

**27 August 2019**  
**[92-19]**

## **Supporting document 1**

Risk assessment – Application A1176

Enzymatic production of Steviol Glycosides

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### **Executive summary**

PureCircle Limited (PureCircle) has applied to amend the Australia New Zealand Food Standards Code (the Code) to permit an enzymatic conversion process to produce steviol glycoside preparations. The process uses three enzymes derived from genetically modified strains of *Escherichia coli* K-12, namely two UDP-glucosyltransferases and sucrose synthase. The resulting steviol glycoside preparations have a high content of Rebaudiosides M and/or D, or Rebaudioside (Reb) AM, subject to the starting material (Reb A or stevioside respectively). The starting material is extracted from the *Stevia rebaudiana* Bertoni (stevia) leaves. Reb M, D and AM are known as ‘minor’ steviol glycosides as they are present in the stevia leaf at low levels compared to other ‘major’ steviol.

Steviol glycosides are currently permitted by the Code to be used in certain foods as food additives up to specified maximum permitted levels. They are used as an intense sweetener or flavour enhancer.

Substances used as food additives must comply with any relevant identity and purity specifications listed in Schedule 3 – Identity and Purity. Schedule 3 of the Code contains a specification for steviol glycosides prepared from the leaves of *Stevia rebaudiana* Bertoni which includes Reb M and Reb D produced by enzymatic conversion (S3—35), however the enzymes used by PureCircle are not included in this specification as the source of the enzymes are different. The enzymes used in the enzymatic conversion process are processing aids for the purposes of the Code. FSANZ has therefore carried out a risk assessment to determine whether there are any potential public health and safety concerns associated with PureCircle’s steviol glycoside preparations produced using the specified enzyme processing aids.

The host strain for the enzyme processing aids, *E. coli* K-12, is not pathogenic or toxigenic and has a history of use for the production of food enzymes. Genes for three enzymes were introduced into *E. coli* K-12, generating three distinct production strains. Molecular characterisation of the production strains has confirmed the enzyme coding sequence is as expected and has not undergone any rearrangement, and the introduced DNA is stably inherited. The production strains have also been shown to be genetically stable.

Previous assessments of steviol glycosides by FSANZ and JECFA have confirmed that steviol glycosides share a metabolic pathway to steviol, which is then glucuronidated and excreted in the urine. The unpublished data presented in the current application confirm that

Reb AM, an isomer of Reb D, is also metabolised to steviol.

Individual steviol glycosides produced using enzymes from genetically modified *E. coli* are chemically the same as the equivalent individual steviol glycoside extracted directly from leaves of *Stevia rebaudiana* Bertoni. Evidence has been provided that proteins used in production have been effectively removed and do not pose an allergenic hazard.

No new evidence of adverse effects of steviol glycosides has been identified that would justify changing the ADI of 0 to 4 mg/kg body weight, expressed as steviol, for steviol glycosides previously established by FSANZ. This is therefore appropriate for steviol glycosides produced by enzymatic conversion using enzymes produced by genetically modified *E. coli* that are the subject of this application.

FSANZ is aware that a number of research papers have reported on possible links between consumption of intense sweeteners and unwanted metabolic effects resulting in weight gain, but considers that the current weight of evidence does not support a causal relationship. FSANZ will continue to monitor the emerging scientific literature in this area.

The novel enzymatic conversion process used by PureCircle is technologically justified in that it yields higher amounts of steviol glycosides that are present in stevia leaves in 'minor' amounts. PureCircle claims these have been shown by taste tests to have preferential taste characteristics compared to preparations containing major individual steviol glycosides alone.

In conclusion, FSANZ's risk assessment has not identified any health or safety concerns associated with PureCircle's steviol glycosides preparations.

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# 1 Introduction

PureCircle Limited (PureCircle) has applied to Food Standards Australia New Zealand (FSANZ) to amend the Australia New Zealand Food Standards Code (the Code) to include a new specification for steviol glycosides produced by an enzymatic conversion process using enzymes derived from genetically modified (GM) strains of *Escherichia coli*. The resulting steviol glycosides preparations have a high Rebaudioside (Reb) M and/or Reb D content, or a high Reb AM content. Reb M, D and AM are known as ‘minor’ steviol glycosides as they are present in the stevia leaf at low levels compared to other ‘major’ steviol.

The enzymatic conversion process uses plant enzymes expressed from GM strains of *E. coli* K-12, namely UDP-glucosyltransferases and sucrose synthase. These novel enzymes used by PureCircle require permission in S3—35 which contains specifications for steviol glycosides.

