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(54) Title: METHOD FOR CULTURING AND PRESERVING *EUBACTERIUM HALLII*

(57) Abstract: In the present document, a method for culturing and preserving the probiotic gut microorganism *Eubacterium hallii* is disclosed. The method provides an *E. hallii* preparation that is suitable for administration or ingestion to humans and that provides high *E. hallii* biomass yields. Preferably, the medium used for culturing *E. hallii* to high biomass yields comprises food grade components only, is free of any animal sources, and/or is kosher.

Title: Method for culturing and preserving *Eubacterium hallii*

5 **Field of the invention**

The present invention is in the field of probiotics, and is particularly concerned with providing a culturing method of *E. hallii* that provides an *E. hallii* composition that is suitable for administration to humans and that gives high biomass yield, and that ideally is suitable for administration to humans regardless of their religion.

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Background of the invention

The human intestinal tract contains a large variety of microorganisms, of which bacteria are the most dominant and diverse. The microbiota is thought to be composed of 500 to 1000 different bacterial species, most of which are anaerobic. Intestinal microbiota contributes to overall metabolism and plays a role in converting food into nutrients and energy. Variations in the composition of microbiota are found between humans, and certain variations can be correlated with certain diseases or conditions.

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WO2013/032328 discloses a causal role of the small intestinal microbiota in insulin resistance and dyslipidemia. 18 male subjects with newly diagnosed metabolic syndrome underwent small intestine biopsies and subsequent polyethylene-glycol bowel lavage through duodenal tube insertion followed by random assignment to either allogenic or autologous faecal transplantation. In the allogenic faecal transplantation group that was performed on 9 subjects, the faecal material was derived from a healthy and lean donor. The autologous transplantation group included the 9 other subjects and these received their own fecal material. It was found that the subjects of the allogenic group were characterized by different sigmoidal gut microbiota compared to those of the autologous group. Fasting levels of TG-rich lipoproteins (TG/ApoB ratio) were significantly reduced in the subjects in the allogenic group with no effect after autologous faeces infusion. Although the weight of the subjects remained stable, 6 weeks after faeces transplantation an improvement in both peripheral (Rd) and hepatic insulin sensitivity (suppression of EGP) was found 6 weeks in the allogenic group while no significant changes were observed in the autologous treatment group.

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Changes in small intestinal microbiota between subjects receiving allogenic or autologous faecal transplantation were identified. Comparing the small intestinal microbiota composition at baseline and after 6 weeks in the allogenic group showed an increased abundance of the butyrate-producing *E. hallii*. Notably, *E. hallii* was almost two-fold reduced following infusion in the autologous group.

Bacteria belonging to *Eubacterium hallii* et rel. include relatively fast-growing anaerobes. They have the metabolic capacity to convert lactate into butyrate in a process that needs acetate (Munoz-Tamayo et al 2011 FEMS Microbiol Ecolo 76: 615-624). Lactate and acetate are abundant metabolites in the upper intestinal tract that is colonized by
5 among others streptococci and lactobacilli that can produce these compounds (Booijink et al 2010, vide supra).

In db/db mice (a model of obesity, diabetes, and dyslipidemia) distinct effect were found of short term oral *E. hallii* L2-7 supplementation to the small intestine on normalization of insulin resistance (as detected by HOMA calculation and postprandial glucose
10 metabolism by AUC of oral glucose tolerance curve) as well as fasting lipid profiles in db/db mice (WO2013/032328).

Culturing obligate anaerobic gut microorganisms is notoriously difficult, and high biomass yields are hardly ever obtained.

Hitherto, *E. hallii* has been cultured in complex media, and relatively low biomass
15 yields have been obtained (WO2013/032328; Duncan et al. Appl. Environm. Microbiol. 70:5810).

There is a need in the art for a method of culturing *E. hallii*, an obligate anaerobe, that provides a composition suitable for administration to humans and that gives high biomass yields. Preferably, such composition should comprise all food grade sources,
20 should be free of animal components, and/or should be kosher. Preferably, the culturing method is based on a simple medium.

Summary of the invention

The present inventors have developed a method of preparing an *E. hallii* preparation
25 that is suitable for administration or ingestion to humans and that gives high biomass yields, preferably yields suitable for commercial production. Ideally, the medium comprises food grade components only, is free of any animal sources, and/or is kosher. The method of the invention is based on a simple medium.

