1 female body weights were recorded weekly until evidence of copulation was observed. Once evidence of mating was observed, female body weights were recorded on Gestation Days 0, 4, 7, 11, 14, 17, and 20 and on Lactation Days 0 (when possible), 1, 4, 7, 11, 14, 17, and 21. F0 and F1 male and female food consumption was measured weekly except during cohabitation. Following the breeding period, individual food consumption for males and for females with no evidence of mating was measured on a weekly basis until the scheduled necropsy. For females with evidence of a positive mating, food consumption was measured during gestation and lactation at the same intervals as body weight measurements. Vaginal lavages were performed daily and evaluated microscopically to determine the stage of the oestrous cycle of each F0 and F1 female for 21 days prior to cohabitation and continuing until evidence of mating was observed or until the end of the mating period. The average oestrous cycle length was determined for each animal. Vaginal lavages were also performed on the day of necropsy to determine the stage of the

oestrous cycle. The F0 and F1 animals were paired on a 1 male to 1 female basis within each treatment group after a minimum of 70 days of treatment. Positive evidence of mating was confirmed and was defined as Gestation Day 0. All females were allowed to deliver naturally and the day of parturition was designated postnatal day (PND) 0. Beginning on PND 0, pups were sexed and examined for gross malformations, and the numbers of stillborn and live pups were recorded. Individual gestation length and mating, fertility, copulation, and conception indices were calculated. Intact offspring that were found dead or euthanized in extremis (by an intraperitoneal injection of sodium pentobarbital) from PND 0 to 4 were necropsied using a fresh dissection technique. A detailed gross necropsy was performed on any pup found dead or euthanized in extremis (by an i.p. injection of sodium pentobarbital) after PND 4 and prior to weaning. To reduce variability among the litters, 8 pups/litter, 4 pups/sex when possible, were randomly selected on PND 4. Each pup received a clinical observation on PND 1, 4, 7, 14, and 21 and individual sex determination was made on PND 0, 4, 14, and 21. Individual pup body weights were obtained on PND 1, 4, 7, 14, 17, and 21. A minimum of 1 male and 1 female F1 pup/litter from each treatment group were randomly selected prior to weaning (PND 21) to comprise the F1 generation (25 rats/sex/group). To assess the maturation of the selected F1 pups, the following developmental landmarks were evaluated.

Each F1 male pup was observed for balanopreputial separation beginning on PND 35 and each F1 female pup was observed for vaginal perforation beginning on PND 25. Body weights were recorded at the age of attainment for these landmarks. Following the completion of weaning of the F1 and F2 offspring, all surviving F0 and F1 adults received a detailed physical examination, females received a vaginal lavage to determine state of oestrous and all were euthanized. Spermatogenic endpoints (sperm motility [including progressive motility], morphology, and numbers) were recorded for all F0 and F1 males, and ovarian primordial follicle counts were recorded for all F1 females in the control and high dose groups and for all F1 females suspected of reduced fertility. For females that delivered or had macroscopic evidence of implantation, the numbers of former implantation sites were recorded. The number of unaccounted-for sites was calculated and the numbers of corpora lutea and former implantation sites were also recorded for females necropsied during gestation through Lactation Day 4. The testing guideline-required list of tissues and organs were weighed and/or retained from all parental F0 and F1 rats. Microscopic evaluations were performed on select tissues for all parental F0 and F1 animals in the control and high-dose groups and for all animals found dead. Reproductive organs of the F0 and F1 adult rats suspected of reduced fertility were examined microscopically.