There are also primary source specifications for steviol glycosides contained within section S3—2, being either S3—2(1)(b) (the FAO JECFA Monograph), S3-2(1)(c) (the Food Chemicals Codex) or S3—2(1)(d) (European Commission Regulation No 231/2012 (EU, 2012) laying down specifications for food additives). Specifications for steviol glycosides in these primary sources also do not include the enzymatic conversion process.

Steviol glycosides are currently permitted by the Code to be added to certain foods as a food additive up to specified maximum permitted levels. PureCircle is not requesting a change to the foods permitted to contain steviol glycosides as a food additive nor do they propose to increase the maximum permitted levels of steviol glycosides in foods.

FSANZ has previously conducted a dietary exposure assessment using the current permissions to use steviol glycosides as a food additive and therefore no dietary exposure assessment was necessary for this Application.

## 1.1 Objectives of the assessment

The objectives of this technical, safety and hazard assessment for the enzymatic production of the purified steviol glycoside preparations with a high content of Rebaudiosides M and/or D, or Reb AM are to:

- confirm the technological purpose of the steviol glycoside mixture and justification for the manufacturing method including use of specific enzyme processing aids
- determine whether there are any potential public health and safety concerns that may arise from the use of the enzyme processing aids derived from genetically modified strains of *E. coli* used for the enzymatic conversion process
- determine whether the proposed production method produces an equivalent product (chemically and metabolically) to that obtained by the traditional extraction method from the *Stevia rebaudiana* Bertoni leaf
- determine whether there are any potential public health and safety concerns that may arise from the consumption of the steviol glycoside mixtures and ultimately whether the current acceptable daily intake (ADI) for steviol is appropriate.

## 2 Food technology assessment

This assessment covers both the method of production for the steviol glycosides mixtures and the enzymes used in the production of those steviol glycosides.

### 2.1 Assessment of the steviol glycosides

#### 2.1.1 Identity and purity of the steviol glycosides

Steviol glycosides are a group of compounds naturally occurring in the *Stevia rebaudiana* Bertoni plant. According to PureCircle, their final purified steviol glycoside preparations are produced by enzymatic conversion of two *Stevia rebaudiana* Bertoni (stevia) leaf extracts (either Reb A or stevioside). There are three different steviol glycosides preparations produced, which differ depending on the starting material, as shown in Table 1.

**Table 1: PureCircle's steviol glycoside preparations**

	Starting material Reb A		Starting material stevioside
Steviol glycoside preparation	Reb D and may contain some Reb A	Reb M and may contain some Reb A	Reb AM and may contain some stevioside and Reb E
Steviol glycosides content	Total >95%. Reb D approximately 89-92%*	Reb M >95%*	Reb AM >95%*

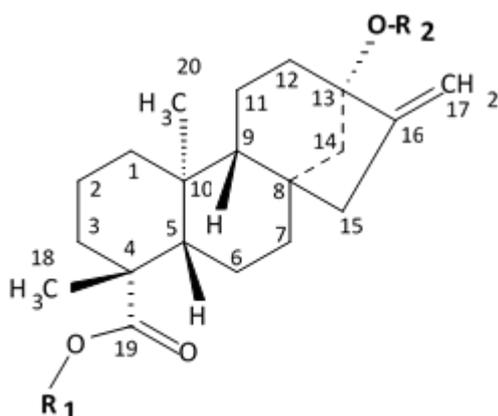
\* From Certificates of analysis for three non-consecutive commercial preparations, dried basis

Results presented from HPLC analyses (JECFA 2017) show that PureCircle's Reb D, Reb M and Reb AM preparations have the same retention times as appropriate controls. This confirms that the enzyme modified steviol glycosides are chemically the same as the plant-extracted forms.

#### **Structural formula:**

All steviol glycosides share the same steviol backbone structure (Figure 1) but have different sugar moieties attached, as conjugated glycosides. These various sugar moieties include glucose, rhamnose, xylose, fructose and deoxyglucose, which can be attached in various combinations, quantity and orientation (JECFA 2017).

*Figure 1 Chemical backbone structure for steviol glycosides*



The chemical information for Reb D, Reb M and Reb AM is provided in Table 2 below. The chemical information for Reb AM was sourced from PureCircle after their application was received by FSANZ and updates the information provided in that application.

