

**METHODS AND COMPOSITIONS FOR PROMOTING HEALTH IN A
SUBJECT**

FIELD OF THE INVENTION

5 The present invention provides methods, compositions and delivery vehicles for promoting health in a subject, comprising administering to a subject a fermented *Brassicaceae* product.

BACKGROUND OF THE INVENTION

10 The prevalence of chronic diseases such as obesity, cardiovascular disease, metabolic syndrome, inflammatory diseases, autoimmune diseases, diabetes, gut health conditions and certain cancers is increasing globally, fuelled particularly by dramatic rises in developing countries where growing affluence is also associated with an expanding adoption of more Westernised diet and lifestyle patterns. While over
15 consumption of high calorie, easily digested foods and beverages plays an important role, there is growing evidence that changes to the 10^{14} microbes, comprising over 10^3 bacterial species (collectively the gut microbiota) of the human large bowel, driven by these dietary patterns also makes a contribution and provides target for preventive and clinical intervention. Diet plays a big part in feeding this hungry gut microbiota, shaping
20 both its structure (the relative proportions of the different species) and its function (genes expressed, metabolites made and their interaction with the subject). Dietary fibre is fermented by the gut microbiota into short chain fatty acids (SCFA). SCFA positively influence the gastrointestinal microenvironment (increases gut health) and other organ sites in the body as they are small enough to enter the blood stream and can be distributed
25 to other sites in the body.

 Accordingly, there is a requirement for supplements and nutritional agents that promote health in a subject.

SUMMARY OF THE INVENTION

30 The present inventors have developed methods, compositions and delivery vehicles for promoting health in a subject.

 In an aspect, the invention provides a method of promoting health in a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from
35 *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an embodiment, the *Brassicaceae* product increases the gastrointestinal level of one or more short chain fatty acids (SCFA) in the subject.

In an embodiment, *Brassicaceae* product increases the production of one or more SCFA in the gastrointestinal tract in the subject. In an embodiment, the *Brassicaceae* product increases the production of one or more SCFA in the lower gastrointestinal tract of the subject. In an embodiment, the *Brassicaceae* product increases the production of one or more SCFA of the colon of the subject. In an embodiment, production of one or more SCFA is increased relative to an unfermented *Brassicaceae* product.

In an embodiment, the *Brassicaceae* product comprises an isothiocyanate.

In an embodiment, the *Brassicaceae* product comprises live lactic acid bacteria from *Brassicaceae*.

In an aspect, the invention provides a method of promoting the health of the gut microbiome in a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an aspect, the invention provides a method of treating and/or preventing microbial dysbiosis in the gastrointestinal tract of a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an aspect, the invention provides a method of treating and/or preventing inflammation in a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an aspect, the invention provides a method of treating and/or preventing diabetes in a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an aspect, the invention provides use of a *Brassicaceae* product fermented with lactic acid bacteria in the manufacture of a medicament for promoting health in a subject, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an aspect, the invention provides use of a *Brassicaceae* product fermented with lactic acid bacteria in the manufacture of a medicament for promoting health of the gut microbiome in a subject, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an aspect, the invention provides use of a *Brassicaceae* product fermented with lactic acid bacteria in the manufacture of a medicament for treating and/or preventing microbial dysbiosis in the gastrointestinal tract of a subject, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an aspect, the invention provides use of a *Brassicaceae* product fermented with lactic acid bacteria in the manufacture of a medicament for treating and/or preventing inflammation in a subject, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an aspect, the invention provides use of a *Brassicaceae* product fermented with lactic acid bacteria in the manufacture of a medicament for treating and/or preventing diabetes in a subject, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an aspect, the invention provides a pharmaceutical composition comprising a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation for use in promoting health in a subject.

In an aspect, the invention provides a pharmaceutical composition comprising a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation for use in promoting health of the gut microbiome in a subject.

In an aspect, the invention provides a pharmaceutical composition comprising a *Brassicaceae* product with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation for treating and/or preventing microbial dysbiosis in the gastrointestinal tract of a subject.

In an aspect, the invention provides a pharmaceutical composition comprising a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria

were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation for treating and/or preventing inflammation in a subject.

5 In an aspect, the invention provides a pharmaceutical composition comprising a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation for treating and/or preventing diabetes in a subject.

10 In an aspect, the invention provides a prebiotic composition comprising a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation, wherein the prebiotic increases the gastrointestinal level of one or more SCFA in a subject.

15 In an aspect, the invention provides a synbiotic composition comprising a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation, wherein the prebiotic increases the gastrointestinal level of one or more SCFA in a subject.

20 In an aspect, the invention provides a combined prebiotic and probiotic composition comprising:

i) a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation; and

ii) live lactic acid bacteria.

25 In an aspect, the invention provides a faecal microbiota suitable for transplantation into a subject, wherein the faecal microbiota is isolated from a subject administered a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

30 In an aspect, the invention provides a delivery vehicle for delivering a bioactive to a subject, wherein the delivery vehicle comprises a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

35 In an embodiment, the bioactive is selected from one or more or all of: i) a fatty acid, ii) oil, iii) a further prebiotic, and iv) a further probiotic.

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

- i) fermenting *Brassicaceae* material with lactic acid bacteria;
- ii) adding a fatty acid and/or oil before or during step i).

5 In an embodiment, the method further comprises forming an emulsion or suspension.

In an embodiment, the *Brassicaceae* material is pre-treated.

10 In an embodiment, pre-treating comprises one or more of: i) heating; ii) macerating; iii) microwaving; iv) exposure to high frequency sound waves (ultrasound), v) pulse electric field processing; and vi) high pressure processing.

In an embodiment, pre-treating comprising heating and maceration. 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.

15 In an embodiment, the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae*.

In an aspect, the present invention provides an emulsion or suspension produced by the methods as described herein.

20 In an aspect, the present invention provides a *Brassicaceae* product comprising the emulsion or suspension as described herein.

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.

25 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.

30 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.

35 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

- 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.
- 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)** Shows the effects of heat treatment condition on the conversion of glucoraphanin into sulforaphane in broccoli matrix.
- 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 (μmol/kg DW) under different heat treatment conditions of broccoli with a sealed bag. **B)** Shows sulforaphane yield (μmol/kg DW) under different heat treatment conditions of broccoli immersed directly in water.
- 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 (μmol/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.
- 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.
- 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.

5 **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.

10 **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.

15 **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 $\mu\text{g}/\text{mg}$), a 23.8 fold increase is observed in sinapic acid (3.6 to 86.6 $\mu\text{g}/\text{mg}$), a 10.5 increase in kaempferol (12.7 to 134.6 $\mu\text{g}/\text{mg}$) and a 0.48 fold decrease is observed in p-coumaric acid.

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

25 **Figure 14.** Shows that a *Brassicaceae* product as described herein increases short chain fatty acid production in an *in vitro* colon fermentation model.

30 **Figure 15.** Shows that a *Brassicaceae* product as described herein increases short chain fatty acid production in an *in vitro* colon fermentation model.

35 **Figure 16.** Shows that a *Brassicaceae* product as described herein increases lactobacilli levels but not *E.coli*, *bifidobacteria* or total bacteria in an *in vitro* colon fermentation model.

Figure 17. A) Shows an oxipres trace showing the stability of tuna oil as compared to tuna oil encapsulated in broccoli and broccoli fermented with oil. B) Shows the stability of EPA and DHA encapsulated in non-fermented and fermented broccoli powders during storage at 25°C. C-To-NF, Control broccoli with tuna oil; C-To-F, Control broccoli

fermented with tuna oil; Ph-To-NF, Preheated broccoli with tuna oil; Ph-To-F, Preheated broccoli fermented with tuna oil.

KEY TO THE SEQUENCE LISTING

- 5 SEQ ID NO:1 - ATCC8014 reference nucleotide sequence position 68529.
SEQ ID NO:2 - ATCC8014 reference nucleotide sequence position 72030.
SEQ ID NO:3 - ATCC8014 reference nucleotide sequence position 10806.
SEQ ID NO:4 - B1 alternate nucleotide sequence position 10806.
SEQ ID NO:5 - B1 alternate nucleotide sequence position 50276.
- 10 SEQ ID NO:6 - ATCC8014 reference nucleotide sequence position 19068.
SEQ ID NO:7 - B1 reference nucleotide sequence position 4326.
SEQ ID NO:8 - ATCC8293 reference nucleotide sequence position 70144.
SEQ ID NO:9 - ATCC8293 reference nucleotide sequence position 341498.
SEQ ID NO:10 - BF2 reference nucleotide sequence position 341498.
- 15 SEQ ID NO:11 - ATCC8293 reference nucleotide sequence position 610344.
SEQ ID NO:12 - BF2 reference nucleotide sequence position 610344.
SEQ ID NO:13 - ATCC8293 reference nucleotide sequence position 843675.
SEQ ID NO:14 - BF2 reference nucleotide sequence position 843675.
SEQ ID NO:15 - ATCC8293 reference nucleotide sequence position 986279.
- 20 SEQ ID NO:16 - BF2 reference nucleotide sequence position 986279.
SEQ ID NO:17 - ATCC8293 reference nucleotide sequence position 1319558.
SEQ ID NO:18 - BF2 reference nucleotide sequence position 1319558.
SEQ ID NO:19 - ATCC8293 reference nucleotide sequence position 1418040.
SEQ ID NO:20 - BF2 reference nucleotide sequence position 1418040.
- 25 SEQ ID NO:21 - ATCC8293 reference nucleotide sequence position 1429917.
SEQ ID NO:22 - BF2 reference nucleotide sequence position 1429917.
SEQ ID NO:23 - ATCC8293 reference nucleotide sequence position 1430314.
SEQ ID NO:24 - BF2 reference nucleotide sequence position 1430314.
SEQ ID NO:25 - ATCC8293 reference nucleotide sequence position 1430785.
- 30 SEQ ID NO:26 - BF2 reference nucleotide sequence position 1430785.
SEQ ID NO:27 - ATCC8293 reference nucleotide sequence position 1444575.
SEQ ID NO:28 - BF2 reference nucleotide sequence position 1444575.
SEQ ID NO:29 - ATCC8293 reference nucleotide sequence position 1629328.
SEQ ID NO:30 - BF2 reference nucleotide sequence position 1629328.
- 35 SEQ ID NO:31 - ATCC8293 reference nucleotide sequence position 1665094.
SEQ ID NO:32 - BF2 reference nucleotide sequence position 1665094.

- SEQ ID NO:33 - ATCC8293 reference nucleotide sequence position 1665337.
SEQ ID NO:34 - BF2 reference nucleotide sequence position 1665337.
SEQ ID NO:35 - ATCC8293 reference nucleotide sequence position 1696196.
SEQ ID NO:36 - BF2 reference nucleotide sequence position 1696196.
5 SEQ ID NO:37 - ATCC8293 reference nucleotide sequence position 1760925.
SEQ ID NO:38 - BF2 reference nucleotide sequence position 1760925.
SEQ ID NO:39 - ATCC8293 reference nucleotide sequence position 1760994.
SEQ ID NO:40 - BF2 reference nucleotide sequence position 1760994.
SEQ ID NO:41 - ATCC8293 reference nucleotide sequence position 1761069.
10 SEQ ID NO:42 - BF2 reference nucleotide sequence position 1761069.
SEQ ID NO:43 - ATCC8293 reference nucleotide sequence position 1857246.
SEQ ID NO:44 - BF2 reference nucleotide sequence position 1857246.
SEQ ID NO:45 - ATCC8293 reference nucleotide sequence position 1887567.
SEQ ID NO:46 - BF2 reference nucleotide sequence position 1887567.
15 SEQ ID NO:47 - ATCC8293 reference nucleotide sequence position 1887711.
SEQ ID NO:48 - BF2 reference nucleotide sequence position 1887711.
SEQ ID NO:49 - ATCC8293 reference nucleotide sequence position 1960134.
SEQ ID NO:50 - BF2 reference nucleotide sequence position 1960134.
SEQ ID NO:51 - ATCC8293 reference nucleotide sequence position 1997007.
20 SEQ ID NO:52 - BF2 reference nucleotide sequence position 1997007.
SEQ ID NO:53 - ATCC8293 reference nucleotide sequence position 986375.
SEQ ID NO:54 - BF2 reference nucleotide sequence position 986375.

DETAILED DESCRIPTION

25 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).

- 30 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
35 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.

As used herein, the term “subject” is any animal. In one example, the animal is a vertebrate. For example, the animal is a mammal, avian, arthropod, chordate, amphibian or reptile. Exemplary subjects include but are not limited to human, fish, prawns, primate, livestock (e.g. sheep, cow, chicken, horse, donkey, pig), companion animals (e.g. dogs, cats), laboratory test animals (e.g. mice, rabbits, rats, guinea pigs, hamsters), captive wild animal (e.g. fox, deer). In one example, the mammal is a human.

As used herein, the terms “treating” or “treatment” include administering a effective amount of a product, composition or delivery vehicle as described herein sufficient to reduce or eliminate at least one symptom of a specified disease or condition.

As used herein, the terms “prevent” or “preventing” include administering a effective amount of a product, composition or delivery vehicle as described herein sufficient to stop or hinder the development of at least one symptom of a specified disease or condition.

As used herein, “microbiome” refers to the microorganisms in a particular environment which can include the body or a part of the body of a subject. For example, the gut microbiome refers to the community of microorganism in the gut.

As used herein, “microorganism” or “microorganisms” refers to microscopic organisms including bacterial, viral, fungal or eukaryotic organisms.

As used herein “gastrointestinal tract” refers to at least a portion of the gastrointestinal tract. In an embodiment, the portion of the gastrointestinal tract is selected from a portion of the stomach, duodenum, small intestine, large intestine, colon, rectum, cecum, and ileum. In an embodiment, the portion of the gastrointestinal tract is selected from a portion of the small intestine, large intestine and colon.

As used herein “lower gastrointestinal tract” refers to at least a portion of the lower gastrointestinal tract. In an embodiment, the portion of the lower gastrointestinal tract is selected from a portion of the large intestine, cecum, colon and rectum.

As used herein “upper gastrointestinal tract” refers to at least a portion of the upper gastrointestinal tract. In an embodiment, the portion of the upper gastrointestinal tract is selected from a portion of the mouth, pharynx, esophagus, stomach, and duodenum.

Promoting health

The present invention provides methods, compositions and delivery vehicles for promoting the health of a subject comprising a *Brassicaceae* product. As used herein

“health” refers to the condition of a subject’s body and the extent to which the subjects body is resistant to an illness or free from an illness.

As used herein “promoting health” refers to increasing, enhancing, inducing, and/or stimulating resistance or resilience to an illness or a reduction in one or more symptoms of an illness.

As used herein “resistance” refers to the insensitivity to a disturbance. As used herein “resilience” refers to the rate of the recovery after a disturbance.

In an embodiment, promoting health comprises treating or preventing a condition in a subject.

In an embodiment, promoting health comprises treating or preventing one or more symptoms of a condition selected from: diabetes, inflammation, metabolic dysfunction, allergy and cancer.

In an embodiment, promoting health comprises promoting one or more of: gut health, immune system health, cardiovascular health, central nervous system function, cognition, metabolic health, skeletal health, liver health, blood sugar control and skin health.

In an embodiment, promoting gut health comprises reducing or preventing one or more symptoms of a gut health associated condition selected from one or more of: irritable bowel syndrome, inflammatory bowel disease, Crohn’s disease, colorectal cancer, gut leakiness, non-alcoholic fatty liver disease, metabolic syndrome, obesity, small intestinal bacterial overgrowth (SIBO), gastroenteritis, gut microbial dysbiosis, reduced gut microbial diversity, antibiotic treatment, post-surgery recovery, food intolerance, diarrhoea, gastritis, diverticulitis, flatulence, constipation, functional gut disorders and functional gastrointestinal and motility disorders.

In an embodiment, the functional gut disorder is selected from one or more of: functional abdominal bloating/distension, functional constipation, functional diarrhoea, unspecified functional bowel disorder, opioid-induced constipation, centrally mediated abdominal pain syndrome, narcotic bowel syndrome, opioid-induced hyperalgesia, functional pancreatic sphincter of oddi disorder, biliary pain, faecal incontinence, functional anorectal pain, and functional defecation disorders.

In an embodiment, the functional gastrointestinal and motility disorders is selected from one or more of: gastroesophageal reflux disease, intestinal dysmotility, intestinal pseudo-obstruction, small bowel bacterial overgrowth, constipation, outlet obstruction type constipation (pelvic floor dyssynergia), diarrhoea, faecal incontinence, hirschsprung's disease, gastroparesis and achalasia.

In an embodiment, promoting health comprises promoting health of the gut microbiome in a subject. In an embodiment, promoting health of the gut microbiome comprises one or more of: increasing the level and/or activity of one or more beneficial bacteria, decreasing or maintaining the level and/or activity of one or more non-beneficial bacteria, increasing the resistance of the gut microbiome, increasing the resilience of the gut microbiome, and increasing the diversity of the gut microbiome. As used herein “resistance of the gut microbiome” refers to the insensitivity of the gut microbiome to a disturbance. As used herein “resilience of the gut microbiome” refers to the rate of the recovery of the gut microbiome after a disturbance (e.g. a disturbance may reduce the number or type of microorganism in the microbiome).

In an embodiment, the beneficial bacteria is selected from one or more or all of: lactic acid bacteria, *Bifidobacteria*, *Bacteroidetes*, *Baciillus*, *Streptococcus*, *Escherichia* and *Enterococcus*.

In an embodiment, the lactic acid bacteria is selected from one or more of the genera selected from: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. In an embodiment, the lactic acid bacteria is selected from one or more or all of: *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactobacillus rhamnosus*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus lactis*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, *Pediococcus pentosaceus* and *Pedicoccus acidilacti*. In an embodiment, the lactic acid bacteria is selected from one or more or all of: 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 vii) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

In embodiment, the *Bifidobacteria* is selected from one or more of: *Bifidobacteria adolescentis*, *Bifidobacteria animalis*, *Bifidobacteria bifidum*, *Bifidobacteria breve*, *Bifidobacteria infantis*, *Bifidobacteria longum*, and *Bifidobacteria thermophilum*.

In embodiment, the *Baciullus* is selected from one or more of: *Baciullus cereus*, *Baciullus clausii*, *Baciullus coagulans*, *Baciullus licheniformis*, *Baciullus pumulis* and *Baciullus subtilis*.

5 In embodiment, the *Streptococcus* is *Streptococcus thermophiles*. In embodiment, the *Escherichia* is beneficial strain of *Escherichia coli*.

In embodiment, the *Enterococcus* is *Enterococcus faecium*.

In an embodiment, the non-beneficial bacteria is a pathogenic strain of bacteria.

