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(54) Title: COMBINATIONS COMPRISING VITAMIN AND BACILLUS COAGULANS

(57) Abstract: The present invention relates to combinations comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans*, and their use for improving gut health in animals and humans. It was found that a combination of vitamin C or vitamin B2, and *Bacillus coagulans*, when delivered to the large intestine, increases the abundance of beneficial *Firmicutes*, *Lachnospiraceae*, and *Anaerostipes hadrus* bacteria in the intestinal tract.

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COMBINATIONS COMPRISING VITAMIN AND BACILLUS COAGULANS

FIELD OF THE INVENTION

The present invention relates to combinations comprising (i) vitamin C or vitamin B2, and
5 (ii) *Bacillus coagulans*, and their uses for improving gut health in animals and humans. It was found that a combination of vitamin C or vitamin B2 and *Bacillus coagulans*, when delivered to the large intestine, increases the abundance of specific beneficial bacteria in the intestinal tract.

BACKGROUND OF THE INVENTION

10 Increasing evidence indicates that imbalances in the human gut microbiota (also referred to as “dysbiosis”) may be associated with Western diseases, including obesity and type 2 diabetes, as well as cardiovascular, autoimmune, and intestinal inflammatory disease. Thus, targeted modulation of the human gut microbiome intended to restore imbalances represents a potential therapeutic and preventive strategy and has attracted the attention of academics as well as those
15 engaged in various industries. Public awareness and acceptance of substances that modulate the human gut microbiome continue to grow.

There is a consensus that certain live microorganisms have beneficial effects on human health. *Firmicutes* is a phylum of bacteria comprising beneficial bacteria commonly found in the gut
20 microbiome of humans and animals. Some members of the *Firmicutes* phylum are probiotic (such as *Lactobacillus*), and/or are known for producing butyrate. Butyrate has many health benefits: it is a source of energy for the cells of the gut lining, stimulates the production of glutathione, is a natural antioxidant, controls intestinal inflammation and supports a strong gut lining, helps prevent cancer by preventing cancer cells from developing, and encourages the production of hormones
25 for a healthy metabolism (Rinninella et al., What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases (2019); Hamer HM et al., The role of butyrate on colonic function (2008)).

The *Lachnospiraceae* family of bacteria belongs to the phylum *Firmicutes* and is a predominant
30 group of bacteria in the gut microbiota of healthy subjects (Vacca, M et al., The Controversial Role of Human Gut Lachnospiraceae (2020); Lozupone, CA et al., Diversity, stability and resilience of the human gut microbiota (2012)). *Lachnospiraceae* form one of the main producers of short-chain fatty acids (SCFAs). SCFAs are bacterial metabolites known to possess several anti-inflammatory and antioxidant effects (Koh, A et al., From Dietary Fiber to Host Physiology: Short-

Chain Fatty Acids as Key Bacterial Metabolites (2016)). *Lachnospiraceae* have been found to be decreased in people with Crohn's disease and ulcerative colitis (Vacca, M et al., The Controversial Role of Human Gut Lachnospiraceae (2020); Sasaki, K et al, Construction of a model culture system of human colonic microbiota to detect decreased Lachnospiraceae abundance and butyrogenesis in the feces of ulcerative colitis patients (2019)).

The genus *Anaerostipes* is among the top fifteen abundant genera in the human intestinal microbiome (Bui TPN et al., Mutual Metabolic Interactions in Co-cultures of the Intestinal *Anaerostipes rhamnosivorans* With an Acetogen, Methanogen, or Pectin-Degrader Affecting Butyrate Production (2019)). *Anaerostipes* are Gram-variable, obligate anaerobes. People with chronic kidney disease exhibit a considerably lower relative abundance of *Anaerostipes* spp. (Mazidi M et al, Adverse Impact of *Desulfovibrio* spp. and Beneficial Role of *Anaerostipes* spp. on Renal Function: Insights from a Mendelian Randomization Analysis (2020)).

Anaerostipes hadrus has been shown to produce butyrate (Allen-Vercor E et al., *Anaerostipes hadrus* comb. nov., a dominant species within the human colonic microbiota; reclassification of *Eubacterium hadrum* (1976)). Butyrate has been demonstrated to have a positive impact on gastrointestinal tract homeostasis, as it promotes the growth of intestinal epithelial cells, increases the expression of tight junction proteins, and acts as an anti-inflammatory agent (Hamer HM et al., The role of butyrate on colonic function (2008); Canani RB et al., Potential beneficial effects of butyrate in intestinal and extraintestinal diseases (2011)). Hence, butyrate-producing bacteria are generally thought to be health promoting. *A. hadrus*-like bacteria were found to be more abundant in the feces of healthy controls in a study concerning diarrhea-predominant irritable bowel syndrome (IBS-D) (Lyra A et al., Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification (2009)).

Recently, it was demonstrated that vitamins may modulate the human gut microbiome. WO2020/043797 discloses that vitamins can be useful to increase the growth of certain beneficial bacteria in the intestine. However, WO2020/043797 does not describe that vitamins can be used in combination with probiotics to increase the abundance of other beneficial bacteria. Moreover, the human gut is home to hundreds of different microbes, and it would be desirable to be able to selectively boost specific beneficial bacteria. In particular, it would be desirable to increase the abundance of *Firmicutes*, *Lachnospiraceae*, and *Anaerostipes* bacteria in the intestine to enhance wellness, improve health, and support the immune system.