**Table 2: Chemical information for steviol glycoside preparations with a high Reb D, Reb M or Reb AM content**

	High Reb D	High Reb M	High Reb AM
<b>Chemical name</b>	13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl)-β-D-glucopyranosyl]oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester	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	13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-18-oic acid, 2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl ester
<b>Chemical formula</b>	C <sub>50</sub> H <sub>80</sub> O <sub>28</sub>	C <sub>56</sub> H <sub>90</sub> O <sub>33</sub>	C <sub>50</sub> H <sub>80</sub> O <sub>28</sub>
<b>Molecular weight</b>	1129	1291	1129
<b>CAS number</b>	63279-13-0	1220616-44-3	2222580-26-7

### 2.1.2 Physical and chemical properties of the steviol glycosides

According to certificates of analysis provided by PureCircle, their steviol glycoside preparations are white to off-white powders that have a clean taste with no abnormal off odour. The preparations have a pH of between 4.5 to 7.0 (1% solution) and are freely soluble in water.

### 2.1.3 Technological purpose of the food additive

PureCircle states that the technological purpose of steviol glycosides as a food additive is that of an intense sweetener which would replace sugar in food in reduced energy or no added sugar products. They also note they may be used as a flavour enhancer.

Steviol glycosides are currently permitted as a food additive at maximum permitted levels (MPL's) in a variety of food categories and at Good Manufacturing Practice (GMP) level for tabletop sweeteners in Schedule 15. PureCircle has not requested any amendments to these MPL's; rather, their application relates to the method of production of steviol glycosides.

### 2.1.4 Technological justification

The primary reason for developing alternative methods for producing steviol glycosides to the traditional extraction methods is that not all steviol glycosides are naturally produced to the same degree in the leaves of *S. rebaudiana* Bertoni. The applicant states that due to recent advances in biotechnology, alternative manufacturing methods have been developed to yield higher amounts of 'minor' steviol glycosides that are present in stevia leaf which have been shown (by taste tests) to have preferential taste characteristics compared to preparations containing major individual steviol glycosides alone. PureCircle utilises an enzymatic conversion process using enzymes unique to them, to produce steviol glycoside preparations from stevia leaf extracts. These are comprised primarily of 'minor' glycosides (Reb M, D and AM).

With regard to the preferential taste characteristics mentioned above, the sensory characteristics of a preparation of steviol glycosides with a high Reb AM content were

assessed using a panel of 30 and results from the study were provided as part of the application (confidential commercial information). Overall, the results of the sensory evaluations demonstrated that when compared with Reb A and other steviol glycosides, Reb AM was associated with decreased bitterness and higher overall liking.

### **2.1.5 Manufacturing process**

PureCircle states in its application that the selected steviol glycosides are enzymatically converted from either a >95% Reb A product or a >95% stevioside product, both of which are produced from stevia leaves using the traditional hot water extraction process, consistent with the process already defined for recognised steviol glycosides (JECFA 2017) (refer to section B.5.3 and Figure B5.1-1 of the application for further details).

UDP-glucosyltransferases and sucrose synthase enzymes are then used as processing aids to catalyse the conversion of Reb A and/or stevioside to Reb D, M or AM. The steviol glycoside purification process following the enzymatic conversion is also consistent with that already defined for recognised steviol glycosides (JECFA 2017).

A detailed description of the manufacturing process, including a flow chart and the raw materials, processing aids and equipment used in the production process is provided in section B.5 of the application.

### **2.1.6 Specification for identity and purity**

Certificates of analyses for nine non-consecutive lots of the enzyme modified steviol glycosides were provided with the application. The results are summarised in Table B.6.1-1 of the application and demonstrate compliance with the majority of the Specification for steviol glycosides from *Stevia rebaudiana* Bertoni in S3—35 (excluding the method of preparation, which is the subject of this application) and with the levels of cadmium and mercury in S3—4. However the sweetness potency of a preparation of steviol glycosides with a high Reb AM content was determined by PureCircle to be 152 times sweeter than sucrose. This is less than that in the specification in S3—35, which is approximately 200-300 times sweeter than sucrose. The methods of analyses used were internationally recognised.

### **2.1.7 Analytical method for detection**

The steviol glycoside purity of the final products that are the subject of this application can be measured using the JECFA HPLC method for steviol glycosides (JECFA 2017).

### **2.1.8 Product stability**

JECFA have previously concluded that steviol glycosides are sufficiently thermally and hydrolytically stable for use in foods, including acidic beverages, under normal conditions of processing and storage (JECFA 2007). The applicant notes that although the stability of each individual steviol glycoside was not specifically addressed during any of the previous JECFA evaluations, it is expected that the stability of steviol glycosides produced by enzymatic conversion would be similar to that of the individual steviol glycosides given the similarities in structure. To confirm this viewpoint, they conducted additional storage stability studies with their enzyme modified steviol glycosides preparations to determine stability under different temperatures and humidity levels as well as stability in solution at various pH levels and temperatures. The results demonstrated that the stability of these steviol glycoside preparations is similar to individual steviol glycosides from stevia leaf extract, consistent with previous stability conclusions made by JECFA.