30 Definitions

As used herein, the term "effective amount" refers to a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect, e.g., an amount, which results in the treatment and/or prevention of the diseases and conditions taught herein. In the context of therapeutic or prophylactic applications, the amount of *E. hallii* bacteria administered to the
35 animal subject, preferably human subject, will depend on the type and severity of the disease or condition and on the characteristics of the subject, such as general health, age, sex, body weight and tolerance to drugs. The skilled artisan will be able to determine

appropriate dosages depending on well-known factors. The bacteria can also be administered in combination with one or more additional therapeutic compounds.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, the verb "to consist" may be replaced by
5 "to consist essentially of" meaning that a composition of the invention may comprise additional component(s) than the ones specifically identified, said additional component(s) not altering the unique characteristics of the invention.

In addition, reference to an element by the indefinite article "a" or "an" does not
10 exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

Detailed description of the invention

Methods for culturing and preserving *E. hallii*

The present invention provides a method of preparing an *E. hallii* preparation suitable for ingestion by a human, said method comprising the steps of:

a) providing a medium comprising about 1-100, such as about 2-80, about 4-60,
20 preferably, about 5-40, more preferably about 10-30, g/kg of a sugar, preferably glucose; about 1-100, such as about 2-80, about 4-60, preferably 5-40, more preferably 10-30 g/kg of a source of nitrogen and growth factors, e.g., yeast extract; about 1-20, such as about 2-16, or about 3-12 g/kg plant peptone, preferably soy peptone; about 0.01-20, such as about 0.1-18, about 0.2-16, about 0.5-14, about 0.75-12, about 1-10, about 1.5-8, about 2-6, or about
25 2.5-4 g/kg of an acetate salt, such as sodium acetate; a buffer system maintaining the pH prior to acidification by *E. hallii* at about 6-8; optionally cysteine; optionally, magnesium ions; optionally, calcium ions; optionally, potassium ions; optionally, vitamins;

b) inoculating said medium with *E. hallii*;

c) allowing said *E. hallii* to grow in said medium; and

30 d) harvesting said *E. hallii* preparation from said medium.

Said medium may comprise about 1-100, such as about 2-80, about 4-60, preferably, about 5-40, more preferably about 10-30, g/kg of a sugar. Said sugar may be selected from the group consisting of glucose, sucrose, galactose, fructose, maltose, and lactose, or any combination thereof. The medium preferably comprises about 1-100, such as about 2-80,
35 about 4-60, preferably, about 5-40, more preferably about 10-30, g/kg of glucose, fructose or sucrose as a carbon source.

The medium may further comprise about 1-100, such as about 2-80, about 4-60, preferably 5-40, more preferably 10-30 g/kg of a source of nitrogen and growth factors such as yeast extract.

The medium taught herein may further comprise from about 0.1-50, such as about 5 0.5-40, about 1-20, about 2-16, or about 3-12 g/kg of a plant peptone. A plant peptone is a plant protein hydrolysate. It may be derived from any plant. The plant peptone may, for example and without limitation, be selected from the group consisting of soy peptone, wheat peptone, cotton peptone, pea peptone, broadbean peptone, lupin peptone, and potato peptone. The medium may, for example, comprise from about 0.1-50, such as about 0.5-40, 10 about 1-20, about 2-16, or about 3-12 g/kg of a soy peptone.

The medium taught herein further comprises about 0.01-20, such as about 0.1-18, about 0.2-16, about 0.5-14, about 0.75-12, about 1-10, about 1.5-8, about 2-6, or about 2.5- 4 g/kg of an acetate salt. The acetate salt is important as it forms a substrate for butyrate formation by *E. hallii*. Additionally, acetate is anti-foaming compound. The acetate salt may, 15 without limitation, be selected from the group consisting of sodium acetate, aluminium acetate, ammonium acetate, and potassium acetate, or any combination thereof. In an embodiment, the medium comprises about 0.01-20, such as about 0.1-18, about 0.2-16, about 0.5-14, about 0.75-12, about 1-10, about 1.5-8, about 2-6, or about 2.5-4 g/kg of sodium acetate.

20 The medium may further comprise a buffer system that maintains the pH of the medium at about 6-8 prior to acidification by *E. hallii*. A suitable buffer system may include sodium bicarbonate, dipotassium HydrogenP and Potassium diHydrogenP as taught herein. The skilled person is perfectly capable of selecting a suitable food grade buffer system for application in the method of the present invention.

