

**ISOTHIOCYANATE CONTAINING *BRASSICACEAE* PRODUCTS AND
METHOD OF PREPARATION THEREOF**

FIELD OF THE INVENTION

5 The present invention relates to methods for producing isothiocyanate containing products from *Brassicaceae* material and lactic acid bacteria for use in such methods. The present invention also relates to isothiocyanate containing products from *Brassicaceae* material produced by such methods.

10 **BACKGROUND OF THE INVENTION**

Brassicaceae family members are rich in glucosinolates which can be converted by the enzyme myrosinase to isothiocyanates which have been noted to have beneficial effects on some types of cancer (Moktari et al., 2017; Capuano et al., 2017; Kim and Park, 2016). Sulforaphane, for example, has been found to reduce hepatic glucose
15 production and improve glucose control in obese patients with type 2 diabetes (Axelsson et al., 2017). However, many *Brassicaceae* family members are highly perishable after harvest with the quality and quantity of nutrients declining rapidly if the product is not stored well.

Brassicaceae are often processed to increase the shelf life which can result in
20 the loss of nutrients. The main methods to obtain a longer shelf life include thermal processing, freezing, modified and controlled atmosphere storage and the addition of chemical preservatives which also would bring undesirable changes in chemical composition.

 These processes can result in the loss of glucosinolates or reduce the ability of
25 the enzyme myrosinase to convert glucosinolates to isothiocyanates. For example, conventional broccoli processing/preservation involves blanching prior to freezing to inactivate quality degrading enzymes such as lipoxygenase. Peroxidase inactivation is commonly used as an indicator of the adequacy of blanching. The condition for inactivation of peroxidase leads to the inactivation of myrosinase and thus the resulting
30 product is devoid of isothiocyanates (Dosz and Jeffery, 2013).

 Accordingly, there remains a need for improved methods for producing *Brassicaceae* products comprising phytonutrients such as isothiocyanates.

SUMMARY OF THE INVENTION

35 The present inventors have developed methods for preparing isothiocyanate containing products from *Brassicaceae* material.

In an aspect, the present invention provides a method of preparing an isothiocyanate containing product from *Brassicaceae* material comprising:

i) pre-treating the *Brassicaceae* material to improve the access of myrosinase to a glucosinolate;

5 ii) fermenting the material obtained by step i) with lactic acid bacteria to form the isothiocyanate containing product.

In an embodiment, pre-treating comprises one or more of the following:

i) heating;

ii) macerating;

10 iii) microwaving;

iv) exposure to high frequency sound waves (ultrasound); or

v) pulse electric field processing

wherein the temperature of the *Brassicaceae* material does not exceed about 75°C during pre-treating.

15 In an embodiment, pre-treating reduces epithiospecifier protein (ESP) activity while maintaining endogenous myrosinase activity.

In an embodiment, pre-treating comprises heating and macerating the *Brassicaceae* material and wherein the temperature of the *Brassicaceae* material does not exceed about 75°C during pre-treating. In an embodiment, heating occurs before macerating or wherein heating and macerating occur at the same time. In an embodiment, pre-treating comprises heating the *Brassicaceae* material to a temperature of about 50°C to about 70°C followed by maceration. In an embodiment, the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a size of about 2 mm or less. In an embodiment, the *Brassicaceae* material is heated in a sealed package.

In an embodiment, the isothiocyanate containing product comprises at least about 10 times more isothiocyanate than macerated *Brassicaceae* material.

In an embodiment, the isothiocyanate containing product comprises at least about 12 times more isothiocyanate than macerated *Brassicaceae* material.

30 In an embodiment, the isothiocyanate containing product comprises at least about 14 times more isothiocyanate than macerated *Brassicaceae* material.

In an embodiment, the isothiocyanate containing product comprises at least about 16 times more isothiocyanate than macerated *Brassicaceae* material.

35 In an embodiment, the isothiocyanate containing product comprises at least about 2 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content.

In an embodiment, lactic acid bacteria was isolated from a broccoli and/or the lactic acid bacteria lacks myrosinase activity.

In an aspect, the present invention provides a method of preparing a isothiocyanate containing product from *Brassicaceae* material comprising:

5 i) pre-treating the *Brassicaceae* material to improve the access of myrosinase to a glucosinolate; and

ii) acidifying the material obtained by step i) forming the isothiocyanate containing product.

10 In an aspect, the present invention provides a method of preparing an isothiocyanate containing product from broccoli material comprising fermenting the material with lactic acid bacteria *Leuconostoc mesenteroides* and/or *Lactobacillus plantarum* to form the isothiocyanate containing product, wherein the method optionally comprises pre-treating the broccoli material to improve the access of myrosinase to a glucosinolate.

15 In an aspect, the present invention provides a method of preparing an isothiocyanate containing product from a *Brassicaceae* material comprising fermenting the material with lactic acid bacteria *Leuconostoc mesenteroides* and/or *Lactobacillus plantarum* isolated from broccoli to form the isothiocyanate containing product, wherein the method optionally comprises pre-treating the *Brassicaceae* material to
20 improve the access of myrosinase to a glucosinolate.

In an aspect, the present invention provides an isolated strain of lactic acid bacteria selected from:

i) BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia; and

25 ii) BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia.

In an aspect, the present invention provides an isolated strain of lactic acid bacteria selected from:

30 i) BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia;

ii) BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia;

iii) B1 deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;

35 iv) B2 deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;

v) B3 deposited under V17/021733 on 25 September 2017 at the National Measurement Institute Australia;

vi) B4 deposited under V17/021734 on 25 September 2017 at the National Measurement Institute Australia; and

5 vii) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

In an aspect, the present invention provides a starter culture for producing an isothiocyanate containing product or a probiotic comprising lactic acid bacteria selected from one or more or all of:

10 i) BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia;

ii) BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia;

15 iii) B1 deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;

iv) B2 deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;

v) B3 deposited under V17/021733 on 25 September 2017 at the National Measurement Institute Australia;

20 vi) B4 deposited under V17/021734 on 25 September 2017 at the National Measurement Institute Australia; and

vii) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

25 In an embodiment, the starter culture comprises lactic acid bacteria at a concentration of at least about 10^8 cfu/mL.

In an aspect, the present invention provides a probiotic composition comprising lactic acid bacteria selected from one or more or all of:

i) BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia;

30 ii) BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia;

iii) B1 deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;

35 iv) B2 deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;

v) B3 deposited under V17/021733 on 25 September 2017 at the National Measurement Institute Australia;

vi) B4 deposited under V17/021734 on 25 September 2017 at the National Measurement Institute Australia; and

5 vii) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

In an aspect, the present invention provides an isothiocyanate containing product obtained by the method as described herein.

10 In an aspect, the present invention provides an isothiocyanate containing product obtainable by the method as described herein.

In an aspect, the present invention provides an isothiocyanate containing *Brassicaceae* product comprising at least about 10 times more isothiocyanate than the macerated *Brassicaceae* material.

15 In an aspect, the present invention provides an isothiocyanate containing *Brassicaceae* product comprising about 10 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material.

In an aspect, the present invention provides an isothiocyanate containing *Brassicaceae* product comprising at least about 2 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content.

20 In an aspect, the present invention provides an isothiocyanate containing *Brassicaceae* product comprising about 2 times to about 4 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content.

In an aspect, the present invention provides an isothiocyanate containing *Brassicaceae* product comprising at least 150 mg/kg dw of isothiocyanate.

25 In an embodiment, the present invention provides an isothiocyanate containing product comprises at least 150 mg/kg dw, at least 200 mg/kg dw, at least 300 mg/kg dw, at least 400 mg/kg dw, or at least 450 mg/kg dw, or at least 500 mg/kg dw, or at least 550 mg/kg dw, or at least 600 mg/kg dw, or at least 650 mg/kg dw, or at least 700 mg/kg dw, or at least 1000 mg/kg dw, or at least 2000 mg/kg dw, or at least 3000
30 mg/kg dw, or at least 4000 mg/kg dw, or at least 5000 mg/kg dw, or at least 6000 mg/kg dw, or at least 7000 mg/kg dw sulforaphane.

In an embodiment, the isothiocyanate containing product comprises *Leuconostoc mesenteroides* and/or *Lactobacillus plantarum*.

35 In an embodiment, the isothiocyanate containing product has one or more or all of the following features:

i) is stable for at least 4 weeks, or for at least 8 weeks, or for at least 12 weeks when stored at about 4°C to about 25°C;

ii) is resistant to yeast, mould and/or coliform growth for at least 4 weeks, or for at least 8 weeks, or for at least 12 weeks when stored at about 4°C to about 25°C; and

5 iii) comprises at least 10^7 CFU/g *Leuconostoc mesenteroides* and/or *Lactobacillus plantarum*.

Any embodiment herein shall be taken to apply *mutatis mutandis* to any other embodiment unless specifically stated otherwise. For instance, as the skilled person would understand examples of lactic acid bacteria outlined above for the methods of the invention equally apply to products of the invention.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

15 Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

20 The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANING DRAWINGS

25 **Figure 1. A)** Shows the pathways of hydrolysis of glucoraphanin to sulforaphane and sulforaphane nitrile. **B)** Shows the effects of maceration and fermentation on sulforaphane content (mg/kg, DW) in broccoli puree. **C)** Shows the effect of fermentation on lactic acid bacteria count (log CFU/gm) of broccoli puree during storage.

30 **Figure 2. A)** Shows the effects of fermentation on the stability of sulforaphane in broccoli puree stored at 4°C and 25°C (RT). **B)** Effects of heat treatment condition on the conversion of glucoraphanin into sulforaphane in broccoli matrix.

35 **Figure 3. A)** Shows the total phenolic content (mg GAE/100 g DW) of raw broccoli and its changes during fermentation and storage at 25°C and 4°C, respectively. **B)** Shows the ORAC (oxygen radical absorbance capacity) antioxidant capacity (μmol

TE/g DW) of raw broccoli and its changes during fermentation and storage at 25°C and 4°C, respectively.

Figure 4. Shows the fermentation time taken to reach a pH of 4.4 or lower for different combinations of lactic acid bacteria strains.

Figure 5. A) Shows sulforaphane yield ($\mu\text{mol/kg DW}$) under different heat treatment conditions of broccoli with a sealed bag. **B)** Shows sulforaphane yield ($\mu\text{mol/kg DW}$) under different heat treatment conditions of broccoli immersed directly in water.

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Figure 6. Shows the comparative effects of the combined effects of maceration, pre-heating and fermentation with just maceration and preheating and maceration, preheating and chemical acidification on sulforaphane yield ($\mu\text{mol/kg DW}$) just after processing and during storage at 4°C and 25°C. Samples were pre-treated at 65°C for 3 min in sealed packs.

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Figure 7. Shows the effect of fermentation and storage on glucoraphanin content. Glucoraphanin content is reduced in fermented samples stored at 25°C and 4°C compared to raw samples.

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Figure 8. PLS-DA score plot showing the difference in polyphenolic metabolite profile of raw and fermented broccoli puree.

Figure 9. Important features differentiating fermented and non-fermented samples identified by PLS-DA. The boxes on the right indicate the relative concentration of the respective metabolites in each group.

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Figure 10. Shows the effect of lactic acid fermentation on metabolite profile of broccoli puree- based on untargeted LC-MS analysis. It demonstrates that fermentation releases bound phytochemicals such as polyphenolic glycosides and glucosinolates and enhances their bioaccessibility.

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Figure 11. Shows a volcano plot indicating metabolites with significant ($p < 0.05$) fold changes after fermentation based on untargeted LC-MS analysis. The top 50 metabolites with significant fold changes and their individual fold changes are recited in Table 8.

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Figure 12. Shows the effect of lactic acid fermentation on broccoli polyphenols based on targeted LC-MS analysis. A 6.6 fold change is observed in chlorogenic acid (2.4 to 15.8 µg/mg), a 23.8 fold increase is observed in sinapic acid (3.6 to 86.6 µg/mg), a 10.5 increase in kaempferol (12.7 to 134.6 µg/mg) and a 0.48 fold decrease is observed in p-coumaric acid.

Figure 13. Shows the *SmaI* and *NotI* restriction enzyme digestion from the genomic DNA of BF1 and BF2 obtained with pulse filed gel electrophoreses.

DETAILED DESCRIPTION

General techniques and definitions

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., enzyme, fermentation, inoculation).

The term "and/or", e.g., "X and/or Y" shall be understood to mean either "X and Y" or "X or Y" and shall be taken to provide explicit support for both meanings or for either meaning.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

As used herein, the term "about", unless stated to the contrary, refers to +/- 10%, more preferably +/- 5%, even more preferably +/- 1%, of the designated value.

An "allele" refers to one specific form of a genetic sequence (such as a gene) within a cell, an individual plant or within a population, the specific form differing from other forms of the same gene in the sequence of at least one, and frequently more than one, variant sites within the sequence of the gene. The sequences at these variant sites that differ between different alleles are termed "variances", "polymorphisms", or "mutations".

Brassicaceae

A person skilled in the art will appreciate that the methods as described herein are suitable for producing an isothiocyanate containing product from any *Brassicaceae* material comprising glucosinolate/s. As used herein, "*Brassicaceae*" refers to members of the Family *Brassicaceae* commonly referred to as mustards, crucifers or the

cabbage family. A person skilled in the art would appreciate that material can be from more than one *Brassicaceae*.

In an embodiment, the *Brassicaceae* is selected from the genus *Brassica* or *Cardamine*. In an embodiment, the *Brassica* is selected from *Brassica balearica*,
 5 *Brassica carinata*, *Brassica elongata*, *Brassica fruticulosa*, *Brassica hilarionis*,
Brassica juncea, *Brassica napus*, *Brassica narinosa*, *Brassica nigra*, *Brassica oleracea*, *Brassica perviridis*, *Brassica rapa*, *Brassica rupestris*, *Brassica septiceps*,
 and *Brassica tournefortii*.

In an embodiment, the *Brassica* is *Brassica oleracea*.

10 In an embodiment, the *Brassica* is selected from *Brassica oleracea* variety
oleracea (wild cabbage), *Brassica oleracea* variety *capitata* (cabbage), *Brassica rapa*
 subsp. *chinensis* (bok choy), *Brassica rapa* subsp. *pekinensis* (napa cabbage), *Brassica*
napobrassica (rutabaga), *Brassica rapa* var. *rapa* (turnip), *Brassica oleracea* variety
 15 *alboglabra* (kai-lan), *Brassica oleracea* variety *viridis* (collard greens), *Brassica*
oleracea variety *longata* (jersey cabbage), *Brassica oleracea* variety *acephala*
 (ornamental kale), *Brassica oleracea* variety *sabellica* (kale), *Brassica oleracea* variety
palmifolia (lacinato kale), *Brassica oleracea* variety *ramose* (perpetual kale), *Brassica*
oleracea variety *medullosa* (marrow cabbage), *Brassica oleracea* variety *costata*
 (trinchuda kale), *Brassica oleracea* variety *gemmifera* (brussels sprout), *Brassica*
 20 *oleracea* variety *gongylodes* (kohlrabi), *Brassica oleracea* variety *italica* (broccoli),
Brassica oleracea variety *botrytis* (cauliflower, Romanesco broccoli, broccoli di
 torbole), *Brassica oleracea* variety *botrytis x italica* (broccoflower), and *Brassica*
oleracea variety *italica* × *alboglabra* (Broccolini).

In an embodiment, the *Brassica* is *Brassica oleracea*, variety *italica* (broccoli).

25 In an embodiment, the *Brassicaceae* is selected from *Cardamine hirsuta*
 (bittercress), *Iberis sempervirens* (candytuft), *Sinapis arvensis* (charlock), *A Armoracia*
rusticana (horseradish), *Pringlea antiscorbutica* (Kerguelen cabbage), *Thlaspi arvense*
 (pennycress), *Raphanus raphanistrum* subsp. *sativus* (radish), *Eruca sativa* (rocket),
Anastatica hierochuntica (rose of Jericho), *Crambe maritima* (sea kale), *Cakile*
 30 *maritima* (sea rocket), *Capsella bursa-pastoris* (shepherd's purse), sweet alyssum,
Arabidopsis thaliana (thale cress), *Nasturtium officinale* (watercress), *Sinapis alba*
 (white mustard), *Erophila verna* (whitlow grass), *Raphanus raphanistrum* (wild
 radish), *Isatis tinctoria* (woad), and *Nasturtium microphyllum* (yellow cress).

In an embodiment, the *Brassicaceae* has a high level of one or more
 35 glucosinolate/s. In an embodiment, the *Brassicaceae* has been selectively bred to have
 a high level of one or more glucosinolate/s. In an embodiment, "high level" of a

glucosinolate can comprise a higher level of a glucosinolate than shown in Table 2 of Verkerk et al. (2009) in the corresponding *Brassicaceae*. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 3400 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 4000 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 5000 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 8000 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 10,000 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 12,000 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 15,000 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 18,000 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 20,000 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 25,000 $\mu\text{mol/kg}$ dry weight. In an embodiment, a high level of glucosinolate is a level of glucosinolate higher than 30,000 $\mu\text{mol/kg}$ dry weight. In an embodiment, the *Brassicaceae* has been genetically modified or subjected to biotic or abiotic stress to have a high level of one or more glucosinolate/s. A person skilled in the art will appreciate that the *Brassicaceae* can be modified by any method known to a person skilled in the art.

In an embodiment, the glucosinolate is glucoraphanin (4-Methylsulphinylbutyl). In an embodiment, the glucosinolate is glucobrassicin (3-Indolylmethyl).

As used herein "*Brassicaceae* material" refers to any part of the *Brassicaceae* which comprises a glucosinolate, including, but not limited to, the leaves, stems, flowers, florets, seeds, and roots or mixtures thereof.

A person skilled in the art will appreciate that the methods as described herein are suitable for use with different volumes of *Brassicaceae* material, for example, but not limited to, at least 30 kg, or at least 50 kg, or at least 80 kg, or at least 100 kg, or at least 1,000 kg, or at least 2,000 kg, or at least 5,000 kg, or at least 8,000 kg, or at least 10,000 kg, or at least 15,000 kg, or at least 20,000 kg.

In an embodiment, the *Brassicaceae* material has been washed. As used herein "washing" removes visible soil and contamination. In an embodiment, the *Brassicaceae* material has been sanitized. As used herein "sanitized" refers to a reduction of pathogens on the *Brassicaceae* material.

In an embodiment, the *Brassicaceae* is mixed with other plant material. In an embodiment, the other plant material is vegetable or fruit material. In an embodiment, the vegetable is a carrot or beetroot.

5 Glucosinolates

As used herein “glucosinolate” refers to a secondary metabolite found at least in the *Brassicaceae* family that share a chemical structure consisting of a β -D-glucopyranose residue linked via a sulfur atom to a (Z)-N-hydroximosulfate ester, plus a variable R group derived from an amino acid as described in Halkier et al. (2006). Examples of glucosinolates are provided in Halkier et al. (2006) and Agerbirk et al. (2012). The hydrolysis of glucosinolate can produce isothiocyanates, nitriles, epithionitrile, thiocyanate and oxazolidine-2-thione (Figure 1A). Many glucosinolates play a role in plant defence mechanisms against pests and disease.

Glucosinolates are stored in *Brassicaceae* in storage sites. As used herein, a “storage site” is a site within the *Brassicaceae* where glucosinolates are present and myrosinase is not present.

As used herein “myrosinase” also referred to as “thioglucosidase”, “sinigrase”, or “sinigrinase” refers to a family of enzymes (EC 3.2.1.147) involved in plant defence mechanisms that can cleave thio-linked glucose. Myrosinases catalyze the hydrolysis of glucosinolates resulting in the production of isothiocyanates. Myrosinase is stored sometimes as myrosin grains in the vacuoles of particular idioblasts called myrosin cells, but have also been reported in protein bodies or vacuoles, and as cytosolic enzymes that tend to bind to membranes. Thus, in an embodiment, myrosinase is stored in a myrosin cell in *Brassicaceae*.

In an embodiment, pre-treating as described herein improves the access of myrosinase to a glucosinolate. As used herein “improves the access” or “access is improved” refers to increasing the availability of glucosinolate to the myrosinase enzyme allowing for the production of an isothiocyanate. In an embodiment, access is improved by the release of a glucosinolate from a glucosinolate storage site. In an embodiment, the glucosinolate storage site is mechanically ruptured (i.e. by maceration) or enzymatically degraded. In an embodiment, glucosinolate is released from a glucosinolate storage site by the activity of one or more polysaccharide degrading enzymes e.g. a cellulase, hemicellulase, pectinase and/or glycosidase. In an embodiment, access is improved by allowing the entry of myrosinase into a glucosinolate storage site. In an embodiment, access is improved by the release of myrosinase from myrosin cells. In an embodiment, about 10% to about 90% of a

glucosinolate is released from a glucosinolate storage site. In an embodiment, about 20% to about 80% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 30% to about 70% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 40% to about 60% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 45% to about 55% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 10% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 20% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 30% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 40% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 50% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 60% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 70% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 80% of a glucosinolate is released from a glucosinolate storage site. In an embodiment, about 90% of a glucosinolate is released from a glucosinolate storage site.

In an embodiment, the *Brassicaceae* material comprises one or more glucosinolate/s selected from an aliphatic, indole or aromatic glucosinolate.

In an embodiment, the aliphatic glucosinolate is selected from one or more of glucoraphanin (4-Methylsulphinylbutyl or glucorafanin), sinigrin (2-Propenyl), gluconapin (3-Butenyl), glucobrassicinapin (4-Pentenyl), progoitrin (2(R)-2-Hydroxy-3-butenyl, epiprogoitrin (2(S)-2-Hydroxy-3-butenyl), gluconapoleiferin (2-Hydroxy-4-pentenyl), glucoibervirin (3-Methylthiopropyl, glucoerucin (4-Methylthiobutyl), dehydroerucin (4-Methylthio-3-butenyl, glucoiberin (3-Methylsulphinylpropyl), glucoraphenin (4-Methylsulphinyl-3-butenyl), glucoalyssin (5-Methylsulphinylpentenyl), and glucoerysolin (3-Methylsulphonylbutyl, 4-Mercaptobutyl).

In an embodiment, the indole glucosinolate is selected from one or more of glucobrassicin (3-Indolylmethyl), 4-hydroxyglucobrassicin (4-Hydroxy-3-indolylmethyl), 4-methoxyglucobrassicin (4-Methoxy-3-indolylmethyl), and neoglucobrassicin (1-Methoxy-3-indolylmethyl).

In an embodiment, the indole glucosinolate is selected from one or more of Glucotropaeolin (Benzyl) and Gluconasturtiin (2-Phenylethyl).

In an embodiment, the *Brassicaceae* material comprises one or more glucosinolate/s selected from benzylglucosinolate, allylglucosinolate and 4-methylsulfinylbutyl. In an embodiment, the glucosinolate is glucoraphanin (4-

Methylsulphinylbutyl). In an embodiment, the glucosinolate is glucobrassicin (3-Indolylmethyl).

In an embodiment, pre-treating as described herein increases the extractable glucosinolate content compared to the extractable glucosinolate content of the *Brassicaceae* material before pre-treatment.

As used herein “extractable glucosinolate content” refers to the level of glucosinolate accessible in the *Brassicaceae* material for conversion to isothiocyanate. Excluding conversion into nitriles and other compounds the expected maximum yield of isothiocyanate from 1 mole of glucosinolate is 1 mole of isothiocyanate (1 mole of glucosinolate can maximally be converted to 1 mole of isothiocyanate, 1 mole of glucose and 1 mole of sulphate ion). Thus, in one example, the extractable glucoraphanin content of a commercial broccoli cultivar is 3400 μmol glucoraphanin/kg dw and the expected maximum yield of sulforaphane from the commercial broccoli cultivar is 3400 μmol sulforaphane /kg dw.

Isothiocyanates

As used herein “isothiocyanate” refers to sulphur containing phytochemicals with the general structure $\text{R}-\text{N}=\text{C}=\text{S}$ which are a product of myrosinase activity upon a glucosinolate and bioactive derivatives thereof. In an embodiment, the isothiocyanate is sulforaphane (1-isothiocyanato-4-methylsulfinylbutane). In an embodiment, the isothiocyanate is allyl isothiocyanate (3-isothiocyanato-1-propene). In an embodiment, the isothiocyanate is benzyl isothiocyanate. In an embodiment, the isothiocyanate is phenethyl isothiocyanate. In an embodiment, the isothiocyanate is 3-Butenyl isothiocyanate. In an embodiment, the isothiocyanate is 5-vinyl-1,3-oxazolidine-2-thione. In an embodiment, the isothiocyanate is 3-(methylthio)propyl isothiocyanate. In an embodiment, the isothiocyanate is 3-(methylsulfinyl)-propyl isothiocyanate. In an embodiment, the isothiocyanate is 4-(methylthio)-butyl isothiocyanate. In an embodiment, the isothiocyanate is 1-methoxyindol-3-carbinol isothiocyanate. In an embodiment, the isothiocyanate is 2-phenylethyl isothiocyanate. In an embodiment, the isothiocyanate is iberin.

In an embodiment, the isothiocyanate containing product, further comprises one or more isothiocyanate bioactive derivative/s or oligomers thereof. In an embodiment, the isothiocyanate bioactive derivative is a derivative of any of the isothiocyanates as described herein. In an embodiment, the isothiocyanate bioactive derivative is a derivative of sulforaphane. In an embodiment, the isothiocyanate bioactive derivative is iberin. In an embodiment, the isothiocyanate bioactive derivative is allyl

isothiocyanate. In an embodiment, the isothiocyanate bioactive derivative is indole-3-caribinol. In an embodiment, the isothiocyanate bioactive derivative is methoxy-indole-3-carbinol. In an embodiment, the isothiocyanate bioactive derivative is ascorbigen. In an embodiment, the isothiocyanate bioactive derivative is neoscorbigen.

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Pre-treatment

As use herein “pre-treatment” or “pre-treating” releases or aids in the release of a glucosinolate from glucosinolate storage site and/or allows myrosinase to enter a glucosinolate storage site in the *Brassicaceae* material. In an embodiment, pre-treating
10 increases the exposure of a glucosinolate to myrosinase allowing myrosinase to convert a glucosinolate to an isothiocyanate.

In an embodiment, pre-treating reduces epithiospecifier protein (ESP) while maintaining endogenous myrosinase activity. As used herein “epithiospecifier protein” or “ESP” refers to a protein that directs myrosinase activity towards the production of
15 nitriles and away from isothiocyanate production. Reducing or inhibiting ESP production (mRNA or protein) or activity can increase production of isothiocyanates.

As used herein, “reduces epithiospecifier protein” refers to decreasing the protein production or activity of ESP. In an embodiment, reducing ESP comprises inactivating (e.g. denaturing) ESP at high temperature. In an embodiment, ESP is
20 denatured at temperatures of about 50°C to about 80°C.

As used herein, “maintaining endogenous myrosinase activity” means not significantly reducing myrosinase activity compared to an untreated control. In an embodiment, endogenous myrosinase activity is not reduced by about 5% or more. In an embodiment, endogenous myrosinase activity is not reduced by about 10% or more.
25 In an embodiment, endogenous myrosinase activity is not reduced by about 15% or more. In an embodiment, endogenous myrosinase activity is not reduced by about 20% or more. In an embodiment, endogenous myrosinase activity is not reduced by about 30% or more. In an embodiment, endogenous myrosinase activity is not reduced by about 40% or more. In an embodiment, endogenous myrosinase activity is not reduced
30 by about 50% or more.

In an embodiment, pre-treating comprises one or more of the following: i) heating; ii) macerating; iii) microwaving; iv) exposure to high frequency sound waves (ultrasound), or v) pulse electric field processing, wherein the temperature of the *Brassicaceae* material does not exceed about 75°C during pre-treating.

35 In an embodiment, the *Brassicaceae* material is heated in a fuel based heating system, an electricity based heating system (i.e. an oven or ohmic heating), radio

frequency heating, high pressure thermal processing or a steam based heating system (indirect or direct application of steam). In an embodiment, the *Brassicaceae* material is heated in a sealed package (e.g. in a retort pouch). In an embodiment, the *Brassicaceae* material is heated in an oven, water bath, bioreactor, stove, water blancher, or steam blancher. In an embodiment, the *Brassicaceae* material is heated via high pressure thermal heating. In an embodiment, the *Brassicaceae* material is via ohmic heating. In an embodiment, the *Brassicaceae* material is via radio frequency heating. In an embodiment, the *Brassicaceae* material is blanched in water. In an embodiment, the *Brassicaceae* material is heated via high pressure thermal processing.

10 In an embodiment, the *Brassicaceae* material is placed in a sealed package for high pressure thermal processing.

In an embodiment, pre-treating comprises heating the *Brassicaceae* material to about 50°C to about 70°C. In an embodiment, pre-treating comprises heating the *Brassicaceae* material to about 50°C to about 65°C. In an embodiment, pre-treating

15 comprises heating the *Brassicaceae* material to about 50°C to about 60°C. In an embodiment, heating comprises heating the *Brassicaceae* material to about 55°C to about 70°C. In an embodiment, heating comprises heating the *Brassicaceae* material to about 60°C to about 70°C. In an embodiment, heating comprises heating the *Brassicaceae* material to about 65°C to about 70°C. In an embodiment, the

20 *Brassicaceae* material is heated for about 30 seconds. In an embodiment, the *Brassicaceae* material is heated for about 1 minute. In an embodiment, the *Brassicaceae* material is heated for about 2 minutes. In an embodiment, the *Brassicaceae* material is heated for about 3 minutes. In an embodiment, the *Brassicaceae* material is heated for about 4 minutes. In an embodiment, the

25 *Brassicaceae* material is heated for about 5 minutes.

In an embodiment, the *Brassicaceae* material is heated in a sealed package for about 1 min at about 60°C. In an embodiment, the *Brassicaceae* material is heated in a sealed package for about 2 mins at about 60°C. In an embodiment, the *Brassicaceae* material is heated in a sealed package for about 3 mins at about 60°C. In an

30 embodiment, the *Brassicaceae* material is heated in a sealed package for about 4 mins at about 65°C. In an embodiment, the *Brassicaceae* material is heated in a sealed package for about 1 min at about 65°C. In an embodiment, the *Brassicaceae* material is heated in a sealed package for about 2 mins at about 65°C. In an embodiment, the *Brassicaceae* material is heated in a sealed package for about 3 mins at about 65°C. In an

35 embodiment, the *Brassicaceae* material is heated in a sealed package for about 4 mins at about 65°C.