## 2.2 Assessment of the enzymes used

### 2.2.1 Identity of the enzymes and manufacturing process

Uridine diphosphate glucosyltransferases (UDP-glucosyltransferases) and sucrose synthase enzymes are produced by microbial fermentation of genetically modified *E. coli* K-12 production strains expressing plant UDP-glucosyltransferases and sucrose synthase genes.

Identification information for these enzymes is as follows:

#### **UDP-glucosyltransferases**

Source (strain):	<i>E. coli</i> K-12 production strains containing DNA sequences encoding UDP-glucosyltransferases from <i>Stevia rebaudiana</i> and <i>Solanum lycopersicum</i>
Common/Accepted Name:	Glucosyltransferase
Enzyme Classification Number of Enzyme Commission (EC) of the International Union of Biochemistry and Molecular Biology (IUBMB):	Not yet fully classified by the IUBMB
Chemical/Systematic Name:	UDP-glucose $\beta$ -D-glucosyltransferase
Chemical Abstracts Service (CAS) Number:	9033-07-2

#### **Sucrose Synthase**

Source (strain):	<i>E. coli</i> K-12 production strain containing DNA sequences encoding sucrose synthase from <i>Arabidopsis thaliana</i>
Common/Accepted Name:	Sucrose synthase
Enzyme Classification Number of Enzyme Commission (EC) of the IUBMB:	2.4.1.13
Chemical/Systematic Name:	NDP-glucose:D-fructose 2- $\alpha$ -D-glucosyltransferase
CAS Number:	9030-05-1

### 2.2.2 Specifics of the enzymatic reactions

UDP-glucosyltransferases catalyse the conversion of highly purified Reb A and stevioside extracted from stevia leaf to produce Reb M and Reb AM respectively, with intermediate production of Reb D and Reb E. Sucrose synthase is utilised in the reaction to regenerate the glucose source, UDP-glucose.

### 2.2.3 Specification for identity and purity for the enzymes

There are international specifications for enzyme preparations used in the production of food. These have been established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in its Compendium of Food Additive Specifications (JECFA 2006) and in the Food Chemicals Codex (FCC 2018). These specifications are included in S3—2 of the Code and enzymes used as a processing aid must meet these specifications. PureCircle provided certificates of analysis indicating that the three enzymes meet these specifications.

## 2.3 Food technology conclusion

PureCircle's steviol glycoside preparations meet the purity specification in S3—35, containing no less than 95% total steviol glycosides. The individual steviol glycosides produced are chemically the same as steviol glycosides produced by hot water extraction of the stevia leaf.

Steviol glycosides are already permitted for use in certain foods as a food additive and are used as an intense sweetener or flavour enhancer, however the current specification for identity and purity for steviol glycosides produced from enzymatic conversion in the Code (S3—35) does not include the enzymes used by PureCircle in their enzymatic conversion process. Additionally, the existing specification is for a preparation approximately 200-300 times sweeter than sucrose, however PureCircle's preparation of steviol glycosides with a high Reb AM content was determined to be less than this, at approximately 150 times sweeter than sucrose. Aside from these aspects, the preparations meet the relevant purity and identity specification in the Code.

## 3 Safety assessment

The enzymatic process used to convert leaf-extracted steviol glycosides to more pure forms (Rebaudiosides D, M and AM) involves three enzymes from three genetically modified sources. The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of these enzymes, specifically by considering the:

- history of use of the host and gene donor organisms (source microorganisms)
- characterisation of the genetic modification(s)
- safety of the novel proteins.

### 3.1 History of use

#### 3.1.1 Host organism

The host strain, *E. coli* K-12 was obtained from the Coli Genetic Stock Center (CGSC) at Yale University. The CGSC number and strain designation are 4474 and W3310, respectively (Bachmann 1972).

*E. coli* K-12 is the most common bacterial laboratory strain in use globally. It was isolated from a human stool sample in 1922 (Bachmann 1996). Comparative genome sequencing and proteomic analysis of the K-12 strain and its derivatives, to well characterised pathogenic strains, have identified differences in the K-12 cell wall structure associated with reduced ability to colonise the human intestinal tract, and absence of adhesive proteins and virulence factors that meet requirements for pathogenicity (Bachmann 1996; EPA 1997; Sahl et al. 2013). These studies have also shown reduced toxin production in K-12 strains and absence of plasmids encoding antibiotic resistance. Under the U.S. National Institutes of Health (NIH) Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH 2019), *E. coli* K-12 is classified as a Risk Group 1 agent which is reserved for organisms which are not associated with disease in healthy adult humans.