25 The medium may further comprise cysteine to render the medium in a reducing state, magnesium ions, calcium ions, potassium ions, and/or vitamins. It was found that these addition of these compounds to the medium taught herein improved biomass yield even further.

In an embodiment, the medium used comprises about 1-100, such as about 2-80, 30 about 4-60, preferably, about 5-40, more preferably about 10-30, g/kg glucose; about 1-100, such as about 2-80, about 4-60, preferably 5-40, more preferably 10-30 g/kg yeast extract; about 1-20, such as about 2-16, or about 3-12 g/kg soy peptone; about 0.01-20, such as about 0.1-18, about 0.2-16, about 0.5-14, about 0.75-12, about 1-10, about 1.5-8, about 2-6, or about 2.5-4 g/kg of an acetate salt, e.g., sodium acetate; a buffer system maintaining the 35 pH prior to acidification by *E. hallii* at about 6-8; optionally cysteine; optionally, magnesium ions; optionally, calcium ions; optionally, potassium ions; and, optionally, vitamins.

The *E. hallii* strain used in the method taught herein may be any human *E. hallii* isolate, such as the *E. hallii* strain designated L2-7 which has been deposited at DSMZ and received deposit no. 17630.

5 Preferably, the medium is free of any animal sources, such as animal products or animal-derived products.

The method taught herein is preferably carried out under anaerobic conditions. Since *E. hallii* is an obligate anaerobic bacterium, its viability is affected by contact with air. Anaerobic conditions may be maintained using well-known methods, which include N₂ flushing.

10 The method as taught herein is preferably carried out at a temperature in the range of 30 – 42 °C, preferably in the range of 34 – 40 °C.

The *E. hallii* preparation may be harvested from said medium using any technique known to the skilled person, particularly a harvesting technique that is amenable to being carried out under anaerobic conditions, such as filtration.

15 To remove or dilute further medium components, harvested *E. hallii* cells may be washed with a physiologically acceptable solution, such as a phosphate buffered saline solution. The skilled person is capable of selecting a suitable physiologically acceptable solution, as well as a washing method achieving the desired result. For example, diafiltration may be used to wash the *E. hallii* cells. Alternatively, the *E. hallii* cells can be centrifuged
20 and resuspended in washing buffer, or they may be directly taken up in a buffer containing cryoprotectants and oxygen scavengers.

The method taught herein may further comprise the step of lyophilizing said *E. hallii* to obtain a lyophilized *E. hallii* preparation. Alternatively or additionally, the method may further comprise the step of encapsulating said *E. hallii* to obtain an encapsulated *E. hallii*
25 preparation.

The technique of encapsulation is well-known in the art for preserving probiotic bacteria (e.g., as reviewed by Serna-Cock and Vallejo-Castillo, 2013. Afr J of Microbiol Res, 7(40): 4743-4753, herein incorporated by reference). Any of the preservation techniques and preservation systems taught by Serna-Cock and Vallejo-Castillo may be employed in
30 the present invention.

The lyophilizing step may be carried out using any method known in the art. Freeze-drying or lyophilization is commonly used to preserve microorganisms. After all, lyophilisates can be stored for decades. Accordingly, many microbes are stored as lyophilisates in strain collections. To overcome the potential viability loss during formulation, it is generally
35 recommended to lyophilize concentrated cultures, e.g., containing at least 10⁷ cells. This ensures the presence of sufficient viable cells after lyophilisation, long-term storage and

reconstitution. For probiotic preparations such as the *E. hallii* preparations taught herein it is important that as many bacteria as possible survive lyophilisation and subsequent storage.

It was found that *E. hallii* is extremely sensitive to freezing and freeze-drying.

Cryoprotectants are often employed to protect probiotic formulations during lyophilisation and to enhance shelf-life. However, the selection of appropriate and compatible
5 cryopreservants has remained a major challenge. Hitherto, no suitable cryoprotectants have been reported for *E. hallii*.

In the method taught herein, a cryoprotectant selected from the group consisting of sucrose, maltose, maltodextrin, trehalose, mannitol, sorbitol, inulin, glycerol, DMSO,
10 ethylene glycol, propylene glycol, 2-methyl-2,4-pentanediol, polyethylene glycol, polyvinylpyrrolidone, polyvinyl alcohol, polyglycerol, skim milk powder, milk protein, whey protein, UHT milk, betaine, adonitol, sucrose, glucose, lactose or any combination thereof, may be employed.