In an embodiment, the *Brassicaceae* material is heated in water for about 1 min at about 60°C. In an embodiment, the *Brassicaceae* material is heated in water for about 2 mins at about 60°C.

In an embodiment, heating comprises steaming the *Brassicaceae* material. In an embodiment, pre-treating comprises steaming the *Brassicaceae* material. In an embodiment, the *Brassicaceae* material is steamed to a temperature of about 50°C to about 70°C. In an embodiment, the *Brassicaceae* material is steamed to a temperature of about 60°C to about 70°C. In an embodiment, the *Brassicaceae* material is steamed for at least about 30 seconds. In an embodiment, the *Brassicaceae* material is steamed for at least about 1 minute. In an embodiment, the *Brassicaceae* material is steamed for at least about 2 minutes. In an embodiment, the *Brassicaceae* material is steamed for at least about 3 minutes. In an embodiment, the *Brassicaceae* material is steamed for at least about 4 minutes. In an embodiment, the *Brassicaceae* material is steamed for at least about 5 minutes.

In an embodiment, pre-treating comprises macerating the *Brassicaceae* material. As used herein “macerating”, “macerated” or “macerate” refers to breaking the *Brassicaceae* material into smaller pieces. In an embodiment, macerating comprising decompartmentalizing at least about 30% to about 90% of the cells of the *Brassicaceae* material to allow myrosinase access to its substrate glucosinolates. In an embodiment, macerating comprising decompartmentalizing at least about 40% to about 90% of the cells of the *Brassicaceae* material. In an embodiment, macerating comprising decompartmentalizing at least about 50% to about 90% of the cells of the *Brassicaceae* material. In an embodiment, macerating comprising decompartmentalizing at least about 60% to about 90% of the cells of the *Brassicaceae* material. In an embodiment, macerating comprising decompartmentalizing at least about 70% to about 90% of the cells of the *Brassicaceae* material. A person skilled in the art will appreciate that decompartmentalizing a cell comprising breaking open the cell wall and disrupting the compartmentalization of organelles within a cell.

In an embodiment, the *Brassicaceae* material is macerated with a blender, grinder or pulveriser. In an embodiment, the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a size of about 2 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a size of about 1 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a size of about 0.5 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a

size of about 0.25 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a size of about 0.1 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a size of about 0.05 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a size of about 0.025 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a size of about 0.01 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that about 50% to about 90% of the *Brassicaceae* material is of a size of about 2 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that about 60% to about 80% of the *Brassicaceae* material is of a size of about 2 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that about 50% to about 90% of the *Brassicaceae* material is of a size of about 1 mm or less. In an embodiment, the *Brassicaceae* material is macerated so that about 60% to about 80% of the *Brassicaceae* material is of a size of about 1 mm or less. In an embodiment, the *Brassicaceae* material is heated to a temperature of about 50°C to about 70°C during maceration. In an embodiment, the *Brassicaceae* material is heated to a temperature of about 55°C to about 70°C during maceration. In an embodiment, the *Brassicaceae* material is heated to a temperature of about 60°C to about 70°C during maceration. In an embodiment, the *Brassicaceae* material is heated to a temperature of about 65°C to about 70°C during maceration.

In an embodiment, pre-treating comprises heating and macerating the *Brassicaceae* material. In an embodiment, pre-treating produces a puree. As used herein a “puree” refers to *Brassicaceae* material blended to the consistency of a creamy paste or liquid.

A person skilled in the art will appreciate that “microwaves” or “microwaving” heats a substance such as *Brassicaceae* material by passing microwave radiation through the substance. In an embodiment, pre-treating comprises microwaving the *Brassicaceae* material. In an embodiment, *Brassicaceae* material is pre-treated in a consumer microwave or industrial microwave. In an embodiment, the industrial microwave is a continuous microwave system, for example, but not limited to the MIP 11 Industrial Microwave Continuous Cooking Over (Ferrite Microwave Technologies). In an embodiment, pre-treating comprises microwaving the *Brassicaceae* material. In an embodiment, the *Brassicaceae* material is microwaved at about 0.9 to about 2.45 GHz. In an embodiment, the *Brassicaceae* material is microwaved for at least about 30 seconds, or at least about 1 minute, or at least about 2 minutes, or at least 3 minutes.

In an embodiment, pre-treating comprises exposing the *Brassicaceae* material at low to medium frequency ultrasound waves. In an embodiment, pre-treating comprises exposing the *Brassicaceae* material with thermosonication (low to medium frequency ultrasound waves with heat of about 30°C to about 60°C). In an embodiment, the
5 ultrasound waves are generated with an industrial scale ultrasonic processor. In an embodiment, the ultrasonic processor is a continuous or batch ultrasonic processor. In an embodiment, the ultrasonic processor is for example, but not limited to, UIP500hd or UIP4000 (Hielscher, Ultrasound Technology). In an embodiment, the ultrasounds waves are at a frequency of about 20 kHz to about 600 kHz. In an embodiment, the
10 *Brassicaceae* material is exposed to sound waves for at least about 30 seconds, or at least about 1 minute, or at least about 2 minutes, or at least about 3 minutes, or about 5 minutes.

In an embodiment, pre-treating comprises exposing the *Brassicaceae* material to pulse electric field processing. Pulse electric field processing is a non-thermal
15 processing technique comprising the application of short, high voltage pulses. The pulses induce electroporation of the cells of the *Brassicaceae* material enhancing the access of myrosinase to glucosinolates. In an embodiment, pulse electric field processing heats the *Brassicaceae* material to a temperature of about 40 to about 70°C. In an embodiment, pulse electric field processing heats the *Brassicaceae* material to a temperature of about 50°C to about 70°C. In an embodiment, pulse electric field processing heats the *Brassicaceae* material to a temperature of about 60°C to about 70°C. In an embodiment, pulse electric field processing comprises treating the *Brassicaceae* material with voltage pulses of about 20 to about 80 kV. In an embodiment, pre-treating converts about 10% to about 90% of a glucosinolate to an
25 isothiocyanate. In an embodiment, pre-treating converts about 20% to about 80% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating converts about 30% to about 70% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating converts about 40% to about 60% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating converts about 10% of a glucosinolate to an isothiocyanate.
30 In an embodiment, pre-treating converts about 20% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating converts about 30% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating converts about 40% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating converts about 50% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating converts about
35 60% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating converts about 70% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating

converts about 80% of a glucosinolate to an isothiocyanate. In an embodiment, pre-treating converts about 90% of a glucosinolate to an isothiocyanate.

Fermentation

5 A person skilled in the art will appreciate that the fermentation method as described herein can comprise the use of any lactic acid bacteria. As used herein, “fermentation” refers to the biochemical breakdown of the *Brassicaceae* material by lactic acid bacteria. In an embodiment, fermentation with lactic acid bacteria is performed using the addition of exogenous lactic acid bacteria. As used herein, “lactic
10 bacteria” or “lactic acid bacteria” are bacteria that produce lactic acid as an end product of carbohydrate fermentation, and can include, but are not limited to including bacteria from the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. In an embodiment, the lactic acid bacteria
15 comprises myrosinase activity. In an embodiment, the lactic acid bacteria is from the genera *Leuconostoc*. In an embodiment, the lactic acid bacteria is from the genera *Lactobacillus*.

In an embodiment, the lactic acid bacteria is selected from one or more of *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactobacillus rhamnosus*,
20 *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactococcus lactis*, *Pediococcus pentosaceus* and *Pedicoccus acidilacti*.

In an embodiment, the lactic acid bacteria was isolated from a *Brassicaceae*. In an embodiment, the lactic acid bacteria was isolated from a *Brassica oleracea*. In an embodiment, the lactic acid bacteria was isolated from broccoli. In an embodiment, the
25 lactic acid bacteria was isolated from broccoli leaves. In an embodiment, the lactic acid bacteria was isolated from broccoli stem. In an embodiment, the lactic acid bacteria was isolated from broccoli puree. In an embodiment, the lactic acid bacteria was isolated from Australian broccoli.

In an embodiment, the lactic acid bacteria lacks myrosinase activity.

30 In an embodiment, the lactic acid bacteria is a *Lactobacillus*.

In an embodiment, the lactic acid bacteria is selected from: i) a *Leuconostoc mesenteroides*; ii) a *Lactobacillus plantarum*; iii) a *Lactobacillus pentosus*; iv) a *Lactobacillus rhamnosus*; v) a combination of i) and ii); vi) a combination of i), ii) and
35 iii); and vii) a combination of i), ii) and iv).

In one embodiment, the lactic acid bacteria is *Leuconostoc mesenteroides*. In an embodiment, the *Leuconostoc mesenteroides* is ATCC8293. In an embodiment, the

Leuconostoc mesenteroides is BF1 and/or BF2. In an embodiment, the *Leuconostoc mesenteroides* lacks myrosinase activity.

In one embodiment, the lactic acid bacteria is *Lactobacillus plantarum*. In an embodiment, the *Lactobacillus plantarum* lacks myrosinase activity.

5 In one embodiment, about 50% of the lactic acid bacteria is *Leuconostoc mesenteroides* and about 50% of the lactic acid bacteria is *Lactobacillus sp.*

In one embodiment, about 50% of the lactic acid bacteria is *Leuconostoc mesenteroides* and about 50% of the lactic acid bacteria is *Lactobacillus plantarum*.

In an embodiment, the *Lactobacillus plantarum* is selected from one or more or
10 all of B1, B2, B3, B4 and B5. In an embodiment, the *Lactobacillus plantarum* is B1. In an embodiment, the *Lactobacillus plantarum* is B2. In an embodiment, the *Lactobacillus plantarum* is B3. In an embodiment, the *Lactobacillus plantarum* is B4. In an embodiment, the *Lactobacillus plantarum* is B5.

In an embodiment, fermentation occurs in the presence of at least 2, or at least 3,
15 or at least 4, or at least 5, or at least 6 strains of lactic acid bacteria selected from BF1, BF2, B1, B2, B3, B4 and B5.

In one embodiment, the lactic acid bacteria is a recombinant bacteria modified to produce a high level of myrosinase activity compared to a control bacteria lacking the modification. A person skilled in the art will appreciate that the recombinant lactic
20 acid bacteria is produced by any technique known to a person skilled in the art.

In an embodiment, the lactic acid bacteria is stressed, for example but not limited to, heat stress, cold stress, sub-lethal ultrasonic waves e.g. about 20 to about 2000 MHz, high pressure, dynamic high pressure or pulsed-electric field, to increase myrosinase activity and the activity of polysaccharide degrading enzymes compared to
25 a control lactic acid bacteria that has not been stressed. In an embodiment, heat stress comprises heating the bacteria to greater than about 40°C to about 75°C. In an embodiment, heat stress comprises heating the bacteria to greater than about 45°C to about 65°C. In an embodiment, heat stress comprises heating the bacteria to greater than about 45°C to about 55°C. In an embodiment, cold stress comprises lower the
30 bacteria to temperature of about 0°C to about 8°C. In an embodiment, cold stress comprises lower the bacteria to temperature of about 2°C to about 6°C. In an embodiment, cold stress comprises lower the bacteria to temperature of about 4°C.

In an embodiment, the *Brassicaceae* material is inoculated with at least about 10⁵ CFU/g of a lactic acid bacteria as described herein. In an embodiment, the
35 *Brassicaceae* material is inoculated with at least 10⁶ about CFU/g of a lactic acid bacteria as described herein. In an embodiment, the *Brassicaceae* material is inoculated

with at least about 10^7 CFU/g of a lactic acid bacteria as described herein. In an embodiment, the *Brassicaceae* material is inoculated with at least about 10^8 CFU/g of a lactic acid bacteria as described herein. In an embodiment, the *Brassicaceae* material has been pre-treated.

5 In an embodiment, fermentation is at about 20°C to about 34°C. In an embodiment, fermentation is at about 22°C to about 34°C. In an embodiment, fermentation is at about 24°C to about 34°C. In an embodiment, fermentation is at about 24°C to about 30°C. In an embodiment, fermentation is at about 34°C to about 34°C. In an embodiment, fermentation is at about 25°C. In an embodiment, fermentation is at about 30°C. In an embodiment, fermentation is at about 34°C.

10 In an embodiment, fermentation is for about 8 hours to about 17 days. In an embodiment, fermentation is for about 8 hours to about 14 days. In an embodiment, fermentation is for about 8 hours to about 7 days. In an embodiment, fermentation is for about 8 hours to about 5 days. In an embodiment, fermentation is for about 8 hours to about 4 days. In an embodiment, fermentation is for about 8 hours to about 3 days. In an embodiment, fermentation is for about 8 hours to about 30 hours. In an embodiment, fermentation is for about 8 to about 24 hours. In an embodiment, fermentation is for about 10 hours to about 24 hours. In an embodiment, fermentation is for about 10 days. In an embodiment, fermentation is for about 9 days. In an embodiment, fermentation is for about 8 days. In an embodiment, fermentation is for about 7 days. In an embodiment, fermentation is for about 4 days. In an embodiment, fermentation is for about 6 days. In an embodiment, fermentation is for about 5 days. In an embodiment, fermentation is for about 72 hours. In an embodiment, fermentation is for about 60 hours. In an embodiment, fermentation is for about 45 hours. In an embodiment, fermentation is for about 30 hours. In an embodiment, fermentation is for about 24 hours. In an embodiment, fermentation is for about 20 hours. In an embodiment, fermentation is for about 18 hours. In an embodiment, fermentation is for about 15 hours. In an embodiment, fermentation is for about 16 hours. In an embodiment, fermentation is for about 14 hours. In an embodiment, fermentation is for about 12 hours. In an embodiment, fermentation is for about 10 hours. In an embodiment, fermentation is for about 8 hours. In an embodiment, the fermentation culture is stirred. In an embodiment, stirring is intermittent. In an embodiment, stirring is continuous. In a particularly preferred embodiment, fermentation is for 15 hours with intermittent stirring. In a particularly preferred embodiment, fermentation is for 24 hours with intermittent stirring.

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In an embodiment, the fermentation reaction is complete when the composition reaches a pH of about 4.5 to about 3.8. In an embodiment, the fermentation reaction is complete when the composition reaches a pH of about 4.5 to about 3.6. In an embodiment, the fermentation reaction is complete when the composition reaches a pH of about 4.5 to about 4.04. In an embodiment, the fermentation reaction is complete when the composition reaches a pH of about 4.3 to about 4.04. In an embodiment, the fermentation reaction is complete when the composition reaches a pH of 4.5 or less, or 4.4 or less, or 4.3 or less, or 4.04 or less, or 3.8 or less. In an embodiment, the fermentation reaction is complete when the composition reaches a pH of 4.5 or less. In an embodiment, the fermentation reaction is complete when the composition reaches a pH of 4.4 or less.

In an embodiment, if present fermentation reduces the number of one or more or all of: *E. coli*, *Salmonella* and *Listeria*. In an embodiment, if present fermentation reduces the CFU/g of one or more or all of: *E. coli*, *Salmonella* and *Listeria*.

In an embodiment, no salt is added to the fermentation culture.

In an embodiment, fermentation increases the extractable glucosinolate content compared to the extractable glucosinolate content in the pre-treated *Brassicaceae* material. In an embodiment, fermentation increases the extractable glucosinolate content compared to the extractable glucosinolate content in the *Brassicaceae* material. In an embodiment, fermentation increases the extractable glucosinolate content is increased by about 100% to about 500% compared to the extractable glucosinolate content in the *Brassicaceae* material. In an embodiment, fermentation increases the extractable glucosinolate content by about 200% to about 450% compared to the extractable glucosinolate content in the *Brassicaceae* material. In an embodiment, fermentation increases the extractable glucosinolate content by about 250% to about 450% compared to the extractable glucosinolate content in the *Brassicaceae* material. In an embodiment, fermentation increases the extractable glucosinolate content by about 300% to about 400% compared to the extractable glucosinolate content in the *Brassicaceae* material. In an embodiment, fermentation increases the extractable glucosinolate content by about 300% compared to the extractable glucosinolate content in the *Brassicaceae* material. In an embodiment, fermentation increases the extractable glucosinolate content by about 400% compared to the extractable glucosinolate content in the *Brassicaceae* material. In an embodiment, the glucosinolate is glucoraphanin.

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Acidification

The pre-treated material can be acidified to improve the microbial safety and stability (susceptibility to microbial degradation) of the product and increase the stability of isothiocyanate in the product. Acidification can be achieved by the addition of organic acids, such as, but not limited to lactic, acetic, ascorbic, and citric acid. In an embodiment, acidification can be achieved with the addition of glucono-delta-lactone. In an embodiment, acidification comprises lowering the pH to a pH of about 4.4 to about 3.4. In an embodiment, acidification comprises lowering the pH to a pH of 4.5, or 4.4, or 4.2, or 4, or 3.8, or 3.6, or 3.4 or less. In an embodiment, acidification comprises lowering the pH to a pH of 4.4 or less.

Isothiocyanate containing product from *Brassicaceae*

An isothiocyanate containing product from *Brassicaceae* as described herein can be produced by the methods as described herein. It will be appreciated by a person skilled in the art that an isothiocyanate containing product produced using the methods as described herein contains higher levels of isothiocyanates, for example sulforaphane, than the *Brassicaceae* material or *Brassicaceae* material subjected to fermentation alone (without pre-treatment as described herein). For example, macerated broccoli from a commercial broccoli cultivar has a sulforaphane concentration of $\sim 800 \mu\text{mol/Kg dw}$ ($\sim 149.8 \text{ mg/Kg dw}$), fermented macerated broccoli has a sulforaphane concentration of $\sim 1600 \mu\text{mol/Kg dw}$ ($\sim 278.8 \text{ mg/Kg dw}$) and pre-treated and fermented broccoli produced using the methods as described herein has a sulforaphane concentration of $\sim 13100 \mu\text{mol/Kg dw}$ ($\sim 2318.7 \text{ mg/Kg dw}$).

In an embodiment, the isothiocyanate containing product comprises at least about 4 times more isothiocyanate than macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 6 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 8 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 10 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 12 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 14 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an

embodiment, the isothiocyanate containing product comprises at least about 17 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises about 4 times to about 17 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises about 4 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises about 8 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises about 10 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises about 12 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises about 14 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate is sulforaphane.

In an embodiment, the level of isothiocyanate present in the isothiocyanate containing product is higher than what would be expected from the extractable glucosinolate content of the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 1 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the isothiocyanate containing product comprises at least about 2 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the isothiocyanate containing product comprises at least about 3 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the isothiocyanate containing product comprises at least about 3.8 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the isothiocyanate containing product comprises at least about 4 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the isothiocyanate containing product comprises about 1 times to about 4 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the isothiocyanate containing product comprises about 1 times to about 3.8 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the isothiocyanate containing product comprises about 2 times to about 3.8 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the

isothiocyanate containing product comprises about 2 times to about 3 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content.

In an embodiment, the level of sulforaphane present in the isothiocyanate containing product is higher than what would be expected from the extractable glucoraphanin content of the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 1 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the isothiocyanate containing product comprises at least about 2 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the isothiocyanate containing product comprises at least about 3 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the isothiocyanate containing product comprises at least about 3.8 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the isothiocyanate containing product comprises at least about 4 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the isothiocyanate containing product comprises about 1 times to about 4 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the isothiocyanate containing product comprises about 1 times to about 3.8 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the isothiocyanate containing product comprises about 1 times to about 3 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the isothiocyanate containing product comprises about 2 times to about 3 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content.

In an embodiment, the isothiocyanate containing product comprises about 100 mg/kg dw to about 7000 mg/kg dw of isothiocyanate. In an embodiment, the isothiocyanate containing product comprises about 500 mg/kg dw to about 7000 mg/kg dw of isothiocyanate. In an embodiment, the isothiocyanate containing product comprises about 1000 mg/kg dw to about 7000 mg/kg dw of isothiocyanate. In an embodiment, the isothiocyanate containing product comprises about 1600 mg/kg dw to about 4000 mg/kg dw of isothiocyanate. In an embodiment, the isothiocyanate containing product comprises about 1600 mg/kg dw to about 3000 mg/kg dw of isothiocyanate. In an embodiment, the isothiocyanate containing product comprises

about 2000 mg/kg dw to about 4000 mg/kg dw of isothiocyanate. In an embodiment, the isothiocyanate containing product comprises about 2000 mg/kg dw of to about 7000 mg/kg dw of isothiocyanate. In an embodiment, the isothiocyanate containing product comprises about 3000 mg/kg dw isothiocyanate to about 7000 mg/kg of isothiocyanate.

- 5 In an embodiment, the isothiocyanate containing product comprises about 2300 mg/kg dw of the isothiocyanate.

In an embodiment, the isothiocyanate containing product comprises at least about 100 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 200 mg/kg dw of the isothiocyanate. In an
10 embodiment, the isothiocyanate containing product comprises at least about 250 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 300 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 350 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at
15 least about 400 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 450 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 500 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 550 mg/kg dw of the isothiocyanate. In an embodiment, the
20 isothiocyanate containing product comprises at least about 600 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 650 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 700 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 1000
25 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 2000 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 3000 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 4000 mg/kg dw of the isothiocyanate. In an
30 embodiment, the isothiocyanate containing product comprises at least about 5000 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 6000 mg/kg dw of the isothiocyanate. In an embodiment, the isothiocyanate containing product comprises at least about 7000 mg/kg dw of the isothiocyanate.

- 35 In an embodiment, the isothiocyanate containing product comprises at least about 100 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing

product comprises at least about 150 mg/kg of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 200 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 250 mg/kg of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 300 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 350 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 400 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 450 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 500 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 550 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 600 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 650 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 700 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 1000 mg/kg of sulforaphane dw. In an embodiment, the isothiocyanate containing product comprises at least about 2000 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 3000 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 4000 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 5000 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 6000 mg/kg dw of sulforaphane. In an embodiment, the isothiocyanate containing product comprises at least about 7000 mg/kg dw of sulforaphane.

In an embodiment, the isothiocyanate containing product comprises at least about 5% more total fibre than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 10% more total fibre than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 15% more total fibre than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 20% more total fibre than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 4% more protein than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 6% more protein than the *Brassicaceae* material. In an embodiment, the

isothiocyanate containing product comprises at least about 8% more protein than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 10% more protein than the *Brassicaceae* material.

In an embodiment, the isothiocyanate containing product comprises at least about 10% less carbohydrate than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 20% less carbohydrate than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 30% less carbohydrate than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 40% less carbohydrate than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 45% less carbohydrate than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises at least about 48% less carbohydrate than the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises about 10% to about 48% less carbohydrate than the *Brassicaceae* material.

In an embodiment, the isothiocyanate containing product comprises an increased level of polyphenolic glycosides compared to the *Brassicaceae* material. In an embodiment, the polyphenolic glycosides are anthocyanin glycosides. In an embodiment, the polyphenolic glycosides are phenolic acid glycosides. In an embodiment, the polyphenolic glycosides are phenolic acids.

In an embodiment, the isothiocyanate containing product comprises an increased level of glucosinolates compared to the *Brassicaceae* material. In an embodiment, the glucosinolate is glucoraphanin. In an embodiment, glucoraphanin is increased at least about 25 fold. In an embodiment, the glucosinolate is glucobrassicin. In an embodiment, the glucobrassicin is increased by 26 times. In an embodiment, the isothiocyanate containing product comprises indole-3-carbinol. In an embodiment, indol-3carbinol is increased at least about 2 fold in the isothiocyanate containing product compared to the macerated *Brassicaceae* material. In an embodiment, indol-3-carbinol is increased at least about 3 fold in the isothiocyanate containing product compared to the macerated *Brassicaceae* material. In an embodiment, the isothiocyanate containing product comprises ascorbigen. In an embodiment, ascorbigen is increased at least about 2 fold in the isothiocyanate containing product compared to the macerated *Brassicaceae* material. In an embodiment, ascorbigen is increased at least about 3 fold in the isothiocyanate containing product compared to the macerated *Brassicaceae* material.

In an embodiment, the isothiocyanate containing product comprises an increased level of one or more of ferullic acid, syringic acid, phenyllactic acid, chlorogenic acid, rutin, sinapic acid, methyl syringate, hesperetin, quercetin and kaempferol compared to the *Brassicaceae* material. In an embodiment, the isothiocyanate containing product
5 comprises an increased level of chlorogenic acid compared to the *Brassicaceae* material. In an embodiment, chlorogenic acid is increased about 6.6 fold. In an embodiment, the isothiocyanate containing product comprises an increased level of sinapic acid compared to the *Brassicaceae* material. In an embodiment, sinapic acid is increased about 23.8 fold. In an embodiment, the isothiocyanate containing product
10 comprises an increased level of kaempferol compared to the *Brassicaceae* material. In an embodiment, kaempferol is increased about 10.5 fold.

In an embodiment, the isothiocyanate containing product comprises an decreased level of one or more of protocatechuic acid, gallic acid, 4,hydroxybenzoic acid, vanillic acid, 2,3dihydroxybenzoic acid, p-cuomeric acid, cinnamic acid, catechin,
15 rosmarinic acid, caffeic acid compared to the *Brassicaceae* material.

In an embodiment, about 40% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an embodiment, about 50% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an
20 embodiment, about 60% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an embodiment, about 70% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an embodiment, about 80% of a glucosinolate present in the *Brassicaceae* material is
25 converted to an isothiocyanate in the isothiocyanate containing product. In an embodiment, about 90% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an embodiment, about 95% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an
30 embodiment, about 97% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an embodiment, about 98% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an
35 embodiment, about 99% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an embodiment, about 100% of a glucosinolate present in the *Brassicaceae* material is

converted to an isothiocyanate in the isothiocyanate containing product. In an embodiment, about 40% to about 100% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing product. In an embodiment, about 40% to about 80% of a glucosinolate present in the *Brassicaceae* material is converted to an isothiocyanate in the isothiocyanate containing *Brassicaceae* product.

In an embodiment, the isothiocyanate in the isothiocyanate containing product is stable for at least a week, or for at least two weeks, or for at least 3 weeks, or for at least 4 weeks, or for at least 6 weeks, or for at least 8 weeks, or for at least 10 weeks, or for at least 12 weeks, or for at least 14 weeks when stored at about 4°C to about 25°C. In an embodiment, the isothiocyanate in the isothiocyanate containing product is stable for at least 4 weeks when stored at about 4°C to about 25°C. In an embodiment, the isothiocyanate in the isothiocyanate containing product is stable for at least 8 weeks when stored at about 4°C to about 25°C. In an embodiment, the isothiocyanate in the isothiocyanate containing product is stable for at least 12 weeks when stored at about 4°C to about 25°C.

As used herein “stable” refers to no decrease or only a minor decrease in isothiocyanate concentration when stored at 4°C for six weeks. In an embodiment, a minor decrease refers to a decrease in isothiocyanate concentration of about 1% to about 30%. In an embodiment, a minor decrease refers to a decrease in isothiocyanate concentration of about 5% or less. In an embodiment, a minor decrease refers to a decrease in isothiocyanate concentration of about 10% or less. In an embodiment, a minor decrease refers to a decrease in isothiocyanate concentration of about 15% or less. In an embodiment, a minor decrease refers to a decrease in isothiocyanate concentration of about 20% or less. In an embodiment, a minor decrease refers to a decrease in isothiocyanate concentration of about 30% or less. Isothiocyanate analysis can be performed by any method known to a person skilled in the art and for example as shown in Example 1 for sulforaphane.

In an embodiment, the isothiocyanate is sulforaphane.

In an embodiment, the isothiocyanate containing product is resistant to yeast, mould and/or coliform growth for at least a week, or for at least two weeks, or for at least 3 weeks, or for at least 4 weeks, or for at least 6 weeks, or for at least 8 weeks, or for at least 10 weeks, or for at least 12 weeks, or for at least 14 weeks when stored at about 4°C to about 25°C.

In an embodiment, the isothiocyanate containing product is resistant to yeast, mould and/or coliform growth for at least 4 weeks when stored at about 4°C to about

25°C. In an embodiment, the isothiocyanate containing product is resistant to yeast, mould and/or coliform growth for at least 8 weeks when stored at about 4°C to about 25°C. In an embodiment, the isothiocyanate containing product is resistant to yeast, mould and/or coliform growth for at least 12 weeks when stored at about 4°C to about 25°C.

As used herein “resistant” to yeast, mould and/or coliform growth means that <1 Log CFU/g of yeast, mould and/or coliform is detectable in the sample after the above listed time periods using the methods described in Example 1. In an embodiment, the isothiocyanate containing product comprises about 20 g/100gdw to about 32 g/100gdw total fibre. In an embodiment, the isothiocyanate containing product comprises about 20 g/100gdw total fibre. In an embodiment, the isothiocyanate containing product comprises about 25 g/100gdw total fibre. In an embodiment, the isothiocyanate containing product comprises about 28 g/100gdw total fibre. In an embodiment, the isothiocyanate containing product comprises about 29 g/100gdw total fibre. In an embodiment, the isothiocyanate containing product comprises about 30 g/100gdw total fibre. In an embodiment, the isothiocyanate containing product comprises about 32 g/100gdw total fibre.

In an embodiment, the isothiocyanate containing product comprises an ORAC antioxidant capacity of about 14000 µmol TE/100 gdw to about 19000 µmol TE/100 gdw. In an embodiment, the isothiocyanate containing product comprises an ORAC antioxidant capacity of about 14000 µmol TE/100 gdw. In an embodiment, the isothiocyanate containing product comprises an ORAC antioxidant capacity of about 15000 µmol TE/100 gdw. In an embodiment, the isothiocyanate containing product comprises an ORAC antioxidant capacity of about 16000 µmol TE/100 gdw. In an embodiment, the isothiocyanate containing product comprises an ORAC antioxidant capacity of about 17000 µmol TE/100 gdw. In an embodiment, the isothiocyanate containing product comprises an ORAC antioxidant capacity of about 18000 µmol TE/100 gdw. In an embodiment, the isothiocyanate containing product comprises an ORAC antioxidant capacity of about 18695 µmol TE/100 gdw. In an embodiment, the isothiocyanate containing product comprises an ORAC antioxidant capacity of about 19000 µmol TE/100 gdw.