*E. coli* K-12 has a long history of use in the human biopharmaceutical industry, with approximately 30% of currently approved recombinant therapeutic proteins in the United States (US) being produced in *E. coli* K-12, starting with the US FDA approval of biosynthetic human insulin in 1983 (Huang et al. 2012; Jozala et al. 2016). The use of this bacterium as a source for the production of food enzymes began in the 1980s (JECFA 1991). *E. coli* K-12 is one source microorganism for the production of chymosin, an enzyme of microbial origin permitted to be used as a processing aid (listed in Schedule 18 of the Food Standards Code). *E. coli* K-12 is considered a model microbial strain and has been thoroughly characterised for use in research and industry, it is therefore considered a safe microorganism.

### 3.1.2 Gene donor organism(s)

Genes for the three enzymes were introduced into *E. coli*, generating three distinct production strains. The genetic material was chemically synthesised, based on the enzyme gene sequences from commonly used food plants (*Stevia rebaudiana* and *Solanum lycopersicum*) and the well characterised model plant *Arabidopsis thaliana*.

#### *Stevia rebaudiana* Bertoni

A UDP-glucosyltransferase (UGT) gene was sourced from *S. rebaudiana* (stevia), commonly known as sweet leaf. Stevia is a member of the daisy family (*Asteraceae*), which also includes lettuce and artichoke. Stevia leaves have been used to prepare sweetened teas for more than 1500 years thus have a long history of safe use.

#### *Solanum lycopersicum*

A second UDP-glucosyltransferase (UGT) gene was sourced from *S. lycopersicum*, the cultivated form of tomato. Earliest records of the use of this plant as food date back to 500 BC, in the Puebla-Veracruz area of Mexico, thus this plant has a long history of safe use. Tomato, being a member of the nightshade family, will produce alkaloid toxicants. Allergenic proteins have also been identified, with global allergy rates to tomato ranging from 1.5 – 16% (OECD, 2008). As the DNA from this source was obtained by chemical synthesis, there is no risk of transfer of the toxicants and allergens to the production strain.

#### *Arabidopsis thaliana*

A sucrose synthase gene was sourced from *A. thaliana*, which is a member of the mustard family (*Brassicaceae*) that includes cabbage, turnip and rapeseed. The common names for this plant are mouse-ear or thale cress. Although this plant is not traditionally used as food, it is ubiquitous in the environment and is not known to be pathogenic, toxicogenic or allergenic to humans.

## 3.2 Characterisation of the genetic modification

### 3.2.1 Description of the introduced DNA

The genes for the three enzymes were chemically synthesised, based on published DNA sequences. The sequence was then modified by standard molecular mutagenesis to introduce specific amino acid substitutions, resulting in changes to the native amino acid sequence of the enzymes. These modifications were performed to increase the specific activity and thermostability of the enzymes, while decreasing the formation of undesirable by-products. Due to this change to the native amino acid sequence, the enzymes are considered to be protein engineered.

After transformation into the host, the genes for each enzyme were located and expressed on plasmids, rather than being integrated into the host genome. The expression plasmid used was a modified commercial system, where the antibiotic selection marker was replaced with an auxotrophy complementation selection system (Vidal et al, 2008). The enzyme expression cassette included the plasmid-based *E. coli* T7 phage promoter and terminator elements, as well as a lac operator and ribosomal binding site sourced from *E. coli*. The plasmids containing the enzyme genes were introduced by electroporation and selection was performed by growth on defined minimal media.

### **3.2.2 Characterisation of the introduced DNA**

Plasmid DNA for each production strain was fully sequenced, before and after transformation. DNA sequence analysis confirmed the enzyme gene sequences in each production strain were as expected. The copy number of the plasmids was also determined by real-time polymerase chain reaction (PCR) analysis, targeting two distinct plasmid backbone regions. The analysis showed the copy number across the two regions analysed was consistent for the two UGT enzyme-containing plasmids. A difference was observed in the copy number for the sucrose synthase-containing plasmid. This indicates there may have been some modification to a primer-binding sequence in some copies of the plasmid. As the sequence data confirmed the enzyme gene was as expected, and subsequent protein expression and enzyme function analyses of sucrose synthase indicate the protein is expressed and is functional, the difference in copy number is of no concern.

### **3.2.3 Stability of the production organisms and inheritance of the introduced DNA**

In order to confirm stability and inheritance of the enzyme genes, the DNA sequence was compared across several generations. Samples were sourced from the initial transformation stock, a master stock and from the end point of three fermentation runs, which covered about 50 generations. The sequence from each sample was as expected confirming that the traits are expressed and inherited in a manner that is stable through several generations, consistent with laws of inheritance.