10 In an embodiment, the non-beneficial bacteria is a pathogenic strain of bacteria selected from one or more of: *Escherichia coli*, *Enterococcus*, *Helicobacter pylori*, *Clostridium*, *Vibrio cholerae*, *Bacteroides fragilis*, *Clostridium*, *Fusobacterium*, *Staphylococcus* (e.g. *pneumoniae*), *Legionella*, *Haemophilus*, *Pseudomonas*, *Prevotella*, *Salmonella*, *Campylobacter*, and *Shigella*, *Listeria*.

15 In an embodiment, non-beneficial bacteria is a pathogenic strain of *Escherichia coli*.

In an embodiment, promoting gut health comprises modulating microbial diversity in the gastrointestinal tract of a subject. In an embodiment, modulating microbial diversity comprises increasing microbial diversity. This may occur, for example after a disturbance which reduces the microbial diversity of the gastrointestinal tract.

20 In an embodiment, promoting gut health comprises treating and/or preventing microbial dysbiosis in the gastrointestinal tract of a subject. As used herein “microbial dysbiosis” refers to an imbalance in the microbiome that is associated with a disease, precedes a disease or occurs as the result of a disease. The imbalance, for example, could be a gain or loss of members of the microbiome community or changes in relative abundance of members of the microbiome community.

25 In an embodiment, promoting gut health comprises increasing the production of one or more short chain fatty acids including salts or esters thereof (SCFA) in the gastrointestinal tract in the subject. In an embodiment, the production of one or more SCFA is increased in the lower gastrointestinal tract of the subject. In an embodiment, the production of one or more SCFA is increased in the colon of the subject. In an embodiment, the production of one or more SCFA is increased in the subject administered a fermented *Brassicaceae* product as described herein compared an unfermented *Brassicaceae* product.

35 It will be appreciated by persons skilled in the art that production of SCFA in the gastrointestinal tract of a subject can be assessed with standard methods in the research field for measuring SCFA in faecal slurries.

Prebiotic

In an embodiment, the *Brassicaceae* product as described herein comprises a prebiotic. As used herein a “prebiotic” refers to a group of nutrients that are degraded by the gut microbiota. Prebiotics result in changes in the composition and/or activity of the gastrointestinal microbiota conferring benefits upon the health of the host (e.g. gut health).

In an embodiment, the prebiotic is converted into one or more SCFA. SCFA positively influence the gastrointestinal microenvironment (increase gut health), in particular the lower gastrointestinal tract including the colon, and distal organ sites as they are small enough to enter the blood and can be delivered to e.g. the central nervous system, immune system and cardiovascular system.

In an embodiment, the prebiotic increases health in the subject by increasing the level of one or more SCFA in the gastrointestinal tract in the subject. In an embodiment, the prebiotic increases the production of one or more SCFA in the gastrointestinal tract in the subject. In an embodiment, the prebiotic increases the production of one or more SCFA in the lower gastrointestinal tract of the subject. In an embodiment, the prebiotic increases the production of one or more SCFA in the colon of the subject.

In an embodiment, the prebiotic increases health in the subject by increasing the level of one or more SCFA in the colon of the subject.

In an embodiment, the SCFA is selected from one or more or all of: butyrate (butanoate), propionate (propanoate), acetate (ethanoate), formate (methanoate), isobutyrate (2-Methylpropanoate), valerate (pentanoate), isovalerate (3-methylbutanoate), caproate (hexanoate), formic acid (methanoic acid), acetic acid (ethanoic acid), propionic acid (propanoic acid), butyric acid (butanoic acid), isobutyric acid (2-methylpropanoic acid), valeric acid (pentanoic acid), isovaleric acid (3-methylbutanoic acid), and caproic acid (hexanoic acid).

In an embodiment, the SCFA is selected from one or more or all of: butyrate, propionate, and acetate. In an embodiment, the SCFA is butyrate. In an embodiment, the SCFA is propionate. In an embodiment, the SCFA is acetate.

In an embodiment, the total SCFA level is increased about 30% to about 70% compared to administration of unfermented *Brassicaceae*. In an embodiment, the total SCFA level is increased about 38% to about 65% compared to administration of unfermented *Brassicaceae*. In an embodiment, the total SCFA level is increased about 40% to about 60% compared to administration of unfermented *Brassicaceae*. In an

embodiment, the total SCFA level is increased about 40% to about 55% compared to administration of unfermented *Brassicaceae*.

In an embodiment, the butyrate level is increased about 30% to about 70% compared to administration of unfermented *Brassicaceae*. In an embodiment, the butyrate is increased about 38% to about 65% compared to administration of unfermented *Brassicaceae*. In an embodiment, the butyrate level is increased about 40% to about 60% compared to administration of unfermented *Brassicaceae*. In an embodiment, the butyrate level is increased about 40% to about 55% compared to administration of unfermented *Brassicaceae*.

In an embodiment, the propionate level is increased about 30% to about 70% compared to administration of unfermented *Brassicaceae*. In an embodiment, the propionate is increased about 38% to about 65% compared to administration of unfermented *Brassicaceae*. In an embodiment, the propionate level is increased about 40% to about 60% compared to administration of unfermented *Brassicaceae*. In an embodiment, the propionate level is increased about 40% to about 55% compared to administration of unfermented *Brassicaceae*.

In an embodiment, the acetate level is increased about 30% to about 70% compared to administration of unfermented *Brassicaceae*. In an embodiment, the acetate is increased about 38% to about 65% compared to administration of unfermented *Brassicaceae*. In an embodiment, the acetate level is increased about 40% to about 60% compared to administration of unfermented *Brassicaceae*. In an embodiment, the acetate level is increased about 40% to about 55% compared to administration of unfermented *Brassicaceae*.

In an embodiment, the prebiotic increases the SCFA level about 5 to about 48 hours after administration. In an embodiment, the prebiotic increases the SCFA level about 10 to about 24 hours after administration.

Probiotic

As used herein a “probiotic” refers to live microorganism which when administered in an adequate amount confers a health benefit to the host (subject). In an embodiment, the *Brassicaceae* product as described herein comprises a probiotic.

In an embodiment, the probiotic is autochthonous to the *Brassicaceae* material. In an embodiment, the probiotic is an autochthonous probiotic present on the *Brassicaceae* material before fermentation. In an embodiment, the probiotic is an allochthonous probiotic added to the *Brassicaceae* material after fermentation. In an embodiment, the probiotic is the same microorganism used for fermentation. In an

embodiment, the probiotic is not active in the fermentation step. In an embodiment, the probiotic is an exogenous probiotic added to the *Brassicaceae* material before or during fermentation. In an embodiment, the probiotic is an exogenous probiotic added to the *Brassicaceae* material after fermentation.

5 In an embodiment, the probiotic is selected from one or more or all of: lactic acid bacteria, *Bifidobacteria*, *Bacteroidetes*, *Baciullus*, *Streptococcus*, *Escherichia*, *Enterococcus*, and *Saccharomyces*.

In an embodiment, the probiotic is selected from one or more of the genera selected from: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*,
 10 *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. In an embodiment, the lactic acid bacteria is selected from one or more or all of: *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactobacillus rhamnosus*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus casei*,
 15 *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus lactis*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, *Pediococcus pentosaceus* and *Pedicoccus acidilacti*. In an embodiment, the lactic acid bacteria is selected from one or more or all of: i) BF1 deposited under V17/021729 on 25 September 2017 at the National Measurement Institute Australia; ii)
 20 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)
 25 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.

In embodiment, the *Bifidobacteria* is selected from one or more of: *Bifidobacteria lactis*, *Bifidobacteria adolescentis*, *Bifidobacteria animalis*, *Bifidobacteria bifidum*,
 30 *Bifidobacteria breve*, *Bifidobacteria infantis*, *Bifidobacteria longum*, and *Bifidobacteria thermophilum*. In embodiment, the *Bifidobacteria* is *Bifidobacteria animalis*. In embodiment, the *Bifidobacteria* is *Bifidobacteria lactis*.

In embodiment, the *Baciullus* is selected from one or more of: *Baciullus cereus*, *Baciullus clausii*, *Baciullus coagulans*, *Baciullus licheniformis*, *Baciullus pumulis* and
 35 *Baciullus subtilis*.

In embodiment, the *Streptococcus* is *Streptococcus thermophiles*. In embodiment, the *Escherichia* is beneficial strain of *Escherichia coli*.

In embodiment, the *Enterococcus* is *Enterococcus faecium*.

In embodiment, the *Saccharomyces* is *Saccharomyces cerevisiae*.

- 5 In an embodiment, the lactic acid bacteria was isolated from a *Brassica oleracea*. In an embodiment, the lactic acid bacteria was isolated from broccoli. A person skilled in the art will appreciate that this includes direct isolation or indirect isolation (e.g. isolated from an original source and cultivated a number of passages, optionally cryogenically stored, before use). In an embodiment, the lactic acid bacteria was isolated from 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.

- 15 In an embodiment, the probiotic is *Faecalibacterium prausnitzii*. In an embodiment, the probiotic is *Akkermansia muciniphila*. In an embodiment, the probiotic is microencapsulated as described in WO 2005030229. In an embodiment, the *Brassicaceae* product as described herein comprises a combined prebiotic and probiotic.

Synbiotic

- 25 In an embodiment, the *Brassicaceae* product as described herein comprises a prebiotic and a probiotic which are synbiotic. As used herein a “synbiotic” refer to a composition comprising a prebiotic and probiotic which results in a synergistic effect. Synbiotics were developed to overcome possible survival difficulties for probiotics. In an embodiment, the synbiotic improves the shelf life of a live microorganism. In an embodiment, a synbiotic improves the delivery of a live microorganism (e.g. passage of the upper gastrointestinal tract). In an embodiment, a synbiotic improves the survival of live microorganism (e.g. by providing a preferred food source for metabolism by the microorganism). In an embodiment, the composition or delivery vehicle as described herein comprises a prebiotic and a probiotic which are synbiotic

Brassicaceae

A person skilled in the art will appreciate that the methods as described herein are suitable for producing a fermented product from any *Brassicaceae* material. 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*, *Brassica carinata*, *Brassica elongate*, *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*.

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 *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 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).

In an embodiment, the *Brassicaceae* is selected from *Cardamine hirsuta* (bittercress), *Iberis sempervirens* (candytuft), *Sinapis arvensis* (charlock), *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 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 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 glucosinolate). In an embodiment, the glucosinolate is glucobrassicin (3-Indolylmethyl glucosinolate).

As used herein “*Brassicaceae* material” refers to any part of the *Brassicaceae*, 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.

- 5 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.

Pre-treatment

- 10 As use herein “pre-treatment” or “pre-treating” the *Brassicaceae* material increases the bioavailability of one or more components in the *Brassicaceae* material.

- In an embodiment, the component is fibre. In an embodiment, the component is a prebiotic and/or prebiotic precursor. In an embodiment, the prebiotic is selected from one or more or all of: dietary fibre (insoluble/soluble), oligosaccharides, cellulose,
15 hemicellulose, pecticoligosaccharide, resistant starch beta-glucans and pectin.

 In an embodiment, the component is an antimicrobial component. In an embodiment, the antimicrobial component is a glucosinolate.

 In an embodiment, the component is a bioactive peptide. In an embodiment, the peptide has angiotensin-converting-enzyme inhibitory activity.

- 20 In an embodiment, the component is a polyphenol. As used herein, “polyphenol” refers to a compound comprising more than one phenolic hydroxyl group. In an embodiment, the polyphenol is selected from one or more of: anthocyanins, dihydrochalcones, flavan-3-ols, flavanones, flavones, flavonols and isoflavones, curcumin, resveratrol, benzoic acid, phenyl acetic acid, hydroxycinnamic acids,
25 coumarins, naphthoquinones, xanthenes, stilbenes, chalcones, tannins, phenolic acids, and catechins (e.g. epigallocatechin gallate (EGCg), epigallocatechin (EGC), epicatechin gallate (ECg), epicatechin (EC), and their geometric isomers gallocatechin gallate (GCg), gallocatechin (GC), catechin gallate (Cg) and catechin.

- In an embodiment, pre-treating alters the activity of one or more indigenous plant
30 enzymes (eg cell-wall degrading enzymes such as pectinase, xylanases, cellulases), with consequent effects of nutritional properties of fibre and the accessibility of plant bioactives.

- 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
35 (ultrasound), v) pulse electric field processing and vi) high pressure processing. In an

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

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. 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 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 *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 *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 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 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
5 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
10 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
15 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
20 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*
25 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
30 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
35 *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 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 ultrasound waves are at a frequency of about 20 kHz to about 600 kHz. In an embodiment, the *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 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 comprises exposing the *Brassicaceae* material to high pressure processing. In an embodiment, the *Brassicaceae* product is in a sealed package during high pressure processing. In an embodiment, high pressure processing comprises treating the *Brassicaceae* material with isostatic pressure at about 100 to about 800 MPa. In an embodiment, high pressure processing comprises treating the *Brassicaceae* material with isostatic pressure at about 100 to about 600 MPa. In an embodiment, high pressure processing comprises treating the *Brassicaceae* product with isostatic pressure at about 350 to about 550 MPa. In an embodiment, high pressure processing comprises treating the *Brassicaceae* 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.

5 In an embodiment, pre-treating converts about 10% to about 90% of a glucosinolate to an 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.

Preparation of an emulsion or suspension

10 In an aspect, the present invention provides a method of preparing a *Brassicaceae* product comprising:

- i) fermenting *Brassicaceae* material with lactic acid bacteria;
- ii) adding a fatty acid and/or oil before or during step i).

15 In an embodiment, the method further comprises forming an emulsion or suspension.

In an embodiment, the *Brassicaceae* material is pre-treated as described herein. In an embodiment, the *Brassicaceae* material is pre-treated by heating as described herein. In an embodiment, the *Brassicaceae* material is heated to about 50°C to about 70°C.

20 In an embodiment, the fatty acid and/or oil is added before step i). In an embodiment, the fatty acid and/or oil is added during step i). In an embodiment, the fatty acid and/or oil is added before pre-treatment. In an embodiment, the fatty acid and/or oil is added during pre-treatment. In an embodiment, the fatty acid and/or oil is added after pre-treatment. In an embodiment, the fatty acid and/or oil is added after pre-treatment and before step i).

25 In an aspect, the present invention provides an emulsion or suspension produced by the method as described herein.

In an aspect, the present invention provides a *Brassicaceae* product comprising the emulsion or suspension as described herein.

30 As used herein “emulsion” refers to a dispersion of droplets/particles of one liquid in another in which it is not soluble or miscible. In one embodiment, the droplets are fatty acid and/or oil dispersed in the aqueous mixture. In an embodiment, the emulsion is a wet emulsion. In an embodiment, the emulsion is dried into powder. In an embodiment, the emulsion is extruded. In an embodiment, the emulsion is extruded with a powder matrix.

35

In an embodiment, droplets produced by the methods described herein are about 0.2 μm to about 10 μm . In an embodiment, droplets produced by the methods described herein are about 1 μm to about 10 μm . In an embodiment, droplets produced by the methods described herein are about 2 μm to about 8 μm . In an embodiment, droplets produced by the methods described herein are about 2 μm to about 4 μm .

In an embodiment, the mean droplet size is about 0.2 μm to about 10 μm . In an embodiment, the mean droplet size is about 1 μm to about 10 μm . In an embodiment, the mean droplet size is about 2 μm to about 8 μm . In an embodiment, the mean droplet size is about 2 μm to about 4 μm .

As used herein "suspension" refers to dispersion of droplets/particles of one substance throughout the bulk of another substance. In one embodiment, the droplets are a fatty acid and/or oil dispersed in the aqueous mixture.

As used herein producing or forming an emulsion or suspension refers to entrapment or encapsulation of a substance in the aqueous mixture reducing the exposure of the substance to degradation. In an embodiment, the substance is a fatty acid and/or oil.

In an embodiment, the fatty acid and/or oil is heated when it is added to the aqueous mixture in step ii) as described herein. In an embodiment, the fatty acid and/or oil is heated to about 30°C to about 80°C. In an embodiment, the fatty acid and/or oil is heated to about 40°C to about 70°C. In an embodiment, the fatty acid and/or oil is heated to about 45°C to about 65°C. In an embodiment, the fatty acid and/or oil is heated to about 50°C to about 60°C.

In an embodiment, forming an emulsion or suspension as described comprises mixing of the fatty acid and/oil with an aqueous mixture comprising the *Brassicaceae* material.

In an embodiment, mixing comprises agitation under high shear. In an embodiment, mixing comprises homogenization to obtain a small droplet size. In an embodiment, droplets produced by homogenization are about 0.2 μm to about 10 μm in diameter. In an embodiment, droplets produced by homogenization are about 1 μm to about 10 μm in diameter. In an embodiment, droplets produced by homogenization are about 2 μm to about 8 μm in diameter. In an embodiment, droplets produced by homogenization are about 2 μm to about 4 μm in diameter. In an embodiment, homogenization forms a homogenous emulsion.

As used herein, the term "fatty acid" refers to a carboxylic acid (or organic acid), often with a long aliphatic tail, either saturated or unsaturated. Typically fatty acids have a carbon-carbon bonded chain of at least 4 carbon atoms (C4) or at least 8 carbon atoms

(C8) in length, more preferably at least 12 carbons in length. Preferred fatty acids of the invention have carbon chains of 18-22 carbon atoms (C18, C20, C22 fatty acids), more preferably 20-22 carbon atoms (C20, C22) and most preferably 22 carbon atoms (C22). Most naturally occurring fatty acids have an even number of carbon atoms because their biosynthesis involves acetate which has two carbon atoms. The fatty acids may be in a free state (non-esterified) or in an esterified form such as part of a triglyceride, diacylglyceride, monoacylglyceride, acyl-CoA (thio-ester) bound or other bound form. The fatty acid may be esterified as a phospholipid such as a phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol or diphosphatidylglycerol forms. In an embodiment, the fatty acid is esterified to a methyl or ethyl group, such as, for example, a methyl or ethyl ester of a C20 or C22 polyunsaturated fatty acid. Preferred fatty acids are the methyl or ethyl esters of eicosatrienoic acid, docosapentaenoic acid or docosahexaenoic acid, or the mixtures eicosapentaenoic acid and docosahexaenoic acid, or eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid, or eicosapentaenoic acid and docosapentaenoic acid.

In an embodiment, the fatty acid is a polyunsaturated fatty acid. As used herein "polyunsaturated fatty acid" refers to a fatty acid that contains more than one double bond in its backbone. In an embodiment, the polyunsaturated fatty acid is selected from one or more of: an omega-3, omega-6, or omega-9. In an embodiment, the polyunsaturated fatty acid is an omega-3. In an embodiment, the polyunsaturated fatty acid is an omega-6. In an embodiment, the polyunsaturated fatty acid is an omega-9. In an embodiment, the omega-3 is selected from one or more of: hexadecatrienoic acid, alpha-linolenic acid, stearidonic acid, eicosatrienoic acid, eicosatetraenoic acid, eicosapentaenoic acid, heneicosapentaenoic acid, docosapentaenoic acid, docosahexaenoic acid, tetracosapentaenoic acid, and tetracosahexaenoic acid. In an embodiment, the omega-3 is selected from one or more or all of eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid. In an embodiment, the omega-6 is selected from one or more of: linoleic acid, gamma-linolenic acid, eicosadienoic acid, dihomo-gamma-linolenic acid, arachidonic acid, docosadienoic acid, adrenic acid, docosapentaenoic acid, tetracosatetraenoic acid, and tetracosapentaenoic acid. In an embodiment, the omega-9 is selected from one or more of: oleic acid, eicosenoic acid, mead acid, erucic acid, and nervonic acid.