In an embodiment, the isothiocyanate containing product comprises a total polyphenol content of about 1750 mg GAE/100gdw to about 2600 mg GAE/100gdw. In an embodiment, the isothiocyanate containing product comprises a total polyphenol content of about 1750 mg GAE/100gdw. In an embodiment, the isothiocyanate containing product comprises a total polyphenol content of about 2000 mg

GAE/100gdw. In an embodiment, the isothiocyanate containing product comprises a total polyphenol content of about 2100 mg GAE/100gdw. In an embodiment, the isothiocyanate containing product comprises a total polyphenol content of about 2200 mg GAE/100gdw. In an embodiment, the isothiocyanate containing product comprises a total polyphenol content of about 2300 mg GAE/100gdw. In an embodiment, the isothiocyanate containing product comprises a total polyphenol content of about 2360 mg GAE/100gdw.

In an embodiment, the isothiocyanate containing product comprises a total titratable acidity of about 0.9% to about 1.1% lactic acid equivalent. In an embodiment, the isothiocyanate containing product comprises a total titratable acidity of about 1.1% lactic acid equivalent.

In an embodiment, the isothiocyanate containing product comprises a total protein content of about 23 g/100gdw to about 39 g/100gdw. In an embodiment, the isothiocyanate containing product comprises a total protein content of about 23 g/100gdw to about 30 g/100gdw. In an embodiment, the isothiocyanate containing product comprises a total protein content of about 25 g/100gdw. In an embodiment, the isothiocyanate containing product comprises a total protein content of about 27 g/100gdw. In an embodiment, the isothiocyanate containing product comprises a total protein content of about 28 g/100gdw. In an embodiment, the isothiocyanate containing product comprises a total protein content of about 29 g/100gdw. In an embodiment, the isothiocyanate containing product comprises a total protein content of about 30 g/100gdw. In an embodiment, the isothiocyanate containing product comprises a total protein content of about 32 g/100gdw.

In an embodiment, the isothiocyanate containing product comprises at least about 100 mg/kg dw of an isothiocyanate and one or more or all of the following.

- i) total fibre at about 29 to about 36g/100gdw;
- ii) an ORAC antioxidant capacity of about 15000 to about 18695 μ mol TE/100 gdw;
- iii) a total polyphenol content of about 2310 to about 2600 mg GAE/100gdw;
- iv) a total titratable acidity of about 0.9 to about 1.1% lactic acid equivalent;
- v) a total protein content of about 27 to about 39 g/100 gdw; and
- vi) *Leuconostoc mesenteroides* and/or *Lactobacillus plantarum*.

In an embodiment, the isothiocyanate containing product is produced from broccoli.

The *Brassicaceae* products as described herein can comprise live lactic acid bacteria which can aid the conversion of glucosinolate present in the isothiocyanate

containing product to an isothiocyanates during digestion of a glucosinolate containing product in a subject (i.e. they act as a probiotic). In an embodiment, the lactic acid bacteria is a *Leuconostoc mesenteroide*. In an embodiment, the lactic acid bacteria is *Lactobacillus sp*. In an embodiment, the lactic acid bacteria is *Lactobacillus plantarum*.

5 In an embodiment, the isothiocyanate containing product comprises lactic acid bacteria at a concentration of at least about 10^2 CFU/g. In an embodiment, the isothiocyanate containing product comprises lactic acid bacteria at a concentration of at least about 10^2 CFU/g. In an embodiment, the isothiocyanate containing product comprises lactic acid bacteria at a concentration of at least about 10^5 CFU/g. In an
10 embodiment, the isothiocyanate containing product comprises lactic acid bacteria at a concentration of at least about 10^6 CFU/g. In an embodiment, the isothiocyanate containing product comprises lactic acid bacteria at a concentration of at least about 10^7 CFU/g. In an embodiment, the isothiocyanate containing product comprises lactic acid bacteria at a concentration of at least about 10^8 CFU/g. In an embodiment, the
15 isothiocyanate containing product comprises lactic acid bacteria at a concentration of at least about 10^9 CFU/g.

In an embodiment, live lactic acid bacteria are present in the isothiocyanate containing product for at least 10 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are present in the isothiocyanate containing
20 product at least 20 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are present in the isothiocyanate containing product at least 30 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are present in the isothiocyanate containing product at least 40 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are
25 present in the isothiocyanate containing product at least 50 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are present in the isothiocyanate containing product at least 60 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are present in the isothiocyanate containing product at least 70 days when stored at about 4°C to about 25°C. In an
30 embodiment, live lactic acid bacteria are present in the isothiocyanate containing product at least 80 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are present in the isothiocyanate containing product at least 85 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are present in the isothiocyanate containing product at least 90 days when
35 stored at about 4°C to about 25°C.

In an embodiment, the lactic acid bacteria is a *Lactobacillus sp.*. In an embodiment, the lactic acid bacteria is *Lactobacillus plantarum*. In an embodiment, the lactic acid bacteria is *Leuconostoc mesenteroides*. In an embodiment, the bacteria are present at a concentration of at least about 10^7 CFU/g.

5 In an embodiment, the isothiocyanate containing product comprises one or more bacteriocin/s produced by lactic acid bacteria. In an embodiment, the bacteriocin is a Class I bacteriocin. In an embodiment, the bacteriocin is a Class II bacteriocin. In an embodiment, the bacteriocin is a Class III bacteriocin. Examples of bacteriocins produced by lactic acid bacteria can be found in Alvarez-Sieiro et al. (2016).

10 In an embodiment, the isothiocyanate containing product is a food product. In an embodiment, the isothiocyanate containing product is a nutraceutical. In an embodiment, the isothiocyanate containing product is a supplement. In an embodiment, the isothiocyanate containing product is a food ingredient. In an embodiment, the isothiocyanate containing product is a probiotic. In an embodiment, the isothiocyanate containing product is an animal feed. The animal can be an aquatic animal such as fish or livestock. In an embodiment, the isothiocyanate containing product is a pesticide. In an embodiment, the isothiocyanate containing product is a cosmeceutical. In an embodiment, the isothiocyanate containing product is topically formulated.

15 In an embodiment, the isothiocyanate containing product is a solid, liquid, puree or a powder. In an embodiment, the isothiocyanate containing product is dried to a powder after fermentation. In an embodiment, the isothiocyanate containing product is freeze dried after fermentation. In an embodiment, the isothiocyanate containing product is microencapsulated as described in WO2005030229 after fermentation. In an embodiment, the isothiocyanate containing product is formulated as a pill.

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Post-treatment

In an embodiment, after fermentation or acidification the isothiocyanate containing product can be post-treated to inactivate microbes that for example contribute to degradation of the product or a pathogenic if consumed.

30 As used herein “post-treatment” or “post-treating” refers to treatment of the isothiocyanate containing product as described herein after fermentation to inactivate microbes. As used herein “microbes” refers to bacterial, viral, fungal or eukaryotic activity that can result in degradation or spoilage of the isothiocyanate containing product. As used herein “inactivate” or “inactivation” of microbes refers to reducing the viable microbes by about 1 to about 7 logs. In an embodiment, the viable microbes are reduced by about 1 to 6 logs. In an embodiment, the viable microbes are reduced by

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about 2 to 6 logs. In an embodiment, the viable microbes are reduced by about 3 to 6 logs.

A person skilled in the art will appreciate that the post treatment can be any method that inactivates microbes, including for example, heat treatment, UV treatment, ultrasonic processing, pulsed electric field processing or high pressure processing. In an embodiment, the isothiocyanate containing product is post-treated with heat processing. In an embodiment, the isothiocyanate containing product is post-treated with high pressure processing. In an embodiment, the isothiocyanate containing product is in a sealed package during post-treatment. In an embodiment, the isothiocyanate containing product is in a sealed package during high pressure processing. In an embodiment, the isothiocyanate containing product is in a sealed package during heat treatment. In an embodiment, high pressure processing comprises treating the isothiocyanate containing product with isostatic pressure at about 300 to about 600 MPa. In an embodiment, high pressure processing comprises treating the isothiocyanate containing product with isostatic pressure at about 350 to about 550 MPa. In an embodiment, high pressure processing comprises treating the isothiocyanate containing product with isostatic pressure at about 300 to about 400 MPa. In an embodiment, heat treatment comprises heating the sample to a temperature of about 60°C to about 121°C. In an embodiment, heat treatment comprises heating the sample to a temperature of about 65°C to about 100°C. In an embodiment, heat treatment comprises heating the sample to a temperature of about 65°C to about 80°C. In an embodiment, heat treatment comprises heating the sample to a temperature of about 65°C to about 75°C.

Isolated strains and starter cultures

In an embodiment, the present invention provides isolated strains of lactic acid bacteria suitable for use in the methods and products as described herein.

In an embodiment, the present invention provides an isolated strain of lactic acid bacteria selected from:

- i) BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia;
- ii) BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia;
- iii) B1 deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;
- iv) B2 deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;

v) B3 deposited under V17/021733 on 25 September 2017 at the National Measurement Institute Australia;

vi) B4 deposited under V17/021734 on 25 September 2017 at the National Measurement Institute Australia; and

5 vii) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

In an embodiment, the present invention provides an isolated strain of *Leuconostoc mesenteroides* comprising genomic DNA which when cleaved with *SmaI* and/or *NotI* produces a *SmaI* and/or *NotI* fingerprint identical to BF1 or BF2. The *SmaI* and *NotI* fingerprints for BF1 and BF2 are shown in Figure 13.

In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising genomic DNA which when cleaved with *SmaI* and/or *NotI* produces a *SmaI* and/or *NotI* fingerprint identical to B1, B2, B3, B4 or B5.

In an embodiment, the present invention provides an isolated strain of
15 *Leuconostoc mesenteroides* comprising one or more or all of the polymorphisms listed in Table 18 or 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 5 or more of the polymorphisms listed in Table 18 or 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 10 or more of the polymorphisms listed in Table
20 18 or 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 15 or more of the polymorphisms listed in Table 18 or 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 19 or more of the polymorphisms listed in Table 18 or 19 that differs from ATCC8293. In an embodiment, the isolated strain of
25 *Leuconostoc mesenteroides* comprises 20 or more of the polymorphisms listed in Table 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 30 or more of the polymorphisms listed in Table 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 50 or more of the polymorphisms listed in Table 19 that
30 differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 80 or more of the polymorphisms listed in Table 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 100 or more of the polymorphisms listed in Table 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 150 or more of the polymorphisms listed in Table 19 that
35 differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc*

mesenteroides comprises 200 or more of the polymorphisms listed in Table 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 300 or more of the polymorphisms listed in Table 19 that differs from ATCC8293. In an embodiment, the isolated strain of *Leuconostoc mesenteroides* comprises 400 or more of the polymorphisms listed in Table 19 that differs from ATCC8293.

In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising one or more or all the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014. In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising 5 or more of the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014. In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising 10 or more of the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014. In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising 15 or more of the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014. In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising 20 or more of the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014. In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising 25 or more of the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014. In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising 30 or more of the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014. In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising 35 or more of the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014. In an embodiment, the present invention provides an isolated strain of *Lactobacillus plantarum* comprising 40 or more of the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014.

In an embodiment, the present invention provides a starter culture for producing an isothiocyanate containing product or a probiotic comprising lactic acid bacteria comprising one or more of the isolated strains as described herein. As used herein a “starter culture” is a culture of live microorganisms for fermentation. In an embodiment, the present invention provides a starter culture for producing an

isothiocyanate containing product or a probiotic comprising lactic acid bacteria selected from one or more or all of:

- i) BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia;
- 5 ii) BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia;
- iii) B1 deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;
- iv) B2 deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;
- 10 v) B3 deposited under V17/021733 on 25 September 2017 at the National Measurement Institute Australia;
- vi) B4 deposited under V17/021734 on 25 September 2017 at the National Measurement Institute Australia; and
- 15 vii) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

In an embodiment, the *Brassicaceae* material is inoculated with at least about 10^5 CFU/g of a starter culture as described herein. In an embodiment, the *Brassicaceae* material is inoculated with at least 10^6 about CFU/g of a starter culture as described
20 herein. In an embodiment, the *Brassicaceae* material is inoculated with at least about 10^7 CFU/g of a starter culture as described herein. In an embodiment, the *Brassicaceae* material is inoculated with at least about 10^8 CFU/g of a starter culture as described herein. In an embodiment, the *Brassicaceae* material is inoculated with at least about 10^{10} CFU/g of a starter culture as described herein. In an embodiment, the *Brassicaceae*
25 material is inoculated with about 10^5 CFU/g to about 10^{10} CFU/g of a starter culture as described herein.

Probiotics

In an embodiment, the present invention provides for a probiotic comprising one
30 or more of the lactic acid bacteria isolated from a *Brassicaceae*. As used herein a “probiotic” refers to a live microorganism which when administered in an adequate amount confers a health benefit to the host. In an embodiment, the lactic acid bacteria was isolated from a *Brassica oleracea*. In an embodiment, the lactic acid bacteria was isolated from broccoli. In an embodiment, the lactic acid bacteria was isolated from
35 Australian broccoli. In an embodiment, the lactic acid bacteria is selected from: i) a *Leuconostoc mesenteroides*; ii) a *Lactobacillus plantarum*; iii) a *Lactobacillus*

pentosus; iv) a *Lactobacillus rhamnosus*; v) a combination of i) and ii); vi) a combination of i), ii) and iii); and vii) a combination of i), ii) and iv). In one embodiment, the lactic acid bacteria is selected from one or more or all of BF1, BF2, B1, B2, B3, B4 and B5. In an embodiment, the lactic acid bacteria is B1. In an embodiment, the lactic acid bacteria is B2. In an embodiment, the lactic acid bacteria is B3. In an embodiment, the lactic acid bacteria is B4. In an embodiment, the lactic acid bacteria is B5. In an embodiment, the probiotic is a capsule, tablet, powder or liquid. In an embodiment, the probiotic is microencapsulated as described in WO 2005030229.

10 **EXAMPLES**

Example 1 - Methods

Chemicals and reagents

HPLC grade methanol, sodium dihydrogen phosphate, sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Merck (Damstadt, Germany). Folin-Ciocalteu's reagent, sodium carbonate (Na₂CO₃), gallic acid, fluorescein sodium salt and dibasic-potassium phosphate were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium dihydrogen phosphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,20-azobis (2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sapphire Bioscience (Redfern, NSW, Australia).

Lactic acid bacteria

Lactic acid bacteria used during fermentation were selected from one or more of:

25 LP: *Lactobacillus plantarum* ATCC8014;

LGG: *Lactobacillus rhamnosus* ATCC53103;

B1: *Lactobacillus plantarum* isolated from broccoli deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;

30 B2: *Lactobacillus plantarum* isolated from broccoli deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;

B3: *Lactobacillus plantarum* isolated from broccoli deposited under V17/021733 on 25 September 2017 at the National Measurement Institute Australia;

B4: *Lactobacillus plantarum* isolated from broccoli deposited under V17/021734 on 25 September 2017 at the National Measurement Institute Australia;

35 B5: *Lactobacillus plantarum* isolated from broccoli deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia;

BF1: *Leuconostoc mesenteroides* isolated from broccoli puree deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia;

BF2: *Leuconostoc mesenteroides* isolated from broccoli puree BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia;

BP: pooled BF1, BF2; and

LAB: pooled B1, B2, B3, B4 and B5.

BF1 and BF2 were identified as *Leuconostoc mesenteroides* via a 16s-RNA sequence (Australian Genome Research Facility; data not shown). B1 to B5 were identified as *Lactobacillus plantarum* based on 16S-RNA sequence. The identity of all the isolates were confirmed by whole genome sequence analysis.

Isolation of lactic acid bacteria from broccoli and broccoli puree

The above *Lactobacillus plantarum* B1, B2, B3, B4 and B5 were isolated from broccoli leaves and stem. The leaves and stem were washed with water and homogenised with added peptone saline using a stomacher. The soaking solution was serially diluted and spread plated on De Man, Rogosa and Sharpe (MRS) agar. The plates were incubated under anaerobic condition for 48 to 72 hrs at 37°C for isolating presumptive mesophilic lactic acid bacteria. Based on different colonial morphology on MRS plates, colonies were isolated, cultivated in MRS broth, screened using staining and biochemical characterisation techniques, and kept frozen with glycerol at -80°C. The isolates were identified at species level using 16s RNA sequencing at AGRF.

For the isolation of *Leuconostoc mesenteroides* BF1 and BF2, broccoli floret puree was used after serial dilution instead of the suspension described above for the isolation from broccoli leaves.

Preparation of starter cultures

The lactic acid bacteria strains, *Leuconostoc mesenteroides* and *Lactobacillus plantarum*, were isolated from broccoli and identified by Australian Genome Research Facility Ltd. To obtain the primary culture, lactic acid bacteria cultures which were stored at -80°C were inoculated into 10 mL of MRS broth (Oxoid, Victoria, Australia) and incubated at 30°C for 24 h to obtain an initial biomass of 8 log colony-forming units per milliliter (CFU/mL). Two mL of each primary inoculum was inoculated into 200 mL of MRS broth and incubated for 24hrs at 30°C. The cultures were collected by centrifugation at 2000g for 15min at 4°C, washed twice with sterile phosphate buffer

saline (PBS), and all the *Lactobacillus plantarum* cultures were mixed together and all the *Leuconostoc mesenteroides* cultures were mixed together. The two culture suspensions were diluted to 10 log CFU/ml and were mixed at the same volumetric proportion and stored with glycerol at -80°C until use as a mixed starter culture for
5 broccoli fermentation.

Fermentation method

Broccoli (*Brassica oleracea* L. ssp. *Italic*; 30 kg) florets were cut approximately 2 cm from the crown, shredded to smaller pieces and, were macerated with Milli-Q
10 water in ratio of 3:2 for 1 min using magic bullet blender. The broccoli slurry, was mixed well and placed into sterile plastic bottles (200 mL) with screw lids. Each bottle of broccoli puree (200 mL) was inoculated with the prepared starter culture at an initial concentration of 8 log CFU/g. The fermentation experiment was carried out in 48 bottles in parallel at 30°C, until a pH value of about 4.0 was reached (Day 4). After the
15 fermentation phase was completed, 3 samples were taken out as the Day 0 storage samples, the other samples were separated to two lots for the storage experiments: one lot was stored in a refrigerator (4°C) and another stored in room thermostated at 25°C. Samples were periodically taken over 12 weeks for microbiological, physicochemical and phytochemical analyses. The fermented broccoli puree was compared with raw
20 broccoli puree which was stored at -20°C after homogenization and puree samples incubated for the same period of time as the fermented samples without inoculation by LAB.

Sampling

25 For time course experiments, sampling was performed at days 10, 20, 30, 40, 50, 60, 70, 80, and 90, and on days 14, 28, 42, 56, 70 and 84 for samples stored at 25°C and 4°C, respectively. Sampling was performed in triplicate with color measured on the surface and pH measured immediately after opening the fermentation bottles. Thereafter, samples were taken for microbiological analysis and titratable acidity
30 analysis. The remaining material was separated into two parts, the first portion was frozen and freeze dried, ground to fine powder and stored in a desiccator for further analyses, and the second part was frozen and kept at -20°C until glucoraphanin and sulforaphane analyses.

35 *Microbiological analysis*

For microbial analysis, three different media were used to measure CFU per g broccoli puree of the different microorganisms; the plate counts for total lactic acid bacteria on DeMan–Rogosa–Sharp (MRS) agar, for total enterobacteria on violet red bile glucose agar (VRBGA), and the yeasts and mould on potato dextrose agar (PDA).

- 5 For each sample, serial dilution of the broccoli suspension in sterilized peptone saline diluent were made and 0.1 mL of the dilutions were plated onto agar plates in duplicates. After aerobic incubation at 25°C for 72 h (PDA), 37°C for 24 h (VRBGA), and anaerobic incubation at 30°C for 72 h (MRS), respectively, the CFU were counted.

10 *Determination of pH and titratable acidity*

The pH value was determined directly in fermentation bottles containing broccoli puree by a pH meter (PHM240, MeterLab). Titratable acidity (TA) of broccoli samples was measured with an Automatic titrator (Titralab 854 titration manager, Radiometric Analytical, France). In brief, diluted broccoli puree (10 mL) was titrated
15 using 0.1 M NaOH to the end point pH = 8.1 and the result obtained was expressed as gram equivalent of lactic acid per liter of sample in accordance with the following equation:

$$\text{TA (g/L)} = \frac{[v \times \text{acid factor} \times 1000]}{\text{sample volume}}$$

where, v is titer volume of NaOH. The acid factor for lactic acid is 0.009.

20

Total protein and color analyses

The total protein content of broccoli samples was determined as total nitrogen content multiplied by 6.25. Total nitrogen content of broccoli was analyzed using a Dumas combustion method with LECO TruMac apparatus (LECO Corporation,
25 Michigan, USA). The color indexes (L, a, b) of fermented broccoli sample were determined using a Chroma meter CR-200 tristimulus colorimeter (Minolta, Osaka, Japan). The color values obtained were expressed as lightness/darkness (as L*), redness/greenness (a*) and yellow/blueness (b*). The total color difference (ΔE) was calculated according to the following equation:

$$\Delta E = [(L^* - L_0)^2 + (a^* - a_0)^2 + (b^* - b_0)^2]^{1/2}$$

30

where, L₀, a₀, b₀ are color values of fresh unfermented broccoli.

35 *Determination of total polyphenol content*

The total phenolic content (TPC) was measured spectrophotometrically using the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965) with modifications. Briefly, 50 mg of broccoli powder was suspended in 10 mL of acidified (1 % HCl) methanol/water (70:30, v/v) solution and extracted in ultrasonic bath (IDK technology Pty Ltd, VIC, Australia) for 8 min. The extracts were kept for 16 h at 4°C and filtered with 0.2 µm filter and stored at 4°C until analysis. 1 mL of 0.2 N Folin-Ciocalteu reagent, 800 µL of sodium carbonate solution (7.5% p/v) and 180 µL Milli-Q grade water were added to the extract (20 µL). After 1 h of incubation in the dark at 37°C, the absorbance was measured at 765 nm in triplicates using a spectrophotometer (UV-1700 Pharma Spec, SHIMADZU). Gallic acid was used as a standard and TPC was expressed as the gallic acid equivalent (GAE) in mg per 100 g of fresh weight (mg GAE/100 g FW) based on a standard curve developed using known concentrations of gallic acid.

15 *Oxygen radical absorbance capacity assay*

Freeze-dried broccoli powder (10 mg) was suspended in 10 mL of methanol/water (80:20, v/v), the extraction solvent. The slurry was extracted at 650rpm on a Heidolph Multi-Reax (John Morris Scientific, NSW, Australia) at room temperature for an hour. Then it was centrifuged at 25,000g for 15 min in 4°C, the supernatant was collected, and was ready for analysis after 100× dilution with 75 mM potassium phosphate buffer (pH 7.4). ORAC analysis was conducted according to the procedure reported by Huang et al. (2002) with minor modifications. The assay was carried out in opaque 96-well plates (dark optical bottom, Waltham, MA, USA). The assay reactants included 81.6 nM of fluorescein, 153 mM of AAPH, Trolox standard of different concentration (100, 50, 25, 12.5, and 6.25 µM), and 75 mM phosphate buffer as the blank. The reactants were added in the following order: 25 µL of diluted sample; either 25 µL of 75 mM phosphate buffer, 25 µL Trolox standard and 150 µL fluorescein. After adding the fluorescein, the plate was incubated at 37°C for 10 min and then the AAPH (25 µL) was added. Immediately after addition of AAPH, the plate was placed in the fluorescence plate reader (BMG Labtech ClarioStar, Germany) and the fluorescence was measured every 3 min until it decreased to less than 5% of original fluorescence. The ORAC values were calculated as the area under the curve (AUC) and expressed as micromoles of trolox equivalent (TE) per gram dry weight of broccoli (µmol TE/g DW). Each sample was assayed triplicate.

35

Sulforaphane analysis

The extraction of sulforaphane from broccoli matrix was conducted following the methods of Li et al. (2012) with some modification. In brief, frozen broccoli (2g) was mixed with 2 mL of Milli-Q water and vortexed for 1 min. Then 20 mL ethyl acetate was added to the slurry followed by sonication for 5 min and shaking for 20 min at 4°C. The slurry was then centrifuged at 15,000g for 10 min, and the supernatant was collected. Then another 15 mL ethyl acetate was added to the precipitate to carry out the second extraction. Pooled extracts from each sample were evaporated to dryness with a vacuum spin dryer (SC250EXP, Thermo Fisher Scientific, CA, USA) at room temperature, and stored at -20°C until analysis. The concentration of sulforaphane was determined using an Acquity™ Ultra Performance LC system (Waters Corporation, Milford, MA, USA), which is equipped with a binary solvent delivery manager and a sample manager. Chromatographic separations were performed on a 2.1 x 50mm, Acquity BEH C18 chromatography column. The mobile phase A and B were 0.1% formic acid in millique water and 0.1% formic acid in acetonitrile, respectively. The gradient elution system consisted of mobile phase A (0.1% formic acid in millique water) and B (0.1% formic acid in acetonitrile) and separation was achieved using the following gradient: 0–2 min, 10% B; 2–5 min, 20% B; 5–10 min, 10% B. The column temperature was kept constant at 30°C. The flow-rate was 0.350 mL/min and the injection volume was 5µL.

Prior to analysis, all samples were dissolved in 1 mL 30% acetonitrile, and filtered through a 0.22µm membrane filter (Merk Millipore, Billerica, MA, USA). The identification of each peak was based on the retention time and the chromatography of authentic standards. The concentrations of each compound were calculated according to a standard curve, and the results were expressed as micromoles per kilogram DW (µmol / kg DW) of broccoli.

Glucoraphanin analysis

The extraction of glucoraphanin from raw or fermented broccoli was carried out according to the method of Cai and Wang (2016) with some modification. Accordingly, to 2 g of frozen broccoli puree, 10 mL of boiling Milli-Q water was added, and the mixture was incubated for 5 min in a boiling water bath. It was then cooled and centrifuged at 15000×g for 15 min, and the supernatant was collected. The precipitate was extracted once more with 8 mL of boiling water. Pooled extracts from each sample were evaporated to dryness with a vacuum spin dryer (Speedvac SC250EXP, Thermo Fisher Scientific, CA, USA) at 3°C, and stored at -20°C until analysis. The concentration of glucoraphanin was quantified using an Alliance HPLC instrument

(Waters Corporation, Milford, MA, USA) equipped with Photo Diode Array Detector 2998. A HPLC column – Luna® 3 μ M Hydrophilic Interaction Liquid Chromatography (HILIC) 200° A (100×4.6 mm; Phenomenex, Torrance, CA, USA) was used for the analysis at a column temperature of 25°C. The mobile phase consisted of an acetonitrile/water (85:15, v/v) with 30mM Ammonium formate (solution A) and acetonitrile (solution B) with the following isocratic flow program: solution A 70%; solution B 30%. Other chromatographic conditions included a constant flow rate of 2.0 mL/ min, an injection volume of 100 μ L, a run time of 8 min, and detection wavelength of 235 nm. Prior to analysis, all samples were dissolved in 1 mL solvent A, and filtered through a 0.22 μ m membrane filter (Merk Millipore, Billerica, MA, USA). The identification of each peak was based on the retention time and the chromatography of an authentic glucoraphanin standard. The concentrations of glucoraphanin were calculated using a standard curve, and the results were expressed as micromoles glucoraphanin per kilogram DW (μ mol/kg DW) of broccoli.

15

Statistical analysis

All experiments were conducted in triplicate and the results were expressed as mean values. A one-way analyses of variance (ANOVA) was applied to evaluate the significance of the differences among the mean values at 0.05 significance level ($p < 0.05$). The statistical analysis was conducted using the statistical software, SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

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Example 2 – Microbial analysis of lactic acid bacteria fermented broccoli florets

The fermentation of broccoli puree was carried out as described in the fermentation section of Example 1. The counts of total lactic acid bacteria were lower for raw broccoli compared to inoculated broccoli as showed in Table 1. After 4 days of fermentation, the pH of the sample reached 4.04 and fermentation was stopped, and the fermented sample before storage experiments was taken as the Day 0 sample. It is clear from Table 1 and Figure 1C that the counts of total lactic acid bacteria of the Day 0 sample were significantly increased (8 log CFU/g) compared to the raw broccoli. During the first two weeks of storage, the viable number of total lactic acid bacteria increased to the highest values of 9 log CFU/g for samples stored at both 25°C and 4°C (Table 1 and Table 2). During storage at 25°C, the total lactic acid bacteria counts increased to 9 log CFU/g at Day 10 and slowly declined during storage to 5 log CFU/g by Day 50, and declined further to almost undetectable level after Day 70. In contrast,

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Table 1: Microbiological and physicochemical changes of fermented broccoli during the storage at room temperature (25°C).