There were no test article-related effects on F0 or F1 parental survival at any dosage level. A few non-test article-related deaths occurred in the F0 and F1 generations which were considered the likely result of the intubation error based on macroscopic findings noted at necropsy (including dark red discoloration and/or areas of the lungs, lungs that were not fully collapsed, and/or foamy tracheal contents) and microscopic finding of inflammation, haemorrhage, and/or edema of the lungs in multiple animals that may have been related to inadvertent pulmonary aspiration of the test article during or following gavage. Test article-related increased incidences of rales and red and/or clear material around the nose and/or mouth were noted in the 150, 500, and 1000 mg/kg/day group F0 and F1 males and females compared to the control group occasionally at the detailed physical examinations or daily examinations, and more frequently at 1-2 hours following dose administration. These findings were generally noted in a dose-related manner throughout the treatment period for both generations. Red and/or clear material findings around the nose and/or mouth are common following oral gavage administration and although dose-responsive, these observations only occasionally persisted to the detailed physical examinations or daily examinations, and therefore were considered non-adverse in the absence of other signs of systemic toxicity. The increased occurrence of rales 1-2 hours following dose administration was attributed to the surfactant properties of the test article combined with oral gavage dosing and was not considered adverse. Similar observations of respiratory distress related to aspiration of an irritant dosing material, especially following treatment with the more concentrated/viscous suspensions at higher doses, were reported for instance in rat and rabbit developmental toxicity studies conducted with ethyl lauroyl arginate (i.e. LAE) via gavage (EFSA, 2007[4]).

No test article-related effects on mean body weights and body weight gains (including body weights during gestation and lactation) were noted in the 150, 500, and 1000 mg/kg/day group F0 males and females or the F1 females. Test article-related lower mean body weight gains were noted in the 150, 500, and 1000 mg/kg/day group F1 males during PND 21–28, when pups were just weaned and first receiving oral gavage

[4] EFSA (European Food Safety Authority). 2007. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commission related to an application on the use of ethyl lauroyl arginate as a food additive. The EFSA Journal 511, 1-27. https://www.efsa.europa.eu/en/efsajournal/pub/511

doses of the test article. Mean body weight gains in these groups were generally similar to the control group throughout the remainder of the study. As a result of the initial lower mean body weight gains during PND 21 28, mean body weights for F1 males were between 4.0% and 7.1% lower than the control group throughout the study. These differences were not considered adverse because the mean body weights in these groups at termination (PND 161) were only 5.4% to 6.1% lower than the control group, demonstrating that the initial effects were ameliorated over the course of the generation.

Mean food consumption evaluated as g/animal/day, and food efficiency in the 150, 500, and 1000 mg/kg/day groups for the F0 and F1 males and females was unaffected by test article administration throughout the study (including gestation and lactation). No test article-related effects on F0 and F1 reproductive performance, including mating and fertility, male copulation and female conception indices, estrous cycle lengths, pre-coital intervals, gestation length, the process of parturition, and spermatogenesis parameters (motility, progressive motility, testicular and epididymal sperm concentration, sperm production rate, and the percentage of morphologically normal sperm), were observed at any dosage level. In addition, there were no test article-related macroscopic or microscopic findings or effects on organ weights noted for F0 and F1 males and females at any dosage level, or on the F1 female primordial follicle counts.

The mean number of pups born, live litter size, percentage of males per litter at birth, and postnatal survival through PND 21 were unaffected by administration of the test article to the F0 and F1 parental animals at all dosage levels. The F1 and F2 pups did not display any effects of test article exposure as evaluated by their general physical condition, body weights, necropsy findings, or organ weights.

Based on the lack of effects on F0 and F1 reproductive performance (mating, fertility, copulation and conception indices, oestrous cyclicity, and spermatogenic endpoints), **the no-observed-adverse-effect level (NOAEL) for parental reproductive toxicity of NATURAL GLYCOLIPIDS when administration orally via gavage to Crl:CD(SD) rats was 1000 mg/kg/day, the highest dosage level tested.** There were no adverse effects on survival, clinical observations, body weight or food consumption parameters, macroscopic or microscopic findings, or organ weights for F0 or F1 males and females at any dosage level. Based on these results, a dosage level of 1000 mg/kg/day was considered to be the NOAEL for F0 and F1 parental systemic toxicity. There were no test article-related effects on F1 and F2 postnatal survival, development, or growth during the pre-weaning period, and therefore, the NOAEL for neonatal toxicity was considered to be 1000 mg/kg/day (Appendix 1-15).

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

There are currently no safety assessment reports prepared by government or international agencies. Dossiers for approval in the EU and Canada are currently in preparation and are expected to be submitted to the relevant authorities during 2019. Natural Glycolipids has been affirmed as GRAS in the USA GRAS GRN 740 (Attachments 6 & 8).

