(54) **Titre : COMPOSITIONS BACTERIENNES DESTINEES AU TRAITEMENT ET A LA PROPHYLAXIE DE MALADIES DEGENERATIVES**

(54) **Title: BACTERIAL COMPOSITIONS FOR PROPHYLAXIS AND TREATMENT OF DEGENERATIVE DISEASE**

Deconjugation of TDCA by free *Lactobacillus*
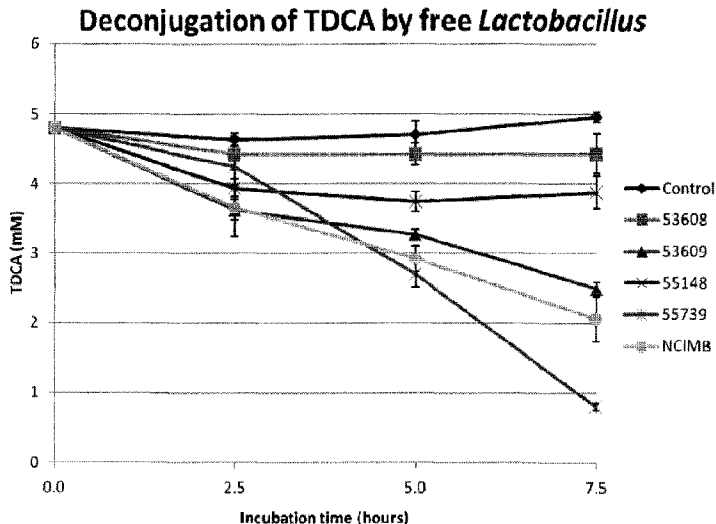
(57) **Abrégé/Abstract:**

The disclosure provides an oral composition for reducing serum cholesterol, serum lipids, body fat, or atherogenic index or for prophylaxis or treatment of atherosclerosis, cardiovascular or cerebrovascular diseases, comprising a highly bsh active bacteria, isolate or supernatant thereof; wherein the highly bsh active bacteria degrades >50µmol glycodeoxycholic acid (GDCA)/gram/hour and >2µmol taurodeoxycholic acid (TDCA)/gram/hour when measured over 1 hour and 5 hours, respectively, or degrades >65 µmol GDCA/g/hr and >7 µmol TDCA/g/hr when measured over 30 minutes.

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(54) **Title:** BACTERIAL COMPOSITIONS FOR PROPHYLAXIS AND TREATMENT OF DEGENERATIVE DISEASE
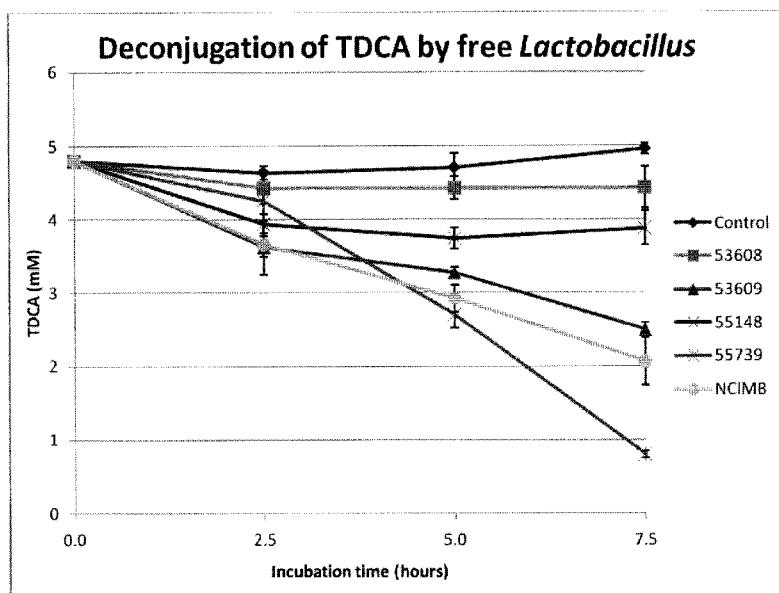
Figure 1

(57) **Abstract**: The disclosure provides an oral composition for reducing serum cholesterol, serum lipids, body fat, or atherogenic index or for prophylaxis or treatment of atherosclerosis, cardiovascular or cerebrovascular diseases, comprising a highly *bsh* active bacteria, isolate or supernatant thereof; wherein the highly *bsh* active bacteria degrades >50μmol glycodeoxycholic acid (GDCA)/gram/hour and >2μmol taurodeoxycholic acid (TDCA)/gram/hour when measured over 1 hour and 5 hours, respectively, or degrades >65 μmol GDCA/g/hr and >7 μmol TDCA/g/hr when measured over 30 minutes.

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TITLE: BACTERIAL COMPOSITIONS FOR PROPHYLAXIS AND TREATMENT OF DEGENERATIVE DISEASE

RELATED APPLICATIONS

[0001]

5

FIELD

[0002] The present disclosure relates to an improved oral composition of a highly *bsh* active bacteria, isolate or supernatant thereof and processes

10 for preparing the improved composition. The present disclosure also relates to methods and uses of the improved oral composition for reducing serum cholesterol, serum lipids, body fat, or atherogenic index and for prophylaxis and treatment of atherosclerosis, cardiovascular or cerebrovascular diseases.

BACKGROUND

15 *Hypercholesterolemia and Coronary Artery Disease*

[0003] Coronary artery disease (CAD) is the leading cause of death, the most common form of heart disease and the most common cause of sudden death in the western world. Clinical and epidemiological evidence

20 have established a clear link between elevated serum cholesterol and CAD. Within apparently healthy populations, there is an exponential relation between serum cholesterol and coronary risk. In middle age, the risk of CAD increases by 2 to 3% for each 1% increase in cholesterol levels.

[0004] An estimated 107 million American adults have total serum

25 cholesterol levels of 5.18 mmol/l (200 mg/dL) and higher. Of these, approximately 37 million have levels of 6.22 mmol/l (240 mg/dL) or above. In adults, total cholesterol levels of 6.22 mmol/l or higher are considered high risk for cardiovascular related events while levels between 5.18 and 6.22 mmol/l are considered borderline high risk. According to the

30 recommendations of the National Cholesterol Education Program's (NCEP) the primary objective of any therapy is the lowering of LDL Cholesterol levels

(Third Report of the NCEP Expert Panel 2002). New guidelines now consider other risk factors such as age, family history, smoking, hypertension, low HDL, and diabetes mellitus, in estimating cut-off levels of cholesterol requiring intervention. LDL goals in primary prevention therefore depend on a patient's

5   absolute risk for CAD related events in the short term or long term. Currently, according to the recently revamped recommendations of the NCEP, an additional 36 million US citizens should be treated for high cholesterol. Currently, less than half of patients who qualify for lipid modifying treatment are receiving it and only a third of treated patients are achieving their LDL

10 cholesterol goal.

Pathogenesis of Atherosclerosis

[0005]   The involvement of elevated LDL cholesterol in atherosclerosis and CAD is well documented. Atherosclerosis is initiated by the retention of

15 apolipoprotein B-containing lipoproteins (e.g. LDL cholesterol) in the arterial wall. Over time, lipoproteins retained in the arterial wall become modified (i.e. aggregated and oxidized) and elicit a cascade of biological responses that develop into a maladaptive inflammatory response (Tabas et al. 2007). In particular, monocytes enter the subendothelium, differentiate into

20 macrophages and ingest the retained modified lipoproteins to become cholesterol-laden foam cells. Eventually, inflammatory cells enter the lesions and help contribute to the aforementioned maladaptive inflammatory response, a process accelerated by amplified retention of lipoproteins in established lesions. A process mediated by cytokines and growth factors

25 causes smooth muscle cells to migrate and form a collagenous fibrous cap (mature atherosclerotic plaque), most likely as a scar-like response to wall off the lesion (Tabas et al. 2007). However, as the lesion progresses, macrophages die, resulting in areas of necrosis containing extracellular debris, cholesterol crystals, proteases and thrombotic material. At this point,

30 fibrous cap thinning, plaque eruption or erosion may occur, potentially leading to acute thrombotic vascular events such as myocardial infarction and stroke.

[0006]   High-density lipoproteins play a key role in "reverse cholesterol transport", a pathway by which excess cholesterol is removed from extrahepatic cells and returned to the liver for excretion from the body. In the peripheral tissues, HDL is believed to remove cellular cholesterol through a variety of mechanisms including interaction of HDL apolipoproteins with cell-surface binding sites or receptors (Tall, 1998). The action of lecithin-cholesterol acyltransferase (LCAT) converts the absorbed cholesterol into cholesterol esters and in turn can increase the absorption capacity of HDL. Upon return to the liver, cholesterol may be metabolized into bile salts and excreted from the body. LDL and HDL cholesterol are the major factors in maintaining the cholesterol balance of the body and a high ratio of HDL to LDL correlate well with a lower incidence of CAD in humans.

[0007]   High serum triglyceride levels are similarly a risk factor for atherosclerosis and CAD. Specific reasons for this include the increased production of atherogenic chylomicron and VLDL remnants, the inverse relationship present between serum triglyceride and HDL, the possible resultant increase in LDL attributable to remnant-reduced hepatic LDL-receptors as well as the formation of more dense and, therefore, more atherogenic LDL, and to the interaction between serum triglyceride and the fibrinolytic/coagulation system. Because of the multiple links between elevated triglyceride levels and risk for atherosclerotic cardiovascular disease, screening for hypertriglyceridemia is important when determining a patient's risk for atherosclerotic cardiovascular disease.

Immune Responses in Atherosclerosis

[0008]   The pathogenesis of atherosclerosis is believed to include dyslipidemia, vascular endothelium dysfunction, and a chronic inflammatory process. Several mediators have been shown to be involved in intercellular signaling in atherosclerosis, including small molecules such as nitric oxide, lipid mediators such as eicosanoids and sterols and cytokines. Inflammation is mediated by cytokines, glyco-proteins involved in cell to cell signaling,

which are produced by macrophages and dendritic cells in the epithelium in response to an antigenic or foreign body stimulus. The immune response is implicated in the formation of early fatty streaks, when the endothelium is activated and expresses chemokines and adhesion molecules leading to

5   monocyte/lymphocyte recruitment and infiltration into the subendothelium. It also acts at the onset of adverse clinical vascular events, when activated cells within the plaque secrete matrix proteases that degrade extracellular matrix proteins and weaken the fibrous cap, leading to rupture and thrombus formation.   Recently, toll-like receptors (TLR) on the surface of the

10   gastrointestinal epithelium have been linked to the induction of an inflammatory response, helping to initiate the start signal for the production of pro-inflammatory cytokines (Tobias and Curtiss, 2007).

[0009]   Specific emphasis is placed on the contribution of pro- and anti-inflammatory cytokines to pathogenic (innate and adaptive) and regulatory

15   immunity in the context of atherosclerosis. Cytokines can be differentiated by those with an essentially pro-inflammatory mode of action, including tumor necrosis factor (TNF-alpha), interleukin-12, IL-18 and interferon gamma from those with anti-inflammatory mode of action, including IL-4, IL-10, IL-13 and the endogenous IL-1 receptor antagonist IL-1ra.   In response to the local

20   milieu of cytokines, CD4$^+$ cells differentiate into the Th1 (pro-inflammatory) or Th2 (anti-inflammatory) lineage. Among the principal inducers of the Th1 and Th2 cells are IL-12 and IL-10, respectively. Cytokines involved in the Th1 process include IL-2, IFN-gamma and TNF, while those involved in the Th2 process include IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13.   Over 30 major

25   members of the interleukin family have been identified, the majority of which play a role in atherogenesis.   Specifically, they have been attributed to primarily anti-atherogenic (IL-1ra, IL-9, IL-10, IL-11) and pro-atherogenic (IL-1, IL-2, IL-6, IL-18) properties.   Modulating these interleukins represent the most readily applicable approach to immunotherapy in atherosclerosis. It is

30   believed that gut bacteria initiate an inflammatory response when epithelium TLRs recognize non-commensal microbial motifs and this cytokine signal may

translate to increased risk of atherosclerosis. The corollary of this response is that commensal microflora are required to maintain gut homeostasis through the recognition of their non-inflammatory motifs by TLRs. Recent research has shown that pro-inflammatory cytokines produced in the gut can be greatly

5   decreased by delivering commensal bacteria (*Lactobacillus acidophilus*) delivered free in saline or in fermented milk (Urbanska et al. 2009). This research showed that *L. acidophilus* decreased IL-6, IL-12, TNF-alpha, and IFN-gamma levels when administered orally in saline and in fermented milk (only IL-6 data was published) (Prakash and Urbanska 2007).

10 **[0010]**   In addition to pro- and anti-inflammatory cytokines, high sensitivity C-reactive protein is arguably the most important serum inflammatory marker of coronary risk. Recent research suggests that patients with elevated basal levels of CRP are at an increased risk of cardiovascular disease as well as diabetes, and hypertension. A clinical study of 700 nurses

15 showed that those in the highest quartile of trans fat consumption had blood levels of C-reactive protein that were 73% higher than those in the lowest quartile (Lopez-Garcia, 2005). Others have shown that CRP can exacerbate ischemic necrosis in a complement-dependent fashion and that CRP inhibition can be a safe and effective therapy for myocardial and cerebral

20 infarcts (Pepys et al. 2006).

Metabolic syndrome

[0011]   Dyslipidemia, atherosclerosis, and chronic inflammation are connected to other degenerative diseases through the metabolic syndrome.

25 Metabolic syndrome is characterized by a group of metabolic risk factors in one individual and increases the individual's risk of developing atherosclerosis, cardiovascular disease, cerebrovascular disease and diabetes. This constellation of signs and symptoms affects one in five people, and prevalence increases with increasing age. Some studies estimate the

30 prevalence in the USA to be up to 25% of the population (Ford et al., 2002). Symptoms and features include: Fasting hyperglycemia — diabetes mellitus

type 2 or impaired fasting glucose, impaired glucose tolerance, or insulin resistance; High blood pressure; Central obesity (also known as visceral, male-pattern or apple-shaped adiposity), overweight with fat deposits mainly around the waist.

5 *Non-alcoholic Fatty Liver Disease (NAFLD)*

[0012] Non-alcoholic fatty liver disease (NAFLD) is considered to be a hepatic manifestation of the metabolic syndrome. NAFLD is defined as fatty inflammation of the liver when this is not due to excessive alcohol use.

10 NAFLD is strongly associated with obesity, dyslipidaemia, insulin resistance (IR) and type II (non-insulin dependent) diabetes mellitus. NAFLD covers the full spectrum of metabolic fatty liver disorders, particularly when histology is undefined. NAFLD can manifest as simple steatosis (fatty liver), at the most clinically indolent extreme, or can progress to steatosis with inflammation or

15 fibrosis, in which case it is termed NASH. However, even stable forms of NAFLD may carry as yet unidentified morbidity since fatty liver typically functions less efficiently than non-fatty liver. NASH likely represents an intermediate stage characterized by steatosis with lobular inflammation. NAFLD is known to affect 10-39% of the general global population with an

20 average incidence of 20% (Angulo 2002).

[0013] There are several risk factors associated with NAFLD. These factors include common life conditions and diseases such as obesity, hyperglycemia, type 2 diabetes mellitus, and hypertriglyceridemia. In addition, NAFLD is strongly associated with central obesity and visceral adiposity.

25 Genetic and racial factors are also associated with NAFLD/NASH. This disorder will therefore contribute substantially to the burden of chronic liver disease in coming decades.

Treatment and Prevention of Hypercholesterolemia and Dyslipidemia

30 **[0014]** Methods for lowering cholesterol levels in humans involve dietary management, behaviour modification, and exercise and drug therapy. Dietary intervention alone is insufficient for most individuals. Studies show

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that complete elimination of dietary cholesterol and limiting fat content to less than ten percent of the daily caloric intake results in only a four percent regression of atherosclerotic plaques after five years when combined with stress management and aerobic exercise (Ornish et al. 1990).

5   [0015]   Additional dietary options for LDL cholesterol lowering have been proposed, including soluble fibres, plant sterols and stanols and soy protein. Recent reports indicate that soluble forms of dietary fibre at 5-10 g per day can reduce LDL cholesterol by approximately 5% (Third Report of the NCEP Expert Panel 2002). Little, no, or inconsistent effects have been
10   reported in regards to HDL cholesterol; however, it appears that modulation of cholesterol and bile metabolic pathways may be required as much evidence from studies that attempt to lower dietary intake or increase cholesterol catabolism result in decreases in HDL unless used in combination with cholesterol lowering medication that affects liver enzymes. Furthermore,
15   insoluble fibre has not been shown to significantly affect circulating cholesterol levels. Animal and human studies show that plant stanols and sterols reduce plasma total cholesterol and low density lipoprotein (LDL) cholesterol levels. Data has shown that plant-derived sterol and stanol esters at dosages of 2-3 g/day decrease LDL cholesterol levels by 6-15 % with no
20   significant change in triglyceride or HDL cholesterol levels (Hallikainen and Uusitupa, 1999). Again, often studies that show no decrease in HDL or an non-statistically significant decrease in HDL have included patients on cholesterol lowering medication that alters liver enzymatic pathways such as Statins. Soy protein included in a diet low in saturated fatty acids and
25   cholesterol has been shown to lower LDL cholesterol by about 5%, however, dosage requirements are not well known (Jenkins et al. 2000).

[0016]   Statins can significantly reduce endogenous cholesterol synthesis, through inhibition of HMG-CoA reductase, and upregulate low-density lipoprotein receptors in the liver, leading to reductions in LDL-C of 20-
30   30%. The efficacy of statins has been thoroughly evaluated in a multitude of clinical trials (Pedersen et al. 1994). Statins, however, have been shown to

exhibit rare, but potentially severe, side-effects. The most predominant of these are myopathy, which may evolve into life-threatening rhabdomyolysis, and polyneuropathy (Gaist et al. 2001; Gaist et al. 2002; Omar and Wilson 2002; Staffa et al. 2002).

5   **[0017]**   Fibrate therapy has also been shown to offer long-term benefits in high-risk patients with low HDL cholesterol-high triglyceride dyslipidemia (Goldenberg et al. 2008). Fibrates, however, are also associated with a variety of adverse effects including increased risk of gall stones, myopathy and stomach upset (Sgro and Escousse, 1991).

10   **[0018]**   Niacin has been used for quite some time now, at doses of 1-2 grams per day, to reduce triglycerides and lower LDL-C. Interestingly, vitamin B3 has been shown to increase HDL-C at these levels as well and has been prescribed to patients with low HDL-C who are at risk of suffering a cardiac event. Unfortunately, uncomfortable and severe side effects including facial

15   and full body flushing are exhibited with regular use.

[0019]   Bile acid sequestrants (BAS) have been used clinically since the 1960s for lowering of LDL cholesterol. Bile acid sequestrants have a low rate of compliance caused, in part, by gastrointestinal side effects (Probstfiled and Rifkind, 1991).