In an embodiment, the fatty acid is in an oil.

As used herein "oil" refers to a viscous liquid that is hydrophobic and lipophilic and not miscible with water.

In an embodiment, the oil is an unsaturated oil.

In an embodiment, the oil is a Plantae oil. In an embodiment, the oil is a vegetable oil. In an embodiment, the oil is an animal oil. In an embodiment, the animal oil is a marine oil or fish oil.

5 In an embodiment, the oil is selected from one or more of: fish oil, krill oil, marine oil, algal oil, microbial oil, canola oil, crustacean oil, mollusc oil, sunflower oil, avocado oil, soya oil, borage oil, evening primrose oil, safflower oil, flaxseed oil, olive oil, pumpkinseed oil, hemp seed oil, wheat germ oil, palm oil, palm oil, palm kernel oil, coconut oil, medium chain triglycerides (MCT) and grapeseed oil. In an embodiment,
10 the canola oil comprises one or more long chain polyunsaturated fatty acids such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) which can be obtained from transgenic *Brassica* encoding the required elongases and desaturases (see, for example, WO 2015/089587).

In an embodiment, the fish oil is selected from one or more of: tuna oil, herring
15 oil, mackerel oil, anchovy oil, sardine oil, cod liver oil, and shark oil.

As used herein “microbial oil” also known as “single cell oil” is an oil produced by a micorbe. For example, the microbe is a yeast, fugus, microalgae or bacteria. In an embodiment, yeast is selected from one or more of: *R. toruloides* 32489, *R. toruloides* ATCC 10788, *Cryptococcus curvatus*, *Candida curvata*, *Cryptococcus albidus*,
20 *Lipomyces starkeyi* and *Rhodotorula glutinis*. In an embodiment, the fungus is selected from one or more of: *Aspergillus oryzae*, *Mortierella isabellina*, and *Humicola lanuginose*. In an embodiment, the microalge is selected from one or more of: *Botryococcus braunii*, *Mucor circinelloides*, *Aspergillus niger*, *Cylindrotheca sp.*, *Chlorella sp.*, *Nitzschia sp.*, *Schizochytrium sp.*, *Crypthecodinium cohnii*,
25 *Nannochloropsis sp.*, *Neochloris oleoabundans*, and *Nannochloris sp.* In an embodiment, the bacteria is selected from one or more of: *Arthrobacter sp.*, *Acinetobacter calcoaceticus* and *Rhodococcus opacus*.

In an embdioment, the mollusc is abalone.

In an embodiment, the essential oil is selected from one or more of: oregano oil,
30 mint oil, basil oil, rosemary oil, tea tree oil, time oil, camphor oil, cardamon oil, citrus oil, clove oil, and/or saffron oil.

In an embodiment, the oil comprises dairy fats.

In an embodiment, the oil is olive oil.

In an embodiment, the oil is sunflower oil.

35 In an embodiment, the oil is canola oil.

In an embodiment, the oil comprises one or more bioactive/s and/or bioactive precursor/s. Thus, in some embodiments, the oil acts as a bioactive carrier. In an embodiment, the bioactive and/or bioactive precursor is added to the oil before the oil is added to the aqueous mixture. In an embodiment, the bioactive and/or bioactive precursor is infused in oil in step ii) of the method as described herein. In an embodiment, the bioactive and/or a bioactive precursor is infused in oil in step iii) of the method as described herein. In an embodiment, the bioactive and/or bioactive precursor is from the biomass and/or further biomass as described herein. In an embodiment, the bioactive and/or bioactive precursor is not from the biomass and/or further biomass.

Fermentation

Brassicaceae material, optionally pre-treated, is fermented as described herein to produce a fermented *Brassicaceae* product. In an embodiment, the *Brassicaceae* material is optionally mixed with a fatty acid and/or oil before fermentation. 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. In an embodiment, fermentation increases the quantity and bioavailability of one or more components in the *Brassicaceae* material. In an embodiment, the component is a prebiotic and/or prebiotic precursor, dietary fibre (insoluble/soluble), oligosaccharides, cellulose, hemicellulose,

In an embodiment, the prebiotic is selected from one or more or all of: dietary fibre, oligosaccharides, exopolysaccharides, oligofructose, cellulose, hemicellulose resistant starch, beta-glucans and dextran. In an embodiment, the oligosaccharides are selected from one or more or all of: gluco-oligosaccharides, fructo-oligosaccharides, galacto-oligosaccharide, trans-galacto-oligosaccharides. In an embodiment, the exopolysaccharides are homopolysaccharides and/or heteropolysaccharides.

In an embodiment, the component is a bioactive peptide. In an embodiment, the peptide is an antimicrobial peptide (e.g. bacteriocins or those described in Pacheco-Cano et al., 2017). In an embodiment, the peptide has angiotensin-converting-enzyme inhibitory activity.

As used herein, “lactic 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 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*,
5 *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactococcus lactis*, *Pediococcus pentosaceus* and *Pediococcus acidilacti*.

In an embodiment, the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae*. In an embodiment, the *Brassicaceae* material has been pre-treated as described herein. In an embodiment, the lactic acid bacteria was derived from an isolate
10 obtained from *Brassica oleracea*. In an embodiment, the lactic acid bacteria was derived from an isolate obtained from broccoli. As used herein “derived from” means isolated directly from or indirectly from a culture derived from an indicated source which has subsequently been passaged in culture (e.g. an isolate initially isolated from broccoli which has subsequently been passaged a number of times in *in vitro* cell culture). In an
15 embodiment, the 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.

20 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 iii); and vii) a combination of i), ii) and iv).

25 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
30 embodiment, the *Lactobacillus plantarum* lacks myrosinase activity.

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*.

35 In an embodiment, the *Lactobacillus plantarum* is selected from one or more or 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.

5 In an embodiment, fermentation occurs in the presence of at least 2, or at least 3, 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 acid
10 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 a control
15 lactic acid bacteria that has not been stressed.

In an embodiment, the *Brassicaceae* material is inoculated with at least about 10^5 CFU/g of a lactic acid bacteria as described herein. In an embodiment, the *Brassicaceae* material is inoculated with at least 10^6 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
20 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.

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.
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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.
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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.

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.

15 Acidification

The pre-treated material can be acidified to improve the microbial safety and stability (susceptibility to microbial degradation) of 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.

25 Post-treatment

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

As used herein “post-treatment” or “post-treating” refers to treatment of the *Brassicaceae* product after fermentation. As used herein “microbes” refers to bacterial, viral, fungal or eukaryotic activity that can result in degradation or spoilage of the *Brassicaceae* 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 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 *Brassicaceae* product is post-treated with heat processing. In an embodiment, the *Brassicaceae* product is post-treated with high pressure processing. In an embodiment, the *Brassicaceae* product is in a sealed package during post-treatment. In an embodiment, the *Brassicaceae* product is in a sealed package during high pressure processing. In an embodiment, the *Brassicaceae* product is in a sealed package during heat treatment. In an embodiment, high pressure processing comprises treating the *Brassicaceae* material with isostatic pressure at about 100 to about 800 MPa. In an embodiment, high pressure processing comprises treating the *Brassicaceae* product with isostatic pressure at about 300 to about 600 MPa. In an embodiment, high pressure processing comprises treating the *Brassicaceae* product with isostatic pressure at about 350 to about 550 MPa. In an embodiment, high pressure processing comprises treating the *Brassicaceae* 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.

A further probiotic may be added to the *Brassicaceae* product before or after post treatment. A person skilled in the art will appreciate that post-treatment can be performed before or after drying as described below.

Sugar/lyoprotectants/cryoprotectants

In an embodiment, a sugar is added to the *Brassicaceae* product as described herein. In an embodiment, the added sugar is about 0.5% to about 40% of the final composition. In an embodiment, the added sugar is about 0.5% to about 30% of the final composition. In an embodiment, the added sugar is about 4% to about 25% of the final composition. In an embodiment, the added sugar is about 6% to about 20% of the final composition. In an embodiment, the added sugar is about 8% to about 18% of the final composition. In an embodiment, the added sugar is about 10% to about 15% of the final composition.

In an embodiment, the sugar may act as a lyoprotectant or cryoprotectant in a drying, cooling or freezing process. In an embodiment, the sugar is a simple sugar. In an embodiment, the sugar is selected from a monosaccharide, disaccharide or polysaccharide.

In an embodiment, a lyoprotectant/cryoprotectant is added to the *Brassicaceae* product as described herein. In an embodiment, the lyoprotectant/cryoprotectant is a monosaccharide, disaccharide or polysaccharide, polyalcohol or a derivative thereof. In an embodiment, the lyoprotectant/cryoprotectant is selected from one or more of:

5 trehalose, sucrose, glycerol, maltodextrin and mannitol.

In an embodiment, the added lyoprotectant/cryoprotectant is about 0.5% to about 40% of the final composition. In an embodiment, the added lyoprotectant/cryoprotectant is about 0.5% to about 30% of the final composition. In an embodiment, the added lyoprotectant/cryoprotectant is about 4% to about 25% of the final composition. In an embodiment, the added lyoprotectant/cryoprotectant is about 6% to about 20% of the final composition. In an embodiment, the added lyoprotectant/cryoprotectant is about 8% to about 18% of the final composition. In an embodiment, the added lyoprotectant/cryoprotectant is about 10% to about 15% of the final composition.

15 Drying

In an embodiment, the *Brassicaceae* product as described herein is partially dried or dried to reduce the water content and/or water activity. In an embodiment, the method as described herein comprises drying the *Brassicaceae* product to reduce the water content to about 1 to about 14%. In an embodiment, the method as described herein comprises drying the *Brassicaceae* product to reduce the water content to about 1 to about 13%. In an embodiment, the method comprises drying the *Brassicaceae* product to reduce the water content to about 1 to about 12%. In an embodiment, the method comprises drying the *Brassicaceae* product to reduce the water content to about 1 to about 10%. In an embodiment, the method comprises drying the *Brassicaceae* product to reduce the water content to about 2 to about 8%. In an embodiment, the method comprises drying the *Brassicaceae* product to reduce the water content to about 2 to about 6%. In an embodiment, the method comprises drying the *Brassicaceae* product to reduce the water content to about 2 to about 4%. In an embodiment, the method comprises drying the *Brassicaceae* product to reduce the water content to about 2 to about 3%.

In an embodiment, the method as described herein comprises drying the *Brassicaceae* product to reduce the water activity to a low water activity to about 0.1 to about 0.7. In an embodiment, the method comprises drying the *Brassicaceae* product to reduce the water activity to a low water activity to about 0.2 to about 0.6. In an embodiment, the method comprises drying the *Brassicaceae* product to reduce the water activity to a low water activity to about 0.2 to about 0.5. In an embodiment, the method

comprises drying the *Brassicaceae* product to reduce the water activity to a low water activity to about 0.3 to about 0.4. In an embodiment, the method comprises drying the *Brassicaceae* product to reduce the water activity to a low water activity of about 0.4.

5 In an embodiment, the method as described herein comprises drying the *Brassicaceae* product to form a powder. Drying may include for example spray drying, freeze-drying (lyophilisation or cryodesiccation), tray drying, drum drying, roller drying, fluid bed drying, impingement drying, refractance windows drying, thin-film belt drying, vacuum microwave drying, ultrasonic-assisted drying, extrusion porosification technology or any other method known to a person skilled in the art.

10 In an embodiment, the *Brassicaceae* product is dried to produce a mean dry particle size of about 10 μM to about 4000 μM . In an embodiment, the *Brassicaceae* product is dried to produce a mean dry particle size of about 10 μM to about 3000 μM . In an embodiment, the *Brassicaceae* product is dried to produce a mean dry particle size of about 20 μM to about 2000 μM . In an embodiment, the *Brassicaceae* product is dried to produce a mean dry particle size of about 10 μM to about 1000 μM . In an embodiment, the *Brassicaceae* product is dried to produce a mean dry particle size of about 10 μM to about 500 μM .

20 In an embodiment, the *Brassicaceae* product is dried by spray drying (e.g. a Drytec laboratory spray dryer) to form a powder. For example, the *Brassicaceae* product is dried using a Drytec laboratory spray dryer with a rotary atomiser, ultrasonic nozzle or twin fluid nozzle at 2.0 – 4.0 bar atomising pressure by heating the feed to 60°C prior to atomisation and the inlet and outlet air temperatures were 180°C and 80°C, respectively. In an embodiment, the spray dryer has a granulation function. In an embodiment, the spray dryer is mounted with a granulation dryer.

25 In an embodiment, spray drying produces individual particles or agglomerates of particles.

30 In an embodiment, spray drying produces a mean dry particle size of about 10 μM to about 3000 μM . In an embodiment, spray drying produces a mean dry particle size of about 20 μM to about 2000 μM . In an embodiment, spray drying produces a mean dry particle size of about 10 μM to about 1000 μM . In an embodiment, spray drying produces a mean dry particle size of about 10 μM to about 500 μM .

35 In an embodiment, the *Brassicaceae* product is dried by freeze-drying to form a powder. In an embodiment, a cryoprotectant is added to the *Brassicaceae* product before freeze drying. In an embodiment, the cryoprotectant is a monosaccharide, disaccharide or polysaccharide, polyalcohol or a derivative thereof. In an embodiment, the

cryoprotectant is selected from one or more of: trehalose, sucrose, glycerol, maltodextrin and mannitol.

In an embodiment, the *Brassicaceae* product is dried by drum drying to form a powder.

- 5 In an embodiment, the powder comprises about 5% to about 50% oil w/w. In an embodiment, the powder about 10% to about 50% oil w/w. In an embodiment, the powder comprises about 20% to about 50% oil w/w. In an embodiment, the powder comprises about 20% to about 50% oil w/w. In an embodiment, the powder comprises about 20% to about 40% oil w/v. In an embodiment, the powder comprises about 20%
10 to about 30% oil w/w.

- In an embodiment, the powder comprises particles of about 20 µm to about 1200 µm. In an embodiment, the powder comprises particles of about 100 µm to about 900 µm. In an embodiment, the powder comprises particles of about 400 µm to about 700 µm. In an embodiment, the powder comprises particles of about 500 µm to about 600
15 µm. In an embodiment, the powder comprises particles of about 1000 µm. In an embodiment, the powder is milled to further reduce the particle size. In an embodiment, milling may reduce the particle size to less than about 10 µm, or less than about 8 µm, or less than about 6 µm, or less than about 4 µm, or less than about 2 µm.

20 Isolated strains and starter cultures

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

- In an embodiment, the present invention provides an isolated strain of lactic acid
25 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;
30 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
35 Measurement Institute Australia;

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.

5 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.

10 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 *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
15 *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 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
20 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 *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 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
30 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 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
35 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.

5 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.

30 In an embodiment, the present invention provides a starter culture for producing a *Brassicaceae* product, a prebiotic, a combined prebiotic and probiotic, or a synbiotic 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 a *Brassicaceae* product, a prebiotic, a combined prebiotic and probiotic, or a synbiotic 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;

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

5 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

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

15 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 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* material is inoculated with about 10^5 CFU/g to about 10^{10} CFU/g of a starter culture as described herein.

25

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
30 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 10% 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, 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 *Brassicaceae* 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 a derivative of iberin. In an embodiment, the isothiocyanate bioactive derivative is a derivative of allyl isothiocyanate. In an embodiment, the isothiocyanate bioactive derivative is indole-3-carbinol. 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.

Fermented *Brassicaceae* product

In an embodiment, a *Brassicaceae* product fermented with lactic acid bacteria (also referred to as a fermented *Brassicaceae* product) as described herein comprises a higher level of a prebiotic and/or a prebiotic precursor compared to the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises a prebiotic as described herein. In an embodiment, the *Brassicaceae* product comprises a prebiotic and a probiotic as described herein. In an embodiment, the *Brassicaceae* product comprises a prebiotic and a probiotic which are synbiotic as described herein.

In an embodiment, fermented *Brassicaceae* product produced by the methods as described herein comprises a higher level of isothiocyanate compared to the *Brassicaceae* material. 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 *Brassicaceae* product comprises at least about 4 times more isothiocyanate than macerated *Brassicaceae* material. In an embodiment, the

Brassicaceae product comprises at least about 6 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 8 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 10 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 12 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 14 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 17 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises about 4 times to about 17 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises about 4 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises about 8 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises about 10 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises about 12 times to about 16 times more isothiocyanate than the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* 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 *Brassicaceae* product is higher than what would be expected from the extractable glucosinolate content of the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 1 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the *Brassicaceae* product comprises at least about 2 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the *Brassicaceae* product comprises at least about 3 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the *Brassicaceae* product comprises at least about 3.8 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the *Brassicaceae* product comprises at least about 4 times the expected maximum yield of isothiocyanate based on the extractable glucosinolate content. In an embodiment, the *Brassicaceae* 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 *Brassicaceae* 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

5 *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* product

10 is higher than what would be expected from the extractable glucoraphanin content of the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 1 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the *Brassicaceae* product comprises at least about 2 times the expected maximum yield of sulforaphane based on the extractable

15 glucoraphanin content. In an embodiment, the *Brassicaceae* product comprises at least about 3 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the *Brassicaceae* product comprises at least about 3.8 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the *Brassicaceae* product comprises at least

20 about 4 times the expected maximum yield of sulforaphane based on the extractable glucoraphanin content. In an embodiment, the *Brassicaceae* 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 *Brassicaceae* product comprises about 1 times to about 3.8 times the expected maximum yield of sulforaphane

25 based on the extractable glucoraphanin content. In an embodiment, the *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* product comprises about 100 mg/kg dw to about 7000 mg/kg dw of isothiocyanate. In an embodiment, the *Brassicaceae* product comprises about 500 mg/kg dw to about 7000 mg/kg dw of isothiocyanate. In an embodiment, the *Brassicaceae* product comprises about 1000 mg/kg dw to about 7000 mg/kg dw of isothiocyanate. In an embodiment, the *Brassicaceae* product comprises

30 about 1600 mg/kg dw to about 4000 mg/kg dw of isothiocyanate. In an embodiment, the *Brassicaceae* product comprises about 1600 mg/kg dw to about 3000 mg/kg dw of

isothiocyanate. In an embodiment, the *Brassicaceae* product comprises about 2000 mg/kg dw to about 4000 mg/kg dw of isothiocyanate. In an embodiment, the *Brassicaceae* product comprises about 2000 mg/kg dw of to about 7000 mg/kg dw of isothiocyanate. In an embodiment, the *Brassicaceae* product comprises about 3000 mg/kg dw isothiocyanate to about 7000 mg/kg of isothiocyanate. In an embodiment, the *Brassicaceae* product comprises about 2300 mg/kg dw of the isothiocyanate.

