FOOD STANDARDS
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Te Mana Kounga Kai – Ahitereiria me Aotearoa

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Supporting document 1

Risk and technical assessment report – Application A1207

Rebaudioside M as a Steviol Glycoside from *Saccharomyces cerevisiae*

Executive summary

This application from Amyris Inc. seeks permission in the Australia New Zealand Food Standards Code (the Code) for rebaudioside M (Reb M) produced from a genetically modified (GM) *Saccharomyces cerevisiae* strain. Permitted steviol glycoside preparations are required to conform to a relevant specification in Schedule 3 of the Code, but the applicant's Reb M currently does not, due to the different method of production.

The food technology assessment concludes that Amyris's Reb M produced from the applicant's production strain of *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes meets the purity parameters of specifications currently listed in the Code, but not the specific method of production. These parameters are also consistent with international purity specifications for steviol glycosides. Its technological purpose matches that of permitted steviol glycoside preparations produced by the currently permitted methods and meets the proposed purpose as an intense sweetener food additive.

The host *S. cerevisiae* strain is neither pathogenic nor toxigenic and has a long history of food use. Analysis of the production strain confirmed the presence and stability of the inserted DNA. The final product does not contain residual protein or DNA and does not give rise to any allergen concerns.

An acceptable daily intake (ADI) of 0-4 mg/kg bodyweight for steviol glycosides, expressed as steviol, was established by FSANZ in 2008. This ADI is appropriate for Reb M produced from fermentation, as it is chemically the same as Reb M extracted traditionally from the leaves of *S. rebaudiana* Bertoni and would therefore follow the same metabolic pathway in humans. No new information has been identified subsequent to FSANZ's previous assessments that would raise concerns regarding the safety of steviol glycosides.

No potential public health and safety concerns have been identified with Amyris's Reb M produced from S. cerevisiae expressing steviol glycoside biosynthesis pathway genes.

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1 Introduction

The application from Amyris Inc. seeks an amendment to the Australia New Zealand Food Standards Code (the Code) for approval of a purified steviol glycoside mixture for use as an intense sweetener, produced from *Saccharomyces cerevisiae* expressing steviol glycoside biosynthesis pathway genes. This purified steviol glycoside product, rebaudioside M (Reb M), is primarily comprised of Reb M and may contain a mixture of other rebaudiosides in low amounts, e.g. Reb D and B.

Permitted steviol glycoside preparations are required to conform to a relevant specification in Schedule 3 of the Code. Schedule 3 includes S3—35 which contains specifications for steviol glycosides extracted from the leaves of *Stevia rebaudiana* Bertoni using hot water extraction and enzymatic conversion. There is also another specification, being S3—39, for steviol glycosides produced by fermentation, but the applicant's Reb M is not listed as a prescribed steviol glycoside, so the applicant's Reb M is currently not a permitted steviol glycoside preparation.

Steviol glycosides, including Reb M, are already permitted for use as a food additive in the Code, with maximum permitted levels (MPL) in a variety of food categories and at Good Manufacturing Practice (GMP) levels in table top sweeteners in Schedule 15. Amyris states that Reb M provides improved sweetness quality when compared to major steviol glycosides derived from leaf, such as Reb A and stevioside. The applicant also claims Reb M has similar stability to other steviol glycoside preparations, making it suitable for a wide variety of applications, functioning as a multi-purpose and low-calorie sweetener.

If approved, Amyris's Reb M will be an alternative sweetener to other steviol glycoside preparations, including other Reb M preparations produced by other methods of production.

1.1 Objectives of the assessment

The objectives of this risk assessment were to:

- determine whether the proposed purpose is clearly stated and that Amyris's Reb M achieves its technological function in the quantity and form proposed to be used as a food additive;
- evaluate any potential public health and safety issues that may arise from the use of Amyris's Reb M produced from *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes.

2 Food technology assessment

The food technology assessment of this application is similar to that of an earlier application, A1170, which also relates to the production of steviol glycosides using a microbial fermentation production method for its steviol glycoside preparation.

2.1 Identity of the substance

Steviol glycosides are a group of compounds naturally occurring in the *Stevia rebaudiana* Bertoni (stevia) plant. Reb M is a minor steviol glycoside that is present in the leaves of the stevia plant at less than 0.1%. There are a large number of steviol glycosides present in stevia leaves, though most are present in very small concentrations. The identity and properties of the various steviol glycosides is very well understood due to recent work in this field (FAO 2017, JECFA 2017).

All steviol glycosides share the same steviol backbone structure (Figure 1) but have different sugar moieties attached, as conjugated glycosides. R1 and R2 can be one or more sugar moieties, including but not limited to glucose, rhamnose, xylose, fructose, galactose and deoxyglucose, which can be attached in various combinations, quantity and orientation (FAO 2017, JECFA 2017). Reb M is a glycoside with a steviol backbone conjugated to six glucose units (three on each of the R1 and R2 positions).
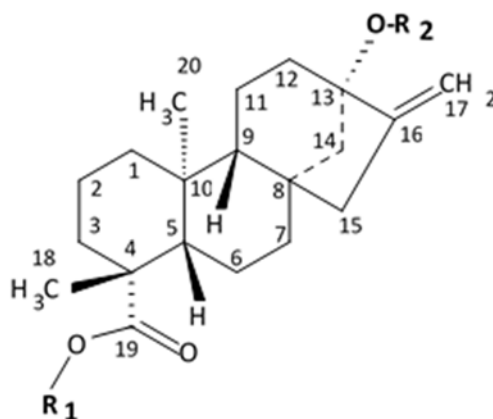

Figure 1   *Chemical backbone structure for steviol glycosides*

The chemical information for Reb M is provided in Table 1 below (FAO 2017, JECFA 2017).

Table 1 *Chemical information for Reb M*

	Reb M
Chemical name	13-[(*O*-β- D-glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl)oxy]-kaur-16-en-18-oic acid (4')-*O*-β- D-glucopyranosyl-(1,2)-*O*-[β- D-glucopyranosyl-(1,3)]-β- D-glucopyranosyl ester
Molecular formula	$C_{56}H_{90}O_{33}$
Molecular weight g mol^{-1}	1291
CAS[1] number	12206-44-2

2.2   Physical and chemical properties

The applicant's Reb M is a white to off-white powder with a characteristic sweet taste, consistent with the description of commercial steviol glycoside preparations in the most recent Chemical and Technical Assessment (CTA) published by the Food and Agriculture Organization of the United Nations (FAO)/JECFA for steviol glycosides (FAO 2016). Reb M is freely soluble in water at room temperature. Purified Reb M meets or exceeds the ≥95% steviol glycoside purity definition for steviol glycosides from *S. rebaudiana* established by JECFA (JECFA 2017).