SUMMARY OF THE INVENTION

The present invention relates to the following items:

- 1) Combination comprising: (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans*.
- 2) Combination according to item 1, wherein said combination comprises: (i) vitamin C or
5 vitamin B2, and (ii) *Bacillus coagulans* BC30.
- 3) Combination according to item 1 or item 2, wherein said combination is for simultaneous administration or delivery or consumption, and preferably wherein said combination is a fixed combination.
- 4) Combination according to item 1 or item 2, wherein said combination is for sequential
10 administration or delivery or consumption, and preferably wherein said combination is a free combination.
- 5) Combination according to any one of items 1-4, wherein said combination is an oral dosage form, and wherein said combination is more preferably a solid oral dosage form.
- 6) Combination according to any one of items 1-5, wherein said combination is for
15 administration or delivery to the large intestine.
- 7) Combination according to any one of items 1-6 for use as a medicament, a dietary supplement, or a food supplement.
- 8) Combination according to any one of items 1-7 for use in the treatment of a patient that is in need of increasing the abundance of Firmicutes, preferably *Lachnospiraceae*, in the large
20 intestine.
- 9) Combination for the use according to item 8, wherein said patient is in need of increasing the abundance of *Lachnospiraceae* and is suffering from ulcerative colitis or Crohn's disease.
- 10) Combination according to any one of items 1-7 for use in the treatment of a patient that is in need of increasing the abundance of *Anaerostipes* in the large intestine.
- 25 11) Combination for the use according to item 10, wherein said patient is suffering from chronic kidney disease.
- 12) Combination according to any one of items 1-7 comprising vitamin C and *Bacillus coagulans* for use in the treatment of a patient that is in need of increasing the abundance of *Anaerostipes hadrus* in the large intestine.
- 30 13) Combination for the use according to item 12, wherein said patient is suffering from irritable bowel syndrome.
- 14) Combination comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans* for use in increasing the abundance of *Firmicutes*, *Lachnospiraceae*, and/or *Anaerostipes* in the large

intestine of an animal, preferably a human, wherein said use comprises delivering the vitamin C or vitamin B2, and the *Bacillus coagulans* to the large intestine.

15) Combination comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans* for the use according to item 14, wherein the vitamin C or the vitamin B2, and the *Bacillus coagulans* are delivered to the large intestine by a delayed-release formulation.

16) Combination comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans* for the use according to item 14 or item 15, wherein said use comprises administering or delivering the vitamin C or the vitamin B2, and the *Bacillus coagulans* simultaneously and/or sequentially to the animal, preferably a human.

17) Combination comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans* for the use according to any one of items 14-16, wherein the animal, including a human, is experiencing a condition selected from ulcerative colitis, Crohn's disease, chronic kidney disease, or irritable bowel syndrome.

18) Combination comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans* for the use according to any one of items 14-17, wherein the *Bacillus coagulans* is *Bacillus coagulans* BC30.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A: Average levels (log cells/mL) of bacteria of the phylum *Firmicutes* in the lumen of the transverse colon upon treatment with *Bacillus coagulans* BC30 in the presence or absence of vitamin B2, at the end of the control period (n=3) and at the end of the treatment period (n=3). Statistically significant differences are indicated with '*' (p<0.05). **FIGURE 1B:** Average levels (log cells/mL) of bacteria of the phylum *Firmicutes* in the lumen of the transverse colon upon treatment with *Bacillus coagulans* BC30 in the presence or absence of vitamin C, at the end of the control period (n=3) and at the end of the treatment period (n=3). Statistically significant differences are indicated with '*' (p<0.05).

FIGURE 2A: Average levels (log cells/mL) of bacteria of the family *Lachnospiraceae* in the lumen of the transverse colon upon treatment with *Bacillus coagulans* BC30 in the presence or absence of vitamin B2 at the end of the control period (n=3) and at the end of the treatment period (n=3). Statistically significant differences are indicated with '*' (p<0.05). **FIGURE 2B:** Average levels (log cells/mL) of bacteria of the family *Lachnospiraceae* in the lumen of the transverse colon upon treatment with *Bacillus coagulans* BC30 in the presence or absence of vitamin C at the end of the

control period (n=3) and at the end of the treatment period (n=3). Statistically significant differences are indicated with '*' (p<0.05).

FIGURE 3: Average levels (log cells/mL) of *Anaerostipes hadrus* in the lumen of the transverse colon upon treatment with *Bacillus coagulans* BC30 in the presence or absence of vitamin C at the end of the control period (n=3) and at the end of the treatment period (n=3). Statistically significant differences are indicated with '*' (p<0.05).

DETAILED DESCRIPTION OF THE INVENTION

Firmicutes, *Lachnospiraceae*, and *Anaerostipes* are bacteria known for their beneficial effects on human health. The present inventors have found that vitamin C or vitamin B2 in combination with *Bacillus coagulans* can boost the growth of *Firmicutes*, *Lachnospiraceae*, and *Anaerostipes hadrus* bacteria in the large intestine, leading to an increase of *Firmicutes*, *Lachnospiraceae*, and *Anaerostipes hadrus* levels in the gut.

Hence, in a first aspect, the present invention relates to combinations comprising (i) vitamin C or vitamin B2 and (ii) *Bacillus coagulans*. Preferably, the *Bacillus coagulans* is the strain *Bacillus coagulans* BC30. The combination is for simultaneous and/or sequential administration.

Patent claims relating to a "combination" are product claims. The product of the present invention comprises two active ingredients: a vitamin (vitamin C or vitamin B2) and a probiotic (*Bacillus coagulans*). For simultaneous and/or sequential administration, see definitions and embodiments below.

Vitamin C, also known as L-ascorbic acid, is a water-soluble vitamin that is required for the biosynthesis of collagen, L-carnitine, and certain neurotransmitters. Vitamin C is also involved in protein metabolism. Further, vitamin C is an important physiological antioxidant. Vitamin C plays an important role in immune function and improves nutrient absorption. Vitamin C can be purchased from DSM GmbH. Alternative suppliers are, for example, TER Chemicals Distribution Group, BIOCHEM Bernburg GmbH, DVA International GmbH, Falken Trade GmbH, and Neupert Ingredients GmbH.

Vitamin B2 (also known as riboflavin) is one of the water-soluble B vitamins which is an essential component of two major coenzymes, flavin mononucleotide (FMN; also known as riboflavin-5'-

phosphate) and flavin adenine dinucleotide (FAD). These coenzymes play major roles in energy production; cellular function, growth, and development; and metabolism of fats, drugs, and steroids. Riboflavin can be purchased from DSM GmbH. Alternative suppliers are TER Chemicals Distribution Group, BIOCHEM Bernburg GmbH, DVA International GmbH, Falken Trade GmbH, and Neupert Ingredients GmbH.

