Title: PROBIOTIC COMPOSITIONS AND USES THEREOF FOR TREATMENT OF OBESITY-RELATED DISORDERS

Abstract: Provided herein are methods for the treatment or prevention of metabolic syndrome and/or insulin resistance, and for the treatment or prevention of obesity an obesity-related disorders, comprising the administration of two or more probiotic bacterial strains selected from Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus. Compositions for use in such methods are also disclosed.
Probiotic compositions and uses thereof for treatment of obesity-related disorders

Field of the Art

[001] The present disclosure relates generally to compositions and methods to provide probiotic supplements useful for the therapeutic and/or prophylactic treatment, amelioration and/or regulation of disease states or pathological conditions. More specifically, the present disclosure relates to methods and compositions for the treatment or prevention of metabolic syndrome, obesity and obesity-related disorders.

Background

[002] Obesity is one of the most common metabolic disorders in the world, rapidly increasing in prevalence both in the developed and the developing world. Obesity is defined as a body weight more than 20% in excess of the ideal body weight, frequently resulting in a significant impairment of health. Obesity may be measured by body mass index, an indicator of adiposity or fatness. Further parameters for defining obesity are waist circumferences, skinfold thickness and bio impedance. Obesity is fast becoming one of the most critical and challenging medical epidemics, we face, not only placing enormous financial burden on health systems, but affecting the quality of life and ultimately life expectancy of sufferers. Obesity is associated with a number of subsequent disorders including cardiovascular disease, type II diabetes, hypertension, hyperlipidemia and various forms of cancer, and is closely correlated with metabolic syndrome.

[003] Metabolic syndrome (also known as syndrome X and insulin resistance syndrome) is a collection of cardiovascular risk factors including abdominal obesity, hypertension, high blood levels of triglycerides, high fasting glucose levels, and low blood levels of HDL cholesterol. Typically the presence of three or more of these risk factors is required for the diagnosis of metabolic syndrome. Insulin resistance also greatly increases the risk of developing metabolic syndrome. Metabolic syndrome often precedes the development of type II diabetes, coronary artery disease and other atherosclerotic conditions such as stroke and peripheral vascular disease. It is estimated that in the United States alone, there are more than 50 million sufferers of metabolic syndrome.
[004] Metabolic syndrome typically begins with obesity and/or insulin resistance. In the early stages, metabolic risk factors such as elevated apolipoprotein B, elevated triglycerides, high small LDL, low HDL, elevated glucose, elevated plasminogen activator inhibitor 1, elevated fibrinogen, elevated factor VII, elevated inflammatory cytokines, vascular dysfunction and vascular inflammation, may be increased slightly. However with time, particularly as obesity increases and other exacerbating factors emerge, the risk factors increase considerably.

[005] Non alcoholic fatty liver disease (NAFLD) is of increasing relevance in today’s society (and is a more commonly diagnosed liver disease than alcoholic liver disease) due to the rapid and increased prevalence and burden of obesity. The prevalence of NAFLD is almost proportional to the global increase in obesity and type 2 diabetes mellitus. Furthermore, NAFLD is the most commonly diagnosed cause of abnormal liver function tests. NAFLD is caused by fat deposited (steatosis) in the liver that is not due to excess alcohol consumption or liver inflammation and its consequences. Disruption of the normal mechanisms for synthesis, transport and removal of fatty acids (LCFA) and triglycerides (TG) underpins the basic mechanism for the development of NAFLD.

[006] The progressive form of NAFLD is designated non–alcoholic steatohepatitis (NASH). NASH is the progression of steatosis to chronic inflammation of the liver with fat accumulation (steatohepatitis). The progression from NAFLD to NASH may lead to cirrhosis and end stage liver disease that is adverse to health and associated with morbidity.

[007] The major risk factor for NAFLD is over consumption of calorie rich foods and the resulting consequences, namely obesity (abdominal visceral obesity), dyslipidemia, insulin resistance and type 2 diabetes mellitus. Diets comprising high amounts of saturated fats or carbohydrates have been associated with increased liver inflammation. Currently, NAFLD has been estimated to affect as much as 34% and NASH between 2–5% of the population. Dividing the population into lean and obese groups further highlights the correlation with obesity. NAFLD has been found in 16% of lean individuals, opposed to 76% of obese individuals. With childhood obesity rates increasing, the prevalence of NAFLD and NASH is expected to rise, increasing the burden of fatty liver disease. In the pathogenesis of NAFLD and NASH, there is hypothesised a strong involvement of the gastrointestinal system, as evidenced by studies on the gut-liver axis. An understanding of the role of the gut microbiota
and intestinal permeability dysfunction in obesity related liver disorders is warranted.

[008] This multifactorial nature of obesity, and obesity-related disorders such as metabolic syndrome, type 2 diabetes, insulin resistance and fatty liver disorders makes treatment a particular challenge. There is growing interest in the development of novel therapeutic strategies that target multiple risk factors more effectively that the current complex multi-drug therapies employed.

**Summary of the Disclosure**

[009] Provided herein are probiotic compositions comprising bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus salivarius*, *Lactobacillus delbrueckii* spp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus johnsonii*, *Lactococcus lactis*, *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*), *Bifidobacterium animalis* subsp. *animalis* (*B. animalis*), *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum*, and uses of such compositions for therapeutic or prophylactic treatment, amelioration and/or regulation of a disease state in a subject.

[0010] In a first aspect, the present disclosure provides a composition for the treatment or prevention of metabolic syndrome and/or insulin resistance, the composition comprising two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*.

[0011] Typically the subject is overweight or obese or is identified as being at risk of becoming overweight or obese. The subject may be diabetic or pre diabetic. The diabetes may be type 2 diabetes mellitus.

[0012] The composition may improve one or more markers of metabolic syndrome and/or insulin resistance, including blood lipid profile, blood triglyceride levels, blood adiponectin levels, leptin levels, HDL:LDL ratio, liver enzyme levels including alanine transaminase
(ALT) and γ-glutamyl transpeptidase (GGT), fasting blood glucose levels, glucose levels as determined by the oral glucose tolerance test, glycosylated haemoglobin (HbA1C) levels, ghrelin levels, n-3 fatty acid levels, n-6 fatty acid levels, n-3/n-6 fatty acid ratio, C reactive protein levels and Vitamin D levels.