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[1] Chemical Abstract Service

2.3 Method of production

Reb M is a purified steviol glycoside mixture that is produced from fermentation of *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes and is manufactured in accordance with current Good Manufacturing Practices (cGMP[2]). Following fermentation, Reb M is purified in accordance with the methodologies outlined in the 2016 CTA (FAO 2016). A schematic overview of the production process for Reb M is presented in Figure 2 below (taken from the application). A more detailed description of the manufacturing process, including the raw materials, processing aids and equipment used in the production process can be found on pages 24-27 of the application.
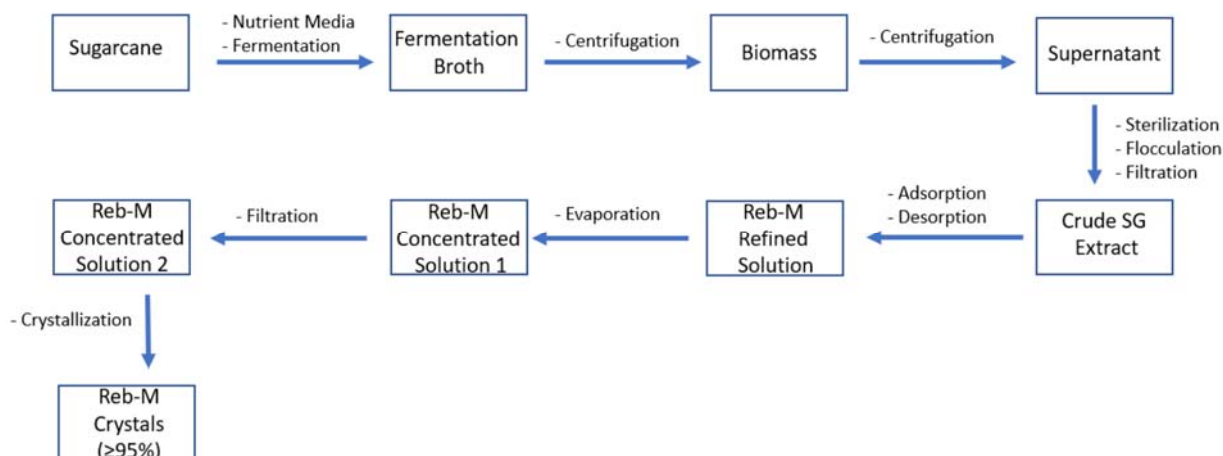

Figure 2   *Schematic of the manufacturing process for the production of Reb M by fermentation (taken from the application)*

2.4 Product stability

JECFA have concluded that "steviol glycosides, including steviol glycosides extract preparations containing higher levels of new glycosides, are thermally and hydrolytically stable for food use, including acidic beverages, under normal conditions of processing/storage" (JECFA, 2007).

The applicant also undertook specific stability testing of its Reb M preparation and confirmed its stability, in solution and as the powder under a variety of storage conditions. Liquid solutions were prepared at pH 2, 5 and 8, and stored at 4°C, 22°C, 40°C and 50°C for times of 2, 4, 6, 8, 10 and 12 weeks. The powder was stored in aluminium food grade bags at two different storage conditions, either 25°C at 60% relative humidity or 40° C at 75% relative humidity for storage times of 4, 8, 12, 24 and 36 weeks. In summary these results showed:

In solution

- at pH 2 Reb M was stable for 12 weeks at 4°C, showed some deterioration stored at 22°C after 12 weeks storage, and significant deterioration at 40°C and 50°C after only 2 weeks storage.
- at pH 5 Reb M was stable for 12 weeks at 4°C, 22°C and 40°C, and showed only some deterioration stored at 50°C after 12 weeks storage.
- at pH 8 Reb M was stable for 12 weeks at 4° C, 22°C, and showed only some deterioration stored at 40°C and further deterioration stored at 50°C after 12 weeks

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[2]cGMP are practices established by the United States Food and Drug Administration (US FDA) within its Code of Federation Regulations to ensure the safe production of food.

storage.

As powder

- some losses were noted when stored at 25°C and 60% relative humidity over the different times out to 36 weeks, but the relative percent of Reb M to total steviol glycosides was consistent.
- some losses were noted when stored at 40°C and 75% relative humidity over the different times out to 36 weeks, but the relative percent of Reb M to total steviol glycosides was consistent.

2.5   Specifications for the substance

International specifications for purity of steviol glycosides are provided within primary sources of specification within section S3—2 of Schedule 3 (Identity and purity). These are S3—2(1)(b) [the FAO JECFA Monograph], S3-2(1)(c) [the Food and Chemicals Codex], or S3—2(1)(d) [European Commission Regulation No 231/2012 (EU 2012) laying down specifications for food additives]. All these international steviol glycosides specifications stipulate that the total percentage of steviol glycosides must be greater than or equal to 95% of the preparation, on a dry basis. This is the case for the applicant's Reb M preparation.

The most current JECFA steviol glycosides monograph is monograph 20 from the 84th JECFA meeting in 2017. It is important to note that the steviol glycosides specifications from the 87th JECFA meeting in 2019 (JECFA 2019a, 2019b, 2020) have not yet been discussed by the Codex Committee on Food Additives (CCFA) or ultimately ratified by the Codex Alimentarius Committee (CAC) due to the cancelling of the CCFA 2020 meeting caused by the COVID-19 pandemic. Therefore these specifications are not yet part of the official JECFA Combined Compendium of Food Additive Specifications.

Schedule 3 also contains a specification relevant to this application, being S3—39 – Specification for steviol glycosides from fermentation. The steviol glycosides preparation of this application does not meet the definition of the 'prescribed steviol glycosides' but the purity requirements are relevant.

Details of the analysis of three non-consecutive lots of the applicant's Reb M are provided in the application and shown to meet specification purity requirements of the international specifications, as well as the purity requirements in S3—39 as summarised in Table 2.

Additional analyses were conducted by the applicant and reported to confirm the method of production and purification steps ensured no residues of protein and DNA coming from the fermentation. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were conducted to check for protein while polymerase chain reaction (PCR) analyses were conducted checking for DNA residues. Both types of analyses confirmed the absence of protein and residual DNA.

Table 2 *Comparison of the applicant's Reb M analyses compared to requirements of JECFA[a], FCC[b], EU[c] and relevant Code (S3—39) purity specifications*

Parameter	Amyris*	JECFA	EU	FCC	Code (S3—39)
Appearance/description	White powder	White to light yellow powder	White to light yellow powder	White to light yellow powder	White to light yellow powder
Purity (%) SG (dried basis)	100, 101, 100	≥ 95	≥ 95	≥ 95	≥ 95
Solubility	Freely soluble	Freely soluble in 50:50 ethanol:water	Freely soluble to slightly soluble in water	Freely soluble in 50:50 ethanol:water	Freely soluble in water
pH (1% solution)	5.5, 5.7, 5.4	4.5-7.0	4.5-7.0	4.5-7.0	4.5-7.0
Total ash (%)	0.01, 0.02, 0.02	≤1	≤1	≤1	≤1
Loss on drying (105°C, 2 hr)	1.02, 1.31, 0.10	≤6	≤6	≤6	≤6
Residual ethanol (mg/kg)	400, <200, 1600	≤5000	≤5000	≤5000	≤5000
Residual methanol (mg/kg	<100, <100, <100	≤200	≤200	≤200	≤200
Lead (mg/kg	0.042, 0.025, 0.017	≤1.0	≤1.0	≤1.0	≤1.0
Arsenic (mg/kg)	0.001, 0.003, 0.003	≤1.0	≤1.0	≤1.0	≤1.0
Cadmium (mg/kg)	0.003, 0.003, <0.002	-	-	-	≤1.0
Mercury (mg/kg)	0.001, 0.004. <0.002	-	-	-	≤1.0
Total (aerobic) plate count (CFU/g)	10, <10, <10	≤1000	-	-	-
Yeast and moulds (CFU/g)	<10, <10, <10	≤200	-	-	-
E. coli	<10, <10, <10	Negative/1 g	-	-	-
Salmonella	ND, ND, ND	Negative/25 g	-	-	-
Protein (ng/ml)	ND, ND, ND		-	-	-
DNA (pg/ul)	ND, ND, ND		-	-	-

