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(54) Title: DIAGNOSTIC AND THERAPEUTIC METHODS FOR TYPE 2 DIABETES

(57) Abstract: Provided herein are methods for diagnosing pre-diabetes or type 2 diabetes based on faecal or colonic samples and determining levels of butyric acid and one or both of acetic acid and propionic acid in the samples, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid. Also provided are methods for increasing the intestinal, colonic or faecal molar ratio of acetic acid to butyric acid, the intestinal, colonic or faecal molar ratio of propionic acid to butyric acid and/or the intestinal, colonic or faecal molar ratio of acetic acid to propionic acid to butyric acid in subjects with type 2 diabetes.



WO 2019/084616 A1

Diagnostic and therapeutic methods for type 2 diabetes

Field of the Art

[001] The present disclosure relates generally to methods for diagnosing type 2 diabetes and related metabolic disorders based on intestinal, colonic or faecal ratios of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid, and to methods for increasing said ratios by administration of a multi-strain probiotic combination.

Background

[002] Type 2 diabetes mellitus is a chronic metabolic disorder characterised by impaired β -cell function, insulin resistance and typically an increasing inability to synthesise sufficient insulin. Type 2 diabetes is a common disease of increasing prevalence worldwide, strongly associated with obesity. Type 2 diabetes is also often associated with macrovascular complications such as cardiovascular disease, and/or microvascular complications such as blindness, neuropathy and/or renal impairment or failure.

[003] Treatment of type 2 diabetes can include the administration of agents that stimulate β -cell function or that enhance the tissue sensitivity to insulin. Agents known to stimulate β -cell function, include, for example, sulfonylureas, such as tolbutamide, glibenclamide, glipizide, chlorpropamide, and gliclazide, and repaglinide. Metformin (N,N-dimethylimidodicarbonimidic diamide or 1,1-dimethylbiguanide) is an antihyperglycemic agent which improves glucose tolerance in patients with type 2 diabetes. It is the most widely used medication to improve glycemic control in type 2 diabetics, and has been shown to reduce body weight and delay the onset of type 2 diabetes in at risk or pre-diabetic individuals.

[004] However existing treatments for type 2 diabetes can often lead to unsatisfactory results for individuals and may be associated with adverse side effects. There is a clear and growing need for the development of alternative or adjunct treatments of type 2 diabetes with the increasing prevalence of the disease.

[005] Previous studies of type 2 diabetes and obesity suggest that metabolites produced by the intestinal microbiota, such as short chain fatty acids, can modulate adipose tissue storage, increase the expression of insulin-sensitising adipokines and improve skeletal muscle glucose uptake. The effects of short chain fatty acids are primarily mediated by the GPR41/43 receptors (GPR41/43) which are expressed in a variety of tissues including adipose tissue, skeletal muscle and the colon. *In vivo*, the activation of these receptors has been shown to reduce lipid accumulation in white adipose tissue, induce fatty acid oxidation and inhibit lipolysis in adipocytes which leads to a decrease in body weight and plasma FFA levels.

[006] Increasing colonic short chain fatty acids and manipulating the fatty acid profile is therefore an attractive potential therapeutic target to manage obesity and insulin resistance, and therefore to treat type 2 diabetes. However little is currently known about the ability of probiotic microorganisms to produce short chain fatty acids and to impact the intestinal short chain fatty acid profile *in vivo*.

Summary of the Disclosure

[007] In a first aspect, the present disclosure provides a method for diagnosing pre-diabetes or type 2 diabetes or a related metabolic disorder in a subject, the method comprising:

- (a) obtaining a faecal or colonic sample from the subject; and
- (b) determining levels of butyric acid and one or both of acetic acid and propionic acid in the sample, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid;

wherein a molar ratio of faecal or colonic acetic acid to butyric acid of less than about 1:1 is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder, a molar ratio of faecal or colonic propionic acid to butyric acid of less than about 1:1 is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder, and/or a molar ratio of faecal or colonic acetic acid to propionic acid to butyric acid of less than about 1:1:1 is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder.

[008] In an embodiment, a molar ratio of faecal acetic acid to butyric acid of about 1:2 is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder. In an embodiment, a molar ratio of faecal propionic acid to butyric acid of about 1:2 is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder. In an embodiment, a molar ratio of faecal acetic

acid to propionic acid to butyric acid of about 1:1:2 is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder.

[009] In a second aspect, the present disclosure provides a method for diagnosing pre-diabetes or type 2 diabetes or a related metabolic disorder in a subject, the method comprising:

- (a) obtaining a faecal or colonic sample from the subject;
- (b) determining levels of butyric acid and one or both of acetic acid and propionic acid in the sample, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid;
- (c) comparing the determined ratio(s) to the molar ratio(s) of faecal or colonic acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid in one or more individuals known not to be pre-diabetic or to have type 2 diabetes;

wherein a lower ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid in the subject than in the one or more individuals is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder in the subject.

[0010] In accordance with the first and second aspects, the molar ratio(s) in the subject and the comparison to the molar ratio(s) in one or more individuals known not to be pre-diabetic or to have type 2 diabetes may be subjected to one or more statistical analyses.

[0011] In a third aspect, the present disclosure provides a method for increasing the intestinal, colonic or faecal molar ratio of acetic acid to butyric acid, the intestinal, colonic or faecal molar ratio of propionic acid to butyric acid and/or the intestinal, colonic or faecal molar ratio of acetic acid to propionic acid to butyric acid in a subject with type 2 diabetes, the method comprising administering to the subject one or more probiotic microorganisms selected from *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

[0012] The increase in the molar ratio(s) is relative to the ratio(s) prior to, and in the absence of, the administration of the one or more probiotic microorganisms. In an embodiment, said administering may result in a faecal acetic acid to butyric acid ratio of about 1:1. In an embodiment, said administering may result in a faecal propionic acid to butyric acid

ratio of about 1:1. In an embodiment, said administering may result in a faecal acetic acid to propionic acid to butyric acid ratio of about 1:1:1.

[0013] In an embodiment, the subject is administered a multi-strain probiotic combination comprising *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

[0014] In an embodiment, the subject is also administered metformin or a derivative or salt thereof.

[0015] In a fourth aspect, the present disclosure provides a method for increasing intestinal, colonic or faecal levels of acetic acid in a subject with type 2 diabetes, the method comprising administering to the subject one or more probiotic microorganisms selected from *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

[0016] The increase in acetic acid levels is relative to the levels prior to, and in the absence of, the administration of the one or more probiotic microorganisms.

[0017] In an embodiment, the subject is administered a multi-strain probiotic combination comprising *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

[0018] In an embodiment, the subject is also administered metformin or a derivative or salt thereof.

[0019] In a fifth aspect, the present disclosure provides a method for treating type 2 diabetes or a metabolic complication or condition associated therewith, or for delaying or inhibiting the onset of type 2 diabetes in a subject, the method comprising:

- (a) obtaining a faecal or colonic sample from the subject;

- (b) determining levels of butyric acid and one or both of acetic acid and propionic acid in the sample, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or of acetic acid to propionic acid to butyric acid, wherein a lower molar ratio(s) in the subject than in one or more individuals known not to be pre-diabetic or to have type 2 diabetes is indicative of pre-diabetes or type 2 diabetes in the subject; and
- (c) where the subject is determined to be pre-diabetic or to have type 2 diabetes, administering to the subject one or more probiotic microorganisms selected from *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

[0020] In an embodiment, the subject is administered a multi-strain probiotic combination comprising *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

[0021] In an embodiment, the subject is also administered metformin or a derivative or salt thereof.

Brief Description of the Drawings

[0022] Embodiments of the disclosure are described herein, by way of non-limiting example only, with reference to the accompanying drawings.

[0023] **Figure 1** Box plots representing the effects of probiotic intervention and placebo on acetic acid to butyric acid ratio (A) and on acetic acid levels (B) in participants with a low acetic acid to butyric acid ratio. *, $p < 0.05$ (as determined by the Mann-Whitney test) between placebo and probiotic groups after treatment of participants with pre-diabetes (left hand box plots for each group) and type 2 diabetes (right hand box plots for each group).

Detailed Description

[0024] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, typical methods and materials are described.

[0025] The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

[0026] In the context of this specification, the term “about,” is understood to refer to a range of numbers that a person of skill in the art would consider equivalent to the recited value in the context of achieving the same function or result.