20   *Probiotics*

[0020]   Probiotics have been reported to be associated with a range of clinically relevant health benefits. Various strains of lactic acid bacteria have been particularly well studied in humans and animals. Placebo controlled

25   clinical trials have shown *L. reuteri*, *L. rhamnosus GG*, *L. casei* and *S. boulardii* to be effective in reducing the duration of acute diarrhea (Huang et al. 2002). *L. rhamnosus GG* administered to infants reduced the risk of nosocomial diarrhea and rotavirus gastroenteritis (Szajewska et al. 2001). Studies by Aso et al. revealed that *L. casei* Shirota increases the percentage

30   of T-helper cells and NK cells in adult colorectal cancer patients and has a protective effect on the recurrence of superficial bladder cancer (Aso et al.,

1995).   In addition, select strains of lactobacilli have been shown to significantly suppress intestinal tumors by chemical mutagens (McIntosh et al. 1999). Lactic acid bacteria have been administered to prevent sepsis in patients with severe acute pancreatitis. A randomized study by Rayes et al.

5   involving liver transplant patients revealed postoperative infections were significantly reduced by feeding live *L. plantarum* cells in comparison to standard antibiotic treatment (Rayes et al. 2002). As a means of preventing allergy, a randomized controlled study by Lodinova-Zadnikova et al. investigated the effect of at birth colonization with nonpathogenic *Escherichia*

10   *coli* Nissle 1917 (Lodinova-Zadnikova and Sonnenborn 1997).   Subjects inoculated with the *E. coli* strain showed significantly reduced colonization of bacterial pathogens as well as significantly lower incidence of allergies after 10 and 20 years in comparison with control subjects. Probiotics have also been used as treatment options for managing Inflammatory Bowel Diseases

15   (IBD) such as Crohn's disease, ulcerative colitis and pouchitis.

[0021]   *L. reuteri* is well-established as one of the most ubiquitous members of the naturally-occurring gut bacteria. Host-specific strains of *L. reuteri* have been documented to confer broad-spectrum protection from an assortment of microbial and chemical associated disease in humans and

20   animals (Dobrogosz, 2005). However, traditional probiotic therapy involves administration of bacteria with the hope that some bacteria will survive the harsh gastric conditions and colonize the colon where the bacteria will reproduce and live indefinitely. Far fewer bacteria survive in the duodenum, jejunum or ileum because of factors such as acidity, immune response and

25   bile concentration. Bacteria must be present in the duodenum or jejunum of the small intestine for lowering cholesterol and in particular bile acid.

SUMMARY

[0022]   The present inventors have determined that highly bile salt hydrolase (*bsh*) active bacteria provide an improved agent for reducing serum

30   cholesterol, serum lipids, body fat, and atherogenic index and for prophylaxis

and treatment of atherosclerosis, cardiovascular and cerebrovascular diseases.

[0023]   Accordingly, in one aspect, the present disclosure provides an oral composition comprising a highly *bsh* active bacteria, isolate or supernatant thereof; wherein the highly *bsh* active bacteria degrades >50 µmol glycodeoxycholic acid (GDCA)/gram/hour and >2 µmol taurodeoxycholic acid (TDCA)/gram/hour when measured over 1 hour and 5 hours, respectively; or degrades >65 µmol GDCA/g/hr and >7 µmol TDCA/g/hr when measured over 30 minutes. In one embodiment, the highly *bsh* active bacteria degrades >300 µmol GDCA/g/hr and >40 µmol TDCA/g/hr when measured over 30 minutes. In another embodiment, the highly *bsh* active bacteria degrades >2000 µmol GDCA/g/hr and >500 µmol TDCA/g/hr when measured over 30 minutes. In yet another embodiment, the highly *bsh* active bacteria degrades >15000 µmol GDCA/g/hr and >2000 µmol TDCA/g/hr when measured over 30 minutes.

[0024]   In one embodiment, the bacteria is *Lactobacillus, Bifidobacteria, Pediococcus, Streptococcus, Enterococcus,* or *Leuconostoc.* In another embodiment, the *Lactobacillus* is *Lactobacillus reuteri,* optionally, *Lactobacillus reuteri* (NCIMB 701359), *Lactobacillus reuteri* (NCIMB 701089), *Lactobacillus reuteri* (ATCC 55148), *Lactobacillus reuteri* (ATCC 23272), *Lactobacillus reuteri* (NCIMB 702655), *Lactobacillus reuteri* (LMG 18238), *Lactobacillus reuteri* (CCUG 32271), *Lactobacillus reuteri* (CCUG 32305), *Lactobacillus reuteri* (CCUG 37470), *Lactobacillus reuteri* (CCUG 44001) or *Lactobacillus reuteri* (CCUG 44144). In another embodiment, the composition further comprises a carrier.

[0025]   In yet another embodiment, the concentration of bacteria is 10^6 - 10^{12} colony forming units (CFU)/gram.

[0026]   The bacteria of the present disclosure is optionally contained in a polymer or in a microcapsule or nanocapsule.

[0027] In another embodiment, the oral composition described herein is grown under fermentation conditions comprising a carbon source, a nitrogen source, a pH of 4-7, optionally 5, and a harvest time of 12-24 hours, optionally 12-16 hours. In one embodiment, the carbon source comprises maltose, sucrose, dextrin, a combination of sorbitol and glucose or a combination of inulin and glucose. In another embodiment, the nitrogen source comprises (i) yeast extract and malt extract, yeast extract and beef extract, or casein hydrolysate and malt extract; and (ii) peptone or tryptone.

[0028] In yet another embodiment, the oral composition described herein is lyophilized with lyoprotectants. In one embodiment, the lyoprotectants comprise a final concentration of 0.2% to 10% maltodextrin and 0.05% to 0.33% yeast extract or 0.05-2.5% inulin and 0.05 to 0.1% yeast extract. In one embodiment, the lyoprotectants comprise a final concentration of 2-4% maltodextrin and 0.1% yeast extract, 0.3% inulin and 0.1% yeast extract, or 0.3% inulin.

[0029] In a further embodiment, the oral composition described herein is stored in liquid, wherein the liquid storage conditions comprise a final preservative solution comprising 2.5-10% growth media, 50-99.99% yogurt or other fermented milk, 50-99.99% culture supernatant or 5% MRS.

[0030] In yet a further embodiment, the oral composition described herein is flash frozen in a final cryoprotectant solution, such as 0.2-10% maltodextrin, optionally 1-3%, maltodextrin and 0.05 to 0.33% yeast extract, optionally 0.1-0.2% yeast extract, 0.05 to 2.5% inulin, optionally at least 0.2% inulin, 0.5M Trehalose, 0.5M fructose, 0.5M lactose, 0.5M maltose or 50-99.99%, optionally 50% spent media.

[0031] In another aspect, the oral composition of the present disclosure further comprises a triglyceride lowering agent, an agent for increasing HDL or limiting HDL decrease, a cholesterol lowering agent, an agent for preserving *bsh* activity, an agent for modulating adipokines or hormones of obesity, a hypoglycemic agent, or a therapeutic for reducing the pro-

inflammatory cytokines IL-1α/ß, IL-2, IL-15, IL-3, IL-6, IL-8, IL-12, IL-17, IFN-gamma, TNF-alpha, or for increasing the level of the anti-inflammatory cytokines IL-1ra, IL-9, IL-10, IL-11.

[0032] In another aspect of the present disclosure, the present inventors provide methods and uses of the oral compositions for reducing serum cholesterol, serum lipids, body fat, or atherogenic index and for prophylaxis and treatment of atherosclerosis, cardiovascular or cerebrovascular diseases in an animal, optionally a mammal, such as a human.

[0033] Also provided herein are processes for producing highly *bsh* active bacteria comprising growing the bacteria under fermentation conditions; lyophilizing the bacteria with lyoprotectant, storing the bacteria under liquid storage conditions and flash freezing the bacteria with cryoprotectants.

[0034]

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] Embodiments of the disclosure will now be described in relation to the drawings in which:

[0036] Figure 1 shows deconjugation of TDCA by free *Lactobacillus reuteri* strains (ATCC 53608, ATCC 53609, ATCC 55148, ATCC 55739, and NCIMB 701359) over time. The experiment was performed in triplicate and error bars represent one standard deviation from the mean.

[0037]   Figure 2 shows deconjugation of GDCA by free *Lactobacillus reuteri* strains (ATCC 53608, ATCC 53609, ATCC 55148, ATCC 55739, and NCIMB 701359) over time. The experiment was performed in triplicate and error bars represent one standard deviation from the mean.

5   [0038]   Figure 3 shows deconjugation of TDCA by free *Lactobacillus reuteri* and *Lactobacillus fermentum* strains over time. The experiment was performed in triplicate and error bars represent one standard deviation from the mean.

[0039]   Figure 4 shows deconjugation of GDCA by free *Lactobacillus* 10 *reuteri* strains (LabMet, NCIMB 701359) and *Lactobacillus fermentum* (ATCC 11976) over time. The experiment was performed in triplicate and error bars represent one standard deviation from the mean.

[0040]   Figure 5 shows deconjugation of TDCA by free *Lactobacillus reuteri* strains (LMG 9213, NCIMB 11951, ATCC 23272, NCIMB 702656, 15 NCIMB 701359, and NCIMB 701089) over time. The experiment was performed in triplicate and error bars represent one standard deviation from the mean.

[0041]   Figure 6 shows deconjugation of GDCA by free *Lactobacillus reuteri* strains (LMG 9213, NCIMB 11951, ATCC 23272, NCIMB 702656, 20 NCIMB 701359, and NCIMB 701089) over time. The experiment was performed in triplicate and error bars represent one standard deviation from the mean.

[0042]   Figure 7 shows the size of precipitation zone of 3 *bsh* active *Lactobacillus reuteri*: Lr010: *Lactobacillus reuteri* (LabMet), Lr052: 25 *Lactobacillus reuteri* (NCIMB 701089), and Lr050: *Lactobacillus reuteri* (NCIMB 701359).

[0043]   Figure 8 shows deconjugation of TDCA by free *Lactobacillus reuteri* strains (ATCC 55148, ATCC 55739, NCIMB 701359, NCIMB 701089, NCIMB 702655, LMG 18238, LMG 22877, LMG 22878, LMG 22879, CCUG

32305, CCUG 37470, CCUG 44001, CCUG 44144, CCUG 47824) over time. The experiment was performed in triplicate and error bars represent one standard deviation from the mean.

[0044] Figure 9 shows deconjugation of GDCA by free *Lactobacillus reuteri* strains (ATCC 55148, ATCC 55739, NCIMB 701359, NCIMB 701089, NCIMB 702655, LMG 18238, LMG 22877, LMG 22878, LMG 22879, CCUG 32305, CCUG 37470, CCUG 44001, CCUG 44144, CCUG 47824) over time. The experiment was performed in triplicate and error bars represent one standard deviation from the mean.

[0045] Figure 10 shows a representative set of microcapsule morphology photomicrographs using different lyoprotectant conditions at 7:3 microcapsule to lyoprotectant for saline, 1M trehalose, and 10% skim milk used for lyophilisation and rehydration of microencapsulated *Lactobacillus*.

DETAILED DESCRIPTION

[0046] The present inventors have shown that highly *bsh* active bacteria provide an improved oral composition for reducing serum cholesterol, serum lipids, body fat, and atherogenic index and for prophylaxis and treatment of atherosclerosis, cardiovascular and cerebrovascular diseases.

Compositions

[0047] Accordingly, there is provided an oral composition for reducing serum cholesterol, serum lipids, body fat, or atherogenic index or for prophylaxis or treatment of atherosclerosis, cardiovascular or cerebrovascular diseases, the composition comprising a highly *bsh* active bacteria, isolate or supernatant thereof; wherein the highly *bsh* active bacteria degrades >50μmol glycodeoxycholic acid (GDCA)/gram/hour and >2μmol taurodeoxycholic acid (TDCA)/gram/hour when measured over 1 hour and 5 hours, respectively; or degrades >65 μmol GDCA/g/hr and >7 μmol TDCA/g/hr when measured over 30 minutes. In one embodiment, the highly *bsh* active bacteria degrades >300 μmol GDCA/g/hr and >40 μmol TDCA/g/hr when measured over 30 minutes. In another embodiment, the highly *bsh* active bacteria degrades >2000 μmol

GDCA/g/hr and >500 μmol TDCA/g/hr when measured over 30 minutes. In yet another embodiment, the highly *bsh* active bacteria degrades >15000 μmol GDCA/g/hr and >2000 μmol TDCA/g/hr when measured over 30 minutes.

[0048]   In one embodiment, the composition further comprises a carrier.

[0049]   Highly *bsh* active bacteria as used herein refers to bacteria that degrade >50μmol GDCA/gram/hour and >2μmol TDCA/gram/hour when measured over 1 hour and 5 hours, respectively; or degrade >65 μmol GDCA/g/hr and >7 μmol TDCA/g/hr when measured over 30 minutes, optionally >300 μmol GDCA/g/hr and >40 μmol TDCA/g/hr when measured over 30 minutes, or >2000 μmol GDCA/g/hr and >500 μmol TDCA/g/hr when measured over 30 minutes or >15000 μmol GDCA/g/hr and >2000 μmol TDCA/g/hr when measured over 30 minutes and are readily identified by a person skilled in the art based on the methods described in the Examples. In one embodiment, the GDCA and TDCA degradation is measured by HPLC. Determination of bile salts by HPLC is described in Scalia 1988 and Jones et al. 2003.

[0050]   The term "isolate" as used herein refers to a separated, isolated, or fractionated portion of the cell culture or fermentation product which may be more pure or more active than the crude sample. The term "supernatant" as used herein refers to the liquid overlying the material deposited by settling, precipitating or centrifuging.

[0051]   In an embodiment, the highly *bsh* active bacteria are live bacteria. The term "live bacteria" as used herein refers to a biomass of nutrient metabolizing and waste excreting bacteria. In one embodiment, the live bacteria are probiotic bacteria. The term "probiotic bacteria" as used herein refers to live microorganisms which when delivered in adequate amounts confer a health benefit to the host.

[0052]   The highly *bsh* active bacteria is optionally *Lactobacillus, Bifidobacteria, Pediococcus, Streptococcus, Enterococcus,* or *Leuconostoc.*

In one embodiment, the *Lactobacillus* is *Lactobacillus reuteri*, optionally, *Lactobacillus reuteri* (NCIMB 701359), *Lactobacillus reuteri* (NCIMB 701089), *Lactobacillus reuteri* (ATCC 55148), *Lactobacillus reuteri* (ATCC 23272), *Lactobacillus reuteri* (NCIMB 702655), *Lactobacillus reuteri* (LMG 18238), *Lactobacillus reuteri* (CCUG 32271), *Lactobacillus reuteri* (CCUG 32305), *Lactobacillus reuteri* (CCUG 37470), *Lactobacillus reuteri* (CCUG 44001) or *Lactobacillus reuteri* (CCUG 44144).

[0053]   In another embodiment, the *Lactobacillus reuteri* adheres to the gastrointestinal epithelial cells, competes for adhesion, or inhibits the binding of other bacteria due to cell surface proteins.

[0054]   In an embodiment, the concentration of bacteria in the oral compositions described herein is 10^6 - 10^{12} colony forming units (CFU)/gram, optionally 10^8 -10^{12} CFU/gram. In another embodiment, the composition provided herein comprises 10^6-10^{14} CFU, optionally 10^8-10^{13} CFU.

[0055]   The term "*bsh*" or "bile salt hydrolase" as used herein refers to an enzyme capable of hydrolyzing bile salts produced by the bacteria.

[0056]   The highly *bsh* active bacteria can be grown under fermentation conditions that improve biomass production and *bsh* activity. In one embodiment, the fermentation conditions comprise inoculation in medium comprising carbon and nitrogen sources and having a pH of 4 to 7 and a harvest time of 12 to 24 hours. In a particular embodiment, the pH of the fermentation conditions is 5. In yet another embodiment, the harvest time is 12 to 16 hours.

[0057]   In one embodiment, the carbon source comprises maltose, sucrose, dextrin, a combination of sorbitol and glucose or a combination of inulin and glucose. In a particular embodiment, the carbon source is maltose. In one embodiment, the carbon sources are added to a final concentration of 2%, for example, if inulin and glucose are used, 1% of each are added to a final concentration of 2%.

[0058]   In another embodiment, the nitrogen source comprises (i) yeast extract and malt extract, yeast extract and beef extract, or casein hydrolysate and malt extract; and (ii) peptone or tryptone. In an embodiment, the nitrogen source further comprises cysteine. The peptone may be any peptone, including without limitation, peptone no. 3, fish peptone, soy peptone, proteose peptone and casein peptone. In a particular embodiment, the peptone is peptone no. 3. In one embodiment, the nitrogen source is added to a total of 2.5%, for example, if a peptone, yeast and malt extract source is used, 1% peptone, 0.5% yeast extract and 1% malt extract are added to a final concentration of 2.5%. In another embodiment, beef extract substitutes for malt extract and casein substitutes for either the peptone or yeast extract. In another embodiment 0.01 to 0.05% cysteine is added, optionally 0.01%.

[0059]   In one embodiment, the highly *bsh* active bacteria are free bacteria. The term "free bacteria" as used herein refers to bacteria that are not immobilized in a polymer or encapsulated by artificial cell microencapsulation.

[0060]   In another embodiment, the highly *bsh* active bacteria are contained or immobilized in a polymer, optionally a *natural polymer*. Natural polymers include, without limitation, alginate, chitosan, agarose, pectin, agaropectin, genipin, and cellulose. In an embodiment, the highly *bsh* active bacteria are immobilized on a film.

[0061]   In yet another embodiment, the highly *bsh* active bacteria are encapsulated. Encapsulation is a term used to include the methods of macroencapsulation, microencapsulation and nanoencapsulation. The terms microencapsulation and nanoencapsulation refer to a subclass of encapsulation, where small, micro- or nano- encapsulated capsules are produced. Encapsulation and microencapsulation techniques are known in the art. Microcapsules are small spherical containers or coated tissues in the 1-999 μm range and nanocapsules range from 1-999 nm, whereas macrocapsules are larger flat-sheet or hollow-fiber membraned vessels.

Macro-, micro- and nano- capsules must contain a cellular environment that is able to support cellular metabolism and proliferation, as the cells they accommodate provide the capsule functionality.

[0062]   Artificial cell microencapsulation or nanoencapsulation is a technique used to encapsulate biologically active materials in specialized ultra thin semi-permeable polymer membranes (see e.g., Chang and Prakash, 1997; Chang, 1964). Methods for preparing artificial cells have been well documented in the pertinent art. Artificial cell membranes are optionally selected or designed for each specific therapeutic device by one of skill in the art, because one may engineer several different membranes for artificial cell preparations with required membrane properties for a desired application. The use of different membranes allows for variation in permeability, mass transfer, mechanical stability, buffering capability, biocompatibility, and other characteristics.   A balance has to be maintained among the physical properties of capsule membranes so as to support the entrapped cell's survival.

[0063]   Microcapsules can be prepared for the bacteria of the invention using techniques as in US Publication No. 2007-0116671 to Prakash and Jones.