Table notes
a Joint FAO/WHO Expert Committee on Food Additives
b Food Chemicals Codex
c European Union, European Commission Regulations No 231/2012 and 2016/1814
* Values for three non-consecutive lots of Amyris's Reb M
ND Not detected

2.6   Analytical methods for detection

There are well established internationally recognised analytical methods of detection for steviol glycosides. The applicant's analytical method of analysis of its Reb M is by reverse-phase high-performance liquid chromatography with diode array detection RP-HPLC-DAD with detection at 210nm. There are also two analytical methods of analysis using HPLC provided within the 2017 JECFA Steviol glycosides specification (JECFA 2017).

2.7   Technological purpose

Steviol glycosides extracted or derived from the leaves of *S. rebaudiana* Bertoni, including Reb M, are already permitted for use as food additives in the Code, with the International Numbering System (INS) assignation 960. The technological purpose of steviol glycosides as a food additive is that of an intense sweetener, which replaces the sweetness normally provided by sugars in food, without contributing significantly to their available energy. Reb M, similar to other already permitted steviol glycoside preparations for use in food and beverages in Australia and New Zealand, will be used as a high-intensity sweetener for the replacement of sucrose in reduced-calorie or no-sugar-added products. Steviol glycosides are permitted at various maximum permitted levels in a variety of food classes and at GMP level for tabletop sweeteners in Schedule 15. The technological purpose of this particular Reb M from the applicant does not differ from currently permitted steviol glycosides, rather it is the production method that differs.

2.8   Technological justification

The primary reason for developing alternative methods to the traditional extraction methods for steviol glycosides is that not all glycosides are produced to the same degree in the leaves of *S. rebaudiana* Bertoni. For example, stevioside is a major glycoside present in the leaves of the plant, constituting about 5 to 10% in dry leaves (JECFA, 1999), whereas Reb M is a minor glycoside and present at much lower levels. Reb M has already been assessed and approved as a steviol glycoside with improved sensory characteristics over major steviol glycosides such as rebaudioside A and stevioside (applications A1108 and A1157). It also has similar stability, making it suitable for a wide variety of food applications, functioning as a multi-purpose and low-calorie sweetener. The assessment of data and information in this application supports the earlier conclusions that Reb M has improved sensory characteristics over other steviol glycosides that are favoured by food manufacturers for use in their products. The applicant's Reb M provides an alternative commercial preparation to food manufacturers.

2.9   Food technology conclusion

The food technology assessment concludes that Amyris's Reb M produced from the applicant's production strain of *S. cerevisiae* expressing steviol glycoside biosynthesis pathway genes meets the purity parameters of specifications currently listed in the Code – but not the specific method of production. These purity parameters are also consistent with international purity specifications for steviol glycosides. The Reb M preparation is also thermally and hydrolytically stable for food use. Its technological purpose matches that of permitted steviol glycosides preparations produced by the currently permitted methods and meets the proposed purpose as an intense sweetener food additive.

3 Risk assessment

3.1 Safety assessment of the genetically modified production strain

Some information relevant to this section is Confidential Commercial Information (CCI), so full details cannot be provided in this public report.

3.1.1 History of use

3.1.1.1 Host organism

Saccharomyces cerevisiae is a yeast with a long history of safe use in the production of food, such as alcoholic beverages (brewer's yeast) and bakery products (baker's yeast). This yeast has been classed as a Biosafety Level 1 organism, based on the United States Public Health Service Guidelines[3] and has been granted Qualified Presumption of Safety[4] (QPS) status by the European Food Safety Authority (with the qualification that for yeast strains able to grow above 37ºC there is absence of resistance to antimycotics used for medical treatment of yeast infection in cases where viable cells are added to the food or feed chain). Reb M is purified after the fermentation process, and the host organism is not present in the final product, so would not enter the food chain. This was confirmed by analysis of three non-consecutive production batches of Reb M, with no protein or residual DNA detected (refer to Section 6.1.6 of the application). Therefore, there is no safety concern.

FSANZ has previously assessed the safety of a different *S. cerevisiae* strain used to produce Rebaudioside MD. In this application, the host organism is *S. cerevisiae* CEN.PK113-7D. This strain is well characterised and used for industry and academic research (Nijkamp et al 2012). The production strain was generated by genetically engineering *S. cerevisiae* CEN.PK113-7D to increase flux to the farnesyl pyrophosphate (FPP) precursor, and then adding the genes necessary to convert FPP to steviol glycosides, primarily Reb M. The taxonomy of the production strain was confirmed as *S. cerevisiae* CEN.PK113-7D via 18s rRNA sequence analysis (100% homology).

3.1.1.2 Gene donor organism(s)

The applicant has provided information about the identity and source of donor genetic material used in the construction of the production organism. All donor genes introduced into the production strain were derived through chemical synthesis where they were codon optimised for expression in *S. cerevisiae*. There is no potential for carryover of any pathogenic, toxigenic or allergenic factors from the donor organisms.

Bacterial Sources

Genes were obtained from three bacterial sources: *Escherichia coli* strain K-12*, Dickeya zeae* and *Zymomonas mobilis*. These bacteria are all considered to be Biosafety Level 1 organisms and are not associated with disease in healthy humans. *E. coli* K-12 has a history of safe use, e.g. Schedule 18 of the Code permits the use of *E. coli* K-12 as the source organism for an enzyme used as a processing aid. *Zymomonas mobilis* has a history of use as a fermenting agent of plant saps to produce alcoholic beverages (Musatti et al

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[3] For more information please see the following CDC webpage:
http://www.cdc.gov/biosafety/publications/bmbl5/index.htm
[4] For more information please see following EFSA webpage:
https://www.efsa.europa.eu/en/topics/topic/qualified-presumption-safety-qps

2018). No evidence was identified of the history of use of *D. zeae* in food.

Fungal Sources

Genetic material was obtained from the yeast *Saccharomyces kluyveri,* the soy fungus *Blakeslea trispora* and the filamentous fungus *Ashbya gossypii.* These are all considered to be Biosafety Level 1 organisms, not known to cause disease in healthy humans. *B. trispora* and *A. gossypii* have a history of use as organisms for production of β-carotene and riboflavin, respectively (EFSA 2018; Aguiar et al 2017; Rodriguez-Saiz et al 2004). *S. kluyveri* has been reported in some food products, such as selroti (fermented rice) batters and nan leaved bread (Yonzan and Tamang 2010).