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

In an embodiment, the *Brassicaceae* product comprises at least about 100 mg/kg dw of sulforaphane. In an embodiment, the *Brassicaceae* product comprises at least about 150 mg/kg of sulforaphane. In an embodiment, the *Brassicaceae* product comprises at least about 200 mg/kg dw of sulforaphane. In an embodiment, the *Brassicaceae* product comprises at least about 250 mg/kg of sulforaphane. In an embodiment, the *Brassicaceae* product comprises at least about 300 mg/kg dw of sulforaphane. In an embodiment, the

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

In an embodiment, the *Brassicaceae* product comprises at least about 5% more total fibre than the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 10% more total fibre than the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 15% more total fibre than the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 20% more total fibre than the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 4% more protein than the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 6% more protein than the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 8% more protein than the *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises at least about 10% more protein than the *Brassicaceae* material.

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

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

In an embodiment, the *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* product comprises indole-3-carbinol. In an embodiment, indol-3carbinol is increased at least about 2 fold in the *Brassicaceae* product compared to the macerated *Brassicaceae* material. In an embodiment, indol-3-carbinol is increased at least about 3 fold in the *Brassicaceae* product compared to the macerated *Brassicaceae* material. In an embodiment, the *Brassicaceae* product comprises ascorbigen. In an embodiment, ascorbigen is increased at least about 2 fold in the *Brassicaceae* product compared to the macerated *Brassicaceae* material. In an embodiment, ascorbigen is increased at least about 3 fold in the *Brassicaceae* product compared to the macerated *Brassicaceae* material.

In an embodiment, the *Brassicaceae* 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 *Brassicaceae* product 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 *Brassicaceae* 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 *Brassicaceae* product 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 *Brassicaceae* 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, rosmarinic acid, caffeic acid compared to the *Brassicaceae* material.

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

30 In an embodiment, the isothiocyanate in the *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* product comprises about 20 g/100gdw to about 32 g/100gdw total fibre. In an embodiment, the *Brassicaceae* product comprises about 20 g/100gdw total fibre. In an embodiment, the *Brassicaceae* product comprises about 25 g/100gdw total fibre. In an embodiment, the *Brassicaceae* product comprises about 28 g/100gdw total fibre. In an embodiment, the *Brassicaceae* product comprises about 29 g/100gdw total fibre. In an embodiment, the *Brassicaceae* product comprises about 30 g/100gdw total fibre. In an embodiment, the *Brassicaceae* product comprises about 32 g/100gdw total fibre.

In an embodiment, the *Brassicaceae* product comprises an ORAC antioxidant capacity of about 14000 $\mu\text{mol TE}/100 \text{ gdw}$ to about 19000 $\mu\text{mol TE}/100 \text{ gdw}$. In an embodiment, the *Brassicaceae* product comprises an ORAC antioxidant capacity of about 14000 $\mu\text{mol TE}/100 \text{ gdw}$. In an embodiment, the *Brassicaceae* product comprises an ORAC antioxidant capacity of about 15000 $\mu\text{mol TE}/100 \text{ gdw}$. In an embodiment, the *Brassicaceae* product comprises an ORAC antioxidant capacity of about 16000 $\mu\text{mol TE}/100 \text{ gdw}$. In an embodiment, the *Brassicaceae* product comprises an ORAC antioxidant capacity of about 17000 $\mu\text{mol TE}/100 \text{ gdw}$. In an embodiment, the *Brassicaceae* product comprises an ORAC antioxidant capacity of about 18000 $\mu\text{mol TE}/100 \text{ gdw}$. In an embodiment, the *Brassicaceae* product comprises an ORAC antioxidant capacity of about 18695 $\mu\text{mol TE}/100 \text{ gdw}$. In an embodiment, the *Brassicaceae* product comprises an ORAC antioxidant capacity of about 19000 $\mu\text{mol TE}/100 \text{ gdw}$.

In an embodiment, the *Brassicaceae* product comprises a total polyphenol content of about 1750 mg GAE/100gdw to about 2600 mg GAE/100gdw. In an embodiment, the *Brassicaceae* product comprises a total polyphenol content of about 1750 mg GAE/100gdw. In an embodiment, the *Brassicaceae* product comprises a total polyphenol content of about 2000 mg GAE/100gdw. In an embodiment, the *Brassicaceae* product comprises a total polyphenol content of about 2100 mg GAE/100gdw. In an embodiment, the *Brassicaceae* product comprises a total polyphenol content of about 2200 mg GAE/100gdw. In an embodiment, the *Brassicaceae* product comprises a total polyphenol content of about 2300 mg GAE/100gdw. In an embodiment, the *Brassicaceae* product comprises a total polyphenol content of about 2360 mg GAE/100gdw.

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

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

embodiment, the *Brassicaceae* product comprises a total protein content of about 32 g/100gdw.

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

- 5 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;
- 10 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 *Brassicaceae* product is produced from broccoli.

In an embodiment, *Brassicaceae* product increases the production of one or more SCFA in the gastrointestinal tract in the subject. In an embodiment, the *Brassicaceae* product increases the production of one or more SCFA in the lower gastrointestinal tract of the subject. In an embodiment, the *Brassicaceae* product increases the production of one or more SCFA in the colon of the subject. In an embodiment, production of one or more SCFA is increased relative to an unfermented *Brassicaceae* product.

In an embodiment, the total SCFA level is increased about 30% to about 70% compared to administration of unfermented *Brassicaceae*. In an embodiment, the total SCFA level is increased about 38% to about 65% compared to administration of unfermented *Brassicaceae*. In an embodiment, the total SCFA level is increased about 40% to about 60% compared to administration of unfermented *Brassicaceae*. In an embodiment, the total SCFA level is increased about 40% to about 55% compared to administration of unfermented *Brassicaceae*.

In an embodiment, the butyrate level is increased about 30% to about 70% compared to administration of unfermented *Brassicaceae*. In an embodiment, the butyrate is increased about 38% to about 65% compared to administration of unfermented *Brassicaceae*. In an embodiment, the butyrate level is increased about 40% to about 60% compared to administration of unfermented *Brassicaceae*. In an embodiment, the butyrate level is increased about 40% to about 55% compared to administration of unfermented *Brassicaceae*.

In an embodiment, the propionate level is increased about 30% to about 70% compared to administration of unfermented *Brassicaceae*. In an embodiment, the propionate is increased about 38% to about 65% compared to administration of unfermented *Brassicaceae*. In an embodiment, the propionate level is increased about

40% to about 60% compared to administration of unfermented *Brassicaceae*. In an embodiment, the propionate level is increased about 40% to about 55% compared to administration of unfermented *Brassicaceae*.

5 In an embodiment, the acetate level is increased about 30% to about 70% compared to administration of unfermented *Brassicaceae*. In an embodiment, the acetate is increased about 38% to about 65% compared to administration of unfermented *Brassicaceae*. In an embodiment, the acetate level is increased about 40% to about 60% compared to administration of unfermented *Brassicaceae*. In an embodiment, the acetate level is increased about 40% to about 55% compared to administration of unfermented
10 *Brassicaceae*.

In an embodiment, the *Brassicaceae* product increases the SCFA level about 5 to about 48 hours after administration. In an embodiment, the *Brassicaceae* product increases the SCFA level about 10 to about 24 hours after administration.

The *Brassicaceae* products as described herein can comprise a live probiotic as
15 described herein. In an embodiment, fermentation of the *Brassicaceae* product as described herein increases the stability of the probiotic in the composition compared to a probiotic in an unfermented *Brassicaceae* product. In an embodiment, the fatty acid as described herein in the *Brassicaceae* product as described herein increases the stability of the probiotic in the *Brassicaceae* product compared to *Brassicaceae* product lacking
20 an added fatty acid.

In an embodiment, the probiotic is a *Bifidobacterim lactis*. In an embodiment, the probiotic is a *Bifidobacterim anamalis*.

The *Brassicaceae* products as described herein can comprise live lactic acid bacteria which can aid the conversion of glucosinolate present in the *Brassicaceae*
25 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*.

In an embodiment, the *Brassicaceae* product comprises lactic acid bacteria at a
30 concentration of at least about 10^2 CFU/g. In an embodiment, the *Brassicaceae* product comprises lactic acid bacteria at a concentration of at least about 10^2 CFU/g. In an embodiment, the *Brassicaceae* product comprises lactic acid bacteria at a concentration of at least about 10^5 CFU/g. In an embodiment, the *Brassicaceae* product comprises lactic acid bacteria at a concentration of at least about 10^6 CFU/g. In an embodiment, the
35 *Brassicaceae* product comprises lactic acid bacteria at a concentration of at least about 10^7 CFU/g. In an embodiment, the *Brassicaceae* product comprises lactic acid bacteria

at a concentration of at least about 10^8 CFU/g. In an embodiment, the *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* product at least 40 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are present in the *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* product at least 70 days when stored at about 4°C to about 25°C. In an embodiment, live lactic acid bacteria are present in the *Brassicaceae* 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 *Brassicaceae* 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 *Brassicaceae* product at least 90 days when 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.

In an embodiment, the *Brassicaceae* product comprises one or more fatty acids or oils as described herein. In an embodiment, the one or more fatty acids or oils are resistant to oxygen degradation. In an embodiment, the one or more fatty acids or oils has a longer IP compared the one or more fatty acids or oils in a non-fermented *Brassicaceae* product. In an embodiment, the one or more fatty acids or oils has a longer IP compared the one or more fatty acids or oils in a non-fermented *Brassicaceae* product, when the oil is added prior to fermentation.

As used herein, the term “resistant to oxygen degradation”, “resistant to degradation by oxygen” or similar phrases, refers to reducing the susceptibility of a fatty acid or an oil to oxidation. In an embodiment, the susceptibility of the fatty acid or oil to oxidation is reduced by entrapping or encapsulating the substance to reduce exposure to oxygen. In an embodiment, this includes entrapping or encapsulating the substance with molecules with oxygen sequestration ability. Assessment of oxidative resistance

may be performed by any method known to a person skilled in the art. For example, the oxidative resistance of a fatty acid or an oil may be based on the oxidation of oil with oxygen under pressure. In such a test, the consumption of oxygen, results in a pressure drop during the test which is due to the uptake of oxygen by the sample during oxidation.

- 5 The oxidation rate is accelerated when carried out at elevated pressure and temperature. In an embodiment, the oxidative resistance is assessed using an Oxipres (e.g. a Mikrolab Aarhus A/S apparatus Højbjerg, Denmark). In an embodiment, an emulsion, suspension and/or powder containing a fatty acid or an oil (e.g. polyunsaturated oils) is exposed to high temperature and high oxygen pressure. In an embodiment, the oxidative resistance
- 10 is assessed at 80°C and 5 bar initial oxygen pressure. In an embodiment, the induction period (IP, h) is determined, which is related to oxidative stability of the samples. A longer IP (h) indicates that the sample is more resistant (more stable in the presence of oxygen) to oxidation during storage. Other methods for measuring oxidation include, for example, peroxide value, para-anisidine value and headspace analysis of volatiles (eg
- 15 aldehydes such as propanal and EE-2,4-heptadienal which are secondary oxidation products from oxidation of omega-3 fatty acids) and change in % individual unsaturated fatty acids (e.g. EPA and DHA) in stored samples.

- In an embodiment, the *Brassicaceae* product comprises one or more bacteriocin/s produced by lactic acid bacteria. In an embodiment, the bacteriocin is a Class I
- 20 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).

- In an embodiment, the *Brassicaceae* product is a food product. In an embodiment, the *Brassicaceae* product is a nutraceutical. In an embodiment, the *Brassicaceae* product
- 25 is a supplement. In an embodiment, the *Brassicaceae* product is a food ingredient. In an embodiment, the *Brassicaceae* product is a probiotic. In an embodiment, the *Brassicaceae* product is an animal feed. In an embodiment, the *Brassicaceae* product is an animal feed is in aquaculture feed. In an embodiment, the *Brassicaceae* product may be added to an animal feed e.g. Novacq prawn feed (CSIRO). The animal can be an
- 30 aquatic animal such as fish, prawns or livestock. In an embodiment, the *Brassicaceae* product is a pesticide. In an embodiment, the *Brassicaceae* product is a cosmeceutical. In an embodiment, the *Brassicaceae* product is topically formulated.

- In an embodiment, the *Brassicaceae* product is a solid, liquid, emulsion, capsule, tablet, pill, puree or a powder. In an embodiment, the *Brassicaceae* product is dried to a
- 35 powder after fermentation. In an embodiment, the *Brassicaceae* product is freeze dried after fermentation. In an embodiment, the *Brassicaceae* product is microencapsulated as

described in WO2005030229 after fermentation. In an embodiment, the *Brassicaceae* product is formulated as a pill.

In an embodiment, the *Brassicaceae* product as described herein may be microencapsulated as described in WO0174175. In an embodiment, the compositions as described herein may be microencapsulated as described in WO2014169315.

Compositions

The present invention provides compositions comprising a fermented *Brassicaceae* product. In an embodiment, the composition is a food product. In an embodiment, the composition is a nutraceutical. In an embodiment, the composition is a supplement. In an embodiment, the composition is a food ingredient. In an embodiment, the composition comprises a prebiotic. In an embodiment, the composition comprises a prebiotic and a probiotic. In an embodiment, the composition is an animal feed. The animal can be an aquatic animal such as fish, prawns or livestock. In an embodiment, the composition is a pharmaceutical composition. In an embodiment, the composition is an emulsion or a suspension. In an embodiment, the pharmaceutical composition comprises one or more pharmaceutically acceptable excipients. Suitable excipients include, for example, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methylcellulose, microcrystalline cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose; or others such as: polyvinylpyrrolidone (PVP or povidone) or calcium phosphate. In an embodiment, the pharmaceutical compositions as described herein may comprise one or more further active ingredients.

Delivery vehicle

In an embodiment, the present invention provides a vehicle for delivering a bioactive to a subject, wherein the vehicle comprises a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

In an embodiment, the *Brassicaceae* product comprises one or more of: i) a prebiotic, ii) a prebiotic and a probiotic, and iii) a prebiotic and a probiotic which are synbiotic

In an embodiment, the delivery vehicle stabilizes (reduces the degradation or loss) of a bioactive during storage and/or delivery. In an embodiment, the delivery vehicle, in

instances where the bioactive is a live microorganism (e.g. a probiotic), improves the viability of the microorganism compared to the microorganism administered without the delivery vehicle.

In an embodiment, the bioactive is selected from one or more or all of:

- 5 i) a fatty acid, ii) oil, iii) a further prebiotic, and iv) a further probiotic.

Examples of fatty acid and oils are described in the “addition of fatty acid and/or oil” section of the specification.

- 10 In an embodiment, the further prebiotic is selected from one or more or all of: dietary fibre, oligosaccharides, exopolysaccharides, oligofructose, cellulose, hemicellulose resistant starch, beta-glucans pectin, inulin and dextran.

In an embodiment, the oligosaccharides are selected from one or more or all of: gluco-oligosaccharides fructo-oligosaccharides galacto-oligosaccharide, pecticoligosaccharide trans-galacto-oligosaccharides.

- 15 In an embodiment, the exopolysaccharides are homopolysaccharides and/or heteropolysaccharides.

In an embodiment, the vehicle improves the viability of a probiotic. In an embodiment, the vehicle improves the viability of the further probiotic.

- 20 As used herein, “viability” refers to a probiotics ability to survive or live successfully. An improvement in the viability of the probiotic can be an improvement during storage (e.g. an increase in hrs or days the probiotic can be stored before use) and/or improvement during delivery of the probiotic to another organism. In an embodiment, an improvement in the viability of the probiotic refers to an increase in viability of the probiotic when passing through the upper gastrointestinal tract. In an embodiment, the viability is increased by about 0.5 log to about 5 log compared to delivery without the vehicle. In an embodiment, the viability is increased by about 0.5 log to about 4 log compared to delivery without the vehicle. In an embodiment, the viability is increased by about 0.5 log to about 3 log compared to delivery without the vehicle. In an embodiment, an improvement in the viability if the probiotic refers to an increase in the delivery of the probiotic to the lower gastrointestinal tract. In an embodiment, delivery of the probiotic to the lower gastrointestinal tract is increased by 0.5 log to about 5 log compared to delivery without the vehicle. In an embodiment, delivery of the probiotic to the lower gastrointestinal tract is increased by 0.5 log to about 4 log compared to delivery without the vehicle. In an embodiment, delivery of the probiotic to the lower gastrointestinal tract is increased by 0.5 log to about 3 log compared to delivery without the vehicle.
- 35

In an embodiment, the vehicle protects the probiotic during passage through the upper gastrointestinal tract. As used herein “protects” or “protecting” refers to reducing the susceptibility of a probiotic to damage and/or death caused by exposure to gastrointestinal digestive enzyme or digestive juices during passage through the upper gastrointestinal tract. In an embodiment, protects refers to reducing the susceptibility of a probiotic to damage or death caused by gastric enzymes, gastric juices and/or bile during passage through the upper gastrointestinal tract. In an embodiment, protecting the probiotic during passage through the upper gastrointestinal tract increases the amount of viable probiotic delivered to the lower gastrointestinal tract.

In an embodiment, the probiotic or further probiotic is autochthonous to the *Brassicaceae* material. In an embodiment, the probiotic or further probiotic is an autochthonous probiotic is present on the *Brassicaceae* material before fermentation. In an embodiment, the probiotic or further probiotic is an allochthonous probiotic added to the *Brassicaceae* material after fermentation.

In an embodiment, the probiotic or further probiotic is selected from one or more or all of: lactic acid bacteria, *Bifidobacteria*, *Bacteroidetes*, *Bacillus*, *Streptococcus*, *Escherichia*, *Enterococcus*, *Saccharomyces*.

In an embodiment, the lactic acid bacteria is selected from one or more of the genera selected from: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus* and *Weissella*. In an embodiment, the lactic acid bacteria is selected from one or more or all of: *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactobacillus rhamnosus*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus lactis*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, *Pediococcus pentosaceus* and *Pediococcus acidilactis*. In an embodiment, the lactic acid bacteria is selected from one or more or all of: 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 vii) B5 deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia.

In embodiment, the *Bifidobacteria* is selected from one or more of: *Bifidobacteria adolescentis*, *Bifidobacteria animalis*, *Bifidobacteria bifidum*, *Bifidobacteria breve*,
5 *Bifidobacteria infantis*, *Bifidobacteria longum*, and *Bifidobacteria thermophilum*.

In embodiment, the *Baciullus* is selected from one or more of: *Baciullus cereus*, *Baciullus clausii*, *Baciullus coagulans*, *Baciullus licheniformis*, *Baciullus pumulis* and *Baciullus subtilis*.