[0064]   The mass transport properties of a membrane are critical since the influx rate of molecules, essential for cell survival, and the outflow rate of metabolic waste ultimately determines the viability of entrapped cells. Any barriers can be potentially applied to enzyme applications. Ordinarily the desired capsule permeability is determined by the molecular weight cut-off (MWCO), and is application dependent. The MWCO is the maximum molecular weight of a molecule that is allowed passage through the pores of the capsule membrane (Uludag et al. (2000) *Adv. Drug Deliv. Rev.* 42:29-64). For transplantation, the MWCO must be high enough to allow passage of nutrients, but low enough to reject antibodies and other immune system molecules. The MWCO range is optionally 3000 D to 950,000 D (Chang and

Prakash, 1998). The MWCO of orally delivered microcapsules must allow for the passage of unwanted metabolites from the plasma into the microcapsule, and then must either facilitate the subsequent removal of the altered molecule or provide for its storage (Uludag et al., 2000). For cells of the present

5   disclosure that are to be administered orally, one optionally uses a retainer that allows passage of nutrients, but blocks antibodies and other immune molecules, for example a semi-permeable membrane having a MWCO 3000 D to 950,000 D (Chang and Prakash, 1998). Alternatively, the lower end of the range may be about: 2000D, 4000D, 5000D or 10,000D and the higher

10   end of the range may be about: 900,000D, 750,000D or 500,000D.

[0065]   The most common type of membrane used for cell therapy is the single alginate based polymer membrane; however, several other substances may be used such as various proteins, polyhemoglobin, and lipids (Uludag et al., 2000; Prakash and Jones, 2002). Yet another approach for

15   membrane composition is to use a biodegradable synthetic polymer such as polylactide, polyglycolic acid, and polyanhydride. Commonly used membranes include hollow fiber Membranes, alginate-polylysine-alginate (APA) membrane, cellulose nitrate, polyamide, lipid-complexed polymer, and lipid vesicles. Established and promising polymers for live cell encapsulation

20   and enzyme encapsulation include alginate-polylysine-alginate (APA), alginate-polymethylene-co-guanidine-alginate (A-PMCG-A), hydroxymethylacrylate-methyl methacrylate (HEMA-MMA), Multilayered HEMA-MMA-MAA, polyacrylonitrilevinylchloride (PAN-PVC), acrylonitirle/sodium methallylsuflonate (AN-69), polyethylene glycol/poly

25   pentamethylcyclopentasiloxane/polydimethylsiloxane (PEG/PD$_5$/PDMS), poly N,N-dimethyl acrylamide (PDMAAm), Siliceous encapsulates, and cellulose sulphate/sodium alginate/polymethylene-co-guanidine (CS/A/PMCG). Other materials that are useful include, without limitation, cellulose acetate phthalate, calcium alginate and k-carrageenan-Locust bean gum gel beads,

30   gellan-xanthan beads, poly(lactide-co-glycolides), carageenan, starch

polyanhydrides, starch polymethacrylates, polyamino acids, enteric coating polymers.

[0066]   The design of a membrane, intended for use in oral live cell therapy, must take into consideration several primary factors so as to minimize microbial death and maximize therapeutic effectiveness. To assure their efficacy, artificially encapsulated cells intended for oral administration must be designed to protect their living cargo against both the acidic environment of the stomach and immunoglobulin released by the intestinal immune response.

[0067]   A useful composition is the encapsulation of calcium alginate beads with poly-L-lysine (PLL) forming alginate-poly-L-lysine-alginate (APA) microcapsules. In the APA membrane microcapsule, alginate forms the core and matrix for the cell and PLL binds to the alginate core. Binding of PLL to alginate is the result of numerous long-chain alkyl-amino groups within PLL that extend from the polyamide backbone in a number of directions and interact with various alginate molecules, through electrostatic interactions. The resulting cross-linkage produces a stable complex membrane that reduces the porosity of the alginate membrane and forms an immunoprotective barrier.

[0068]   Alternatively, Alginate/Poly-l-lysine/Pectin/Poly-l-lysine/Alginate (APPPA), Alginate/Poly-l-lysine/Pectin/Poly-l-lysine/Pectin (APPPP), and Alginate/Poly-L-lysine/Chitosan/Poly-l-lysine/Alginate (APCPA) membranes are used for encapsulation. These multi-layer membrane compositions perform well in GI stability tests, providing for increased resistance to complete dissolution in water, dilute acids and base, as well as in the presence of ion chelators, while allowing for more precise control over membrane permeability.

[0069]   There are various methods available for preparing artificial cells containing live cells for therapy. For example, for preparation of the classic alginate-polylysine-alginate (APA) membrane, the live cells, such as bacterial

cells, are suspended in a matrix of the natural polymer alginate (1.5%). The viscous polymer-bacterial suspension is passed through a 23-gauge needle using a syringe pump. Sterile compressed air, passed through a 16-gauge coaxial needle, is then used to shear the droplets coming out of the tip of the

5   23-gauge needle. The droplets are allowed to gel for 15 minutes in a gently stirred ice-cold solution of solidifying chemicals, such as $CaCl_2$ (1.4 %). After gelation in the $CaCl_2$, the beads are then washed with HEPES (0.05 % in HEPES, pH 7.20), coated with polylysine (0.1% for 10 min) and washed again in HEPES (0.05 % in HEPES, pH 7.20). The resultant capsules are then

10   coated by reaction with alginate (0.1% for 10 min) and washed with appropriate chemicals to dissolve their inner core content. For this step a 3.00 % citrate bath (3.00 % in 1:1 HEPES-buffer saline, pH 7.20) is often used. The microcapsules formed can then be stored at 4°C in minimal solution (10% cell nutrient to 90% water).

15   **[0070]**   Accordingly, in one embodiment, the highly *bsh* active bacteria are encapsulated in polymeric semi permeable microcapsules (1-999μm) or nanocapsules (1-999nm). In one embodiment, polymeric semi permeable microcapsules or nanocapsule comprise Alginate/Poly-l-lysine/Alginate (APA), Alginate/Chitosan/Alginate (ACA) or Alginate/Genipin/Alginate (AGA)

20   membranes. In another embodiment, the microcapsule or nanocapsule comprises Alginate/Poly-l-lysine/Pectin/Poly-l-lysine/Alginate (APPPA), Alginate/Poly-l-lysine/Pectin/Poly-l-lysine/Pectin (APPPP), Alginate/Poly-L-lysine/Chitosan/Poly-l-lysine/Alginate (APCPA), alginate-polymethylene-co-guanidine-alginate (A-PMCG-A), hydroxymethylacrylate-methyl methacrylate

25   (HEMA-MMA), Multilayered HEMA-MMA-MAA, polyacrylonitrilevinylchloride (PAN-PVC), acrylonitirle/sodium methallylsuflonate (AN-69), polyethylene glycol/poly   pentamethylcyclopentasiloxane/polydimethylsiloxane (PEG/PD5/PDMS) or poly N,N-dimethyl acrylamide (PDMAAm) membranes. In yet another embodiment, the microcapsule or nanocapsule comprises

30   hollow fiber, cellulose nitrate, polyamide, lipid-complexed polymer, a lipid vesicle   a   siliceous   encapsulate,   cellulose   sulphate/sodium

alginate/polymethylene-co-guanidine (CS/A/PMCG), cellulose acetate phthalate, calcium alginate, k-carrageenan-Locust bean gum gel beads, gellan-xanthan beads, poly(lactide-co-glycolides), carageenan, starch polyanhydrides, starch polymethacrylates, polyamino acids or enteric coating polymers.

[0071]   In a further embodiment, the polymeric microcapsules or nanocapsules are resistant to gastro-intestinal conditions, such as pH 1-8 and/or bile [1-30mmol]).

[0072]   The oral compositions disclosed herein are optionally lyophilized, heat dried, spray dried or freeze-dried. Alternatively, the oral compositions are optionally prepared wet.

[0073]   In an embodiment, the oral compositions described herein are lyophilized with lyoprotectants to ensure viability and improved *bsh* activity over time. Typical lyoprotectants include, without limitation, a final concentration of 0.2% to 10% maltodextrin and 0.05% to 0.33% yeast extract or 0.05-2.5% inulin and 0.05 to 0.1% yeast extract. In one embodiment, the lyoprotectants comprise a final concentration of 2-4% maltodextrin and 0.1% yeast extract, 0.3% inulin and 0.1% yeast extract, or 0.3% inulin.

[0074]   In another embodiment, the oral compositions described herein are stored in liquid to ensure viability and improved *bsh* activity. Typical liquid storage conditions include, without limitation, a final concentration of preservative solution comprising 2.5-10% growth media (as described herein), 50-99.99% yogurt or other fermented milk, 50-99.99% culture supernatant or 5% MRS.

[0075]   In yet another embodiment, the oral compositions described herein are flash frozen to ensure viability and improved *bsh* activity. Typical flash freezing conditions include, without limitation, a final concentration of cryoprotectant solution comprising 0.2-10% maltodextrin, optionally 1-3%, maltodextrin and 0.05 to 0.33% yeast extract, optionally 0.1-0.2% yeast extract, 0.05 to 2.5% inulin, optionally at least 0.2% inulin, 0.5M Trehalose,

0.5M fructose, 0.5M lactose, 0.5M maltose or 50-99.99%, optionally 50% spent media.

[0076]   The term "carrier" as used herein refers to an acceptable carrier that facilitates administration to the subject. For example, an acceptable carrier that facilitates oral administration includes, without limitation, a supplement, food product, beverage, functional food or nutraceutical, or excipient. "Nutraceutical" means a product isolated or purified from foods (or sources used to make food, such as plants, animals or other organisms) that is thought to have a health benefit, such as a medicinal, physiological or prophylactic effect. "Functional Food Product" means it is food, is consumed as part of a diet and has health benefits, such as medicinal, physiological or prophylactic benefits beyond basic nutritional function of supplying nutrients.

[0077]   In another embodiment, the carrier comprises a capsule, pill, gel capsule, liquid, or dissolvable film.

[0078]   The oral compositions disclosed herein for reducing serum cholesterol, serum lipids, body fat, or atherogenic index or for prophylaxis or treatment of atherosclerosis, cardiovascular or cerebrovascular diseases optionally further comprise other agents or therapeutics for such indications. Accordingly, in one embodiment, the oral composition further comprises a triglyceride lowering agent, optionally, squalene synthase inhibitors, microsomal triglyceride transfer protein inhibitors, statins, bile acid sequestrants, cholesterol absorption inhibitors, fibrates and other PPAR alpha agonists, dual PPAR agonists, lipase inhibitors, protein tyrosine phosphatase 1B inhibitors, pancreatic peptide $YY_{3\text{-}36}$, recombinant and other cannabinoid receptor antagonists or 5-HT2c agonists, such as lorcaserin. In another embodiment, the composition further comprises an agent for increasing HDL or limiting HDL decrease, optionally, statins, bile acid sequestrants, cholesterol absorption inhibitors, fibrates and other PPAR alpha agonists, dual PPAR agonists, lipase inhibitors, protein tyrosine phosphatase 1B inhibitors, pancreatic peptide $YY_{3\text{-}36}$, recombinant and other cannabinoid

receptor antagonists, or 5-HT2c agonists, such as lorcaserin. In yet another embodiment, the oral composition further comprises a cholesterol lowering agent, optionally, a bile acid sequestrant (BAS), a statin, ezetimibe, alpha-Linoleitic acid, omega-3,6,9, eicosapentaenoic acid (EPA), docosahexaenoic

5   acid (DHA), fibrates, soluble fibre, polyphenol, gama-oryzanol hesperetin metabolite, phytochemical, other probiotic, psyllium, phytosterol, phytostanol, vitamin, antioxidant or antibiotic. The statin may be selected from the group consisting of Lovastatin, Pravastatin, Zocor™, Fluvastatin, Mevastatin, Pitavastatin, Cerivastatin, Simvastatin, Rosuvastatin and Atorvastatin. The

10   BAS may be colestyramine, colestipol or colesevelam. The fibrate may be clofibrate, bezafibrate, gemfibrozil or fenofibrate. In a further embodiment, the oral composition further comprises an agent for preserving bsh activity, optionally, inulin, trealose, maltodextran, yeast extract, polyethylene glycol, glycerol, lipid, emulsified fat, a dairy product, glucose, fructose, sucrose, a

15   poly sugar, anhydrobiosis, a polycosanol, polyethlylene glycol (PEG), a plant sterol, a plant stanol, or an omega fatty acid. The polycosanol may be octacosanol, triacontanol, behenyl alcohol, lignoceryl alcohol, ceryl alcohol, 1-heptacosanol, 1-nonacosanol, 1-dotriacontanol, or geddyl alcohol. In yet a further embodiment, the oral composition further comprises an agent for

20   modulating adipokines or hormones of obesity, optionally, leptin, ghrelin, resistin, adiponectin, chemerin, Il-6, visfatin, retinol binding protein 4 or plasminogen activator inhibitor-1. In yet another further embodiment, the oral composition further comprises a hypoglycemic agent, optionally, Metformin, Rosiglitazone, Pioglitazone, Glyburide, Gliclazide, Glimepiride, Glipizidebile

25   Glibenclamide, Acarbose, Miglitol, Voglibose, Sitagliptin, Nateglinide, Repaglinide, Mitiglinide, Alogliptin, Saxagliptin, Vildagliptin and Dapagliflozin. In an even further embodiment, the oral composition further comprises a therapeutic for reducing the pro-inflammatory cytokines IL-1α/ß, IL-2, IL-15, IL-3, IL-6, IL-8, IL-12, IL-17, IFN-gamma, TNF-alpha, or for increasing the

30   level of the anti-inflammatory cytokines IL-1ra, IL-9, IL-10, IL-11.

[0079] In yet another embodiment, the oral composition further comprises vitamin B12. In a further embodiment, the oral composition further comprises conjugated linoleic acid (CLA). In yet a further embodiment, the oral composition further comprises reuterin and/or reutericyclin.

5   **Methods and Uses**

[0080] The disclosure includes methods and uses of the oral compositions disclosed herein. In one embodiment, there is provided a method of therapy comprising administration of an oral composition disclosed herein to an animal in need thereof. Also provided is a use of an oral

10   composition disclosed herein for therapy of an animal. Further provided is a use of an oral composition disclosed herein in the preparation of a medicament for therapy. Also provided is the oral composition disclosed herein for use in therapy.

[0081] The compositions described herein are useful for lowering

15   serum cholesterol of an animal in need thereof. Accordingly, in one aspect, the present disclosure provides a method of lowering serum cholesterol in an animal in need thereof comprising administering a bile acid lowering amount of an oral composition disclosed herein. There is also provided a use of an oral composition disclosed herein for lowering serum cholesterol in an animal

20   in need thereof. Also provided is a use of an oral composition disclosed herein in the preparation of a medicament for lowering serum cholesterol in an animal in need thereof. Further provided is an oral composition disclosed herein for use in lowering serum cholesterol in an animal in need thereof.

[0082] In one embodiment, the animal in need thereof has a disease or

25   disorder characterized by increased cholesterol accumulation in serum and/or tissue causing pathology or having excessive cholesterol as a risk factor. Cholesterol disorders include familial hypercholesterolemia or inherited cholesterol disorder (ICD), defects in the gene products of cholesterol metabolism e.g. 7-alpha-hydroxylase, and various forms of xanthomas.

30   Increased levels of serum cholesterol may indicate atherosclerosis, biliary

cirrhosis, familial hyperlipidemias, high-cholesterol diet, hypothyroidism, myocardial infarction, nephritic syndrome and uncontrolled diabetes. "Excessive cholesterol" means outside the typical (normal) cholesterol range. Typical cholesterol level is less than 200 mg/dL. Borderline High is 200-239

5   mg/dL and anything over 240 mg/dL is high. The National Cholesterol Education Program NCEP III report on cholesterol includes "Full Report" and a "Drug Therapy" section. This provides a review of examples of cholesterol management by statins, bile acid sequestrants, diet, etc. and it relates to cholesterol levels and risk factors (eg. see Tables IV.1-1 VI.1-1; VI.1-2, VI.1-

10   3). The compositions described herein are similar to bile acid sequestrants in that they reduce bile levels. The NCEP report provides guidance on use of pharmaceutical therapy in relation to the presence of other risk factors. There are two types of cholesterol, HDL cholesterol (sometimes called good cholesterol) and LDL cholesterol (sometimes called bad cholesterol).

15   "Excessive cholesterol" may also be determined with respect to LDL. For example, drug therapy is optionally considered for individuals with multiple risk factors (2 or more) when LDL cholesterol is: >100mg/dL (eg. with a goal to reduce LDL cholesterol to <100mg/dL), at least 130mg/dL (eg. with a goal to reduce LDL cholesterol to less than 130mg/dL), at least 160mg/dL (eg. with

20   a goal to reduce LDL cholesterol to less than 130mg/dL). Furthermore, drug therapy is also optionally considered for individuals with 0-1 risk factors when LDL cholesterol is at least 190mg/dL (eg. with a goal to reduce LDL cholesterol to less than 160mg/dL). Normal values tend to increase with age, and premenopausal women have somewhat lower levels than men of the

25   same age.

[0083]   In another embodiment, the methods and uses for lowering serum cholesterol also increase or limit the reduction of serum high density lipoproteins (HDL-C) of the animal. In a further embodiment, the methods and uses for lowering serum cholesterol also decrease serum triglycerides of an

30   animal. In yet a further embodiment, the methods and uses for lowering serum cholesterol also reduce atherosclerotic risk factors of the animal.

Atherosclerotic risk factors, include, without limitation serum homocystine, fibrinogen, C-reactive protein, lipoprotein(a), uric acid, matrix metallopeptidase 9 (MMP-9), plasminogen activator inhibitor-1 (PAI-1) or its antigen, tissue plasminogen activator (tPA), TNF alpha, IL-6, P-selectin,

5   monocyte chemotactic protein-1 (MCP-1), soluble CD40 ligand (sCD40L), inter-cellular adhesion molecule 1 (ICAM-1), myeloperoxidase (MPO), adiponectin, leptin, lipoprotein-associated phospholipase A and insulin.

[0084]   In yet another embodiment, the methods and uses for lowering serum cholesterol also produce and deliver vitamin B12 to the animal. In a

10   further embodiment, the methods and uses for lowering serum cholesterol also produce and deliver conjugated linoleic acid (CLA) to the animal. In yet a further embodiment, the methods and uses for lowering serum cholesterol also produce and deliver reuterin and reutericyclin to the animal.

[0085]   The oral compositions described herein are also useful for

15   decreasing serum triglycerides in an animal in need thereof. Accordingly, in one aspect, the present disclosure provides a method of decreasing serum triglycerides in an animal in need thereof comprising administering a bile acid lowering amount of an oral composition disclosed herein. There is also provided a use of an oral composition disclosed herein for decreasing serum

20   triglycerides in an animal in need thereof. Also provided is a use of an oral composition disclosed herein in the preparation of a medicament for decreasing serum triglycerides in an animal in need thereof. Further provided is an oral composition disclosed herein for use in decreasing serum triglycerides in an animal in need thereof.