PART 3Information related to the dietary exposure to the food additive

3.1 Food groups and foods proposed to contain the food additive, or changes to currently permitted foods

S15-5 Food Category Number	**Food group**
14.1	Non-alcoholic beverages and brewed soft drinks
14.1.2	Fruit and vegetable juices and fruit and vegetable juice products
14.1.3	Water based flavoured drinks, including electrolyte drinks, brewed soft drinks and formulated caffeinated beverages
14.1.4	Formulated Beverages
14.1.5	Ready to drink coffee, coffee substitutes, tea, herbal infusions and similar products
14.2	Non-alcoholic beers*.

* Non-alcoholic beers are included on the premise that The ANZ Food Standards Code allows products with identical composition to be represented and sold as either brewed soft drinks or non-alcoholic beers. This is relevant in a retail context due to potential differences between State & Territory liquor licensing laws regarding the sale of non-alcoholic beer products through un-licenced outlets. The technological justification is identical *for these beverages* irrespective of the label representation.

3.2 The maximum proposed level or the concentration range of the food additive for each food group or food, or the proposed changes to the currently permitted levels

Preferred option (based on ADI “not specified”

S16-2	Additives permitted at GMP	GMP

Alternative option based on numerical ADI

S15-5 category number	Food category name	Proposed max level of use (mg/kg)	Details of use in specific foods within category			
					Maximum use level (mg/kg)	Average use level (mg/kg)
14.1.2	Fruit and vegetable juices and fruit and vegetable juice products	100	14.1.2.1	*Fruit and vegetable juices (clear)*	20	8
				Fruit and vegetable Juices (turbid)	100	75
				Fruit and vegetable nectars (clear)	20	4
				Fruit and vegetable juices (turbid)	100	55
			14.1.2.2	*Fruit and vegetable juices products*	50	15

				(carbonated drinks with >7% juice)		
				Fruit and vegetable juices products (non-carbonated drinks with >7% juice)	50	15
14.1.3	Water based flavoured drinks	50	14.1.3	*Carbonated soft drinks, sports drinks and electrolyte drinks, Formulated caffeinated beverages.*	20	5
				Non-carbonated soft drinks, sport drinks and electrolyte drinks, Formulated caffeinated beverages.	20	5
				Carbonated fruit juice drinks	50	15
				Non-carbonated fruit juice drinks	50	15
				Cordials and concentrates (liquid & solid) for water based flavoured drinks	20	5
			14.1.3.1	Brewed soft drink	30	7
14.1.4	Formulated Beverages	20		*Formulated Beverages*	20	5
14.1.5	Coffee, coffee substitutes, tea, herbal infusions and similar products	10		*Non-carbonated ready to drink teas*	20	5
14.2.1	Beer and related products	100		*Non-alcoholic beer (<0.5% abv)*	100	

The use of Natural Glycolipids as a preservative is self-limiting at levels well below those found to be safe through animal studies, as discussed in Part 2.2 above. Within each identified beverage category above, the actual level of use required varies based on the beverage matrix.

3.3 Information on the likely level of consumption for foods or food groups not currently listed in the most recent Australian or New Zealand National Nutrition Surveys (NNSs)

The application does not seek approval for food groups not included in the most recent Australian or New Zealand NNS.

3.4 The percentage of the food group in which the food additive is proposed to be used or the percentage of the market likely to use the food additive

The product is expected to be launched in 2019 in the US. At present there are no real time data on the use of Natural Glycolipids. However, it is anticipated that a majority of foods in the market will continue to use existing preservatives or processing technologies.

3.5 Information relating to the use of the food additive in other countries, if applicable

Natural Glycolipids is approved for use in select non-alcoholic beverages in the USA (GRN 740) but as indicated above has not yet been introduced to the market.

There are published reports of the consumption of the fruiting bodies of *Dacryopinax spathularia*, also known as sweet osmanthus ear, *Cantharellus spathularius or Guepinia spathularia*, across the world (Boa 2004) and particularly in India (Ao *et al.,* 2016), China (Zhishu *et al.,* 1993, Malaysia (Lee *et al.,* 2009), South Cameroon (VanDijk *et al,.* 2003) and the USA (Meuninck 2017). However, the mushroom is not available as a commercial product. Its use as food is restricted to collection of wild growing mushrooms in the aforementioned countries. The glycolipids described in this application are also present in the edible fruiting bodies (internal data, not published yet).