[0013] The composition may comprise three or more, four or more, five or more, six or more, or seven or more of said strains.

[0014] In an exemplary embodiment, the composition comprises or consists of Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus.

[0015] In an exemplary embodiment, the composition comprises or consists of Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus.

[0016] The composition may further comprise one or more yeast strains. The yeast may be a Saccharomyces species, such as S. cerevisiae or S. cerevisiae subsp. boulardii. In an exemplary embodiment, the composition comprises or consists of Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis), Streptococcus thermophilus and Saccharomyces cerevisiae subsp. boulardii.

[0017] The composition may further comprise one or more prebiotic components. In an exemplary embodiment the prebiotic is FOS or a resistant starch.

[0018] In a second aspect, the present disclosure provides a composition for the treatment or prevention of obesity or an obesity-related disorder, the composition comprising two or more probiotic bacterial strains selected from Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus.
[0019] The subject may have metabolic syndrome or insulin resistance. The subject may be diabetic or pre-diabetic. The diabetes may be type 2 diabetes mellitus. The subject may be susceptible to, or be identified as being at risk of developing obesity or an obesity-related disorder.

[0020] The obesity-related disorder may be a fatty liver disorder, such as non-alcoholic fatty liver disease. The non-alcoholic fatty liver disease may be characterised by, or associated with, steatosis and/or non-alcoholic steatohepatitis.

[0021] The composition may improve one or more markers of metabolic syndrome and/or insulin resistance, including blood lipid profile, blood triglyceride levels, blood adiponectin levels, leptin levels, HDL:LDL ratio, liver enzyme levels including alanine transaminase (ALT) and γ-glutamyl transpeptidase (GGT), fasting blood glucose levels, glucose levels as determined by the oral glucose tolerance test, glycosylated haemoglobin (HbA1C) levels, ghrelin levels, n-3 fatty acid levels, n-6 fatty acid levels, n-3/n-6 fatty acid ratio, C reactive protein levels and Vitamin D levels.

[0022] The composition may comprise three or more, four or more, five or more, six or more, or seven or more of said strains.

[0023] In an exemplary embodiment, the composition comprises or consists of Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus.

[0024] In an exemplary embodiment, the composition comprises or consists of Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus.

[0025] The composition may further comprise one or more yeast strains. The yeast may be a Saccharomyces species, such as S. cerevisiae or S. cerevisiae subsp. boulardii. In an exemplary embodiment, the composition comprises or consists of Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis), Streptococcus thermophilus and Saccharomyces cerevisiae subsp. boulardii.
[0026] The composition may further comprise one or more prebiotic components. In an exemplary embodiment the prebiotic is FOS or a resistant starch.

[0027] In a third aspect, the present disclosure provides a method for treating or preventing a condition selected from metabolic syndrome, insulin resistance, obesity and an obesity-related disorder, the method comprising administering to a subject in need thereof a multi-strain probiotic combination comprising two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*.

[0028] Typically the subject is overweight or obese or is identified as being at risk of becoming overweight, obese or of developing an obesity-related disorder. The subject may be diabetic or pre diabetic. The diabetes may be type 2 diabetes mellitus.

[0029] The obesity-related disorder may be a fatty liver disorder, such as non-alcoholic fatty liver disease. The non-alcoholic fatty liver disease may be characterised by, or associated with, steatosis and/or non-alcoholic steatohepatitis.

[0030] The treatment or prevention may comprise improving one or more markers of metabolic syndrome and/or insulin resistance, including blood lipid profile, blood triglyceride levels, blood adiponectin levels, leptin levels, HDL:LDL ratio, liver enzyme levels including alanine transaminase (ALT) and γ-glutamyl transpeptidase (GGT), fasting blood glucose levels, glucose levels as determined by the oral glucose tolerance test, glycosylated haemoglobin (HbA1C) levels, ghrelin levels, n-3 fatty acid levels, n-6 fatty acid levels, n-3/n-6 fatty acid ratio, C reactive protein levels and Vitamin D levels.

[0031] The method may comprise administering to the subject three or more, four or more, five or more, six or more, or seven or more of said strains.

[0032] In an exemplary embodiment, the method comprises or consists of administering to the subject *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*. 
[0033] In an exemplary embodiment, the method comprises or consists of administering to the subject *Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*.

[0034] The method may further comprise administering to the subject one or more yeast strains. The yeast may be a *Saccharomyces* species, such as *S. cerevisiae* or *S. cerevisiae* subsp. *boulardii*. The one or more yeast strains may be present in the same composition or formulation as one or more of the probiotic strains or may be present in a different composition or formulation.

[0035] In an exemplary embodiment, the method comprises or consists of administering to the subject *Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis* subsp. *lactis* (*B. lactis*), *Streptococcus thermophilus* and *Saccharomyces cerevisiae* subsp. *boulardii*.

[0036] The method may further comprise administering to the subject one or more prebiotic components. In an exemplary embodiment the prebiotic is FOS or a resistant starch. The prebiotic may be present in the same composition or formulation as one or more of the probiotic strains or may be present in a different composition or formulation.

[0037] In a fourth aspect, the present disclosure provides a composition for improving one or more markers of metabolic syndrome and/or insulin resistance, the composition comprising two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*.

[0038] Typically the subject is overweight or obese or is identified as being at risk of becoming overweight or obese. The subject may be diabetic or pre diabetic. The diabetes may be type 2 diabetes mellitus.

[0039] The one or more markers of metabolic syndrome and/or insulin resistance may be selected from blood lipid profile, blood triglyceride levels, blood adiponectin levels, leptin levels, HDL:LDL ratio, liver enzyme levels including alanine transaminase (ALT) and γ-glutamyl transpeptidase (GGT), fasting blood glucose levels, glucose levels as determined by
the oral glucose tolerance test, glycosylated haemoglobin (HbA1C) levels, ghrelin levels, n-3 fatty acid levels, n-6 fatty acid levels, n-3/n-6 fatty acid ratio, C reactive protein levels and Vitamin D levels.

[0040] The composition may comprise three or more, four or more, five or more, six or more, or seven or more of said strains.

[0041] In an exemplary embodiment, the composition comprises or consists of *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*.

[0042] In an exemplary embodiment, the composition comprises or consists of *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*.

[0043] The composition may further comprise one or more yeast strains. The yeast may be a *Saccharomyces* species, such as *S. cerevisiae* or *S. cerevisiae* subsp. *boulardii*. In an exemplary embodiment, the composition comprises or consists of *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*), *Streptococcus thermophilus* and *Saccharomyces cerevisiae* subsp. *boulardii*.