10 In embodiment, the *Streptococcus* is *Streptococcus thermophiles*. In embodiment, the *Escherichia* is beneficial strain of *Escherichia coli*.

In embodiment, the *Enterococcus* is *Enterococcus faecium*.

In embodiment, the *Saccharomyces* is *Saccharomyces cerevisiae*

Administration

15 A variety of routes of administration are possible for the methods, compositions and delivery vehicles as described herein, including but not limited to enteral, dietary, parenteral, and topically. In an embodiment, the *Brassicaceae* product is administered enterally. As used herein “enterally” or “enteral” comprises passing through the gastrointestinal tract. In an embodiment, enteral administration comprises oral
20 administration. In an embodiment, enteral administration comprises rectal administration. In an embodiment, rectal administration may be selected from one or more of: suppository, enema, via colonoscope or other medical equipment and faecal transplantation. In an embodiment, the *Brassicaceae* product as described herein is administered parenterally. In an embodiment, the *Brassicaceae* product as described
25 herein is administered topically.

In an embodiment, the present invention provides a faecal microbiota suitable for transplantation into a subject, wherein the faecal microbiota was isolated from a subject administered a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the
30 *Brassicaceae* product was pre-treated prior to fermentation.

In an embodiment, the present invention provides a digesta microbiota suitable for transplantation into a subject, wherein the faecal microbiota was isolated from a subject administered a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or
35 the *Brassicaceae* product was pre-treated prior to fermentation.

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:

- LP: *Lactobacillus plantarum* ATCC8014;
- 15 LGG: *Lactobacillus rhamnosus* ATCC53103;
- B1: *Lactobacillus plantarum* isolated from broccoli deposited under V17/021731 on 25 September 2017 at the National Measurement Institute Australia;
- B2: *Lactobacillus plantarum* isolated from broccoli deposited under V17/021732 on 25 September 2017 at the National Measurement Institute Australia;
- 20 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;
- B5: *Lactobacillus plantarum* isolated from broccoli deposited under V17/021735 on 25 September 2017 at the National Measurement Institute Australia;
- 25 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;
- 30 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

5 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
10 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.

15 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

20 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
25 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
30 proportion and stored with glycerol at -80°C until use as a mixed starter culture for broccoli fermentation.

Fermentation method

35 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 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 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 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

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 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.

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). 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.

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 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:

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

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

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
10 Dumas combustion method with LECO TruMac apparatus (LECO Corporation, 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
15 calculated according to the following equation:

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

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

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
25 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
30 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.

Oxygen radical absorbance capacity assay

- Freeze-dried broccoli powder (10 mg) was suspended in 10 mL of methanol/water
35 (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.

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 5 µL.

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 µM 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 µL, 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 µm 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 (µmol/kg DW) of broccoli.

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).

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

5 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
10 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
15 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, the LAB count in
the samples stored at 4°C remained high (6 log CFU/g) even after storage for 84 days.

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 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 ~10⁶ 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 10⁸ 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.

- 5 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.

10 **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

- 15 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.

5 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
10 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
15 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
20 (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
25 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
30 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
35 significantly increased the ORAC value by ~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 µmol TE/g, respectively. Similar results were obtained for samples stored at 4°C. The ORAC value was 172.0±15.5 µmol TE/g at the beginning of storage, which increased to a maximum value of (188.7±12.9 µmol TE/g) after storage.

10 **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 10⁷ CFU/g or 10⁸ 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.

25 **Table 4: Composition of the fermented broccoli product.**

Quality attributes	Value
Total fibre	~29.5g/100gdw
ORAC antioxidant capacity	18695 µmol TE/100 gdw
Total polyphenol content	2369 mg GAE/100gdw
Total titratable acidity	1.1% lactic acid equiv.
Lactic acid bacteria count	~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 ± 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 ± 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 ± 39.7 $\mu\text{mol/kg}$ (Figure 7). After fermentation, the glucoraphanin content sharply decreased to 712.4 ± 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

5 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
10 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
15 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
20 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
25 broccoli puree, after mixing well, was aliquoted into sterile plastic containers (100 mL) with screw lids (Technoplast Australia) for further experiments.

Fermentation

Broccoli puree samples (pre-heated and untreated) were inoculated with the LAB
30 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 experiments were conducted on pre-heated and untreated broccoli puree using glucono-
35 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.

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 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 analyses.

Sulforaphane analysis and statistical analysis

Was performed as described in Example 1.

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 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±240.7µmol/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±35.1µmol/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
5 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
10 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
15 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
20 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
25 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
30 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 (~6%) was observed during storage at 4°C whereas the decline during storage at 25°C was ~70%.

Table 6. Sulforaphane yield (μmol/Kg DW) of broccoli before and after processing.

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

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

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.

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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

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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%).

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 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.

5 **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-dehydroatesterone	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
glucomalcommen	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-riboseylaminopyrimidin-43H-one	0.3594	-1.4763	0.020453	1.6893
S-4-hydroxymandelonitrile	0.32867	-1.6053	0.00375	2.426
22alpha-hydroxy-campester-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-coumaroyltriacytic acid lactone	0.18436	-2.4394	0.0122	1.9136
6"-O-Acetyldaidzin	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
glucoiberverin	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-geranylinalool	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- α -D-manno-octulosonate	6.6012	2.7227	0.027652	1.5583
Anhydro-secoisolaricresinol	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
3 β -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-pentonate	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	2.7008	1.4334	0.048201	1.3169
7-O-rhamnoside	6.3398	2.6644	0.049365	1.3066
pheophorbide a	4.8949	2.2913	0.049727	1.3034
Chrysoeriol 7-O-6"-malonyl-glucoside	10.972	3.4557	0.050008	1.301
allantoate	12.072	3.5936	0.052404	1.2806
Ligstroside-aglycone	3.4926	1.8043	0.052645	1.2786
cycloeucalenone	3.5807	1.8403	0.053727	1.2698
Unidentified metabolite No. 3	0.42811	-1.224	0.05399	1.2677
laricitrin	11.488	3.5221	0.05455	1.2632
Sulfadimethoxine	5.2839	2.4016	0.054583	1.2629
3,4-Diferuloylquinic acid	0.47952	-1.0603	0.054637	1.2625
glucotropeolin	5.2663	2.3968	0.055218	1.2579
5,6-dihydroxyindole-2-carboxylate	2.8697	1.5209	0.055638	1.2546
S-laudanine	0.39854	-1.3272	0.057257	1.2422
L-nicotianamine	0.30202	-1.7273	0.057551	1.2399
5-methylthiopentyl-thiohydroximate	2.6643	1.4138	0.05785	1.2377
aldehydo-D-galacturonate	0.34888	-1.5192	0.058188	1.2352
R-mevalonate 5-phosphate	2.142	1.099	0.05845	1.2332
6-Hydroxyluteolin 7-O-rhamnoside	3.5705	1.8361	0.061441	1.2115
L-aspartate	2.4481	1.2916	0.063269	1.1988
--Epicatechin 3-O-gallate	0.23586	-2.084	0.065585	1.1832
glycine	2.4077	1.2677	0.065876	1.1813
Episesaminol	3.7782	1.9177	0.068376	1.1651
6alpha-hydroxy-castasterone	11.846	3.5664	0.070966	1.149
alpha-D-galacturonate 1-phosphate	2.995	1.5825	0.071057	1.1484
R-2,3-dihydroxy-3-methylpentanoate	2.0686	1.0487	0.07128	1.147
cyanidin-3-O-beta-D-glucoside	3.7463	1.9054	0.07247	1.1398
D-erythrose 4-phosphate	617.84	9.2711	0.073728	1.1324
CDP-choline	2.0623	1.0442	0.074004	1.1307
adenine	5.5593	2.4749	0.074387	1.1285
raphanusamate	2.4046	1.2658	0.075102	1.1243
3-Methoxysinensetin	3.5234	1.817	0.075291	1.1233
betaine aldehyde	2.2972	1.1999	0.076906	1.114
E-7-methylthioheptanaldoxime	5.5473	2.4718	0.077579	1.1103
6-methylthiohexyl-thiohydroximate	0.16741	-2.5786	0.080677	1.0933
6"-O-Malonylglycitin	2.0677	1.048	0.081844	1.087
monodehydroascorbate radical	3.0289	1.5988	0.082088	1.0857
anthranilate	0.43234	-1.2098	0.082209	1.0851
Hydroxycaffeic acid	2.3978	1.2617	0.086518	1.0629
Myricetin 3-O-arabinoside	0.18331	-2.4477	0.088998	1.0506
cis-aconitate				

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 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 12). Decreases in protocatechuic acid, gallic acid, 4-hydroxybenzoic acid, vanillic acid, 2,3-dihydroxybenzoic acid, p-coumaric 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 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

10⁴ 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 x 10⁸ cfu/mL in NB overnight at 37°C, static. The cultures were combined (1 mL of each) and combined culture diluted to 10⁴ 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.

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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 10^3 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

- 5 **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
- 10 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

- 15 **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
- 20 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 Dalmasso (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*

- 5 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.

10 *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.

15

Day 5

Gels stained ~30 min in GelRed, destained, visualised

Results

- 20 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).

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

25

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.

30

Example command was :

```
snippy --cpus 24 --outdir B5 --ref B1_S1mod.gbk --pe1
B5_S17_L001_R1_001.fastq.gz --pe2 B5_S17_L001_R2_001.fastq.gz
```

35

Calculated individual comparisons and core using B1 gbk as reference
snippy-core --prefix core B1 B2 B3 B4 B5

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.

Results

10 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
15 which are summarized in Table 19.

Example 17 - Short chain fatty acid assessment in an *in vitro* colonic fermentation model

Samples

20 Raw: untreated broccoli (blended, and freeze dried into powder with no fermentation). Broccoli florets were homogenized with water (3 parts broccoli to 2 parts of water) for 1 min using a kitchen scale magic bullet blender (Nutribullet pro 900 series, LLC, USA).

Raw fermented: raw broccoli which has been fermented and then freeze dried.

25 Preheat fermented: broccoli that has been subject to a heat pre-treatment prior to fermentation and freeze drying. Broccoli florets were cut at approximately 2 cm below the head and packed in retort pouches, sealed and pre-heated in a thermostated water batch maintained at 65°C (Core temperature 65°C for 3 min), and immediately following the heat treatment, the samples were cooled in ice water and homogenised as above and
30 the homogenized samples were incubated in dark for 4 h at 25 °C.

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	JBMIHLAL_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	JBMIHLAL_00337	lacR_1
68529	del	TATTAATG GCTCGCGT CATTAA	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	JBMIHLAL_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	JBMIHLAL_00353	
72030	del	CGCTCAAC CAGATTAG TACCCAG	CG	CG:91 CGCTCAACACAGAT TAGTACCCAG:0	CDS	-	978/996	320/331	inframe_deletion c.958_978delCTGGGT ACTAATCTGGTTGAG p.Leu320_Glu326del	JBMIHLAL_00354	lacR_3
136221	snp	C	A	A:178 C:1	CDS	-	559/127 2	187/423	missense_variant c.559G>T p.Ala187Ser	JBMIHLAL_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	JBMIHLAL_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	JBMIHLAL_01118	VjJP_1
188850	snp	A	C	C:241 A:0	CDS	-	491/161 7	164/538	missense_variant c.491T>G p.Ile164Ser	JBMIHLAL_01179	oppA_2
2322	snp	A	G	G:107 A:1	CDS	-	397/474	133/157	missense_variant c.397T>C p.Phe133Leu	JBMIHLAL_01186	adcR
111662	ins	CAA	CAAA	CAAA:133 CAA:11	CDS	+	10/876	4/291	frameshift_variant c.9dupA p.Ser4fs	JBMIHLAL_01302	mntB

11376	snp	G	A	A:115 G:0	CDS	-	1831/19 47	611/648	synonymous_variant c.1831C>T p.Leu611Leu	JBIMIHLAL_01356	
115510	snp	G	A	A:199 G:1	CDS	-	95/411	32/136	missense_variant c.95C>T p.Thr32Ile	JBIMIHLAL_01453	
143457	snp	G	C	C:264 G:0	CDS	+	1122/14 16	374/471	synonymous_variant c.1122G>C p.Val374Val	JBIMIHLAL_01479	pepD
111973	snp	G	A	A:118 G:1	CDS	-	731/131 7	244/438	missense_variant c.731C>T p.Ala244Val	JBIMIHLAL_01603	murA1
27553	snp	C	T	T:104 C:1	CDS	-	472/109 2	158/363	missense_variant c.472G>A p.Gly158Ser	JBIMIHLAL_01677	wbnH
80888	snp	T	C	C:84 T:0	CDS	+	256/258	86/85	stop_lost&splice_region_variant c.256T>C p.Ter86Glnext*?	JBIMIHLAL_01727	ytIR_1
133147	snp	A	C	C:76 A:0	CDS	-	443/663	148/220	missense_variant c.443T>G p.Phe148Cys	JBIMIHLAL_01777	yjbM
74711	snp	C	T	T:212 C:1	CDS	+	874/138 9	292/462	missense_variant c.874C>T p.Leu292Phe	JBIMIHLAL_01855	murF_2
19793	snp	T	C	C:114 T:1	CDS	-	925/110 7	309/368	missense_variant c.925A>G p.Asn309Asp	JBIMIHLAL_01907	sigA
60643	snp	C	T	T:89 C:1	CDS	-	242/186 9	81/622	missense_variant c.242G>A p.Ser81Asn	JBIMIHLAL_01945	dnaK
10806	ins	GTTTTTTT G	GTTTTTTT G	GTTTTTTTGG:49 GTTTTTTTG:1							
50276	complex	CG	CACCACCAGG CCGATTGTGG CGA	CACCACCAGCCG ATTGTGGCGA:39 CG:0	CDS	-	341/555	114/184	missense_variant&inframe_insertion c.341delCinsTCGCCAC AATCGGCGCTGGTGGT p.Ala114delInsValAlaThrIleGlyLeuValVal	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	-	2516/26 04	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	-	1312/1728	438/575	missense_variant c.1312A>C p.Lys438Gln	JBIMIHLAL_02365	pgcA
21706	ins	TAT	TAAT	TAAT:122 TAT:1	CDS	+	872/2604	291/867	frameshift_variant c.871dupA p.Ile291fs	JBIMIHLAL_02489	mprF
29454	del	TGA	TA	TA:73 TGA:0	CDS	+	94/132	32/43	frameshift_variant c.94delG p.Asp32fs	JBIMIHLAL_02559	
27619	snp	A	G	G:134 A:1	CDS	-	78/588	26/195	synonymous_variant c.78T>C p.Gly26Gly	JBIMIHLAL_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	JBIMIHLAL_02963	tcaR
19068	del	CTTGCCGA AATTCGAC AAACAACC CTCGGATT GT	CT	CT:51 CTTGCCGAAATTC GACAAACAACCT CGGATTGT:0	CDS	+	154/564	52/187	frameshift_variant c.154_185delGAAATT CGACAAACAACCTCG GATTGTTGCC p.Glu52fs	JBIMIHLAL_02974	
17533	ins	ATTTTITG	ATTTTITG	ATTTTITG:220 ATTTITG:2							

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

POS	TYPE	REF	ALT	EVIDENCE	FTYPE	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	JBIMIHLAL_02984	

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

POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	NT_POS	AA_P OS	EFFECT	LOCUS_TAG	GENE
4326	del	TATAAAAAAGCG ACCCCGTTCATTA ACGGTGCCGCTCA CAGATCATTATTAG TGAAAAATCACCCG GCA	TA	TA:31 TATAAAAAAGCGACC CCCGTTCATTAAACGGT GCCGCTCACAGATCAT TATTAGTGAAATATCAC 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	

Table 16. Polymorphism identified by variant analysis B4 compared to B1.

POS	TYPE	REF	ALT	EVIDENCE	FTYPE E	STRAND	NT_POS	AA_P OS	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 E	STRAND	NT_POS	AA_P OS	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	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	LEUM_0777	
796683	del	GCC	GC	GC:207 GCC:0	CDS	+	2986/3009	996/1002	frameshift_variant p.Glu997fs	LEUM_0814	
953160	snp	G	T	T:178 G:0	CDS	+	805/843	269/280	missense_variant p.Ala269Ser	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	FTV 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.IleSer211IleSer	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 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 p.ThrAsp68AlaAsn	LEUM_0076	
70144	GGTATGG GATGGGA	CGTATGGG A	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 p.ValAsn330ValAsn	LEUM_0153	
138092	AACT	GACC	GACC:278 AACT:0	CDS	-	975/1431	324/476	synonymous_variant 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	CAAAAAAG	CAAAAAAA	CAAAAAAA:178 CAAAAAAG: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	gitX
162834	TGAT	GGAC	GGAC:260 TGAT:0	CDS	+	2400/248 1	800/826	missense_variant c.2400_2403delTGATinsGGAC p.AspAsp800GluAsp	LEUM_0185	
192260	ATAAA	GTAAC	GTAAC:301 ATAAA: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 c.52_55delATAGinsGTAT p.IleAla18ValSer	LEUM_0234	
196918	AATA	GATG	GATG:246 AATA:0					synonymous_variant c.108_111delCAGGinsTACC p.AspThr36AspThr	LEUM_0256	nrdF
216494	CACG	TACC	TACC:230 CACG:0	CDS	+	108/978	36/325	missense_variant c.553_557delATCTCinsGTCTT p.IleSer185ValLeu	LEUM_0276	
231792	ATCTC	GTCTT	GTCTT:235 ATCTC:0	CDS	+	553/1728	185/575	synonymous_variant c.573_576delGCTCinsACTT p.AlaLeu191AlaLeu	LEUM_0276	
231812	GCTC	ACTT	ACTT:229 GCTC:0	CDS	+	573/1728	191/575	synonymous_variant c.573_576delGCTCinsACTT p.AlaLeu191AlaLeu	LEUM_0276	
234250	ACTT	CCTG	CCTG:217 ACTT:0	CDS	+	336/642	112/213	synonymous_variant c.336_339delACTTinsCCTG p.GlyLeu112GlyLeu	LEUM_0279	tmk
242029	CTAT	TTAC	TTAC:265 CTAT:0	CDS	-	664/966	221/321	missense_variant c.661_664delATAGinsGTAA p.IleAla21ValThr	LEUM_0287	
244287	GACT	AACC	AACC:251 GACT:0	CDS	+	1436/196 2	479/653	missense_variant c.1436_1439delGACTinsAACC p.ArgLeu479LysPro	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 c.358_360delTTAinsCTG p.Leu120Leu	LEUM_0318	
288308	ATA	AC	AC:232 ATA:0							
318676	GATTAG	AATCAA	AATCAA:121 GATTAG:0	CDS	+	14/306	5/101	missense_variant c.14_19delGATTAGinsAATCAA p.GlyLeuVal5GluSerIle	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 c.957_961delACATCinsGCATT p.LysHisLeu319LysHisLeu	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 c.1032_1038delGTTGGCCinsATTAGCA p.LysLeuAla344LysLeuAla	LEUM_0406	lysS
366971	ATTGTGA	GTTGCGT	GTTGCGT:225 ATTGTGA:0	CDS	+	1107/148 8	369/495	synonymous_variant c.1107_1113delATTGTGTAinsGTTGCGT p.GluPheVal369GluPheVal	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_224delTGGTinsCGGC p.MetVal74ThrAla	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	-	1922/208 8	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_144delGCGinsACA p.Ala48Thr	LEUM_0524	
493487	TGGT	CGGC	CGGC:189 TGGT:0	CDS	+	168/834	56/277	synonymous_variant c.168_171delTGGTinsCGGC 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	+	1776/203 1	592/676	synonymous_variant c.1776_1779delCGAAinsTGAG p.ValGlu592ValGlu	LEUM_0536	