[0027] Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

[0028] In the context of this specification, the term “probiotic” is to be given its broadest construction and is understood to refer to a microbial cell population or preparation, or component of a microbial cell population or preparation, which when administered to a subject in an effective amount promotes a health benefit in the subject.

[0029] In the context of this specification, the term “prebiotic” is to be given its broadest construction and is understood to refer to any non-digestible substance that stimulates the growth and/or activity of bacteria in the digestive system.

[0030] In the context of this specification, the terms “food”, “foods”, “beverage” or “beverages” include but are not limited to health foods and beverages, functional foods and beverages, and foods and beverages for specified health use. When such foods or beverages of

the present invention are used for subjects other than humans, the terms can be used to include a feedstuff.

[0031] The term "subject" as used herein refers to any mammal, including, but not limited to, livestock and other farm animals (such as cattle, goats, sheep, horses, pigs and chickens), performance animals (such as racehorses), companion animals (such as cats and dogs), laboratory test animals and humans. Typically the subject is a human.

[0032] As used herein, the term "effective amount" refers to an amount of metformin or a derivative or salt thereof, or of one or more probiotic microorganisms or a multi-strain probiotic combination that is sufficient to effect one or more beneficial or desired outcomes. An "effective amount" can be provided in one or more administrations. The exact amount required will vary depending on factors such as the form of metformin administered, the identity and number of individual probiotic strains employed, the subject being treated, the nature of the disease(s) or condition(s) suffered by the subject that is to be treated and the age and general health of the subject, and the form in which the composition is administered. Thus, it is not possible to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation.

[0033] As used herein the terms "treating", "treatment" and the like refer to any and all applications which remedy, or otherwise hinder, retard, or reverse the progression of, a disease or disorder or at least one symptom of a disease or disorder, including reducing the severity of a disease or disorder. Thus, treatment does not necessarily imply that a subject is treated until complete recovery from a disease or disorder. Similarly, the terms "preventing", "prevention" and the like refer to any and all applications that prevent the establishment of a disease or disorder or otherwise delay the onset of a disease or disorder.

[0034] The term "optionally" is used herein to mean that the subsequently described feature may or may not be present or that the subsequently described event or circumstance may or may not occur. Hence the specification will be understood to include and encompass embodiments in which the feature is present and embodiments in which the feature is not present, and embodiments in which the event or circumstance occurs as well as embodiments in which it does not.

Diagnosis

[0035] Embodiments of the present disclosure provide for the determination of butyric acid, acetic acid and propionic acid levels in the intestine, colon or faeces of a subject and thereby determining one or more of the molar ratio of acetic acid to butyric acid, the molar ratio of propionic acid to butyric acid, and the molar ratio of acetic acid to propionic acid to butyric acid in the intestine, colon or faeces, and thereby diagnosing pre-diabetes or type 2 diabetes in the subject.

[0036] Accordingly, provided herein are methods for diagnosing pre-diabetes or type 2 diabetes or a related metabolic disorder, comprising:

- (a) obtaining a faecal or colonic sample from a subject; and
- (b) determining levels of butyric acid and one or both of acetic acid and propionic acid in the sample, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid;

wherein a molar ratio of faecal or colonic acetic acid to butyric acid of less than about 1:1 is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder, a molar ratio of faecal or colonic propionic acid to butyric acid of less than about 1:1 is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder, and/or a molar ratio of faecal or colonic acetic acid to propionic acid to butyric acid of less than about 1:1:1 is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder.

[0037] Also provided are methods for diagnosing pre-diabetes or type 2 diabetes or a related metabolic disorder, comprising:

- (a) obtaining a faecal or colonic sample from a subject;
- (b) determining levels of butyric acid and one or both of acetic acid and propionic acid in the sample, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid;
- (c) comparing the determined ratio(s) to the molar ratio(s) of faecal or colonic acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid in one or more individuals known not to be pre-diabetic or to have type 2 diabetes;

wherein a lower ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid in the subject than in the one or more individuals is indicative of pre-diabetes or type 2 diabetes or related metabolic disorder in the subject.

[0038] In the context of the above embodiments, metabolic disorders related to or associated with type 2 diabetes are well known to those skilled in the art, and include for example, metabolic syndrome, insulin resistance, hyperglycaemia, hypoglycaemia, hyperinsulinemia and hypoinsulinemia.

[0039] In exemplary embodiments, a faecal or colonic molar ratio of acetic acid to butyric acid of about 1:3, 1:2.8, 1:2.6, 1:2.4, 1:2.2, 1:2, 1:1.8, 1:1.6, 1:1.4, or 1:1.2 may be indicative of pre-diabetes or type 2 diabetes. In a particular embodiment a faecal or colonic ratio of acetic acid to butyric acid of about 1:2 is indicative of pre-diabetes or type 2 diabetes.

[0040] In exemplary embodiments, a faecal or colonic molar ratio of propionic acid to butyric acid of about 1:3, 1:2.8, 1:2.6, 1:2.4, 1:2.2, 1:2, 1:1.8, 1:1.6, 1:1.4, or 1:1.2 may be indicative of pre-diabetes or type 2 diabetes. In a particular embodiment a faecal or colonic ratio of propionic acid to butyric acid of about 1:2 is indicative of pre-diabetes or type 2 diabetes.

[0041] In exemplary embodiments, a faecal or colonic molar ratio of acetic acid to propionic acid to butyric acid of about 1:1:3, 1:1:2.8, 1:1:2.6, 1:1:2.4, 1:1:2.2, 1:1:2, 1:1:1.8, 1:1:1.6, 1:1:1.4, or 1:1:1.2 may be indicative of pre-diabetes or type 2 diabetes. In a particular embodiment a faecal or colonic ratio of acetic acid to propionic acid to butyric acid of about 1:1:2 is indicative of pre-diabetes or type 2 diabetes.

[0042] The skilled person will appreciate that acetic acid, propionic acid and butyric acid levels may be determined in any suitable tissue or fluid using a variety of techniques well known in the art. Levels and ratios of other short chain fatty acids may also be determined. By way of example only, and as exemplified herein, acetic acid, propionic acid and butyric acid may be measured using gas chromatography-mass spectrometry. The skilled person will also appreciate that any suitable tissue or fluid comprising a faecal or colonic sample may be obtained from a subject using a variety of known methods in the art. Alternatively, acetic acid

propionic acid and butyric acid levels may be determined *in vivo (in situ)* in the subject. Thus, the present disclosure contemplates diagnostic methods as disclosed above, but in which a sample is not required to be obtained from the subject in order to determine levels of acetic, propionic and butyric acids or the molar ratios therebetween.

[0043] In particular embodiments of the present disclosure, analysis of acetic acid, propionic acid and butyric acid levels or ratios are subjected to one or more statistical analyses. Such statistical analyses may include receiver operating characteristic (ROC) analysis, logistical regression analysis, Spearman's rank-order correlation analysis and the Mann-Whitney U test. ROC analysis may comprise determining ROC ranges for individual ROC variables, cut off limits, compound ROC variables and/or continuous variables, equations involving ROC variables and/or continuous variables. Suitable statistical analyses and methods for carrying them out will be well known to those skilled in the art.

[0044] The present disclosure also provides kits suitable for use in accordance with the diagnostic methods of the disclosure. Such kits may include for example diagnostic kits for assaying biological samples, comprising an agent(s) for detecting acetic acid, propionic acid and butyric acid levels and reagents useful for facilitating the determination of acetic acid, propionic acid and butyric acid levels. Kits according to the present disclosure may also include other components required to conduct the methods of the present invention, such as buffers and/or diluents. The kits typically include containers for housing the various components and instructions for using the kit components in the methods of the present disclosure.

Treatment

[0045] The findings described herein offer novel opportunities for the development of therapeutic treatments for type 2 diabetes and associated metabolic disorders and for monitoring the progress or efficacy of therapeutic treatments.

[001] Accordingly, provided herein is a method for evaluating the efficacy of a treatment regime in a subject with type 2 diabetes, the method comprising:

- (a) treating the subject with a treatment regime for type 2 diabetes for a period sufficient to evaluate the efficacy of the regime;

- (b) determining one or more of the intestinal, colonic or faecal molar ratio of acetic acid to butyric acid, the intestinal, colonic or faecal molar ratio of propionic acid to butyric acid, and the intestinal, colonic or faecal molar ratio of acetic acid to propionic acid to butyric acid in the subject or a sample derived therefrom in accordance with aspects described hereinbefore;
- (c) repeating step (b) at least once over a period of time; and
- (d) determining whether the ratio(s) changes over the period of time.