## **3.3 Safety of the novel proteins**

A relevant factor to consider in relation to the safety of the novel proteins is whether they will be present in the final rebaudioside product. As described in the application (section B.5.3), after the enzymatic conversion step, the reaction mix is heated to between 80-100°C for 10 minutes to inactivate the enzymes, followed by several purification steps (flocculation, filtration and resin purification). These steps would effectively remove any residual protein.

Subsequent analysis of five batches of purified steviol glycoside product, using an amount of the product that would be added to food, did not detect protein. The analysis was performed using the bicinchoninic acid (BCA) assay (limit of detection 5 ppm). Data was also presented from protein gel electrophoresis analysis (limit of detection 100 ng) showing absence of protein, however the level of product analysed was significantly less than the amount commonly added to food.

### **3.3.1 History of safe use**

The source of the genes that encode the novel enzyme processing aids come from either common food plants (stevia and tomato) or a model plant (thale cress), with no known adverse effects in humans. Even though the enzymes have been protein engineered, the enzyme sequences are 95-99% identical to the endogenous proteins found in the source organisms. This 1-5% difference is of no public health concern considering the large variation that naturally exists in the enzyme homologs found throughout our food supply.

### **3.3.2 Assessment of enzyme toxicity**

The applicant provided results from *in silico* analyses comparing the amino acid sequences of the three enzyme processing aids to known protein toxins and virulence factors. The toxins and virulence factors were first identified from the NCBI Protein database and saved as a user-generated sample file. The sample file of known toxins contained 6715 samples and the database of known virulence factors contained 2845 samples. A subsequent BLAST 2 Sequences search (v2.8.0) found no biologically significant similarity between the three enzymes with the identified toxins or virulence factors.

### **3.4 Safety assessment conclusion**

The host strain, *E. coli* K-12 is not pathogenic or toxigenic and has a history of use for the production of food enzymes. The applicant has provided data showing that three production strains have been generated each expressing a distinct polypeptide, including two glycosyltransferases and a sucrose synthase. Molecular characterisation of the production strains has confirmed the introduced enzyme coding sequences are as expected and has not undergone any rearrangement, and the introduced DNA is stably inherited. The production strains have also been shown to be genetically stable.

## **4 Hazard Assessment**

### **4.1 Previous FSANZ assessments**

FSANZ established an ADI of 0-4 mg/kg bw/day steviol in 2008 (Application A540). At the time, the only known steviol glycosides were stevioside, dulcoside, steviolbioside, rubudioside, and rebaudiosides A through to F. The ADI was derived by applying a 100-fold safety factor to the NOEL of 970 mg/kg bw per day (equivalent to 383 mg/kg bw per day steviol) in a two-year rat study.

FSANZ updated the hazard assessment for steviol glycosides as a part of Applications A1037, A1108, A1132, A1157 and A1172. These assessments extended permissions to newly discovered steviol glycosides, but did not identify a need to change the ADI.

### **4.2 Characteristics of steviol glycosides produced using enzymes from *E. coli***

Individual steviol glycosides produced using enzymes from genetically modified *E. coli* are chemically the same as the equivalent individual steviol glycoside extracted directly from leaves of *Stevia rebaudiana* Bertoni. The steviol glycoside preparations that are the subject of this application have a purity of  $\geq 95\%$ . Certificates of Analysis for three separate batches of each preparation produced by this process show that protein is below the limit of detection, supporting the conclusion that enzymes and other proteins used in production are effectively removed and do not pose an allergenic hazard.

### 4.3 Toxicological data

Two unpublished *in vitro* studies were submitted by the Applicant to demonstrate that steviol glycosides produced by the method that is the subject of this application undergo a similar metabolic fate to steviol glycosides extracted directly from *Stevia rebaudiana* Bertoni.

No new published studies designed to assess the toxicity of steviol glycosides were submitted by the applicant, or located from other sources, or located by literature search using EBSCO or PubMed.

Three studies not primarily designed to assess toxicity, including one rat study (Nettleton *et al.* 2019), one human study (Higgins and Mattes 2019) and one review article (Lobach *et al.* 2019), addressing non-toxicological endpoints have been reviewed.