The most common *Bacillus coagulans* strain is *Bacillus coagulans* BC30 (also known as *Bacillus coagulans* GBI-30, 6086; or "BC30"). BC30 can be purchased, for example, from KERRY (Kerry Group, Ireland), or from Ganeden Biotech, USA. GanedenBC30™ is contained in dietary supplements which are available, for example, from Schiff (Digestive Advantage, Daily Probiotic Capsule).

Alternative *Bacillus coagulans* strains are, for example, *Bacillus coagulans* MTCC 5856 and *Bacillus coagulans* 15B. *B. coagulans* MTCC 5856 has been in the market as a dietary ingredient for nearly two decades, under the trade name LactoSpore®. The active product *B. coagulans* MTCC 5856 is supplied, for example, by Sabinsa Corporation, Utah, USA. *Bacillus coagulans* 15B can be purchased, for example, from Green Jeeva, USA.

In one embodiment, the combination of the present invention is for simultaneous administration. Preferably, the combination for simultaneous administration is a fixed combination. However, for simultaneous administration, a free combination can also be used.

In another embodiment, the combination is for sequential administration. The combination for sequential administration is a free combination.

Preferably, the combination is an oral dosage form; more preferably, it is a solid oral dosage form.

The combination of the present invention can be, for example, a pharmaceutical combination or composition, a dietary supplement, or a food supplement.

In another aspect, the present invention relates to vitamin C or vitamin B2 and *Bacillus coagulans* (i.e., a combination of (i) vitamin C or vitamin B2 and (ii) *Bacillus coagulans*) for use as a medicament.

In one embodiment, the combinations of the invention (e.g. the pharmaceutical combinations) are for use in the treatment of a patient that is in need of increasing the abundance of *Firmicutes*, preferably *Lachnospiraceae* in the large intestine. Preferably, said patient is suffering from ulcerative colitis or Crohn's disease.

5

In another embodiment, the (pharmaceutical) combinations of the invention are for use in the treatment of a patient that is in need of increasing the abundance of *Anaerostipes* in the large intestine. Preferably, said patient is suffering from chronic kidney disease.

10 In another preferred embodiment, the (pharmaceutical) combinations of the invention are for use in the treatment of a patient that is in need of increasing the abundance of *Anaerostipes hadrus* in the large intestine. Preferably, said patient is suffering from irritable bowel syndrome. Preferably, the vitamin in the combination is vitamin C.

15 In a further aspect, the present invention relates to vitamin C or vitamin B2, and *Bacillus coagulans* (i.e., a combination of (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans*) for use in improving gut health in an animal. Said improvement comprises or consists of increasing the abundance of *Firmicutes*, *Lachnospiraceae* and/or *Anaerostipes* in the large intestine of said animal. Specifically, the vitamin C or vitamin B2 and *Bacillus coagulans* is for use in increasing the
20 abundance of *Firmicutes*, *Lachnospiraceae* and/or *Anaerostipes* in the large intestine (colon) of an animal, wherein said use preferably comprises delivering the vitamin C or vitamin B2 and *Bacillus coagulans* to the large intestine. Preferably, the animal is a human. Preferably, the *Bacillus coagulans* used is *Bacillus coagulans* BC30. In a preferred embodiment, the combination comprises vitamin C as the vitamin, and is for use in increasing the abundance of *Anaerostipes*
25 *hadrus*.

To achieve an increase of the abundance of *Firmicutes*, *Lachnospiraceae*, *Anaerostipes* and/or *Anaerostipes hadrus* in the large intestine, the vitamin C or vitamin B2 and *Bacillus coagulans* is preferably directly delivered to the large intestine. That is, the vitamin is delivered/ administered
30 in a manner such that the vitamin is not absorbed in the stomach and/or small intestine; rather the vitamin and the probiotic is delivered/ administered to the distal intestinal tract, preferably the large intestine (colon). This is preferably done by delivering/ administering the vitamin C or vitamin B2 and *Bacillus coagulans* in a delayed-release formulation. Oral administration is preferred.

In a preferred embodiment, the animal (including a human) is experiencing a condition selected from the group consisting of: ulcerative colitis, Crohn's disease, chronic kidney disease, and irritable bowel syndrome.

5 In another aspect, the present invention relates to a method of increasing the abundance of *Firmicutes*, *Lachnospiraceae*, *Anaerostipes* and/or *Anaerostipes hadrus* in the intestine, preferably the large intestine, comprising administering to the animal an effective dose of vitamin C or vitamin B2 and *Bacillus coagulans* (preferably, *Bacillus coagulans* BC30). The method is for improving intestinal health in an animal, including a human, wherein said improvement comprises
10 increasing the abundance of *Firmicutes*, *Lachnospiraceae*, *Anaerostipes* and/or *Anaerostipes hadrus* in the large intestine. Preferably, the animal is a human. Preferably, the vitamin C or vitamin B2 and the *Bacillus coagulans* is delivered directly to the large intestine. Delivery to the large intestine can be achieved by administering the vitamin C or vitamin B2 and the *Bacillus coagulans* as a delayed-release formulation.

15

The methods of the invention can be used to treat, prevent, and/or lessen the symptoms in an animal, including a human, in need thereof, of one or more of the following: ulcerative colitis, Crohn's disease, chronic kidney disease, and irritable bowel syndrome.

20 In a further aspect, the present invention relates to the use of vitamin C or vitamin B2 and *Bacillus coagulans* (i.e., a combination of (i) vitamin C or vitamin B2 and (ii) *Bacillus coagulans*) for increasing the abundance of *Firmicutes*, *Lachnospiraceae*, *Anaerostipes* and/or *Anaerostipes hadrus* in the large intestine of an animal, preferably a human, wherein said use comprises delivering the vitamin C or vitamin B2 and the *Bacillus coagulans* to the large intestine. Preferably,
25 the use comprises delivering the vitamin C or vitamin B2 and *Bacillus coagulans* to the large intestine by a delayed-release formulation. Preferably, the animal, including a human, is experiencing one or more condition(s) selected from the group consisting of: ulcerative colitis, Crohn's disease, chronic kidney disease, and irritable bowel syndrome.