[002] Also provided herein is a method for identifying an agent suitable for use in the treatment of type 2 diabetes, the method comprising:

- (a) determining one or more of the intestinal, colonic or faecal molar ratio of acetic acid to butyric acid, the intestinal, colonic or faecal molar ratio of propionic acid to butyric acid, and the intestinal, colonic or faecal molar ratio of acetic acid to propionic acid to butyric acid in the subject or a sample derived in accordance with aspects described hereinbefore;
- (b) administering to the subject a candidate agent for the treatment of type 2 diabetes at least once;
- (c) repeating step (a) at least once over a period of time during and/or after the course of administration of the candidate agent; and
- (d) determining whether the ratio(s) changes over the period of time.

[0046] Also provided herein are methods for increasing the intestinal, colonic or faecal molar ratio of acetic acid to butyric acid, the intestinal, colonic or faecal molar ratio of propionic acid to butyric acid, and/or the intestinal, colonic or faecal molar ratio of acetic acid to propionic acid to butyric acid in a subject with type 2 diabetes, comprising administering to the subject one or more probiotic microorganisms selected from *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

[0047] Also provided herein are methods for increasing intestinal, colonic or faecal acetic acid levels in a subject with type 2 diabetes, comprising administering to the subject one or more probiotic microorganisms selected from *Lactobacillus plantarum*, *Lactobacillus*

bulgaricus, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

[0048] Also disclosed herein is a method for treating type 2 diabetes, or a metabolic complication or condition associated therewith, or for delaying or inhibiting the onset of type 2 diabetes in a subject, the method comprising:

- (a) obtaining a faecal or colonic sample from the subject;
- (b) determining levels of butyric acid and one or both of acetic acid and propionic acid in the sample, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or of acetic acid to propionic acid to butyric acid, wherein a lower molar ratio(s) in the subject than in one or more individuals known not to be pre-diabetic or to have type 2 diabetes is indicative of pre-diabetes or type 2 diabetes in the subject; and
- (c) where the subject is determined to be pre-diabetic or to have type 2 diabetes, administering to the subject one or more probiotic microorganisms selected from *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

[0049] Metabolic complications and conditions associated with type 2 diabetes are well known to those skilled in the art, and include for example, diabetic hypoglycaemia, diabetic hyperglycemia, autoimmune dysfunction, microvascular conditions such as nephropathy, neuropathy and retinopathy, macrovascular conditions such as peripheral arterial disease, transient ischemic attacks, angina pectoris and myocardial infarction. Also encompassed by metabolic complications and conditions associated with type 2 diabetes as referred to herein include metabolic disorders related to, and associated with, type 2 diabetes such as metabolic syndrome, insulin resistance, hyperglycaemia, hypoglycaemia, hyperinsulinemia and hypoinsulinemia.

[0050] Optionally, in accordance with methods of the present disclosure the subject is administered a multi-strain probiotic combination comprising *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and

Saccharomyces boulardii. Optionally, the subject is also administered metformin or a derivative or salt thereof.

[0051] In particular embodiments, the probiotic microorganisms are administered as a multi-strain probiotic combination comprising *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*. Optionally the one or more probiotic microorganisms or the multi-strain combination may be present in a composition as specially selected strains as a culture concentrate or as part of a multi-strain blend, optionally with a variety of excipients.

[0052] Methods of the present disclosure may further comprise the administration of one or more additional probiotic microorganisms selected from, for example, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus salivarius*, *Lactobacillus delbrueckii* spp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus johnsonii*, *Lactococcus lactis*, *Bifidobacterium animalis* subsp. *animalis* (*B. animalis*), *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum*.

[0053] The amounts of individual microorganisms to be administered to subjects or to be included in compositions disclosed herein will depend on a variety of factors including the identity and number of individual strains employed, the condition or disease to be treated or against which the composition is designed to be used, and the form in which a composition is administered. For any given case, appropriate amounts may be determined by one of ordinary skill in the art using only routine experimentation. By way of example only, the amount of each microbial strain present in a single dose of a composition disclosed herein may be from about 1×10^2 cfu to about 1×10^{11} cfu, and may be about 1×10^3 cfu, about 2.5×10^3 cfu, about 5×10^3 cfu, about 7.5×10^3 cfu, 1×10^4 cfu, about 2.5×10^4 cfu, about 5×10^4 cfu, about 7.5×10^4 cfu, about 1×10^5 cfu, about 2.5×10^5 cfu, about 5×10^5 cfu, about 7.5×10^5 cfu, about 1×10^6 cfu, about 2.5×10^6 cfu, about 5×10^6 cfu, about 7.5×10^6 cfu, about 1×10^7 cfu, about 2.5×10^7 cfu, about 5×10^7 cfu, about 7.5×10^7 cfu, about 1×10^8 cfu, about 2.5×10^8 cfu, about 5×10^8 cfu, about 7.5×10^8 cfu, about 1×10^9 cfu, about 2.5×10^9 cfu, about 5×10^9 cfu, about 7.5×10^9 cfu, about 1×10^{10} cfu, about 2.5×10^{10} cfu, about 5×10^{10} cfu, about 7.5×10^{10} cfu, and about 1×10^{11} cfu.

[0054] Also contemplated by the present disclosure are variants of the microorganisms described herein. As used herein, the term "variant" refers to both naturally occurring and specifically developed variants or mutants of the microbial strains disclosed and exemplified herein. Variants may or may not have the same identifying biological characteristics of the specific strains exemplified herein, provided they share similar advantageous properties in terms of their ability to be used as probiotic strains. Illustrative examples of suitable methods for preparing variants of the microbial strains exemplified herein include, but are not limited to, culturing under selective growth conditions, gene integration techniques such as those mediated by insertional elements or transposons or by homologous recombination, other recombinant DNA techniques for modifying, inserting, deleting, activating or silencing genes, intraspecific protoplast fusion, mutagenesis by irradiation with ultraviolet light or X-rays, or by treatment with a chemical mutagen such as nitrosoguanidine, methylmethane sulfonate, nitrogen mustard and the like, and bacteriophage-mediated transduction. Suitable and applicable methods are well known in the art and are described, for example, in J. H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1972); J. H. Miller, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1992); and J. Sambrook, D. Russell, *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2001), *inter alia*.

[0055] Also encompassed by the term "variant" as used herein are microorganisms or strains phylogenetically closely related to microorganisms or strains disclosed herein and microorganisms or strains possessing substantial sequence identity with the microorganisms and strains disclosed herein at one or more phylogenetically informative markers such as rRNA genes, elongation and initiation factor genes, RNA polymerase subunit genes, DNA gyrase genes, heat shock protein genes and *recA* genes. For example, the 16S rRNA genes of a "variant" microorganism or strain as contemplated herein may share about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a strain disclosed herein.

[0056] The microorganisms to be employed in accordance with the present disclosure may be cultured according to any suitable method known to the skilled addressee and may be prepared for addition to a composition by, for example, freeze-drying, spray-drying or

lyophilisation. Thus, in embodiments of the present disclosure the microorganisms may be in a dried form (such as lyophilized or sporulated form) in a suitable carrier medium, for example a FOS medium or other soluble fiber, sugar, nutrient or base material for the composition, with which the bacterial strains can be presented in an orally administrable form. One or more of the strains may be encapsulated in, for example, a suitable polymeric matrix to improve long-term stability and storage of the compositions. In one example, encapsulation may comprise alginate beads, although those skilled in the art will appreciate that any suitable encapsulation material or matrix may be used. Encapsulation may be achieved using methods and techniques known to those skilled in the art.

[0057] In some embodiments, methods of the present disclosure may comprise administration to the subject of one or more prebiotic components. Similarly, in some embodiments, compositions of the present disclosure may further comprise at least one prebiotic component. Suitable prebiotics include polydextrose, inulin, fructooligosaccharides (FOS), xylooligosaccharides (XOS), galactooligosaccharides (GOS), mannan oligosaccharides, arabinogalactans (such as larch arabinogalactans), resistant starches (such as Hi-Maize resistant starch), protein-based green lipped mussel extract, and various prebiotic-containing foods such as raw onion, raw leek, raw chickory root and raw artichoke. In certain embodiments the prebiotic component is a fructooligosaccharide. Those skilled in the art will appreciate that other prebiotics may be added to the compositions.