Prebiotics such as starch and wheat bran may further be added before lyophilisation
15 to enhance the efficacy of the *E. hallii* preparation taught herein. Addition of antioxidants such as riboflavin, riboflavin phosphate or a physiologically acceptable salt thereof, glutathione, ascorbate, glutathione and cysteine to the lyophilisation mixture may further enhance the viability of the *E. hallii* preparation taught herein during storage.

Lyophilisation methods include, without limitation, slow, gradual freezing to -40°C
20 before drying, rapid freezing by placing at -80°C before drying, or ultra rapid freezing by dripping cells with cryoprotector in liquid nitrogen before drying.

In order to render *E. hallii* more resistant to freezing, it may be beneficial to subject the *E. hallii* cells to mild to moderate stress treatments during culturing to prepare these for adverse conditions. For example, during culturing the cells may be subjected to mild
25 oxidative stress, e.g., by adding a low dose of hydrogen peroxide, by limiting certain nutrient, by culturing at low pH value, and the like.

E. hallii preparation

The present invention also relates to an *E. hallii* preparation obtainable by the
30 method as taught herein. Said preparation is characterized by being suitable for ingestion by a human.

The *E. hallii* preparation taught herein preferably comprises an effective amount of *E. hallii*, in conjunction with a physiologically acceptable carrier.

Preferably, the *E. hallii* preparation comprises between about 10^6 and about 10^{12} ,
35 preferably between about 10^8 and about 10^{12} , viable *E. hallii* bacteria. Preferably, said bacteria are contained in a daily dose.

Preferably, the *E. hallii* preparation taught herein is for enteral or oral administration. A composition for enteral or oral administration may be either a food composition, feed composition, or a pharmaceutical composition.

The *E. hallii* preparation taught herein will usually comprise a carrier, such as a pharmaceutical carrier, preferably an inert carrier. The preferred form depends on the intended mode of administration and (therapeutic) application. A pharmaceutical carrier can be any compatible, nontoxic substance suitable to deliver *E. hallii* bacteria to the gastrointestinal-tract of a subject. For example, sterile water, or inert solids may be used as a carrier usually complemented with a pharmaceutically acceptable adjuvant, buffering agent, dispersing agent, and the like. A composition will either be in liquid form, e.g. a stabilized suspension of *E. hallii* bacteria, or in solid form, e.g. a powder of lyophilized *E. hallii* bacteria. For oral administration, *E. hallii* bacteria can be administered in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. *E. hallii* bacteria may, for example, be encapsulated in capsules such as gelatin capsules, together with inactive ingredients and powdered carriers, such as e.g. glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like.

The *E. hallii* preparation taught herein may be in the form of a food supplement or a food or food composition (herein jointly referred to as "food composition"), which may, besides *E. hallii* bacteria, also contain a suitable food base. Alternatively, such preparation may be in the form of a feed supplement or a fodder or feed composition (herein jointly referred to as "feed composition"). A food or food composition or feed composition is herein understood to include a liquid for human or non-human animal consumption, i.e., a drink or beverage. A food or food composition or feed composition may be a solid, semi-solid and/or liquid food or food composition, and in particular may be a dairy product, such as a fermented dairy product, including but not limited to, a yoghurt, a yoghurt-based drink or buttermilk. Such a food or food composition or feed composition may be prepared in a manner known per se, for example, by adding *E. hallii* bacteria to a suitable food, food base, or feed base, in a suitable amount. Similarly, this may include the use of these bacteria in capsulated form as described above since they have to pass the low pH of the stomach. This may also be a preferred way as to reduce the traces of butyrate that are associated with the growth of *E. hallii* bacteria and may produce off-flavor in a food or food composition. In another embodiment, *E. hallii* bacteria may be used in or for the preparation of a food or food composition or feed composition, e.g. by fermentation. In doing so, *E. hallii* bacteria may be used in a manner known per se for the preparation of such fermented foods or food compositions or fermented feed compositions, e.g. in a manner known per se for the

preparation of fermented dairy products using lactic acid bacteria. In such methods, *E. hallii* bacteria may be used in addition to a micro-organism usually used, and/or may replace one or more or part of a micro-organism usually used.

Preferably, the *E. hallii* preparation taught herein may contain *E. hallii* bacteria in amounts that allow for convenient (oral) administration as indicated above, e.g., as or in one or more doses per day or per week. In particular, a preparation may contain a unit dose of *E. hallii*.