30 In the combinations, uses, and methods of the invention, preferably, the vitamin C (ascorbic acid) dose is up to 2000 mg/day, preferably 100-2000 mg/day; more preferably 200-1000 mg/day. In one embodiment, vitamin C is dosed/ administered in an amount such that its local concentration in the colon is at least 0.05 g/L, preferably at least 0.1 g/L, most preferably at least 0.33 g/L. Preferred local concentrations in the colon range from about 0.05 g/L to about 1.5 g/L, more

preferably from about 0.1 g/L to about 1 g/L, most preferably from about 0.2 g/L to about 0.5 g/L.

In the combinations, uses, and methods of the invention, preferably, the Vitamin B2 dose is up to 200 mg/day, preferably 5-100 mg/day, more preferably from 10-50 mg/day. In one embodiment, vitamin B2 is dosed/ administered in an amount such that its local concentration in the colon is at least 0.001 g/L, preferably at least 0.01 g/L more preferably at 0.02 g/L. Preferred local concentrations in the colon range from about 0.001 g/L to about 0.5 g/L or from about 0.005 g/L to about 0.2 g/L, preferably about 0.01 to about 0.02 g/L.

The dosage of the *Bacillus coagulans* can be up to 5E+10 cfu/day. Preferably, the dosage range is from 1E+08 to 1E+10 cfu/day, more preferably from 1E+09 to 5E+10 cfu/day. Preferably, the *Bacillus coagulans* is *Bacillus coagulans* BC30.

Definitions and embodiments

As used throughout, the following definitions apply:

Patent claims relating to a “combination” or “pharmaceutical combination” are product claims. The product of the present invention comprises two active ingredients: a vitamin (vitamin C and/or vitamin B2) and a probiotic (*Bacillus coagulans*).

A “combination for simultaneous administration” or a “combination for simultaneous consumption” is a combination that is suitable for simultaneous administration or consumption, respectively. By “simultaneous administration” or “simultaneous consumption”, it is meant that the vitamin and the probiotic bacteria are administered/consumed on the same day (i.e., within 24 hours). Said two active ingredients can be administered/consumed at the same time (for fixed combinations) or one at a time (for free combinations). For example, the vitamin can be administered/consumed in one pill or tablet, while the probiotic is administered/consumed in another pill or tablet, wherein both pills/tablets are administered/consumed within 24 hours. In another example, the vitamin and the probiotic are formulated in the same composition and are administered/consumed at exactly the same time.

A “combination for sequential administration or consumption” is a combination that is suitable for sequential administration or consumption, respectively. By “sequential administration” or “sequential consumption”, it is meant that during a period of two or more days of continuous

treatment, only one of the vitamin and the probiotic is administered/consumed on any given day. By way of example, the vitamin can be administered/consumed on day one, and the probiotic is administered/consumed only the next day (i.e., after more than 24 hours), or even later. The active ingredients can be administered/consumed in any order.

5

A "fixed combination" is a combination that delivers both actives (i.e., the vitamin and the probiotic) at the same time to a patient. A solid oral dosage form (e.g., a tablet or capsule) comprising both, the vitamin and the probiotic, is an example of a fixed combination. A liquid oral dosage form (e.g., oral drops) comprising both, the vitamin and the probiotic, is another example of a fixed combination.

10

A "free combination" is a combination that allows to administer/consume both actives (i.e., the vitamin and the probiotic) separately, i.e. one at a time. Treatment regimens in which the vitamin and the probiotic are not administered/consumed by the same route and/or are not administered/consumed at the same time require free combinations.

15

Simultaneous administration/consumption can be done both by using a fixed combination and a free combination. Sequential administration/consumption requires a free combination; fixed combinations are not suitable for sequential administration/consumption. Hence, free combinations are more versatile: they are suitable for sequential administration/consumption and - if both actives are administered/consumed on the same day - also for simultaneous administration/consumption. Fixed combinations are only suitable for simultaneous administration/consumption if both ingredients (i.e., the vitamin and the probiotic) are to be administered/consumed at the same time of the same day; if, however, the vitamin and the probiotic are to be administered/consumed on the same day but separately, fixed combinations are not suitable.

20

25

By "separate administration/consumption", it is meant that the vitamin and the probiotic are administered one at a time. Thus, separate administration/consumption can refer to both sequential administration/consumption and - when referring to the administration/consumption of both actives on the same day but one at a time - also to simultaneous administration/consumption.

30

"Administering" or "administration" means to give or to deliver an active to a human or animal; likewise, the human or animal can take (consume) the active.

The term "vitamin C" which can be used interchangeably with "ascorbic acid" also includes pharmaceutically acceptable salts thereof (e.g., sodium ascorbate and calcium ascorbate) and pharmaceutically acceptable esters thereof (in particular ascorbyl palmitate) and other pharmaceutically acceptable forms.

The term "vitamin B2", which is used interchangeably with "riboflavin", includes riboflavin and esters thereof, in particular riboflavin-5'-phosphate and other pharmaceutically acceptable forms.

To "increase the abundance" of *Firmicutes*, *Lachnospiraceae*, *Anaerostipes*, or *Anaerostipes hadrus* means to increase the level (or the amount, or number, or the population size) of *Firmicutes*, *Lachnospiraceae*, *Anaerostipes*, or *Anaerostipes hadrus* compared to the respective control (i.e., the level/ amount/ number/ population size of *Firmicutes*, *Lachnospiraceae*, *Anaerostipes*, or *Anaerostipes hadrus* when the combination of vitamin C and *Bacillus coagulans* has not been added).

The term "intestine" (or "gut") as used herein refers to the portion of the gastrointestinal tract consisting of the small intestine and the large intestine. The "large intestine" (*intestinum crassum*) is the lower part of the gastrointestinal tract and is also referred to herein as "colon".