25   [0086]   In another aspect, the present disclosure provides a method for reducing the atherogenic index of an animal, comprising administering to the animal a bile acid lowering amount of an oral composition of the disclosure. Also provided is a use of a bile acid lowering amount of a composition of the disclosure for reducing the atherogenic index of an animal. Also provided is a

30   use of a bile acid lowering amount of an oral composition of the disclosure in

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the preparation of a medicament for reducing the atherogenic index of an animal. Further provided is a bile acid lowering amount of an oral composition of the disclosure for use in reducing the atherogenic index of an animal. Atherogenic index is calculated using at least one of the equations shown in

5   Table 1.

[0087]   In yet another aspect, the disclosure provides a method for prophylaxis or treatment of atherosclerosis or a degenerative disorder caused by atherosclerosis in an animal comprising administering to the animal a bile acid lowering amount of an oral composition of the disclosure. Also provided

10   is a use of a bile acid lowering amount of an oral composition of the disclosure for prophylaxis or treatment of atherosclerosis or a degenerative disorder caused by atherosclerosis. Also provided is a use of a bile acid lowering amount of an oral composition of the disclosure in the preparation of a medicament for prophylaxis or treatment of atherosclerosis or a

15   degenerative disorder caused by atherosclerosis. Further provided is a bile acid lowering amount of an oral composition of the disclosure for use in the prophylaxis or treatment of atherosclerosis or a degenerative disorder caused by atherosclerosis. Degenerative disorders include, without limitation cerebrovascular disease, stroke, vascular disease, coronary artery disease,

20   myocardial infarction, thrombosis, angina, unstable angina, intermittent claudication, transient ischemic attack, or renal failure.

[0088]   The term "prophylaxis or treatment of" refers to decreasing the likelihood of a condition or ameliorating a condition.

[0089]   In a further aspect, the disclosure provides a method for

25   lowering total body fat or treating obesity or pre-obesity in an animal, comprising administering to the animal a bile acid lowering amount of an oral composition of the disclosure. Also provided is a use of a bile acid lowering amount of an oral composition of the disclosure for lowering total body fat or treating obesity or pre-obesity in an animal. Also provided is a use of a bile

30   acid lowering amount of an oral composition of the disclosure in the

preparation of a medicament for lowering total body fat or treating obesity or pre-obesity in an animal. Further provided is a bile acid lowering amount of an oral composition of the disclosure for use in lowering total body fat or treating obesity or pre-obesity in an animal. The term "obesity" as used herein

5   refers to a disease, medical condition or disorder and is defined as a body mass index (BMI)>30. A BMI of 25-30 is pre-obese.

[0090]   In yet a further aspect, the disclosure provides a method for prophylaxis or treatment of a metabolic disease or disorder in an animal comprising administering to the animal a bile acid lowering amount of an oral

10   composition of the disclosure. Also provided is a use of a bile acid lowering amount of an oral composition of the disclosure for prophylaxis or treatment of a metabolic disease or disorder in an animal. Also provided is a use of a bile acid lowering amount of an oral composition of the disclosure in the preparation of a medicament for prophylaxis or treatment of a metabolic

15   disease or disorder in an animal. Further provided is a bile acid lowering amount of an oral composition of the disclosure for use in the prophylaxis or treatment of a metabolic disease or disorder in an animal. Metabolic diseases and disorders include, without limitation, hyperlipidemia, hyperglycemia, hyperlipoproteinemia, impaired glucose tolerance (IGT), insulin resistance,

20   pre-diabetes, type I diabetes, type II diabetes, and metabolic syndrome.

[0091]   In another aspect, the disclosure provides a method for prophylaxis or treatment of a liver disease or disorder associated with high serum or hepatic lipid and triglyceride concentrations, hepatic inflammation, non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease (AFLD),

25   non-alcoholic steatohepatitis (NASH), liver cirrhosis, liver steatosis, liver fibrosis, abnormally high serum ALT, AST, GGT, or Alk-P levels, Epstein-Barr virus, hepatitis, autoimmune hepatitis, hepatic granulomatus disease, cholangitis, hepatocellular cancer, cholangiocarcinoma, metabolic liver disease in an animal, comprising administering to the animal bile acid

30   lowering amount of an oral composition of the disclosure. Also provided is a use of a bile acid lowering amount of an oral composition of the disclosure for

prophylaxis or treatment of a liver disease or disorder associated with high serum or hepatic lipid and triglyceride concentrations, hepatic inflammation, non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease (AFLD), non-alcoholic steatohepatitis (NASH), liver cirrhosis, liver steatosis, liver

5   fibrosis, abnormally high serum ALT, AST, GGT, or Alk-P levels, Epstein-Barr virus, hepatitis, autoimmune hepatitis, hepatic granulomatus disease, cholangitis, hepatocellular cancer, cholangiocarcinoma, metabolic liver disease in an animal. Also provided is a use of a bile acid lowering amount of an oral composition of the disclosure in the preparation of a medicament for

10   prophylaxis or treatment of a liver disease or disorder associated with high serum or hepatic lipid and triglyceride concentrations, hepatic inflammation, non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease (AFLD), non-alcoholic steatohepatitis (NASH), liver cirrhosis, liver steatosis, liver fibrosis, abnormally high serum ALT, AST, GGT, or Alk-P levels, Epstein-Barr

15   virus, hepatitis, autoimmune hepatitis, hepatic granulomatus disease, cholangitis, hepatocellular cancer, cholangiocarcinoma, metabolic liver disease in an animal. Further provided is a bile acid lowering amount of an oral composition of the disclosure for use in the prophylaxis or treatment of a liver disease or disorder associated with high serum or hepatic lipid and

20   triglyceride concentrations, hepatic inflammation, non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease (AFLD), non-alcoholic steatohepatitis (NASH), liver cirrhosis, liver steatosis, liver fibrosis, abnormally high serum ALT, AST, GGT, or Alk-P levels, Epstein-Barr virus, hepatitis, autoimmune hepatitis, hepatic granulomatus disease, cholangitis,

25   hepatocellular cancer, cholangiocarcinoma, metabolic liver disease in an animal.

[0092]   Other agents or therapeutics can be coadministered or used in combination with the oral compositions disclosed herein. Accordingly, in an embodiment, the methods and uses disclosed herein further comprise

30   administering a triglyceride lowering agent, optionally, squalene synthase inhibitors, microsomal triglyceride transfer protein inhibitors, statins, bile acid

sequestrants, cholesterol absorption inhibitors, fibrates and other PPAR alpha agonists, dual PPAR agonists, lipase inhibitors, protein tyrosine phosphatase 1B inhibitors, pancreatic peptide YY_{3-36}, recombinant and other cannabinoid receptor antagonists or 5-HT2c agonists, such as lorcaserin. In another

5   embodiment, the methods and uses disclosed herein further comprise administering an agent for increasing HDL or limiting HDL decrease, optionally, statins, bile acid sequestrants, cholesterol absorption inhibitors, fibrates and other PPAR alpha agonists, dual PPAR agonists, lipase inhibitors, protein tyrosine phosphatase 1B inhibitors, pancreatic peptide YY_{3-}

10     $_{36}$, recombinant and other cannabinoid receptor antagonists, or 5-HT2c agonists, such as lorcaserin. In yet another embodiment, the methods and uses disclosed herein further comprise administering a cholesterol lowering agent, optionally, a bile acid sequestrant (BAS), a statin, ezetimibe, alpha-Linoleitic acid, omega-3,6,9, eicosapentaenoic acid (EPA), docosahexaenoic

15   acid (DHA), fibrates, soluble fibre, polyphenol, gama-oryzanol hesperetin metabolite, phytochemical, other probiotic, psyllium, phytosterol, phytostanol, vitamin, antioxidant or antibiotic. The statins, include, without limitation, Lovastatin, Pravastatin, Zocor, Fluvastatin, Mevastatin, Pitavastatin, Cerivastatin, Simvastatin, Rosuvastatin and Atorvastatin. The BAS include,

20   without limitation colestyramine, colestipol and colesevelam. The fibrates include, without limitation, clofibrate, bezafibrate, gemfibrozil and fenofibrate. In yet another embodiment, the methods and uses disclosed herein further comprise administering an agent for preserving *bsh* activity, optionally, inulin, trealose, maltodextran, yeast extract, polyethylene glycol, glycerol, lipid,

25   emulsified fat, a dairy product, glucose, fructose, sucrose, a poly sugar, anhydrobiosis, a polycosanol, polyethlylene glycol (PEG), a plant sterol, a plant stanol, or an omega fatty acid. The polycosanol includes, without limitation, octacosanol, triacontanol, behenyl alcohol, lignoceryl alcohol, ceryl alcohol, 1-heptacosanol, 1-nonacosanol, 1-dotriacontanol, and geddyl

30   alcohol. In a further embodiment, the methods and uses disclosed herein further comprise administering an agent for modulating adipokines or

hormones of obesity, optionally, leptin, ghrelin, resistin, adiponectin, chemerin, Il-6, visfatin, retinol binding protein 4 or plasminogen activator inhibiotor-1. In yet a further embodiment, the methods and uses disclosed herein further comprise administering a hypoglycemic agent, optionally,

5   Metformin, Rosiglitazone, Pioglitazone, Glyburide, Gliclazide, Glimepiride, Glipizidebile Glibenclamide, Acarbose, Miglitol, Voglibose, Sitagliptin, Nateglinide, Repaglinide, Mitiglinide, Alogliptin, Saxagliptin, Vildagliptin and Dapagliflozin. In another embodiment, the methods and uses disclosed herein further comprise administering a therapeutic for reducing the pro-inflammatory

10   cytokines IL-1α/ß, IL-2, IL-15, IL-3, IL-6, IL-8, IL-12, IL-17, IFN-gamma, TNF-alpha, or for increasing the level of the anti-inflammatory cytokines IL-1ra, IL-9, IL-10, IL-11.

[0093]   The term "animal" as used herein refers to any member of the animal kingdom, optionally, a mammal, such as a human.

15   **[0094]**   Administration of an "effective amount" or "bile acid lowering amount" of the agents described described herein is defined as an amount effective at dosages and for periods of time necessary to achieve the desired result. The effective amount of the highly *bsh* active bacterial composition is optionally adjusted according to factors such as the disease state, age, sex,

20   and weight of the animal. Dosage regimens are readily adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. In one embodiment, the compositions may be administered or used 1 to 4 times per day.

25   **[0095]**   The compositions can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the cell is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example in Remington's Pharmaceutical Sciences

30   (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton,

Pa., USA 2003 – 20th Edition) and in The United States Pharmacopeia: The National Formulary (USP 24 NF19) published in 1999).

Processes for growing and preparing bacteria

[0096]   Also provided herein are processes for preparing a highly *bsh* active bacteria comprising growing *bsh*-producing bacteria under fermentation conditions that support development of high *bsh* activity. Suitable fermentation conditions have been described in the compositions section above. For example, the fermentation conditions optionally comprise a carbon source, a nitrogen source, a pH of 4 to 7 and a harvest time of 12 to 24 hours. In one embodiment, the carbon source is a sugar that optionally comprises maltose, sucrose, dextrin, a combination of sorbitol and glucose or a combination of inulin and glucose. In a particular embodiment, the carbon source is maltose. In another embodiment, the nitrogen source comprises (i) yeast extract and malt extract, yeast extract and beef extract, or casein hydrolysate and malt extract; and (ii) peptone or tryptone. In another embodiment, the nitrogen source further comprises cysteine. In a particular embodiment, the peptone is peptone no. 3. Typical concentrations of carbon and nitrogen sources are as described in the compositions section above.

[0097]   In a further embodiment, the process further comprises lyophilizing the free or microencapsulated bacterial compositions with lyoprotectants as described herein. In one embodiment, the lyoprotectants comprise a final concentration of 0.2% to 10% maltodextrin and 0.05% to 0.33% yeast extract or 0.05-2.5% inulin and 0.05 to 0.1% yeast extract. In one embodiment, the lyoprotectants comprise a final concentration of 2-4% maltodextrin and 0.1% yeast extract, 0.3% inulin and 0.1% yeast extract, or 0.3% inulin.

[0098]   In an alternate embodiment, the process further comprises storing the highly bsh active free or microencapsulated bacterial composition under liquid storage conditions. In one embodiment, the liquid storage conditions comprise a final preservative solution comprising 2.5-10% growth

media, 50-99.99% yogurt or other fermented milk, 50-99.99% culture supernatant or 5% MRS.

[0099]   In yet another embodiment, the process further comprises flash freezing the free or microencapsulated composition in cryoprotectant solution as described herein. In one embodiment, the cryoprotectant solution comprises a final concentration of 0.2-10% maltodextrin, optionally 1-3%, maltodextrin and 0.05 to 0.33% yeast extract, optionally 0.1-0.2% yeast extract, 0.05 to 2.5% inulin, optionally at least 0.2% inulin, 0.5M Trehalose, 0.5M fructose, 0.5M lactose, 0.5M maltose or 50-99.99%, optionally 50% spent media. Flash freezing as used herein refers to subjecting the composition to temperatures below -80 degrees Celsius, for example, by subjecting the free or microencapsulated composition to liquid nitrogen such as at a temperature of -196 degrees Celsius, or freezing the composition at ultra low temperatures, such as -130 degrees Celsius, or using dry ice.

[00100]   The above disclosure generally describes the present disclosure. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for the purpose of illustration and are not intended to limit the scope of the disclosure. Changes in form and substitution of equivalents are contemplated as circumstances might suggest or render expedient.

[00101]   The following non-limiting examples are illustrative of the present disclosure:

EXAMPLES

Deconjugation of TDCA and GDCA by free *Lactobacillus reuteri* strains (Figures 1 and 2)

Results

[00102]   Figures 1 and 2 show the deconjugation of taurodeoxycholic acid and glycodeoxycholic acid, as measured by HPLC, in an *in-vitro* assay using 0.4 grams of free *Lactobacillus reuteri* (ATCC 53608, ATCC 53609,

ATCC 55148, ATCC 55739, and NCIMB 701359) over time. The NCIMB 701359 and ATCC 55739 *Lactobacillus reuteri* strains have the highest *bsh* activity as measured by HPLC. One should take into consideration the limitations to the assay "resolution" (using 0.4 grams and sampling over 2.5 hours); however, these strains were still shown to be considerably more *bsh* active than the others tested in this experiment and as the level of GDCA is not measurable at 2.5 hours there is an even greater difference in *bsh* activity, as the substrate became limiting.

Materials and Methods

Bacteria and culture conditions

[00103]   Four *Lactobacillus reuteri* strains obtained from ATCC (53609, 53608, 55148 and 55739) and NCIMB 701359 were cultivated in sterile de Man, Rogosa, Sharpe (MRS) broth at 37°C for 20 hours. Grown cultures were isolated by centrifugation and collected bacteria cells were used in the following BSH assay.

Measurement of BSH activity

[00104]   To measure the BSH activity, collected bacteria were added into 100% MRS supplemented with a combination of sodium glycodeoxycholate and sodium taurodeoxycholate both at 5mM (0.4g bacteria cells/20ml MRS supplemented with GDCA and TDCA). Bacteria were then incubated in the reaction broth anaerobically at 37°C with minimal shaking (100rpm), and supernatant was sampled at intervals of 2.5 hours and processed to determine the conjugated bile salt concentrations. Briefly, 500μl samples were acidified with 5ul of 6N HCl after removing bacteria cells by centrifugation at 10000g for 3min. The supernatants were then supplemented with 500μl of Methanol containing 4mM GCA (glycocholic acid) as internal standard. The samples were vortexed for 10min and centrifuged at 1000g for 15min. The samples were filtered through 0.22μM filter before being analyzed by HPLC. HPLC analysis of bile salts followed the procedure described by Jones et al. 2003.

Bile salt HPLC analysis

[00105]   HPLC analysis of bile salts followed the procedure described by Jones et al. 2003. The analysis was performed on a reverse-phase C-18 column: LiChrosorb RP-18, 5μm, 250 x 4.6mm. The HPLC system comprised

5   of two ProStar 210 solvent delivery modules, a ProStar 320 UV-VIS detector, a ProStar 410 autosampler and Galaxie Chromatography Data system (version 1.9.3.2). A mixture of methanol and 50mM sodium acetate buffer adjusted to pH4.3 with o-phosphoric acid (70:30, v/v) was applied as mobile phase with a flow rate of 1.0ml/min. The detector was set at 210nm and all

10   the measurements were performed at room temperature.

Deconjugation of TDCA and GDCA by free *Lactobacillus reuteri* and *Lactobacillus fermentum* (Figures 3 and 4)

<u>Results</u>

15   **[00106]**   Figures 3 and 4 show the deconjugation of taurodeoxycholic acid and glycodeoxycholic acid as measured by HPLC in an *in-vitro* assay of free *Lactobacillus reuteri* (Lab Met, NCIMB 701359) and *Lactobacillus fermentum* (ATCC 11976) over time. *Lactobacillus reuteri* (NCIMB 701359) has a much greater *bsh* activity than *Lactobacillus reuteri* (LabMet) or

20   *Lactobacillus fermentum* (ATCC 11976) and even a fraction (1/8th) of the quantity of *Lactobacillus reuteri* (NCIMB 701359) cells outperform *Lactobacillus reuteri* (LabMet) *in-vitro*.

<u>Materials and Methods</u>

Bacteria and culture conditions

25   **[00107]**   The bacterial strains used in this study are *L. reuteri (LabMet, NCIMB 701359)* and *L. fermentum* (ATCC 11976). The bacteria were cultivated in sterile de Man, Rogosa, Sharpe (MRS) broth at 37°C for 20 hours. Grown cultures were isolated by centrifugation and collected bacteria cells were used in the following BSH assay.

Measurement of BSH activity

[00108]   To measure the BSH activity, collected bacteria were added into 100% MRS supplemented with a combination of sodium glycocholate and sodium taurocholate at 5mM (0.4g or 0.05g bacteria /20ml MRS supplemented with GDCA and TDCA). Bacteria were then incubated anaerobically at 37°C, and supernatant was sampled at intervals of 3 hours and processed to determine the conjugated bile salt concentrations in the reaction tubes. Briefly, 500μl samples were acidified with 5μl of 6N HCl after removing bacteria cells by centrifugation at 10000g for 3min. The supernatants were then supplemented with 500μl of Methanol containing 4mM GCA (glycocholic acid) as internal standard. The samples were vortexed for 10min and centrifuged at 1000g for 15min. The samples were filtered through 0.22μM filter before being analyzed by HPLC. HPLC analysis of bile salts followed the procedure described by Jones et.al.

Bile salt HPLC analysis

[00109]   HPLC analysis of bile salts followed the procedure described by Jones et al. 2003. The analysis was performed on a reverse-phase C-18 column: LiChrosorb RP-18, 5μm, 250 x 4.6mm. The HPLC system comprised of two ProStar 210 solvent delivery modules, a ProStar 320 UV-VIS detector, a ProStar 410 autosampler and Galaxie Chromatography Data system (version 1.9.3.2). A mixture of methanol and 50mM sodium acetate buffer adjusted to pH4.3 with o-phosphoric acid (70:30, v/v) was applied as mobile phase with a flow rate of 1.0ml/min. The detector was set at 210nm and all the measurements were performed at room temperature.