[0044] The composition may further comprise one or more prebiotic components. In an exemplary embodiment the prebiotic is FOS or a resistant starch.

[0045] In a fifth aspect, the present disclosure provides a method for improving one or more markers of metabolic syndrome and/or insulin resistance, the method comprising administering to a subject in need thereof a multi-strain probiotic combination comprising two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*.
[0046] Typically the subject is overweight or obese or is identified as being at risk of becoming overweight, obese or of developing an obesity-related disorder. The subject may be diabetic or pre diabetic. The diabetes may be type 2 diabetes mellitus.

[0047] The one or more markers of metabolic syndrome and/or insulin resistance may comprise blood lipid profile, blood triglyceride levels, blood adiponectin levels, leptin levels, HDL:LDL ratio, liver enzyme levels including alanine transaminase (ALT) and γ-glutamyl transpeptidase (GGT), fasting blood glucose levels, glucose levels as determined by the oral glucose tolerance test, glycosylated haemoglobin (HbA1C) levels, ghrelin levels, n-3 fatty acid levels, n-6 fatty acid levels, n-3/n-6 fatty acid ratio, C reactive protein levels and Vitamin D levels.

[0048] The method may comprise administering to the subject three or more, four or more, five or more, six or more, or seven or more of said strains.

[0049] In an exemplary embodiment, the method comprises or consists of administering to the subject Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus.

[0050] In an exemplary embodiment, the method comprises or consists of administering to the subject Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus.

[0051] The method may further comprise administering to the subject one or more yeast strains. The yeast may be a Saccharomyces species, such as S. cerevisiae or S. cerevisiae subsp. boulardii. The one or more yeast strains may be present in the same composition or formulation as one or more of the probiotic strains or may be present in a different composition or formulation.

[0052] In an exemplary embodiment, the method comprises or consists of administering to the subject Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis), Streptococcus thermophilus and Saccharomyces cerevisiae subsp. boulardii.
[0053] The method may further comprise administering to the subject one or more prebiotic components. In an exemplary embodiment the prebiotic is FOS. The prebiotic may be present in the same composition or formulation as one or more of the probiotic strains or may be present in a different composition or formulation.

[0054] Compositions according to the above aspects are typically in a form suitable for oral administration. The composition may be a solid or liquid composition. The composition may be in unit dosage form. In one embodiment, the unit dosage form is a capsule. The composition may be in the form of a beverage or food supplement.

[0055] In accordance with methods of the present disclosure probiotic compositions may be administered to subjects in need thereof as food or drink supplements. The compositions may be also be administered as an adjunct to one or more other treatments or therapies for metabolic syndrome or obesity.

[0056] Also provided herein is the use of two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (B. lactis) and *Streptococcus thermophilus*, for the manufacture of a probiotic composition for treating or preventing metabolic syndrome or insulin resistance, for improving one or more markers of metabolic syndrome and/or insulin resistance, or for treating or preventing obesity or an obesity-related disorder.

**Brief Description of the Drawings**

[0057] Exemplary embodiments of the present disclosure are shown in the accompanying drawings, in which:

[0058] **Figure 1** shows Swiss roll histological staining of the large intestine for ZO-1. A) Chow fed mouse; B) HFD fed mouse; C) Mouse fed HFD and supplemented with probiotics.

[0059] **Figure 2** shows Swiss roll histological staining score of the large intestine for ZO-1 and ZO-2.
[0060] **Figure 3** shows liver histology for: A, Mouse fed a standard chow diet with very little fat deposits; B, Mouse fed a HFD with large fat droplets; C, Mouse fed a HFD with probiotics supplementation showing significant reduction in fat droplets.

[0061] **Figure 4** shows hepatic triglyceride concentrations in mice fed a standard chow diet, mice fed a HFD and mice fed a HFD with probiotics supplementation.

[0062] **Figure 5** shows serum biochemical data with three outliers removed in mice fed a standard chow diet, mice fed a HFD and mice fed a HFD with probiotics supplementation. A, ALT; B, AST.

[0063] **Figure 6** shows differences in fat pad mass between mice fed a standard chow diet, mice fed a HFD and mice fed a HFD with probiotics supplementation. *,#, - statistical significance; *, p<0.01.

[0064] **Figure 7** illustrates a box and blister pack of capsules in accordance with Example 2.

**Detailed Description**

[0065] 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.

[0066] 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.

[0067] 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.
[0068] 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.

[0069] 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.

[0070] 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.

[0071] 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.

[0072] 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.

[0073] As used herein, the term "effective amount" refers to an amount of a probiotic composition 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 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.
[0074] 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.

[0075] The term "improve” in the context of markers of metabolic syndrome and insulin resistance means that a composition is administered to a subject, or a method is used, for a period of time effective to improve one or more markers of metabolic syndrome and/or insulin resistance as determined by comparison with the same one or more markers in the subject absent the administration of the composition or method. Typically such “improvement” comprises normalization (e.g. of the level) of a marker, wherein normalization means restoring the (e.g. level) of the marker to (or towards) that expected, or observed in individuals, in the absence of metabolic syndrome or insulin resistance. Any suitable method(s) of assessing markers can be used to determine whether an improvement occurs, as will be readily appreciated by those skilled in the art.

[0076] 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.

[0077] Provided herein are methods for treating or preventing metabolic syndrome, insulin resistance, obesity and obesity-related disorders. In particular embodiments the methods comprise administering to a subject in need thereof a multi-strain probiotic combination comprising two or more probiotic bacterial strains selected from Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus. Optionally the method may further comprise
the administration of one or more yeast strains such as, for example, *Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* subsp. *boulardii*.

[0078] Also provided are methods for improving one or more markers of metabolic syndrome and/or insulin resistance. In particular embodiments the methods comprises administering to a subject in need thereof a multi-strain probiotic combination comprising two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B*. *lactis*) and *Streptococcus thermophilus*. Optionally the method may further comprise the administration of one or more yeast strains such as, for example, *Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* subsp. *boulardii*.

[0079] Methods of the present disclosure may further comprise the administration of one or more additional probiotic strains selected from, for example, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus salvarius*, *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*.