Said *E. hallii* preparation is suitable for use as a medicament, particularly for use in preventing and/or treating insulin resistance and/or insulin resistance-related complications such as metabolic syndrome, dyslipidemia, type 2 diabetes mellitus, non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatosis hepatitis (NASH), and insulin resistance in endocrine diseases (e.g., obese subjects with type 1 diabetes mellitus, Cushing's disease and lipodystrophy syndromes), for preventing and/or treating lactic-acidosis, short bowel syndrome or inflammatory bowel disease, for preventing, reducing the incidence or reducing the severity of colorectal cancer or colitis.

The present invention also teaches a method to promote butyric acid formation in the intestine of a mammal, said method comprising the step of administering an *E. hallii* preparation as taught herein to a subject in need thereof.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

It will be clear that the above description is included to illustrate some embodiments of the invention, and not to limit the scope of protection. Starting from this disclosure, many more embodiments that are within the scope of protection and the essence of this invention and that are obvious combinations of prior art techniques and the disclosure of this patent, will be evident to a skilled person.

Examples

Culturing of E. hallii

Medium was prepared using the following components and concentrations:

Medium 1

Component	Concentration (g/kg)
Yeast extract	20
Soy peptone Amisoy BF	4

Sodium bicarbonate	8
Sodium acetate	2.7
dipotassium HydrogenP	0.9
Potassium diHydrogenP	0.9
Ammonium chloride	0.9
Sodium chloride	0.9
Glucose	20
Demi water	940

The medium was subjected to autoclavation for 20 minutes at 121°C, followed by flushing with N₂. The pH was adjusted to 6.9±0.1.

- 5 About 20 ml lab culture of *E. hallii* was added to about 1 L of medium and cultured during 18 hrs at 37°C. 500 ml of this culture was subsequently added to 20 L of media, and cultured during 14-18 hrs at 37°C. All steps were executed aseptically and under N₂ (flush).

10 Cells grew to an optical density (OD₆₀₀) of 8.5 while acidifying the medium from pH 6.9 to pH 5.0.

Medium 2

Component	Concentration (g/kg)
Yeast extract	20
Soy peptone Amisoy BF	4
Sodium bicarbonate	8
Sodium acetate	2.7
dipotassium HydrogenP	0.9
Potassium diHydrogenP	0.9
Ammonium chloride	0.9
Sodium chloride	0.9
Magnesium sulphate	0.9
Calcium chloride	0.26
Glucose	20
Demi water	940

To medium 2, the following filtersterilised anaerobic solutions were added:

Cysteine, N ₂ degassed	0.05%
Vitamin solution I (1000 x concentrated), N ₂ degassed	1

Vitamin solution I (1000x concentrated)

Biotin	10 mg
Cobalamin	10 mg
PABA	30 mg
Folic acid	50 mg
Pyridoxamine	150 mg
Demi water	1 L

The medium was subjected to autoclavation for 20 minutes at 121°C, followed by flushing
5 with N₂. The pH was adjusted to 6.9±0.1.

About 20 ml lab culture of *E. hallii* was added to about 1 L of medium and cultured during 18
hrs at 37°C. 500 ml of this culture was subsequently added to 20 L of medium, and cultured
10 during 14-18 hrs at 37°C. 10 L of said culture was added to 320 L of medium and cultured
during 14-16 hrs at 37°C. All steps were executed aseptically and under N₂ (flush).

Cells grew to an optical density (OD₆₀₀) of 9.4 (1.4 x 10⁹ cells/ml) while acidifying the medium
from pH 6.9 to pH 5.0.

15 The cells were harvested using concentration with hollow fibre membranes (Koch membrane
systems; HF3043-25-43-PM500; HF3043-16-106-PM500) and diafiltration using PBS .
Diafiltration continued until the level of medium components and fermentation products
reached about 2% of their initial concentration. The sample was concentrated about 20-fold,
yielding about 15 L of concentrated *E. hallii* cells in PBS. About 99.8% of the medium
20 compounds were removed.

Samples were filled inside a disinfected laminar flow cabinet either in glycerol or in glycerol
and PBS (pre-autoclaved, pre-cooled and pre-flushed with N₂).

25 ***Freeze-drying of cells***

Centrifugation bowls comprising 800 mL end culture were centrifugated at 4000 rpm⁻¹ for 30
minutes. The supernatant was removed in an anaerobic chamber.