Plant Sources

Genes encoding steviol glycoside biosynthesis enzymes were obtained from *Stevia rebaudiana,* a member of the daisy family (*Asteraceae*), which includes lettuce and artichoke. Evidence suggests the leaves from the Stevia plant have been used in South America to prepare sweetened teas for more than 1500 years. Thus, this plant has a long history of safe use. The leaves of *S. rebaudiana* contain steviol glycosides, which provide the sweetness to the tea.

Genes were obtained from three common food crops: *Oryza sativa* (rice), *Pisum sativum* (pea) and *Setaria italica* (foxtail millet). All three crops have a long history of safe use. For example, rice has been cultivated since ~15,000 BC (OECD, 1999). Rice and pea have been associated with food allergy in some individuals, these reactions being attributed to specific groups of proteins (OECD, 2016; Abrams and Gerstner, 2015). The proteins derived from rice and pea and found in the production organism do not raise any allergenicity concerns (refer to Section 3.1.5 below).

Genes were also obtained from *Arabidopsis thaliana* and *Picea glauca*, commonly known as mouse cress and white spruce, respectively. Although *A. thaliana* is not traditionally used as food, it is ubiquitous in the environment and is not known to be pathogenic, toxigenic or allergenic to humans. *Picea glauca* has a history of consumption in a particular sub-population in North America (USDA NRCS 2003).

3.1.2   Description DNA to be introduced and method of transformation

Genes required for the production of Reb M were introduced into the *S. cerevisiae* host using multiple DNA constructs. Each construct contained one or more expression cassettes and each expression cassette contained a gene under the control of a fungal promoter and terminator. The introduced genes encode enzymes that are used to convert FPP to steviol glycosides, primarily Reb M (Table 3), as well as improving the production efficiency of steviol glycosides.

Standard yeast transformation methods were used to insert the DNA constructs into the host's genome (Rothstein 1991). Each DNA construct contained 5' and 3' *S. cerevisiae* genomic DNA homologous to the upstream and downstream DNA sequence of a desired locus of integration in the host's genome. This allowed for site-specific integration of the DNA construct via homologous recombination (Sung and Klein 2006). Sites of integration included non-coding regions in the genome or specific genes. For example, the *HO* gene in the host was deleted by construct integration and results in a haploid negative production strain (Jensen et al 1983). This ensures the production strain will not undergo any mating events and unwanted genetic rearrangement.

Following transformations, intermediary strains were selected if they showed resistance to

antibiotics and PCR was used to verify correct integration. The parental strain was auxotrophic for histidine, leucine, tryptophan, uracil and adenine due to mutations in the HIS3, LEU2, TRP1, URA3, and ADE1 genes, respectively. In the development of the production strain, functional copies of these genes were introduced to restore prototrophy.

Table 3 *Introduced steviol glycoside enzymes and their functions*

Enzyme	Function
Geranylgeranyl pyrophosphate (GGPP) synthase	Converts FPP to GGPP
Copalyl diphosphate (CDP) synthase	Converts GGPP to CDP
Kaurene synthase (KS)	Converts CDP to kaurene
Kaurene oxidase (KO)	Converts kaurene to kaurenoic acid
Kaurenoic acid hydroxylase (KAH)	Converts kaurenoic acid to steviol
Cytochrome P450 reductase (CPR)	Works in conjunction with the P450 enzymes in pathway (KO and KAH)
UDP-glucosyl transferases (UGTs)	Adds a glucose to steviol or steviol glycosides

3.1.3   Characterisation of inserted DNA

Whole genome sequencing was performed on the final production organism. Analysis of this data showed that the genes required for steviol glycoside biosynthesis were integrated into the host genome at the specified locations, with no unintended genome rearrangements or insertions.

The final production strain was unable to grow on media containing any of the antibiotics used during transformation. This shows that all antibiotic resistance genes have been successfully removed from the production strain.

3.1.4   Genetic stability of the inserted genes

The stability of the inserted steviol glycoside biosynthesis pathway genes in the production strain was examined by colony PCR analysis. Samples before and after a 7-day fermentation were compared. It can be concluded that steviol glycoside biosynthesis pathway genes have been stably integrated into the host's genome.

3.1.5   Safety of novel proteins

Analytical results of three non-consecutive batches of the Reb M produced by fermentation that is the subject of this application, were provided by the applicant, and show that the final product does not contain residual protein or DNA.

Bioinformatics searches of the specific engineered gene constructs for Reb M production were conducted using the AllergenOnline database. Evaluation of sequence identity over a sliding 80-mer amino acid window indicated that several gene sequences had greater than 35% similarity to known allergens, but none of the sequences shared greater than 35% identity with any identified allergens over their full sequence length, indicating the unlikely potential for cross-reactivity to any known allergens. A search was also done looking for contiguous six amino acid matches with known allergens, however given the propensity for false positives using this approach, the results were not included in this assessment. It was concluded that the potential for allergenicity of any residual protein that might occur in this

Reb M is low.

3.2   Toxicological assessment

FSANZ established an ADI for steviol glycosides of 0-4 mg/kg bw/day steviol in 2008 under application A540 (FSANZ 2008). The ADI was derived by applying a 100-fold safety factor to the NOEL of 970 mg/kg bw/day (equivalent to 383 mg/kg bw/day steviol) in a two-year rat study.

The FSANZ ADI is consistent with the ADI established by JECFA at the 69th meeting held in 2008, and published in 2009. JECFA re-assessed steviol glycosides at the 82nd meeting in 2016 and confirmed the existing ADI. The assessments confirmed that steviol glycosides share a metabolic pathway to steviol. The ADI, expressed as steviol, is therefore appropriate for all steviol glycosides.

FSANZ updated the hazard assessment for steviol glycosides as a part of applications A1037, A1108, A1132, A1157, A1172, A1176 and A1183 (FSANZ 2011, FSANZ 2015, FSANZ 2017, FSANZ 2018, FSANZ 2019a, FSANZ 2019b, FSANZ 2020). These assessments did not identify a need to change the ADI.

Briefly, all known steviol glycosides share a common metabolic pathway, and are hydrolysed to steviol at similar rates. The number of sugar moieties in the glycosides, and the sugars present in those moieties, do not have any marked effect on the rate of hydrolysis. Steviol is metabolised by conjugation to steviol glucuronide, which in humans is predominantly excreted in the urine.

A number of genotoxicity studies have been conducted, using a range of assays. There is no evidence that steviol glycosides are genotoxic. On the basis of a two-year rat study of stevioside, JECFA concluded that there is no indication of carcinogenic potential. There is no evidence that purified steviol glycosides are likely to be allergenic.

3.2.1   Toxicity

The Applicant submitted a number of studies, all of which have been reviewed by FSANZ as part of previous hazard assessments of steviol glycosides.

FSANZ also conducted a literature search in PubMed and EBSCO using the search terms 'steviol' or 'rebaudioside' and 'toxic-', 'safety' or 'hazard'. No new general toxicology studies of steviol glycosides were located. However a number of *in vitro*, animal and human studies were located that address specific aspects of steviol glycoside metabolism and effects, as well as a small number of reviews. The new publications are briefly reviewed below.