[0080] Those skilled in the art will appreciate that a range of diseases and disorders associated with obesity, may be treated in accordance with the present disclosure. By way of example, the obesity-related disorder may be selected from metabolic syndrome, insulin resistance, type 2 diabetes or a fatty liver disorder, such as non-alcoholic fatty liver disease or alcoholic liver disease. The non-alcoholic fatty liver disease may be characterised by, or associated with, steatosis and/or non-alcoholic steatohepatitis. Assessment of treatment of obesity and obesity-related disorders may be achieved by one of a number of means well known to those skilled in the art. For example, in the case of a fatty liver disorder, such as non-alcoholic fatty liver disease, assessment of successful treatment may include improvements in liver histology, as determined, for example, by MRI or biopsy.

[0081] Subjects to be treated in accordance with the present disclosure may be overweight or obese or be identified as being at risk of becoming overweight or obese. Identifying subjects at risk of becoming overweight or obese may comprise the measurement or
determination of one or more known risk factors for obesity and/or an evaluation of aspects of the subjects lifestyle and diet. Such risk factors, measurements, determinations and evaluations are well within the knowledge and capabilities of the person skilled in the art and do not require undue experimentation. The subjects may be diabetic or pre diabetic. The diabetes may be type 2 diabetes mellitus.

[0082] In accordance with methods of the present disclosure, the probiotic strains 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. Accordingly, novel probiotic compositions for treating or preventing metabolic syndrome ad insulin resistance, for improving one or more markers of metabolic syndrome and insulin resistance, and for treating or preventing obesity and obesity-related disorders are provided herein. Probiotic compositions of the present disclosure typically comprise or consist of two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (B. lactis) and *Streptococcus thermophilus*. Optionally the composition may further comprise one or more yeast strains such as, for example, *Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* subsp. *boulardii*.

[0083] Compositions of the present disclosure may further comprise one or more additional probiotic strains 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*.

[0084] The amounts of individual microbial strains 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 $1 \times 10^2$ cfu to about $1 \times 10^{11}$ cfu, and may be about $1 \times 10^3$ cfu, about $2.5 \times 10^3$ cfu, about $5 \times 10^3$ cfu, about $7.5 \times 10^3$ cfu, $1 \times 10^4$ cfu, about $2.5 \times 10^4$ cfu, about $5 \times 10^4$ cfu, about $7.5 \times 10^4$ cfu, about $1 \times 10^5$ cfu, about $2.5 \times 10^5$ cfu, about $5 \times 10^5$ cfu, about $7.5 \times 10^5$ cfu, about $1 \times 10^6$ cfu, about $2.5 \times 10^6$ cfu, about $5 \times 10^6$ cfu, about $7.5 \times 10^6$ cfu, about $1 \times 10^7$ cfu, about $2.5 \times 10^7$ cfu, about $5 \times 10^7$ cfu, about $7.5 \times 10^7$ cfu, about $1 \times 10^8$ cfu, about $2.5 \times 10^8$ cfu, about $5 \times 10^8$ cfu, about $7.5 \times 10^8$ cfu, about $1 \times 10^9$ cfu, about $2.5 \times 10^9$ cfu, about $5 \times 10^9$ cfu, about $7.5 \times 10^9$ cfu, about $1 \times 10^{10}$ cfu, about $2.5 \times 10^{10}$ cfu, about $5 \times 10^{10}$ cfu, about $7.5 \times 10^{10}$ cfu, and about $1 \times 10^{11}$ cfu.

[0085] Also contemplated by the present disclosure are variants of the microbial strains 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.

[0086] Also encompassed by the term “variant” as used herein are microbial strains phylogenetically closely related to strains disclosed herein and strains possessing substantial sequence identity with the 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” 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.

[0087] The bacterial strains 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 bacterial strains 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.

[0088] 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 chicory 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.

[0089] 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.
[0090] 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.

[0091] 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.

[0092] Compositions of the present disclosure may further comprise vitamins and/or 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.
[0093] Compositions of the invention 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.

[0094] Treatments in accordance with the invention may comprise improving one or more markers of metabolic syndrome and/or insulin resistance. Such markers include, by way of example only, blood lipid profile, blood triglyceride levels, blood adiponectin levels, leptin levels, HDL:LDL ratio, liver enzyme levels including alanine transaminase (ALT) and γ-glutamyl transpeptidase (GGT), fasting blood glucose levels, glucose levels as determined by the oral glucose tolerance test, glycosylated haemoglobin (HbA1C) levels, ghrelin levels, n-3 fatty acid levels, n-6 fatty acid levels, n-3/n-6 fatty acid ratio, C reactive protein levels and Vitamin D levels. Marker levels may be determined using any suitable method known to those skilled in the art. Those skilled in the art will also appreciate that other markers of metabolic syndrome and insulin resistance may be determined in accordance with the invention. Reference may be made, for example, to the disclosures in Gray et al., Nutr Res, 2013, 33:781-788 and Gray et al., Eu J Clin Nutr, 2013, 1-9.

[0095] Contemplated herein are packages comprising a probiotic composition according to the present disclosure in one unit dosage form and a prebiotic in a separate unit dosage form, wherein the separate unit dosage forms are intended to be co-administered. The unit dosage forms may be, for example, capsules. In an exemplary embodiment, the probiotic composition may be administered in a daily dose of 2000 mg of a multi-strain combination (200 billion CFU bacteria) and 3000 mg prebiotic (such as FOS), to be taken, for example, as 2 x 50 mg probiotic capsules and 2 x 750 mg prebiotic capsules in the morning, followed by 2 x 500 mg probiotic capsules and 2 x 750 mg prebiotic capsules at night (8 capsules per day). A package may contain blister packs of probiotic and prebiotic capsules, each blister pack providing one, two or more days supply.

[0096] Compositions of the invention 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.

[0097] In particular embodiments, compositions of the present disclosure may be gluten free and dairy free, and suitable for ingestion by vegetarians.

[0098] Compositions of the invention can be readily manufactured by those skilled in the art using known techniques and processes. For example, the microbial strains 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.

[0099] Compositions of the present disclosure may be suitably formulated 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.

[00100] 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.

[00101] 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.
[00102] 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.

[00103] 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.

[00104] The probiotic strains 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.
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 the circumstances and on the condition 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 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.