[0058] In accordance with particular embodiments of the invention the at least one prebiotic component may be administered or be present in a composition in an amount of from about 1 mg to about 100 g, or more typically between about 5 mg to about 50 g. Alternatively, the composition may comprise about 10 mg, 100 mg, 1 g, 5 g, 10 g, 15 g, 20 g, 25 g, 30 g, 35 g, 40 g or 45 g of prebiotic.

[0059] Metformin can exist as a free base, or may form salts, including pharmaceutically acceptable salts, such as a hydrochloride salt (e.g., a mono-hydrochloride salt. Accordingly, in accordance with the present disclosure, the metformin may be administered as the free base form, or as a hydrochloride salt, a mono-hydrochloride salt or other a pharmaceutically acceptable salt of metformin. Suitable metformin salts include, but are not limited to, metformin hydrochloride, metformin glycinate salts, metformin fumarate salts and metformin succinate salts. Other representative pharmaceutically acceptable salts include, for example,

acetate, benzenesulfonate, benzoate, bicarbonate, bisulfate, bitartrate, borate, bromide, calcium edetate, camsylate, carbonate, chloride, clavulanate, citrate, dihydrochloride, edetate, edisylate, estolate, esylate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexylresorcinate, hydrabamine, hydrobromide, hydroxynaphthoate, iodide, isethionate, lactate, lactobionate, laurate, malate, maleate, mandelate, mesylate, methylbromide, methylnitrate, methylsulfate, monopotassium maleate, mucate, napsylate, nitrate, N-methylglucamine, oxalate, pamoate (embonate), palmitate, pantothenate, phosphate/diphosphate, polygalacturonate, potassium, salicylate, sodium, stearate, subacetate, tannate, tartrate, teoclate, tosylate, triethiodide, trimethylammonium and valerate. The skilled addressee will appreciate that the scope of the present disclosure is not limited by reference to specific salts of metformin.

[0060] Also contemplated herein are derivatives of metformin, including synthetic derivatives of metformin such as HL010183 and other derivatives such as those described in US Patent No. 8,853,259 (the disclosure of which is incorporated herein by reference). The skilled addressee will appreciate that the scope of the present disclosure is not limited by reference to specific derivatives of metformin.

[0061] The metformin or derivative or salt thereof may be included in any suitable dosage form. For example, the metformin may exist in a powder, a tablet, a capsule, or the like. Such dosage forms may, in some embodiments, also include specialized coatings, matrices, and the like to give effect a sustained release, a controlled release, enteric release, etc. In some embodiments, the metformin or derivative or salt thereof may be present in a dosage form with the one or more probiotic microorganisms or multi-strain probiotic combination.

[0062] The metformin or derivative or salt thereof may be administered in any dosage suitable for the treatment of type 2 diabetes. Such dosages will be well known to the skilled person. For example, in an immediate or controlled release formulation, the metformin or derivative or salt thereof may be administered in an initial dose of between about 500 mg to 1000 mg daily, increasing to a maintenance dose of about 2000 mg daily. The daily amount may be administered as a single dose or in divided doses two or three times a day. By way of example only, the daily dose of metformin or derivative or salt thereof administered in accordance with the present disclosure may be between about 500 mg and about 2500 mg, such as about 500 mg, about 700 mg, about 900 mg, about 1100 mg, about 1300 mg, about 1500 mg, about 1700 mg, about 1900 mg, about 2100 mg, about 2300 mg or about 2500 mg.

[0063] In particular embodiments, the probiotic microorganisms are administered as a multi-strain probiotic combination comprising *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*. Optionally the one or more probiotic microorganisms or the multi-strain combination may be present in a composition as specially selected strains as a culture concentrate or as part of a multi-strain blend, optionally with a variety of excipients.

[0064] In some embodiments, methods of the present disclosure may comprise administration to the subject of a source of fibre. Similarly, in some embodiments, compositions of the present disclosure may further comprise a source of fibre. The source of fibre may comprise soluble fibre, insoluble fibre, or a combination of both soluble and insoluble fibre. Suitable sources of soluble fibre include, but are not limited to, psyllium, dextrins, fructans, oligosaccharides (e.g. inulins) and polysaccharides. Exemplary polysaccharides include resistant starches and arabinogalactans. An exemplary arabinogalactan is Larch arabinogalactan. The resistant starch may be an RS1, RS2,, RS3 or RS4 resistant starch. An exemplary resistant starch is Hi-Maize resistant starch. Arabinogalactans are complex proteoglycans of arabinose and galactose, often classified as plant or microbial arabinogalactan.

[0065] Molecules such as acetyl-l-carnitine, alpha-lactalbumin, beta-lactoglobulin, glycomacropeptides, immunoglobulin G, and bovine serum albumin may augment the health of the gut individually and/or in combination with each other as well as with the administration of probiotic bacteria, and optionally a prebiotic, as disclosed herein. This augmented health benefit may translate in a reduction of risk of chronic disease progression via the gastrointestinal control of obesity. Accordingly, embodiments of the present invention contemplate the addition of carnitine, such as acetyl-l-carnitine, and/or a protein-containing component to compositions disclosed herein, and the administration thereof to subjects in accordance with methods of the invention. The protein-containing component may comprise a protein powder, such as a milk powder. The milk powder may be skim milk powder. The protein-containing component may comprise one or more of colostrum or a protein-containing fraction thereof, alpha-lactalbumin, beta-lactoglobulin, glycomacropeptides, lactoferrin, immunoglobulin G and/or bovine serum albumin.

[0066] In some embodiments, methods of the present disclosure may comprise administration to the subject of suitable vitamins, minerals and/or amino acids. Similarly, in some embodiments, compositions of the present disclosure may further comprise suitable vitamins, minerals and/or amino acids. The vitamins and minerals may be selected from, but not limited to: vitamins A, B₁, B₂, B₃, B₅, B₆, B₉, B₁₂, C, D, E and calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, phosphorus, potassium, selenium, sodium and zinc. The amino acids may be selected from, but are not limited to: alanine, leucine, valine, isoleucine, arginine, aspartic acid, cystine, glycine, histidine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan and tyrosine.

[0067] In some embodiments, methods of the present disclosure may comprise administration to the subject of one or more antioxidants. Similarly, in some embodiments, compositions of the present disclosure may further comprise one or more antioxidants. The antioxidants may be water-soluble or lipid-soluble antioxidants. Exemplary water soluble antioxidants include sodium ascorbate, calcium ascorbate, potassium ascorbate, ascorbic acid, glutathione, lipoic acid and uric acid. Exemplary lipid soluble antioxidants include tocopherols, tocotrienols, phenols, polyphenols and the like.

[0068] Compositions of the disclosure may further comprise any suitable additives, carriers, additional therapeutic agents, bioavailability enhancers, side-effect suppressing components, diluents, buffers, flavouring agents, binders, preservatives or other ingredients that are not detrimental to the efficacy of the composition. In some embodiments, the probiotic strains may comprise from about 50% to about 90% by weight of the composition, based on the total weight of the composition including a carrier medium, or from about 60% to about 80% by weight of the composition. In particular embodiments, compositions may be gluten free and dairy free, and suitable for ingestion by vegetarians.

[0069] Compositions of the disclosure can be readily manufactured by those skilled in the art using known techniques and processes. For example, the probiotic microorganisms can be seeded from standard stock into a reactor and grown in standardized media until a predetermined cfu/g concentration is reached. The bulk material can then be drained from the reactor and dried by spray drying, lyophilization, or flatbed oven drying. The dried bacterial material can then be blended with the carrier medium and the resulting mixture can be pressed

into tablets, filled into foil pouches as a granular solid, or introduced into gelatin capsules as a particulate material.

[0070] Microorganisms and the metformin or derivative or salt thereof may be suitably formulated, separately or in a single composition, for oral administration, and may be prepared according to conventional methods well known in the pharmaceutical and nutraceutical industries, such as those described in Remington's Pharmaceutical Handbook (Mack Publishing Co., NY, USA) using suitable excipients, diluents and fillers.

[0071] Compositions suitable for oral administration may be presented as discrete units (i.e. dosage forms) such as gelatine or HPMC capsules, cachets or tablets, each containing a predetermined amount of each component of the composition as a powder, granules, as a solution or a suspension in an aqueous liquid or a non-aqueous liquid, or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion.