	Microbial loads (Log CFU/g)					Color				
	MRS	PDA	VRBGA	pH	TA (g/L)	TP (mg/g, FW)	L	a	b	ΔE
Raw broccoli	2.4±0.2	2.5±0.1	3.4±0.1	6.33±0.00	4.8±0.2	26.9±0.0	48.4±0.4	-13.2±0.1	17.2±0.2	-
Day 0	8.4±0.2	<1	<1	4.04±0.00	10.7±0.7	29.6±0.8	48.5±0.7	-2.1±0.1	13.6±0.6	11.7
Days 10	9.4±0.1	<1	<1	3.87±0.02	14.4±0.2	27.8±0.8	47.7±0.8	-1.1±0.2	12.2±0.5	13.1
Days 20	6.2±0.3	<1	<1	3.76±0.02	14.7±0.2	30.5±0.8	47.1±0.5	-1.1±0.0	12.5±0.2	13
Days 30	6.2±0.1	<1	<1	3.78±0.00	15.1±0.3	29.7±1.2	47.2±0.2	-1.0±0.1	10.9±0.5	13.8
Days 40	6.1±0.4	<1	<1	3.79±0.02	15.1±0.4	28.8±1.1	46.3±0.5	-0.8±0.1	11.0±0.9	14
Days 50	5.1±0.6	<1	<1	3.75±0.00	15.2±0.5	28.5±0.1	45.8±0.5	-0.9±0.1	11.0±0.2	14
Days 60	2.4±0.1	<1	<1	3.76±0.01	15.4±0.3	27.3±0.6	45.4±0.1	-0.9±0.1	10.5±0.1	14.3
Days 70	1.5±0.1	<1	<1	3.76±0.01	15.7±0.1	27.7±0.2	45.3±0.5	-0.9±0.1	9.9±0.4	14.7
Days 80	<1	<1	<1	3.76±0.01	15.7±0.7	28.3±0.2	45.9±0.1	-0.9±0.1	9.7±0.1	14.6
Days 90	<1	<1	<1	3.71±0.01	15.7±0.3	28.7±0.4	45.0±0.0	-0.8±0.2	9.3±0.2	15.1

Each value was expressed as mean ± standard deviation (n = 3). “-” not available.

MRS, de Man-Rogosa-Sharpe agar for LAB; PDA, potato dextrose agar for total yeasts and moulds; VRBGA, violet red bile glucose agar for Enterobacteriaceae; TA, titratable acidity; TP: total protein; ΔE: total color difference.

Table 2: Microbiological and physicochemical changes of fermented broccoli during the storage at 4°C.

	Microbial loads (Log CFU/g)					Color				
	MRS	PDA	VRBGA	pH	TA (g/L)	TP (mg/g, FW)	L	a	b	ΔE
Raw broccoli	2.4±0.2	2.5±0.1	3.4±0.1	6.33±0.00	4.8±0.2	26.9±0.0	48.4±0.4	-13.2±0.1	17.2±0.2	-
Day 0	8.4±0.2	<1	<1	4.04±0.00	10.7±0.7	29.6±0.8	48.5±0.7	-2.1±0.1	13.6±0.6	11.7
Days 14	9.0±0.1	<1	<1	4.04±0.03	12.6±0.8	32.5±1.2	47.2±1.1	-1.9±0.5	12.4±1.5	12.3
Days 28	8.0±0.1	<1	<1	3.95±0.02	13.5±0.8	32.0±0.7	45.9±0.7	-2.2±0.3	13.8±2.5	11.8
Days 42	7.6±0.1	<1	<1	3.89±0.03	13.8±0.2	32.0±0.8	46.7±0.2	-1.5±0.1	12.6±0.5	12.7
Days 56	6.5±0.4	<1	<1	3.89±0.02	13.8±0.5	29.9±0.3	46.6±0.4	-1.7±0.1	13.1±0.5	12.4
Days 70	6.3±0.4	<1	<1	3.86±0.01	13.7±0.1	31.6±0.2	46.7±0.8	-1.6±0.2	12.2±0.4	12.7
Days 84	6.0±0.8	<1	<1	3.85±0.01	13.8±0.1	32.0±0.5	47.6±0.9	-1.9±0.2	14.0±0.6	11.8

5 Each value was expressed as mean ± standard deviation (n = 3). “-” not available.

MRS, de Man-Rogosa-Sharpe agar for LAB; PDA, potato dextrose agar for total yeasts and moulds; VRBGA, violet red bile glucose agar for Enterobacteriaceae; TA, titratable acidity; TP: total protein; ΔE: total color difference.

the LAB count in the samples stored at 4°C remained high (6 log CFU/g) even after storage for 84 days.

The total counts of yeast and moulds in the raw broccoli sample was 2 log CFU/g. The Enterobacteriaceae count in the raw broccoli with 3 log CFU/g. No fungi, moulds and enterobacteria were detected after fermentation or on the fermented samples after storage at both temperature conditions. No pathogenic and spoilage organisms were detected following fermentation and during storage. The results indicate that the fermentation process resulted in a safe and stable product with undetectable level of potentially pathogenic enterobacteriaceae and spoilage yeast and mould, which maintained high levels of total lactic acid bacteria when stored at 4°C. There are $\sim 10^6$ CFU/g lactic acid bacteria after ~ 3 months at 4°C.

Example 3 – Assessment of pH and titratable acidity after storage of lactic acid bacteria fermented broccoli florets

The pH and titratable acidity (TA) of raw broccoli, fermented broccoli and fermented broccoli after storage at 25°C and 4°C was analyzed as described in Example 1. The determination of TA was used to estimate the amount of lactic acid and acetic acid, the main acids produced by lactic acid bacteria, during fermentation. During fermentation, the acids produced by the lactic acid bacteria decrease the pH of the sample. As shown in Table 1, the TA was increased to 10.7 g/L in Day 0 samples. When stored in 25°C, the pH was decreased to 3.87 during storage after 10 days, along with the significantly increased values of TA which reached 14.4 g/L ($p < 0.05$; see Table 1). The results indicate that there were still substrates present for lactic acid bacteria to consume and further produce acid during the early days of storage. Neither the pH nor TA value were significantly changed during the remaining storage period (Table 1).

Decreasing the temperature to 4°C reduced the rate of decrease of pH and TA in the stored samples due to the decreased activity of the lactic acid bacteria at the lower temperature (see Table 2). After nearly 3 months storage at 4°C, the pH was 3.85 and the TA value was 13.7 g/L.

Example 4 – Assessment of broccoli maceration and fermentation on the conversion of glucoraphanin into sulforaphane

Broccoli florets were cut into small pieces, mixed with water at 3:2 broccoli: water ratio and the mixture was macerated into a puree using a blender. Puree samples (200 gm) were aliquoted into sterile plastic bottles. The samples were inoculated at 10^8

CFU/gm with pooled culture of lactic acid bacteria (*Leuconostoc mesenteroides* and *Lactobacillus plantarum*) isolated from Australian broccoli. Samples were incubated in a water bath maintained at 30°C until the pH dropped to ~4.0, which was attained after four days of fermentation. Control non-inoculated samples were immediately frozen after maceration. A second set of non-inoculated control samples, to which sodium benzoate was added to inhibit microbial growth, were incubated with the inoculated samples at 30°C for four days until the fermentation of the inoculated samples was completed. Experiments were conducted in triplicate. All samples were kept frozen until sulforaphane and glucoraphanin analysis. As shown in Figure 1B and Table 3 maceration followed by fermentation increased the sulforaphane yield compared to just maceration and incubation alone.

Table 3: Effects of maceration and fermentation on sulforaphane content in broccoli puree.

25°C	SF(mg/kg, DW)	4°C	SF (mg/Kg, DW)
<i>Raw material</i>	149.8±12.4	<i>Raw material</i>	149.8±12.4
<i>Control incubated</i>	86.8±0.6	<i>Control incubated</i>	86.8±0.6
<i>0 days</i>	278.4±1.8	<i>0 days</i>	278.4±1.8
<i>10 days</i>	189±8.8	<i>14 days</i>	288.6±3.1
<i>20 days</i>	136.6±6.2	<i>28 days</i>	218.8±4.3
<i>30 days</i>	122.2±12.2	<i>42 days</i>	199.4±14.7
<i>40 days</i>	116.3± 5.0	<i>56 days</i>	190± 7.1
<i>50 days</i>	112.3±4.0	<i>70 days</i>	190.8±10.7
<i>60 days</i>	111.9±11.0	<i>84days</i>	179.6±10.2
<i>70days</i>	108.8± 15.8		
<i>80days</i>	102.6±14.7		
<i>90days</i>	87.6±3.7		

Example 5 – Assessment of total protein content and color after storage of lactic acid bacteria fermented broccoli florets

The total protein content and color of lactic acid fermented broccoli florets after fermentation was assessed as described above in the methods section. Compared to raw broccoli (26.9±0.03), the total protein content of fermented broccoli was significantly increased (29.6±0.8 mg/g; p<0.05). This could be due to the high number of lactic acid bacteria inoculated into the sample and the growth during fermentation and protein synthesis by the lactic acid bacteria. The total protein content stayed stable during storage both at 25°C and 4°C (Table 1 and Table 2), with no significant difference between samples.

The color values (L, a, b) and the total color difference (ΔE) of broccoli samples are summarized in Table 1 and Table 2. As presented in Table 1 and Table 2, significant differences in the color parameters and the total color difference value (ΔE) were recorded between raw and fermented samples. The L* value (lightness) did not change significantly, whereas a* (greenness) and b* (yellowness) values decreased after the fermentation of broccoli puree. The decrease in a* and b* values may be attributed to the degradation in the color pigmented compounds, such as chlorophyll which would convert to pheophytins under the low pH. The high ΔE value (12.5) of Day 0 sample indicate that the color of broccoli puree was significantly changed after fermentation, which was visually noticeable. During storage (Table 1 and Table 2) there was no significant change in the ΔE value in neither 25°C nor 4°C samples.

Broccoli after fermentation with LAB+BP (*Lactobacillus plantarums* B1, B2, B3, B4, B5 and *Leuconostoc mesenteroides* BF1, BF2 isolated from broccoli) had a brighter, more intense green color more similar in color to raw macerated broccoli compared to broccoli fermented with LAB only (the *Lactobacillus plantarums* isolated from broccoli (B1, B2, B3, B4, B5)).

Example 6 – Changes of total phenolic content and antioxidant activity of lactic acid bacteria in fermented broccoli florets

The total phenolic content (TPC) and antioxidant activity of lactic acid fermented broccoli florets after fermentation was assessed as described above in the methods section. The TPC of raw broccoli was 127.6±12.4 mg GAE/100 g (Figure 3A) of fresh weight. The values of TPC on Day 0 significantly increased to 236.9±23.4 mg GAE/100 g (p<0.05) compared to raw broccoli. There was no significant difference between samples stored at 25°C and 4°C in the TPC after storage (Figure 3A). When stored at 25°C, the value of TPC in fermented broccoli was 246.2±19.3 mg GAE/100 g

on Days 10, and 248.1 ± 25.0 mg GAE/100 g on Days 90. When stored at 4°C , the values of TPC was 274.1 ± 20.2 and 267.2 ± 3.3 mg GAE/100 g for Days 14 and Days 84, respectively.

The antioxidant activities of sample expressed as ORAC values are shown in Figure 3B. The ORAC value of the raw sample was 110.1 ± 0.05 $\mu\text{mol TE/g}$. Fermentation significantly increased the ORAC value by $\sim 70\%$ to 186.9 ± 3.3 $\mu\text{mol TE/g}$ when compared to raw broccoli. This result suggested that antioxidant compounds may have increased during fermentation and was consistent with the change in TPC after fermentation.

During storage, the antioxidant activity of fermented broccoli did not change significantly. As shown in Figure 3B, when stored at 25°C , the values of ORAC at Days 10 and Days 90 were 173.0 ± 14.4 and 150 ± 5.5 $\mu\text{mol TE/g}$, respectively. Similar results were obtained for samples stored at 4°C . The ORAC value was 172.0 ± 15.5 $\mu\text{mol TE/g}$ at the beginning of storage, which increased to a maximum value of 188.7 ± 12.9 $\mu\text{mol TE/g}$ after storage.

Example 7 – Assessment of fermentation time for different combinations of lactic acid bacteria

Macerated broccoli was prepared as described above in the methods section with a broccoli to water ratio of 3:2 and a maceration time of 1 min. The broccoli material was inoculated with either 10^7 CFU/g or 10^8 CFU/g with one of: LGG, LAB (*Lactobacillus plantarum* (B1, B2, B3, B4, B5) isolated from Australian broccoli, LAB+LP (*Lactobacillus plantarum* isolated from broccoli and *Lactobacillus sp.* ATCC 8014), BP (*Leuconostoc mesenteroides* isolated from broccoli), LAB+BP (a mixture of the two groups as described in the methods sections) and fermented at either 25°C , 30°C or 34°C to reach a target pH of 4.4. As shown in Figure 4 the addition of lactic acid bacteria isolated from broccoli and/or broccoli puree significantly reduced the time taken for the fermentation with the combination of LAB +BP reaching a pH of 4.4 after fermenting for about 4 days. An example composition of fermented broccoli product is shown in Table 4.

Table 4: Composition of the fermented broccoli product.

Quality attributes	Value
Total fibre	~29.5g/100gdw
ORAC antioxidant capacity	18695 $\mu\text{mol TE}/100 \text{ gdw}$
Total polyphenol content	2369 mg GAE/100gdw
Total titratable acidity	1.1% lactic acid equiv.
Lactic acid bacteria count	$\sim 10^8$ CFU/gm
Total protein	30 g/100 gdw
Broccoli to water ratio in puree by mass	3 to 2

Example 8: Effect of storage on sulforaphane content of fermented broccoli

Figure 2A shows the effects of storage at 4 and 25°C on sulforaphane content of fermented broccoli puree. As can be seen in the Figure 2A, the sulforaphane content of samples stored at 25°C dramatically decreased to $770.7 \pm 34.9 \mu\text{mol/kg}$ (a 52% loss) after 20 days storage, followed by a slower decline during the rest of the storage period, reaching a total loss of 69.5%. Interestingly, no statistically significant change in sulforaphane content was observed during the first 2 weeks of storage of fermented broccoli samples at 4°C. A significant decrease of ~23.7 % occurred during the subsequent two weeks followed by a slow degradation during the rest of the storage period. At the end of the storage (Day 84), the sulforaphane content was $1012.9 \pm 57.6 \mu\text{mol/kg}$ in samples stored at 4°C, making the total loss of sulforaphane ~37.4 % compared to the Day 0 samples. The sulforaphane content during the first two weeks of storage was maintained perhaps due to simultaneous production and degradation of sulforaphane since some decrease in glucoraphanin content was observed in the 4°C stored samples over the same period.

Example 9: Effect of fermentation and storage on glucoraphanin content

Figure 7 shows the effect of maceration and fermentation on glucoraphanin content and its stability during storage at 4°C and 25°C. The glucoraphanin content of raw broccoli was $3423.7 \pm 39.7 \mu\text{mol/kg}$ (Figure 7). After fermentation, the glucoraphanin content sharply decreased to $712.4 \pm 64.2 \mu\text{mol/kg}$ (Day 0 sample). Glucoraphanin is relatively stable in intact tissue and the degradation in this case can be attributed to myrosinase catalyzed hydrolysis due to increased enzyme-substrate

interaction in the macerated tissue during fermentation. The period of sharp decrease in glucoraphanin coincided with the fermentation period.

No significant change in glucoraphanin content was observed in fermented samples during storage at 25°C and 4°C. However, slightly higher glucoraphanin content was observed in samples stored at 25°C. This could be related to the faster decline in pH of the samples stored at 25°C (pH 3.87 at the second time point) compared to samples stored at 4°C (pH 4.04 at the second time point). The optimal pH for myrosinase catalyzed hydrolysis of glucoraphanin ranges from 5 to 6 decreasing to the lowest value at pH 3.0 (Dosz & Jeffery, 2013). The relatively higher pH of the samples stored at 4°C may have contributed to the slightly higher degradation of glucoraphanin during storage at 4°C compared to 25°C.

Example 10 – Assessment of heat treatment conditions to maximise conversion of glucoraphanin into sulforaphane in broccoli matrix

Broccoli florets packed in retort pouches were subjected to thermal processing at temperatures ranging from 60°C to 80°C and treatment times of 0 to 5 minutes. The treatment involved pre-heating to the experimental temperature in a water bath maintained at 5°C higher than the experimental temperature followed by incubation in a second water bath maintained at the experimental temperature. Following thermal treatment, samples were cooled in ice-water and were macerated with water added at 2:3 water to broccoli ratio as described above. The macerated samples were incubated for 1 hr at 30°C and kept frozen until sulforaphane analysis. Results are shown in Figure 2B and Table 5. As shown in Table 5 pre-heating the sample at 60°C, 65°C or 80°C followed by maceration increased the sulforaphane yield relative to raw broccoli floret which was macerated without pre-heating.

Table 5: Effects of heat treatment on sulforaphane production in broccoli matrix.

Temperature	Heat treatment time (minute)	Sulforaphane (μmol/kg, DW)	Sulforaphane (mg/kg, DW)	Sulforaphane (mg/g, DW)
Raw broccoli floret	-	817.5±9.29	145±1.6	0.145±0.002
60°C	0	2343.5±124.1	415.5±22.0	0.415±0.022
	1	2661.5±10.9	471.9±1.9	0.472±0.002
	3	2780.9±270.7	493.0±48.0	0.493±0.048
	5	3147.6±148	558.1±26.2	0.558±0.026

65°C	0	3585.9±119.2	635.8±21.1	0.636±0.021
	1	3673±144.8	651.2±25.7	0.651±0.026
	3	3983.4±30.5	706.3±5.4	0.706±0.005
	5	3620.1±240.7	641.8±42.7	0.642±0.043
80°C	0	1451.5±43.5	257.3±7.7	0.257±0.008
	1	1446.8±17.5	256.5±3.1	0.257±0.003
	2	1043.1±94.2	184.9±16.7	0.185±0.017
	3	981.2±35.1	174±6.2	0.174±0.006

Example 11 – Assessment of preheating prior to lactic acid bacterial fermentation on the sulforaphane content of broccoli

This study evaluated the impact of mild preheating treatment of broccoli florets to inactivate the Epithiospecifier protein (ESP) combined with lactic acid bacteria on sulforaphane content of broccoli puree.

Materials

Broccoli (cv. 'Viper') was purchased from a local supermarket (Coles, Werribee South, VIC, Australia). DeMan–Rogosa–Sharp (MRS) broth (1823477, CM0359, Oxoid) was purchased from Thermo Fisher Scientific (Australia). DL-Sulforaphane was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All the other chemical and biochemical reagents were analytical grade or higher and were purchased from local chemical vendors.

Experiments to optimize the mild pre-heating conditions to maximize sulforaphane yield

Broccoli florets were cut at approximately 2 cm below the head, and each 30g of randomly mixed broccoli florets were used in the pre-heating experiments. Two types of pre-heating experiments were conducted; in-pack processing and direct water blanching. In the case of the in-pack experiments, broccoli florets were packed in retort pouches (Caspak Australia, Melbourne), sealed and pre-heated for various time points in a thermostated water batch maintained at 60°C, 65°C and 80°C. The temperature of the broccoli samples at the slowest heating point was measured by using a thermometer. Time 0 was defined as the time for the core temperature to reach the designated experimental temperature. The treatment time were 0, 1, 3, and 5 min for 60°C and 65°C and 0, 1, 2, 3 min for 80°C. With the direct water-blanching

experiments, the broccoli florets were immersed in Milli-Q water in a glass beaker that was heated in a thermostated water-bath. The direct water blanching experiments were conducted at 60°C and 65°C. The temperature of the broccoli samples was continuously measured using a thermometer and timing started once the temperature at the slowest heating point attained the designated experimental temperature as described above. All thermal treatment experiments were carried out in triplicate. Unheated broccoli florets were used as controls. Immediately following the heat treatment, the samples were cooled in ice water and were homogenized with Milli-Q water in ratio of 3 parts broccoli to 2 parts of water for 1 min using a kitchen scale magic bullet blender (Nutribullet pro 900 series, LLC, USA). The homogenized samples were incubated in the dark for 4 h at 25°C to allow the enzymatic hydrolysis of glucoraphanin. After incubation, all the samples were frozen in -20°C until sulforaphane analysis.

Preparation of starter cultures

Pooled cultures of *Leuconostoc mesenteroides* (BF1, BF2) and *Lactobacillus plantarum* (B1, B2, B3, B4, B5) isolated from broccoli as described in the methods in Example 1. were used in the fermentation experiments. The lactic acid bacteria stock cultures, which were stored at -80°C, were activated by inoculation into 10mL MRS broth (Oxoid, Victoria, Australia) and incubation at 30°C for 24 hours to get the primary inoculum. 2 mL of the primary cultures were inoculated into 200 mL of MRS broth to obtain the secondary cultures. After 24 h incubation, the 6 secondary cultures were centrifuged, washed twice with sterile phosphate buffer saline (PBS) and each of the culture was resuspended in Milli-Q water at a concentration of 10 log colony-forming units per millilitre (CFU/mL) to obtain an initial biomass of 8 log CFU/mL in 100 gm broccoli puree samples. The *L. plantarum* cultures were mixed with the *L. mesenteroides* cultures at 1:1 proportion prior to inoculation into the broccoli puree samples.

Sample preparation

Broccoli florets were cut at approximately 2 cm below the crown and were separated into two lots; heat treated and non-treated. After heat treatment at the optimal condition selected based on the results of the experiments as described above, the samples were cooled in ice-water, shredded and homogenized with Milli-Q water in ratio of 3:2 for 1 min using a kitchen scale magic bullet blender (Nutribullet pro 900 series, LLC, USA). The non-treated broccoli were also homogenized in a similar way.

The broccoli puree, after mixing well, was aliquoted into sterile plastic containers (100 mL) with screw lids (Technoplast Australia) for further experiments.

Fermentation

5 Broccoli puree samples (pre-heated and untreated) were inoculated with the LAB culture prepared as described above in this example. Preheating of broccoli florets was conducted in-pack at 65 °C for 3 min based on the result of the experiment to optimise the pre-heating condition. In order to evaluate the impact of acidification without fermentation on conversion of glucoraphanin into sulforaphane, acidification
10 experiments were conducted on pre-heated and untreated broccoli puree using glucono-delta-lactone (GDL) to attain the pH of the fermented broccoli puree. Preheated broccoli puree and untreated broccoli puree without further treatment were used as controls.

15 For the fermentation experiment, each broccoli puree sample was inoculated with the prepared starter culture at an initial level of 8 log CFU/g. The fermentation experiment was carried out at 30°C until the pH reached ~ 4.0 after 15 hrs of incubation. Once the fermentation was completed, 3 samples (day 0 samples) of each fermented group were taken and stored at -20°C until analysis. The rest of the ferments were randomly separated into two lots for the storage trials: one lot was stored under
20 refrigerated condition (4°C) and the second lot was stored at 25°C for the assessment of the sulforaphane stability of the samples after 14 days storage. Similarly, the untreated broccoli puree, preheated broccoli puree and the preheated-GDL treated broccoli puree were also sampled at time zero and stored at 25 and 4°C for the 14 days storage trials. After 14 days storage, all the samples were frozen and kept at -20°C until sulforaphane
25 analyses.

Sulforaphane analysis and statistical analysis

Was performed as described in Example 1.

30 *Optimization of heat treatment conditions for improving sulforaphane yield*

The influence of heat treatment on the formation of sulforaphane of the heated-in-pack broccoli florets at three different temperatures (60, 65 and 80°C) for various processing times (0, 1, 3 and 5 min for 60 or 65°C; 0, 1, 2 and 3 min for 80°C) are shown in Figure 5A. The results showed that compared to the raw broccoli the
35 sulforaphane yield increased in all of the heat treated samples. Time 0 designate samples that were heated until their core reached the experimental temperature.

As shown in Figure 5A, an increase in sulforaphane yield occurred when the packed broccoli samples were heated at 60°C for 0, 1, 3, and 5 min. The concentration of sulforaphane in these samples were 2343.5±124.1, 2661.5±10.9, 2780.9±270.8, and 3147.7±148.0 µmol/kg DW, respectively. On the other hand, when broccoli was processed at 65°C, the sulforaphane yield initially increased with processing time from 3585.9±119.2 (0 min) to the highest value of 3983.4±30.5 µmol/kg DW (3 min). Further increase in treatment time resulted in lower yield with the lowest value of 3620.1±240.7 µmol/kg observed after 5 min treatment time. In contrast to treatments at 60 and 65°C, for samples that were processed at 80°C, a steady decrease in sulforaphane yield was observed with longer treatment times; with sulforaphane content of 1451.5±43.5, 1446.8±17.5, 1043.1±94.2, and 981.2±35.1 µmol/kg DW after 0 min, 1 min, 2 min and 3 min treatment respectively. Overall, the highest yield of sulforaphane (3983.4±30.5 µmol/kg) for in-pack treatment of broccoli was obtained for samples pre-heated at 65°C for 3 min, which is ~5 fold higher than raw broccoli (817.5±9.3 µmol/kg DW). In contrast, heating broccoli directly in water, generally resulted in a lower yield of sulforaphane compared to in-pack processing as shown in Figure 5B. For direct water blanching at 60°C, the sulforaphane yield increased with treatment time from 1698.00±121.9 µmol/kg DW (0 min), to 2833.3±118.6 µmol/kg DW (1 min) and then steadily decreased to the lowest value of 2345.8±57.7 µmol/kg DW for 5 min treatment at 60°C. A sharp drop in sulforaphane yield compared to 60°C was observed when samples were blanched at 65°C. The sulforaphane yield was 503.7±23.8 µmol/kg DW of broccoli after 5 min thermal treatment at 65°C, which was even lower than the value obtained for raw broccoli. The reason could be the leaching of glucoraphanin into the blanching water resulting in low yield of sulforaphane. For direct water blanching, the optimum treatment temperature for maximizing sulforaphane yield was 60°C compared to 65°C for the in-pack processing.

In this study, the highest yield of sulforaphane was obtained for broccoli florets processed in-pack for 3 min at 65°C, indicating that the condition favors the inactivation of ESP to a larger extent while maintaining sufficient myrosinase activity resulting in optimal conversion into sulforaphane. Under this condition, it seems that most of the extractable glucoraphanin is converted to sulforaphane assuming 1 to 1 conversion, since the glucoraphanin content of the broccoli samples were determined to be 3423.7±39.7 µmol/kg DW.

The observation that the exposure of the heat-treated broccoli to fermentation resulted in higher levels of sulforaphane than would be predicted from the level of extractable glucoraphanin from raw broccoli suggests heat-treatment may have

increased the accessibility of glucoraphanin to myrosinase, resulting in higher sulforaphane yield than would be expected based on the quantifiable amount of glucoraphanin present in the untreated broccoli.

Less sulforaphane yield was obtained for broccoli florets directly blanched in water, most probably due to leaching into the blanching water, since glucoraphanin is soluble in water. It is also interesting to note that when broccoli florets were heated directly in water, the maximum amount of sulforaphane was obtained by heating at 60°C for 1 min compared to 65°C for 3 min when heat treatment of broccoli florets was done in-pack. This may be due to the higher leaching rate into the blanching water at 65°C which counteracted the effects of higher level of inactivation of ESP at 65°C.

The effect of LAB fermentation and chemical acidification on sulforaphane yield

Broccoli florets were pre-heated in-pack at the best treatment condition selected above (65°C, 3 min). Samples were then either fermentation by lactic acid bacteria or acidified using the acidulant (GDL). Consistent with the pre-treatment experiments, the sulforaphane value of broccoli significantly increased ($p < 0.05$) after the heat treatment; with 806.2 ± 7.0 $\mu\text{mol/kg DW}$ and 3536.0 ± 136.9 $\mu\text{mol/kg DW}$ of sulforaphane yield for raw and pre-heated broccoli, respectively. The value of 3536 $\mu\text{mol/kg DW}$ obtained with this separate batch of broccoli preheated prior to fermentation is of the same order obtained when a different batch of broccoli was used, where 3983 $\mu\text{mol/kg DW}$ was obtained indicating slight batch to batch variation.

As shown in Table 6, after the fermentation, the sulforaphane content of broccoli samples varied depending on the treatment of the broccoli prior to fermentation. The sulforaphane content of raw broccoli puree after fermentation (1617.4 ± 10.2 $\mu\text{mol/kg DW}$) was approximately twice the sulforaphane content of raw broccoli puree. Pre-heating of broccoli prior to pureeing resulted in much higher increase in sulforaphane content after fermentation. The sulforaphane content of preheated-fermented broccoli (13121.3 ± 440.8 $\mu\text{mol/kg DW}$) was about 8 times of the raw-fermented broccoli puree. The observed sulforaphane yield after the combined preheating-fermentation treatment is much higher than what would be expected based on the quantifiable amount of glucoraphanin (3423.7 ± 39.7 $\mu\text{mol/kg}$) in the raw broccoli sample. It seems that the combined preheating and fermentation process enhances the release and accessibility of glucoraphanin for conversion over and above the inactivation of ESP by the pre-heating process. The pre-heating process coupled with microbial cell wall degrading enzymes may have enhanced the disruption of the cell compartment and release of bound glucosinolates in the matrix, that were not

extractable or accessible in the raw broccoli. Some lactic acid strains produce polysaccharide degrading enzymes such as cellulases and pectinases capable of degrading the cell wall structure and enhance the release of wall bound components.

In contrast, chemical acidification of preheated broccoli puree by GDL resulted in a significantly lower ($p < 0.05$) content of sulforaphane compared to pre-heated and preheat-fermented samples (Table 6). The sulforaphane content of the GDL acidified samples were 2169.4 ± 176.0 $\mu\text{mol/kg DW}$, which is 40% lower than the preheated broccoli sample (3536.0 ± 136.9 $\mu\text{mol/kg DW}$) ($P < 0.05$). It appears that the fast reduction to pH 4.04 during acidification may have reduced the conversion of glucoraphanin into sulforaphane in the GDL samples. It is well known that the conversion of glucosinolates is highly dependent on pH and acidic pH favours conversion into nitriles (Latte et al., 2011).

In the case of the pre-heated fermented samples, the acidification occurs gradually over a period of > 15 hr enabling the conversion of glucoraphanin mainly to sulforaphane since the activity of ESP is expected to be significantly reduced after preheating at 65°C for 3 min.

Changes of sulforaphane content during storage

The concentration of sulforaphane of all the samples declined after 14 days storage at 25°C (see Table 6 and Figure 6). Interestingly, an increase in sulforaphane content was observed in all samples except the fermented samples during 14 days storage at 4°C . The sulforaphane content of the raw puree almost doubled during storage at 4°C . Similarly, the sulforaphane content of the pre-heated samples increased by ~ 2.6 times whereas the sulforaphane content of the preheated GDL samples increased by ~ 2.3 times, which suggests continuous release of glucoraphanin from the matrix during storage allowing further conversion to sulforaphane and increase in concentration counteracting the consequence of sulforaphane degradation during storage. With respect to the preheated-fermented samples, reduction in sulforaphane content was observed during storage at both temperatures. All the accessible glucoraphanin may have been converted to sulforaphane during fermentation so much so that no further conversion occurred during storage but rather degradation albeit to a different extent depending on the temperature. As such, only a slight decline ($\sim 6\%$) was observed during storage at 4°C whereas the decline during storage at 25°C was $\sim 70\%$.