3.6 For foods where consumption has changed in recent years, information on likely current food consumption

The beverage categories identified in Part 3.1 above have well established patterns of consumption.

PART 4Assessment procedure

The applicant considers that assessment under the general level procedure would the appropriate for this application.

PART 5Confidential Information

5.1 Confidential commercial information (CCI)

The application does not contain Commercial Confidential Information.

5.2 Other confidential information

The application does not contain other, non-CCI, confidential information.

PART 6Exclusive capturable commercial benefit (ECCB)

The applicant does not hold intellectual property rights in Australia or New Zealand to the preparation of Natural Glycolipids nor their use as a preservative. Consequently, this application is not expected to confer an exclusive capturable commercial benefit on the applicant.

PART7 International and other national standards

7.1 International Standards

There are currently no Codex standards relating to the use of Natural Glycolipids as a food additive preservative.

Dacryopinax spathularia is listed in the Food and Agriculture Organization of the United Nations (FAO) compendium on edible mushrooms[5].

7.2 Other national standards or regulations

Other than GRAS status is the USA, there are currently no other national standards or regulations approving the use of Natural Glycolipids.

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[5] http://www.fao.org/docrep/018/y5489e/y5489e.pdf

PART 8Statutory declaration

Statutory declarations meeting both Australian and New Zealand legislative requirements are included in Appendix 3

PART 9Checklists & Ministerial Policy Guidelines

General requirements (3.1.1)

Check	Page No.	Mandatory requirements
		A Form of application
		☑ *Application in English*
		☑ *Executive Summary (separated from main application electronically)*
☑		☑ *Relevant sections of Part 3 clearly identified*
		☑ *Pages sequentially numbered*
		☑ *Electronic copy (searchable)*
		☑ *All references provided*
☑	1	B Applicant details
☑	2	C Purpose of the application
		D Justification for the application
☑	2	☑ *Regulatory impact information*
		☑ *Impact on international trade*
☑	3-23	E Information to support the application
		☑ *Data requirements*
		F Assessment procedure
		☑ *General*
☑	24	☐ *Major*
		☐ *Minor*
		☐ *High level health claim variation*
		G Confidential commercial information
☑		☐ *CCI material separated from other application material*
	na	☐ *Formal request including reasons*
		☐ *Non-confidential summary provided*
		H Other confidential information
☐	na	☐ *Confidential material separated from other application material*
		☐ *Formal request including reasons*
☑	24	I Exclusive Capturable Commercial Benefit
		☑ *Justification provided*
		J International and other national standards
☑	24	☐ *International standards*
		☐ *Other national standards*
☑	25	K Statutory Declaration

☑	26	L Checklist/s provided with application ☑*3.1.1 Checklist* ☑*All page number references from application included* ☑*Any other relevant checklists for Chapters 3.2–3.7*

Food additives (3.3.1)

Check	Page No.	Mandatory requirements
☑	3	A.1 Nature and technological purpose information
☑	6	A.2 Identification information
☑	7	A.3 Chemical and physical properties
☑	9	A.4 Impurity profile
☑	9	A.5 Manufacturing process
☑	10	A.6 Specifications
☑	11	A.7 Food labelling
☑	11	A.8 Analytical detection method
☑	11	A.9 Additional functions
☑	12	B.1 Toxicokinetics and metabolism information
☑	13	B.2 Toxicity information
☑	20	B.3 Safety assessments from international agencies
☑	21	C.1 List of foods likely to contain the food additive
☑	21	C.2 Proposed levels in foods
☑	22	C.3 Likely level of consumption
☑	22	C.4 Percentage of food group to contain the food additive
☑	23	C.5 Use in other countries (if applicable)
☐	n/a	C.6 Where consumption has changed, information on likely consumption

Ministerial Policy Guideline Addition to Food of Substances other than Vitamins and Minerals

Specific Order Policy Principles – Technological Function

The addition of substances other than vitamins and minerals to food where the purpose of the addition is to achieve a solely technological function should be permitted where:

Policy Criteria	Section	Page
the purpose for adding the substance can be articulated clearly by the manufacturer as achieving a solely technological function (ie the 'stated purpose'); and	1.2 2.1.1	2 3
the addition of the substance to food is safe for human consumption; and	2.2	11

the amounts added are consistent with achieving the technological function ; and	2.1.1 3.2	3 21
the substance is added in a quantity and a form which is consistent with delivering the stated purpose; and	2.1	3