[0072] When the composition is formulated as capsules, the components of the composition may be formulated with one or more pharmaceutically acceptable carriers such as starch, lactose, microcrystalline cellulose and/or silicon dioxide. Additional ingredients may include lubricants such as magnesium stearate and/or calcium stearate. The capsules may optionally be coated, for example, with a film coating or an enteric coating and/or may be formulated so as to provide slow or controlled release of the composition therein.

[0073] Tablets may be prepared by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the components of the composition in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant (for example magnesium stearate or calcium stearate), inert diluent or a surface active/dispersing agent. Moulded tablets may be made by moulding a mixture of the powdered composition moistened with an inert liquid diluent, in a suitable machine. The tablets may optionally be coated, for example, with a film coating or an enteric coating and/or may be formulated so as to provide slow or controlled release of the composition therein.

[0074] The compositions may be provided to the user in a powder form. For oral administration, the composition may then be mixed with a suitable volume of an aqueous

medium, typically with agitation, to dissolve the components, or produce a suspension, suitable for ingestion. Thus, the compositions may be provided to a user in a powder form, which powder may then be added by the user to any type of aqueous medium (for example water or fruit juice) and consumed there after. Alternatively, the composition may be provided as a beverage, pre-mixed with an aqueous medium such as water. In another embodiment the compositions may be added in powder form by the user to any type to a food product (for example, yoghurt) and consumed there after. In another embodiment, the compositions may simply be consumed as a powder in the absence of a drink or additional food product.

[0075] The probiotic microorganisms may be conveniently incorporated in a variety of food and/or beverage products, nutraceutical products, probiotic supplements, food additives, pharmaceuticals and over-the-counter formulations. The food or food additive may be a solid form such as a powder, or a liquid form. Specific examples of the types of beverages or foods include, but are not limited to water-based, milk-based, yoghurt-based, other dairy-based, milk-substitute based such as soy milk or oat milk, or juice-based beverages, water, soft drinks, carbonated drinks, and nutritional beverages, (including a concentrated stock solution of a beverage and a dry powder for preparation of such a beverage); baked products such as crackers, breads, muffins, rolls, bagels, biscuits, cereals, bars such as muesli bars, health food bars and the like, dressings, sauces, custards, yoghurts, puddings, pre-packaged frozen meals, soups and confectioneries.

[0076] Those skilled in the art will appreciate that single or multiple administrations of compositions disclosed herein can be carried out with dose levels and dosing regimes being determined as required depending on circumstances and requirements of the subject to be treated. The skilled addressee can readily determine suitable dosage regimes. A broad range of doses may be applicable. Dosage regimens may be adjusted to provide the optimum therapeutic response. Those skilled in the art will appreciate that the exact amounts and rates of administration of the metformin or derivative or salt thereof and of the probiotic microorganisms will depend on a number of factors such as the particular composition being administered, the age, body weight, general health, sex and dietary requirements of the subject, as well as any drugs or agents used in combination or coincidental with the compositions. For example, several divided doses may be administered hourly, daily, weekly, monthly or at other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies

of the situation. Based on the teaching herein those skilled in the art will, by routine trial and experimentation, be capable of determining suitable dosage regimes on a case-by-case basis.

[0077] Methods of the present disclosure may be employed as an adjunct to other therapies or treatments for type 2 diabetes, pre-diabetes, metabolic syndrome, insulin resistance, obesity and obesity-related disorders. Accordingly compositions and methods disclosed herein may be co-administered with other agents that may facilitate a desired therapeutic outcome, for example sulfonylureas such as such as tolbutamide, glibenclamide, glipizide, chlorpropamide, or gliclazide. By “co-administered” is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. By “sequential” administration is meant a time difference of from seconds, minutes, hours or days between the administration of the agents, compositions or treatments. Sequential administration may be in any order. Similarly, methods of the present disclosure may be employed in conjunction with lifestyle changes by the subject, such as a healthy diet and adequate exercise.

[0078] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

[0079] The present disclosure will now be described with reference to the following specific examples, which should not be construed as in any way limiting the scope of the invention.

Examples

[0080] The following examples are illustrative of the invention and should not be construed as limiting in any way the general nature of the disclosure of the description throughout this specification.

Example 1 – Double-blind, randomised, placebo-controlled trial

Study protocol

[0081] The study described herein was a pilot, single site, randomised, double blind, placebo-controlled trial. Ethical approval was granted by the Sydney Local Health District Human Research Ethics Committee, RPA Hospital, Sydney, Australia. The study was carried out according to the Declaration of Helsinki, the National Health and Medical Research Council National Statement on Ethical Conduct in Research Involving Humans and the Notes for Guidance on Good Clinical Practice as adopted by the Australian Therapeutic Goods Administration and the International Conference on Harmonisation Good Clinical Practise guidelines. Written informed consent was obtained from participants before enrolment.

[0082] Individuals were eligible for the study if the following criteria were met: (i) aged ≥ 18 years; (ii) BMI ≥ 25 kg/m²; (iii) pre-diabetes or type 2 diabetes mellitus diagnosed within the previous 12 months (criteria for the diagnosis of pre-diabetes and type 2 diabetes were based on the guidelines of the American Diabetes Association); (iv) treated by diet alone or diet plus metformin and; (v) willingness to adhere to the study protocol (no yogurt, fermented food, dietary supplements, probiotics or prebiotics) for the duration of the study.

[0083] Individuals were excluded from the study if any of the following factors applied: (i) type 1 diabetes mellitus; (ii) type 2 diabetes mellitus diagnosed for more than 12 months; (iii) taking anti-obesity drugs or blood glucose-lowering medications (i.e. sulfonylureas, alpha-glucosidase inhibitors, thiazolidinediones and glucagon-like peptide-1 analogues) other than metformin; (iv) presence of concomitant gastrointestinal disorders (irritable bowel syndrome, inflammatory bowel disease and coeliac disease); (v) recent use (within the previous four weeks) of antibiotics and dietary supplements (fish oil, probiotics, prebiotics, multivitamins, minerals, nutraceuticals and herbal preparations); (vi) pregnancy, breast feeding or planning to become becoming pregnant; (vii) alcohol abuse or the use of any illicit drugs and; (viii) clinical evidence of active infection or any severe illness unrelated to diabetes.

[0084] The active product administered was a capsule containing 5×10^{10} colony forming units (cfu) of a multi-strain probiotic combination of *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis subsp.*

lactis, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*. The placebo was a capsule containing 50 mg maltodextrin. The probiotic and placebo capsules were opaque white in colour and looked and smelled identical.

[0085] Participants were randomised to the probiotic (intervention) or placebo group without stratification using computer-generated random numbers (FileMaker Pro). Participants and study investigators were blinded to treatment allocation. Blinding remained intact until the statistical testing of the outcomes.

[0086] Enrolled participants in each group were required to: take two capsules twice per day (20 minutes before breakfast and dinner) with cold non-carbonated water (not to be mixed or taken with hot drinks or foods (as heat and stomach acids can reduce the stability of the probiotic bacteria) for the 12 week course of the study; store the capsules in the refrigerator at 4–6° C; record the number of capsules taken each day; bring all capsules remaining in the bottle to the subsequent visit and; avoid eating/drinking yoghurt, fermented food, dietary supplements (i.e. vitamins, minerals, nutraceuticals, herbal preparations, probiotics, prebiotics or fish oils) and antibiotics (unless recommended by a health professional). Capsule counting, at weeks six and 12, was used to assess the participant's compliance. A participant was deemed to be non-compliant if they took less than 80% of the study product on both occasions.

[0087] Changes were assessed in the following biomarkers and measurements from baseline (week 0 at initiation of the study) to 12 weeks: (i) plasma HbA1c, 2-h post-load glucose, triglycerides, free fatty acids, total cholesterol, HDL-c, LDL-c, high-sensitive CRP, LPS and zonulin; (ii) insulin resistance measured by the homeostatic model assessment for insulin resistance (HOMA-IR) and insulin sensitivity index of Matsuda (ISI-M); (iii) faecal microbial profile and short chain fatty acids; (iv) weight, BMI, waist circumference and waist and hip ratio; (v) gastrointestinal symptoms and; (vi) blood pressure. Differences in all biomarkers/measurements were determined between participants with pre-diabetes and participants with type 2 diabetes in the intervention (probiotic) group and the placebo group from baseline to 12 weeks. Differences were determined between participants taking and not taking metformin in the intervention (probiotic) group and the placebo group from baseline to 12 weeks on glycaemic parameters, insulin resistance, LPS, zonulin, faecal microbial profile, short chain fatty acid levels and gastrointestinal symptoms.