"Direct delivery" or "directly delivered" means that the vitamin is formulated in a manner such that the vitamin is not absorbed in the stomach and/or small intestine; rather the vitamin is made available in the distal intestinal tract, preferably the large intestine (colon), where it is available to the microbiome. The vitamin is not part of a person's usual daily nutritional requirements (generally obtained through diet and conventional vitamin supplementation), and is administered in excess thereof. For human use, the preferred method according to the present invention is through a form which delays release until the large intestinal tract (colon) is reached. Alternatively, a large enough dose can be administered, so that only a portion of the administered vitamin is absorbed in the proximal small intestine, and the remainder, which is an effective dose, is available to the large intestinal tract; although not preferred, the latter method of delivery can be used for humans as well. With respect to the probiotic, "direct delivery" or "directly delivered" means that the probiotic is formulated in a manner such that it is not released in the stomach and/or small intestine but rather it is made available in the distal intestinal tract, preferably the large intestine (colon).

A used herein, "delayed release" refers to the release of the vitamin and/or the probiotic at a time later than immediately after administration. Preferably, "delayed release" means delivery of the vitamin (and/or probiotic), upon oral administration, to the large intestine (colon) in a delayed manner relative to an immediate release formulation.

An "enteric layer" or "enteric coating" is a layer surrounding a core, wherein the core comprises the active agent and the layer confers resistance to gastric juice.

"Prevent" can include lessening the risk of an adverse condition occurring, lessening the symptoms of an adverse condition, lessening the severity of an adverse condition, and prolonging the time for occurrence of an adverse condition.

"Oral formulation" means that the vitamin and/or probiotic is formulated for oral administration/consumption.

"Co-administering" or "co-administration" means that the vitamin and/or the probiotic is delivered/administered/ consumed simultaneously (i.e., together), or separately but within a time frame of 24 hours. The vitamin can be delivered/ administered/ consumed first. Likewise, the probiotic can be delivered/ administered/ consumed first.

Doses

Preferably, vitamin C is administered in an amount such that its local concentration in the colon is at least 0.05 g/L, preferably at least 0.1 g/L, most preferably at least 0.33 g/L. Preferred local concentrations in the colon range from about 0.05 g/L to about 1.5 g/L, more preferably from about 0.1 g/L to about 1 g/L, most preferably from about 0.2 g/L to about 0.5 g/L. Specific dosages per day can range up to 2000 mg/day, preferably 100-2000 mg/day; more preferably 200-1000 mg/day.

Preferably, vitamin B2 can be administered in an amount such that its local concentration in the colon is at least 0.001 g/L, preferably at least 0.01 g/L more preferably at 0.02 g/L. Preferred local concentrations in the colon range from about 0.001 g/L to about 0.5 g/L or from about 0.005 g/L to about 0.2 g/L, preferably about 0.01 to about 0.02 g/L. Specific dosages per day can range up to 200 mg/day, preferably 5-100 mg/day, more preferably from 10-50 mg/day.

The dosage of the probiotic can be up to 5×10^{10} cfu/day. Preferably, the dosage range of the probiotic is from 1×10^8 to 1×10^{10} cfu/day, more preferably from 1×10^9 to 5×10^{10} cfu/day.

5 Formulations

The vitamin (vitamin C or vitamin B2) and/or the probiotic (*Bacillus coagulans*), preferably both, is (are) preferably present in a formulation which allows the vitamin (and/or probiotic) to be available predominantly in the large intestine.

- 10 Oral formulations are preferred. Other formulations include non-oral routes, such as via suppositories or injections.

For human use, the preferred method is through a delayed-release form which delays delivery until the intestinal tract is reached. For non-human animals, a preferred delivery includes a
15 method of administering a large enough dose so that only a portion of the vitamin and/or probiotic delivered is absorbed in the stomach, and the remainder, which is an effective dose, is available to the intestinal tract; although not preferred, this method of delivery can be used for humans as well.

- 20 Delayed-release formulations are known in the art. Preferably, the delayed-release formulations have an enteric coating (also referred to as enteric layer).

In one embodiment of the present invention, the vitamin and/or probiotic, preferably both, is in a formulation comprising an enteric capsule, filled with a composition comprising the vitamin and/or
25 probiotic. The enteric capsule confers resistance against the acidic environment of the stomach. For example, soft gel formulations may deliver the active agent in solution and yet offer advantages of solid dosage forms.

In another embodiment, the formulation is a tablet comprising (i) a core comprising the vitamin
30 and/or the probiotic, and (ii) a delayed-release coating such as an enteric coating. This may be a hard gel capsule.

Alternatively, for direct colon delivery, a matrix-based delivery system can be used. Matrix based systems have no discrete layer of coating material, but the active agent (i.e., the vitamin and/or

the probiotic) is more or less homogenously distributed within the matrix. Further, there are colon-release systems that embed the active agent in e.g. in a fiber matrix (enzyme-triggered) and an enteric coating on top.

- 5 The release of the vitamin and/or probiotic may be delayed until the small intestine. In another embodiment, the release is delayed until the distal small intestine. In yet another, preferred embodiment, the release of the vitamin and/or probiotic is delayed until the colon (large intestine).

10 In a preferred embodiment for humans, the vitamin and/or probiotic is formulated in a solid dosage form for oral administration. The formulation may be in the form of a capsule, pellet, bead, sphere, mini spheres, tablet, mini tablet, or granule, optionally coated with a delayed release coating that prevents the release of the active agent before the small intestine, preferably before the colon.

Coating, or matrix materials for the delayed release of the vitamin and/or probiotic, in particular
15 for targeted release in the ileum or the large intestine upon oral administration are known in the art. They can be subdivided into coating materials that disintegrate above a specific pH, coating materials that disintegrate after a specific residence time in the gastrointestinal tract and coating materials that disintegrate due enzymatic triggers specific to the microflora of a specific region of the intestines. Coating materials from different categories are commonly used in combinations.
20 Coating materials of the different categories for targeting to the large intestine have been reviewed for example in Bansal et al. (Polim. Med. 2014, 44, 2, 109-118). In one embodiment of the present invention, the delayed-release coating comprises at least one component selected from coating materials that disintegrate pH-dependently, coating materials that disintegrate time-dependently, coating materials that disintegrate due to enzymatic triggers in the intestinal environment (e.g., in
25 the intestinal environment of the ileum and the large intestine), and combinations thereof.