Deconjugation of TDCA and GDCA by free *Lactobacillus reuteri* strains (Figures 5 and 6)

Results

[00110]   Figures 5 and 6 show the deconjugation of taurodeoxycholic acid and glycodeoxycholic acid as measured by HPLC in *in-vitro* assay using

0.2 grams of free *Lactobacillus reuteri* (NCIMB 701359, NCIMB 701089, NCIMB 702656, NCIMB 11951, ATCC 23272 and LMG 9213) over time. NCIMB 701359, NCIMB 701089 and ATCC 23272 strains have the greatest degree of *bsh* activity as measured by HPLC, again limited by the resolution

5   of the study.

Materials and Methods

Bacteria and culture conditions

[00111]   The bacterial strains used in this study are *L. reuteri NCIMB*

10   *701359*, *L. reuteri NCIMB 701089*, *L. reuteri NCIMB 702656*, *L. reuteri NCIMB 11951*, *L. reuteri ATCC 23272*, and *L. reuteri LMG 9213*. The bacteria were inoculated from single colony and passaged for twice with 1% inoculums. The bacteria were cultivated in sterile de Man, Rogosa, Sharpe (MRS, Difco) broth at 37°C for 20 hours every time. Grown cultures were

15   isolated by centrifugation and collected bacteria cells were used in the following BSH assay.

Measurement of BSH activity

[00112]   To measure the BSH activity, 0.2g of collected bacteria were added into 100% MRS supplemented with a combination of sodium

20   glycocholate and sodium taurocholate at 5mM (0.2g of bacteria /20ml MRS supplemented with 5mM GDCA and 5mM TDCA). Bacteria were then incubated anaerobically at 37°C, and supernatant was sampled after 1, 3, 5, and 7 hours to determine the conjugated bile salt concentrations in the reaction tubes. Briefly, 500µl samples were acidified with 5µl of 6N HCl after

25   removing bacteria cells by centrifugation at 10000g for 3min. The supernatants were then supplemented with 500µl of Methanol containing 4mM GCA (glycocholic acid) as internal standard. The samples were vortexed for 10min and centrifuged at 1000g for 15min. The samples were filtered through 0.45µM filter before being analyzed by HPLC. HPLC analysis of bile

30   salts followed the procedure described by Jones et.al.

Highly *bsh* active *L. reuteri* and reduction of cholesterol

Materials and Methods

[00113]   The *bsh* activity of *Lactobacilli* strains was calculated by the
5   standard HPLC assay for TDCA and GDCA described in materials and
methods sections above. Rates of xDCA removal were calculated by taking
the concentration of GDCA or TDCA removed from simulated intestinal
contents at the endpoint and subtracting the baseline value. The amount of
DCA produced, or xDCA removed, was divided by the mass of microcapsules
10   used and multiplied by the volume of simulated intestinal contents used and
divided by the time elapsed, in hours, from baseline to endpoint. This was
done by the equation: xDCA reduced = μmol DCA produced or xDCA
reduced/g microcapsule/h.

Results
15

[00114]   Table 2 shows the *bsh* activity of *Lactobacillus reuteri* strains
tested preclinically or clinically and expressed as a rate (μmol DCA/g/hr)
measured over 5 hours and over 30 minutes. Although, *Lactobacillus reuteri*
(LabMet) was shown to lower cholesterol preclinically, high doses of *L. reuteri*
20   and high frequency dosing was required. The more *bsh* active *Lactobacillus
reuteri* (NCIMB 701359) which lowered cholesterol in preclinical studies was
confirmed to reduce cholesterol significantly in human clinical trials. The
higher rate of *bsh* activity was presumed to be responsible for the activity and
there is considerable evidence supporting the claim that a threshold level of
25   *bsh* activity is required for free cells delivered at high daily doses. It appears
that organisms that degrade >50μmol GDCA/gram/hour and >2μmol
TDCA/gram/hour measured over 1 hour and 5 hours, respectively and that
are delivered in the quantity of 10^6-10^{12} organisms are sufficient for
cholesterol lowering. For this reason, the even more *bsh* active *Lactobacillus
30   reuteri* (NCIMB 701089) are predicted to reduce cholesterol and perform well
in preclinical and clinical studies. In addition, b*sh* activity was measured with
the same HPLC assay for TDCA and GDCA; however, the average rate was

calculated over a 30 minute period. This provides an accurate determination of the true enzymatic rate, as the xDCA deconjugation curve is more linear between 0 and 30 minutes and the reaction is not limited by the low availability of substrate seen at later time points.

5   [00115]   Materials and Methods are the same as for the HPLC assay section described above.

Efficacy and Safety of highly _bsh_ active microencapsulated _Lactobacillus reuteri_ (NCIMB 701359) in Syrian Golden F1B Hamsters (Table 3)

10

Results

[00116]   Table 3 shows the lipid endpoint percent change from control values for F1B hamsters induced to be hypercholesterolemic and then treated

15   by gavages with either microencapsulated or free _Lactobacillus reuteri_ (NCIMB 701359). While there are similar reductions of total cholesterol between groups, there is increased LDL-C reduction, less HDL-C reduction, and improved triglyceride removal with the free organism in this model. This results in a dramatic difference in overall atherogenic index (AI) and shows

20   that while there are advantages to microencapsulation in terms of improved delivery and survival, there are also advantages to delivering highly _bsh_ active free organism in that an improved lipid profile may be obtained.

Materials and Methods

25   [00117]   A total of 38, 7-8 week old Bio F1B Syrian golden hamsters were purchased from Biobreeders, USA. Upon delivery the animals were given a week of acclimatization. Animals were housed one per cage in a temperature and humidity controlled room with a twelve hour inverted light-dark cycle with food and water available ad libidum. After acclimatization the

30   animals were weighed and blood was collected to assess baseline lipids as described below.

[00118]   Hypercholesterolemia was induced by five weeks of feeding with Test diet containing 0.05% cholesterol. Food consumption and weight gain

was monitored weekly. Blood was collected after four and five weeks of induction and analyzed for lipid levels. Additionally, safety markers were assessed prior to initiation of treatment (five weeks of induction). Fecal samples were collected on the final day of the induction period and assessed

5   for bile acid content.

[00119] Any animals that did not show hypercholesterolemia after five weeks of feeding with the hypercholesterolemia inducing diet were excluded (5 animals). The remaining animals were then assigned to one of three treatment groups by block randomization based on serum LDL levels and

10   adjusted to equalize the average weight of each group (n=11 for each group). After randomization, animals were administered treatment by gavage for six weeks.

[00120] During the treatment period feeding with the hypercholesterolemic test diet continued. Food intake and weight was

15   monitored on a weekly basis and blood lipid levels were measured on a biweekly basis. After six weeks of treatment endpoint fecal samples were collected and the animals were sacrificed by carbon dioxide. Blood was collected by cardiac puncture for analysis of endpoint lipids, safety markers and hematology. During necropsy, livers from representative animals of each

20   group were collected for histological analysis.

Efficacy and Safety of less *bsh* active microencapsulated *Lactobacillus reuteri* (LabMet) containing yogurt in lowering lipids (Table 4)

Results

25

[00121] Table 4 shows the percent change in fasting lipids of mildly hypercholesterolemic subjects in response to consumption of microencapsulated *Lactobacillus reuteri* (LabMet) over a 6-week treatment period. The result shows some change in serum cholesterol over control with

30   reduced levels of serum triglycerides.

Materials and Methods

[00122]   This study examined the efficacy of the less *bsh* active microencapsulated *Lactobacillus reuteri (LabMet)* containing probiotic yogurt compositions on health parameters related to degenerative disease in humans.

[00123]   It was anticipated that consumption of the probiotic product would induce favourable shifts in risk markers for several debilitating diseases of increasing age and that probiotic consumption would favourably alter lipids *versus* conventional treatments in hyperlipidemic individuals.

[00124]   The study design was a multi phase/washout, randomized, double blinded, controlled trial in which subjects received control yogurts or test yogurts over a 6 week period followed by a 6 week washout prior to following phases.

[00125]   A total of 30 healthy males and females, aged 18-60 yr, were randomized with plasma LDL-C 130-260 mg/dl, TG levels below 400 mg/dl, and a body mass index (BMI) of 22-32 kg/m^2.

[00126]   Metabolic diets of precisely known composition were provided to subjects under strict supervision at a clinical research facility. Diets were nutritionally adequate and provided 100% of energy requirements. In addition, the subjects received one test yogurt per day for the treatment period.

[00127]   Twelve-hour fasting blood samples were collected at the beginning and end of each of the phases of the trial. Blood samples obtained on day 1 and 2 were used to measure baseline values for different study measurements, whereas blood samples obtained on the last days were used to measure final values for serum lipid levels.

Efficacy and Safety of highly *bsh* active microencapsulated *Lactobacillus reuteri* (NCIMB 701359) containing yogurt in lowering lipids (Tables 5 and 6)

<u>Results</u>

[00128]   Tables 5 and 6 show the percent change in fasting lipids in hypercholesterolemic subjects in response to consumption of the more highly *bsh* active *Lactobacillus reuteri* (NCIMB 701359) over a 6-week treatment period. The result shows significant decreases in total and LDL cholesterol as well as in ApoB as would be predicted from the *bsh* activity values (26.4 µmol GDCA/gram/hour and 182.6 µmol TDCA/gram/hour). This randomized, double blinded, parallel arm study was well powered and well controlled; thus, any cholesterol lowering was due to the probiotic ingredient.

<u>Materials and Methods</u>

[00129]   This study examined the efficacy of the highly *bsh* active microencapsulated *Lactobacillus reuteri (NCIMB 701359)* containing probiotic yogurt formulations on health parameters related to degenerative disease in humans.

[00130]   The objective was to evaluate the effects of consumption of a yogurt formulation containing alginate poly-L-lysine alginate (APA) microencapsulated highly bile salt hydrolase *(bsh)*-active *Lactobacillus reuteri (NCIMB 701359)* on plasma lipids levels in hypercholesterolemic adults and access the relative changes of plasma LDL-cholesterol concentration in hypercholesterolemic adults after 6 weeks of product consumption versus control product.

[00131]   The experiment involved a multi-centric (5 centers) double-blinded randomized parallel-arm placebo controlled trial. Subjects were instructed to follow Health Canada dietary recommendations, which are intended to help reduce risks of obesity and heart disease. The study duration was 10 weeks including 2-week wash-out, 2-week run-in periods and a 6-week treatment period. During the wash-out period, subjects followed dietary instructions only. During the run-in period, placebo was taken. The treatment or placebo product was taken during the whole treatment period.

[00132]   A total of 120 healthy males and females between the ages 18-74, with LDL-Cholesterol >3.4 mmol/L, and TG levels <4.0 mmol/L, and a BMI range 22-32 kg/m^2 were randomized and 109 subjects were evaluated as-per-protocol.

5   [00133]   Twelve-hour fasting blood samples were collected at the beginning and end of the treatment period. Blood samples obtained on day 1 and 2 were used to measure baseline values for different study measurements, whereas blood samples obtained on the last days were used to measure final values for serum lipid levels.

10   **Identifying highly *bsh* active bacteria (Figure 7 and Table 7)**

Results

[00134]   The MRS-TDCA-plate precipitation zone screening assay show that, as a method for determining *bsh* activity TDCA, plating is crude and may not be sufficient for identifying highly *bsh* active candidate cholesterol
15   lowering probiotics. As can be seen, the more *bsh* active cultures have larger zones of precipitation; however, in cases in which the DCA precipitate is more densely concentrated, screening with TDCA plates alone does not identify the most highly *bsh* active organisms as potential candidates. For this reason an assay with more resolution, such as an HPLC assay, which quantifies the *bsh*
20   activity for glyco- and tauro- conjugates may be required.

[00135]   Figure 7 shows three *bsh* active *Lactobacillus reuteri* grown anaerobically on an MRS-TDCA plate for 24 hours. The size of precipitation zone and density of precipitation are clearly different for each organism.

[00136]   Table 7 shows the diameter of precipitation (mm) of deoxycholic
25   acid (DCA) as measured on MRS-TDCA plates after 24 hours of anaerobic growth by filter discs impregnated with culture. The values are averages of triplicate measurements on 3 MRS-TDCA agar plates. Comparison of the results from Table 7 with those from Table 2 shows that the zone of precipitation assay does not always differentiate highly *bsh* active bacteria as

it is clear that LR050 has a larger precipitation zone than LR052 but HPLC shows that in fact LR052 is more highly *bsh* active.

Materials and Methods

[00137] *Lactobacillus* cultures were grown overnight in MRS media at 37°C. 500µl of each culture was centrifuged in a pre-weighed Eppendorf™. The supernatant was removed and the pellets were weighed. The pellets were resuspended with MRS to get same 1:10 w/v ratio and 10µl of each culture was added to filter disks in duplicate on different MRS-TDCA agar plates. The plates were incubated anaerobically at 37°C and measurements were made.

Deconjugation of TDCA and GDCA by free *Lactobacillus reuteri* strains (Figures 8 and 9)

Results

[00138] Figures 8 and 9 show the deconjugation of taurodeoxycholic acid and glycodeoxycholic acid, as measured by HPLC, in an *in-vitro* assay using 0.2 grams of free *Lactobacillus reuteri* (ATCC 55148, ATCC 55739, NCIMB 701359, NCIMB 701089, NCIMB 702655, LMG 18238, LMG 22877, LMG 22878, LMG 22879, CCUG 32271, CCUG 32305, CCUG 37470, CCUG 44001, CCUG 44144, CCUG 47824) over time. The results show that strains ATCC 55148, NCIMB 701359, NCIMB 701089, NCIMB 702655, LMG 18238, CCUG 32271, CCUG 32305, CCUG 37470, CCUG 44001 and CCUG 44144 have particularly high *bsh* activity as measured by HPLC.

Materials and Methods

Bacteria and culture conditions

[00139] The *Lactobacillus reuteri* strains were cultivated in sterile de Man, Rogosa, Sharpe (MRS) broth at 37°C for 20 hours. Grown cultures were isolated by centrifugation and collected bacteria cells were used in the following BSH assay.

Measurement of BSH activity

[00140]   To measure the BSH activity, collected bacteria were added into 100% MRS supplemented with a combination of sodium glycodeoxycholate and sodium taurodeoxycholate both at 5mM (0.2g bacteria cells/20ml MRS supplemented with GDCA and TDCA). Bacteria were then incubated in the reaction broth anaerobically at 37°C with minimal shaking (100rpm), and supernatant was sampled at intervals of 2.5 hours and processed to determine the conjugated bile salt concentrations. Briefly, 500μl samples were acidified with 5ul of 6N HCl after removing bacteria cells by centrifugation at 10000g for 3min. The supernatants were then supplemented with 500μl of Methanol containing 4mM GCA (glycocholic acid) as internal standard. The samples were vortexed for 10min and centrifuged at 1000g for 15min. The samples were filtered through 0.22μM filter before being analyzed by HPLC. HPLC analysis of bile salts followed the procedure described by Jones et al. 2003.

Bile salt HPLC analysis

[00141]   HPLC analysis of bile salts followed the procedure described by Jones et al. 2003. The analysis was performed on a reverse-phase C-18 column: LiChrosorb RP-18, 5μm, 250 x 4.6mm. The HPLC system comprised of two ProStar 210 solvent delivery modules, a ProStar 320 UV-VIS detector, a ProStar 410 autosampler and Galaxie Chromatography Data system (version 1.9.3.2). A mixture of methanol and 50mM sodium acetate buffer adjusted to pH4.3 with o-phosphoric acid (70:30, v/v) was applied as mobile phase with a flow rate of 1.0ml/min. The detector was set at 210nm and all the measurements were performed at room temperature.

Example 2 – High BSH activity

General Materials and Methods

[00142]   *Bacterial seeding and growth:* the surfaces of frozen glycerol bacterial stocks were scratched with a sterile wooden stick to streak MRS agar plates. After an overnight incubation at 37°C under anaerobic conditions,

a single colony of *L. reuteri* NCIMB 701359 was picked with a metallic loop under sterile conditions and transferred into a tube containing 10 mL of MRS. The cultures were incubated overnight at 37°C for experimental use.

[00143]   *Microencapsulation of L. reuteri NCIMB 701359:* microcapsules were prepared with an 8% cell load and a 1.75% alginate concentration using a 200µm sized nozzle. The coating process was the following: first, alginate beads were drained of $CaCl_2$; second, alginate beads were washed in 0.85% (w/v) NaCl for 10 minutes; third, alginate beads were coated in 0.1% (w/v) ε-PLL for 20 minutes; fourth, alginate-PLL microcapsules were washed with 0.85% (w/v) NaCl for 10 minutes; fifth, alginate-PLL microcapsules were coated with 0.1% (w/v) alginate for 20 minutes; and finally the alginate-PLL-alginate microcapsules were washed with 0.85% (w/v) NaCl for 10 minutes.

[00144]   *BSH assay for frozen and lyophilized free cells:* frozen free cells were thawed, centrifuged, and washed and were added (0.05g) to 20 ml of MRS containing 5 mM TDCA and 5 mM GDCA. Lyophilized free cells were added (0.15g) to 20 ml of MRS containing 5 mM TDCA and 5 mM GDCA. Samples were taken out after 30 min and were analyzed with HPLC. Controls were the medium alone and freshly prepared microcapsules grown in MRS.

[00145]   *BSH assay for microcapsules:*   thawed and washed microcapsules (sample between 0.3g and 2.5g, depending on relative activity) were added to 20 ml of MRS containing 5 mM TDCA and 5 mM GDCA. Samples were taken out after 30 min and were analyzed with HPLC. Controls were the medium alone and freshly prepared microcapsules grown in MRS. 0.3 g of microcapsules contained 0.03 g pellet of free cells.

[00146]   *HPLC assay for BSH activity:*   analyses were performed on a reverse-phase C-18 column (LiChrosorb RP-18 250mn x 4.6mm, 5µm) at a flow rate of 1.0ml/min. The mobile phase was a mixture of methanol and 50mM sodium acetate buffer (pH 4.3 adjusted with o-phosphoric acid) in 70:30 ratio and detection was measured at 210nm. The *bsh* activity was

evaluated by the amount of deconjugated GDCA and TDCA in samples per hour per gram microcapsules.

A. Improved bsh activity through fermentation (Tables 8-10)

Materials and Methods

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[00147]   *Increase of bsh activity based on carbon and nitrogen sources:* *Lactobacillus reuteri* NCIMB 701359 cells were grown by the general method described above. 1% of *L. reuteri* NCIMB 701359 was inoculated into modified MRS medium with different sources of carbon and nitrogen.

10   Inoculated cultures were incubated at 37°C for 24h. Following incubation time, 0.05g or 0.1g of cell pellet was added to 20ml of MRS containing 5mM TDCA and 5mM GDCA. Samples were removed after 0.5h and 1.5h, and were analyzed with HPLC. MRS was used as the control growth media. Analyses were performed by HPLC as described in the general methods

15   above.

[00148]   *Increase of BSH activity based on pH and harvest time:* *Lactobacillus reuteri* NCIMB 701359 cells were grown by the general method described above. 1% of *L. reuteri* NCIMB 701359 was inoculated into growth medium with carbon and nitrogen sources and pH adjusted to pH 5, 6 and 6.8

20   by adding NaOH or HCl. Inoculated cultures at different pH conditions were incubated at 37°C for harvest times of 12h to 48h. Following incubation time, 0.05g or 0.1g of cell pellet was added to 20ml of MRS containing 5mM TDCA and 5mM GDCA. Samples were removed after 0.5h and 1.5h, and were analyzed with HPLC as described in the general methods above. Controls

25   were grown in non-modified MRS medium.