In general, compositions of the present disclosure may be administered in any suitable dose amount that is effective as a health supplement, food supplement, food additive, and/or therapeutic agent to achieve the desired health outcome. In some embodiments, an effective dose of a composition may be in a range of from 1 g to 30 g for an adult subject per day, between about 2 g and about 20 g per day, or between about 5 g and about 15 g per day. Pediatric dosages may be in the range of 15% to 90% of adult dosages. In therapeutic applications a constant dosage of the composition can be administered over time.

For the various circumstances in which a composition of the present disclosure may be administered, those skilled in the art will appreciate that the amount of the consumed, and the number of discreet consumptions per unit time will vary depending on the actual use to which the composition is put, the condition of the subject who will consume the beverage, the subject's age, sex, weight and general health. Typically, several doses may be administered in hourly, daily, twice daily or at other suitable time intervals, and/or the dose may be proportionally reduced or increased as indicated by the exigencies of the situation. In exemplary embodiments disclosed herein, where the composition is provided in capsule form, a daily dose may comprise one or two capsules.

Compositions and methods of the present disclosure may be employed as an adjunct to other therapies or treatments for 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 one more cardiovascular or anti-obesity drugs. 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.

[00109] 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.

[00110] 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

[00111] 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 – Attenuation of non-alcoholic fatty liver disease in mice fed a high fat diet**

[00112] The inventors investigated the effect of a multi–strain probiotic preparation containing a combination of probiotic strains including *Bifidobacteria* and *Lactobacilli* species and a *Streptococcus* species in a mouse model of high fat diet/obesity induced liver steatosis.

*Study design*

[00113] Mice were fed either a standard chow diet or a high fat diet (HFD) for a total of 20 weeks. At the end of week 10, the HFD group of mice were divided into 2 groups and probiotics were added to the drinking water for the remaining 10 weeks for one group of mice.
At the end of week 20 all mice were euthanized, dissected and samples stored for later analysis.

**Animals**

[00114] Young wild type (WT) mice on a C57B1/6J background were randomly divided into one of three groups: 1) a control group, receiving a standard diet (n=10); 2) a HFD fed group (n=10); and 3) HFD fed group supplemented with probiotics (n=10; 1 $\times$ $10^{8.9}$ Colony Forming Units (CFU)/mL). All animals were housed in a SPF facility maintained at 20°C on a 12 hour light/dark cycle with access to clean water and food.

**Bacterial Strains**

[00115] The multi-strain probiotic preparation consisted of nine strains of probiotics represented here as percentages of total CFU/mL: *Lactobacillus rhamnosus* / *Lactobacillus casei* / *Lactobacillus acidophilus* / *Lactobacillus plantarum* / *Lactobacillus fermentum* comprised 87% of the total colony forming units; *Bifidobacterium lactis* / *Bifidobacterium breve* / *Bifidobacterium bifidum* comprised 13% of the total colony forming units; and *Streptococcus thermophilus* comprised 5% of the total colony forming units. All strains were lyophilized, water soluble and added to the drinking water as a combination probiotic. The total concentration of probiotic blend added to the drinking water was 1 $\times$ $10^{8.9}$ CFU/mL. This dosage was chosen as it represents a dose per kilogram of body mass equivalent for a human.

**Diet**

[00116] The standard chow and HFD were purchased from Specialty Feeds (WA, Australia; HFD product no. SF03-020). The chow diet contained 4.8% total fat (mono unsaturated fat 2%, polyunsaturated fat 1.77% and saturated fat 0.74%) providing 14 MJ/Kg of energy while the HFD contained 23% total fat (mono unsaturated fat 7.59%, polyunsaturated fat 2.04% and saturated fat 12.6%) providing 20 MJ/Kg of energy.

**Dissection**

[00117] At the end of week 20, mice were anaesthetized using an intraperitoneal injection of pentobarbital and xylene. Once anaesthetized, blood was collected using a cardiac puncture and allowed to clot at room temperature before serum was removed and stored.
Tissue was collected by first removing the pancreas followed by the liver, fat pads, thigh muscle, small intestine, large intestine, spleen and the heart. The mass of the liver and fat pads was recorded. Tissue samples from the different sites were snap frozen in liquid nitrogen, fixed in OCT fluid or placed in formalin for histology. Tissue placed in formalin for fixing was transferred to a 70% ethanol solution after 24 hrs.

Large Intestine / Swiss Roll

[00118] The large intestine was cut longitudinally from the caecum to the rectum and opened. The intestinal tract was cleared of faecal matter using a cotton bud and cut longitudinally down the centre again providing two separate longitudinal segments. One segment was carefully rolled on a wooden toothpick starting from the colon end with the mucosa on the outside of the roll. The resulting roll was then carefully placed in formalin for histology. The second segment was cut into two or three pieces and frozen in liquid nitrogen.

Blood Biochemistry

[00119] Serum was analysed spectrophotometrically for alanine transaminase (ALT), aspartate transaminase (AST), albumin, glucose, cholesterol and triglycerides. Analysis was performed using a Cobas Integra 400, reagents and calibrators supplied by Roche Diagnostics (NSW, Australia).

Hepatic Triglycerides Quantification

[00120] Liver tissue was homogenized in a 1.5% potassium chloride solution (2.3 g KCl in 200 mL water). 500 μl of homogenate was extracted using a 2:1 chloroform/methanol mixture. Extracts were dried and stored at -80°C until analysis. For analysis, samples were reconstituted using 2% triton-x with the aid of sonication. Samples were further diluted with 2% triton-x for a final concentration of 1:6 ready for analysis. Samples were measured spectrophotometrically (Cobas Mira, Roche Diagnostics, Australia) using a kit and calibrators supplied by Novachem (Victoria, Australia).

Protein Quantification

[00121] Proteins were measured as per manufacturer’s direction using a Pierce BCA protein assay kit supplied by Thermo Scientific (Victoria, Australia).
Tight Junction Proteins – ZO-1, ZO-2

[00122] Tight junction proteins zonulin occludens 1 (ZO-1) and ZO-2 rabbit anti-human polyclonal antibodies were purchased from Lifespan Biosciences (WA, USA). A polymer HRP anti-rabbit secondary antibody was purchased from Daco (Vic, Australia). ZO-1 and ZO-2 were analysed on large intestine swiss roll histology slides as per manufacturer’s direction. Optimisation of the antibodies gave optimal concentrations of 2.5 µg/ml and 10 µg/ml for ZO-1 and ZO-2 respectively.