This study showed that pre-heating coupled with lactic acid bacteria fermentation substantially enhances the sulforaphane content of broccoli based

products. In-pack pre-heating treatment of broccoli florets at 65°C for 3 min followed by maceration and fermentation resulted in as much as ~16 times higher yield of sulforaphane compared to raw broccoli puree. Preheating under this condition increased the sulforaphane yield in broccoli puree from 806 $\mu\text{mol/KgDW}$ (dry weight) in the untreated broccoli to 3536 $\mu\text{mol/KgDW}$, indicating that the treatment substantially inhibits ESP while maintaining sufficient myrosinase activity for the conversion of glucoraphanin into sulforaphane. The best preheating condition during direct water blanching was 1 min at 60°C and resulted in sulforaphane yield of 2833 $\mu\text{mol/KgDW}$. The lower yield during direct blanching can be attributed to leaching of the water-soluble glucoraphanin into the blanching media. Preheating of broccoli florets in-pack (65°C/3min) combined with lactic acid bacteria fermentation further enhanced the sulforaphane content to 13121 $\mu\text{mol/KgDW}$, which is ~16 times increase compared to raw broccoli. Chemical acidification of in-pack preheated (65°C, 3min) combined with acidification of the broccoli puree by glucono-delta-lactone resulted in sulforaphane yield of 2169 $\mu\text{mol/KgDW}$, which is lower than pre-heating alone. The sulforaphane content of the preheated-fermented puree remained stable (~94% retention) during two weeks storage at 4°C.

Table 6. Sulforaphane yield ($\mu\text{mol/Kg DW}$) of broccoli before and after processing.

	Sulforaphane ($\mu\text{mol/kg, DW}$)				
	Raw	Raw-Fermented	Preheatnot GDL	Preheat GDL	Preheat-Fermented
Day 0	806.2 $\pm$ 7.0	1617.4 $\pm$ 10.2	3536.0 $\pm$ 136.9	2169.4 $\pm$ 176.0	13121.3 $\pm$ 440.8
Days 14_4°C	1409.8 $\pm$ 82.7	1627.7 $\pm$ 17.5	9149.4 $\pm$ 63.6	4994.8 $\pm$ 291.2	12301.3 $\pm$ 443.5
Days 14_25°C	1268.2 $\pm$ 0.1	1065.8 $\pm$ 49.8	3338.2 $\pm$ 93.9	2593.1 $\pm$ 97.7	3974.2 $\pm$ 71.2

DW: dry weight, GDL: acidified using glucono-delta-lactone. Preheating was conducted at 65°C in pack for 3 minutes.

Example 12 – Effect of lactic acid bacteria fermentation on polyphenolic profile of broccoli

In order to determine the effects of fermentation on the polyphenolic metabolites of broccoli samples, targeted liquid chromatography-mass spectrometry (LC-MS) based metabolomic analysis of the raw and fermented broccoli puree samples

was conducted. The resulting multivariate data was analysed using Metaboanalyst software (Metaboanalyst 3.0, Xia and Wishart, 2016). Fermentation resulted in a significant change in the metabolite profile of the broccoli samples. The partial least square discriminant analysis (PLS-DA) of the data shows a clear distinction between the polyphenolic profile of the fermented and the non-fermented samples (Figure 8).

The top 15 metabolites that were identified to be responsible for the differences between the two groups are shown in Figure 9. They are phenolic acids and phenolic aglycones, with higher bioactivity and bioavailability compared to their phenolic acid ester and phenolic glycoside precursors. The concentrations of most of these metabolites showed substantial increase following fermentation indicating the beneficial effect of fermentation on the polyphenol profile of broccoli puree. The fold changes for some of the metabolites are shown in Table 7.

A substantial increase in sinapic acid and kaempferol, 24 fold and 16 fold respectively was observed following fermentation. Similarly, fermentation induced an 8 fold increase in chlorogenic acid and phenyllactic acid. The concentrations of hesperetin, quercetin, methyl syringate and syringic acid also increased substantially after fermentation. The increase in the concentration of aglycones such as kaempferol, hesperetin and quercetin can be attributed to conversion of their glycoside precursors by the activity of microbial glycosidases. The increase in the concentration of phenolic acids such as sinapic acid could be due to the conversion of phenolic acid esters in broccoli by the activity of microbial esterases. Some decrease in caffeic acid and gallic was observed following fermentation. The activity of microbial decarboxylases convert caffeic acid into the corresponding vinyl catechol and gallic acid into pyrogallol, which may be responsible for the decrease in their concentration (Filanino et al., 2015; Guzman-Lopez et al., 2009).

Table 7. Fold changes in the top 13 polyphenols responsible for differences between fermented and non-fermented broccoli puree.

	Compounds	Fold change (FC)	Log ₂ (FC)
1	Sinapic acid	24.1	4.6
2	Kaempferol	16.1	4.0
3	Chlorogenic acid	8.3	3.1
4	Phenyllactic acid	7.9	3
5	Hesperetin	3.7	1.9
6	Methyl syringate	3.3	1.7

7	Syringic acid	3.3	1.7
8	Caffeic acid	0.32	-1.6
9	Ferullic acid	2.7	1.4
10	4, hydroxybenzoic acid	0.4	-1.4
11	Quercetin	2.6	1.3
12	Rutin	2.5	1.3
13	Gallic acid	0.5	-1.1

Example 13 – Identification of metabolites produced by lactic acid bacteria fermentation of broccoli by targeted and untargeted LC MS analyses of samples

The fermented and non-fermented broccoli puree samples were frozen and freeze dried. The samples (100 mg freeze dried powder each) were extracted using 1 ml of ice-cold methanol and Milli-Q water (50:50, v:v), which comprised 100 mg/ml of caffeine as an internal standard. The samples were then vortexed for 2 minutes prior to being sonicated (40 Hz) for 30 minutes. Samples were then centrifuged at 20,000 rpm at 4°C for 30 minutes, and the supernatant transferred to clean silanised LC-MS vials.

Samples were analyzed by injecting 1.4 µl into an Agilent 6410 LC-QQQ HPLC (Agilent Technologies, Santa Clara, California, USA). The analyses were performed using a reversed-phase Agilent Zorbax Eclipse Plus C18, Rapid Resolution HD, 2.1 x 50 mm, 1.8 µm (Agilent Technologies, Santa Clara, California, USA), with a column temperature of 30°C and a flow rate of 0.3 ml/min. The mobile phase was operated isocratically for 1 min 95:5 (A:B) then switched to 1:99 (A:B) for a further 12 min before returning back to 95:5 (A:B) for an additional 2 min; providing a total run time of 15 min. Mobile phase 'A' consisted of 100% H₂O and 0.1% formic acid, and mobile phase 'B' contained 75% acetonitrile, 25% isopropanol and 0.1% formic acid. The MS was collecting data in the mass range 50–1000 m/z. Qualitative identification of the compounds was performed according to the Metabolomics Standard Initiative (MSI) Chemical Analysis Workgroup using several online LC-MS metabolite databases, including Massbank and METLIN. Overall, the instrumental conditions were similar for both positive electrospray (+ESI) and negative electrospray (–ESI) modes. Scan time was 500, the source temperature was maintained at 350°C, the gas flow was 12 L/min and the nebuliser pressure was 35 psi.

For the identification of compounds in the untargeted analysis, the criteria was set at >90% match rate. Where the match rate dropped to between 70-89%, the compounds are identified with brackets (for example, if a compound was between 70-

89% they are annotated as “<name>”). Any matches below 70% were removed. In total, there was ca. 1000-1500 features to identify; many were poorly matched (and removed) or were less than 10 x S/N ratio from the baseline. As such, the compounds/peaks used were actual peaks and the IDs are fairly strong (i.e. >70%).

- 5 Untargeted LC-MS metabolomics study showed a 2 to 360 fold increase in certain polyphenolic glycosides including anthocyanin glycosides, phenolic acid glycosides, phenolic acids, a 5 to 60 fold increase in some glucosinolates with glucoraphanin increasing 27 fold and about a 3 to 4 fold increase in indol-3carbinol and ascorbigen. Results are summarised in Table 8 and are shown in Figure 10 and in a
10 volcano plot in Figure 11. The top 50 metabolites that increased after fermentation include several polyphenol glycosides and glucosinolates indicating that the process enhances their extractability and bioaccessibility.

15 **Table 8. Fold changes in different metabolites between fermented and non-fermented broccoli puree based on untargeted LC-MS analysis.**

Metabolite	FC	log2(FC)	raw.pval	(-LOG10(p))
Benzoic acid	4670.1	12.189	5.50E-08	7.2593
Cyanidin 3-O-rutinoside	361.03	8.496	0.011951	1.9226
Cyanidin 3-O-6"-p-coumaroyl-glucoside	271.87	8.0868	0.011465	1.9406
molybdopterin	149.51	7.2241	0.00915	2.0386
5-methylthiopentylglucosinolate	59.335	5.8908	0.005835	2.234
5-methylthioribulose 1-phosphate	46.001	5.5236	0.000334	3.4757
Ellagic acid arabinoside	42.956	5.4248	0.002845	2.546
thiamine phosphate	42.436	5.4072	0.005123	2.2905
2-carboxy-D-arabinitol 1-phosphate	41.06	5.3597	0.013093	1.883
N-acetyl-D-glucosamine 1,6-bisphosphate	40.636	5.3447	0.001824	2.739
S-norreticuline	32.883	5.0393	0.000362	3.4412
5-formamido-1-5-phospho-D-ribosyl-imidazole-4-carboxamide	30.585	4.9348	8.28E-06	5.0817
4-methylumbelliferone 6'-O-malonylglucoside	30.436	4.9277	0.001329	2.8765
Hydroxytyrosol 4-O-glucoside	28.971	4.8565	0.001319	2.8798
glucoraphanin	27.475	4.7801	0.014685	1.8331
glucobrassicin	26.746	4.7413	0.00441	2.3556
5-hydroxy-CMP	25.864	4.6929	0.004277	2.3689
4alpha-formyl,4beta,14alpha-dimethyl-9beta,19-cyclo-5alpha-ergost-24241-en-3beta-ol	18.8	4.2326	0.003497	2.4563
indole-3-acetyl-phenylalanine	17.44	4.1243	2.37E-06	5.6245
N-hydroxypentahomomethionine	16.92	4.0807	0.000559	3.2529
Cyanidin 3-O-arabinoside	16.098	4.0088	0.000413	3.3837
tetrahydrobiopterin	15.412	3.946	0.015746	1.8028

orotidine 5'-phosphate	14.737	3.8813	0.001699	2.7699
2-2'-methylthiopentylmaleate	14.621	3.87	0.005417	2.2662
S-adenosyl 3-methylthiopropylamine	14.564	3.8644	0.00177	2.752
4-methylthiobutyl glucosinolate	14.183	3.8261	0.011178	1.9516
salicylate	13.59	3.7644	0.000221	3.6556
N-hydroxyhomomethionine	12.902	3.6896	0.004311	2.3654
4'-phosphopantetheine	11.775	3.5576	0.003073	2.5124
5-phospho-beta-D-ribosylamine	10.643	3.4119	0.003185	2.497
D-erythro-imidazole-glycerol-phosphate	10.288	3.3629	0.019147	1.7179
a reduced flavodoxin	10.108	3.3374	0.005373	2.2698
Cyanidin 3-O-6"-dioxalyl-glucoside	9.9207	3.3104	0.000299	3.5242
8-oxo-GMP	9.8883	3.3057	0.008524	2.0694
3-dehydroteasterone	8.985	3.1675	8.33E-09	8.0793
indolylmethylisothiocyanate	7.7651	2.957	0.018337	1.7367
choline	7.7212	2.9488	0.023412	1.6306
carbamoyl phosphate	7.7098	2.9467	0.009139	2.0391
homogentisate	7.6608	2.9375	0.00153	2.8153
S-adenosyl-L-methionine	7.3817	2.8839	2.85E-05	4.5445
oxaloacetate	7.3494	2.8776	0.000538	3.2694
urate	7.2329	2.8546	0.000803	3.0951
coniferaldehyde glucoside	7.1826	2.8445	0.016973	1.7702
pyridoxal 5'-phosphate	7.0734	2.8224	0.021829	1.661
dTMP	6.9501	2.797	0.018743	1.7272
2-oxoglutarate	6.8749	2.7813	0.00019	3.7216
coniferaldehyde	6.6643	2.7365	1.46E-05	4.8345
Petunidin 3-O-rhamnoside	6.0484	2.5965	0.002487	2.6043
6-phospho D-glucono-1,5-lactone	5.8171	2.5403	0.019384	1.7126
dTDP	5.6526	2.4989	0.000837	3.0774
propane-1,3-diamine	5.5793	2.4801	0.001873	2.7275
benzoate	5.4402	2.4437	0.005218	2.2825
xi-progoitrin	5.091	2.3479	0.000107	3.9715
2-phospho-D-glycerate	5.0613	2.3395	0.001146	2.941
R-4'-phosphopantothenoyl-L-cysteine	4.8855	2.2885	0.01357	1.8674
L-arogenate	4.782	2.2576	0.018843	1.7248
L-phenylalanine	4.5585	2.1886	0.000213	3.671
Phenol	4.4651	2.1587	0.002537	2.5956
Gardenin B	4.3888	2.1338	0.012372	1.9076
glucomalcommiin	4.1855	2.0654	0.014526	1.8378
Sulfachloropyridazine	4.1627	2.0575	0.013676	1.864
4-methyl-2-oxopentanoate	3.906	1.9657	0.004372	2.3593
ascorbigen	3.7819	1.9191	0.017398	1.7595
2-naphthol	3.6366	1.8626	0.01404	1.8526

Medioresinol	3.6131	1.8532	0.007717	2.1125
E-2-pentenol	3.5473	1.8267	0.012466	1.9043
N-feruloyltyramine	3.3648	1.7505	0.004573	2.3399
2-methyl-6-phytyl-1,4-benzoquinol	3.3442	1.7417	0.000245	3.6101
pyridoxal	3.0278	1.5983	0.00016	3.7954
1D-myo-inositol 1-monophosphate	2.784	1.4771	0.005472	2.2618
N-monomethylethanolamine	2.7546	1.4618	1.55E-05	4.8092
3,4-Dicaffeoylquinic acid	2.7368	1.4525	0.012553	1.9013
Cirsilineol	2.6151	1.3868	0.001515	2.8197
S-methylmalonate-semialdehyde	2.5477	1.3492	0.012237	1.9123
benzaldehyde	2.5268	1.3373	0.01558	1.8074
Unidentified metabolite No. 1	2.3799	1.2509	7.84E-05	4.1056
Isorhamnetin	2.2605	1.1766	0.001828	2.738
AMP	2.1939	1.1335	0.002464	2.6083
2-Hydroxybenzoic acid	2.1338	1.0935	0.006072	2.2167
butan-1-al	2.0853	1.0602	3.16E-07	6.5005
7-Hydroxymatairesinol	2.0626	1.0445	0.008034	2.095
Dimethylmatairesinol	0.43475	-1.2018	0.000284	3.5464
trans-zeatin	0.39207	-1.3508	0.008484	2.0714
Unidentified metabolite No. 2	0.38059	-1.3937	0.000721	3.1421
coniferyl alcohol	0.37824	-1.4026	0.011806	1.9279
papaverine	0.36651	-1.4481	0.012288	1.9105
2,5-diamino-6-5-phospho-D-ribosylaminopyrimidin-43H-one	0.3594	-1.4763	0.020453	1.6893
S-4-hydroxymandelonitrile	0.32867	-1.6053	0.00375	2.426
22alpha-hydroxy-campesterol-4-en-3-one	0.32674	-1.6138	0.004969	2.3037
3-cyano-L-alanine	0.32471	-1.6228	0.013212	1.879
Ellagic acid glucoside	0.32466	-1.623	0.022951	1.6392
2-naphthol 6'-O-malonylglucoside	0.30641	-1.7064	0.000709	3.1492
pelargonidin	0.30629	-1.707	0.010379	1.9838
2S-naringenin	0.30353	-1.7201	0.019827	1.7027
8-methylthiooctyl-thiohydroximate	0.28257	-1.8233	0.002811	2.5512
Stigmastanol ferulate	0.28168	-1.8279	0.017703	1.752
Pinosylvin	0.26912	-1.8937	0.01535	1.8139
germacra-110,4,1113-trien-12-ol	0.23506	-2.0889	0.022511	1.6476
indole-3-acetyl-glutamine	0.20278	-2.302	0.006425	2.1921
2-7'-methylthioheptylmalate	0.19682	-2.3451	0.001077	2.968
p-coumaroyltriatic acid lactone	0.18436	-2.4394	0.0122	1.9136
6"-O-Acetylaidzin	0.15801	-2.6619	0.008935	2.0489
indole-3-acetyl-glutamate	0.15472	-2.6922	0.003623	2.441
Isorhamnetin 3-O-glucoside 7-O-rhamnoside	0.15357	-2.703	0.002647	2.5773
olivitol	0.13094	-2.933	0.005902	2.229

N-hydroxy-L-phenylalanine	0.1141	-3.1316	0.000812	3.0905
R-pantothenate	0.10725	-3.221	1.36E-05	4.8679
glucoiberberin	0.087316	-3.5176	0.00014	3.8538
6-O-methylnorlaudanoline	0.055734	-4.1653	6.96E-05	4.1575
carlactone	0.052932	-4.2397	2.93E-05	4.5332
E,E-geranyllinalool	0.018254	-5.7757	0.004044	2.3932
UDP-alpha-D-xylose	13.367	3.7407	0.0235	1.6289
Z-1-glutathione-S-yl-2-phenyl-acetohydroximate	19.906	4.3151	0.026163	1.5823
Apigenin 7-O-6"-malonyl-apiosyl-glucoside	0.38092	-1.3925	0.02641	1.5782
4alpha-formyl-stigmasta-7,24241-dien-3beta-ol	58.691	5.8751	0.026582	1.5754
soyasapogenol B	0.35836	-1.4805	0.027448	1.5615
dihydroconiferyl alcohol glucoside	5.6248	2.4918	0.027644	1.5584
3-deoxy-alpha-D-manno-octulosonate	6.6012	2.7227	0.027652	1.5583
Anhydro-secoisolariciresinol	2.3975	1.2616	0.027928	1.554
3-isopropyl-7-methylthio-2-oxoheptanoate	0.30287	-1.7232	0.028072	1.5517
Kaempferide	0.15749	-2.6666	0.0281	1.5513
2-aminoprop-2-enoate	2.0003	1.0002	0.029166	1.5351
isoliquiritigenin	2.8505	1.5112	0.029212	1.5344
m-Coumaric acid	2.187	1.129	0.029331	1.5327
indole-5,6-quinone	2.6937	1.4296	0.02956	1.5293
2-4'-methylthiobutylmalate	0.43617	-1.197	0.030711	1.5127
7-methylthioheptyl glucosinolate	0.42422	-1.2371	0.030739	1.5123
camalexin	0.27584	-1.8581	0.030778	1.5118
3-Methoxynobiletin	8.9717	3.1654	0.031528	1.5013
8-methylsulfinyloctyl glucosinolate	0.1694	-2.5615	0.031733	1.4985
ent-cassa-12,15-diene	0.33285	-1.587	0.032806	1.484
Catechol	4.0005	2.0002	0.033382	1.4765
L-aspartate-semialdehyde	2.9298	1.5508	0.033499	1.475
10-methylthio-2-oxodecanoate	4.5655	2.1908	0.033543	1.4744
indole-3-carbinonium ion	2.7807	1.4754	0.033654	1.473
laurate	0.33955	-1.5583	0.034205	1.4659
malonate	9.0975	3.1855	0.035699	1.4473
1-aci-nitro-8-methylsulfanyloctane	8.8356	3.1433	0.035865	1.4453
2-hydroxy-5-methylthio-3-oxopent-1-enyl 1-phosphate	13.56	3.7612	0.036727	1.435
glyoxylate	16.835	4.0734	0.037951	1.4208
Feruloyl tartaric acid	5.5489	2.4722	0.038578	1.4137
3beta-hydroxyparthenolide	8.1691	3.0302	0.038749	1.4117
22R,23R-22,23-dihydroxycampesterol	2.0564	1.0401	0.039305	1.4056
Gallic acid 4-O-glucoside	2.515	1.3306	0.039605	1.4023
E-phenylacetaldoxime	2.1608	1.1116	0.040641	1.391

18-hydroxystearate	0.14519	-2.784	0.042027	1.3765
5'-phosphoribosyl-4-N-succinocarboxamide-5-aminoimidazole	0.4281	-1.224	0.042243	1.3742
3-Feruloylquinic acid	3.3496	1.744	0.042655	1.37
2-carboxy-L-threo-pentionate	2.0447	1.0319	0.043	1.3665
trans-zeatin riboside	0.40453	-1.3057	0.044527	1.3514
4-fumaryl-acetoacetate	5.0298	2.3305	0.044744	1.3493
2-cis-abscisate	76.81	6.2632	0.044918	1.3476
4-Hydroxycoumarin	0.48212	-1.0525	0.045785	1.3393
Biochanin A	2.1017	1.0716	0.046533	1.3322
S-2,3,4,5-tetrahydrodipicolinate	4.1401	2.0497	0.046976	1.3281
26,27-dehydrozymosterol	14.846	3.892	0.047042	1.3275
N-methylethanolamine phosphate	10.038	3.3273	0.047416	1.3241
Kaempferol 3-O-2"-rhamnosyl-galactoside 7-O-rhamnoside	2.7008	1.4334	0.048201	1.3169
pheophorbide a	6.3398	2.6644	0.049365	1.3066
Chrysoeriol 7-O-6"-malonyl-glucoside	4.8949	2.2913	0.049727	1.3034
allantoate	10.972	3.4557	0.050008	1.301
Ligstroside-aglycone	12.072	3.5936	0.052404	1.2806
cycloeucalenone	3.4926	1.8043	0.052645	1.2786
Unidentified metabolite No. 3	3.5807	1.8403	0.053727	1.2698
laricitrin	0.42811	-1.224	0.05399	1.2677
Sulfadimethoxine	11.488	3.5221	0.05455	1.2632
3,4-Diferuloylquinic acid	5.2839	2.4016	0.054583	1.2629
glucotropeolin	0.47952	-1.0603	0.054637	1.2625
5,6-dihydroxyindole-2-carboxylate	5.2663	2.3968	0.055218	1.2579
S-laudanine	2.8697	1.5209	0.055638	1.2546
L-nicotianamine	0.39854	-1.3272	0.057257	1.2422
5-methylthiopentyl-thiohydroximate	0.30202	-1.7273	0.057551	1.2399
aldehydo-D-galacturonate	2.6643	1.4138	0.05785	1.2377
R-mevalonate 5-phosphate	0.34888	-1.5192	0.058188	1.2352
6-Hydroxyluteolin 7-O-rhamnoside	2.142	1.099	0.05845	1.2332
L-aspartate	3.5705	1.8361	0.061441	1.2115
--Epicatechin 3-O-gallate	2.4481	1.2916	0.063269	1.1988
glycine	0.23586	-2.084	0.065585	1.1832
Episesaminol	2.4077	1.2677	0.065876	1.1813
6alpha-hydroxy-castasterone	3.7782	1.9177	0.068376	1.1651
alpha-D-galacturonate 1-phosphate	11.846	3.5664	0.070966	1.149
R-2,3-dihydroxy-3-methylpentanoate	2.995	1.5825	0.071057	1.1484
cyanidin-3-O-beta-D-glucoside	2.0686	1.0487	0.07128	1.147
D-erythrose 4-phosphate	3.7463	1.9054	0.07247	1.1398
CDP-choline	617.84	9.2711	0.073728	1.1324
adenine	2.0623	1.0442	0.074004	1.1307

raphanusamate	5.5593	2.4749	0.074387	1.1285
3-Methoxysinensetin	2.4046	1.2658	0.075102	1.1243
betaine aldehyde	3.5234	1.817	0.075291	1.1233
E-7-methylthioheptanaldoxime	2.2972	1.1999	0.076906	1.114
6-methylthiohexyl-thiohydroximate	5.5473	2.4718	0.077579	1.1103
6"-O-Malonylglycitin	0.16741	-2.5786	0.080677	1.0933
monodehydroascorbate radical	2.0677	1.048	0.081844	1.087
anthranilate	3.0289	1.5988	0.082088	1.0857
Hydroxycaffeic acid	0.43234	-1.2098	0.082209	1.0851
Myricetin 3-O-arabinoside	2.3978	1.2617	0.086518	1.0629
cis-aconitate	0.18331	-2.4477	0.088998	1.0506
5-phospho-alpha-D-ribose 1-diphosphate	0.47829	-1.064	0.089065	1.0503
Malvidin 3-O-glucoside	0.48171	-1.0538	0.089472	1.0483
N6-delta2-isopentenyl-adenosine 5'-monophosphate	44.241	5.4673	0.092566	1.0335
Quercetin 3-O-6"-acetyl-galactoside 7-O-rhamnoside	2.9914	1.5808	0.093824	1.0277
cholesterol	2.816	1.4936	0.095163	1.0215
9-methylthiononyl-thiohydroximate	15.416	3.9464	0.098598	1.0061

In order to determine the effects of fermentation on the polyphenolic metabolites of broccoli samples, targeted liquid chromatography-mass spectrometry (LC-MS) based metabolomic analysis of the raw and fermented broccoli puree samples was conducted. Statistical analysis was performed without preprocessing. Fermentation

5 resulted in a significant change in the metabolite profile of the broccoli samples.

In the targeted LC-MS analysis, polyphenol standards were used for the identification and quantification of the metabolites. Increases in chlorogenic acid, ferullic acid, syringic acid, phenyllactic acid, rutin, sinapic acid, methyl syringate, hesperetin, quercetin and kaempferol were confirmed in fermented broccoli (Figure

10 12). Decreases in protocatechuic acid, gallic acid, 4,hydroxybenzoic acid, vanillic acid, 2,3dihydroxybenzoic acid, p-cuomarcic acid, cinnamic acid, catechin, rosmarinic acid, caffeic acid were confirmed in fermented broccoli (Figure 12). Of note is that a 6.6 fold change in chlorogenic acid (2.4 to 15.8 µg/mg), a 23.8 fold increase is in sinapic acid (3.6 to 86.6 µg/mg), a 10.5 increase in kaempferol (12.7 to 134.6 µg/mg) and a 0.48

15 fold decrease in p-Coumaric acid occurred in fermented samples (Figure 12).

Example 14 – Assessment of the broccoli fermentation culture to inhibit the growth of intentionally introduced microorganisms

A challenge study was conducted to assess the ability of the broccoli fermentation culture to inhibit the growth of intentionally introduced microorganisms which are often observed and of concern in food preparation.

Lab culture/starter culture

10 ml of 10^{10} cfu/mL of an inoculum comprising B1, B2, B3, B4, B5, BF1 and BF2 to achieve 10^8 CFU/gm of sample in the ferment.

Pathogen cultures

E. coli isolates FSAW 1310, FSAW 1311, FSAW 1312, FSAW 1313 and FSAW 1314 were grown separately to $1-4 \times 10^8$ cfu/mL in NB (nutrient broth) overnight at 37°C, static. The cultures were combined (1 mL of each) and the combined culture diluted to 10^4 with MRD (maximum recovery diluent) for first two dilutions and water for last two dilutions.

Salmonella strains *S. Infantis* 1023, *S. Singapore* 1234, *S. Typhimurium* 1657 (PT135), *S. Typhimurium* 1013 (PT9) and *S. Virchow* 1563 were grown separately to $1-4 \times 10^8$ cfu/mL in NB overnight at 37°C, static. The cultures were combined (1 mL of each) and combined culture diluted to 10^4 with MRD for first two dilutions and water for last two dilutions.

Listeria isolates Lm2987 (7497), Lm2965 (7475), Lm2939 (7449), Lm2994 (7537) and Lm2619 (7514) were grown separately in 10 mL BHI (brain heart infusion broth) overnight at 37°C under agitation. All cultures were then combined (1 mL of each) and this cocktail was diluted using MRD for first two (1/10) dilutions and sterile deionised water for last two dilutions.

B. cerus spore crops were prepared from isolates B3078, B2603, 2601, 7571 and 7626.

Method

Broccoli puree was prepared prior to preparing the inoculums, Broccoli: Sterile Tap Water 3:2 (900 g broccoli: 600 g water). Broccoli heads were rinsed in tap water, the stalks were cut off the broccoli with a sterile knife on a cutting board sanitised with 80% ethanol. Broccoli florets (900 g) were cut into small pieces. 450 g of broccoli pieces were placed into Thermomix bowl with all 600 g of the water. The translucent Thermomix cup/lid was sanitised with 80 % ethanol and placed over the lid hole. The

broccoli was chopped at speed 4 for 1 min. The second 450 g of broccoli pieces were added to the Thermomix bowl and chopped at speed 4 for 1 min. The contents were chopped for a further 5 min at speed 10 (max). After making sure the puree was indeed smooth enough, the Thermomix bowl was placed in the cool room to cool down the contents for 30 min. Following this, the bowl was put in the incubator and equilibrated to 30°C. Meanwhile the starter culture and pathogen culture (*E. coli*, *B. cereus*, *Salmonella*, *Listeria monocytogenes*) were prepared. 10 mL of LAB culture and 7.5 mL of the 10⁻⁴-diluted challenge microorganism cocktail (10⁴ cfu/mL culture in water) were added into the broccoli puree (10⁵ of *B. cereus*). Foil was held down over the large hole in the Thermomix lid prior to mixing culture. The cultures were mixed into the puree for 1 min on maximum speed. The heat setting for the Thermomix was switched off and the Thermomix was placed inside the 30°C incubator and the fermentation started at 10:45 am. pH and temperature measurements were taken every hour up until 7 h (end of work time) after mixing the puree for 1 min speed 4.5. The pH meter was calibrated and sanitised using 80% ethanol. The temperature probe was also sanitised prior to measurements with 80% ethanol.