*In vitro anaerobic metabolism study of rebaudiosides D and M, produced by bioconversion, in human fecal homogenates (Biopharmaceutical Research Inc., 2018). Regulatory status: Non-GLP*

Test articles for this study were rebaudioside D (Reb D) and rebaudioside M (Reb M), both produced by enzymatic conversion. Reb D made up 90.9% of a mixture composed of 97.8% steviol glycosides, while the Red M was 95.4% of a mixture composed of 97.2% steviol glycosides. The test system was prepared from stool samples from six healthy adults of each sex. By pooling fecal samples from two adults of the same sex, three homogenates from male subjects, and three from female subjects, were prepared by diluting, centrifugation and collection of the supernatant. The test articles were mixed to contain equal quantities of Reb D and Reb M, and triplicate samples of this mixture were mixed with each fecal homogenate and incubated at 37°C, under anaerobic conditions, for 0, 4, 8, 12, 16, 24, 48 or 72 h. Triplicate samples of Reb A were mixed with each homogenate and incubated under the same conditions for the same time intervals. Metabolism of Reb D, Reb M and Reb A was determined using LC/MS, with mogrol and siamenoside as the internal standards.

Results showed that both Reb D and Reb M underwent rapid deglycosylation to steviol. Deglycosylation was nearly complete within 12 hours, and the sex of the donors of the fecal samples from which the test systems were prepared had no effect on the rate of metabolism. Reb A used as the positive control showed a similar rate of metabolism.

*In vitro anaerobic metabolism study of steviol glycosides extracted from Stevia leaf, in human fecal homogenates (Biopharmaceutical Research Inc. 2019). Regulatory status: Non-GLP*

Metabolism was determined for Reb AM (from enzymatic conversion), Reb W2, Reb Y, Reb U2, Reb V Reb N and Reb O, with Reb A as the positive control. Reb AM is an isomer of Reb D. Test systems were male and female homogenates as described in the previous study. The incubation details were similar to those in the previous study, although time points were 0, 4, 8, 12, 16, 24 and 48 h. All incubations were conducted in triplicate. All steviol glycosides were rapidly metabolised to steviol with deglycosylation largely complete within 12 hours. There were no differences in metabolism based on the sex of the donors of fecal homogenates. No deglycosylation of rebaudiosides, or generation of steviol, was observed in negative control assays in which fecal homogenate was not added.

Considered together, these two studies show that the metabolism of Reb D, Reb M and Reb AM synthesised using enzymatic conversion is closely similar to that of rebaudiosides extracted directly from stevia leaf.

*Study of effects of rebaudioside A on gut microbiota (Nettleton et al. 2019). Regulatory status: Non-GLP*

This study was not a toxicology study, but examined the effect of exposure to rebaudioside A, with and without co-administration of prebiotic, on young male Sprague-Dawley rats. Rats were 3 weeks old and were randomized to four groups of 8 rats/group. Details of husbandry, housing, and randomization procedures were lacking. The control group was provided with plain drinking water and standard rat chow. The Rebaudioside A group was administered 2-3 mg/kg bw Reb A in the drinking water. The prebiotic group was provided with a diet containing 10% w/w oligofructose-enriched inulin in the diet as a prebiotic, and the fourth group was administered both the Reb A and the dietary prebiotic. Treatment was continued for 9 weeks. At the end of treatment, rats were subject to oral glucose and insulin tolerance tests, a gut permeability test by means of oral gavage with fluorescein isothiocyanate-dextran-4000 followed an hour later by blood collection for measurement of fluorescence in plasma. Lean and fat mass, body fat and bone mineral density were quantified by dual X-ray absorptiometry while rats were under anesthesia. Finally, rats were killed for collection of liver, cecum, caecal contents, colon and brain. Caecal contents were analysed for microbiota analysis and short chain fatty acid analysis.

The authors found that Reb A alone did not affect food intake, water intake, weight gain, glucose tolerance or insulin sensitivity, but resulted in an alteration in gut microbiota and was associated with lower tyrosine hydroxylase and dopamine transporter mRNA in the nucleus accumbens, when compared to controls. Dietary prebiotic, with or without Reb A, was associated with reduced food intake, fat mass, gut permeability and caecal short chain fatty acid concentration. Caecal weight of rats administered both Reb A and prebiotic was significantly lower than that of rats administered only prebiotic.

FSANZ considers the significance of these findings to be of uncertain relevance to human health. There are a number of relevant details absent from both the Materials and Methods and the Results sections of the paper. For example, it is not clear that the diet containing 10% prebiotic had the same energy content as the standard diet. However, FSANZ notes that administration of 2-3 mg/kg bw Reb A was not associated with any adverse effects in the rats.