Sampling and analysis

[0088] Stool (faecal) samples were collected at baseline and 12 weeks using a stool specimen collection kit. This collection kit included an instruction booklet for the stool sample collection and transportation, ice packs, gloves, a sterile container, sealed plastic pouch, cool box and an AnaeroGen™ Compact sachet that preserved the microbiological characteristics of the sample for 72 hours. Participants brought the stool sample to the clinic and a one-gram sample was stored at -80°C for faecal microbial and short chain fatty acid analysis.

[0089] Gas chromatography-mass spectrometry (GC-MS), with slight modifications from the protocol of Garcia-Villalba *et al.* (*J Sep Sci* (2012) 35:1906-13), was employed to determine the presence of small chain fatty acids in faecal samples. 0.1 g of each faecal sample was weighted and suspended in 1 mL of distillate water with 0.5% phosphoric acid per 0.1 g of sample, vortexed for 2 min and centrifuged for 10 minutes at 17000 x g. A liquid-liquid extraction of small chain fatty acids was then obtained by adding 1 mL of ethyl acetate into 1 ml of water supernatant of the sample, vortexed for 2 min and centrifuged for 10 minutes at 17000 x g. Analysis was performed using an Agilent 7890A gas chromatography system coupled to an Agilent 5975C inert XL EI/CI mas spectrometric detector (MSD, Agilent Technologies). 1 µl of the organic layer of each sample was injected in split mode with a ratio of 10:1 into a high polarity, polyethylene glycol, fused silica capillary column DB-WAXETR (30 m × 0.25 mm id × 0.25 µm film thickness; Technomind). The solvent delay time was set to 3 min. The initial oven temperature was 90°C, then increased to 150°C at a rate of 15°C/min, to 170°C at a rate of 5°C/min, and finally held at 250°C at a rate of 20 °C/min for 2 min (total time 14 min). Helium was used as a carrier gas at a constant flow rate of 1 mL/min though the column. The temperature of the ion source, quadrupole, and interface were set at 230, 150, and 280°C, respectively. The electron energy was 70 eV, and the mass spectral data was collected in SIM mode (m/z 60). Identification of acetic, propionic, isobutyric, butyric, isovaleric and valeric acids was based on the retention time of standard compounds and quantified using Agilent Mass Hunter Quantative software. Quantification of each small chain fatty acid was based on calibration curves obtained from increasing concentrations of standards diluted in ethyl acetate.

[0090] Statistical analyses were conducted using intention-to-treat and missing data was imputed with baseline values for a conservative estimate (i.e. no change). All data was checked for normal distribution using the Shapiro-Wilk test. Descriptive statistics were presented as mean $\pm$ standard deviation or median with interquartile range (IQR), as appropriate. HbA1c and insulin resistance were analysed using generalised linear models and the covariates of age, sex and baseline values were added to the model. Other outcomes were analysed using independent Student's *t* test or the Mann-Whitney test to compare the study groups at week 12. Paired Student's *t* test or Wilcoxon matched-pairs signed-rank was used to analyse differences between baseline and endpoint values. Three subgroup analyses were completed between those participants taking metformin, participants classified as having pre-diabetes or type 2 diabetes, and those participants defined as compliant. Statistical analyses were performed using SPSS Statistics version 22 (IBM).

Baseline characteristics

[0091] Participants were on average 59 years old, their mean BMI was above 30 kg/m², 97% of the participants had central obesity, 68% had insulin resistance (HOMA-IR ≥ 2.5), 40% had plasma total cholesterol concentrations >5.2 mmol/L, 58% had plasma LDL-cholesterol >2.6 mmol/L, 50% of women and 68% of men had low plasma HDL-cholesterol (<1.4 mmol/L and <1.15 mmol/L, respectively), 20% had plasma triglyceride concentrations >2.3 mmol/L, 34% had plasma free fatty acids >720 μ mol/L, 50% had systolic blood pressure >130 mmHg, 30% had diastolic blood pressure >80 mmHg, 25% had elevated plasma hs-CRP >5 mg/L. Moreover, 68% had plasma LPS above values considered normal in adults (0.15 - 0.35 EU/ml) and 34% had plasma zonulin concentrations above the normal range (30 - 200 mg/mL). A total of 20 participants (10 in each group) were classified as non-compliant as they took less than 80% of the study product. Therefore, sub-group analysis was performed on 40 compliant participants (20 in each group).

Table 1 – Baseline characteristics of participants by group and condition/disease

	Placebo			Probiotic		
	All (n=30)	Pre-diabetes (n=19)	T2DM (n=11)	All (n=30)	Pre-diabetes (n=17)	T2DM (n=13)
Gender						
Female	19 (63%)	15 (79%)	4 (36%)	13 (43%)	7 (41%)	6 (46%)
Male	11 (37%)	4 (21%)	7 (64%)	17 (57%)	10 (59%)	7 (54%)
Age (years)	56.1±12.3	56.5±11.1	55.5±14.7	61.4±8.9	62.9±9.3	59.3±8.3
Medications						
Metformin	14 (47%)	7 (37%)	7 (64%)	14 (47%)	4 (24%)	10 (77%)
Lipid-lowering	8 (27%)	5 (26%)	3 (27%)	15 (50%)	10 (59%)	5 (38%)
BP-lowering ^a	14 (47%)	9 (47%)	5 (45%)	13 (43%)	7 (41%)	6 (46%)
Body composition						
Height (m)	1.7±0.1	1.6±0.1	1.7±0.1	1.7±0.1	1.7±0.1	1.7±0.1
Body weight	101.7±21.9	97.5±19.6	108.9±24.9	100.1±20.4	97.3±14	103.8±26.8
BMI (kg/m ²)	36.3±7.5	35.9±5.7	37.2±10.0	35.5±6.2	33.9±3.9	37.6±8.0
Glycaemia						
FPG (mmol/L)	6.3±1.6	5.6±0.6	7.4±2.0	7.0±3.0	5.9±0.6	8.6±4.1
HbA1c (%)	6.3±1.1	5.8±0.4	7.0±1.4	6.6±1.4	5.8±0.3	7.6±1.6
Lipids						
Total cholesterol (mmol/L)	5.4±1.3	5.2±1.2	5.6±1.2	4.9±1.1	5.0±0.3	4.8±0.9
Triglycerides (mmol/L)	1.9±1.2	1.8±1.1	2.1±1.3	1.8±0.9	1.6±0.6	2.1±1.1
Blood pressure						
Systolic (mmHg)	127.8±12.5	126.4±14	130±9.3	133.0±10.8	133.7±7.9	132.1±14.1
Diastolic (mmHg)	81.6±6.3	80.4±6.4	83.6±5.9	79.8±7.3	79.6±7.8	79.9±6.8
Inflammation / intestinal permeability						
hs-CRP (mg/L)	2.7 (5.2)	2.6 (5.5)	3.0 (5.3)	3.0 (3.4)	2.5 (2.2)	4.7±11.7
LPS (EU/mL)	0.6±0.3	0.5±0.4	0.6±0.3	0.5±0.2	0.5±0.2	0.5±0.3
Zonulin (mg/dL)	207.8±172.9	217.2±183.9	191.5±159.3	203.6±171.9	171.3±106.2	249.2±234.6

Results are mean±SD or median (IQR) or number and percentage where appropriate. FPG: fasting plasma glucose; LPS: lipopolysaccharide; ^ablood pressure lowering medication: ACE inhibitors, angiotensin II receptor blockers, beta-blockers and calcium channel blockers

Outcomes

[0092] Example 2 below details outcomes determined from the present study.

Example 2 – Effect of metformin and a multi-strain probiotic combination on intestinal microbiota in type 2 diabetic and pre-diabetic subjects

[0093] Faecal SCFA concentrations were quantified by GC-MS. Statistical analysis revealed a high butyric acid concentration in relation to the other SCFA in both the probiotic and placebo groups at the commencement of the study. The concentrations of faecal acetic, propionic and butyric acids were found in a molar ratio of approximately 1:1:2 thus a low acetic to butyric acid ratio was found in the placebo and probiotic groups at baseline.