Histological Scoring

[00123] Histology was blindly scored on large intestinal swiss roll sections with ZO-1 and ZO-2 staining and liver sections with H and E and oil red O staining. Swiss rolls were scored for the expression of ZO-1 and ZO-2. Liver sections were scored for diagnosis, NAFLD activity score, steatosis grade and percentage, portal inflammation, lobular inflammation and ballooning, mallory’s hyaline, fibrosis stage, portal score and centrilobular score.

Statistical analysis

[00124] All data is presented as mean ± standard deviation unless otherwise stated. Data was tested for normality of the distribution, and analysis was performed with the statistical software GraphPad Prism. Comparison between groups was carried out using a 1-way analysis of variance with a Tukey’s multiple comparison post hoc test or a Kruskal-Wallis test for non-parametric data with a Dunn’s multiple comparisons post hoc test of significance between individual groups. Differences were considered significant when $P$ was less than 0.05 (* = $P < 0.05$; # = $p < 0.01$; § = $p < 0.001$). All significant differences between groups were determined using the HFD group as the reference group.

Results

[00125] Histological examination of the large intestine Swiss rolls for tight junction proteins ZO-1 and ZO-2 showed mice fed a HFD had reduced expression for ZO-1 (0.01±0.01 vs. 0.38±0.08) and ZO-2 (0.17±0.08 vs. 0.81±0.19) compared to the chow group. Mice fed a HFD and supplemented with probiotics showed significant recovery in ZO-1 (0.24±0.04 vs. 0.01±0.01) and ZO-2 (0.44±0.12 vs. 0.17±0.08) compared to high fat fed mice (Figure 1A-C and Figure 2). Histological examination of livers from HFD mice demonstrated the development of steatosis with large fat droplets present. Chow fed mice showed no steatosis
development (Table 1) or fat droplet accumulation. Compared to mice fed a HFD, mice fed a HFD and supplemented with probiotics showed a reduction in steatosis grade (2.44 ± 0.73 and 2.00 ± 1.25 respectively; p = 0.50) and steatosis percentage (72.78% ± 27.17 & 59.2% ± 38.74; p = 0.9) respectively (Table 1) and visible reductions in fat droplets (Figure 3A-C). Without wishing to be bound by theory, the inventors suggest that the reduction in fat deposits observed in mice fed a HFD and supplemented with probiotics may be due to, or influenced by, the ability of the probiotic strains to alleviate gut dysbiosis (a gut barrier associated abnormality that increases permeability) and restore, in whole or in part, homeostasis in the gastrointestinal tract.

**Table 1: Liver histology grading**

<table>
<thead>
<tr>
<th></th>
<th>Chow (n=9)</th>
<th>HFD (n=9)</th>
<th>HFD + probiotics (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Steatosis Grade</strong></td>
<td>0.0 ± 0.0</td>
<td>2.4 ± 0.7*</td>
<td>2.0 ± 1.3*</td>
</tr>
<tr>
<td><strong>Steatosis %</strong></td>
<td>0.1 ± 0.3</td>
<td>72.8 ± 27.2*</td>
<td>59.2 ± 38.7*</td>
</tr>
<tr>
<td><strong>Portal Inflammation</strong></td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><strong>Lobular Inflammation</strong></td>
<td>0.6 ± 0.7</td>
<td>1.9 ± 0.9*</td>
<td>1.7 ± 1.1</td>
</tr>
<tr>
<td><strong>Ballooning</strong></td>
<td>0.2 ± 0.4</td>
<td>1.3 ± 0.7*</td>
<td>1.1 ± 0.6*</td>
</tr>
</tbody>
</table>

[00126] Confirming liver histology scores and observation, mice supplemented with probiotics had a two and a half fold reduction in hepatic triglyceride concentrations compared to HFD mice (Figure 4; p<0.001).

[00127] After the removal of outliers, serum ALT and AST concentrations were significantly reduced in HFD mice with probiotic supplementation compared to HFD fed mice (Figure 5; p<0.05). As shown in Figure 6, fat pad mass in the HFD mice and in mice fed the HFD supplemented with probiotics was significantly greater than in the chow fed mice (0.90 ± 0.33, 2.60 ± 0.47 and 2.14 ± 0.56; p <0.01), while the reduction in fat pad mass in mice fed the HFD supplemented with probiotics versus HFD mice was also statistically significant.
[00128] The results of this study demonstrate that mice fed a HFD followed by the administration of a multi-strain probiotic formulation maintained tight junction proteins (i.e., decreased intestinal epithelial cell permeability) and reduced hepatic triglyceride concentrations compared to mice fed a HFD alone. Furthermore, the study demonstrated that supplementation with a multi-strain probiotic preparation non-significantly reduced the effects of a HFD by attenuating the progression of steatosis / NAFLD as evidenced in part from liver tissue histology, with more than half the mice showing visible reductions in fat droplets.

[00129] Histological analysis of the large intestine showed that the administration of probiotics partly preserved the HFD induced sub-optimal expression of tight junction protein ZO-1 and ZO-2. Without wishing to be bound by theory, the inventors suggest that by maintaining the integrity of the gastrointestinal tract epithelial layer through the administration of probiotics, the liver may have been spared further development / progression of NAFLD, and hypothesise that this is in part the result of reducing the inflammatory response that a HFD triggered dysbiotic gastrointestinal tract would promote from the effects of exogenous pathogens / pathobiont and by-product translocations across the epithelial gut barrier.