The growth of the challenge microorganisms was assessed by counts on growth on the selective media MRS, DRBX and NA +S of raw broccoli, before fermentation (T0) and after fermentation commenced at 4 hours (T4) and 22 hours (T22).

Results

The yeast and mould were significantly reduced by 4 hours, and were not detected at the end of fermentation (T22). *E. coli* and *Salmonella* were never detected at the end of fermentation (T22). *Listeria* was detected in low numbers at the end of fermentation, with a starting inoculum just over 10³ cfu/mL. *B. cereus* spores were generally not affected by the fermentation, but did not germinate. The result of the challenge study indicates that the lactic acid bacteria strains that we isolated from broccoli are able to completely inactivate *Salmonella* and *E. coli* and inhibit the growth of the most acid resistant strains of *Listeria*. They are also able to inhibit the sporulation of *B. cereus* spores.

Table 9. Example of microbial challenge study with *E. coli*. *E. coli* (mix of 5 *E. coli* strains EC1605, EC1606, EC1607, EC1608 inoculated (2.2×10^2 CFU/gm) into the macerated broccoli (3:2 broccoli-water ratio) ferment to evaluate if the fermentation starter (a consortia of B1, B2, B3, B4, B5, BF1, BF2) inhibits the growth of *E. coli*. Experiments were repeated three times. Fermentation was conducted at 30°C for 22 hrs to pH below 4.0.

Time (hrs)	Lactic acid bacteria (CFU/gm)	Yeast and mould (CFU/gm)	<i>E. coli</i> (CFU/gm)
0	1.6×10^8	2.4×10^3	1.6×10^2
4	1.5×10^8	3×10	1.2×10^2
22	3.6×10^9	<10	<1

Table 10. Example of microbial challenge study with *Salmonella*. *Salmonella* (A mix of 5 strains *S. Infantis* 1023, *S. Singapore* 1234, *S. Typhimurium* 1657 (PT135), *S. Typhimurium* 1013 (PT9), *S. Virchow* 1623) inoculated (1.1×10^3) into macerated broccoli (3:2 broccoli-water ratio) ferment to evaluate if the fermentation starter (a consortia of B1, B2, B3, B4, B5, BF1, BF2) inhibits the growth of *Salmonella*. Experiments were repeated three times. Fermentation was conducted at 30°C for 22 hrs to pH below 4.0.

Time (hrs)	Lactic acid bacteria (CFU/gm)	Yeast and mould (CFU/gm)	<i>Salmonella</i> (CFU/gm)
0	3.5×10^8	1.4×10^3	6.4×10^2
4	4.2×10^8	2×10	3.3×10^2
22	1.4×10^9	<10	<10

Table 11. Example of microbial challenge study with *Listeria monocytogenes*. *Listeria monocytogenes* (A mix of 5 strains Lm2987 (7497), Lm2965 (7475), Lm2939 (7449), Lm2994 (7537), Lm2919 (7514)) inoculated (1.9×10^3) into macerated broccoli (3:2 broccoli-water ratio) ferment to evaluate if the fermentation starter (a consortia of B1, B2, B3, B4, B5, BF1, BF2) inhibits the growth of acid resistant *Listeria*. Experiments were repeated three times and the final *Listeria* count at the end of fermentation ranged from <10 (undetected) to 1.1×10^2 CFU/gm. Fermentation was conducted at 30°C for 22 hrs to pH below 4.0.

Time (hrs)	Lactic acid bacteria (CFU/gm)	Yeast and mould (CFU/gm)	<i>Listeria</i> (CFU/gm)
0	5.6×10^8	5.2×10^4	2.1×10^3
4	4.1×10^8	3.6×10^3	2.8×10^3
22	5.1×10^9	<10	2×10

- Table 12. Example of microbial challenge study with *Bacillus cereus*.** *Bacillus cereus* (A mix of 5 strains B3078, B2603, B2601, B7571, B7626) inoculated (1.9×10^3) into macerated broccoli (3:2 broccoli-water ratio) ferment to evaluate if the fermentation starter (a consortia of B1, B2, B3, B4, B5, BF1, BF2) inhibits the growth of acid resistant *Listeria*. Experiments were repeated three times. Fermentation was conducted at 30°C for 22 hrs to pH below 4.0.

Time (hrs)	Lactic acid bacteria (CFU/gm)	Yeast and mould (CFU/gm)	<i>Listeria</i> (CFU/gm)
0	2.4×10^8	1.2×10^3	3.1×10^3
4	3.3×10^8	9.5×10	2.3×10^3
22	1.9×10^9	<10	1.7×10^3

Example 15 - Pulse filed gel electrophoreses of *Leuconostoc mesenteroides* isolates

Leuconostoc mesenteroides from vegetables was assessed with *SmaI* and *NotI* restriction enzyme digestion with pulse filed gel electrophoreses as described in Chat and Dalmaso (2015) with modification.

Methods:

Day 1

Assessed isolates were inoculated into 10 mL MRS broth and incubated overnight at 30°C in incubator (16 h).

Day 2

Isolates were centrifuge at 3500 g for 10 min and the supernatant discarded. The pellet was mixed and washed with 5 mL deionised water and centrifuged at 3500 g for 10 min and the supernatant discarded. The pellet was mixed with 5 mL TES (1 mM EDTA, 10 mM Tris-HCl, 0.5 M saccharose) and vortexed. Next the samples were centrifuged at 3500 g for 15 min and the supernatant discarded. 700 µL of Lysis solution (TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0, sterilise as normal) with

lysozyme at 10 mg/mL) was added to the pellet and mixed and incubated at 56 °C for 2 h to lyse bacteria. Next, 700 µL of agarose (1 % SeaChem Gold agarose with 50 µL EDTA/100 mL) was added to the cell mixture, mix and dispensed into plug moulds and 2 mL of deproteinisation (660 µL of proteinase K buffer, 11 µL proteinase K) solution added all plugs for one sample placed in the tube and incubated at 55 °C overnight.

Day 3

Next the plugs were heated in 100 mL of sterile deionised water at 55 °C, the deproteinisation solution was removed and the plugs transferred to 15 mL centrifuge tubes, washed with 4 mL of sterile deionised water and heated to 55 °C for 10 min at room temperature followed by washing four times with 4 mL TE buffer for 10 min at room temperature.

Restriction digests

2mm slice off plug was placed in an eppendorf tube with 100 µL 1X restriction buffer, incubated for 20 min at room temperature, restriction buffer was removed and replaced with 40-100 µL of *SmaI* (20 U) or *NotI* in restriction buffer and incubated for 4 h at the optimum temperature (25 °C).

Day 4

Separation of restriction fragments

1mL 0.5X TBE buffer to each tube and allowed to sit for at least 15 min to stop reaction and the bacteriophage λ DNA ladder (New England Biolab) was incubated in TBE buffer. The buffer was removed and the slices loaded onto comb, with the ladder in every five lanes. 1.0 % ultra-pure DNA grade agarose (pulsed field certified agarose) was prepared in 0.5X TBE running buffer.

Electrophoresis conditions

Buffer maintained at 14°C (model 1000 Mini-chiller, BioRad). BioRad “Chef Mapper™”, select Two State Program (not Auto Algorithm). Pulse time ramped linearly (press enter when “a” appears) from 2 to 25 s. Gradient 6 V/cm (voltage), Included angle 120°, Running time of 24 h.

Day 5

Gels stained ~30 min in GelRed, destained, visualised

Results

The restriction fingerprint for BF1 was distinct but similar to *Leuconostoc mesenteroides* isolated from carrot (Figure 13). The restriction fingerprint for BF2 was distinct from all *Leuconostoc mesenteroides* strains assessed (Figure 13).

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Example 16 - Variant analysis of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* isolates

For the SNP analysis of the *Lactobacillus plantarum* isolates (B1 to B5), B1 Prokka gbk was used as reference for Snippy SNP analysis - standard method. Single comparisons were performed using read data for each strain. B1 reads were ran as a control.

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Example command was :

snippy --cpus 24 --outdir B5 --ref B1_S1mod.gbk --pe1

15 B5_S17_L001_R1_001.fastq.gz --pe2 B5_S17_L001_R2_001.fastq.gz

Calculated individual comparisons and core using B1 gbk as reference

snippy-core --prefix core B1 B2 B3 B4 B5

20

Comparisons were also performed between B1 and the reference strain read data downloaded from the SRA for *Lactobacillus plantarum* ATCC 8014 (SRR1552613). Downloading was performed using standard method with prefetch and conversion to fastq using - sratoolkit.2.9.2-win64. Similar approaches were used for comparison of the *Leuconostoc mesenteroides* isolates BF1 and BF2 with *Leuconostoc mesenteroides* ATCC 8293 as reference.

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Results

Variants (41) were observed between B1 and ATCC 8014 (Table 13). Variants (1 to 4) were observed between B1 and the other B isolates B2, B3, B4 and B5 (Table 14 to 17). BF1 and BF2 are very different from one another. Variants (19) were observed between BF1 and ATCC 8293 (Table 18). Variants (~7000) were observed between BF2 and ATCC 8293. 459 complex variants were identified between BF2 and ATCC8293 which are summarized in Table 19.

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Table 13. Polymorphisms identified by variant analysis B1 compared to ATCC8014.

POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE
292863	comple	GTCG	ATCT	ATCT:96 GTCG:0	CDS	+	292/477	98/158	missense_variant c.292_295delGTCGins ATCT p.ValAla98IleSer	JBMHILAL_00290	ohrR_1
21413	snp	C	T	T:204 C:1							
49138	snp	T	G	G:226 T:2	CDS	+	771/101 1	257/336	missense_variant c.771T>G p.Asn257Lys	JBMHILAL_00337	lacR_1
68529	del	TATTAATG GCTCGCGT CATTA	TA	TA:97 TATTAATGGCTCG CGTCATTAA:0							
70435	snp	G	A	A:199 G:1	CDS	-	95/1959	32/652	missense_variant c.95C>T p.Thr32Ile	JBMHILAL_00352	lacS_2
70584	snp	T	C	C:154 T:1							
71677	snp	T	C	C:201 T:0	CDS	-	209/102 9	70/342	missense_variant c.209A>G p.Tyr70Cys	JBMHILAL_00353	
72030	del	CGCTCAAC CAGATTAG TACCCAG	CG	CG:91 CGCTCAACCAGAT TAGTACCCAG:0	CDS	-	978/996	320/331	inframe_deletion c.958_978delCTGGGT ACTAATCTGGTTGAG p.Leu320_Glu326del	JBMHILAL_00354	lacR_3
136221	snp	C	A	A:178 C:1	CDS	-	559/127 2	187/423	missense_variant c.559G>T p.Ala187Ser	JBMHILAL_00407	gatC_1
15092	snp	C	A	A:102 C:1							
153210	snp	G	T	T:117 G:1	CDS	-	385/136 5	129/454	missense_variant c.385C>A p.Gln129Lys	JBMHILAL_00681	gabR
38124	snp	C	T	T:264 C:1							
128067	snp	G	A	A:261 G:1	CDS	-	208/134 4	70/447	missense_variant c.208C>T p.Arg70Cys	JBMHILAL_01118	yjP_1
188850	snp	A	C	C:241 A:0	CDS	-	491/161 7	164/538	missense_variant c.491T>G p.Ile164Ser	JBMHILAL_01179	oppA_2
2322	snp	A	G	G:107 A:1	CDS	-	397/474	133/157	missense_variant c.397T>C p.Phe133Leu	JBMHILAL_01186	adcR
111662	ins	CAA	CAAA	CAAA:133 CAA:11	CDS	+	10/876	4/291	frameshift_variant c.9dupA p.Ser4fs	JBMHILAL_01302	mntB
11376	snp	G	A	A:115 G:0	CDS	-	1831/19 47	611/648	synonymous_variant c.1831C>T	JBMHILAL_01356	

115510	snp	G	A		A:199 G:1	CDS	-		95/411	32/136	p.Leu611Leu	JBIMIHLAL_01453	
143457	snp	G	C		C:264 G:0	CDS	+		1122/1416	374/471	missense_variant c.95C>T p.Thr32Ile	JBIMIHLAL_01479	pepD
111973	snp	G	A		A:118 G:1	CDS	-		731/1317	244/438	synonymous_variant c.1122G>C p.Val374Val	JBIMIHLAL_01603	murA1
27553	snp	C	T		T:104 C:1	CDS	-		472/1092	158/363	missense_variant c.731C>T p.Ala244Val	JBIMIHLAL_01677	wbnH
80888	snp	T	C		C:84 T:0	CDS	+		256/258	86/85	missense_variant c.472G>A p.Gly158Ser	JBIMIHLAL_01727	ytIR_1
133147	snp	A	C		C:76 A:0	CDS	-		443/663	148/220	stop_lost&splice_region_variant c.256T>C p.Ter86Glnext*?	JBIMIHLAL_01777	yjBM
74711	snp	C	T		T:212 C:1	CDS	+		874/1389	292/462	missense_variant c.874C>T p.Leu292Phe	JBIMIHLAL_01855	murF_2
19793	snp	T	C		C:114 T:1	CDS	-		925/1107	309/368	missense_variant c.925A>G p.Asn309Asp	JBIMIHLAL_01907	sigA
60643	snp	C	T		T:89 C:1	CDS	-		242/1869	81/622	missense_variant c.242G>A p.Ser81Asn	JBIMIHLAL_01945	dnaK
10806	ins	GTTTTTTT G	GTTTTTTT G		GTTTTTTT GTTTTTTT G:49 G:1								
50276	complex	CG	CACCACCAGG CCGATTGTGG CGA		CACCACCAGGCG ATTGTGGCGA:39 CG:0	CDS	-		341/555	114/184	missense_variant&inframe_insertion c.341delCinsTCGCCAC AATCGGCTGGTGGT p.Ala114delinsValAlaT hrleGlyLeuValVal	JBIMIHLAL_02031	ribU
50325	snp	A	C		C:99 A:1	CDS	-		293/555	98/184	stop_gained c.293T>G p.Leu98*	JBIMIHLAL_02031	ribU
64233	snp	A	G		G:77 A:1	CDS	-		2516/2604	839/867	missense_variant c.2516T>C p.Val839Ala	JBIMIHLAL_02043	clpB
79046	snp	G	C		C:140 G:1	CDS	+		394/765	132/254	missense_variant c.394G>C p.Ala132Pro	JBIMIHLAL_02139	ygaZ_2
14904	snp	G	A		A:82 G:0	CDS	-		113/876	38/291	missense_variant c.113C>T p.Pro38Leu	JBIMIHLAL_02340	

45542	snp	T	G	G:158 T:0	CDS	-	1312/17 28	438/575	missense_variant c.1312A>C p.Lys438Gln	JBMIHLAL_02365	pgcA
21706	ins	TAT	TAAAT	TAAT:122 TAT:1	CDS	+	872/260 4	291/867	frameshift_variant c.871dupA p.Ile291fs	JBMIHLAL_02489	mprF
29454	del	TGA	TA	TA:73 TGA:0	CDS	+	94/132	32/43	frameshift_variant c.94delG p.Asp32fs	JBMIHLAL_02559	
27619	snp	A	G	G:134 A:1	CDS	-	78/588	26/195	synonymous_variant c.78T>C p.Gly26Gly	JBMIHLAL_02812	
4360	snp	C	T	T:96 C:1							
8851	del	CGG	CG	CG:117 CGG:0	CDS	-	82/513	28/170	frameshift_variant c.82delC p.Pro28fs	JBMIHLAL_02963	tcaR
19068	del	CTTGCCGA AATTCGAC AAACAACC CTCGGATT GT	CT	CT:51 CTTGCCGAAATTC GACAAACACCCCT CGGATTGT:0	CDS	+	154/564	52/187	frameshift_variant c.154_185delGAAATT CGACAAACACCCCTCG GATTGTTGCC p.Glu52fs	JBMIHLAL_02974	
17533	ins	ATTTTGTG	ATTTTGTG	ATTTTGTG:220 ATTTTGTG:2							

Table 14. Polymorphism identified by variant analysis B2 compared to B1.

POS	TYPE	REF	ALT	EVIDENCE	FTYP	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE
8417	snp	C	T	T:105 C:0	CDS	+	105/264	35/87	synonymous_variant c.105C>T p.Asp35Asp	JBMIHLAL_02984	

Table 15. Polymorphisms identified by variant analysis B3 compared to B1.

POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE
4326	del	TATAAAAAAGCG ACCCCGTTCATTA ACGGTGCGGCTCA CAGATCATATTAG TGAAAATCACCCG GCA	TA	TA:31 TATAAAAAAGCGACC CCCGTTCATTAAACGGT GCCGCTCACAGATCAT TATTAGTAAAAATCAC CCGGCA:0							

8417	snp	C	T	T:135 C:0	CDS	+	105/264	35/8 7	synonymous_variant c.105C>T p.Asp35Asp	JBMIHLAL_02984	
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Table 16. Polymorphism identified by variant analysis B4 compared to B1.

POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE
8417	snp	C	T	T:93 C:0	CDS	+	105/264	35/8 7	synonymous_variant c.105C>T p.Asp35Asp	JBMIHLAL_02984	

Table 17. Polymorphisms identified by variant analysis B5 compared to B1.

POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE
199035	snp	T	C	C:124 T:0	CDS	+	368/120 6	123/ 401	missense_variant c.368T>C p.Val123Ala	JBMIHLAL_00 946	
143457	snp	G	C	C:158 G:0	CDS	+	1122/14 16	374/ 471	synonymous_variant c.1122G>C p.Val374Val	JBMIHLAL_01 479	pepD
23797	snp	A	C	C:146 A:0	CDS	+	71/666	24/2 21	missense_variant c.71A>C p.Gln24Pro	JBMIHLAL_02 490	immR_1
8417	snp	C	T	T:131 C:0	CDS	+	105/264	35/8 7	synonymous_variant c.105C>T p.Asp35Asp	JBMIHLAL_02 984	

Table 18. Polymorphisms identified by variant analysis BF1 compared to ATCC8293.

POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE
197592	del	TGT	TT	TT:178 TGT:0							
269841	del	TGG	TG	TG:305 TGG:0	CDS	+	33/306	11/101	frameshift_variant p.Asn12fs	LEUM_0316	
338699	snp	G	T	T:239 G:0	CDS	+	764/1719	255/572	missense_variant p.Trp255Leu	LEUM_0385	
410044	snp	C	A	A:210 C:0	CDS	+	2229/2457	743/818	synonymous_variant p.Thr743Thr	LEUM_0448	pheT
558511	ins	CAT	CAAT	CAAT:140 CAT:0	CDS	+	204/261	68/86	frameshift_variant p.His68fs	LEUM_0587	

559188	snp	A	G	G:169 A:0	CDS	+	601/981	201/326	missense_variant p.Ile201Val	c.601A>G	LEUM_0588	
615572	del	TCC	TC	TC:245 TCC:5								
755527	snp	A	T	T:196 A:0	CDS	+	351/993	117/330	missense_variant p.Leu117Phe	c.351A>T	LEUM_0777	
796683	del	GCC	GC	GC:207 GCC:0	CDS	+	2986/3009	996/1002	frameshift_variant p.Glu997fs	c.2986delC	LEUM_0814	
953160	snp	G	T	T:178 G:0	CDS	+	805/843	269/280	missense_variant p.Ala269Ser	c.805G>T	LEUM_0952	
1009293	snp	C	A	A:1652 C:171	CDS	+			no annotation		LEUM_1009	
1094250	snp	T	A	A:188 T:0	CDS	+			no annotation		LEUM_1090	
1236979	snp	G	T	T:194 G:1								
1237016	del	CAA	CA	CA:183 CAA:6								
1291050	del	CGT	CT	CT:177 CGT:0								
1600218	del	AGG	AG	AG:168 AGG:2								
1624087	ins	GA	GTA	GTA:205 GA:0								
1693283	snp	T	A	A:247 T:0	CDS	-			no annotation		LEUM_1724	
1993032	snp	G	A	A:209 G:0	CDS	-			no annotation		LEUM_2026	

Table 19. Polymorphisms identified by variant analysis BF2 compared to ATCC8293.

POS	REF	ALT	EVIDENCE	FTY PE	STR AND	NT_POS	AA_POS	EFFECT	LOCUS_TAG	GENE
1737	TTCA	ATCC	ATCC:151 TTCA:0	CDS	+	63/1137	21/378	synonymous_variant p.IleSer21IleSer	LEUM_0002	
11810	CATG	TATA	TATA:216 CATG:0	CDS	+	144/1626	48/541	missense_variant p.AsnMet48AsnIle	LEUM_0010	
12635	ACGT	GCGC	GCGC:255 ACGT:0	CDS	+	969/1626	323/541	synonymous_variant p.GlnArg323GlnArg	LEUM_0010	
20351	TCT	GCG	GCG:230 TCT:0	CDS	+	172/795	58/264	missense_variant p.Ser58Ala	LEUM_0017	

22033	AGCTA	GGCTG	GGCTG:214 AGCTA:0	CDS	+	1047/118 5	349/394	missense_variant c.1047_1051delAGCTAinsGGCTG p.GluAlaAsn349GluAlaAsp	LEUM_0018	
36499	TATT	CATC	CATC:289 TATT:0	CDS	+	564/1062	188/353	synonymous_variant c.564_567delTATTinsCATC p.ArgIle188ArgIle	LEUM_0044	
45902	GTAATGT GA	CCACATTAC	CCACATTAC:251 GTAATGTGA:0							
47145	TAT	TTCAG	TTCAG:241 TAT:0							
64340	CTGT	TTGC	TTGC:335 CTGT:0	CDS	-	205/915	68/304	missense_variant c.202_205delACAGinsGCAA p.ThrAsp68AlaAsn	LEUM_0076	
70144	GGTATGG GATGGGA A	CGTATGGG	CGTATGGGA:233 GGTATGGGATGGGA :0							
75797	AGAG	GGAT	GGAT:179 AGAG:0	CDS	+	51/171	17/56	missense_variant p.LeuGlu17LeuAsp	LEUM_0091	
97951	TAAT	CAAG	CAAG:197 TAAT:0	misc _bin ding	+			no annotation		
138065	GGCG	TGCA	TGCA:279 GGCG:0	CDS	-	1002/143 1	333/476	synonymous_variant c.999_1002delCGCCinsTGCA p.ValAla333ValAla	LEUM_0153	
138074	ATTG	GTTC	GTTC:276 ATTG:0	CDS	-	993/1431	330/476	synonymous_variant c.990_993delCAATinsGAAC p.ValAsn330ValAsn	LEUM_0153	
138092	AACT	GACC	GACC:278 AACT:0	CDS	-	975/1431	324/476	synonymous_variant c.972_975delAGTTinsGGTC p.ProVal324ProVal	LEUM_0153	
140746	GGGT	AGGC	AGGC:196 GGGT:0	CDS	+	366/540	122/179	synonymous_variant c.366_369delGGGTinsAGGC p.GluGly122GluGly	LEUM_0156	
140797	CGCC	TGCT	TGCT:208 CGCC:0	CDS	+	417/540	139/179	synonymous_variant c.417_420delCGCCinsTGCT p.AspAla139AspAla	LEUM_0156	
142611	GTT	CTG	CTG:135 GTT:0	CDS	+	271/375	91/124	missense_variant c.271_273delGTTinsCTG p.Val91Leu	LEUM_0158	
142687	CAAAAAG	CAAAAAAA	CAAAAAAA:178 CAAAAAG:0	CDS	+	353/375	118/124	frameshift_variant&missense_variant c.353delGinsAA p.Ser118fs	LEUM_0158	
145324	CAG	AAA	AAA:292 CAG:0	CDS	+	505/1497	169/498	missense_variant c.505_507delCAGinsAAA p.Gln169Lys	LEUM_0161	gltx
162834	TGAT	GGAC	GGAC:260 TGAT:0	CDS	+	2400/248 1	800/826	missense_variant c.2400_2403delTGATinsGGAC p.AspAsp800GluAsp	LEUM_0185	
192260	ATAAAA	GTAAC	GTAAC:301 ATAAAA:0	CDS	+	433/768	145/255	missense_variant c.433_437delATAAAinsGTAAC p.IleAsn145ValThr	LEUM_0228	truA

196751	CTAT	ATAC	ATAC:138 CTAT:0	CDS	-	55/204	18/67	missense_variant p.IleAla18ValSer	c.52_55delATAGinsGTAT	LEUM_0234	
196918	AATA	GATG	GATG:246 AATA:0								
216494	CACG	TACC	TACC:230 CACG:0	CDS	+	108/978	36/325	synonymous_variant p.AspThr36AspThr	c.108_111delCACGinsTACC	LEUM_0256	nrdF
231792	ATCTC	GTCCT	GTCCT:235 ATCTC:0	CDS	+	553/1728	185/575	missense_variant p.IleSer185ValLeu	c.553_557delATCTCinsGTCCT	LEUM_0276	
231812	GCTC	ACTT	ACTT:229 GCTC:0	CDS	+	573/1728	191/575	synonymous_variant p.AlaLeu191AlaLeu	c.573_576delGCTCinsACTT	LEUM_0276	
234250	ACTT	CCTG	CCTG:217 ACTT:0	CDS	+	336/642	112/213	synonymous_variant p.GlyLeu112GlyLeu	c.336_339delACTTinsCCTG	LEUM_0279	tmk
242029	CTAT	TTAC	TTAC:265 CTAT:0	CDS	-	664/966	221/321	missense_variant p.IleAla221ValThr	c.661_664delATAGinsGTAA	LEUM_0287	
244287	GACT	AACC	AACC:251 GACT:0	CDS	+	1436/196 2	479/653	missense_variant p.ArgLeu479LysPro	c.1436_1439delGACTinsAACC	LEUM_0288	
250392	GGCG	AGCT	AGCT:182 GGCG:0	CDS	+	345/1242	115/413	synonymous_variant c.345_348delGGCGinsAGCT	p.ValAla115ValAla	LEUM_0295	proA
271910	TTA	CTG	CTG:297 TTA:0	CDS	+	358/843	120/280	synonymous_variant p.Leu120Leu	c.358_360delTTAinsCTG	LEUM_0318	
288308	ATA	AC	AC:232 ATA:0								
318676	GATTAG	AATCAA	AATCAA:121 GATTAG:0	CDS	+	14/306	5/101	missense_variant p.GlyLeuVal5GluSerIle	c.14_19delGATTAGinsAATCAA	LEUM_0366	
341498	GTTTTTTT TTA	GTTTTTTTC	GTTTTTTTC:114 GTTTTTTTAA:0								
359500	GCAAG	ACAAC	ACAAC:238 GCAAG:0	CDS	+	3034/354 0	1012/117 9	missense_variant c.3034_3038delGCAAGinsACAAC	p.AlaSer1012Thr	LEUM_0399	
366821	ACATC	GCATT	GCATT:250 ACATC:0	CDS	+	957/1488	319/495	synonymous_variant p.LysHisLeu319LysHisLeu	c.957_961delACATCinsGCATT	LEUM_0406	lysS
366884	AGAAGCA	GGATGCG	GGATGCG:217 AGAAGCA:0	CDS	+	1020/148 8	340/495	missense_variant c.1020_1026delAGAAGCAinsGGATGCG	p.GluGluAla340GluAspAla	LEUM_0406	lysS
366896	GTTGGCC	ATTAGCA	ATTAGCA:225 GTTGGCC:0	CDS	+	1032/148 8	344/495	synonymous_variant p.LysLeuAla344LysLeuAla	c.1032_1038delGTTGGCCinsATTAGCA	LEUM_0406	lysS
366971	ATTGTGA	GTTTCGT	GTTTCGT:225 ATTGTGA:0	CDS	+	1107/148 8	369/495	synonymous_variant p.GluPheVal369GluPheVal	c.1107_1113delATTGTTCinsGTTTCGT	LEUM_0406	lysS

371223	CTTC	ATTT	ATTT:226 CTTC:0	CDS	+	273/1449	91/482	synonymous_variant c.273_276delCTTCinsATTT p.GlyPhe91GlyPhe	LEUM_0414	
395520	CTCT	ATCC	ATCC:206 CTCT:0	CDS	-	525/942	174/313	missense_variant c.522_525delAGAGinsGGAT p.IleGlu174MetAsp	LEUM_0436	
395821	ACCA	GCCG	GCCG:177 ACCA:0	CDS	-	224/942	74/313	missense_variant c.221_224delTTGGTinsCGGC p.MetVal174ThrAla	LEUM_0436	
410847	CGGT	TGGC	TGGC:232 CGGT:0	CDS	+	495/1287	165/428	synonymous_variant c.495_498delCGGTinsTGGC p.ValGly165ValGly	LEUM_0449	
420486	CGCAC	AGCAT	AGCAT:187 CGCAC:0	CDS	+	200/609	67/202	missense_variant c.200_204delCGCACinsAGCAT p.AlaHis67GluHis	LEUM_0457	
455735	GTG	CTT	CTT:112 GTG:0	CDS	-	1922/2088	640/695	missense_variant c.1920_1922delCACinsAAG p.AsnThr640LysSer	LEUM_0497	
457087	GCCAT	ACCAC	ACCAC:262 GCCAT:0	CDS	-	570/2088	189/695	missense_variant c.566_570delATGGCinsGTGGT p.AspGly189GlyGly	LEUM_0497	
490235	GCG	ACA	ACA:136 GCG:0	CDS	+	142/738	48/245	missense_variant c.142_144delGCGGinsACA p.Ala48Thr	LEUM_0524	
493487	TGGT	CGGC	CGGC:189 TGGT:0	CDS	+	168/834	56/277	synonymous_variant c.168_171delTTGGTinsCGGC p.ArgGly56ArgGly	LEUM_0527	
500830	GCT	ACC	ACC:176 GCT:0	CDS	+	352/2031	118/676	missense_variant c.352_354delGCTinsACC p.Ala118Thr	LEUM_0536	
502254	CGAA	TGAG	TGAG:214 CGAA:0	CDS	+	1776/2031	592/676	synonymous_variant c.1776_1779delCGAAinsTGAG p.ValGlu592ValGlu	LEUM_0536	
502272	CATTC	TCTCT	TCTCT:187 CATTC:0	CDS	+	1794/2031	598/676	missense_variant c.1794_1798delCATTCinsTCTCT p.PheLeu598PheLeuLeu	LEUM_0536	
502291	TTG	CTA	CTA:215 TTG:0	CDS	+	1813/2031	605/676	synonymous_variant c.1813_1815delTTGinsCTA p.Leu605Leu	LEUM_0536	
505441	AGG	GGA	GGA:156 AGG:4	CDS	+	826/834	276/277	missense_variant c.826_828delAGGinsGGA p.Arg276Gly	LEUM_0540	
507015	ACCAC	GCCAA	GCCAA:199 ACCAC:0	CDS	-	507/1098	168/365	missense_variant c.503_507delTTGGTinsTTGGC p.SerGly168IleGly	LEUM_0543	
508582	TGCT	CGCG	CGCG:163 TGCT:0	CDS	+	861/1008	287/335	synonymous_variant c.861_864delTGCTinsCGCG p.ProAla287ProAla	LEUM_0544	
509588	TTG	CTA	CTA:171 TTG:0	CDS	+	751/1866	251/621	synonymous_variant c.751_753delTTGinsCTA p.Leu251Leu	LEUM_0545	
510386	GTCAATA	ATCTTG	ATCTTG:158 GTCAATA:0	CDS	+	1549/1866	517/621	missense_variant c.1549_1554delGTCAATAinsATCTTG p.ValIle517IleLeu	LEUM_0545	