Subsequently, the *E. hallii* cells were lyophilized. Five different cryoprotectant solutions were tested:

Cryoprotectant solution 1:

	Concentration (g/L)
Maltodextrine	100
Trehalose	100
L-cysteine HCl	9
Water	

5

Cryoprotectant solution 2:

	Concentration (g/L)
Mannitol	100
Trehalose	100
L-cysteine HCl	9
Water	

Cryoprotectant solution 3:

	Concentration (g/L)
Sucrose	100
Mannitol	100
Water	

10 Cryoprotectant solution 4:

	Concentration (g/L)
Sucrose	150
Maltose	150
Water	

Cryoprotectant solution 5:

	Concentration (g/L)
Sucrose	100
Trehalose	100
Water	

80 mL cryoprotectant solution was added to the centrifugation bowl containing cell pellet. The bowls were shaken in order to homogenize the biomass and cryoprotectant solution. The resultant was spread in 2 plates containing 50 mL in an anaerobic chamber. These plates were put in a hermetically-sealed box and deep-frozen at -40°C. The plates were left
5 in the freeze-dryer for 1 to 2 days. After collection, lyophilisates were grinded manually and packed in bags under anaerobic conditions. Afterwards, they were stored at -4 °C.

E. hallii could be re-grown after lyophilisation, demonstrating that the bacteria remained viable after lyophilisation. In this example the highest viability following lyophilisation was
10 found for cryoprotectant solution 1. It was found that viability of the lyophilisates were better when the entire process was carried out under anaerobic conditions to the extent possible.

Example 3 – Comparative example

15 The growth yields on the kosher media describe in examples 1 and 2 were much higher than those described for growth of *E. hallii* on conventional media, which contained components from animal sources. A typical example is YCFA medium as described by Duncan et al 2003 (Appl Environ Microbiol 69: 1136-42) with added glucose or lactate. This YCFA medium is widely used to support growth of anaerobic and intestinal bacteria (see Browne et al 2016
20 Nature 533: 543-46). However, the growth of *E. hallii* L2-7 was described to reach only an OD₆₀₀ of 0.8-1.0 on YCFA with added glucose or other compounds (Duncan et al 2004 Appl Environ Microbiol 70: 5810–17). When the animal derived products hemin and casiton were added to YCFA with glucose, the growth was only slightly better and a maximum OD₆₀₀ of 1.8 was reached (Engels et al 2016 Front Microbiol 7: 713).

25

It should also be noted that the growth yield described above of an OD₆₀₀ for medium 2 of 9.4 was obtained from not yet fully outgrown cultures. To show that the developed kosher media are superior to other hitherto described media, *E. hallii* strain L2-7 was cultured for 35-40 hours in 200 ml volume of medium 2 in anaerobic chambers (Exp #1 and Exp #2; see
30 Table below). Moreover, *E. hallii* L2-7 was also grown on a large scale in a 2.5 liter fermenter on medium 2 (Exp 2.5 liter; see Table below). The results show that on small and large scale fermentation high yields of OD₆₀₀ close to 20 can be obtained.

Translating the OD₆₀₀ to a specific number of cells is dependent on several factors. We
35 determined the number of cells by flow cytometry and found an OD₆₀₀ of 1 to correspond to approximately 1×10^8 – 1.3×10^8 cells per ml. However, it is not only the number of cells that determines the yield but also the size of the cells. It is known that the dimensions of *E. hallii*

can vary considerably and cells have been described to be 0.8-2.4 micrometer in diameter and 4.7 to 25.0 micrometer in length (De Vos P et al., Bergey's Manual of Systematic Bacteriology Volume 3 Sec Ed p. 881). Phase contrast microscopy showed cells grown in medium 2 to be rather long, resulting in relatively low cell counts per OD₆₀₀ unit. Hence in the comparative experiments OD₆₀₀ values were used.

Growth of *E. hallii* L2-7

Experiment	OD ₆₀₀
Exp #1	16.2
Exp #2	19.5
Exp #2.5 L	17.2

The usefulness of the media taught herein in supporting growth of *E. hallii* was also illustrated by the observation that efficient growth of single cells was obtained on medium 2 supplemented with 1 % agar with a high efficiency of plating (over 10 %). It is notoriously difficult to enumerate anaerobes and usually most probable number (MPN) approaches are used to estimate the number of viable cells, which is a tedious procedure. Moreover, the usefulness of a medium that supported a high plating efficiency of *E. hallii* was also used to select for derivatives or mutants of *E. hallii* strain L2-7.