Results

[00149]   The most favourable results from a carbon source for *Lactobacillus reuteri* NCIMB 701359 fermented in modified MRS, increasing

30   *bsh* activity and yield, was maltose. GDCA and TDCA were deconjugated at rates of 2,253 (μmol/g/h) and 173 (μmol/g/h) respectively and a yield of 0.015

g/ml was maintained (Table 8). The most favourable results from nitrogen sources for *Lactobacillus reuteri* NCIMB 701359 fermented with this carbon source (maltose) and looking for increased GDCA (μmol/g/h) deconjugation, TDCA (μmol/g/h) deconjugation (HPLC), and yield (g/ml) was a combination

5   of peptone No. 3, yeast extract, malt extract, and cysteine. GDCA and TDCA were deconjugated at rates of 21,185 (μmol/g/h) and 2,323 (μmol/g/h) respectively and a yield of 0.013 g/ml was maintained (Table 9). The most favourable results for harvest time and initial pH for *Lactobacillus reuteri* NCIMB 701359 fermented in either the above-noted media (maltose +

10   peptone No. 3 + yeast extract + malt extract + cysteine) or MRS, looking for increases for GDCA (μmol/g/h) deconjugation, TDCA (μmol/g/h) deconjugation (HPLC), and yield (g/ml) was a pH of 5 at a harvest time of 12-20 hours (Table 10).

[00150]   These results show that conditions including carbon and

15   nitrogen sources, pH, and harvest time achieve high *bsh* activity and cell yield with *Lactobacillus reuteri* NCIMB 701359; these conditions produce a highly active product, which maintains high *bsh* levels over a shelf life in supplement or functional food format, and is cost effective to produce commercially. This data shows that probiotic cells with *bsh* producing machinery are readily

20   fermented with defined media and conditions to achieve therapeutic levels of enzymatic activity and commercially viable levels of biomass. This process is useful to make *bsh* active probiotics commercially viable for lipid lowering and other applications.

B. Improved bsh activity by lyophilization (Table 11 and Figure 10)

25   Materials and Methods

[00151]   *Lyophilization storage conditions for high BSH activity.* Microcapsules containing *Lactobacillus reuteri* NCIMB 701359 were prepared by the general method described above. Microcapsules were stored in a 7:3

30   ratio of microcapsules to lyoprotectant solution as follows: 1M trehalose, 10% maltodextrin, 1% inulin, 10% maltodextrin and 0.33% yeast extract, 1M

trehalose and 0.33% yeast extract, 1% inulin and 0.33% yeast extract, 10% maltodextrin and 1% casein hydrolysate, and 10% skim milk. Slurries containing microcapsules and lyoprotectant solution were lyophilized and stored at 4°C for 0, 1, 2, 3, 4, 5 and 6 weeks in individual aliquots. At each timepoint, duplicate samples of lyophilized microcapsules containing each lyoprotectant were rehydrated with saline. The *bsh* assay for microcapsules described above was used to prepare samples for HPLC analysis by the general method above.

[00152]   Free *Lactobacillus reuteri* NCIMB 701359 was prepared by the general method described above. Lyoprotectants were added to the free cell slurry at a final concentration of: 10% maltodextrin and 0.33% yeast extract. Slurries were lyophilized and stored at 4°C or RT for 0, 1, 2, and 3 months in individual aliquots. At each time point, duplicate samples of lyophilized cells containing each lyoprotectant were rehydrated with saline. The *bsh* assay for lyophilized material described above was used to prepare samples for HPLC analysis by the general method above.

Results

[00153]   The most favourable results from a lyoprotectant for *Lactobacillus reuteri* NCIMB 701359, increasing *bsh* activity were 10% maltodextrin and 0.33% yeast extract, 1% inulin and 0.33% yeast extract, and 1% inulin (Table 11).

[00154]   These results show that lyoprotectants maintain high *bsh* activity for *Lactobacillus reuteri* NCIMB 701359; the lyoprotectant maintains microcapsule morphology upon rehydration (Figure 10), maintains high *bsh* levels over a shelf life in supplement or functional food format, and is cost effective to produce commercially. This data shows that free or microencapsulated probiotic cells with *bsh* producing machinery are readily lyophilized with lyoprotectants to maintain therapeutic levels of enzymatic activity. This process is useful to make lyophilized *bsh* active probiotics commercially viable for lipid lowering and other applications.

C. Improved bsh activity by liquid storage (Table 12)

<u>Materials and Methods</u>

[00155]   *Liquid storage conditions for BSH activity:* Microcapsules

5   containing *Lactobacillus reuteri* NCIMB 701359 were prepared by the general method described above. Microcapsules were stored in a 1:1 ratio of microcapsules to preservative solutions as follows: 5% growth media, 10% growth media, 20% growth media, 10% MRS media, yogurt, culture supernatant, 1% maltose, 0.85% saline, 1% malt extract, 1% inulin, 10%

10   sorbitol, 0.33% yeast extract, 1% inulin and 0.33% yeast extract and 1M fructose.   The resulting microcapsules were stored in preservative solutions at 4 degrees for short-term storage of 4 days in individual aliquots. Duplicate samples of microcapsules containing each liquid preservative solution were removed from storage medium and washed with saline. The *bsh* assay for

15   microcapsules described above was used to prepare samples for HPLC analysis by the general method above.

<u>Results</u>

[00156]   The most favourable results from a liquid storage condition for

20   *Lactobacillus reuteri* NCIMB 701359, considering *bsh* activity after 4 days liquid storage, were yogurt (1:1), 5% growth media (1:1), 10% growth media, 20% growth media (1:1), culture supernatant (1:1), and 10% MRS (1:1) (Table 12).

[00157]   These results show liquid storage conditions maintain high *bsh*

25   activity with *Lactobacillus reuteri* NCIMB 701359; these conditions result in production of a highly active product, which maintains high *bsh* levels during temporary liquid storage, and which is cost effective to use in the commercial process. This data shows that specific storage conditions are beneficial for storage in liquid media to achieve therapeutic levels of enzymatic activity.

30   This process is useful in maintaining *bsh* activity during short term storage and makes the production process commercially viable for producing *bsh*

active probiotics for lipid lowering and other metabolic disease therapeutic applications.

D. Improved bsh activity through flash freezing (Table 13)

Materials and Methods

[00158]   *Flash freezing storage conditions for bsh activity:* Microcapsules containing *Lactobacillus reuteri* NCIMB 701359 were prepared by the general method above. Microcapsules were stored in a 1:1 ratio of microcapsules to cryoprotectant solution as follows: 1M trehalose, 1M fructose, 1% inulin, 1M maltose, 1M lactose, 1M sucrose, 10% PEG 8000, 0.85% saline, 10% skim milk, 10% starch or 10% fructooligosaccharides.   Slurries containing microcapsules and cryoprotectant solution were slowly passed through a sterile syringe to form spherical droplets which were suspended in liquid nitrogen. The resulting flash frozen pellets were isolated from liquid nitrogen and stored at -80°C in individual aliquots. Immediately and after 3 weeks storage, duplicate samples of pelleted microcapsules containing each cryoprotectant solution were removed from storage medium and washed with saline. The *bsh* assay for microcapsules described above was used to prepare samples for HPLC analysis described in the general methods above.

[00159]   Free *Lactobacillus reuteri* NCIMB 701359 were prepared by the general method above. Free cell pellet was re-suspended in spent media at a 1:1 ratio of cells to spent media. The resulting cell slurry was slowly passed through a sterile syringe to form spherical droplets which were suspended in liquid nitrogen. The resulting flash frozen pellets were isolated from the liquid nitrogen and stored at -80°C. Duplicate samples of pelleted cells containing spent media cryoprotectant solution were removed from storage medium. The *bsh* assay for free cells described above was used to prepare samples for HPLC analysis described in the general methods above.

Results

[00160]   The most favourable results from cryopreservative media for *Lactobacillus reuteri* NCIMB 701359, flash frozen in liquid nitrogen, when considering capsule morphology and/or % remaining *bsh* activity immediately post flash freezing and at 3 weeks, included 1:1 free or microencapsulated bacteria to cryopreservative solution with maltodextrin and yeast extract, inulin, trehalose, fructose, sucrose, lactose, maltose and spent media at indicated concentrations (Table 13).

[00161]   These results show that cryopreservation conditions when flash freezing in liquid nitrogen for frozen storage achieve high *bsh* activity and good microcapsule morphology with encapsulated *Lactobacillus reuteri* NCIMB 701359; allowing production of a product with high *bsh* levels over an extended shelf life in supplement or functional food format, and which is cost effective for commercial production. This data shows that free or microencapsulated probiotic cells with high *bsh* activity are readily prepared under several cryopreservative conditions and flash frozen in liquid nitrogen achieves therapeutic levels of enzymatic activity and excellent microcapsule morphology. This process is useful to make *bsh* active probiotics commercially viable for lipid lowering and other applications.

[00162]   The scope of the claims should not be limited by the preferred embodiments and examples, but should be given the broadest interpretation consistent with the description as a whole.

[00163]

Table 1: Two commonly used equations for determining atherogenic index (AI) representing atherogenic risk and used as prognostic indicators patients at risk of developing atherosclerosis

Atherogenic Index (AI) = Log(Triglycerides/HDL-Cholesterol)

Atherogenic Index (AI) = (TC-HDL-Cholesterol)/(HDL-Cholesterol)

Table 2: BSH activity of free *Lactobacillus reuteri* as measured by HPLC is shown in μmol DCA per gram per hour (μmol DCA/g/hr). Taurodeoxycholic acid = TDCA, Glycodeoxycholic acid = GDCA, Deoxycholic acid = DCA.

	TDCA (μmol DCA/g/hr) Average rate (0-5h)	GDCA (μmol DCA/g/hr) Average rate (0-1h)	TDCA (μmol DCA/g/hr) Average rate (0-0.5h)	GDCA (μmol DCA/g/hr) Average rate (0-0.5h)
Free *L. reuteri* (LabMet)	1.5	47.8	7.2	65.3
Free *L. reuteri* (NCIMB 701359)	26.4	182.6	44.0	372.0
Free *L. reuteri* (NCIMB 701089)	77.5	424.0	93.0	805.0

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Table 3: Lipid endpoint values (% change from control) are shown for F1B hamsters induced to be hypercholesterolemic (0.5% dietary cholesterol) (5 weeks) and then treated (6 weeks) by gavages with either microencapsulated or free *Lactobacillus reuteri* (NCIMB 701359) (n=33).

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	LDL-C (%)	Total Cholesterol (%)	HDL-C (%)	TG (%)	AI (%)
Microencapsulated *L. reuteri* (NCIMB 701359)	-23.60	-16.83	-11.11	-5.05	-11.27
Free *L. reuteri* (NCIMB 701359)	-27.43	-16.94	-6.26	-11.63	-18.53

Table 4: Percent change in fasting lipid levels (over control) in mildly
10 hypercholesterolemic human subjects in response to consumption of the less *bsh* active APA microencapsulated *Lactobacillus reuteri* (LabMet) over a 6 week treatment period (n=30, as-per-protocol).

	LDL-C (%)	Total Cholesterol (%)	HDL-C (%)	TG (%)
Microencapsulated *L. reuteri* (LabMet)	-0.07	-3.63	-2.37	-12.51

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Table 5: Fasting lipid levels are given, as percent difference from palcebo, in mildly hypercholesterolemic human subjects in response to consumption of highly *bsh* active microencapsulated *Lactobacillus reuteri* (NCIMB 701359) over a 6 week treatment period (n=109, as-per-protocol).

Microencapsulated *L. reuteri* (NCIMB 701359)				
Lipid Parameter	% Change (3 wks)	P-Value	% Change (6 wks)	P-Value
TC	-2.89	0.2321	-4.86	0.0501
LDL-C	-3.83	0.1660	-9.23	0.0061
HDL-C	+0.14	0.9697	+0.49	0.9101
TG	-23.69	0.0275	+21.05	0.0869
ApoB-100	-3.84	0.2056	-6.66	0.0405

Table 6: Fasting lipid levels are given, as a percent change from placebo, in hypercholesterolemic human subjects, at high risk and very high risk, in response to consumption of the highly *bsh* active APA microencapsulated *Lactobacillus reuteri* (NCIMB 701359) over a 6 week treatment period (n=65).

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Microencapsulated *L. reuteri* (NCIMB 701359)		
Lipid Parameter	% Change (6 wks)	P-Value
TC	-5.53	0.101
LDL-C	-10.22	0.024
HDL-C	-0.19	0.97
TG	+11.09	0.54
ApoB-100	-10.69	0.0082

Table 7: Diameter of precipitation (mm) of deoxycholic acid (DCA) as
10   measured on MRS-TDCA plates after 24 hours of anaerobic growth by filter discs impregnated with culture. The values are averages of triplicate measurements on 3 MRS-TDCA agar plates.

	Lr010	Lr050	Lr052
2 days	12.6	17.3	17
3 days	13	20	18
4 days	13.2	20.3	18.2

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Table 8: Experimental results for determining the carbon source for increasing GDCA (μmol/g/h) deconjugation, TDCA (μmol/g/h) deconjugation (HPLC), and yield (g/ml) for *Lactobacillus reuteri* NCIMB 701359 fermented in modified MRS (yeast extract + beef extract + peptone No. 3) while varying the carbon source.

	Yeast extract + Beef extract + Peptone No. 3		
	GDCA (μmol/g/h)	TDCA (μmol/g/h)	Yield (g/ml)
Sucrose	950	117	0.012
Xylose	225	75	0.008
Inulin + Glucose	2050	825	0.008
Lactose	145	18	0.011
Dextrin	1640	180	0.01
Sorbitol + Glucose	1889	689	0.009
Glucose (MRS)	957	57	0.014
Maltose	**2253**	**173**	**0.015**

Table 9: Experimental results for determining the nitrogen sources increasing for GDCA (μmol/g/h) deconjugation, TDCA (μmol/g/h) deconjugation (HPLC), and yield (g/ml) for *Lactobacillus reuteri* NCIMB 701359 fermented in the carbon source (maltose) while varying sources of nitrogen.

Malltose (Carbon source) +	Yeast extract + Beef extract			Casein acid hydrolysate + Malt extract			Yeast extract + **Malt extract**		
	GDCA (μmol/ g/h)	TDCA (μmol/ g/h)	Yield (g/ml)	GDCA (μmol/ g/h)	TDCA (μmol/ g/h)	Yield (g/ml)	GDCA (μmol/ g/h)	TDCA (μmol/ g/h)	Yield (g/ml)
Peptone No. 3	2253	173	0.015	2156	167	0.018	3322	933	0.018
Tryptone				843	514	0.014	1013	67	0.015
Fish peptone				680	80	0.005	1067	107	0.015
Soy peptone				367	67	0.012	689	78	0.018
Peptone No. 3 + Cysteine							**21185**	**2323**	**0.013**
Tryptone + Cysteine	6271	1271	0.014						
Proteose peptone + Cysteine							2786	671	0.014
Casein peptone + Cysteine	6271	1414	0.014						
Fish peptone + Cysteine	8415	1446	0.013						
Soy peptone + Cysteine	1779	179	0.019						

Table 10: Experimental results for determining the harvest time and initial pH increasing for GDCA (µmol/g/h) deconjugation, TDCA (µmol/g/h) deconjugation (HPLC), and yield (g/ml) for *Lactobacillus reuteri* NCIMB 701359 fermented with either growth media (maltose + peptone No. 3 + yeast extract + malt extract + cysteine) or MRS media at various initial pH values and harvest times.

	Growth media pH 5			Growth media pH 6			Growth media pH 6.8			MRS media pH 6.8		
	GDCA (µmol/g/h)	TDCA (µmol/g/h)	OD (600 nm)	GDCA (µmol/g/h)	TDCA (µmol/g/h)	OD (600 nm)	GDCA (µmol/g/h)	TDCA (µmol/g/h)	OD (600 nm)	GDCA (µmol/g/h)	TDCA (µmol/g/h)	OD (600 nm)
12h	19350	3475	3.12	15775	3063	3.09	18752	2288	2.69	201	0	1.20
16h	19078	3772	3.18	16163	3547	3.06	18173	2448	2.60	168	0	1.62
20h	17750	3463	3.21	18013	3001	3.02	15765	2175	2.72	186	0	1.57
24h	19187	3711	3.10	18860	3048	2.99	16362	2362	2.66	483	33	1.63
36h										961	80	1.82
48h										1704	339	1.77

Table 11: Experimental results for lyoprotectants, at a 7:3 microcapsule to lyoprotectant solution ratio, and % of original *bsh* activity for *Lactobacillus reuteri* NCIMB 701359 at averaged values for 2 and 3 as well as 5 and 6 weeks.

Lyoprotectant solutions shown to retain microcapsule morphology after lyophilization and rehydration	% of Original activity after lyophilization and storage at 4°C	
	Weeks 2 and 3	Weeks 5 and 6
Free cells: (final concentration: 10% maltodextrin + 0.33% yeast extract)		100% (1, 2, 3 months at 4°C and RT)
Microcapsules: 1M Trehalose (7:3) (final conc. 0.3M)	70.4%	67.4%
Microcapsules: 10% Maltodextrin (7:3) (final conc. 3%)	<25%	<25%
Microcapsules: 1% Inulin (7:3) (final conc. 0.3%)	83.1%	85.3%
Microcapsules: 10% Maltodextrin + 0.33% Yeast extract (7:3) (final conc. 3% + 0.1%)	100%	100%
Microcapsules: 1M Trehalose + 0.33% Yeast extract (7:3) (final conc. 0.3M + 0.1%)	69.2%	65.2%
Microcapsules: 1% Inulin + 0.33% Yeast extract (7:3) (final conc. 0.3% + 0.1%)	75.2%	80.5%
Microcapsules: 10% Maltodextrin + 1% Casein hydrolysate (7:3) (final conc. 3% + 0.3%)	<25%	<25%

Table 12: Experimental results for liquid storage conditions based on *bsh* activity (% original) at 4 days.

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Storage condition	% of original *bsh* activity after short term storage (4 days) at 4°C
Microcapsules: Yogurt (3:97)	87.1% (1 wks)
Microcapsules: Yogurt (3:97)	54.6% (4 wks)
Microcapsules: Yogurt (3:97)	53.5% (6wks)
Microcapsules: Yogurt (1:1)	96.0%
Microcapsules: 5% Growth media (1:1) (final conc. 2.5%)	92.6%
Microcapsules: 10% Growth media (1:1) (final conc. 5%)	92.4%
Microcapsules: 20% Growth media (1:1) (final conc. 10%)	88.2%
Microcapsules: 100% Culture supernatant (1:1) (final conc. 50%)	87.0%
Microcapsules: 10% MRS (1:1) (final conc. 5%)	81.5%
Microcapsules: 1% Maltose (1:1) (final conc. 0.5%)	22.0%
Microcapsules: 1% Malt extract (1:1) (final conc. 0.5%)	<15%
Microcapsules: 1% Inulin (1:1) (final conc. 0.5%)	<15%
Microcapsules: 10% Sorbitol (1:1) (final conc. 5%)	<15%
Microcapsules: 0.33% Yeast extract (1:1) (final conc. 0.165%)	<15%
Microcapsules: 1% Inulin + 0.33% Yeast extract (1:1) (final conc. 0.5% + 0.165%)	<15%
Microcapsules: 1M Fructose (1:1) (final conc. 0.5M)	<15%
Microcapsules: No liquid	<15%
Microcapsules: 0.85% Saline (1:1) (final conc. 0.425%)	<15%

Table 13: Experimental results for cryopreservative solution, when flash freezing and storing at -80°C, for *Lactobacillus reuteri* NCIMB 701359 microcapsules determined by microscopy for microcapsule morphology (% original quality) and HPLC for *bsh* activity (% original activity) data immediately after flash freezing in liquid nitrogen and after 3 weeks storage at -80°C.