**Example 2 – Exemplary probiotic compositions**

[00130] An exemplary probiotic composition according to the present disclosure comprises or consists of the following per capsule:

- *Lactobacillus plantarum* 4 billion cfu
- *Lactobacillus bulgaricus* 4 billion cfu
- *Lactobacillus gasseri* 20 billion cfu
- *Bifidobacterium breve* 4 billion cfu
- *Bifidobacterium animalis* subsp. *lactis* 4 billion cfu
- *Bifidobacterium bifidum* 4 billion cfu
- *Streptococcus thermophilus* 5 billion cfu
- *Saccharomyces cerevisiae* subsp. *boulardii* 5 billion cfu

[00131] An exemplary probiotic composition according to the present disclosure comprises or consists of the following per capsule:
Lactobacillus rhamnosus 9 billion cfu
Lactobacillus acidophilus 3.75 billion cfu
Lactobacillus plantarum 1.575 billion cfu
Bifidobacterium animalis subsp. lactis 3 billion cfu
Bifidobacterium breve 1.75 billion cfu
Bifidobacterium bifidum 500 million cfu
Streptococcus thermophilus 1.5 billion cfu

[00132] A further exemplary probiotic combination according to the present disclosure comprises or consists of the following probiotic strains:

Lactobacillus rhamnosus \((10^9\text{ CFU/g})\)
Lactobacillus gasseri \((10^9\text{ CFU/g})\)
Lactobacillus paracasei \((10^9\text{ CFU/g})\)
Streptococcus thermophilus \((10^8\text{ CFU/g})\)
Bifidobacterium animalis subsp. lactis \((10^9\text{ CFU/g})\)
Bifidobacterium breve \((10^8\text{ CFU/g})\)

[00133] A further exemplary probiotic combination according to the present disclosure comprises or consists of the following probiotic strains:

Lactobacillus rhamnosus \((10^9\text{ CFU/g})\)
Lactobacillus acidophilus \((10^9\text{ CFU/g})\)
Lactobacillus casei \((10^9\text{ CFU/g})\)
Lactobacillus plantarum \((10^9\text{ CFU/g})\)
Lactobacillus fermentum \((10^9\text{ CFU/g})\)
Streptococcus thermophilus \((10^8\text{ CFU/g})\)
Bifidobacterium animalis subsp. lactis \((10^9\text{ CFU/g})\)
Bifidobacterium breve \((10^8\text{ CFU/g})\)
Bifidobacterium bifidum \((10^9\text{ CFU/g})\)

[00134] A further exemplary probiotic combination according to the present disclosure comprises or consists of the following probiotic strains in capsular form:
*Lactobacillus plantarum* (10 mg/capsule)
*Lactobacillus bulgaricus* (53.75 mg/capsule)
*Lactobacillus gasseri* (58.75 mg/capsule)
*Bifidobacterium breve* (31.25 mg/capsule)
*Bifidobacterium animalis subsp.lactis* (11.25 mg/capsule)
*Bifidobacterium bifidum* (25 mg/capsule)
*Streptococcus thermophiles* (56.25 mg/capsule)
*Saccharomyces boulardi* (58.5 mg/capsule)

[00135] When a combination of probiotic organisms as described in any of the above paragraphs is formulated as a single composition, the composition may further comprise:

**Additional active components:**
Acetyl leucarnitine hydrochloride (acetyl-l-carnitine) 1,000 mg
Alpha-lactalbomin 3.1 g
Beta-lactoglobulin 8 g
Glycomacropeptides 4 g
Immunoglobulin G 1,000 mg
Bovine serum albumin 459.1 mg

**Carrier components:**
magnesium oxide
magnesium gluconate
glutathione
fructose

**Additional excipients:**
anhydrous citric acid
flavouring
colouring

[00136] The probiotic combination may be formulated for co-administration with a prebiotic composition, e.g. comprising FOS (750 mg/capsule) or Hi-maize resistant starch.
[00137] Products comprising probiotic compositions such as those described above will be clinically tested in a combination matrix as shown in Figure 7. As illustrated, each 500 mg capsule (size 0) will contain 50 billion CFU multi-strain probiotic as described above, and each 750 mg capsule (size 00) will contain prebiotic (FOS). A daily dose will be 2000 mg probiotic (200 billion CFU) and 3000 mg prebiotic, to be taken as 2 x 50 mg probiotic capsules and 2 x 750 mg prebiotic capsules in the morning, followed by 2 x 500 mg probiotic capsules and 2 x 750 mg prebiotic capsules at night (8 capsules per day). A box may contain 11 blister packs of 16 capsules (total 176 capsules), each blister pack providing two days supply.
Claims

1. A composition for the treatment or prevention of metabolic syndrome and/or insulin resistance, the composition comprising two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*.

2. A composition for the treatment or prevention of obesity or an obesity-related disorder, the composition comprising two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) and *Streptococcus thermophilus*.

3. A composition according to claim 2, wherein the obesity-related disorder is a fatty liver disorder.

4. A composition according to claim 3, wherein the fatty liver disorder is a non-alcoholic fatty liver disease.

5. A composition according to any one of claims 1 to 4, wherein the composition improves one or more markers of metabolic syndrome and/or insulin resistance.

6. A composition according to claim 5, wherein the one or more markers are selected from blood lipid profile, blood triglyceride levels, blood adiponectin levels, leptin levels, HDL:LDL ratio, liver enzyme levels including alanine transaminase (ALT) and γ-glutamyl transpeptidase (GGT), fasting blood glucose levels, glucose levels as determined by the oral glucose tolerance test, glycosylated haemoglobin (HbA1C) levels, ghrelin levels, n-3 fatty acid levels, n-6 fatty acid levels, n-3/n-6 fatty acid ratio, C reactive protein levels and Vitamin D levels.

7. A composition according to any one of claims 1 to 6, comprising three or more, four or more, five or more, six or more, or seven or more of said strains.

8. A composition according to any one of claims 1 to 7, comprising or consisting of *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium*
breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis and Streptococcus thermophilus.

9. A composition according to any one of claims 1 to 7, comprising or consisting of Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis and Streptococcus thermophilus.

10. A composition according to any one of claims 1 to 9, further comprising one or more yeast strains.

11. A composition according to claim 10, wherein the yeast strain is Saccharomyces cerevisiae or Saccharomyces cerevisiae subsp. boulardii.

12. A composition according to any one of claims 1 to 7, comprising or consisting of Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis, Streptococcus thermophilus and Saccharomyces cerevisiae subsp. boulardii.

13. A composition according to any one of claims 1 to 12, further comprising one or more prebiotic components.

14. A composition according to any one of claims 1 to 13, wherein the composition is for administration to a subject that is overweight, obese, or identified a being at risk of becoming overweight or obese.

15. A method for treating or preventing a condition selected from metabolic syndrome, insulin resistance, obesity and an obesity-related disorder, the method comprising administering to a subject in need thereof a multi-strain probiotic combination comprising two or more probiotic bacterial strains selected from Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus gasseri, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium bifidum, Bifidobacterium animalis subsp. lactis (B. lactis) and Streptococcus thermophilus.
16. A method according to claim 15, wherein the subject is overweight or obese or is identified as being at risk of becoming overweight, obese or of developing an obesity-related disorder.