502272	CATTC	TCTCT	TCTCT:187 CATTC:0	CDS	+	1794/203 1	598/676	missense_variant c.1794_1798delCATTCinsTCTCT p.Phe11eLeu598Phe11eLeu	LEUM_0536	
502291	TTG	CTA	CTA:215 TTG:0	CDS	+	1813/203 1	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_507delGTGGTTinsTTGGC p.SerGly168IleGly	LEUM_0543	
508582	TGCT	CGCG	CGCG:163 TGCT:0	CDS	+	861/1008	287/335	synonymous_variant c.861_864delTGCTinsCCGC 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	GTCATA	ATCTTG	ATCTTG:158 GTCATA:0	CDS	+	1549/186 6	517/621	missense_variant c.1549_1554delGTCATinsATCTTG p.Val1517IleLeu	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_675delAATinsGAC 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	CAGCTGCAAGTG:12 7 TAGCTGCAAGTGCT GCAAGTG:0	CDS	+	854/1464	285/487	missense_variant&inframe_deletion c.854_864delTAGCTGCAAGTinsCA 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_1107delGACGinsAACCA 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.AspPhe199AsnPhe	LEUM_0686	
658429	TCG	GCA	GCA:147 TCG:0	CDS	+	622/4314	208/1437	missense_variant c.622_624delTCCGinsGCA 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 c.2160_2162delAAATinsCAG p.Leulle720PheSer	LEUM_0723	
700692	ACCC	CCCT	CCCT:206 ACCC:0	CDS	+	378/1527	126/508	synonymous_variant c.378_381delACCCinsCCCT p.GlyPro126GlyPro	LEUM_0727	purH
700713	AGCT	TGCC	TGCC:209 AGCT:0	CDS	+	399/1527	133/508	synonymous_variant c.399_402delAGCTinsTGCC p.AlaAla133AlaAla	LEUM_0727	purH
701025	CGGCAAA	TGGTAAG	TGGTAAG:121 CGGCAAA:0	CDS	+	711/1527	237/508	synonymous_variant c.711_717delCGGCAAAinsTGGTAAG p.HisGlyLys237HisGlyLys	LEUM_0727	purH
723536	CACTG	TACTC	TACTC:162 CACTG:0	CDS	+	326/534	109/177	missense_variant c.326_330delCACTGinsTACTC p.ThrLeu109IleLeu	LEUM_0746	
726007	ATAAA	TTTAT	TTTAT:130 ATAAA:0							
745561	ATAAT	GTAAC	GTAAC:87 ATAAT:0							
751089	ACTG	GCTA	GCTA:157 ACTG:0	CDS	+	2232/833 9	744/1112	synonymous_variant c.2232_2235delACTGinsGCTA p.GluLeu744GluLeu	LEUM_0774	
769650	GCCA	ACCG	ACCG:139 GCCA:0	CDS	-	27/834	8/277	synonymous_variant c.24_27delTGGCinsCGGT p.AspGly8AspGly	LEUM_0791	
784937	CCCG	TCCA	TCCA:96 CCCG:0	CDS	-	1608/167 4	535/557	synonymous_variant c.1605_1608delCCGGinsTGA p.IleGly535IleGly	LEUM_0807	
787928	AAACG	GAACC	GAACC:132 AAACG:0	CDS	+	1190/170 1	397/566	missense_variant c.1190_1194delAAACGinsGAACC p.GlnThr397ArgThr	LEUM_0808	

788232	TATCATC	CATCTTG	CATCTTG:120 TATCATC:0	CDS	+	1494/170 1	498/566	missense_variant c.1494_1500delTATCATCinsCATCTTG p.ThrIleIle498ThrIleLeu	LEUM_0808	
796989	ATTAGGC	GCTGGGT	GCTGGGT:149 ATTAGGC:0							
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 c.900_904delACAATinsGCAAG p.ProGlnSer300ProGlnAla	LEUM_0818	
829273	CATTAT	AAGTAC	AAGTAC:116 CATTAT:0	CDS	+	211/909	71/302	missense_variant c.211_216delCATTATinsAAGTAC p.HisTyr71LysTyr	LEUM_0842	
831087	TAGC	CAAT	CAAT:103 TAGC:0	CDS	-	408/897	135/298	synonymous_variant c.405_408delGCTAinsATTG p.ValLeu135ValLeu	LEUM_0844	
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_1175delGAGGinsCAGT 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	LEUM_0849	
838832	GGTAC	AGCAT	AGCAT:131 GGTAC:0	CDS	+	185/330	62/109	missense_variant c.185_189delGGTACinsAGCAT p.GlyTyr62GluHis	LEUM_0850	
843675	CAGATT ACG	AAAATCAAA A	AAAATCAAAA:133 CAGATTACG:0	CDS	+	256/1620	86/539	missense_variant c.256_265delCAGATTAAACGinsAAAAATCAAAA 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_663delAACGinsGACG p.ThrThr220ThrThr	LEUM_0857	
853659	ATA	GTG	GTG:201 ATA:0	CDS	+	6734/846 6	2245/282 1	missense_variant c.6734_6736delATATinsGTG 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_1514delGAGAGinsAAGG p.ArgGlu504LysGly	LEUM_0885
893725	TAA	CAG	CAG:132 TAA:0	CDS	+	1167/225 9	389/752	missense_variant c.1167_1169delTTAAinsCAG p.Alalys389AlaArg	LEUM_0894
894794	AAA	GAG	GAG:173 AAA:0	CDS	+	2236/225 9	746/752	missense_variant c.2236_2238delAAAAinsGAG p.Lys746Glu	LEUM_0894
895508	CAAG	TAAA	TAAA:112 CAAG:0	CDS	+	675/687	225/228	synonymous_variant c.675_678delCAAGinsTAAA p.IleLys225IleLys	LEUM_0895
895583	ATTAAGC G	GTCAAGTT	GTCAAGTT:92 ATTAAGCG:0	CDS	-	996/1008	330/335	missense_variant c.989_996delCGCTTAATinsAACTTGAC p.ThrLeuAsn330LysLeuAsp	LEUM_0896
895607	CGGT	TGGG	TGGG:101 CGGT:0	CDS	-	972/1008	323/335	synonymous_variant c.969_972delACCCGinsCCCCA p.ValPro323ValPro	LEUM_0896
903892	CTTTGCCT T	TTTACCTC	TTTACCTC:158 CTTTGCCTT:0	CDS	+	1215/183 9	405/612	missense_variant c.1215_1223delCTTTGCCTTinsTTTTACCTC p.AlaPheAlaLeu405AlaPheThrSer	LEUM_0901
907285 911930	GCTAC CAGC	ACTAT TAGT	ACTAT:127 GCTAC:0 TAGT:94 CAGC:0	CDS	+	39/822	13/273	synonymous_variant c.39_42delCAGCinsTAGT p.SerSer13SerSer	LEUM_0909
933210	CAGGGC	GAGCGT	GAGCGT:156 CAGGGC:0	CDS	+	1909/199 2	637/663	missense_variant c.1909_1914delCAGGGCinsGAGCGT p.GlnGly637GluArg	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 c.168_171delCATTinsTATC p.HisLe56HisLe	LEUM_0972

980203	TTAGTA	CTGGTG	CTGGTG:85 TTAGTA:0	CDS	+	220/513	74/170	synonymous_variant c.220_225delTTAGTAinsCTGGTG p.LeuVal74LeuVal	LEUM_0980	
980531	TCATTA	CAATTG	CAATTG:125 TCATTA:0							
982914	AGCT	GGCA	GGCA:58 AGCT:0	CDS	+			no annotation	LEUM_0984	
986252	GGTCC	TGCT	TGCT: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 AAAAA	ATGTCGGA AGAG	ATGTCGGAAGAG:2 5 GTTTCAGAAAAA:0	CDS	+			no annotation	LEUM_0988	
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	ATTG	ATTG: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	

[illegible]

1415294	AAGT	TAGC	TAGC:163 AAGT:0	CDS	-				no annotation	LEUM_1420	
1415554	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	GAGAA GT	GAGAA GT: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 CTTGTCA:0	CDS	-				no annotation	LEUM_1423	
1418013	GCCA	ACCG	ACCG:208 GCCA:0	CDS	-				no annotation	LEUM_1423	
1418025	GGCG	AGCA	AGCA:180 GGCG:0	CDS	-				no annotation	LEUM_1423	
1418040	TAAAGCC	CAGAGCAG	CAGAGCAGCTTC:88	CDS	-				no annotation	LEUM_1423	
1418061	TCTTG	CTTC	TAAAGCCTCTTG:0								
1418061	TTC	CTC	CTC:91 TTC:0	CDS	-				no annotation	LEUM_1423	
1418069	GACCGGC	ACCTGCG	ACCTGCG:89 GACCGGC: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	CGCG	TGCA	TGCA:197 CGCG: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	TTGCC	TTGCC:208 GTGCT:0	CDS	-				no annotation	LEUM_1424	
1419226	GTTA	ATTG	ATTG:221 GTTA:0	CDS	-				no annotation	LEUM_1424	
1419311	TGC	GCC	GCC:230 TGC:0	CDS	-				no annotation	LEUM_1424	
1419388	GCTT	ACTG	ACTG:223 GCTT:0	CDS	-				no annotation	LEUM_1424	
1419438	TTTTAG	GTTC	GTTC:162 TTTAG:0	CDS	-				no annotation	LEUM_1424	
1429917	TGGCTCC TCTATTG TCTTT	AGGCACCTT TAGTCGTTT TA	AGGCACCTT:173 GTTT:173 TGGCTCCTCTATTG TCTTT:0	CDS	-				no annotation	LEUM_1434	
1429993	TGTG	CGTA	CGTA:204 TGTG:0	CDS	-				no annotation	LEUM_1434	
1430085	AGAGT	GGAGC	GGAGC:169 AGAGT:0	CDS	-				no annotation	LEUM_1434	
1430128	GTTC	ATTA	ATTA:172 GTTC: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	GGCAGTCA	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	TTAGAAAT C	GTAGGATT	GTAGGATT:180 TTAGAAATC:0	CDS	-				no annotation	LEUM_1435	

1431011	CTTTT	TCTTTC	TCTTTC: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	ACAAT	ACAAT: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	AGATTG	AGATTG: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	CAAAAAAACAAC: 127 CAAAAAA 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	CDS	-				no annotation	LEUM_1502	
			AGATC:0								
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	AGAAC	AGAAC:112	CDS	+				no annotation	LEUM_1546	
			CAAACT:0								
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	CDS	-				no annotation	LEUM_1578	
			TACTGCA:0								
1589522	ACTGC	GCCGT	GCCGT:119	CDS	-				no annotation	LEUM_1586	
			ACTGC:0								
1622472	TTATAT	ACGTAC	ACGTAC:247	CDS	-				no annotation	LEUM_1624	
			TTATAT:0								
1624045	AGCCTAC	GCCCGAT	GCCCGAT:111	CDS	-				no annotation	LEUM_1627	
			AGCCTAC:0								
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	CDS	-					no annotation		LEUM_1628
1624234	GCGCCA	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	GCGG	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	ATAATTTG C	GTAGCTTGT	GTAGCTTGT:206 ATAATTTGC: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	TGTGTC AAAGAT	CATATTAG AGAC	CATATTAGAGAC:1 59 TGTGTCAAAGAT: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 GTTCA	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	TTTTAAAC CGTCA	CTTCAAATC ATCG	CTTCAAATCATCG:1 64 TTTTAAACCGTCA: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 GAAAGAGCCT: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	TAAACGT	TAAACGT:139 CAAGCGC:0	CDS	+				no annotation		LEUM_1690	
1665484	ACCTACC	GCCAACT	GCCAACT: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	ATCTT	ATCTT:153 GTCTC:0							
1695377	ACCG	GCCA	GCCA:273 ACCG:0	CDS	-			no annotation	LEUM_1726	
1696196	GGCCGCT AGCATG	TGCAGCCAA CATA	TGCAGCCAA:1 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							
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	
1719885	GGA	AGG	AGG:289 GGA:2	CDS	-			no annotation	LEUM_1748	
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	CAGCT:95 TAGCC:0	CDS	-				no annotation	LEUM_1752	
1726251	CAGCT	TAGCC	TAGCC	TAGCC:143 CAGCT:2	CDS	-				no annotation	LEUM_1752	
1756654	TCTAC	GCTAT	GCTAT	GCTAT:128 TCTAC:0								
1756824	ATC	GTA	GTA	GTA:145 ATC:0	CDS	-				no annotation	LEUM_1786	
1757247	GAAA	AAG	AAG	AAG:196 GAAA:0	CDS	-				no annotation	LEUM_1786	
1759552	TACT	CACC	CACC	CACC:256 TACT:0	CDS	+				no annotation	LEUM_1788	
1759606	GGCG	AGCA	AGCA	AGCA:266 GGCG:0	CDS	+				no annotation	LEUM_1788	
1760925	ACCCGAT GGGTTGT ATT	GCCACTAG GCTGCAT	GCCACTAG GCTGCAT	GCCACTAGGCTGCA T:37 ACCCGATGGGTTGT ATT:0								
1760955	CAATGA	TAAGTGG	TAAGTGG	TAAGTGG:35 CAATGA:0	CDS	-				no annotation	LEUM_1791	
1760994	GGCTGCA AACGCTG CACGCAG GCGCAGC	AGCAGCGA AAGCAGCG CGTAAACG AAGT	AGCAGCGA AAGCAGCG CGTAAACG AAGT	AGCAGCGAAAAGCAG CGCGTAAACGAAAGT :37 GGCTGCAAAACGCTG CACGCAGGCGCAGC :0	CDS	-				no annotation	LEUM_1791	
1761057	CTTGCGG	TTTGGT	TTTGGT	TTTGGT:167 CTTGCGG:0	CDS	-				no annotation	LEUM_1791	
1761069	CTGGGGT ATCAAAA CGGTTAC A	TTGTGGAAT TAATACTGT CACT	TTGTGGAAT TAATACTGT CACT	TTGTGGAATTAATAC TGTCACT:168 CTGGGGTATCAAAA CGGTTACA:0	CDS	-				no annotation	LEUM_1791	
1761096	GTTA	ATTG	ATTG	ATTG:166 GTTA:0	CDS	-				no annotation	LEUM_1791	
1761107	CTGCCTG C	TTGCTTGT	TTGCTTGT	TTGCTTGT:173 CTGCCTGC:0	CDS	-				no annotation	LEUM_1791	
1764663	TTC	CTG	CTG	CTG:125 TTC:0	CDS	+				no annotation	LEUM_1793	
1766295	TAA	CAG	CAG	CAG:302 TAA:0	CDS	-				no annotation	LEUM_1794	
1776537	CGA	AGC	AGC	AGC:191 CGA:0	CDS	-				no annotation	LEUM_1803	
1790033	CTGT	TTGC	TTGC	TTGC:198 CTGT:0	CDS	-				no annotation	LEUM_1817	
1824412	CAA	AAG	AAG	AAG:178 CAA:0	CDS	-				no annotation	LEUM_1850	
1830003	GAGA	AAGG	AAGG	AAGG:208 GAGA:0								

1842065	ACCA	GCCC	GCCC:231 ACCA:0	CDS	-						LEUM_1868	atpC
1857246	ATTACCTT TGATAAC	GTTATCAAA GGTAAT	GTTATCAAAAGGTAA T:71 ATTACCTTTGATAAC :0									
1860337	AGA	GGG	GGG:145 AGA:0	CDS	-						LEUM_1886	
1861225	CTTTGCA	TTTTACG	TTTTACG:221 CTTTGCA:0	CDS	-						LEUM_1888	
1875169	ATT	GTC	GTC:252 ATT:0	CDS	-						LEUM_1900	
1878574	ACG	AA	AA:157 ACG:1									
1878900	GCAAGT	ATAAGC	ATAAGC:121 GCAAGT:0	CDS	+						LEUM_1905	
1878918	GTG	TTT	TTT:121 GTG:0	CDS	+						LEUM_1905	
1878926	CTTT	TTTC	TTTC:114 CTTT:0	CDS	+						LEUM_1905	
1878938	ATAGA	GTA	GTA:113 ATAGA:0	CDS	+						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	+						LEUM_1917	
1887546	TAGG	CAAA	CAAA:137 TAGG:0	CDS	-						LEUM_1919	
1887555	ACGTGTT	TCGCGTA	TCGCGTA:147 ACGTGTT:0	CDS	-						LEUM_1919	
1887567	CAATGAA CCG	TAGAGAGC CA	TAGAGAGCCA:147 CAATGAACCG:0	CDS	-						LEUM_1919	
1887582	TTCA	CTCG	CTCG:153 TTCA:0	CDS	-						LEUM_1919	
1887645	GGCT	AGCC	AGCC:249 GGCT:0	CDS	-						LEUM_1919	
1887654	CTTG	TTTA	TTTA:252 CTTG:0	CDS	-						LEUM_1919	
1887666	ACGAAGC	GCGCAAT	GCGCAAT:172 ACGAAGC:0	CDS	-						LEUM_1919	

1887684	CTGG	TTGT	TTGT:196 CTGG:0	CDS	-				no annotation	LEUM_1919
1887711	TGTCACCTG GA	AGTTACCTG G	AGTTACCTGG:239 TGTCACCTTGA:0	CDS	-				no annotation	LEUM_1919
1887732	GGCG	ACCA	ACCA:275 GGCG: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	GGCG	ACGT	ACGT:286 GGCG: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	GCCA	TCCCG	TCCCG:175 GCCA:0	CDS	+				no annotation	LEUM_1960
1933941	GTCT	ATT	ATT:134 GTCT:0							
1934018	ATATTAC	TTGTTAT	TTGTTAT:133 ATATTAC:0							
1934029	ACAA	GTAT	GTAT:135 ACAA:0							
1934072	GTAA	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	AAGGA	AAGGA:39 TTTGGG:0								
1960134	TGTGTTA AATAC	AGTGTATA TTT	AGTGTATATT:34 TGTGTTAAATAC:0	CDS	-					LEUM_1991	
1960163	GTCA	ATCT	ATCT:36 GTCA:0	CDS	-					LEUM_1991	
1960179	ATTGC	CTTAA	CTTAA:39 ATTGC:0	CDS	-					LEUM_1991	
1960376	TGCT	AGCA	AGCA:107 TGCT:0	CDS	-					LEUM_1991	
1960390	GTCTT	ACCTC	ACCTC:106 GTCTT:0	CDS	-					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	+					LEUM_2001	
1969941	GTTTA	ATTTT	ATTTT:173 GTTTA:0	CDS	+					LEUM_2001	
1970013	TTAT	CTGC	CTGC:152 TTAT:0	CDS	+					LEUM_2001	
1978224	AGTAT	GGTAC	GGTAC:277 AGTAT:0	CDS	-					LEUM_2010	
1980589	CTTGT	TTTGC	TTTGC:192 CTTGT:0								
1994040	TAATT	GAATC	GAATC:291 TAATT:0	CDS	-					LEUM_2027	
1996966	GTGG	ATGA	ATGA:363 GTGG:0	CDS	-					LEUM_2030	
1996984	GATT	AATC	AATC:258 GATT:0	CDS	-					LEUM_2030	
1996993	GGCAGGC	AGCTGGT	AGCTGGT:241 GGCAGGC:0	CDS	-					LEUM_2030	
1997007	GACCCGG TTCAGGC	ATCCTCGCT CCGGT	ATCCTCGCTCCGGT: 235 GACCCCGTTCAGGC: 0	CDS	-					LEUM_2030	
1997032	CACA	AACG	AACG:318 CACA:0	CDS	-					LEUM_2030	
2025691	GCTA	ACTG	ACTG:240 GCTA:0	CDS	-					LEUM_2060	
2025829	AACA	GACG	GACG:213 AACA:0	CDS	-					LEUM_2060	
2026633	GCAG	ACAA	ACAA:327 GCAG:0	CDS	-					LEUM_2061	
2036598	GCCT	ACCC	ACCC:291 GCCT:0	CDS	-					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

Fermentation

Preparation of starter cultures: Pooled cultures of *Leuconostoc mesenteroides* (BF1, BF2) and *Lactobacillus plantarum* (B1, B2, B3, B4, B5) isolated from broccoli (described in fermentation patent and deposited) were used for fermentation. 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 *Leu. mesenteroides* cultures at 1:1 proportion prior to inoculation into the broccoli puree samples. Broccoli puree samples were inoculated with the cultures. 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.