511743	CAGC	AAGT	AAGT:187 CAGC:0	CDS	+	927/1347	309/448	synonymous_variant c.927_930delCAGCinsAAGT p.LeuSer309LeuSer	LEUM_0546
519040	TCGT	CCGC	CCGC:165 TCGT:0	CDS	+	210/1371	70/456	synonymous_variant c.210_213delTCGTinsCCGC p.GlyArg70GlyArg	LEUM_0553
530354	TTGG	GTGA	GTGA:118 TTGG:0	CDS	+	193/1728	65/575	missense_variant c.193_196delTTGGinsGTGA p.LeuVal65ValMet	LEUM_0562
536863	AAGA	GAGG	GAGG:178 AAGA:0	CDS	+	1959/230 1	653/766	synonymous_variant c.1959_1962delAAGinsGAGG p.SerArg653SerArg	LEUM_0566
560132	AAC	TAT	TAT:202 AAC:0	CDS	+	423/882	141/293	missense_variant c.423_425delAACinsTAT p.ValThr141ValMet	LEUM_0589
603339	AAT	GAC	GAC:238 AAT:0	CDS	+	673/1944	225/647	missense_variant c.673_675delAAATinsGAC p.Asn225Asp	LEUM_0636
607531	GAGC	AAGT	AAGT:217 GAGC:0	CDS	+	438/894	146/297	missense_variant c.438_441delGAGCinsAAGT p.MetSer146IleSer	LEUM_0640
610263	TAACA	CAACG	CAACG:174 TAACA:0	CDS	+	773/1464	258/487	missense_variant c.773_777delTAACAinsCAACG p.LeuThr258SerThr	LEUM_0643
610344	TAGCTGC AAGTGCT GCAAGTG	CAGCTGCAA GTG	CAGCTGCAAAGTG:12 7 TAGCTGCAAAGTGCT GCAAGTG:0	CDS	+	854/1464	285/487	missense_variant&inframe_deletion c.854_864delTAGCTGCAAGTGinsCA p.Ile285_Ser288delinsThr	LEUM_0643
613023	CGGC	AGGT	AGGT:209 CGGC:0	CDS	+	801/1143	267/380	synonymous_variant c.801_804delCGGCinsAGGT p.ProGly267ProGly	LEUM_0645
613326	GACG	AACA	AACA:160 GACG:0	CDS	+	1104/114 3	368/380	synonymous_variant c.1104_1107delGACGinsAACA p.AlaThr368AlaThr	LEUM_0645
615534	GTTG	ATTA	ATTA:217 GTTG:0						
615580	GCCC	CCCT	CCCT:199 GCCC:0						
641900	TCCG	CCCA	CCCA:199 TCCG:0	CDS	+	417/570	139/189	synonymous_variant c.417_420delTCCGinsCCCA p.TyrPro139TyrPro	LEUM_0673
642442	CAGTA	TAGCG	TAGCG:148 CAGTA:0	CDS	+	282/684	94/227	missense_variant c.282_286delCAGTAinsTAGCG p.GlySerThr94GlySerAla	LEUM_0674
654478	CTTC	TTTT	TTTT:217 CTTC:0	CDS	+	597/795	199/264	synonymous_variant c.597_600delCTTCinsTTTT p.AsnPhe199AsnPhe	LEUM_0686
658429	TCG	GCA	GCA:147 TCG:0	CDS	+	622/4314	208/1437	missense_variant c.622_624delTCGinsGCA p.Ser208Ala	LEUM_0689
671357	CAGTTAT	AAGCTAC	AAGCTAC:180 CAGTTAT:0	CDS	+	432/891	144/296	synonymous_variant c.432_438delCAGTTATinsAAGCTAC p.LeuSerTyr144LeuSerTyr	LEUM_0698

697054	AAT	CAG	CAG:204 AAT:0	CDS	+	2160/221 7	720/738	missense_variant p.Leu720PheSer	c.2160_2162delAAATinsCAG	LEUM_0723	
700692	ACCC	CCCT	CCCT:206 ACCC:0	CDS	+	378/1527	126/508	synonymous_variant p.GlyPro126GlyPro	c.378_381delAACCCinsCCCT	LEUM_0727	purH
700713	AGCT	TGCC	TGCC:209 AGCT:0	CDS	+	399/1527	133/508	synonymous_variant p.AlaAla133AlaAla	c.399_402delAGCTinsTGCC	LEUM_0727	purH
701025	CGGCAAA	TGGTAAG	TGGTAAG:121 CGGCAAA:0	CDS	+	711/1527	237/508	synonymous_variant c.711_717delCGGCAAAinsTGGTAAG		LEUM_0727	purH
723536	CACTG	TACTC	TACTC:162 CACTG:0	CDS	+	326/534	109/177	missense_variant p.HisGlyLys237HisGlyLys	c.326_330delCACTGinsTACTC	LEUM_0746	
726007	ATAAA	TTTAT	TTTAT:130 ATAAA:0					p.ThrLeu109IleLeu			
745561	ATAAT	GTAAC	GTAAC:87 ATAAT:0								
751089	ACTG	GCTA	GCTA:157 ACTG:0	CDS	+	2232/333 9	744/1112	synonymous_variant c.2232_2235delACTGinsGCTA		LEUM_0774	
769650	GCCA	ACCG	ACCG:139 GCCA:0	CDS	-	27/834	8/277	synonymous_variant p.GluLeu744GluLeu	c.24_27delTTGGCinsCGGT	LEUM_0791	
784937	CCCG	TCCA	TCCA:96 CCCG:0	CDS	-	1608/167 4	535/557	synonymous_variant c.1605_1608delCCGGGinsTGA	p.IleGly535IleGly	LEUM_0807	
787928	AAACG	GAACC	GAACC:132 AAACG:0	CDS	+	1190/170 1	397/566	missense_variant c.1190_1194delAAACGinsGAACC		LEUM_0808	
788232	TATCATC	CATCTTG	CATCTTG:120 TATCATC:0	CDS	+	1494/170 1	498/566	p.GlnThr397ArgThr	c.1494_1500delTATCATCinsCATCTTG	LEUM_0808	
796989	ATTAGGC	GCTGGGT	GCTGGGT:149 ATTAGGC:0					p.ThrIle498ThrIleLeu			
797082	GGGA	TGGG	TGGG:154 GGGA:0								
797274	TAAAA	GAAAC	GAAAC:136 TAAAA:0								
800184	ACAAT	GCAAG	GCAAG:171 ACAAT:0	CDS	+	900/4521	300/1506	missense_variant p.ProGlnSer300ProGlnAla	c.900_904delACAAATinsGCAAG	LEUM_0818	
829273	CATTAT	AAGTAC	AAGTAC:116 CATTAT:0	CDS	+	211/909	71/302	missense_variant c.211_216delCATTATinsAAGTAC		LEUM_0842	
831087	TAGC	CAAT	CAAT:103 TAGC:0	CDS	-	408/897	135/298	p.HisTyr71LysTyr	c.405_408delGCTAinsATTG	LEUM_0844	
								synonymous_variant p.ValLeu135ValLeu			

831917	GAACAGG T	AAACCGGC	AAACCGGC:130 GAACAGGT:0	CDS	+	300/2025	100/674	synonymous_variant c.300_307delGAACAGGTinsAAACCGGC p.GlyAsnArgLeu100GlyAsnArgLeu	LEUM_0845
832789	GAGC	CAGT	CAGT:158 GAGC:0	CDS	+	1172/202 5	391/674	missense_variant c.1172_1175delGAGCinsCAGT p.GlyAla391AlaVal	LEUM_0845
833573	TATGG	CATGA	CATGA:172 TATGG:0	CDS	+	1956/202 5	652/674	missense_variant c.1956_1960delTATGGinsCATGA p.HisMetAla652HisMetThr	LEUM_0845
835366	GCAT	ACAA	ACAA:139 GCAT:0	CDS	+	459/1149	153/382	missense_variant c.459_462delGCATinsACAA p.GlyHis153GlyGln	LEUM_0847
838604	AAGT	GAGC	GAGC:132 AAGT:0	CDS	+	687/729	229/242	synonymous_variant c.687_690delAAGTinsGAGC p.GlySer229GlySer p.GlyTyr62GluHis	LEUM_0849
838832	GGTAC	AGCAT	AGCAT:131 GGTAC:0	CDS	+	185/330	62/109	missense_variant c.185_189delGGTACinsAGCAT p.GlyTyr62GluHis	LEUM_0850
843675	CAGATTA ACG	AAAATCAAA A	AAAATCAAA:133 CAGATTAACG:0	CDS	+	256/1620	86/539	missense_variant c.256_265delCAGATTAACGinsAAAAATCAAAA p.GlnIleAsnAla86LysIleLysThr	LEUM_0854
843731	GAAT	AAAC	AAAC:158 GAAT:0	CDS	+	312/1620	104/539	synonymous_variant c.312_315delGAATinsAAAC p.LysAsn104LysAsn	LEUM_0854
847585	AACA	GACG	GACG:149 AACA:0	CDS	+	660/8466	220/2821	synonymous_variant c.660_663delAACAinsGACG p.ThrThr220ThrThr	LEUM_0857
853659	ATA	GTG	GTG:201 ATA:0	CDS	+	6734/846 6	2245/282 1	missense_variant c.6734_6736delATAinsGTG p.AsnAsn2245SerAsp	LEUM_0857
863407	GTAA	TTGC	TTGC:77 GTAA:0						
870920	TC	TAT	TAT:106 TC:0						
876892	ATAGCTC A	CTAGATCG	CTAGATCG:171 ATAGCTCA:0	CDS	+	367/2223	123/740	missense_variant c.367_374delATAGCTCAinsCTAGATCG p.IleAlaHis123LeuAspArg	LEUM_0882
877704	CGCC	TGCT	TGCT:185 CGCC:0	CDS	+	1179/222 3	393/740	synonymous_variant c.1179_1182delCGCCinsTGCT p.TyrAla393TyrAla	LEUM_0882
880042	ACTAT	TCTAC	TCTAC:151 ACTAT:0	CDS	+	77/1506	26/501	missense_variant c.77_81delACTATinsTCTAC p.AsnTyr26IleTyr	LEUM_0884
883034	ACCACTT	GCCGCTC	GCCGCTC:136 ACCACTT:0	CDS	+	1422/225 3	474/750	missense_variant c.1422_1428delACCACTTinsGCCGCTC p.IleProLeu474MetProLeu	LEUM_0885
883123	GAGA	AAGG	AAGG:126 GAGA:0	CDS	+	1511/225 3	504/750	missense_variant c.1511_1514delGAGinsAAGG p.ArgGlu504LysGly	LEUM_0885
893725	TAA	CAG	CAG:132 TAA:0	CDS	+	1167/225	389/752	missense_variant c.1167_1169delTAAinsCAG p.Alalys389AlaArg	LEUM_0894

894794	AAA	GAG		GAG:173 AAA:0	CDS	+	9	746/752	missense_variant p.Lys746Glu	c.2236_2238delIAAAinsGAG	LEUM_0894
895508	CAAG	TAAA		TAAA:112 CAAG:0	CDS	+	675/687	225/228	synonymous_variant p.IleLys225IleLys	c.675_678delCAAGinsTAAA	LEUM_0895
895583	ATTAAAGC G	GTC AAGTT		GTC AAGTT:92 ATTAAGCG:0	CDS	-	996/1008	330/335	missense_variant c.989_996delCGCTTAATinsAACTTGAC		LEUM_0896
895607	CGGT	TGGG		TGGG:101 CGGT:0	CDS	-	972/1008	323/335	p.ThrLeuAsn330LysLeuAsp synonymous_variant	c.969_972delACCGinsCCCA p.ValPro323ValPro	LEUM_0896
903892	CTTTGCCT T	TTTTACCTC		TTTTACCTC:158 CTTTGCCTT:0	CDS	+	1215/183 9	405/612	missense_variant c.1215_1223delCTTTGCCTTinsTTTACCTC		LEUM_0901
907285	GCTAC	ACTAT		ACTAT:127 GCTAC:0							
911930	CAGC	TAGT		TAGT:94 CAGC:0	CDS	+	39/822	13/273	synonymous_variant p.SerSer13SerSer	c.39_42delCAGCinsTAGT	LEUM_0909
933210	CAGGGC	GAGCGT		GAGCGT:156 CAGGGC:0	CDS	+	1909/199 2	637/663	missense_variant c.1909_1914delCAGGGCinsGAGCGT		LEUM_0929
945839	TAG	TAAA		TAAA:60 TAG:0							
945853	GAT	AAC		AAC:61 GAT:0							
972869	CATT	TATC		TATC:142 CATT:0	CDS	+	168/480	56/159	synonymous_variant p.HisIle56HisIle	c.168_171delCATTinsTATC	LEUM_0972
980203	TTAGTA	CTGGTG		CTGGTG:85 TTAGTA:0	CDS	+	220/513	74/170	synonymous_variant c.220_225delTTAGTAinsCTGGTG		LEUM_0980
980531	TCATTA	CAATTG		CAATTG:125 TCATTA:0							
982914	AGCT	GGCA		GGCA:58 AGCT:0	CDS	+			no annotation		LEUM_0984
986252	GGTCC	TGTCT		TGTCT:31 GGTCC:0	CDS	+			no annotation		LEUM_0987
986279	CGAAACG CTCATTC	TGAGACACT AATTA		TGAGACACTAATTA: 30 CGAAACGCTCATTC: 0	CDS	+			no annotation		LEUM_0987
986308	GGTC	AGAT		AGAT:30 GGTC:0							
986319	ATT	GTC		GTC:31 ATT:0	CDS	+			no annotation		LEUM_0988
986356	CGTT	TGTG		TGTG:30 CGTT:0	CDS	+			no annotation		LEUM_0988
986375	GTTTCAG	ATGTCGGA		ATGTCGGAAGAG:2	CDS	+			no annotation		LEUM_0988

	AAAAA	AGAG	5 GTTTCAGAAAAA:0							
1008480	CAAG	TAAA	TAAA:14 CAAG:0	CDS	+				no annotation	LEUM_1008
1008786	CCTG	TCTA	TCTA:1619 CCTG:0	CDS	+				no annotation	LEUM_1009
1008954	ACCC	GCCA	GCCA:1877 ACCC:0	CDS	+				no annotation	LEUM_1009
1022214	TTTG	ATTA	ATTA:76 TTTG:0							
1135118	TGG	CGA	CGA:83 TGG:0							
1135159	TCGT	CCGC	CCGC:83 TCGT:0							
1135269	TTAC	CTAT	CTAT:123 TTAC:0	CDS	+				no annotation	LEUM_1138
1138281	GTTT	ATTC	ATTC:201 GTTT:0	CDS	-				no annotation	LEUM_1142
1139585	CAACC	TAACT	TAACT:197 CAACC:0	CDS	-				no annotation	LEUM_1143
1155368	AGCG	GGCA	GGCA:141 AGCG:0	CDS	-				no annotation	LEUM_1157
1157871	ATTT	GTTG	GTTG:155 ATTT:0	CDS	-				no annotation	LEUM_1161
1169465	GTCG	TTCT	TTCT:178 GTCG:0	CDS	-				no annotation	LEUM_1172
1170652	GCG	TCA	TCA:135 GCG:0	CDS	-				no annotation	LEUM_1173
1170669	TATC	CATT	CATT:124 TATC:0	CDS	-				no annotation	LEUM_1173
1170980	TTTA	CTCG	CTCG:123 TTTA:0	CDS	-				no annotation	LEUM_1174
1174201	GAC	AAT	AAT:87 GAC:0							
1174261	CGTG	AGTA	AGTA:130 CGTG:0	CDS	-				no annotation	LEUM_1177
1183816	GGTA	AGTG	AGTG:139 GGTA:0	CDS	-				no annotation	LEUM_1187
1194019	GCAAT	ACAAC	ACAAC:139 GCAAT:0	CDS	-				no annotation	LEUM_1195
1238393	GGCAGG	AGTAGA	AGTAGA:81 GGCAGG:0							
1238441	TAAT	GATA	GATA:47 TAAT:0							
1258437	CTT	TTG	TTG:43 CTT:0							
1263043	TGGG	CGGA	CGGA:194 TGGG:0	CDS	+				no annotation	LEUM_1275
1267583	TGGGCAG	GGGTCAA	GGGTCAA:131 TGGGCAG:0	CDS	+				no annotation	LEUM_1279
1289296	TCTC	CCTT	CCTT:197 TCTC:0	CDS	-				no annotation	LEUM_1302
1294486	ACAA	GCA	GCA:189 ACAA:0							
1296449	CAGCTGT A	TATCCGTG	TATCCGTG:188 CAGCTGTGA:0	CDS	-				no annotation	LEUM_1309 aspS
1302442	TCCG	ACCA	ACCA:161 TCCG:0	CDS	-				no annotation	LEUM_1314
1303222	AGTA	GGTG	GGTG:220 AGTA:0	CDS	-				no annotation	LEUM_1314
1306063	TACC	GACA	GACA:193 TACC:0	CDS	-				no annotation	LEUM_1316 lacZ

1319719	TACAGCA A	CACATCAC	CACATCAC:135 TACAGCAA:0						
1319558	ATTTAAGT TCAGTCA CA	CTACAATAT CACTTCCC	CTACAATATCACATT C:109 ATTTAAGTTCAGTCA CA:0						
1319611	ACGTCT	CCGTTTC	CCGTTTC:146 ACGTC:0						
1319951	ACGC	GGGT	GGGT:150 ACGC:0	CDS	+			no annotation	LEUM_1334
1345228	ACTTG	GCTTA	GCTTA:204 ACTTG:0	CDS	-			no annotation	LEUM_1363
1346846	TGGG	CGGA	CGGA:191 TGGG:0	CDS	-			no annotation	LEUM_1363
1392214	TAAA	AAGC	AAGC:157 TAAA:0	CDS	-			no annotation	LEUM_1404
1396399	CGC	TGT	TGT:177 CGC:0	CDS	-			no annotation	LEUM_1408
1407216	TGA	AGC	AGC:120 TGA:0	CDS	-			no annotation	LEUM_1412
1407234	TGTTAGT	AGCTAAC	AGCTAAC:94 TGTTAGT:0	CDS	-			no annotation	LEUM_1412
1407252	AATG	GATA	GATA:112 AATG:0	CDS	-			no annotation	LEUM_1412
1410440	GCTT	ACTC	ACTC:158 GCTT:0	CDS	-			no annotation	LEUM_1415
1410471	CTT	ATC	ATC:162 CTT:0	CDS	-			no annotation	LEUM_1415
1415069	TTTC	CTTA	CTTA:140 TTTC:0	CDS	-			no annotation	LEUM_1420
1415084	CACT	AACA	AACA:142 CACT:0	CDS	-			no annotation	LEUM_1420
1415294	AAGT	TAGC	TAGC:163 AAGT:0	CDS	-			no annotation	LEUM_1420
1415654	GTAC	ATAA	ATAA:203 GTAC:0	CDS	-			no annotation	LEUM_1420
1415711	AGCT	CGCC	CGCC:184 AGCT:0	CDS	-			no annotation	LEUM_1420
1415881	AAC	GAA	GAA:192 AAC:0						
1416065	GCCT	TCCA	TCCA:207 GCCT:0	CDS	-			no annotation	LEUM_1421
1416263	GTTT	ATTA	ATTA:191 GTTT:0	CDS	-			no annotation	LEUM_1421
1416317	GATG	AATA	AATA:199 GATG:0	CDS	-			no annotation	LEUM_1421
1416380	CAAA	TAAG	TAAG:211 CAAA:0	CDS	-			no annotation	LEUM_1421
1416695	TGTT	GGTC	GGTC:168 TGTT:0	CDS	-			no annotation	LEUM_1421
1417341	ATTG	GTTA	GTTA:195 ATTG:0	CDS	-			no annotation	LEUM_1422
1417434	ATTA	GTTG	GTTG:217 ATTA:0	CDS	-			no annotation	LEUM_1422
1417596	CAG	TAA	TAA:222 CAG:0	CDS	-			no annotation	LEUM_1423
1417722	AAGGAGA	GAGAAGT	GAGAAGT:134 AAGGAGA:0	CDS	-			no annotation	LEUM_1423

1417734	CAACGTT	GTGTGTC	GTGTGTC:128 CAACGTT:0	CDS	-				no annotation	LEUM_1423	
1417782	GTCT	ATCC	ATCC:185 GTCT:0	CDS	-				no annotation	LEUM_1423	
1417965	CTTGTC	TTTATCG	TTTATCG:206 CTTGTC:0	CDS	-				no annotation	LEUM_1423	
1418013	GCCA	ACCG	ACCG:208 GCCA:0	CDS	-				no annotation	LEUM_1423	
1418025	GCGG	AGCA	AGCA:180 GCGG:0	CDS	-				no annotation	LEUM_1423	
1418040	TAAAGCC	CAGAGCAG	CAGAGCAGCTTC:88	CDS	-				no annotation	LEUM_1423	
1418061	TCCTG	CTTC	TAAAGCCTCTTG:0	CDS	-				no annotation	LEUM_1423	
1418069	TTG	CTC	CTC:91 TTG:0	CDS	-				no annotation	LEUM_1423	
1418069	GACCGGC A	ACCCTGCG	ACCCTGCG:89 GACCGGCA:0	CDS	-				no annotation	LEUM_1423	
1418094	TCCC	ACCT	ACCT:100 TCCC:0	CDS	-				no annotation	LEUM_1423	
1418103	TAAG	CAGA	CAGA:87 TAAG:0	CDS	-				no annotation	LEUM_1423	
1418148	GCGG	TGCA	TGCA:197 GCGG:0	CDS	-				no annotation	LEUM_1423	
1418160	GCCA	ACCG	ACCG:194 GCCA:0	CDS	-				no annotation	LEUM_1423	
1418193	GTGCAA	ATTAG	ATTAG:162 GTGCAA:0	CDS	-				no annotation	LEUM_1423	
1418208	ATGG	CTGA	CTGA:175 ATGG:0	CDS	-				no annotation	LEUM_1423	
1418271	TTTT	ATCC	ATCC:170 TTTT:0	CDS	-				no annotation	LEUM_1423	
1418322	TTTA	CTTG	CTTG:167 TTTA:0	CDS	-				no annotation	LEUM_1423	
1418385	AGAG	GGAA	GGAA:118 AGAG:0	CDS	-				no annotation	LEUM_1423	
1418582	ACC	GCT	GCT:210 ACC:0	CDS	-				no annotation	LEUM_1424	
1418878	TGCCTCG	AGTCTCA	AGTCTCA:149 TGCCTCG:0	CDS	-				no annotation	LEUM_1424	
1418950	ACTC	GCTT	GCTT:163 ACTC:0	CDS	-				no annotation	LEUM_1424	
1419097	CCTA	TCTG	TCTG:175 CCTA:0	CDS	-				no annotation	LEUM_1424	
1419197	GTGCT	TGCG	TGCG:208 GTGCT:0	CDS	-				no annotation	LEUM_1424	
1419226	GTTA	ATTG	ATTG:221 GTTA:0	CDS	-				no annotation	LEUM_1424	
1419311	TCG	GCC	GCC:230 TCG:0	CDS	-				no annotation	LEUM_1424	
1419388	GCTT	ACTG	ACTG:223 GCTT:0	CDS	-				no annotation	LEUM_1424	
1419438	TTTAG	GTG	GTG:162 TTTAG:0	CDS	-				no annotation	LEUM_1424	
1429917	TGGCTCC TCTATTG TCTTT	AGGCACCTT TAGTCGTTT TA	AGGCACCTTTAGTC GTTTAA:173 TGGCTCTCTATTG	CDS	-				no annotation	LEUM_1434	

1429993	TGTG	CGTA	TCCTT:0	CDS	-				no annotation	LEUM_1434
1430085	AGAGT	GGAGC	CGTA:204 TGTG:0 GGAGC:169 AGAGT:0	CDS	-				no annotation	LEUM_1434
1430128	GTTG	ATTA	ATTA:172 GTTG:0	CDS	-				no annotation	LEUM_1434
1430143	AGACGTG	GGCTGTA	GGCTGTA:153 AGACGTG:0	CDS	-				no annotation	LEUM_1434
1430176	CTCT	TTCA	TTCA:177 CTCT:0	CDS	-				no annotation	LEUM_1434
1430203	CCCG	TCCA	TCCA:186 CCCG:0	CDS	-				no annotation	LEUM_1434
1430314	AGCTGTG ACC	GGCAGTCA CT	GGCAGTCACT:192 AGCTGTGACC:0	CDS	-				no annotation	LEUM_1434
1430344	CAAC	TAAG	TAAG:206 CAAC:0	CDS	-				no annotation	LEUM_1434
1430374	TTCG	CTCA	CTCA:216 TTCG:0	CDS	-				no annotation	LEUM_1434
1430413	TAAA	CAAG	CAAG:214 TAAA:0	CDS	-				no annotation	LEUM_1434
1430623	CTCT	TTCA	TTCA:192 CTCT:0	CDS	-				no annotation	LEUM_1435
1430785	AACCAAT CCT	TACAAAACC A	TACAAAACCA:159 AACCAATCCT:0	CDS	-				no annotation	LEUM_1435
1430806	CAA	TAG	TAG:183 CAA:0	CDS	-				no annotation	LEUM_1435
1430942	TTAGAAT C	GTAGGATT	GTAGGATT:180 TTAGAATC:0	CDS	-				no annotation	LEUM_1435
1431011	CTTTT	TCCTTC	TCCTTC:161 CTTTT:0	CDS	-				no annotation	LEUM_1435
1431073	CTTA	TTTT	TTTT:160 CTTA:0	CDS	-				no annotation	LEUM_1435
1431088	CAGA	TAGG	TAGG:142 CAGA:0	CDS	-				no annotation	LEUM_1435
1431356	AAC	TAT	TAT:129 AAC:0	CDS	-				no annotation	LEUM_1435
1431525	TTT	CTC	CTC:143 TTT:0	CDS	-				no annotation	LEUM_1436
1431755	CACC	TACT	TACT:154 CACC:0	CDS	-				no annotation	LEUM_1436
1431803	CGTA	TGTG	TGTG:139 CGTA:0	CDS	-				no annotation	LEUM_1436
1432287	GCAAA	ACAAAT	ACAAAT:162 GCAAA:0							
1432326	AAAC	TACT	TACT:140 AAAC:0							
1432336	TAAAA	GAAAG	GAAAG:143 TAAAA:0							
1432349	TATG	CATA	CATA:141 TATG:0	CDS	-				no annotation	LEUM_1437
1432378	CTGA	TTGG	TTGG:207 CTGA:0	CDS	-				no annotation	LEUM_1437

1432717	AAT	CAC	CAC:213 AAT:0	CDS	-				no annotation	LEUM_1437	
1433379	CCA	GCG	GCG:209 CCA:0	CDS	-				no annotation	LEUM_1438	
1433417	GGACTTA	AGATTTG	AGATTTG:205 GGACTTA:0	CDS	-				no annotation	LEUM_1438	
1433441	CACA	TACG	TACG:222 CACA:0	CDS	-				no annotation	LEUM_1438	
1433984	CGTG	TGTA	TGTA:206 CGTG:0	CDS	-				no annotation	LEUM_1438	
1436006	AAAG	GAAA	GAAA:254 AAAG:0	CDS	-				no annotation	LEUM_1440	
1436796	CAA	TAC	TAC:92 CAA:0								
1437736	CAAA	TAAG	TAAG:245 CAAA:0	CDS	-				no annotation	LEUM_1443	
1437751	CTTA	TTTG	TTTG:249 CTTA:0	CDS	-				no annotation	LEUM_1443	
1441725	CGCTT	TGCTTT	TGCTTT:165 CGCTT:0								
1444575	CAAAAAA AAAAAAA C	CAAAAAA ACAAAC	CAAAAAA:127 CAAAAAA:127 CAAAAAA:127 C:0								
1447932	AAAC	GAAT	GAAT:203 AAAC:0	CDS	-				no annotation	LEUM_1454	
1474016	TTAAC	CTAAT	CTAAT:171 TTAAC:0	CDS	-				no annotation	LEUM_1480	
1475011	TAGT	CAGC	CAGC:175 TAGT:0	CDS	-				no annotation	LEUM_1481	
1475048	TGTG	CGTT	CGTT:194 TGTG:0	CDS	-				no annotation	LEUM_1481	
1475219	TTGT	CTGC	CTGC:188 TTGT:0	CDS	-				no annotation	LEUM_1481	
1477474	TTAAC	CTAAA	CTAAA:148 TTAAC:0	CDS	-				no annotation	LEUM_1481	
1501570	AGATC	GCATG	GCATG:145 AGATC:0	CDS	-				no annotation	LEUM_1502	
1501590	ACA	GCG	GCG:140 ACA:0	CDS	-				no annotation	LEUM_1502	
1510576	TAAT	CAAA	CAAA:199 TAAT:0	CDS	-				no annotation	LEUM_1513	
1518189	AGGC	GGGT	GGGT:152 AGGC:0	CDS	-				no annotation	LEUM_1520	engB
1519140	AGCA	GGCT	GGCT:222 AGCA:0	CDS	-				no annotation	LEUM_1521	clpX
1519209	GGAG	AGAT	AGAT:236 GGAG:0	CDS	-				no annotation	LEUM_1521	clpX
1527336	GTCC	ATCT	ATCT:171 GTCC:0	CDS	-				no annotation	LEUM_1529	
1539200	GAAA	AAAG	AAAG:234 GAAA:0	CDS	-				no annotation	LEUM_1539	
1548015	CAAACT	AGAACA	AGAACA:112 CAAACT:0	CDS	+				no annotation	LEUM_1546	
1553910	AATT	GATA	GATA:154 AATT:0	CDS	-				no annotation	LEUM_1554	
1563023	ATAG	TTAA	TTAA:147 ATAG:0								