*Review of the in vivo data concerning low- or no-calorie sweeteners and intestinal microbiota (Lobach et al. 2019) Regulatory status: Non-GLP*

The authors conducted a review of the literature concerning potential changes to gastrointestinal microbiota associated with acesulfame K, aspartame, cyclamate, neotame, saccharin, sucralose and Rebaudioside A. Both rodent studies and human studies were included in the review. Only one study of the effects of Reb A was found. It was a four-week study conducted in SPF BALB-c mice. Mice, 5/group, were administered Reb A daily by oral gavage at a dose of 0, 5.5 mg/kg bw/day, or 139 mg/kg bw/day. There were no significant effects on total numbers of anaerobic bacteria, enterococci, enterobacteria or lactobacilli, and also no effects on *Enterobacteriaceae* distribution. At the high dose of Reb A, there was a significant increase in the diversity of lactobacilli species, but this finding was considered to be of limited relevance to human health risk assessment because the dose was very much higher than the ADI for steviol glycosides. Overall, the authors found that effects of low or no-calorie sweeteners on the microbiome were found only at high doses that bear no relevance to human consumption, and that there is no evidence of an actual adverse effect on human health. The authors also concluded that there was clear evidence that changes in the diet unrelated to sweetener consumption were likely the major determinants of observed changes in microbiota.

*Randomised controlled trial of the effects of four low-calorie sweeteners and sucrose on*

*bodyweight of overweight or obese adults (Higgins and Mattes 2019). Regulatory status: Non-GLP*

The objective of this study was to compare the effects of consumption of four low-calorie sweeteners on bodyweight, eating behaviour, and glucose tolerance, using sucrose as the control. The study was conducted over 12 weeks. The study was conducted with a total of 154 subjects between the ages of 18 and 60 years, with a BMI between 25 and 40. Participants were required to be healthy, with low consumption of low-calorie sweeteners, and weight-stable. Prior to commencement of the intervention, baseline energy intake and expenditure, appetite, glucose tolerance and anthropomorphic indices, including BMI, total body water, total fat mass, total fat-free mass, total tissue percentage fat, android fat mass and gynoid fat mass, were determined. Participants were randomly assigned to consume, on a daily basis, 1.25 to 1.75 L of beverage sweetened with sucrose (n=39), aspartame (n=30), saccharin (n=29), sucralose (n=28) or rebaudioside A (n=28) for 12 weeks. Bodyweight was measured every 2 weeks and energy intake, energy expenditure and glucose tolerance were measured every 4 weeks.

A total of 123 participants completed the intervention. Both sucrose consumption and saccharin consumption were associated with statistically significant weight gain ( $1.85 \pm 3.6$  kg,  $P < 0.001$  and  $1.18 \pm 0.036$  kg,  $P < 0.02$  respectively), and sucralose consumption was associated with weight loss, although this was not statistically significant. No significant weight change was discovered in the aspartame group or the Reb A group. There were no adverse effects observed in the Reb A group.

#### **4.4 Assessments by other regulatory agencies**

There have been no new assessments by other regulatory agencies since FSANZ reviewed A1172 in 2019. FSANZ has previously reviewed the assessments of JECFA, Health Canada and EFSA.

The FSANZ ADI is consistent with the ADI established by JECFA at the 69th meeting held in the same year, and published in 2009. JECFA re-assessed steviol glycosides at the 82nd meeting in 2016 and confirmed the existing ADI.

#### **4.5 Hazard assessment discussion and conclusion**

The host strain for the enzyme processing aids, *E. coli* K-12, is not pathogenic or toxigenic and has a history of use for the production of food enzymes. Genes for three enzymes were introduced into *E. coli* K-12, generating three distinct production strains. Molecular characterisation of the production strains has confirmed the enzyme coding sequence is as expected and has not undergone any rearrangement, and the introduced DNA is stably inherited. The production strains have also been shown to be genetically stable.

Previous assessments of steviol glycosides by FSANZ and JECFA have confirmed that steviol glycosides share a metabolic pathway to steviol, which is then glucuronidated and excreted in the urine. The unpublished data presented in the current application confirm that Reb AM, an isomer of Reb D, is also metabolised to steviol. A group ADI, expressed as steviol, is therefore appropriate to all steviol glycosides.

Steviol glycosides synthesised using enzymes derived from *E. coli* are chemically the same as those extracted directly from leaves of *Stevia rebaudiana* Bertoni. Evidence has been provided that proteins used in production have been effectively removed. *In vitro* metabolism studies provide evidence that the steviol glycosides are metabolised to steviol by fecal bacteria with comparable efficiency to those extracted directly from the leaves of *S. rebaudiana* Bertoni.

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 steviol glycosides produced by enzymatic conversion, using enzymes produced by genetically modified *E. coli*, that are the subject of this application.

Further to the assessment presented above, FSANZ is aware that a number of research papers have reported on possible links between consumption of intense sweeteners and unwanted metabolic effects resulting in weight gain, but considers that the current weight of evidence does not support a causal relationship. FSANZ will continue to monitor the emerging scientific literature in this area.

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