Methods

The raw and processed broccoli's were assessed for their potential to improve gut health by examining their ability to influence gut microbial activity and stimulate the production of beneficial short chain fatty acids (SCFA) using an *in vitro* model. That is, broccoli samples of interest were added to tubes containing human stool (from healthy donors collected as described in Charoensiddhi et al. (2016) diluted in media and subsequently incubated to allow fermentation under anaerobic conditions for 24 hours. The broccoli products tested were added to the fermentation medium at 1.5% w/v. The ferment mixtures were then analysed for levels of SCFA, especially the main forms acetic acid, propionic acid and butyric acid, and also analysed using Q-PCR and sequencing methods to ascertain any changes in the microbial populations in response to the different broccoli substrates. The method comprises converting salts and esters of short chain fatty acids to short chain fatty acids and measuring acetic acid, propionic acid and butyric acid. Thus, the levels assessed are indicative of an increase in: acetic acid and acetate; propionic acid and propionate; and butyric acid and butyrate. The *in vitro* fermentation method, SCFA analysis and Q-PCR methods (for the targeted measure of changes in *Lactobacillus*, *Bifidobacterium*, *E. coli* and total bacteria) are as described in Charoensiddhi et al. (2016). A broad assessment of microbial population changes was

carried by PCR amplification of the 16S rRNA region of DNA extracted from the ferment samples and the sequencing of the amplified DNA.

Results

- 5 As shown in Figure 14 and 15 fermented broccoli and fermented pre-treated broccoli increased short chain fatty acid production 10 and 24 hours after addition compared to unfermented broccoli control and a cellulose control. As shown in Figure 16 fermented broccoli and fermented pre-treated broccoli has an increased number of lactic acid bacteria (*Lactobacillus*) compared to the unfermented broccoli control and the
10 cellulose control.

Example 18 - Fermented broccoli as delivery vehicle for omega-3 fatty acids

Methods

Sample preparation

- 15 Fresh broccoli (cv. Solitaire) was obtained from a local farm (FreshSelect, Werribee South). Following washing, the broccoli florets were cut at approximately 2 cm from the head and were divided into two lots. The first lot was steamed in a steam oven pre-heated to 100°C (Rational combi oven) to a core temperature of ~65°C and held at that temperature for 3 min to inactivate the protein co-factor Epithispecifier protein
20 (ESP) followed by cooling in ice-water. Following cooling, the broccoli florets were mixed with water (3 parts broccoli and 2 parts water) and were pureed for 1 min using a kitchen blender (Nutribullet pro 900 series, LLC, USA). The second lot was similarly processed into puree without preheating. Both the control and the preheated broccoli puree were further divided into two lots; fish oil was added at 50% loading to one lot
25 from each group. The fish oil was added at 6% (w/w) since the total solid contents of the broccoli puree samples were ~6%. Following the addition of oil, the mixture was homogenised into a coarse emulsion using a laboratory scale mixer (Silverson, Model: L4R, USA) at a stirring speed ranging from 2500 rpm to 6000 rpm over 5 minutes. Samples from each lot were further divided into two; one for use as control and the
30 second for subsequent fermentation. The control and the preheated-control samples with or without added oil were immediately frozen after preparation for subsequent freeze drying. The samples and their designation are provided in Table 20.

Table 20. Processed broccoli samples and their designation.

Sample type	Sample designation
Control broccoli	C-NF
Control broccoli with tuna oil	C-To-NF
Control fermented broccoli	C-F
Control broccoli fermented with tuna oil	C-To-F
Preheated broccoli	Ph-NF
Preheated broccoli with tuna oil	Ph-To-NF
Preheated fermented broccoli	Ph-F
Preheated broccoli fermented with tuna oil	Ph-To-F

Preparation of starter culture for fermentation

A cocktail of seven lactic acid bacteria strains isolated from broccoli i.e. 5
 5 *Lactobacillus plantarum* strains (B1, B2, B3, B4, B5) and two *Leuconostoc mesenteroides* (BF1, BF2) were used as a starter for the fermentation of broccoli puree samples. To obtain the primary inoculum, lactic acid bacteria cultures which were stored at -80°C were inoculated into 10mL of MRS broth (Oxoid, Victoria, Australia) and incubated at 30°C for 18 hrs. This was followed by a secondary culture where 2mL of
 10 the primary culture was inoculated into 200mL of MRS broth and incubated for 18 hrs at 30°C . The cultures were collected by centrifugation at 3500g for 15min at 4°C , washed twice with sterile phosphate buffer saline (PBS), and were suspended in Milli-Q water at a concentration of $10 \log \text{CFU/mL}$. Then, all the *L. plantarum* and *Leu. mesenteroides* cultures were mixed together, glycerol was added to the mixture, the pooled culture was
 15 stored at -80°C until use as a starter culture for broccoli puree fermentation. Prior to the fermentation experiment, the cultures were thawed, washed twice with PBS and re-suspended in Milli-Q water.

Fermentation experiments

20 Each broccoli puree and emulsion sample (~450g) was inoculated with the prepared starter culture at a dose of $8 \log \text{CFU/g}$. The fermentation experiment was carried out at 30°C until the pH reached ~ 4.0 , which was from 15 hrs to 48 hours of incubation depending on the sample. Once the fermentation was completed, samples (labelled as day 0 samples) were taken for microbial, physicochemical and chemical
 25 analyses. The rest of the ferments were packed in aluminium foil bags and were flat frozen (~2cm thick) and stored at -20°C until freeze drying. The samples were freeze dried using Cuddon freeze dryer (New Zealand) at 25°C and 2.5 mbar vacuum within 2 days. The freeze-dried powders were used in subsequent analyses and storage stability

studies. Following freeze drying, samples were aliquoted for the storage stability trial as described below and the rest of the samples were kept at -80°C until microbial and chemical analyses. All experiments were conducted in duplicates.

5 *Storage stability study*

For the storage stability study, 1 g powder samples were aliquoted into amber glass vials, flushed with nitrogen and tightly capped and stored at 25°C. Samples were taken every 10 days for FAME analysis.

10 *Microbial analysis*

The microbiological analyses of the samples were conducted following standard methods in literature (Cai et al., 2019). Accordingly, total lactic acid bacteria (LAB) was enumerated by plating on De Man, Rogosa and Sharpe agar (MRS), total Enterobacteriaceae on VRBGA (Violet Red Blue Glucose Agar), and yeasts and mould
15 on PDA (Potato Dextrose Agar) agar plates with the pH adjusted to 3.5 using 10% tartaric acid. For each sample, the broccoli suspension was serially diluted with sterilized peptone saline diluent and 0.1 mL of the diluted samples were plated onto the agar plates in duplicate. After aerobic incubation at 25°C for 72h (PDA), 37°C for 24h (VRBGA), and anaerobic incubation at 37°C for 72h (MRS), respectively, the colony forming units
20 (CFU) were counted.

Fatty Acid Methyl Ester (FAME) analysis

The oxidative stability of the fish oil encapsulated in freeze-dried broccoli powders (fermented/unfermented broccoli) was evaluated after freeze drying and during
25 storage at 25°C by direct methylation of microencapsulated powder. The content of Eicosapentaenoic acid (EPA, C20:5 ω3) and Docosahexaenoic acid (DHA, C22:6 ω3) (mg per g powder) was calculated from the FAME data. The remaining EPA & DHA content (%) during the storage time for each powder was also analysed. Each sample was analysed in triplicate. Fatty acid composition was determined by gas chromatography
30 (GC). A direct methylation of the powder for fatty acid analysis was conducted in accordance with a previously reported method (Zhou et al., 2009).

Fatty acid methyl esterification and extraction was conducted as follows. A mixture of the powder (10 ± 0.01 mg) with the 75 µl internal standards (0.75 mg of 17:0 Triheptadecanoin, TAG in toluene,) were suspended in 0.9 mL 1N methanolic HCl and
35 0.1 mL of Dichloromethane in argon-flushed 2 mL GC vial. The mixture was subsequently incubated in a shaker water bath (100 rpm) at 80°C for 2 hr. FAME were

extracted with 0.3 mL hexane. Transesterified fatty acids were added with 0.3 ml hexane for GC analysis (Shen et al., 2014).

The samples were quantified by GC following previously described method with some modifications (Shen et al., 2014). FAME solution (1 μ L) was injected at a split ratio of 1:40 into a GC column (BPX 70 fused silica column, 30 m, 0.25 mm id and 0.25 μ m films, SGE, Australia), installed in a model 7890A GC system equipped with a model 7693 autosampler (Agilent Technologies Australia Pty Ltd., Mulgrave, Victoria 3170, Australia). The GC column temperature was increased from 60 to 170°C at a rate of 20°C/min, then to 192°C at a rate of 1°C/min and finally to 220 °C at 20°C/min. The injector and detector (FID) were held at 220 and 250°C, respectively. Agilent Chemstation software [B.04.02 SP2 (256)] was used to integrate GC peak areas. Individual polyunsaturated fatty acids (i.e. Eicosapentaenoic acid, EPA, C20:5 ω 3 and Docosahexaenoic acid, DHA, C22:6 ω 3 in the powder (mg fatty acid/g dry weight) were calculated as described in the AOCS official method (AOCS, Method Ce 1b-892009).

Oxipres analysis

Oxipres analysis of the oil powder samples was conducted in order to determine the oxidative stability of the fish oil encapsulated in the broccoli matrix under accelerated conditions. It involves exposing the samples to oxygen at high temperature and analysing the rate of oxygen consumption as a measure of oxidation. The induction point for oxidation (IP) is used as a measure of the oxidation stability of the oil powder and is evaluated as the time point in which an inflection is observed in the oxygen pressure versus time plot. The Oxipres IP analysis was conducted using ML OXIPRES (Mikrolab Aarhus A/S, Højbjerg, Denmark). About 8 grams of samples (containing about 4 g of oil) were used in the analysis. The analysis was conducted at 80°C and 5 bar oxygen pressure.

Results

Oxidative Stability of omega-3 fatty acids in freeze dried non-fermented-oil powder and broccoli fermented with oil powder as measured by Oxipres

Broccoli-oil, preheated broccoli-oil, broccoli fermented with oil and preheated broccoli fermented with oil had substantially lower oxygen absorption rates compared to the neat oil, indicating that encapsulation of omega-3 fatty acids with all broccoli-based matrices improve the stability of omega-3 fatty acids. Example data comparing the Oxipres trace of neat tuna oil, and broccoli-tuna oil powder and broccoli fermented with oil powder are given in Figure 17A. The IP of this batch of neat tuna oil was less ~10

hrs, whereas the IP of the broccoli encapsulated tuna oil powder was ~158 hrs. No clear IP was observed for the broccoli fermented with oil powder and preheated broccoli fermented with oil powder for up to 350 hrs indicating that addition of oil prior to fermentation further enhances the stabilisation effects on omega-3 fatty acids.

5

Stability of EPA and DHA in broccoli fermented with oil and non-fermented broccoli-oil powders during storage at 25°C

The levels of omega-3 fatty acids in the different broccoli powders were evaluated during one month storage at 25°C for samples stored in amber bottles flushed with nitrogen. The levels of both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) remained stable during storage of the broccoli powders (Figure 17B), indicating that both non-fermented broccoli and broccoli fermented with oil can be used for delivery of omega-3 fatty acids.

Impact of tuna oil on growth of lactic acid bacteria during fermentation of broccoli samples and survival during freeze drying

The addition of tuna oil into the non-preheated and pre-heated broccoli puree did not inhibit the growth of lactic acid bacteria and the fermentation process. It was observed that the lactic acid bacteria count in the oil samples was slightly higher in both control and preheated samples (Table 21). However, the difference was not statistically significant. There was on average 2.51, 1.68, 1.84 and 2.25 log reduction in lactic acid bacteria count after freeze drying in the control fermented, control fermented with oil, preheated fermented and preheated fermented with oil samples, respectively. The presence of tuna oil improved the survival of lactic acid bacteria in the control fermented samples.

25

Example 19 - Fermented broccoli as a delivery system for probiotic bacteria

Methods

Sample preparation

Farm fresh broccoli was sourced from a local farm (Fresh Select). Untreated control (C-NF) and preheated (Ph-NF) broccoli puree samples were prepared as described in Example 18. Fermentation of the control and the preheated puree samples were conducted as described in Example 18. The final pH of the control fermented and the preheated fermented broccoli were 3.93 and 3.72, respectively. Following fermentation, *Bifidobacterium animalis* subsp. *Lactis* powdered culture (Chrstian Hansen), as a model probiotic microorganism, was added into the fermented purees (C-

35

F and Ph-F) at 10% dry weight basis. The samples were thoroughly mixed and frozen and freeze dried as described in Example 18. All experiments were conducted in duplicates.

5 **Table 21. Lactic acid bacteria count in fermented broccoli samples with and without added tuna oil (log CFU/g) dry weight basis.**

Sample	Fermented puree (log CFU/g dry weight)	Freeze dried fermented powder (log CFU/g dry weight)
Fermented control broccoli (C-F)	8.6±0.17	6.09±0.12
Control broccoli fermented with tuna oil (C-To-F)	8.72±0.14	7.05±0.33
Preheated fermented broccoli (Ph-F)	10.84±0.90	9.09±0.13
Preheated broccoli fermented with tuna oil (Ph-To-F)	11.33±0.39	9.0±0.0

Microbial analysis

Freeze-dried probiotic powders were rehydrated by dispersing in Buffered peptone water (BPT) in a shaking water bath (37°C, 100 rpm, 1 h). The rehydrated samples were then diluted with Maximum recovery diluent (MRD). De Man, Rogosa and Sharpe agar (MRS agar, Oxoid Ltd, UK) was used for enumeration LAB from the samples as described in Example 18. RCA (reinforced clostridial agar, pH 6.8, Oxoid Ltd, UK) agar was used for enumeration of *Bifidobacterium lactis* in the samples. The inoculated plates were incubated under anaerobic conditions at 37°C for 48 h. Each viable unit (cells) grown as a colony on the plates was counted as a colony forming unit (CFU) and calculated for the number of CFU per gram of the powder (CFU/g). The viable counts were transformed into log₁₀ value and loss of the count in the powders were calculated and compared with the control probiotic powders as well as with the value of each powder.

Simulated in vitro digestion

Broccoli powders (C-F-Bifido, and Ph-F-bifido powders) were sequentially exposed to simulated gastric fluid (SGF) and simulated intestinal fluids (SIF). The SGF solution (pH adjusted to 1.2 with HCl) was comprised of sodium chloride (2 g) and pepsin (1.6 g) made up to 1000 mL with Milli-Q water. The SIF (pH adjusted to 6.8 using

NaOH) contained anhydrous potassium dihydrogen phosphate (17 g) and pancreatin (3.15 g) made up to 1000 mL with Milli-Q water. For sequential exposure to (SGF+SIF), the freeze-dried powders (0.2 g in 9.8 g of deionized water) was added to SGF solution (12.5 mL) and incubated in a shaking water bath (37°C for 2h). The SGF-digested sample was adjusted to pH 6.8 with 1M NaOH, combined with 10 mL of SIF solution and incubated for 20 min prior to addition of CaCl₂ (0.05 M, 2.5 mL) and incubation for a further 2 h and 40 min. The survival of the lactic acid bacteria and the added bifido bacteria cells with/without broccoli matrices were evaluated in the simulated digestive fluids (SGF, and SIF) by plating in the respective media as described above in the microbial analysis section. For sequential exposure to simulated SGF and SIF with bile extract, similar procedure of incubation with 10 mL of SIF solution (with added bile extract (6.25±0.01 g/L) to the SIF solution) was applied to the SGF-digested samples, following which the viability of the lactic acid bacteria and the *Bifidobacterium* were assessed.

15

Results

Survival of Bifidobacterium animalis subsp. lactis in fermented broccoli and preheated fermented broccoli matrices during freeze drying

The initial viable counts of the samples and the viable counts after the powders production are given in Table 22. Before freeze drying, the fermented broccoli with Bifido powders had 2.60E+10 CFU/g powder. After freeze-drying, the viable count of cells in the fermented broccoli-bifido (C-F-Bifido) powder was 1.65E+09 CFU/g powder and that of the preheated fermented bifido (Ph-F-Bifido) powder was 2.88E+10 CFU/g powder. This represents a loss of 1.2 log CFU/g during freeze-drying process for the control fermented sample whereas no loss was observed in the case of the preheated fermented sample.

25

Table 22. Changes in the viable count of *Bifidobacterium animalis* subsp. *lactis* after freeze-drying of broccoli powders.