[0094] At the initiation of the study, baseline concentrations of faecal acetic and butyric acids were surprisingly found to be in a molar ratio of approximately 1:2 in participants with pre-diabetes and type 2 diabetes. This contrast to previous studies which revealed a molar ratio of 3:1 in lean individuals (den Besten *et al.*, *J Lipid Res* (2013) 54:2325-40) and in obese individuals (Schwartz *et al.*, *Obesity* (2010) 18:190-5).

[0095] At the conclusion of the study, a trend towards more balanced ratios of acetic acid to butyric acid, of propionic acid to butyric acid and of acetic acid to propionic acid to butyric acid were found in the probiotic group but not the placebo group. For example, subgroup analysis showed a significant improvement in the acetic to butyric acid ratio in those with type 2 diabetes in comparison to the placebo group (1:1 vs. 1:2; $p=0.01$; Figure 1A). The effect of probiotic supplementation on acetic acid levels was assessed in type 2 diabetic participants who had a low acetic to butyric acid ratio at baseline. This analysis showed an increase in acetic acid median levels in the probiotic group in comparison to the placebo group, after the intervention (1047.5 [806.0] vs. 591.3 [586.4]; $p<0.05$; Figure 1B).

Claims

1. A method for diagnosing pre-diabetes or type 2 diabetes in a subject, the method comprising:

- (a) obtaining a faecal or colonic sample from the subject; and
- (b) determining levels of butyric acid and one or both of acetic acid and propionic acid in the sample, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid;

wherein a molar ratio of faecal or colonic acetic acid to butyric acid of less than about 1:1 is indicative of pre-diabetes or type 2 diabetes, a molar ratio of faecal or colonic propionic acid to butyric acid of less than about 1:1 is indicative of pre-diabetes or type 2 diabetes, and a molar ratio of faecal or colonic acetic acid to propionic acid to butyric acid of less than about 1:1:1 is indicative of pre-diabetes or type 2 diabetes.

2. A method according to claim 1, wherein a molar ratio of acetic acid to butyric acid of about 1:2 is indicative of pre-diabetes or type 2 diabetes.

3. A method according to claim 1, wherein a molar ratio of propionic acid to butyric acid of about 1:2 is indicative of pre-diabetes or type 2 diabetes.

4. A method according to claim 1, wherein a molar ratio of acetic acid to propionic acid to butyric acid of about 1:1:2 is indicative of pre-diabetes or type 2 diabetes.

5. A method for diagnosing pre-diabetes or type 2 diabetes in a subject, the method comprising:

- (a) obtaining a faecal or colonic sample from the subject;
- (b) determining levels of butyric acid and one or both of acetic acid and propionic acid in the sample, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid;
- (c) comparing the determined ratio(s) to the molar ratio(s) of faecal or colonic acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid in one or more individuals known not to be pre-diabetic or to have type 2 diabetes;

wherein a lower ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or acetic acid to propionic acid to butyric acid in the subject than in the one or more individuals is indicative of pre-diabetes or type 2 diabetes in the subject.

6. A method for increasing the intestinal, colonic or faecal molar ratio of acetic acid to butyric acid, the intestinal, colonic or faecal molar ratio of propionic acid to butyric acid and/or the intestinal, colonic or faecal molar ratio of acetic acid to propionic acid to butyric acid in a subject with type 2 diabetes, the method comprising administering to the subject one or more probiotic microorganisms selected from *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

7. A method according to claim 6, wherein the increase in the molar ratio(s) is relative to the ratio(s) prior to, and in the absence of, the administration of the one or more probiotic microorganisms.

8. A method according to claim 6, wherein said administering results in a faecal acetic acid to butyric acid ratio of about 1:1.

9. A method according to claim 6, wherein said administering results in a faecal propionic acid to butyric acid ratio of about 1:1.

10. A method according to claim 6, wherein said administering results in a faecal acetic acid to propionic acid to butyric acid ratio of about 1:1:1.

11. A method for increasing intestinal, colonic or faecal levels of acetic acid in a subject with type 2 diabetes, the method comprising administering to the subject one or more probiotic microorganisms selected from *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

12. A method according to claim 11, wherein the increase in acetic acid levels is relative to the levels prior to, and in the absence of, the administration of the one or more probiotic microorganisms.
13. A method according to any one of claims 6 to 12, wherein the subject is administered a multi-strain probiotic combination comprising *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.
14. A method according to any one of claims 6 to 13, wherein the subject is also administered metformin or a derivative or salt thereof.
15. A method for treating type 2 diabetes, or a metabolic complication or condition associated therewith, or for delaying or inhibiting the onset of type 2 diabetes in a subject, the method comprising:
- (a) obtaining a faecal or colonic sample from the subject;
 - (b) determining levels of butyric acid and one or both of acetic acid and propionic acid in the sample, and thereby determining the molar ratio of acetic acid to butyric acid, propionic acid to butyric acid and/or of acetic acid to propionic acid to butyric acid, wherein a lower molar ratio(s) in the subject than in one or more individuals known not to be pre-diabetic or to have type 2 diabetes is indicative of pre-diabetes or type 2 diabetes in the subject; and
 - (c) where the subject is determined to be pre-diabetic or to have type 2 diabetes, administering to the subject one or more probiotic microorganisms selected from *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.
16. A method according to claim 15, wherein the subject is administered a multi-strain probiotic combination comprising *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Saccharomyces boulardii*.

17. A method according to claim 15 or 16, wherein the subject is also administered metformin or a derivative or salt thereof.