3.2.1.1 Special Studies in Laboratory Animals

Of the new studies located by literature search, some relate to possible therapeutic effects of steviol glycosides and are not relevant to hazard assessment. Jia *et al* (2019) found that stevioside, at doses of 75 and 150 mg/kg bw/day, ameliorated hyperlipidaemic fatty liver in male Sprague Dawley rats in a six-week study, and a dose-response relationship was evident. No adverse effects of stevioside were observed. Similarly, a hepatoprotective effect of rebaudioside A (Reb A) was reported by Xi et al (2020) in mice with obesity induced by a high fat diet. The study was of 15 weeks duration. Although Reb A, administered in drinking water at 194 mg/L had no effect on weight gain or energy balance, when compared to fructose or sucrose added to water to equivalent sweetness, it reduced the severity of hepatic steatosis and fibrosis, reduced the levels of hepatic enzymes in serum, and also had beneficial effects on fasting glucose levels, insulin sensitivity, pancreatic islet cell mass and

other parameters of uncertain significance. Rizwan et al (2019) used gentamycin to induce nephrotoxicity in male Sprague Dawley rats, and assessed the effects of 200 mg/kg bw/day stevia (82% w/w rebaudioside A and 18% w/w stevioside), losartan, valsartan or amlodipine on renal and hepatic parameters, in a 30-day study. Treatment with stevia was associated with significant decrease in the group mean values for AST, total cholesterol and LDL, relative to rats with gentamycin-induced nephrotoxicity that received no other treatment (positive controls). Rats treated with stevia also exhibited less tubular injury and inflammation in the kidneys when compared to positive controls, and also less portal expansion, inflammation and fibrosis in the liver. The authors concluded that stevia has some renoprotective properties.

Eight-week drinking water study of low- or non-caloric sweeteners on inflammatory response and behaviour in mice (Schiano et al 2019). Regulatory status: Not GLP

The Stevia-derived sweetener used in this study was rebaudioside A. Other sweeteners included sucrose, glucose, fructose, aspartame, and sodium cyclamate. Results for glucose, fructose, aspartame and sodium cyclamate are not relevant to this hazard assessment and not summarized further. The test subjects were 12 week old male C57BL/6 mice, individually housed under standard laboratory environmental conditions. Mice were acclimatised for one week before study start. Mice were assigned to groups of 6/group. All doses were administered by oral gavage. Two control groups were administered water. Sucrose groups were administered 0.75 or 1.50 g/kg bw/day, glucose and fructose groups were both administered 0.8 or 1.6 g/kg bw/day, and low- or non-caloric sweeteners were administered at doses that corresponded in sweetness to the glucose doses. The doses of rebaudioside A were 2.8 and 5.6 mg/kg bw/day. Bodyweights were recorded on Study Days 0, 21 and 56. Blood pressure was measured before and after sweetener administrations, 20 times in each mouse with the mean of the last 10 measurements used for analysis, but the schedule of measurements is not clear. After eight weeks of treatment, carrageenan-induced paw oedema was evaluated, high-dose mice were subject to behavioural evaluations, blood was collected and mice were killed. Liver, kidney, brain and intraabdominal fat were excised, weighed and stored frozen. Blood collected prior to killing was analysed for numbers of endothelial progenitor cells (EPC) in peripheral blood and for clinical chemistry (aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, total cholesterol, high-density lipoprotein, triglyceride, total bilirubin, alkaline phosphatase, glucose). Neurological assessments included tail suspension test, forced swimming test, wire hang and marble-burying, and Y maze. Treatment with rebaudioside A was associated with a dose-related decrease in circulating EPC. Numerical data concerning EPC were not presented, but bar graphs indicate that the decrease was in the approximate range of 40 to 50% below that of controls. However following induction of inflammation by the injection of carrageenan into a paw, rebaudioside A was associated with a significant increase in EPC. Rebaudioside A was not associated with any adverse effects on inflammatory response or performance on neurological tests. The authors interpreted the reduced EPC numbers as a possible adverse effect, but the promotion of EPC numbers in response to an inflammatory stimulus to be a beneficial effect.

The relevance of this study to hazard assessment of steviol glycosides is uncertain, because measurement of EPC is not a standard parameter in risk assessment. It is not clear that the changes in group mean values of circulating EPC are of biological relevance.

10-week drinking water study of the effects of Stevia leaf extract on gut microbiota and glucose tolerance in mice (Becker et al 2020) Regulatory status: Not GLP

In this study, the effects of Stevia leaf extract were compared to those of saccharin in mice fed a high fat (60% kCal from fat) diet. The Stevia leaf extract is not well described. The experiment was conducted on C57BL/6J mice, 5/sex/group. The age of the mice at time of

receipt is not stated. Mice were individually housed under standard laboratory environmental conditions and acclimatised on a low-fat diet for 8 days prior to the start of the study. During the acclimatisation period, baseline bodyweights, food consumption and water consumption were recorded daily, faecal samples were collected for baseline characterisation of microbiota, and glucose tolerance was determined in 10 randomly selected mice. At the end of the acclimatisation period, a negative control group remained on the low-fat diet while the remaining three groups were changed to the high-fat diet. After six days on the high-fat diet, one group was supplied with drinking water containing saccharin for an intended dose of 5 mg/kg bw/day, and another was supplied with drinking water containing Stevia leaf extract for an intended dose of 5 mg/kg bw/day. A third group was given plain drinking water and acted as the positive control group. The experiment continued for 10 weeks, with food intake, body weight and water intake of each mouse recorded three times per week. At the end of the 10 week in-life phase, glucose tolerance tests were conducted on all mice, and faecal samples were collected from all mice to examine microbiota using PCR.

One female mouse in the stevia group refused food and died in Week 3 of study, but all other mice survived until the end of the study. Treatment with saccharin or stevia had no effect on group mean calorie consumption, group mean water consumption, bodyweight gain or glucose response when compared to the positive control group. Group mean water consumption was higher for the mice on the low fat diet than mice on the high-fat diet, and it was suggested that this difference might reflect higher fibre intake. Mice on the low-fat diet gained less weight than mice in other groups, as expected. Relative to mice on the low-fat diet, mice on the high-fat diet developed subclinical glucose intolerance. Feeding a high-fat diet had a greater effect on microbiota than administration of a sweetener. Consumption of a high-fat diet and a sweetener was associated with a significant increase in *Firmicutes/Bacteroidetes* ratio (F/B ratio) when compared to the feeding of a low-fat diet. Sex of the mice was also an important factor, with changes in microbiota more pronounced in females than in males. Only two taxonomic groups differed significantly between the Stevia-treated group and the saccharin-treated group. One *Lactococcus* species was more abundant in Stevia-treated mice than in saccharin-treated mice, and *Akkermansia* was more abundant in saccharin-treated mice than Stevia-treated mice. The authors concluded that supplementation with Stevia does not prevent the effects of a high-fat diet on glucose tolerance or microbiota.

This study does not contribute to the hazard assessment of steviol glycosides because the Stevia leaf extract was inadequately characterised, and because there is no evidence that the changes in glucose tolerance and in intestinal microbiota had any adverse effects on mice.