Attachments

Attachment 1 Efficacy data for Jelly Mushroom Glycolipids (AM-1) (Attachment 1- AM-1_Efficacy data_20180711.pdf)

Attachment 2 AM-1 Certificates of Analysis and Stability (Attachment 2 - AM-1 Certificates of Analysis and Stability.pdf)

Attachment 3 Stability of Jelly Mushroom Glycolipids (AM-1) in Beverage Applications (Attachment 3 - AM-1_Stability in beverage applications_20180711.pdf)

Attachment 4 Glycolipid quantification (Attachment 4 - Nagardo Quantification.pdf)

Attachment 5 Quantification of Nagardo™ in beverages (Attachment 4 - Method_Quantification of Nagardo in beverages_RoW_20181221.pdf)

Attachment 6 GRN 740 GRAS Notification for Long-Chain Glycolipids from *Dacryopinax spathularia* (Attachment 6 - AM-1_GRAS Notice_26Oct2017.pdf)

Attachment 7 Characterization and Safety Evaluation of *Dacryopinax spathularia* MUCL 53181 (Attachment 7 - AM-1_Characterization and safety of the producer organism_20180711.pdf)

Attachment 8 GRN 740 – FDA letter - no further questions

Attachment 9 AM-1_Food use of Dacryopinax spathularia_201807111

Appendices

Appendix 1 Toxicology Studies Original Reports

1-1 Pharmacokinetics, Excretion Balance, and Tissue Distribution of [14C]-AM-1 and [14C]-LCFA following Administration to Rats

1-2 Acute oral toxicity in rats (Appendix 1-2 - IM-11_3_Acute oral toxicity.pdf)

1-3 Acute Dermal Toxicity and Irritation Study in human cultured lymphocytes (Appendix 1-3 Usta 2012.pdf)

1-4 *In vitro* Skin Corrosion Test (Appendix 1-4 Skin corrosion_OECD431_1561500_final-report.pdf)

1-5 Dermal sensitisation guinea pig (Appendix 1-5 Buehler-test_report_35537_final_signed.pdf)

1-6 Dermal Sensitization (Appendix 1-6 Skin irritationHRIPT_AMA_M8804O.50.INS_signed-report.pdf)

1-7 Acute Eye Irritation (Appendix 1-7 HCE_Eye-irritation_VR 51-11 B.pdf)

1-8 Phototoxicity (Appendix 1-8 Phototoxicity_OECD432_VR 54-11 B.pdf)

1-9 Bacterial reverse mutation (Ames) test (Appendix 1-9 AmesTest_Final Report_TNO V9905-07.pdf)

1-10 Human lymphoma micronucleus test (Appendix 1-10 MicronucleusTest_P9917-05 final signed report.pdf)

1-11 Evaluation of the mutagenic activity of IMD AM-1 in an in vitro mammalian cell gene mutation test with l5178y mouse lymphoma cells (Appendix 1-11 Verspeek-Rip 2016.pdf)

1-12 90 Day oral toxicity study in rats (Appendix 1-12 90 Day Oral Rats- July 20, 2015.pdf)

1-13 90 Day oral toxicity study in dogs (Appendix 1-13 294503_90-day-oral-dog_Final Report.pdf)

1-14 Oral (Gavage) Developmental Toxicity Study of IMD AM-1 in Rats (Appendix 1-14 294507_Developmental toxicity_Final Report.pdf)

1-15 Two-Generation Reproductive Toxicity in Rats (Appendix 1-15 Two-Generation Reproductive Toxicity Rats.pdf)

Appendix 2 - Original References

Ao *et al.,* 2016

Bitzer *et al*., 2017a

Bitzer *et al*., 2017b

Bitzer *et al*., 2017c

Bitzer *et al.,* 2018a

Bitzer *et al.,* 2018b

Boa 2004

Lee *et al.,* 2009

Leray 2015

Meuninck 2017

Pariza and Johnson 2001

VanDijk *et al,.* 2003

Zhishu *et al.,* 1993

Appendix 3 – Statutory Declarations & Letters of Support

3.1 Statutory Declaration – Australia

3.2 Statutory Declaration – New Zealand

3.3 Letters of Support