Coating materials that disintegrate pH-dependently include polyvinyl acetate phthalate, cellulose acetate trimellitate, hydroxypropyl methylcellulose phthalate HP-50, HP-55 or HP-55S, cellulose acetate phthalate, shellac, hydroxypropyl methylcellulose acetate succinate (HPMCAS),
30 poly(methacrylic acid, ethyl acrylate) 1:1 (Eudragit® L100-55, Eudragit® L30D-55), poly(methacrylic acid, methyl methacrylate) 1:1 (Eudragit® L-100, Eudragit® L12.5), poly(methacrylic acid, methyl methacrylate) 1:2 (Eudragit® S-100, Eudragit® S12.5, and Eudragit® FS30D). Coating materials that disintegrate time-dependently include Eudragit® RL, Eudragit®RS, and ethylcellulose. Coating materials that disintegrate due to enzymatic triggers in

the large intestinal environment include chondroitin sulfate, pectin, guar gum, chitosan, inulin, lactulose, raffinose, stachyose, alginate, dextran, xanthan gum, locust bean gum, arabinogalactan, cyclodextrin, pullulan, carrageenan, scleroglucan, chitin, curdulan, levan, amylopectin, starch, amylose, resistant starch, and azo compounds being degraded by azo bonds splitting bacteria.

The following non-limiting examples are presented to better illustrate the invention.

EXAMPLES

The aim of this study was to investigate the effect of a combination of vitamin C and *Bacillus coagulans*, and a combination of vitamin B2 and *Bacillus coagulans* on the composition of the gut microbiota in a long-term continuous fermentation experiment.

Materials and Methods

Design of the long-term SHIME fermentation experiment (Colon Model)

The typical reactor setup of the SHIME®, representing the gastrointestinal tract of the adult human, was described by Molly et al. (1993) Applied Microbiology and Biotechnology 39(2):254-258. Inoculum preparation, retention time, pH, temperature settings and reactor feed composition were previously described by Possemiers et al. (2004) FEMS Microbiol Ecol. 49(3):495-507. Compared to the typical setup of the SHIME, the long-term SHIME experiment used for this example included some adaptations. In one reactor, at first, the conditions of the stomach were simulated, before being changed by the computer to conditions simulating the small intestine. The suspension was then added to the colonic reactors mimicking the transverse colon (pH 6.15-6.4; retention time = 32h; volume of 800 mL).

The SHIME® experiment for this study consisted of three stages:

1. Stabilization period: After inoculation of the colon reactors with an appropriate fecal sample, a two-week stabilization period allowed the microbial community to differentiate in the different reactors depending on the local environmental conditions. During this period the basic nutritional matrix was provided to the SHIME to support the maximum diversity of the gut microbiota originally present in the faecal inoculum.
2. Control period: During this two-week reference period, the standard SHIME nutrient matrix was further dosed to the model for a period of 14 days. Analysis of samples in this period

allowed to determine the baseline microbial community composition and activity in the different reactors, which is used as a reference for results obtained during the treatment.

3. Treatment: During this three-week period, the SHIME was operated under nominal conditions, but the appropriate probiotic strain and vitamin(s) were supplemented to the appropriate reactors. The probiotic strain was added to the reactors at a concentration of 1×10^{10} CFU/reactor. Vitamin B2 (Riboflavin, DSM) and vitamin C (Ascorbic acid, DSM) were added to the reactors at a dose of 10 mg/day and 200 mg/day, respectively.

The probiotic strain used in this experiment was *Bacillus coagulans* GBI-30, 6086, also known as BC30, and was purchased from Schiff (Digestive Advantage, Daily Probiotic Capsule). The probiotic was isolated from the capsule and added as an overnight grown pure culture.

Quantitative Microbial Community Analysis by 16S rRNA Gene Sequencing and Flow Cytometry

Samples for quantitative 16S-targeted Illumina sequencing were collected 3x/week during the last week of the control and treatment period. Next-generation 16S rRNA gene amplicon sequencing of the V3–V4 region was performed by LGC Genomics GmbH (Berlin, Germany) on samples from the medium-term SHIME experiment. Library preparation and sequencing were performed on an Illumina MiSeq platform with v3 chemistry. The 341F (50-CCTACGGGNGGCWGCAG-30) and 785R (50-GACTACHVGGGTATCTAAKCC-30) primers were used as described by De Paepe et al. (2017) with the reverse primer being adapted to increase coverage. Quality control PCR was conducted using Taq DNA Polymerase with the Fermentas PCR Kit according to the manufacturers' instructions (Thermo Fisher Scientific, Waltham, MA, USA). The DNA quality was verified by electrophoresis on a 2% (w/v) agarose gel for 30 min at 100 V. Bioinformatics analysis of amplicon data was performed as described by De Paepe et al. (2017). The obtained high-resolution proportional phylogenetic information (i.e., proportional abundances (%)) was combined with an accurate quantification of total bacterial cells via flow cytometry to obtain quantitative data at phylum, family, and species level. This was done by multiplying the proportional abundances with absolute cell numbers (cells/mL) obtained via flow cytometry. For flow cytometry analysis, 10-fold serial dilutions were prepared in Dulbecco's phosphate-buffered Saline (DPBS) (Sigma-Aldrich, Bornem, Belgium) of all samples and stained with 0.01mM SYTO24 (Life Technologies Europe, Merelbeke, Belgium) for 15' at 37°C in the dark. Samples were analyzed on a BD FACSVerse (BDBiosciences, Erembodegem, Belgium) using the high-flow-

rate setting and bacteria were separated from medium debris and signal noise by applying a threshold level of 200 on the SYTO channel.

Results

5 Supplementation of a combination of *Bacillus coagulans* BC30 and vitamin B2 increased abundance of Firmicutes

As can be taken from Figure 1A, supplementation of *Bacillus coagulans* BC30 alone did not significantly change the abundance of *Firmicutes* compared to the control. In contrast, the combination of *Bacillus coagulans* BC30 and vitamin B2 significantly increased the abundance of Firmicutes compared to the control.