Cryopreservation Conditions	% of Original Quality	% of Original BSH activity	
	Microcapsule Morphology	Post-Flash freeze and thaw process	Post-Flash freeze + storage (3 weeks)
Free cells: cells + 100% spent media (1:1) (final conc. 50%)		100%	
Microcapsules: 1% maltodextrin + 0.23% yeast extract (1:1) (final conc. 0.5% + 0.115%)		77.8%	
Microcapsules: 2% maltodextrin + 0.23% yeast extract (1:1) (final conc. 1% + 0.115%)		100%	
Microcapsules: 10% maltodextrin + 0.33% yeast extract (1:1) (final conc. 5% + 0.165%)		100%	
Microcapsules: 1% Inulin (1:1) (final conc. 0.5%)	100%	98.5%	90.3%
Microcapsules: 1M Trehalose (1:1) (final conc. 0.5M)	98.3%	100%	88.3%
Microcapsules: 1M Fructose (1:1) (final conc. 0.5M)	98.2%	<50%	<50%
Microcapsules: 1M Sucrose (1:1) (final conc. 0.5M)	97.1%	97.7%	93.3%
Microcapsules: 1M Lactose (1:1) (final conc. 0.5M)	95.0%	100%	92.3%
Microcapsules: 1M Maltose (1:1) (final conc. 0.5M)	90.0%	94.2%	92.1%
Microcapsules: 10% FOS (1:1) (final conc. 5%)	70.0%	Not tested due to morphology	
Microcapsules: 10% PEG8000 (1:1) (final conc. 5%)	28.1%	Not tested due to morphology	
Microcapsules: 10% Skim milk (1:1) (final conc. 5%)	25.5%	Not tested due to morphology	
Microcapsules: 10% Starch (1:1) (final conc. 5%)	24.4%	Not tested due to morphology	
Microcapsules: 0.85% Saline (1:1) (final conc. 0.425%)	12.0%	Not tested due to morphology	

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CLAIMS:

1. An oral composition comprising a highly bile salt hydrolase (*bsh*) active bacteria; wherein the highly *bsh* active bacteria have a high *bsh* activity that degrades >2000 µmol Glycodeoxycholic acid (GDCA)/gram wet cell bacterial
5   pellet/hour and >500 µmol taurodeoxycholic acid (TDCA)/gram wet cell bacterial pellet/hour when measured over 30 minutes, wherein said high *bsh* activity is stabilized for therapeutic delivery and wherein said high *bsh* activity results from their fermentation conditions, wherein the oral composition further comprises a carrier, and wherein the highly *bsh* active bacteria is *Lactobacillus*.

10   2. The oral composition of claim 1, wherein the highly *bsh* active bacteria degrades >15000 µmol GDCA/gram wet cell bacterial pellet/hour and >2000 µmol TDCA/gram wet cell bacterial pellet/hour when measured over 30 minutes.

3. The oral composition of claim 1, wherein the bacteria is live.

15   4. The oral composition of any one of claims 1 to 3, wherein the bacteria is free.

5. The oral composition of any one of claims 1 to 4, wherein the *Lactobacillus* is *Lactobacillus reuteri*.

6. The oral composition of claim 5, wherein the *Lactobacillus reuteri* is
20   *Lactobacillus reuteri* (NCIMB 701359), *Lactobacillus reuteri* (NCIMB 701089), *Lactobacillus reuteri* (ATCC 55148), *Lactobacillus reuteri* (ATCC 23272), *Lactobacillus reuteri* (NCIMB 702655), *Lactobacillus reuteri* (LMG 18238), *Lactobacillus reuteri* (CCUG 32271), *Lactobacillus reuteri* (CCUG 32305), *Lactobacillus reuteri* (CCUG 37470), *Lactobacillus reuteri* (CCUG 44001) or
25   *Lactobacillus reuteri* (CCUG 44144).

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7. The oral composition of any one of claims 1-6, wherein the concentration of bacteria is 10^6 - 10^{12} CFU/gram wet cell bacterial pellet.

8. The oral composition of any one of claims 1-7, wherein the fermentation conditions comprise growth medium comprising:

(a) a carbon source comprising maltose or a combination of inulin and glucose;

(b) a nitrogen source comprising yeast extract, malt extract and peptone; and

(c) a pH of 5-7.

9. The oral composition of claim 8, wherein the nitrogen source further comprises cysteine.

10. The oral composition of any one of claims 1-9, wherein the bacteria is immobilized in a polymer.

11. The oral composition of claim 10, wherein the polymer comprises alginate, chitosan, agarose, pectin, agaropectin, genipin, or cellulose.

12. The oral composition of claim 10, wherein the polymer forms semi permeable microcapsules or nanocapsules.

13. The oral composition of claim 12, wherein said polymeric microcapsules or nanocapsules are resistant to gastro-intestinal conditions.

14. The oral composition of any one of claims 1-13, wherein said stabilization is accomplished by lyophilization, heat drying, spray drying or freeze drying the composition.

15. The oral composition of claim 14, wherein the composition is lyophilized with lyoprotectants comprising (a) 0.2 to 10% maltodextrin and 0.05 to 0.33% yeast extract, (b) 0.05 to 2.5% inulin and 0.1% yeast extract, or (c) 0.3% inulin.

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16. The oral composition of any one of claims 1-13, wherein the composition is stabilized in liquid, wherein the liquid storage conditions comprise a final preservative solution comprising (a) 2.5-10% growth media, (b) 50-99.99% yogurt or other fermented milk, (c) 50-99.99% culture supernatant or (d) 5% Man, Rogosa and Sharpe (MRS) solution.

17. The oral composition of any one of claims 1-13, wherein said stabilization is accomplished by flash freezing the oral composition in a cryoprotectant solution.

18. The oral composition of claim 17, wherein the cryoprotectant solution comprises a final concentration of (a) 0.2-10% maltodextrin and 0.05 to 0.33% yeast extract, (b) 0.05 to 2.5% inulin, (c) 0.5M Trehalose, (d) 0.5M fructose, (e) 0.5M lactose, (f) 0.5M maltose or (g) 50-99.99% spent media.

19. The oral composition of any one of claims 1-18, wherein the carrier comprises a supplement, food product, beverage, functional food or nutraceutical.

20. The oral composition of any one of claims 1-18, wherein the carrier comprises a capsule, pill, gel capsule, liquid, or dissolvable film.

21. The oral composition of any one of claims 1-20, wherein the composition further comprises a therapeutic selected from the group consisting of a triglyceride lowering agent, an agent for increasing HDL, an agent for limiting HDL decrease, a cholesterol lowering agent, an agent for modulating adipokines, an agent for modifying obesity hormones, a hypoglycemic agent, a therapeutic for reducing pro-inflammatory cytokines, vitamin B12, conjugated linoleic acid (CLA), reuterin and reutericyclin.

22. The oral composition of any one of claims 1-21, wherein said *bsh* activity is stabilized by inulin, trehalose, maltodextrin, yeast extract, polyethylene glycol, glycerol, lipid, emulsified fat, a dairy product, glucose, fructose, sucrose, a poly

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sugar, anhydrobiosis, a polycosanol, polyethlylene glycol (PEG), a plant sterol, a plant stanol or an omega fatty acid.

23. A use of a bile acid lowering amount of the oral composition of any one of claims 1-22 for lowering serum cholesterol of an animal.

24. The use of claim 23, for increasing or limiting the reduction of serum high density lipoproteins (HDL-C) of the animal.
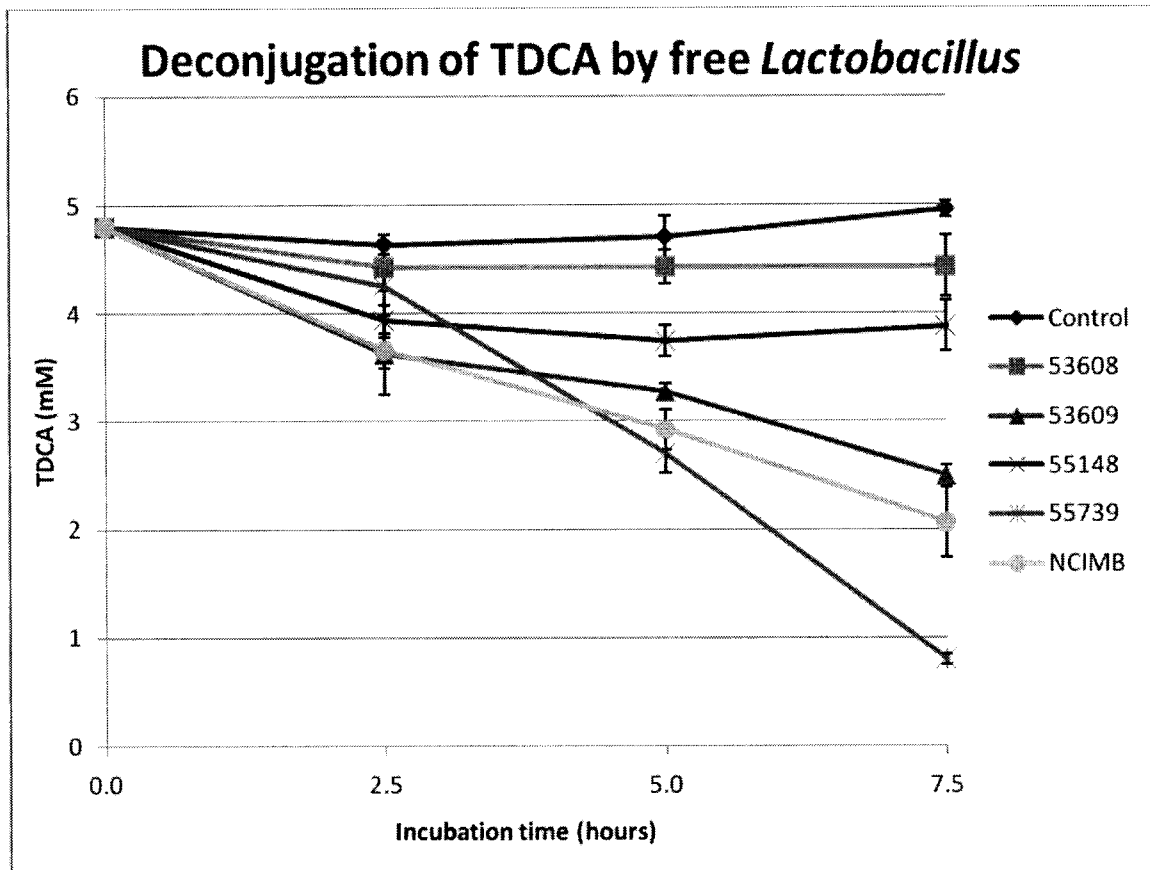
25. The use of claim 23, for decreasing serum triglycerides of the animal.

26. The use of claim 23, for reducing atherosclerotic risk factors of the animal, wherein the atherosclerotic risk factors are selected from serum homocystine, fibrinogen, C-reactive protein, lipoprotein(a), uric acid, matrix metallopeptidase 9 (MMP-9), plasminogen activator inhibitor-1 (PAI-1) or its antigen, tissue plasminogen activator (tPA), TNF alpha, IL-6, P-selectin, monocyte chemotactic protein-1 (MCP-1), soluble CD40 ligand (sCD40L), inter-cellular adhesion molecule 1 (ICAM-1), myeloperoxidase (MPO), adiponectin, leptin, lipoprotein-associated phospholipase A, and insulin.

27. A use of a bile acid lowering amount of the oral composition of any one of claims 1-22 for reducing the atherogenic index of an animal.

28. The use of claim 27, wherein atherogenic index (AI) is calculated by at least one of the following equations: AI = Log(Triglycerides/HDL-C) or AI = TC-HDL-C/HDL-C.

29. A use of a bile acid lowering amount of the oral composition of any one of claims 1-22 for prophylaxis or treatment of atherosclerosis or a degenerative disorder caused by atherosclerosis in an animal.
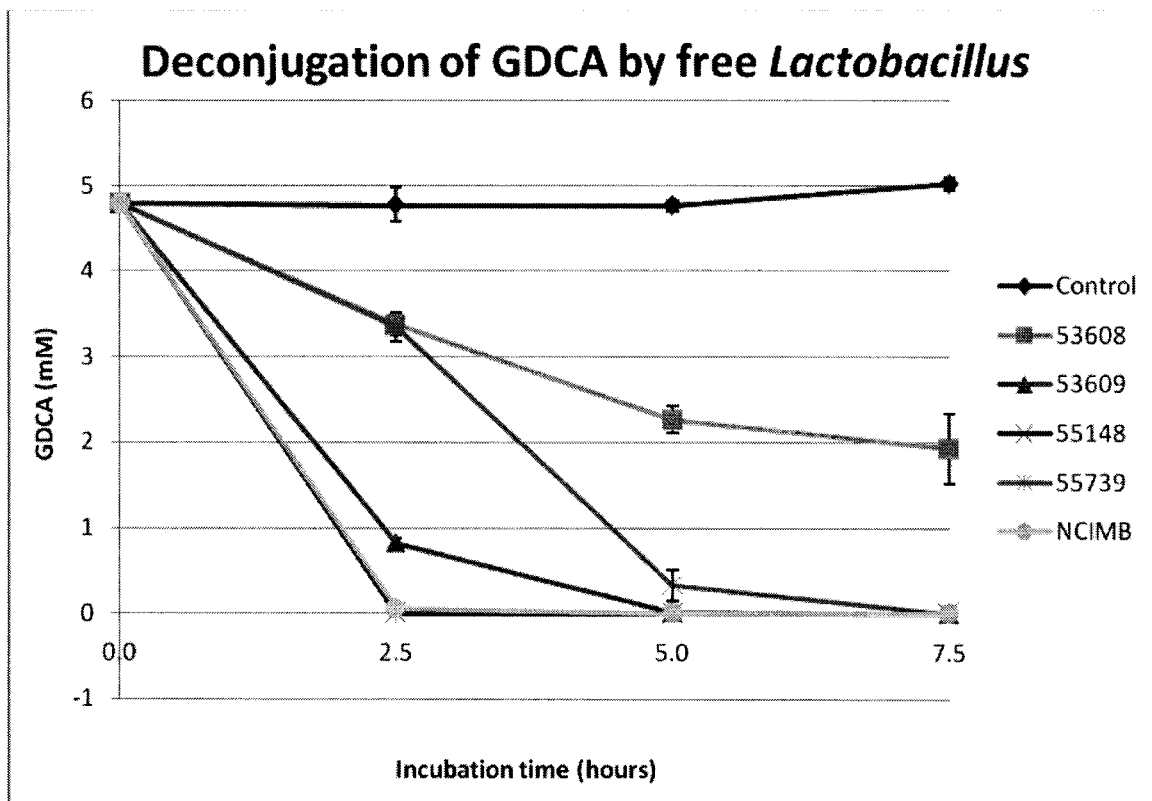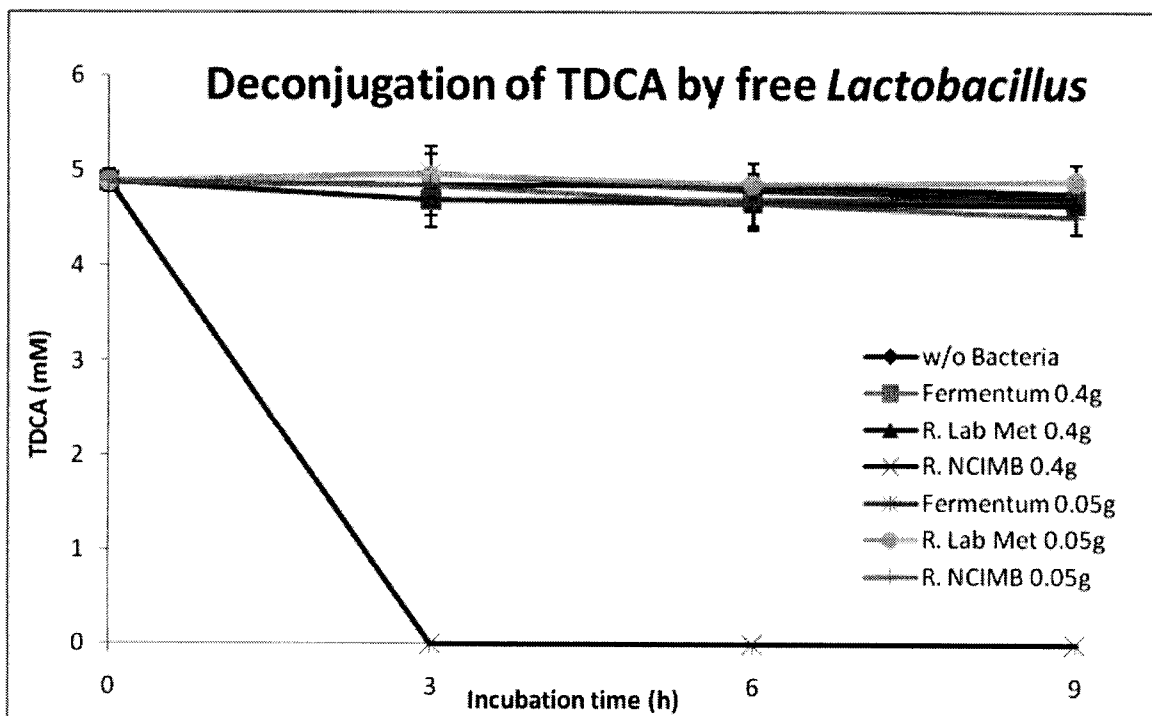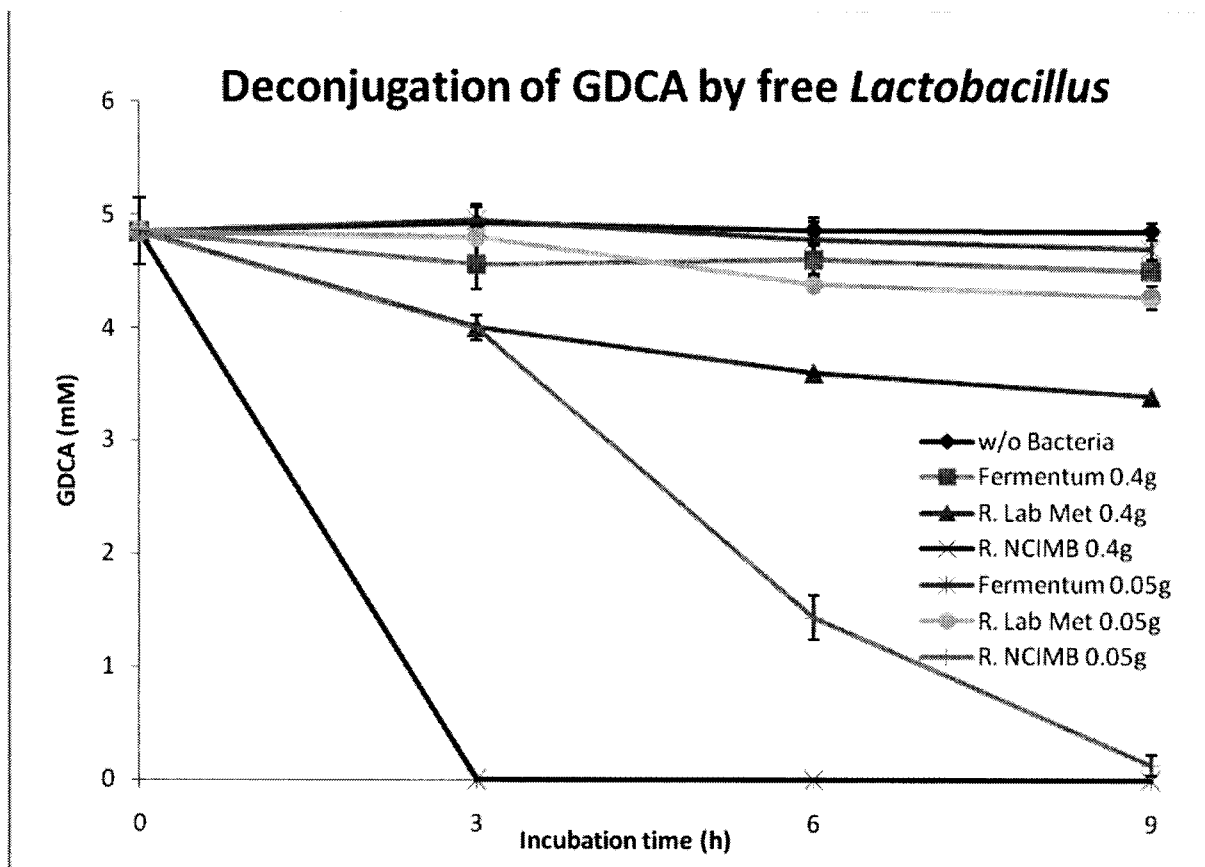
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Figure 1