1563156	CCCC	TCCT	TCCT:161 CCCC:0	CDS	-				no annotation	LEUM_1564
1563399	ACCG	GCCC	GCCC:202 ACCG:0	CDS	-				no annotation	LEUM_1564
1570912	GGGA	AGGG	AGGG:201 GGGA:0	CDS	-				no annotation	LEUM_1569
1575438	GCAAA	ACAAG	ACAAG:118 GCAAA:0							
1576436	TTCT	CTCC	CTCC:188 TTCT:0	CDS	-				no annotation	LEUM_1575
1576450	GTATA	ATATC	ATATC:188 GTATA:0	CDS	-				no annotation	LEUM_1575
1576582	CCTC	ACTT	ACTT:201 CCTC:0	CDS	-				no annotation	LEUM_1575
1582261	CACA	GACG	GACG:210 CACA:0	CDS	-				no annotation	LEUM_1578
1582441	TACTGCA	CACCGCG	CACCGCG:178 TACTGCA:0	CDS	-				no annotation	LEUM_1578
1589522	ACTGC	GCCGT	GCCGT:119 ACTGC:0	CDS	-				no annotation	LEUM_1586
1622472	TTATAT	ACGTAC	ACGTAC:247 TTATAT:0	CDS	-				no annotation	LEUM_1624
1624045	AGCCTAC	GCCCGAT	GCCCGAT:111 AGCCTAC:0	CDS	-				no annotation	LEUM_1627
1624058	CAAG	GAGA	GAGA:110 CAAG:0	CDS	-				no annotation	LEUM_1627
1624079	TATT	AATCA	AATCA:164 TATT:0							
1624096	ATTA	GTTG	GTTG:184 ATTA:0							
1624117	TAG	CAA	CAA:203 TAG:0							
1624234	GCCGCCA	ACCACCG	ACCACCG:231 GCCGCCA:0	CDS	-				no annotation	LEUM_1628
1624336	TTGA	CTGG	CTGG:149 TTGA:0	CDS	-				no annotation	LEUM_1628
1624351	ATTACCA	GTTCCCG	GTTCCCG:149 ATTACCA:0	CDS	-				no annotation	LEUM_1628
1624431	TGTTG	AGTTA	AGTTA:98 TGTTG:0	CDS	-				no annotation	LEUM_1628
1624459	CTTA	TTGT	TTGT:84 CTTA:0	CDS	-				no annotation	LEUM_1628
1624574	TTG	GTA	GTA:149 TTG:0	CDS	-				no annotation	LEUM_1628
1624609	GCCG	TCCA	TCCA:180 GCCG:0	CDS	-				no annotation	LEUM_1628
1624618	TCCG	GCCA	GCCA:193 TCCG:0	CDS	-				no annotation	LEUM_1628
1624654	GTTGGAA	ATTTGAG	ATTTGAG:220 GTTGGAA:0	CDS	-				no annotation	LEUM_1628
1624720	TAA	CAT	CAT:230 TAA:0	CDS	-				no annotation	LEUM_1628
1624729	AGCG	GGCA	GGCA:229 AGCG:0	CDS	-				no annotation	LEUM_1628

1624843	TAG	CAA	CAA:250 TAG:0	CDS	-			no annotation	LEUM_1628	
1624858	ATTA	GTTG	GTTG:243 ATTA:0	CDS	-			no annotation	LEUM_1628	
1624900	TGCG	AGCA	AGCA:250 TGCG:0	CDS	-			no annotation	LEUM_1628	
1624918	GGCTAGC	AGCCAGT	AGCCAGT:239 GGCTAGC:0	CDS	-			no annotation	LEUM_1628	
1624978	CACCGAG	GACTGAA	GACTGAA:222 CACCGAG:0	CDS	-			no annotation	LEUM_1628	
1625140	AAACGAA	GAATGAG	GAATGAG:202 AAACGAA:0	CDS	-			no annotation	LEUM_1628	
1625152	ATAATTG C	GTAGCTTGT	GTAGCTTGT:206 ATAATTGTC:0	CDS	-			no annotation	LEUM_1628	
1625209	CACG	TACA	TACA:233 CACG:0	CDS	-			no annotation	LEUM_1628	
1629235	GATG	TATA	TATA:176 GATG:0	CDS	-			no annotation	LEUM_1635	
1629250	ATTA	GTTG	GTTG:180 ATTA:0	CDS	-			no annotation	LEUM_1635	
1629328	TGTGTT AAAGAT	CATATTTAG AGAC	CATATTTAGAGAC:1 59 TGTGTTCAAAGAT:0	CDS	-			no annotation	LEUM_1635	
1629619	TAATGCG	CAGTGCA	CAGTGCA:203 TAATGCG:0	CDS	-			no annotation	LEUM_1635	
1629658	TATC	GATT	GATT:223 TATC:0	CDS	-			no annotation	LEUM_1635	
1629722	ACACCTG	TCTGCTAA	TCTGCTAA:130 ACACCTG:0	CDS	-			no annotation	LEUM_1635	
1629759	ATGA	GTGC	GTGC:191 ATGA:0	CDS	-			no annotation	LEUM_1635	
1650708	TAAC	AAAT	AAAT:59 TAAC:0	CDS	-			no annotation	LEUM_1656	
1650750	AGGAATC GTTC	ATAGATTGG CTCG	ATAGATTGGCTCG:3 5 AGGAATCGTTCA:0							
1650948	ACGCATT	GCGCCTC	GCGCCTC:199 ACGCATT:0	CDS	-			no annotation	LEUM_1657	
1651008	ATTG	GTTA	GTTA:221 ATTG:0	CDS	-			no annotation	LEUM_1657	
1651041	TAT	CAC	CAC:223 TAT:0	CDS	-			no annotation	LEUM_1657	
1651098	ATA	GTC	GTC:188 ATA:0							
1651117	GTGCA	GATA	GATA:133 GTGCA:0							
1651140	GCCA	ACCG	ACCG:210 GCCA:0							
1651201	TTCC	CTCT	CTCT:224 TTCC:0	CDS	-			no annotation	LEUM_1658	
1656232	GCCT	ACCC	ACCC:197 GCCT:0	CDS	-			no annotation	LEUM_1671	

1661069	CACT	AACC	AACC:262 CACT:0	CDS	-				no annotation	LEUM_1680	
1665094	TTTAAAC CGTCA	CTTCAAATC ATCG	CTTCAAATCATCG:1 64 TTTAAACCGTCA:0	CDS	+				no annotation	LEUM_1690	
1665117	CTTCC	ATTCA	ATTCA:176 CTTCC:0	CDS	+				no annotation	LEUM_1690	
1665274	GTACGGC	ATATGGG	ATATGGG:200 GTACGGC:0	CDS	+				no annotation	LEUM_1690	
1665286	CCAC	TCAT	TCAT:208 CCAC:0	CDS	+				no annotation	LEUM_1690	
1665328	CGGA	TGGC	TGGC:200 CGGA:0	CDS	+				no annotation	LEUM_1690	
1665337	GAAAGAC GCT	AAAGGATG CC	AAAGGATGCC:196 GAAAGACGCT:0	CDS	+				no annotation	LEUM_1690	
1665424	GAAA	AAAG	AAAG:171 GAAA:0	CDS	+				no annotation	LEUM_1690	
1665436	GTATG	ATACA	ATACA:144 GTATG:0	CDS	+				no annotation	LEUM_1690	
1665448	CAAGCGC	TAAAGT	TAAAGT:139 CAAGCGC:0	CDS	+				no annotation	LEUM_1690	
1665484	ACCTACC	GCCAAT	GCCAAT:153 ACCTACC:0	CDS	+				no annotation	LEUM_1690	
1665529	TTTA	ATTG	ATTG:168 TTTA:0	CDS	+				no annotation	LEUM_1690	
1665572	AGAAC	GGAAT	GGAAT:198 AGAAC:0	CDS	+				no annotation	LEUM_1690	
1665664	GGG	AGA	AGA:206 GGG:0	CDS	+				no annotation	LEUM_1690	
1665752	TTACAA	CTGCAG	CTGCAG:201 TTACAA:0	CDS	+				no annotation	LEUM_1690	
1665790	GATTACT	AATAACA	AATAACA:195 GATTACT:0	CDS	+				no annotation	LEUM_1690	
1665814	TAGT	CAGC	CAGC:202 TAGT:0	CDS	+				no annotation	LEUM_1690	
1666025	TTAT	ATAC	ATAC:134 TTAT:0								
1667151	TAAAAAA T	TAAAAAA G	TAAAAAAAG:78 TAAAAAAT:0								
1669413	AAACA	GAACG	GAACG:158 AAACA:0	CDS	+				no annotation	LEUM_1695	
1670484	ACCT	TCCC	TCCC:177 ACCT:0	CDS	-				no annotation	LEUM_1696	
1672983	ACTGG	GCTGT	GCTGT:189 ACTGG:0	CDS	+				no annotation	LEUM_1698	

1684163	GTCTC	ATCIT	ATCT:153 GTCTC:0	CDS	-					no annotation	LEUM_1726	
1695377	ACCG	GCCA	GCCA:273 ACCG:0	CDS	-					no annotation	LEUM_1726	
1696196	GGCCGCT AGCATG	TGCAGCCAA CATA	TGCAGCCAA 89 GGCCGCTAGCATG:0	CDS	-					no annotation	LEUM_1726	
1696244	TCGCAA	CCGTAG	CCGTAG:215 TCGCAA:0	CDS	-					no annotation	LEUM_1726	
1716146	TAATT	CAATC	CAATC:45 TAATT:0		-					no annotation	LEUM_1748	
1717930	ATCA	GTCT	GTCT:47 ATCA:0	CDS	-					no annotation	LEUM_1748	
1717975	ATCGATG	GTCTATA	GTCTATA:22 ATCGATG:0	CDS	-					no annotation	LEUM_1748	
1718317	ATCG	GTCT	GTCT:10 ATCG:0	CDS	-					no annotation	LEUM_1748	
1718353	ATT	GTTC	GTTC:22 ATT:0	CDS	-					no annotation	LEUM_1748	
1719685	GGA	AGG	AGG:289 GGA:2	CDS	-					no annotation	LEUM_1752	
1725927	TAGCC	CAGCT	CAGCT:186 TAGCC:1	CDS	-					no annotation	LEUM_1752	
1726130	GCTA	TCTG	TCTG:43 GCTA:0	CDS	-					no annotation	LEUM_1752	
1726179	TATCC	CAGCT	CAGCT:65 TATCC:0	CDS	-					no annotation	LEUM_1752	
1726202	GCTA	TCTG	TCTG:90 GCTA:0	CDS	-					no annotation	LEUM_1752	
1726215	TAGCC	CAGCT	CAGCT:95 TAGCC:0	CDS	-					no annotation	LEUM_1752	
1726251	CAGCT	TAGCC	TAGCC:143 CAGCT:2	CDS	-					no annotation	LEUM_1752	
1756654	TCTAC	GCTAT	GCTAT:128 TCTAC:0		-					no annotation	LEUM_1786	
1756824	ATC	GTA	GTA:145 ATC:0	CDS	-					no annotation	LEUM_1786	
1757247	GAAA	AAAG	AAAG:196 GAAA:0	CDS	-					no annotation	LEUM_1788	
1759552	TACT	CACC	CACC:256 TACT:0	CDS	+					no annotation	LEUM_1788	
1759606	GGCG	AGCA	AGCA:266 GGCG:0	CDS	+					no annotation	LEUM_1788	
1760925	ACCCGAT GGGTTGT ATT	GCCACTAG GCTGCAT	GCCACTAGGCTGCA T:37 ACCCGATGGTTGT ATT:0									
1760955	CAATGA	TAAGTGG	TAAGTGG:35 CAATGA:0	CDS	-					no annotation	LEUM_1791	
1760994	GGCTGCA AACGCTG	AGCAGCGA AAGCAGCG	AGCAGCGAAAGCAG CGCGTAAACGGAAGT	CDS	-					no annotation	LEUM_1791	

1761057	CACGAG GCGCAGC	CGTAAACG AAGT	:37 GGCTGCAACGCTG CACGAGGCGCAGC :0	CDS	-				no annotation	LEUM_1791	
1761069	CTGGGGT ATCAAAA CGGTAC A	TTTGGT TTGTGGAAT TAATACTGT CACT	TTTGGT:167 CTTGGGG:0 TTGTGGAATTAATAC TGTCAC:168 CTGGGTATCAAAA CGGTACA:0	CDS	-				no annotation	LEUM_1791	
1761096	GTGA	ATTG	ATTG:166 GTTA:0	CDS	-				no annotation	LEUM_1791	
1761107	CTGCTG C	TTGCTTGT	TTGCTTGT:173 CTGCTGC:0	CDS	-				no annotation	LEUM_1791	
1764663	TTC	CTG	CTG:125 TTC:0	CDS	+				no annotation	LEUM_1793	
1766295	TAA	CAG	CAG:302 TAA:0	CDS	-				no annotation	LEUM_1794	
1776537	CGA	AGC	AGC:191 CGA:0	CDS	-				no annotation	LEUM_1803	
1790033	CTGT	TTGC	TTGC:198 CTGT:0	CDS	-				no annotation	LEUM_1817	
1824412	CAA	AAG	AAG:178 CAA:0	CDS	-				no annotation	LEUM_1850	
1830003	GAGA	AAGG	AAGG:208 GAGA:0								
1842065	ACCA	GCCC	GCCC:231 ACCA:0	CDS	-				no annotation	LEUM_1868	atpC
1857246	ATTACCTT TGATAAC	GTTATCAAA GGTAAT	GTTATCAAAAGGTAA T:71 ATTACCTTTGATAAC :0								
1860337	AGA	GGG	GGG:145 AGA:0	CDS	-				no annotation	LEUM_1886	
1861225	CTTTGCA	TTTACG	TTTACG:221 CTTTGCA:0	CDS	-				no annotation	LEUM_1888	
1875169	ATT	GTC	GTC:252 ATT:0	CDS	-				no annotation	LEUM_1900	
1878574	ACG	AA	AA:157 ACG:1								
1878900	GCAAGT	ATAAGC	ATAAGC:121 GCAAGT:0	CDS	+				no annotation	LEUM_1905	
1878918	GTG	TTT	TTT:121 GTG:0	CDS	+				no annotation	LEUM_1905	
1878926	CTTT	TTTC	TTTC:114 CTTT:0	CDS	+				no annotation	LEUM_1905	
1878938	ATAGA	GTAAT	GTAAT:113 ATAGA:0	CDS	+				no annotation	LEUM_1905	
1878945	TCCC	GACG	GACG:112 TCCC:0								

1878959	GTAT	TTAA	TTAA:139 GTAT:0						
1879309	CCTAGCC A	TCTGGCCT	TCTGGCCT:176 CCTAGCCA:0						
1882947	AGTAGT	GGTTGC	GGTTGC:244 AGTAGT:0						
1882969	TACAT	GACAC	GACAC:243 TACAT:0						
1886783	CCAATCA	TCGATCG	TCGATCG:207 CCAATCA:0	CDS	+			no annotation	LEUM_1917
1887546	TAGG	CAAA	CAAA:137 TAGG:0	CDS	-			no annotation	LEUM_1919
1887555	ACGTGTT	TCGCGTA	TCGCGTA:147 ACGTGTT:0	CDS	-			no annotation	LEUM_1919
1887567	CAATGAA CCG	TAGAGAGC CA	TAGAGAGCCA:147 CAATGAACCG:0	CDS	-			no annotation	LEUM_1919
1887582	TTCA	CTCG	CTCG:153 TTCA:0	CDS	-			no annotation	LEUM_1919
1887645	GGCT	AGCC	AGCC:249 GGCT:0	CDS	-			no annotation	LEUM_1919
1887654	CTTG	TTTA	TTTA:252 CTTG:0	CDS	-			no annotation	LEUM_1919
1887666	ACGAAGC	GCGCAAT	GCGCAAT:172 ACGAAGC:0	CDS	-			no annotation	LEUM_1919
1887684	CTGG	TTGT	TTGT:196 CTGG:0	CDS	-			no annotation	LEUM_1919
1887711	TGTCACT GA	AGTTACCTG G	AGTTACCTGG:239 TGTCACTTGA:0	CDS	-			no annotation	LEUM_1919
1887732	GCCG	ACCA	ACCA:275 GCCG:0	CDS	-			no annotation	LEUM_1919
1887771	CTTC	TTTT	TTTT:299 CTTC:0	CDS	-			no annotation	LEUM_1919
1887795	CGCTCCA	TGCACCG	TGCACCG:316 CGCTCCA:0	CDS	-			no annotation	LEUM_1919
1887821	ATTTA	GCTTG	GCTTG:277 ATTTA:0	CDS	-			no annotation	LEUM_1919
1887831	GTTTCCA	ATTACCG	ATTACCG:281 GTTTCCA:0	CDS	-			no annotation	LEUM_1919
1887852	GTGA	ATGT	ATGT:312 GTGA:0	CDS	-			no annotation	LEUM_1919
1887867	ACTG	GCTA	GCTA:324 ACTG:0	CDS	-			no annotation	LEUM_1919
1887897	TAG	CAA	CAA:307 TAG:0	CDS	-			no annotation	LEUM_1919
1887906	AGCA	GGCG	GGCG:305 AGCA:0	CDS	-			no annotation	LEUM_1919
1896684	TCAGC	CCAGA	CCAGA:220 TCAGC:0	CDS	-			no annotation	LEUM_1927

1897538	GCGC	ACGT	ACGT:286 GCGC:0	CDS	-				no annotation	LEUM_1928
1915818	AGTT	GGTC	GGTC:305 AGTT:0	CDS	-				no annotation	LEUM_1944
1917475	TTA	CTC	CTC:134 TTA:0	CDS	-				no annotation	LEUM_1945
1933246	TCA	CCG	CCG:225 TCA:0	CDS	+				no annotation	LEUM_1960
1933618	CATT	TATA	TATA:200 CATT:0	CDS	+				no annotation	LEUM_1960
1933723	GCCCA	TCCCG	TCCCG:175 GCCCA:0	CDS	+				no annotation	LEUM_1960
1933941	GTCT	ATT	ATT:134 GTCT:0							
1934018	ATATTAC	TGTTAT	TGTTAT:133 ATATTAC:0							
1934029	ACAA	GTAT	GTAT:135 ACAA:0							
1934072	GTA	ATA	ATA:142 GTAA:0							
1934080	ATGTGGC	GTGTTGT	GTGTTGT:142 ATGTGGC:0							
1952692	GAATA	TAATG	TAATG:97 GAATA:0							
1952721	GAAG	AAAT	AAAT:82 GAAG:0							
1952732	GTGTT	TCGTC	TCGTC:78 GTGTT:0							
1953810	CGGTG	TTGTA	TTGTA:462 CGGTG:0							
1960043	CAATT	TAATC	TAATC:36 CAATT:0							
1960073	TTTGGG	AAGGGA	AAGGGA:39 TTTGGG:0							
1960134	TGTGTTA AATAC	AGTGCTATA TTT	AGTGCTATA:34 TGTGTTAATAC:0	CDS	-				no annotation	LEUM_1991
1960163	GTCA	ATCT	ATCT:36 GTCA:0	CDS	-				no annotation	LEUM_1991
1960179	ATTGC	CTTAA	CTTAA:39 ATTGC:0	CDS	-				no annotation	LEUM_1991
1960376	TGCT	AGCA	AGCA:107 TGCT:0	CDS	-				no annotation	LEUM_1991
1960390	GTCIT	ACCTC	ACCTC:106 GTCIT:0	CDS	-				no annotation	LEUM_1991
1960567	AAA	CAC	CAC:136 AAA:0							
1960585	CTGCA	TTGCG	TTGCG:122 CTGCA:0							
1960664	TGTC	CGTT	CGTT:161 TGTC:0							
1969902	GTC	ATT	ATT:182 GTC:0	CDS	+				no annotation	LEUM_2001
1969941	GTTTA	ATTTT	ATTTT:173 GTTTA:0	CDS	+				no annotation	LEUM_2001
1970013	TTAT	CTGC	CTGC:152 TTAT:0	CDS	+				no annotation	LEUM_2001

1978224	AGTAT	GGTAC	GGTAC:277 AGTAT:0	CDS	-				no annotation	LEUM_2010	
1980589	CTTGT	TTTGC	TTTGC:192 CTTGT:0								
1994040	TAATT	GAATC	GAATC:291 TAATT:0	CDS	-				no annotation	LEUM_2027	
1996966	GTGG	ATGA	ATGA:363 GTGG:0	CDS	-				no annotation	LEUM_2030	
1996984	GATT	AATC	AATC:258 GATT:0	CDS	-				no annotation	LEUM_2030	
1996993	GGCAGGC	AGCTGGT	AGCTGGT:241 GGCAGGC:0	CDS	-				no annotation	LEUM_2030	
1997007	GACCCG TTCAGGC	ATCCTCGCT CCGGT	ATCCTCGCTCCGGT: 235 GACCCCGTTCAGGC: 0	CDS	-				no annotation	LEUM_2030	
1997032	CACA	AACG	AACG:318 CACA:0	CDS	-				no annotation	LEUM_2030	
2025691	GCTA	ACTG	ACTG:240 GCTA:0	CDS	-				no annotation	LEUM_2060	
2025829	AACA	GACG	GACG:213 AACA:0	CDS	-				no annotation	LEUM_2060	
2026633	GCAG	ACAA	ACAA:327 GCAG:0	CDS	-				no annotation	LEUM_2061	
2036598	GCCT	ACCC	ACCC:291 GCCT:0	CDS	-				no annotation	LEUM_2072	
2037136	TCGA	CCGT	CCGT:198 TCGA:0								
2037152	TAACA	GAACG	GAACG:210 TAACA:0								
2037383	TCCA	CCCT	CCCT:285 TCCA:0	CDS	-				no annotation	LEUM_2073	
2037417	CGT	TGC	TGC:259 CGT:0	CDS	-				no annotation	LEUM_2073	
2037438	GTATC	TTATT	TTATT:286 GTATC:0	CDS	-				no annotation	LEUM_2073	

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

This application claims priority from Australian Provisional Application No. 2017903944 entitled "Isothiocyanate containing Brassicaceae products and method of preparation thereof" filed on 28 September 2017, the entire contents of which are hereby incorporated by reference.

All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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CLAIMS

1. A method of preparing an isothiocyanate containing product from *Brassicaceae* material comprising a glucosinolate comprising:
 - 5 i) pre-treating the *Brassicaceae* material to improve the access of myrosinase to a glucosinolate;
 - ii) fermenting the material obtained by step i) with lactic acid bacteria to form the isothiocyanate containing product, and
 - wherein at least one of the following apply:
 - 10 a) when pre-treating comprises maceration the *Brassicaceae* material is heated to a temperature that does not exceed about 75°C;
 - b) the material obtained by step i) is fermented for about 8 to about 24 hours at about 20°C to about 34°C; and
 - c) the pre-treating comprises heating the *Brassicaceae* material to about
 - 15 50°C to about 70°C reducing epithiospecifier protein (ESP) activity while maintaining endogenous myrosinase activity.
2. The method of claim 1, wherein the pre-treating comprises one or more of the following:
 - 20 i) heating;
 - ii) macerating;
 - iii) microwaving;
 - iv) exposure to high frequency sound waves (ultrasound); or
 - v) pulse electric field processing.
- 25 3. The method of claim 1 or claim 2, wherein the pre-treating comprises heating the *Brassicaceae* material to about 50°C to about 70°C reducing epithiospecifier protein (ESP) activity while maintaining endogenous myrosinase activity.
- 30 4. The method of any one of claims 1 to 3, wherein pre-treating comprises one or both of:
 - a) heating occurs before macerating or wherein heating and macerating occur at the same time; or

b) heating the *Brassicaceae* material to a temperature of about 50°C to about 70°C followed by maceration.

5. The method of any one of claims 1 to 4, wherein at least one of the following
5 apply:

a) the *Brassicaceae* material is macerated so that at least about 80% of the *Brassicaceae* material is of a size of about 2 mm or less;

b) the isothiocyanate containing product comprises at least about 10 times more isothiocyanate than macerated *Brassicaceae* material; and

10 c) the isothiocyanate containing product comprises at least about 2 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content of the *Brassicaceae* material.

6. The method of any one of claims 1 to 5, which comprises one or more of the
15 following:

a) acidification of the isothiocyanate containing product from step ii) to a pH of about 4.4 or less;

b) wherein the fermentation in step ii) is at about 22°C to about 34°C;

c) wherein the fermentation in step ii) is at about 30°C; and

20 d) post-treating the isothiocyanate containing product to inactivate microbes.

7. The method of any one of claims 1 to 6, wherein the lactic acid bacteria is:

25 i) the genera selected from *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus* and *Weissella*;

ii) the species selected from *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactococcus lactis*, *Pediococcus pentosaceus*, *Lactobacillus rhamnosus* and *Pedicoccus acidilacti*;

iii) the lactic acid bacteria lacks myrosinase activity;

30 iv) a) a combination of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*;

b) a combination of *Leuconostoc mesenteroides*, *Lactobacillus plantarum* and *Lactobacillus pentosus*; or

c) a combination of *Leuconostoc mesenteroides*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus*;

v) is BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia and/or BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia; or

vi) one or more of:

- a) B1 deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;
- b) B2 deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;
- c) B3 deposited under V17/021733 on 25 September 2017 at the National Measurement Institute Australia
- c) B4 deposited under V17/021734 on 25 September 2017 at the National Measurement Institute Australia; and
- e) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

8. The method of any one of claims 1 to 7, wherein the lactic acid bacteria is an isolated strain of *Leuconostoc mesenteroides* comprising one or more or all of the polymorphisms listed in Table 18 or 19 that differs from ATCC8293.

9. The method of any one of claims 1 to 8, wherein the lactic acid bacteria is an isolated strain of *Lactobacillus plantarum* comprising one or more or all of the polymorphisms listed in Table 13, Table 14, Table 15, Table 16 or Table 17 that differs from ATCC8014.

10. The method of any one of claims 1 to 9, wherein the lactic acid bacteria is B1 deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia.

11. The method of any one of claims 1 to 10, wherein the isothiocyanate containing product has one or more or all of the following features:

a) is resistant to yeast, mould and/or coliform growth for at least 4 weeks, or at least 8 weeks, or at least 12 weeks when stored at about 4°C to about 25°C;

- b) comprises lactic acid bacteria at a concentration of at least 10^8 CFU/g; and
- c) comprises an isothiocyanate bioactive derivative.

12. The method of any one of claims 1 to 11, wherein pre-treating comprises
5 maceration and the isothiocyanate containing product comprises 10 times more isothiocyanate than macerated *Brassicaceae* material.

13. The method of any one of claims 1 to 12, wherein the glucosinolate is selected
10 from one of: glucoraphanin (4-methylsulphinylbutyl), sinigrin (2-propenyl), gluconapin (3-butenyl), glucobrassicinapin (4-pentenyl), progoitrin (2(R)-2-hydroxy-3-butenyl), epiprogoitrin (2(S)-2-hydroxy-3-butenyl), gluconapoleiferin (2-hydroxy-4-pentenyl), glucoiberin (3-methylthiopropyl), glucoerucin (4-methylthiobutyl), dehydroerucin (4-methylthio-3-butenyl, glucoiberin (3-methylsulphinylpropyl), glucoraphenin (4-methylsulphinyl-3-butenyl), glucoalyssin (5-methylsulphinylpentenyl), glucoerysolin
15 (3-methylsulphonylbutyl), 4-mercaptobutyl, glucobrassicin (3-indolylmethyl), 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl), 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl), neoglucobrassicin (1-methoxy-3-indolylmethyl), glucotropaeolin (benzyl), and gluconasturtiin (2-phenylethyl).

20 14. The method of any one of claims 1 to 13, wherein the isothiocyanate is sulforaphane.

15. The method of any one of claims 1 to 14, the *Brassicaceae* is a *Brassica*.

25 16. The method of any one of claims 1 to 15, wherein the *Brassicaceae* is selected from *Brassica oleracea*, *Brassica balearica*, *Brassica carinata*, *Brassica elongata*, *Brassica fruticulosa*, *Brassica hilarionis*, *Brassica juncea*, *Brassica napus*, *Brassica narinosa*, *Brassica nigra*, *Brassica perviridis*, *Brassica rapa*, *Brassica rupestris*, *Brassica septiceps* and *Brassica tournefortii*.

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17. An isolated strain of lactic acid bacteria selected from:

- a) BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia;

b) BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia;

c) B1 deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;

5 d) B2 deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;

e) B3 deposited under V17/021733 on 25 September 2017 at the National Measurement Institute Australia;

10 f) B4 deposited under V17/021734 on 25 September 2017 at the National Measurement Institute Australia; and

g) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

18. A probiotic composition comprising lactic acid bacteria selected from one or more
15 or all of:

a) BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia;

b) BF2 deposited under V17/021730 on 25 September 2017 at the National Measurement Institute Australia;

20 c) B1 deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;

d) B2 deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;

25 e) B3 deposited under V17/021733 on 25 September 2017 at the National Measurement Institute Australia;

f) B4 deposited under V17/021734 on 25 September 2017 at the National Measurement Institute Australia; and

g) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

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