Supplementation of a combination of *Bacillus coagulans* BC30 and vitamin C increased abundance of Firmicutes

As can be taken from Figure 1B, supplementation of *Bacillus coagulans* BC30 alone did not significantly change the abundance of Firmicutes compared to the control. In contrast, the combination of *Bacillus coagulans* BC30 and vitamin C significantly increased the abundance of Firmicutes compared to the control.

20 Supplementation of a combination of *Bacillus coagulans* BC30 and vitamin B2 increased abundance of *Lachnospiraceae*

As can be taken from Figure 2A, supplementation of *Bacillus coagulans* BC30 alone did not significantly change the abundance of *Lachnospiraceae* compared to the control. In contrast, the combination of *Bacillus coagulans* BC30 and vitamin B2 significantly increased the abundance of *Lachnospiraceae* compared to the control.

25 Supplementation of a combination of *Bacillus coagulans* BC30 and vitamin C increased abundance of *Lachnospiraceae*

As can be taken from Figure 2B, supplementation of *Bacillus coagulans* BC30 alone did not significantly change the abundance of *Lachnospiraceae* compared to the control. In contrast, the combination of *Bacillus coagulans* BC30 and vitamin C significantly increased the abundance of *Lachnospiraceae* compared to the control.

Supplementation of a combination of *Bacillus coagulans* BC30 and vitamin C increased abundance of *Anaerostipes hadrus*

As can be taken from Figure 3, supplementation of *Bacillus coagulans* BC30 alone did not significantly change the abundance of *Anaerostipes hadrus* compared to the control. In contrast,
5 the combination of *Bacillus coagulans* BC30 and vitamin C significantly increased the abundance of *Anaerostipes hadrus* compared to the control.

Claims

1. Combination comprising:
 - i) vitamin C or vitamin B2, and
 - 5 ii) *Bacillus coagulans*.
2. Combination according to claim 1, wherein said combination comprises:
 - i) vitamin C or vitamin B2, and
 - 10 ii) *Bacillus coagulans* BC30.
3. Combination according to claim 1 or claim 2, wherein said combination is for simultaneous administration or consumption.
4. Combination according to claim 1 or claim 2, wherein said combination is for sequential
15 administration or consumption.
5. Combination according to any one of claims 1-4, wherein said combination is an oral dosage form, and wherein said combination is more preferably a solid oral dosage form.
- 20 6. Combination according to any one of claims 1-5, wherein said combination is for administration to the large intestine.
7. Combination according to any one of claims 1-6 for use as a medicament, a dietary supplement, or a food supplement.
- 25 8. Combination according to any one of claims 1-7 for use in the treatment of a patient that is in need of increasing the abundance of *Firmicutes*, preferably *Lachnospiraceae*, in the large intestine.
- 30 9. Combination for the use according to claim 8, wherein said patient is in need of increasing the abundance of *Lachnospiraceae* and is suffering from ulcerative colitis or Crohn's disease.
- 35 10. Combination according to any one of claims 1-7 for use in the treatment of a patient that is in need of increasing the abundance of *Anaerostipes* in the large intestine.

11. Combination for the use according to claim 10, wherein the combination comprises vitamin C and *Bacillus coagulans*, further wherein the said patient is in need of increasing the abundance of *Anaerostipes hadrus*, and is suffering from irritable bowel syndrome.

5

12. Combination comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans* for use in increasing the abundance of *Firmicutes*, *Lachnospiraceae* and/or *Anaerostipes* in the large intestine of an animal, preferably a human, wherein said use comprises delivering the vitamin C or vitamin B2, and the *Bacillus coagulans* to the large intestine.

10

13. Combination comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans* for the use according to claim 12, wherein the vitamin C or the vitamin B2, and the *Bacillus coagulans* are delivered to the large intestine by a delayed-release formulation.

15

14. Combination comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans* for the use according to claim 12 or claim 13, wherein said use comprises administering the vitamin C or the vitamin B2, and the *Bacillus coagulans* simultaneously and/or sequentially to the animal, preferably a human.

20

15. Combination comprising (i) vitamin C or vitamin B2, and (ii) *Bacillus coagulans* for the use according to any one of claims 12-14, wherein the animal, including a human, is experiencing a condition selected from ulcerative colitis, Crohn's disease, chronic kidney disease, and irritable bowel syndrome.