1/2

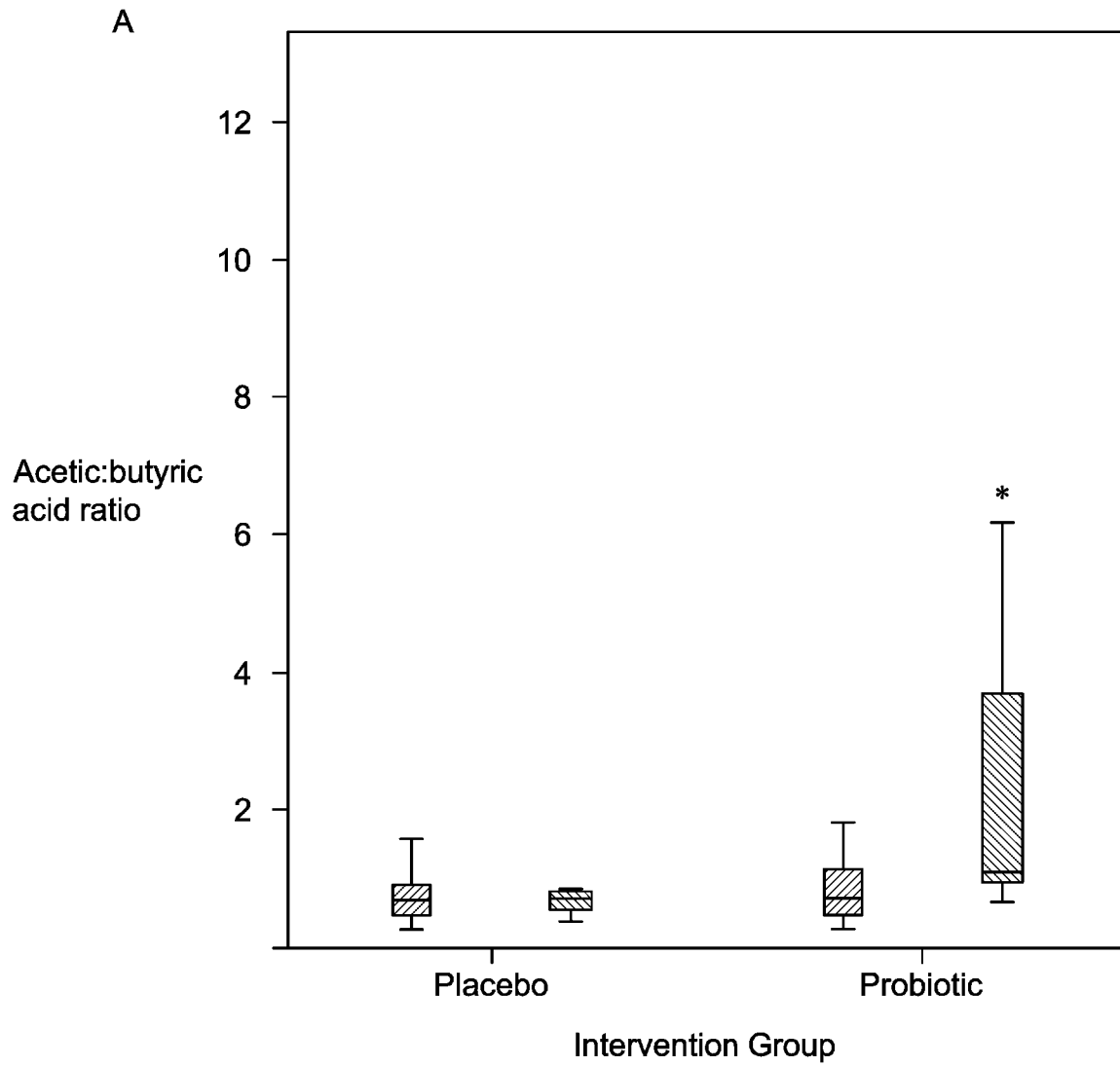


Figure 1

2/2

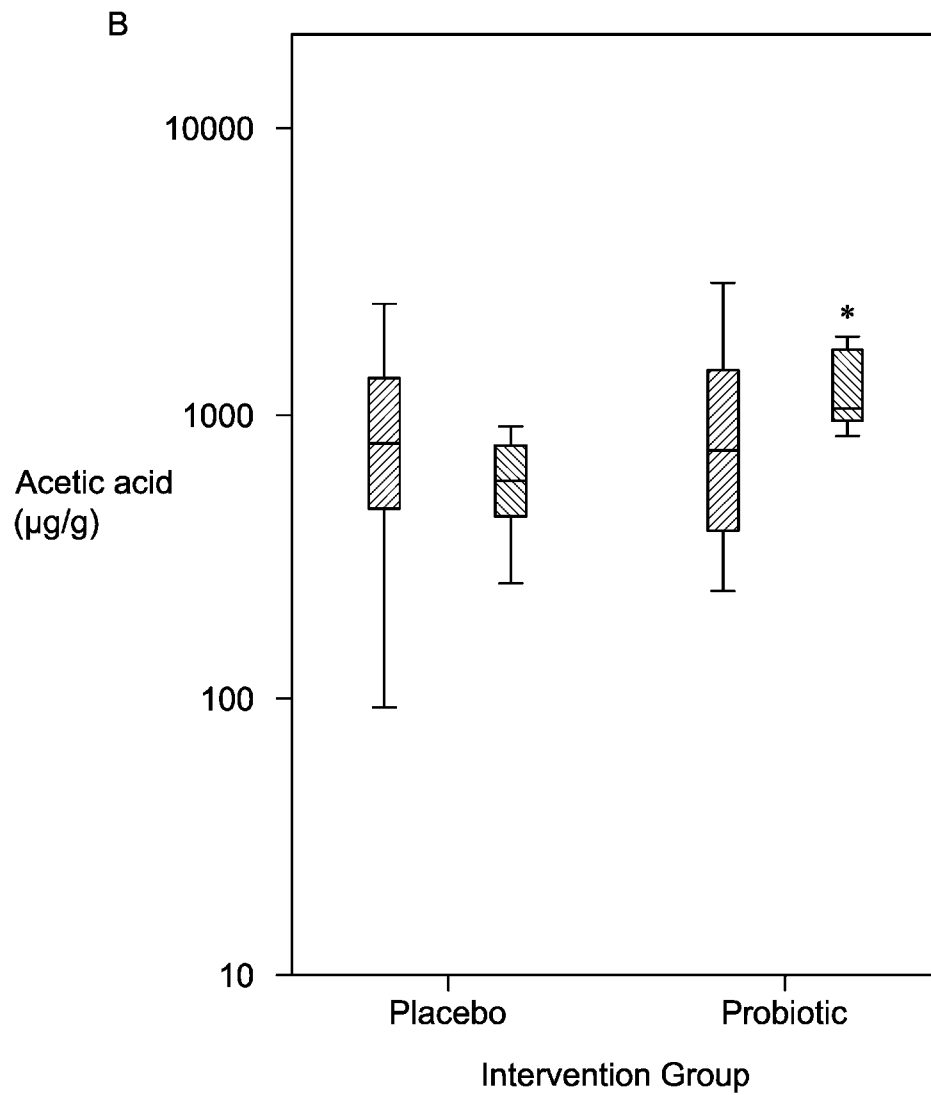


Figure 1(cont'd)

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/AU2018/051182

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/50 (2006.01) **A61K 35/741 (2015.01)** **A61K 35/744 (2015.01)** **A61K 35/745 (2015.01)**
A61K 35/747 (2015.01) **A61K 36/064 (2006.01)** **A61P 3/10 (2006.01)**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CAPLUS, EMBASE, BIOSIS (diagnosing, determining, detecting, predicting, diabetes, insulin resistance, metabolic syndrome, hyperglycaemia, hypoglycaemia, glucose intolerance, impaired glucose tolerance, butyric acid, acetic acid, propionic acid, intestinal, colonic, faecal, ratio, plantarum, bulgaricus, gasseri, breve, lactis, bifidum, thermophiles, boulardii, probiotic, treating, pharmaceutical, medicine, therapy, and other synonyms and like terms); PATENW (G01N2800/042, A61P3/10, and keywords listed above); GOOGLE (pre-diabetes, butyric acid, ratio); ESPACENET, PUBMED (Applicant and Inventor Search)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 19 December 2018		Date of mailing of the international search report 19 December 2018	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au		Authorised officer Damian Triffett AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832845	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2018/051182
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/172191 A1 (MEDLAB IP PTY LTD) 19 November 2015 See whole document especially Claims 17, 26 and 27; Example 2, [00130]	6-14
X	LI, C. et al., "Carrot Juice Fermented with <i>Lactobacillus plantarum</i> NCU116 Ameliorates Type 2 Diabetes in Rats", Journal of Agricultural and Food Chemistry, 22 October 2014, Vol. 62, pages 11884-11891 See whole document especially Abstract; Page 11886, SCFA Composition in Faeces	11-12
X	TONUCCI, L. B. et al., "Clinical application of probiotics in type 2 diabetes mellitus: A randomized, double-blind, placebo-controlled study", Clinical Nutrition, February 2017, Vol. 36, Issue 1, pages 85-92 See whole document especially Abstract; Page 88, 3.5 Effect of probiotics on faecal SCFA analysis	6, 11, 14
A	TEIXEIRA, T. F. S. et al., "High level of faecal SCFA in women correlates with metabolic syndrome risk factors", 2013, Vol. 109, pages 914-919 See whole document especially Abstract; Page 915, Faecal SCFA analysis; Page 917, left hand column, lines 2-5; Page 917, right hand column, first paragraph	1-17

Form PCT/ISA/210 (fifth sheet) (January 2015)

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

- Invention 1 (Claims 1-5 and 15-17). The feature of correlating a lower molar ratio of faecal or colonic acetic acid to butyric acid, propionic acid to butyric acid, and/or acetic acid to propionic acid to butyric acid, with pre-diabetes or type 2 diabetes is specific to this group of claims.
- Invention 2 (Claims 6-10). The feature of increasing the intestinal, colonic or faecal molar ratio of acetic acid to butyric acid, propionic acid to butyric acid, and/or acetic acid to propionic acid to butyric acid, in a subject with type 2 diabetes, by administering one or more of eight probiotic species defined in claim 6, is specific to this group of claims.
- Invention 3 (Claims 11-14). The feature of increasing the intestinal, colonic or faecal acetic acid levels in a subject with type 2 diabetes, by administering one or more of eight probiotic species defined in claim 11, is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art. When there is no special technical feature common to all the claimed inventions there is no unity of invention. In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship.

The only feature common to Invention 2 and Invention 3 and which provides a technical relationship among them is increasing the intestinal, colonic or faecal acetic acid levels in a subject with type 2 diabetes, by administering one or more of eight probiotic species defined in claim 11.

However this feature does not make a contribution over the prior art because it is disclosed in at least D2 and D3.

Therefore in the light of these documents this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/AU2018/051182	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2015/172191 A1	19 November 2015	WO 2015172191 A1 AU 2015258769 A1	19 Nov 2015 01 Dec 2016
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(January 2015)			