Deconjugation of GDCA by free *Lactobacillus*

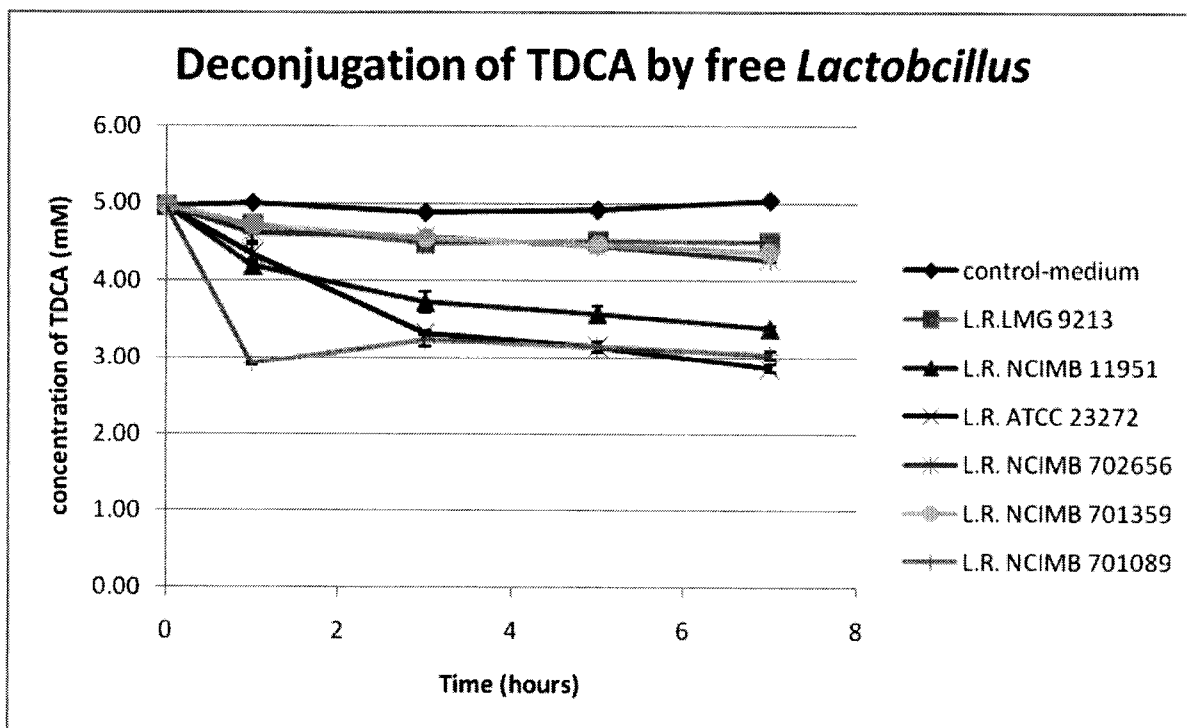**Figure 2**

Deconjugation of TDCA by free *Lactobacillus*

Figure 3

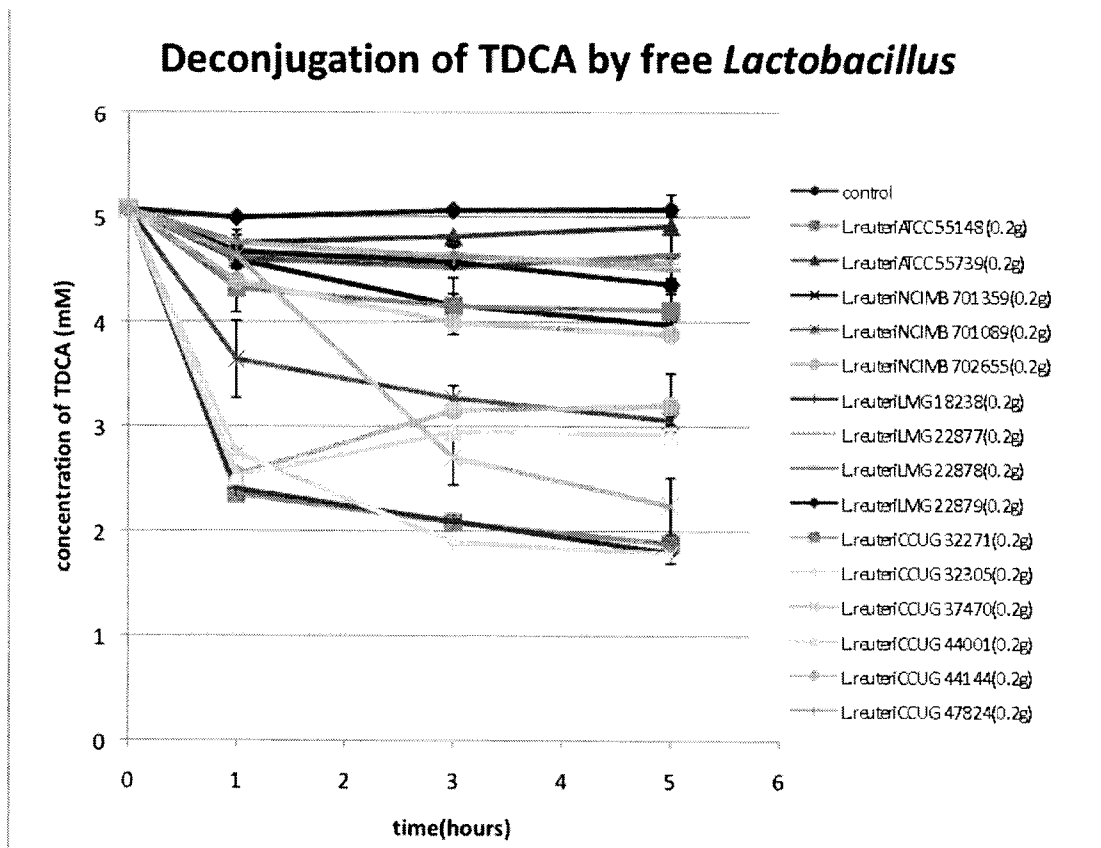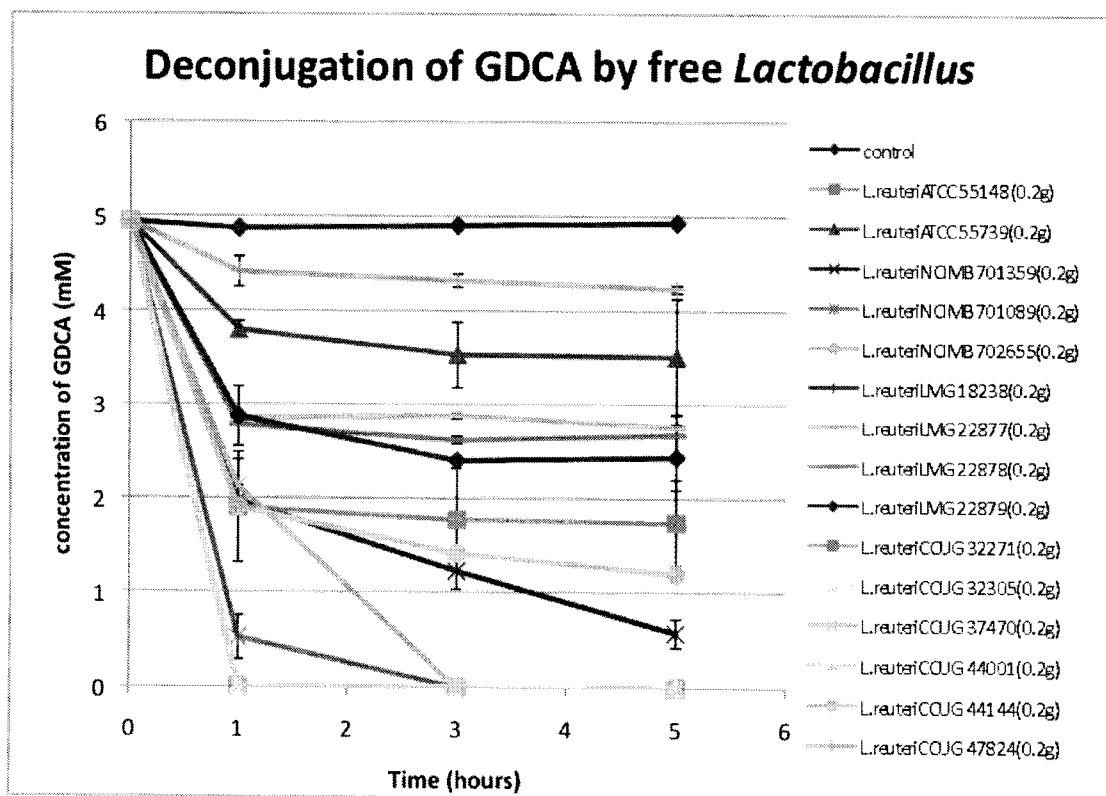**Figure 4**
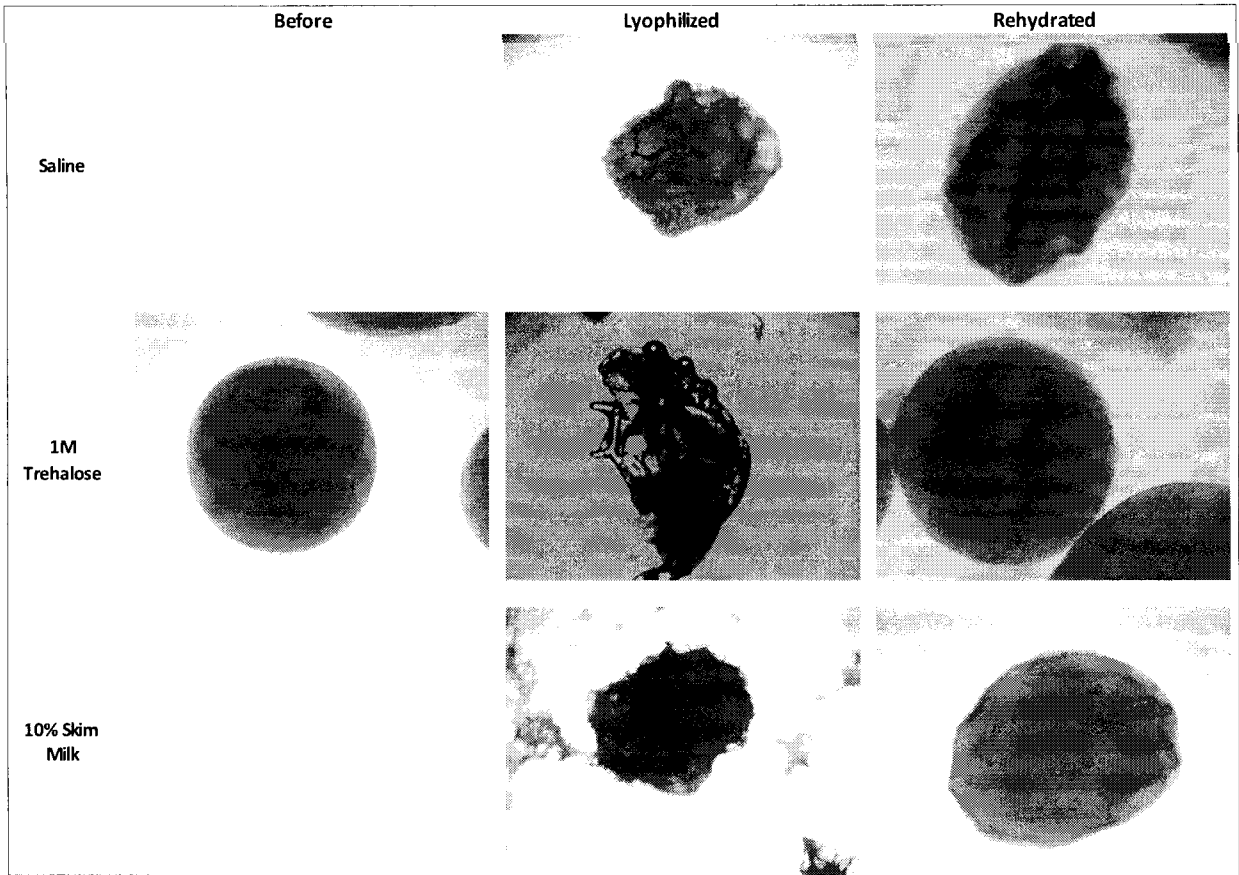

Figure 5
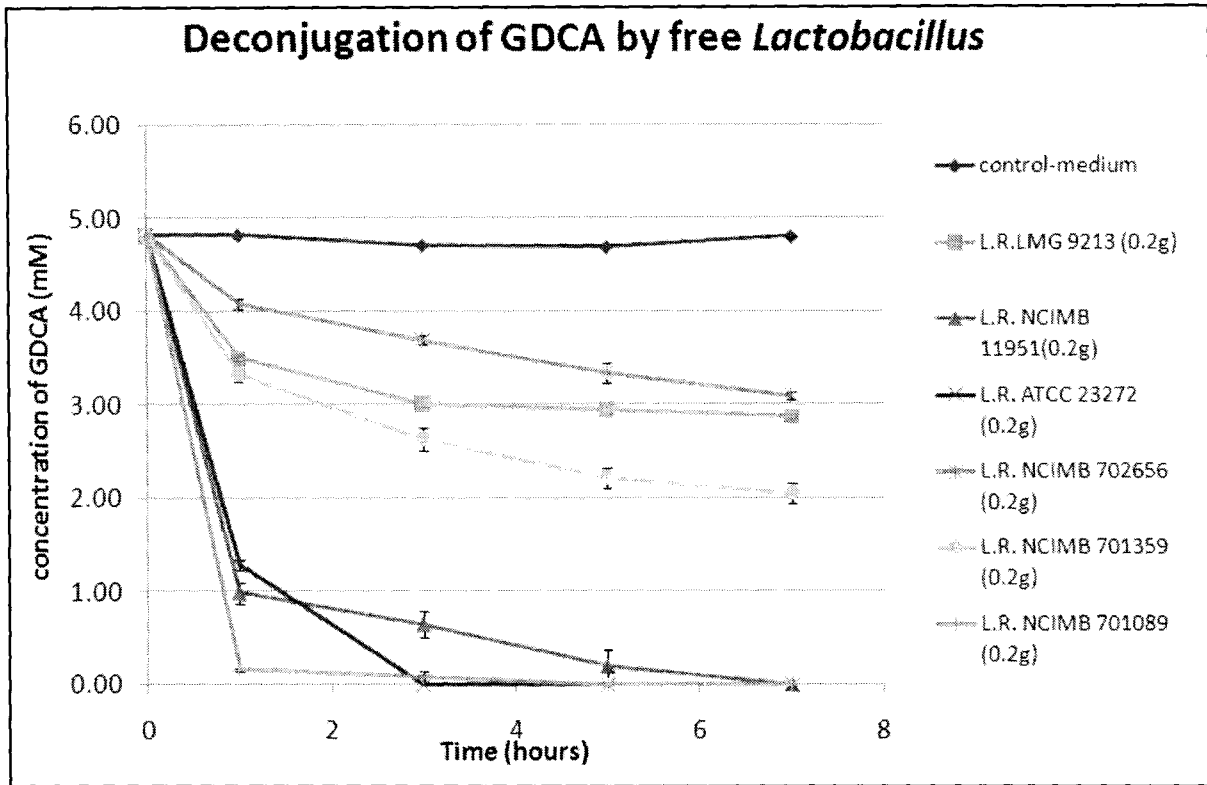
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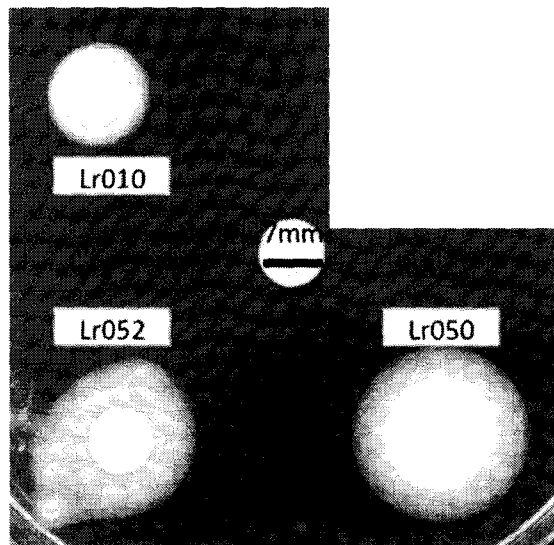
Figure 7

Figure 8

Figure 9

Figure 10

Deconjugation of TDCA by free *Lactobacillus*