Fig. 1A

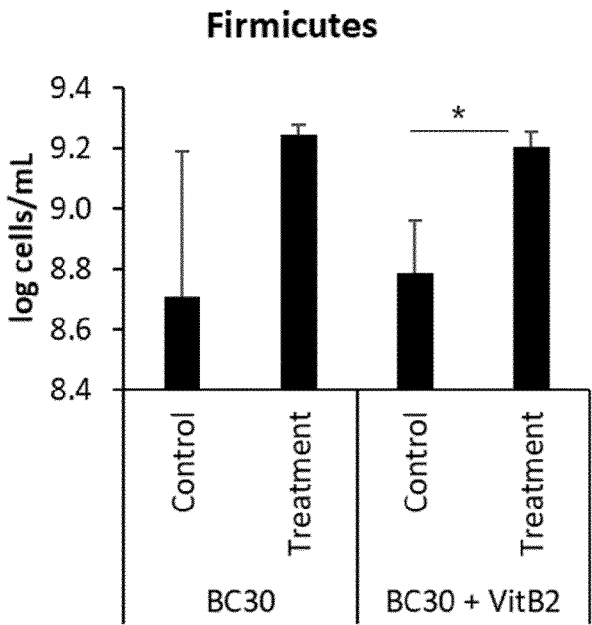


Fig. 1B

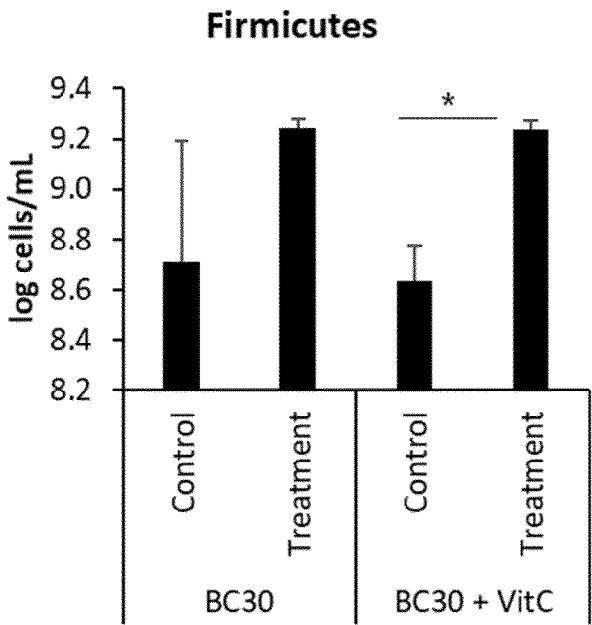


Fig. 2A

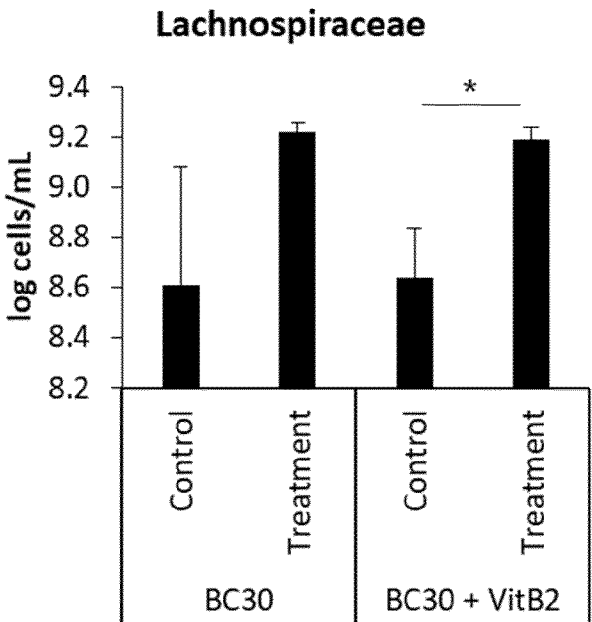


Fig. 2B

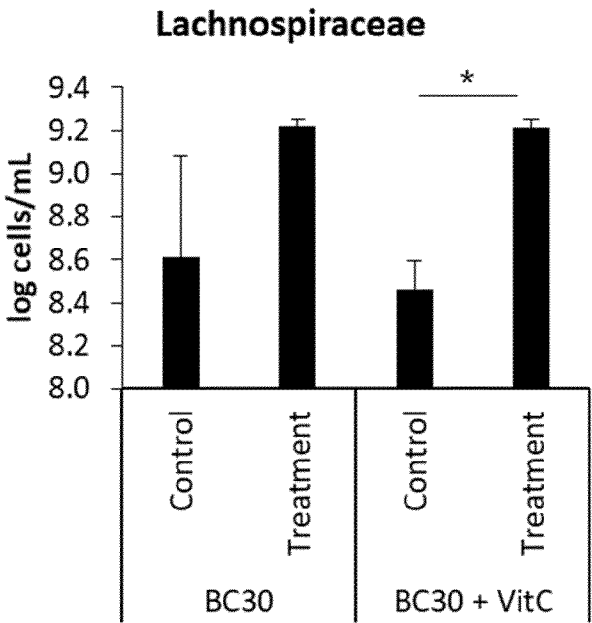
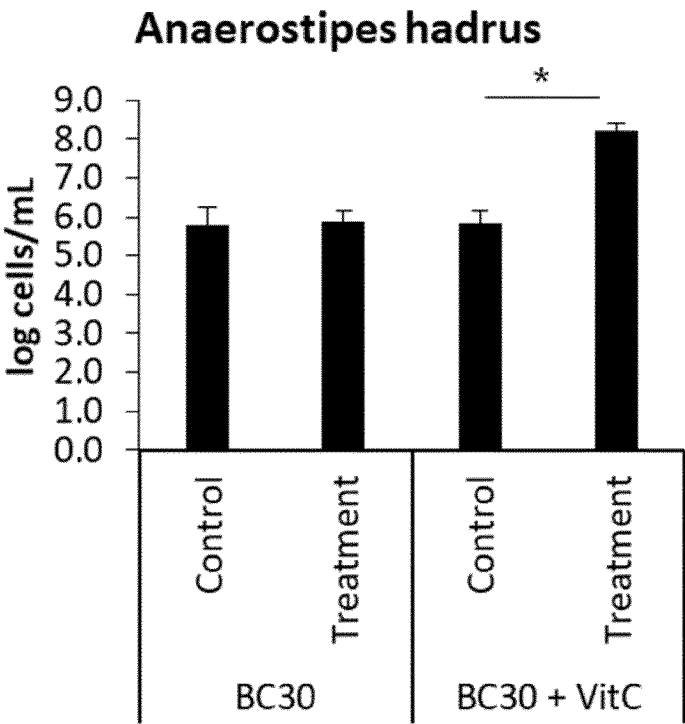


Fig.3



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2023/065396

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	A61K35/742	A61P1/00
	A61K31/525	A61P13/12
		A61P1/04
		A61P37/00
		A61K31/375
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)		
A61K A61P		
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, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 109 222 106 A (GUANGZHOU LAIKEFU BIOTECHNOLOGY CO LTD) 18 January 2019 (2019-01-18) claims 1-10	1-15

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☒ 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		Date of mailing of the international search report
12 September 2023		18/09/2023
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 Rodrigo-Simón, Ana

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2023/065396

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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	claims 1-8	4, 8-15
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INTERNATIONAL SEARCH REPORT

International application No

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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ALAM MOHAMMAD TAUQEER ET AL: "Microbial imbalance in inflammatory bowel disease patients at different taxonomic levels", GUT PATHOGENS, vol. 12, no. 1, 1 December 2020 (2020-12-01), XP093080045, DOI: 10.1186/s13099-019-0341-6 Retrieved from the Internet: URL:https://gutpathogens.biomedcentral.com/counter/pdf/10.1186/s13099-019-0341-6.pdf > page 10, paragraph 2 - page 12, paragraph 3 -----	1-15

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Information on patent family members

International application No

PCT/EP2023/065396

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			BR 112021003633 A2 27-07-2021
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