17. A method according to claim 15 or 16, wherein the subject is diabetic, optionally type 2 diabetic, or pre diabetic.

18. A method according to any one of claims 15 to 17, wherein the obesity-related disorder is a fatty liver disorder.

19. A method according to claim 18, wherein the fatty liver disorder is a non-alcoholic fatty liver disease.

20. A method according to any one of claims 15 to 19, wherein the treatment improves one or more markers of metabolic syndrome and/or insulin resistance.

21. A method according to claim 20, wherein the one or more markers are selected from blood lipid profile, blood triglyceride levels, blood adiponectin levels, leptin levels, HDL:LDL ratio, liver enzyme levels including alanine transaminase (ALT) and \( \gamma \)-glutamyl transpeptidase (GGT), fasting blood glucose levels, glucose levels as determined by the oral glucose tolerance test, glycosylated haemoglobin (HbA1C) levels, ghrelin levels, n-3 fatty acid levels, n-6 fatty acid levels, n-3/n-6 fatty acid ratio, C reactive protein levels and Vitamin D levels.

22. A method according to any one of claims 15 to 21, comprising administering to the subject three or more, four or more, five or more, six or more, or seven or more of said strains.

23. A method according to any one of claims 15 to 22, comprising administering to the subject \textit{Lactobacillus plantarum}, \textit{Lactobacillus bulgaricus}, \textit{Lactobacillus gasseri}, \textit{Bifidobacterium breve}, \textit{Bifidobacterium bifidum}, \textit{Bifidobacterium animalis} subsp. \textit{lactis} and \textit{Streptococcus thermophilus}.

24. A method according to any one of claims 15 to 22, comprising administering to the subject \textit{Lactobacillus plantarum}, \textit{Lactobacillus rhamnosus}, \textit{Lactobacillus acidophilus}, \textit{Bifidobacterium breve}, \textit{Bifidobacterium bifidum}, \textit{Bifidobacterium animalis} subsp. \textit{lactis} and \textit{Streptococcus thermophilus}.
25. A method according to any one of claims 15 to 24, further comprising administering to the subject one or more yeast strains.

26. A method according to claim 25, wherein the yeast strain is *Saccharomyces cerevisiae* or *Saccharomyces cerevisiae* subsp. *boulardii*.

27. A method according to any one of claims 15 to 22, comprising administering to the subject *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis*, *Streptococcus thermophilus* and *Saccharomyces cerevisiae* subsp. *boulardii*.

28. A method according to any one of claims 15 to 27, further comprising administering to the subject one or more prebiotic components.

29. A method according to claim 15, comprising administering to the subject a composition according to any one of claims 1 to 13.

30. Use of two or more probiotic bacterial strains selected from *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus gasseri*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium animalis* subsp. *lactis* (B. lactis) and *Streptococcus thermophilus*, for the manufacture of a probiotic composition for treating or preventing metabolic syndrome or insulin resistance, for improving one or more markers of metabolic syndrome and/or insulin resistance, or for treating or preventing obesity or an obesity-related disorder.
Figure 1

Figure 2

Swiss Roll Histology Score

- **ZO1**
  - chow: *
  - HFD: *
  - Probiotic: *

- **ZO2**
  - chow: *
  - HFD: *
  - Probiotic: #
Figure 5

Figure 6
INTERNATIONAL SEARCH REPORT


A. CLASSIFICATION OF SUBJECT MATTER

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)

AUSPAT, inventor and applicant search.

EPDOC, WPIAP, CAPLUS, MEDLINE. For keywords see detailed search strategy, too many to list here.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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Documents are listed in the continuation of Box C

Further documents are listed in the continuation of Box C

See patent family annex

Date of the actual completion of the international search
10 August 2015

Date of mailing of the international search report
10 August 2015

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
Email address: pct@ipaustralia.gov.au

Authorised officer

Geoffrey Peters
AUSTRALIAN PATENT OFFICE
(ISO 9001 Quality Certified Service)
Telephone No. 0262832184

Form PCT/ISA/210 (fifth sheet) (July 2009)
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<td>US 2014/0079676 A1 (PRO Thera, INC.) 20 March 2014 Whole Document.</td>
<td>1, 2, 5-7, 10, 11, 14-17, 20-22, 25, 26, 29-30, 8, 9, 12, 23, 24, 27</td>
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<td>X</td>
<td>VINCENT WAI-SUN WONG et al &quot;TREATMENT OF NONALCOHOLIC STEATOHEPATITIS WITH PROBIOTICS. A PROOF-OF-CONCEPT STUDY&quot; ANNALS OF HEPATOLOGY, 2013; VOL 12 No 2; 256-262. Whole Document.</td>
<td>1-4, 6, 7, 14, 15, 16, 18-22, 29-30, 8, 9, 12, 23, 24, 27</td>
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<td>ZATOLLAH ASEMI et al &quot;EFFECT OF MULTISPECIFIC PROBIOTIC SUPPLEMENTS ON METABOLIC PROFILES, hs-CRP, and OXIDATIVE STRESS IN PATIENTS WITH TYPE 2 DIABETES&quot;. ANNALS OF NUTRITION &amp; METABOLISM 2013; 63: 1-9. Whole Document.</td>
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<td>ZATOLLAH ASEMI et al &quot;EFFECTS OF MULTISPECIFIC PROBIOTIC SUPPLEMENTS ON SERUM MINERALS, LIVER ENZYMES AND BLOOD PRESSURE ON PATIENTS WITH TYPE 2 DIABETES&quot;. INTERNATIONAL JOURNAL OF DIABETES DEVELOPING COUNTRIES (April-June 2015) 35(2): 90-95. Published online 12 April 2014. Whole Document.</td>
<td>1, 2, 5-7, 15, 17, 21, 29-30</td>
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<td>Y</td>
<td>JUN-E LIU et al &quot;PROBIOTIC YOGURT EFFECTS ON INTESTINAL FLORA OF PATIENTS WITH CHRONIC LIVER DISEASE&quot; &quot;NURSING RESEARCH NOVEMBER/DECEMBER 2010, VOL 59, No 6, 426-432 Whole Document.</td>
<td>8, 9, 12, 23, 24, 27</td>
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Form PCT/ISA/210 (fifth sheet) (July 2009)
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:

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.
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<td>US 5, 716,615 A</td>
<td>10 February 1998</td>
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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) (July 2009)