This all shows the usefulness of the media taught herein that allow efficient growth to high density of *E. hallii*.

CLAIMS

1. A method of preparing an *E. hallii* preparation suitable for ingestion by a human, said method comprising the steps of:
- 5 a) providing a medium comprising:
- 1-100, preferably 5-40, g/kg of a sugar, preferably glucose,
 - 1-100, preferably 5-40, g/kg of a source of nitrogen and growth factors, such as yeast extract,
 - 0.1-50 g/kg plant peptone, preferably soy peptone,

10 - acetate,

 - a buffer system at pH6-8,
 - optionally, cysteine,
 - optionally, magnesium ions,
 - optionally, calcium ions,

15 - optionally, potassium ions,

 - optionally, vitamins;
- b) inoculating said medium with *E. hallii*;
- c) allowing said *E. hallii* to grow in said medium; and
- d) harvesting said *E. hallii* preparation from said medium.
- 20
2. The method of claim 1, which is carried out under anaerobic conditions.
3. The method of claim 1 or 2, which is carried out at a temperature in the range of 30 – 42 °C, preferably in the range of 34 – 40 °C.
- 25
4. The method of any of claims 1 – 3, wherein said *E. hallii* preparation is harvested from said medium using filtration.
5. The method of claim 4, wherein the filtrate is further washed with phosphate buffered saline solution.
- 30
6. The method of any of claims 1 – 5, which further comprises the step of lyophilizing said *E. hallii* to obtain a lyophilized *E. hallii* preparation.
- 35
7. The method of claim 6, wherein said lyophilizing is carried out using a cryoprotectant selected from the group consisting of sucrose, maltose, maltodextrin, trehalose, mannitol, inuline, glycerol, DMSO, ethylene glycol, propylene glycol, 2-methyl-2,4-pentanediol,

polyethylene glycol, polyvinylpyrrolidone, polyvinyl alcohol, polyglycerol, or any combination thereof.

8. *E. hallii* preparation obtainable by the method of any of the preceding claims.

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9. *E. hallii* preparation of claim 8 for use as a medicament.

10. *E. hallii* preparation of claim 8 for use in preventing and/or treating insulin resistance and/or insulin resistance-related complications such as metabolic syndrome, dyslipidemia, type 2 diabetes mellitus, non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatosis hepatitis (NASH), and insulin resistance in endocrine diseases (e.g., obese subjects with type 1 diabetes mellitus, Cushing's disease and lipodystrophy syndromes), for preventing and/or treating lactic-acidosis, short bowel syndrome or inflammatory bowel disease, for preventing, reducing the incidence or reducing the severity of colorectal cancer or colitis.

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11. A method to promote butyric acid formation in the intestine of a mammal, said method comprising the step of administering an *E. hallii* preparation according to claim 8 to a subject in need thereof.

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2017/050001

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/20
ADD.

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

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2013/032328 A1 (AMC AMSTERDAM [NL]; UNIV WAGENINGEN [NL]; NIEUWDORP MAX [NL]; DE VOS W) 7 March 2013 (2013-03-07) cited in the application page 2, line 9 - line 32; example 2 -----	1-11
Y	HEENAN C N ET AL: "GROWTH MEDIUM FOR CULTURING PROBIOTIC BACTERIA FOR APPLICATIONS IN VEGETARIAN FOOD PRODUCTS", LWT- FOOD SCIENCE AND TECHNOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 35, no. 2, 1 January 2002 (2002-01-01), pages 171-176, XP008012512, ISSN: 0023-6438, DOI: 10.1006/FSTL.2001.0833 tables 2,3-5 ----- -/--	1-11

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 3 March 2017	Date of mailing of the international search report 13/03/2017
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer van Heusden, Miranda

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2017/050001

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	<p>----- WO 2014/145958 A2 (SERES HEALTH INC [US]) 18 September 2014 (2014-09-18) paragraphs [0008], [0176], [0315]</p>	1-11
Y,P	<p>----- WO 2016/070151 A1 (WHOLE BIOME INC [US]) 6 May 2016 (2016-05-06) figure 14; example 1; table 7</p> <p>-----</p>	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/NL2017/050001

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