18-week study of effects of Stevia on obese Sprague Dawley rats and their pups (Nettleton et al 2020). Regulatory status: Not GLP

The maternal generation for this study were Sprague Dawley rats, 8 weeks old at time of receipt. They were kept under standard laboratory environmental conditions, and underwent a 10-week induction phase of a high fat/high sucrose diet. Study subjects were selected from the rats that gained the most weight, and assigned to three groups of 15 rats/group. One group was supplied with plain water to drink while the other two groups were provided with either aspartame (5-7 mg/kg bw) or rebaudioside A (2-3 mg/kg bw). The rats were then bred to Sprague Dawley males and maintained on the diet and assigned drinking solution throughout pregnancy and lactation. Litters were culled to 10 offspring (5/sex when possible) at birth. At 21 days of age, pups were weaned onto control diet and water, and maintained until 18 weeks of age. Maternal and pup body weights were recorded weekly. Food and fluid intake of dams was measured prior to mating and in the first and third weeks of pregnancy and lactation. Food intake of pups was measured at 6, 12 and 18 weeks of age. Oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT) were performed on dams between

days 13 and 16 of both pregnancy and lactation, and on pups at 8 and 17 weeks of age. Dams underwent dual-energy X-ray absorptiometry at weaning and were then killed, and samples were collected from caecum and brain. Cohorts of pups underwent dual-energy X-ray absorptiometry and were then killed at 3, 12 and 18 weeks of age and similarly sampled. Caecal matter collected from male pups from all three groups was gavaged into male germ-free mice aged 8-10 weeks, (10/group). Body weights were recorded at baseline and 7, 10 and 14 days after gavage. On Day 15, mice were given an OGTT scan, underwent dual-energy X-ray absorptiometry, and were killed.

In dams, treatment with aspartame or rebaudioside A (Reb A) had no effect on body weight or body composition. Pup bodyweights at birth were similar between the groups. At weaning, group mean bodyweights of female pups of dams given sweeteners were significantly greater than those of dams given water. For the Reb A group, the difference was an increase of approximately 20%. Both male and female pups in the Reb A group had increased body fat percentage when compared to the water group. However treatment with Reb A did not have a long term effect on pup bodyweight, in contrast to aspartame. Reb A treatment was associated with altered faecal microbiota in both dams and pups, although differences in microbiota between treated and untreated groups declined after weaning. Dams treated with Reb A had slightly increased blood glucose at 120 min after an insulin load, compared to dams given water, but no difference in insulin sensitivity was found during lactation. During the OGTT, male pups from dams treated with Reb A had higher glucose at 0 and 15 min than male pups from dams given water, although female pups were not similarly affected. From weaning until the end of the in-life phase at 18 weeks, male pups from Reb A-treated dams had increased levels of ventral tegmental area (VTA) dopamine transporter (DAT) mRNA, and at 18 weeks, they had greater nucleus accumbens (NAc) D2 receptor expression, as determined by mRNA levels, when compared to male pups of dams given water. Female pups of Reb A-treated dams had increased expression of NAc D2 receptors, as determined by mRNA levels, at weaning. The authors interpreted these changes as alterations in the mesolimbic reward system. Germ-free mice gavaged with caecal matter from pups of dams treated with Reb A had greater body weight gain than those gavaged with matter from pups of untreated dams, and also exhibited reduced glucose tolerance and greater deposition of body fat.

FSANZ does not consider this study provides grounds to revise the risk assessment of steviol glycosides, because there is a lack of evidence that maternal exposure to Reb A had a lasting detrimental effect on either dams or pups. Although differences in some measured parameters were observed, it is not clear that they are of any biological significance, or that similar changes would occur in human beings. Other developmental and reproductive studies of steviol glycosides in rodents, conducted according to OECD guidelines and reviewed by FSANZ as part of assessments of previous applications, have not shown any adverse effects on either dams or pups.

28-day study of the effects of sodium saccharin and rebaudioside A on ovaries of guinea pigs (Li et al 2020). Regulatory status: Not GLP

This study was conducted on female Harley-white guinea pigs, approximately 242 g at time of receipt. Guinea pigs were acclimatised to standard laboratory environmental conditions and assigned to groups of 5 animals/group. Sweeteners were administered in the drinking water. A control group was provided with plain drinking water. Sodium saccharin (SS) groups were provided with water containing 1.5 mM or 7.5 mM SS, and Reb A groups were provided with water containing 0.5 mM or 2.5 mM Reb A. Doses of sweetener were chosen to be approximately equivalent in sweetness. Food intake and water consumption were recorded each morning. Bodyweights were recorded on days 1, 7, 14, 21 and 28. Guinea pigs were examined daily and the day of vaginal opening was recorded, after which the oestrous cycle was monitored daily by vaginal sears. At the end of the in-life phase, guinea pigs were

anaesthetised with CO_2, blood was collected by cardiac puncture, guinea pigs were killed and fresh samples of uterus and ovary were collected. The right ovary and uterine horn were fixed for histopathology and immunohistochemistry, and the left ovary and uterine horn were frozen for later Western blot analysis.

Group mean food intake of the control group was significantly lower in Week 1, than that of groups given sweeteners, although numerical data are not provided. By the end of the in-life phase, there were no significant differences in group mean values for food consumption. The group provided with the high dose of SS consumed significantly more water throughout the study than the other groups, and the high-dose Reb A group consumed significantly less water than the other groups in Weeks 2 to 4. All groups administered sweeteners had higher group mean values for bodyweight gain than the control group, although the difference was not always statistically significant, and numerical data are not provided. Group mean ovary weights of treated guinea pigs were also higher than those of controls, although it is not clear whether the differences were statistically significant. Treatment with sweetener had no effect on timing of vaginal opening. At 28 days, group mean serum progesterone values of the low-dose Reb A group and the high-dose SS group were significantly higher than that of controls, although data are presented as bar graphs rather than numerical tables and differences appear to be small. There was no significant difference in group mean serum oestradiol levels between treated groups and controls. The taste receptor subunit T1R2 was markedly expressed in ovary and uterus of guinea pigs whereas the taste receptor subunit T1R3 were rarely expressed in either tissue. There was no consistent, dose-related effect of either sweetener on T1R2 expression in either tissue, relative to that observed in the control group. Expression of T1R3 in lutein cells of ovary was greater in Reb A-treated guinea pigs, and atresia of antral follicles, were more common in ovaries of Reb A-treated guinea pigs than controls, but it is not clear whether there was a dose-response relationship for either parameter. Numbers of corpora lutea were significantly increased ($p < 0.05$) in ovaries of high-dose Reb A guinea pigs, compared to controls. Treatment with Reb A had no effect on uterine morphology. Expression of T1R2 in epithelial and stromal cells of the uterus was greater in high-dose Reb A guinea pigs than in controls.

FSANZ considers that the results of this study are of uncertain relevance to risk assessment of steviol glycosides. The lack of clearly presented numerical data makes it difficult to determine whether statistically significant differences were biologically significant. The relevance of expressions of taste receptor subunits is unclear. Other developmental and reproductive studies of steviol glycosides, conducted in rodents according to OECD guidelines and reviewed by FSANZ as part of assessments of previous applications, have not shown any adverse effects on either dams or pups.