	Viable LAB count/g powder (log ₁₀ transformed value)		Loss of LAB (log ₁₀ transformed value)
	Before freeze- drying	After freeze- drying	
C-F-Bifido	10.41±0.10	9.22±0.74	1.19
Ph-F-Bifido	10.41±0.10	10.46±0.23	No loss

30

Survival of Bifidobacterium animalis subsp. lactis in fermented and preheated fermented broccoli matrices following in vitro digestion

The survival of *Bifidobacterium animalis* subsp. *Lactis* following simulated *in-vitro* digestion of the fermented and unfermented samples were evaluated. The viable counts prior to and after *in vitro* digestion (SGF+SIF) are presented in Table 23. The broccoli matrices protected the probiotic bacteria against inactivation during the simulated digestion. The control fermented broccoli provided the best protection with the least viability loss whereas the preheated fermented broccoli provided the least protection for the probiotic organism.

Table 23. Survival of *Bifidobacterium animalis* subsp. *Lactis* cells as is and in fermented broccoli matrices after sequential exposure to simulated gastric fluid (SGF) for 2 h and simulated intestinal fluid (SIF) for 3 h (without added bile).

Sample	Viable count/g powder (log ₁₀ transformed value)		Loss of LAB compared to Freeze-dried powder (log ₁₀ transformed value)
	Before <i>in-vitro</i> digestion	After <i>in-vitro</i> digestion	
Bifido control	10.41±0.10	4.55±0.21	5.86
C-F-Bifido	9.22±0.74	5.83±0.29	3.39
Ph-F-Bifido	10.46±0.23	5.08±0.14	5.39

The survival of *Bifidobacterium animalis* subsp. *Lactis* after simulated *in-vitro* digestion of the fermented samples with added bile were also evaluated. The data are presented in Table 24. Overall, higher loss of viability was observed in this case compared to *in vitro* digestion without added bile. Higher survival was observed in the fermented samples with the control fermented broccoli providing the best protection compared to the bifido control. The preheated fermented samples was less effective than the control fermented sample perhaps due to its lower pH (pH 3.72 compared to pH 3.93 of the control fermented product). The result indicates that fermented broccoli can be used for protected delivery of probiotic microorganisms into the gastrointestinal tract.

Survival of lactic acid bacteria (autochthonous and starter culture in the fermented samples) following in vitro digestion

The survival of lactic acid bacteria in fermented broccoli matrices during simulated *in vitro* digestion with and without bile were evaluated. Data are presented in Table 25 and 26. The lactic acid bacteria in the broccoli samples survived simulated *in vitro* digestion with and without bile, indicating that the products can be used for

delivering beneficial lactic acid bacteria into the gut for probiotic benefit. Lower survival of lactic acid bacteria was observed in the case of the pre-heated fermented sample compared to the control fermented sample perhaps due to its lower pH compared to all the other non-fermented and fermented samples in this study.

5

Table 24. Survival of *Bifidobacterium animalis* subsp. *Lactis* cells as is and in fermented broccoli matrices after sequential exposure to simulated gastric fluid (SGF) for 2 h and simulated intestinal fluid (SIF) with added bile for 3 h.

Sample	Viable count/g powder (log ₁₀ transformed value)		Loss of LAB compared to Freeze-dried powder (log ₁₀ transformed value)
	Before <i>in-vitro</i> digestion	After <i>in-vitro</i> digestion	
Bifido control	10.41±0.10	4.24±0.09	6.17
C-F-Bifido	9.22±0.74	4.42±0.09	4.80
Ph-F-Bifido	10.46±0.23	4.78±0.18	5.68

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Table 25. Survival of total lactic acid bacteria (autochthonous and starter culture in fermented samples) after *in-vitro* digestion without bile.

	Viable LAB count/g powder (log ₁₀ transformed value)		Loss of LAB compared to Freeze-dried powder (log ₁₀ transformed value)
	Before <i>in-vitro</i> digestion	Before <i>in-vitro</i> digestion	
C-F-Bifido	6.20±0.28	5.19±0.21	1.01
Ph-F-Bifido	8.53±0.06	5.05±0.11	3.48

15

Table 26. Survival of total lactic acid bacteria (autochthonous and starter culture in fermented samples) after *in-vitro* digestion with bile.

	Viable LAB count/g powder (log ₁₀ transformed value)		Loss of LAB compared to Freeze-dried powder (log ₁₀ transformed value)
	Before <i>in-vitro</i> digestion	Before <i>in-vitro</i> digestion	
C-F-Bifido	6.20±0.28	3.70±0.00	2.50
Ph-F-Bifido	8.53±0.06	4.00±0.00	4.53

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. 2019901142 entitled "Methods and compositions for promoting health in a subject" filed on 3 April 2019, 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 promoting health in a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.
2. The method of claim 1, wherein the *Brassicaceae* product increases the gastrointestinal level of one or more short chain fatty acids (SCFA) in the subject.
3. The method of claim 1 or claim 2, wherein the *Brassicaceae* product increases the production of one or more SCFA in the gastrointestinal tract of the subject.
4. The method of any one of claims 2 to 3, wherein the *Brassicaceae* product increases the production of one or more SCFA in the colon of the subject.
5. The method of any one of claims 2 to 4, wherein the SCFA is selected from one or more or all of: butyrate (butanoate), propionate (propanoate), acetate (ethanoate), formate (methanoate), isobutyrate (2-Methylpropanoate), valerate (pentanoate), isovalerate (3-methylbutanoate), caproate (hexanoate), formic acid (methanoic acid), acetic acid (ethanoic acid), propionic acid (propanoic acid), butyric acid (butanoic acid), isobutyric acid (2-methylpropanoic acid), valeric acid (pentanoic acid), isovaleric acid (3-methylbutanoic acid), and caproic acid (hexanoic acid).
6. The method of any one of claims 2 to 5, wherein the SCFA is selected from one or more or all of: butyrate, propionate and acetate.
7. The method of any one of claims 1 to 6, wherein the *Brassicaceae* product comprises an isothiocyanate.
8. The method of any one of claims 1 to 7, wherein the *Brassicaceae* product comprises live lactic acid bacteria from *Brassicaceae*.
9. The method of any one of claims 1 to 8, wherein promoting health comprises promoting one or more of: gut health, immune system health, cardiovascular health,

central nervous system function, cognition, metabolic health, skeletal health, liver health, blood sugar control and skin health.

10. The method of any one of claims 1 to 9, wherein promoting health comprises
5 treating or preventing one or more symptoms of a condition selected from: diabetes, inflammation, metabolic dysfunction, allergy and cancer.
11. The method of claim 10, wherein promoting gut health comprises reducing or preventing one or more symptoms of a gut health associated condition selected from one
10 or more of: irritable bowel syndrome, inflammatory bowel disease, Crohn's disease, colorectal cancer, gut leakiness, non-alcoholic fatty liver disease, metabolic syndrome, obesity, small intestinal bacterial overgrowth (SIBO), gastroenteritis, gut microbial dysbiosis, reduced gut microbial diversity, antibiotic treatment, post-surgery recovery, food intolerance, diarrhea, gastritis, diverticulitis, flatulence, constipation, functional gut
15 disorders and functional gastrointestinal and motility disorders.
12. The method of any one of claims 1 to 11, wherein promoting health comprises promoting health of the gut microbiome in a subject.
- 20 13. The method of claim 12, wherein promoting health of the gut microbiome comprises one or more of: increasing the level and/or activity of one or more beneficial bacteria, decreasing or maintaining the level and/or activity of one or more non-beneficial bacteria, increasing the resistance of the gut microbiome, increasing the resilience of the gut microbiome, and increasing the diversity of the gut microbiome.
25
14. The method of claim 13, wherein the beneficial bacteria is lactic acid bacteria.
15. The method of claim 13, wherein the non-beneficial bacteria is a pathogenic strain of *E. coli*.
30
16. The method of any one of claims 1 to 15, wherein the *Brassicaceae* product does not increase the total level of gastrointestinal bacteria in a subject.
17. The method of any one of claims 1 to 16, wherein the *Brassicaceae* product
35 comprises a prebiotic or a prebiotic and a probiotic.

18. The method of any one of claims 1 to 17, wherein the *Brassicaceae* product comprises a prebiotic and a probiotic which are synbiotic.
19. The method of any one of claims 1 to 18, wherein the subject is an animal.
- 5 20. The method of claim 19, wherein the subject is a human.
21. A method of promoting the health of the gut microbiome in a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.
- 10 22. A method of treating and/or preventing microbial dysbiosis in the gastrointestinal tract of a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.
- 15 23. A method of treating and/or preventing inflammation in a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.
- 20 24. A method of treating and/or preventing diabetes in a subject, comprising administering to the subject a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.
- 25 25. Use of a *Brassicaceae* product fermented with lactic acid bacteria in the manufacture of a medicament for promoting health in a subject, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.
- 30 26. Use of a *Brassicaceae* product fermented with lactic acid bacteria in the manufacture of a medicament for promoting health of the gut microbiome in a subject,
- 35

wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.

27. Use of a *Brassicaceae* product fermented with lactic acid bacteria in the
5 manufacture of a medicament for treating and/or preventing microbial dysbiosis in the
gastrointestinal tract of a subject, wherein the lactic acid bacteria were derived from an
isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior
to fermentation.
- 10 28. Use of a *Brassicaceae* product fermented with lactic acid bacteria in the
manufacture of a medicament for treating and/or preventing inflammation in a subject,
wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae*
and/or the *Brassicaceae* product was pre-treated prior to fermentation.
- 15 29. Use of a *Brassicaceae* product fermented with lactic acid bacteria in the
manufacture of a medicament for treating and/or preventing diabetes in a subject,
wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae*
and/or the *Brassicaceae* product was pre-treated prior to fermentation.
- 20 30. The use of any one of claims 25 to 29 wherein the medicament comprises one or
more or all of: i) a prebiotic, ii) a prebiotic and a probiotic, and iii) a prebiotic and a
probiotic which are synbiotic.
31. A pharmaceutical composition comprising a *Brassicaceae* product fermented
25 with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate
obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to
fermentation for use in promoting health in a subject.
32. A pharmaceutical composition comprising a *Brassicaceae* product fermented
30 with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate
obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to
fermentation for use in promoting health of the gut microbiome in a subject.
33. A pharmaceutical composition comprising a *Brassicaceae* product with lactic
35 acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from

Brassicaceae and/or the *Brassicaceae* product was pre-treated prior to fermentation for treating and/or preventing microbial dysbiosis in the gastrointestinal tract of a subject.

34. A pharmaceutical composition comprising a *Brassicaceae* product fermented
5 with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation for treating and/or preventing inflammation in a subject.
35. A pharmaceutical composition comprising a *Brassicaceae* product fermented
10 with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation for treating and/or preventing diabetes in a subject.
36. The pharmaceutical composition of any one of claims 31 to 35, wherein the
15 composition comprises one or more or all of: i) a prebiotic, ii) a combined prebiotic and a probiotic, and iii) a prebiotic and a probiotic which are synbiotic.
37. A prebiotic composition comprising a *Brassicaceae* product fermented with lactic
20 acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation, wherein the prebiotic increases the gastrointestinal level of one or more SCFA in a subject.
38. A combined prebiotic and probiotic composition comprising:
25 i) a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation; and
ii) live lactic acid bacteria.
39. The composition of claim 37 or claim 38, wherein the composition further
30 comprises an isothiocyanate.
40. The composition of any one of claims 31 to 36, 38 or 39, wherein the composition
35 increases the gastrointestinal level of one or more short chain fatty acids (SCFA) in a subject.

41. The composition of any one of claims 31 to 40, wherein the *Brassicaceae* product increases the production of one or more SCFA in the gastrointestinal tract of the subject.
42. The composition of any one of claims 31 to 41, wherein the *Brassicaceae* product increases the production of one or more SCFA in the colon of the subject.
43. The composition of claim 37 or claims 40 to 42, wherein the SCFA is selected from one or more or all of: butyrate (butanoate), propionate (propanoate), acetate (ethanoate), formate (methanoate), isobutyrate (2-Methylpropanoate), valerate (pentanoate), isovalerate (3-methylbutanoate), caproate (hexanoate), formic acid (methanoic acid), acetic acid (ethanoic acid), propionic acid (propanoic acid), butyric acid (butanoic acid), isobutyric acid (2-methylpropanoic acid), valeric acid (pentanoic acid), isovaleric acid (3-methylbutanoic acid), and caproic acid (hexanoic acid).
44. The composition of claim 37, or claims 40 to 43, wherein the SCFA is selected from one or more or all of: butyrate, propionate and acetate.
45. The composition of any one of claims 36 to 44, wherein the *Brassicaceae* product protects the probiotic during passage through the upper gastrointestinal tract.
46. The composition of any one of claims 31 to 45, wherein the *Brassicaceae* product increases the gastrointestinal level of live lactic acid bacteria in a subject.
47. The composition of any one of claims 31 to 46, wherein the *Brassicaceae* product does not increase the gastrointestinal level of *E. coli* in a subject.
48. The composition of any one of claims 31 to 47, wherein the *Brassicaceae* product does not increase the total level of gastrointestinal bacteria in a subject.
49. The method or composition of any one of claims 1 to 48, wherein the lactic acid bacteria was isolated from a broccoli.
50. The method or composition of any one of claims 1 to 49, wherein the lactic acid bacteria is selected from one or more of the genera selected from: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*,

Enterococcus, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus* and *Weissella*.

51. The method or composition of any one of claims 1 to 50, wherein the lactic acid
5 bacteria is selected from one or more of: *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus brevis*, *Lactococcus lactis*, *Pediococcus pentosaceus*, *Lactobacillus rhamnosus* and *Pedicoccus acidilacti*.
52. The method or composition of any one of claims 1 to 51, wherein the lactic acid
10 bacteria is selected from:
- i) *Leuconostoc mesenteroides*;
 - ii) *Lactobacillus plantarum*;
 - iii) *Lactobacillus pentosus*;
 - iv) *Lactobacillus rhamnosus*;
 - 15 v) a combination of i) and ii);
 - vi) a combination of i), ii) and iii); and
 - vii) a combination of i), ii) and iv).
53. The method or composition of any one of claims 1 to 52, wherein the lactic acid
20 bacteria is selected from one or more of:
- 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;
 - 25 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
30 Measurement Institute Australia;
 - 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.
- 35

54. The method or composition of any one of claims 1 to 53, wherein the pre-treating comprises one or more of the following:
- i) heating;
 - ii) macerating;
 - 5 iii) microwaving;
 - iv) exposure to high frequency sound waves (ultrasound),
 - v) pulse electric field processing; and
 - vi) high pressure processing.
- 10 55. The method or composition of claim 54, wherein heating comprises heating the *Brassicaceae* product to about 50°C to about 70°C.
56. The method or composition of any one of claims 1 to 55, wherein the *Brassicaceae* is selected from *Brassica oleracea*, *Brassica balearica*, *Brassica carinata*,
- 15 *Brassica elongate*, *Brassica fruticulosa*, *Brassica hilarionis*, *Brassica juncea*, *Brassica napus*, *Brassica narinosa*, *Brassica nigra*, *Brassica perviridis*, *Brassica rapa*, *Brassica rupestris*, *Brassica septiceps* and *Brassica tournefortii*.
57. The method or composition of claim 56, wherein the *Brassicaceae* is *Brassica*
- 20 *oleracea*.
58. The method or composition of any one of claims 1 to 57, wherein the *Brassicaceae* product is administered enterally.
- 25 59. The method or composition of claim 58, wherein administration is oral or rectal.
60. The method or composition of any one of claims 1 to 59, wherein the *Brassicaceae* product is administered topically.
- 30 61. Faecal microbiota suitable for transplantation into a subject, wherein the faecal microbiota has been isolated from a subject administered a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.
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62. A vehicle for delivering a bioactive to a subject, wherein the vehicle comprises a *Brassicaceae* product fermented with lactic acid bacteria, wherein the lactic acid bacteria were derived from an isolate obtained from *Brassicaceae* and/or the *Brassicaceae* product was pre-treated prior to fermentation.
- 5 63. The vehicle of claim 62, wherein the *Brassicaceae* product comprises one or more or all of: i) a prebiotic, ii) a prebiotic and a probiotic, and iii) a prebiotic and a probiotic which are synbiotic.
- 10 64. The vehicle of claim 62 or claim 63, wherein the bioactive is selected from one or more or all of:
- i) a fatty acid,
 - ii) oil,
 - iii) a further prebiotic, and
 - 15 iv) a further probiotic.
65. The vehicle of claim 64, wherein the fatty acid is selected from an omega-3 or and omega-6 fatty acid.
- 20 66. The vehicle of claim 65, wherein the omega-3 fatty acid is selected from one or more or all of: of α -linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).
- 25 67. The vehicle of claim 65, wherein the oil is selected from one or more of: fish oil, krill oil, marine oil, algal oil, microbial oil, canola oil, crustacean oil, mollusc oil, sunflower oil, avocado oil, soya oil, borage oil, evening primrose oil, safflower oil, flaxseed oil, olive oil, pumpkinseed oil, hemp seed oil, wheat germ oil, palm oil, palm oil, palm kernel oil, coconut oil, medium chain triglycerides and grapeseed oil.
- 30 68. The vehicle of claim 67, wherein the fish oil or marine oil is selected from: tuna oil, herring oil, mackerel oil, sardine oil, cod liver oil, menhaden oil, shark oil, squid oil, and squid liver oil.
- 35 69. The vehicle of claim 64, wherein the further prebiotic is selected from one or more or all of: fructo-oligosaccharides galacto-oligosaccharide, trans-galacto-

oligosaccharides, oligofructose, pecticoligosaccharide, resistant starch, pectin, glucosinolate and inulin.

70. The vehicle of claim 64, wherein the further probiotic comprises one or more
5 probiotics selected from: lactic acid bacteria, *Bifidobacteria*, *Bacteroidetes*, *Baciullus*,
Streptococcus, *Escherichia*, *Enterococcus* and *Saccharomyces*.

71. The method, composition or vehicle of any one of claims 1 to 64, wherein the
10 *Brassicaceae* product is in a form selected from a: liquid, emulsion, powder, capsule,
tablet.

72. A method of preparing a *Brassicaceae* product comprising:
i) fermenting *Brassicaceae* material with lactic acid bacteria;
ii) adding a fatty acid and/or oil before or during step i).
15

73. The method of claim 72, wherein the method further comprises forming an
emulsion or suspension.

74. The method of claim 72 or claim 73, wherein the *Brassicaceae* material is pre-
20 treated.

75. The method of claim 74, wherein the *Brassicaceae* material is pre-treated with
heating to about 50°C to about 70°C.

25 76. The method of any one of claims 72 to 75, wherein the lactic acid bacteria were
derived from an isolate obtained from *Brassicaceae*.

77. An emulsion or suspension produced by any one of claims 72 to 76.

30 78. A *Brassicaceae* product comprising the emulsion or suspension of claim 77.