3.2.1.2 Other Studies

Two recent *in vitro* studies related to steviol glycosides were located. Park *et al* (2019) investigated the effects of non-nutritive sweeteners on the endoplasmic reticulum in a hypothalamic cell line (mHypoE-N43/5). They reported that Reb A had only moderate effects on endoplasmic reticulum stress and no adverse effects on axon outgrowth or caspase 3/7 activity, in contrast to sucralose, aspartame and acesulfame potassium. Schiano *et al* (2020) treated human umbilical vein endothelial cells for 48 hours with different sweeteners including glucose, fructose, aspartame, cyclamate, Reb A, steviol and stevioside, and examined cell morphology, angiogenesis and array gene expression. In contrast to glucose and fructose, the steviol glycosides did not damage endothelial cells.

3.2.1.3 Studies in Humans

Three-arm crossover trial of effects of Stevia extract on postprandial glucose response, satiety and energy intake (Farhat et al *2019). Regulatory status: Not GLP*

The test article for this study was described as "stevia extract" but not further characterised. Test subjects were 10 male and 20 female volunteers, ranging in age from 18 to 65 years (mean of 26 years), and in BMI from 18.5 to 29.9 kg/m^2. Participants received one of three different preloads (300 mL) on three different days, separated by washout periods of 4 to 5 days. On each test day, participants attended the clinic at 9:00 am after an 8 h fast and were given a standardised 360 kCal breakfast. Three h later (12:00 noon), they were given one of the three preloads, followed 30 min later by a pizza lunch *ad libitum*. Participants were asked to rate their hunger, desire to eat, fullness and satiety over a 180 min period before the lunch, and every 30 min after the lunch for 120 min. The preloads were water and citric acid, water and 60 g sugar, or water and 1 g stevia. Blood samples were collected at 12:00, 12:30, 13:00, 13:30, 14:00 and 14:30.

All participants completed the study. The sugar preload resulted in a higher AUC for blood glucose than the other two preloads. There was no difference in glucose AUC between the citric acid preload and the stevia preload. Postprandial blood glucose levels were significantly higher after the sugar preload but after correction for the blood glucose level measured after the preload, there was no significant difference between blood glucose levels between groups. There was no significant difference in energy intake at lunch between the different preloads. There were no significant differences in scores for satiety or fullness between preloads, after adjustment for baseline values. However participants reported higher hunger and desire to eat after the water preload than after the sugar or stevia preloads, both before and after the lunch. There were no significant differences in hunger scores or desire to eat between sugar and stevia preloads. It was concluded that stevia did not lead to energy compensation during lunch, and resulted in lower postprandial blood glucose levels than sugar.

The value of this study is reduced by the lack of adequate characterisation of the stevia extract.

3.2.1.4 Reviews

Three recent reviews were located by literature search, authored by Anker *et al* (2019), Plaza-Diaz *et al* (2020), and Ray *et al* (2020).

Anker et al (2019) conducted a systematic review and meta-analysis of studies of the effects of steviol glycosides on human health, with emphasis on markers of Type 2 diabetes. They concluded that compared to placebo, steviol glycosides were associated with significant reduction in systolic blood pressure, non-significant reductions in diastolic blood pressure, total cholesterol, and HDL-C, and non-significant increases in LDL-C and triglycerides. There was no significant effect on glycated haemoglobin.

The review by Plaza-Diaz *et al* (2020) concerned possible interactions of low- and non-calorie sweeteners with intestinal microbiota. Sweeteners included in their literature search included aspartame, acesulfame-K, cyclamate, sucralose, saccharin, steviol glycosides, erythritol, isomalt, lactitol, maltitol, sorbitol, mannitol and xylitol. The authors did not find any reports that indicated that steviol glycosides could have negative effects on intestinal flora.

Ray *et al* (2020) reviewed effects of steviol glycosides on glucose homeostasis, blood pressure and inflammation. They conclude that there is compelling evidence that steviol glycosides have beneficial effects on glucose homeostasis, markers of inflammation, lipid profiles and blood pressure.

3.3   Assessments by other regulatory agencies

There have been no new assessments by other regulatory agencies since FSANZ reviewed

A1183 in 2020. FSANZ has previously reviewed the most recent assessments of JECFA, Health Canada and the European Food Safety Authority (EFSA).

3.4 Risk assessment discussion and conclusion

No potential public health and safety concerns were identified with Amyris's Reb M as a steviol glycoside from *S. cerevisiae*.

The host *S. cerevisiae* has a long history of safe use and is neither toxigenic or pathogenic. Molecular characterisation of the genetically modified production strain confirmed both presence and stable inheritance of the inserted steviol glycoside biosynthesis pathway genes.

Residual proteins are unlikely to be present in the finished Reb M preparation. The risk of allergenicity from any residual proteins that might be present is considered to be low.

No new evidence of adverse effects of steviol glycosides has been identified that would justify a change in the ADI of 0 to 4 mg/kg bw, expressed as steviol, for steviol glycosides established by FSANZ in 2008 and JECFA at their 69th meeting and confirmed at their 82nd meeting in 2016. The ADI of 0 to 4 mg, when expressed as steviol, is therefore appropriate for the Reb M produced by enzymatic conversion that is the subject of this application.

4   References

Abrams EM, Gerstner TV (2015) Allergy to cooked, but not raw, peas: a case series and review. Allergy Asthma Clin Immunol 11(1):10

Aguiar TG, Silva R, Domingues (2017) New biotechnological applications for *Ashbya gossypii*: Challenges and perspectives. Bioengineered 8(4):309-315

Anker CCB, Rafiq S and Jeppesen PB (2019) Effect of Steviol Glycosides on Human Health with Emphasis on Type 2 Diabetic Biomarkers: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Nutrients 11:1965 doi:10.3390/nu11091965

Becker SL, Chiang E, Plantinga A, Carey HV, Suen G and Swoap SJ (2020) Effect of stevia on the gut microbiota and glucose tolerance in a murine model of diet-induced obesity. FEMS Microbiology Ecology, 96, doi:10.1093/femsec/fiaa079

EC (2012) COMMISSION REGULATION (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. Accessed 21 September 2020

EC (2016) COMMISSION REGULATION (EU) 2016/1814 of 13 October 2016 amending the Annex to Regulation (EU) No 231/2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council as regards specifications for steviol glycosides (E 960). Accessed 21 September 2020

EFSA 2018 Safety and efficacy of vitamin B 2 (riboflavin) produced by *Ashbya gossypii* ■■■■■ for all animal species based on a dossier submitted by BASF SE. EFSA J 12;16(7):e05337

FAO (2017) Steviol glycosides. In: *84th JECFA - Chemical and Technical Assessment (CTA)* [84th meeting held June 2017]. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO) / Geneva, Switz.: Joint FAO/WHO Expert Committee on Food Additives Meeting (JECFA). Available at: http://www.fao.org/3/BU609en/bu609en.pdf Accessed 21 September 2020

Farhat G, Berset V and Moore L (2019) Effects of Stevia Extract on Postprandial Glucose Response, Satiety and Energy Intake: A Three-Arm Crossover Trial Nutrients 11: 3036 doi:10.3390/nu11123036

Food Chemicals Codex 2018, United States Pharmacopeial Convention (2018) 11th ed, United States Pharmacopeial Convention, Rockville, MD

JECFA (2017) Steviol glycosides from Stevia *rebaudiana Bertoni* [New specifications prepared at the 84th JECFA, 2017), Superseding tentative specifications prepared at the 82nd JECFA (2016)]. In: *Compendium of Food Additive Specifications*. 84th Meeting, Rome, 6-15 June 2017 (FAO JECFA Monographs 20). Rome. pp. 50-69. http://www.fao.org/documents/card/en/c/4b06cdda-3e70-4c80-b7e5-56034601836b/ Accessed 21 September 2020

JECFA (2019a) Joint FAO/WHO Expert Committee on Food Additives, Eighty-seventh meeting, Rome, 4-13 June 2019. Summary and Conclusions. https://www.who.int/foodsafety/areas_work/chemical-risks/JECFA87_Summary_Report.pdf?ua=1 Accessed 21 September 2020

JECFA (2019b) World Health Organization & Joint FAO/WHO Expert Committee on Food Additives. Evaluation of certain food additives: eighty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series 1020. World Health Organization. https://apps.who.int/iris/handle/10665/330612. Accessed 21 September 2020

JECFA (2020) FAO and WHO (2020) *Compendium of Food Additive Specifications. Joint FAO/WHO Expert Committee on Food Additives (JECFA), 87th Meeting June 2019.* Contains (Framework for) Steviol Glycosides, and four annexes. FAO JECFA Monographs 23. Rome. pp 62-84 http://www.fao.org/3/ca7513en/CA7513EN.pdf Accessed 21 September 2020.

Jensen R, Sprague GF Jr, Herskowitz (1983) Regulation of yeast mating-type interconversion: feedback control of HO gene expression by the mating-type locus. Proc Natl Acad Sci USA 80(10): 3035-3039

Jia C-H, Zhang J-Y, Shen W, Zhao X and Xie M-L (2019) Attenuation of high-fat diet-induced fatty liver through PPARα activation by stevioside. Journal of Functional Foods 57: 392-398

Li J, Shen T, Shi F and Fu Y (2020) Influences of non-nutritive sweeteners on ovarian and uterine expression of T1R2 and T1R3 in peripubertal female guinea pigs. Animal Science Journal DOI: 10.1111/asj.13348

Musatti A, Mapelli C, Rollini M, oschino R, Picozzi C (2018) Can *Zymomonas mobilis* substitue *Saccharomyces cerevisiae* in ceral dough leavening? Foods 7, 61. doi:10.3390/foods7040061

Nettleton JE, Cho NA, Klancic T, Nicolucci AC, Shearer J, Borgland SL, Johnston LA, Ramay HR,4 Tuplin EN, Chleilat F,1 Thomson C, Mayengbam S, McCoy KD and Reimer RA (2020) Maternal low-dose aspartame and stevia consumption with an obesogenic diet alters metabolism, gut microbiota and mesolimbic reward system in rat dams and their offspring. Gut 0:1–11. doi:10.1136/gutjnl-2018-317505

Njkamp JF, van der Broek M, Datema E, de Kok S, Bosman L, Luttik MA, Daran-Lapujade P, Vongsangnak W, Nielsen J, Heijne WHM, Klassen P, Paddon CJ, Platt D, Kotter P, van Ham RC, Reinders MJT, Pronk JT, de Ridder D, Daran JM (2012) *De novo* sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. Microbial Cell Factories 11:36

OECD (1999) Consensus document on the biology of Oryza sativa (rice). Report No. ENV/JM/MONO(99)26. Organisation for Economic Co-operation and Development, Paris. https://www.oecd.org/env/ehs/biotrack/46815658.pdf. Accessed 18 September 2020

OECD (2016) Revised consensus document on compositional considerations for new varieties of rice (Oryza sativa): key food and feed nutrients, anti-nutrients and other constituents. ENV/JM/MONO(2016)38. Organisation for Economic Co-operation and Development, Paris. Accessed 8 September 2020

Park S, Sethi S and Bouret SG (2019) Non-nutritive Sweeteners Induce Hypothalamic ER Stress Causing Abnormal Axon Outgrowth. Frontiers in Endocrinology https://doi.org/10.3389/fendo.2019.00876

Plaza-Diaz J, Pastor-Villaescusa B, Rueda-Robles A, Abadia-Molina F and Ruiz-Ojeda FJ (2020) Plausible Biological Interactions of Low- and Non-Calorie Sweeteners with the Intestinal Microbiota: An Update of Recent Studies. Nutrients 12: 1153 doi:10.3390/nu12041153

Ray J, Kumar S, Laor D, Shereen N, Nwamaghinna F, Thomson A, Perez JP, Soni L and McFarlane SI (2020) Effects of *Stevia Rebaudiana* on Glucose Homeostasis, Blood Pressure and Inflammation: A Critical Review of Past and Current Research Evidence. International Journal of Clinical Research & Trials 5 . doi:10.15344/2456-8007/2020/142.

Rizwan F, Yesmine S, Banu SG, Chowdhury IA, Hasan R and Chatterjee TK (2019) Renoprotective effects of stevia (*Stevia rebaudiana* Bertoni), amlodipine,valsartan, and losartan in gentamycin-induced nephrotoxicity in the rat model: Biochemical, hematological and histological approaches. Toxicology Reports 6: 683-691

Rodriguez-Saiz M, Paz B, de la Fuente JL, Lopez-Nieto MJ, Cabri W, Barredo JL (2004) *Blakeslea trispora* genes for carotene biosynthesis. Applied and Environmental Microbiology 70(9):5589-5594

Rothstein R (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. In: Guthrie C, Fink GR, editors. Guide to Yeast Genetics and Molecular Biology. (Methods in Enzymology, vol 194). Amsterdam, The Netherlands: Elsevier/Academic Press, pp. 281-301

Schiano C, Grimald Vi, Boccella S, Iannotta M,o Zullo A, Luongo L, Mancini FP, Maione S and Napoli C (2019) Sweeteners modulate bioactivity of endothelial progenitor cells but not induce detrimental effects both on inflammation and behavioural changes, International Journal of Food Sciences and Nutrition, 70(6), 725-737, DOI: 10.1080/09637486.2018.1563052

Schiano C, Grimaldi V, Franzese M, Fiorito C, de Nigris F, Donatelli F, Doricelli A, Salvatore M and Napoli C (2020) Non-nutritional sweeteners effects on endothelial vascular function. Toxicology In Vitro 62: 104694

Sung P, Klein H (2006) Mechanism of homologous recombination: mediators and helicases take on regulatory functions. Nature reviews: Molecular Cell Biology 7:739-750

USDA NRCS (2003) Plant guide: White spruce *Picea glauca* (Moench) Voss. United States Department of Agriculture, Natural Resources Conservation Service, Baton Rouge https://plants.usda.gov/plantguide/pdf/pg_pigl.pdf Assessed 18 September 2020

Xi D, Bhattacharjee J, Salazar-Gonzalez R-M, Park S, Jang A, Warren M, Merritt R, Michail S, Bouret S and Kohli R (2020) Rebaudioside affords hepatoprotection ameliorating sugar sweetened beverage-induced nonalcoholic steatohepatitis. Nature Research Scientific Reports https://www.nature.com/articles/s41598-020-63688-z

Yon H, Tamang JP (2010) Microbiology and nutritional value of *selroti*, an ethnic fermnted cereal food of the Himalyas. Food Biotechnology 24(3):227-247