

SELECTION AND USE OF MELATONIN SUPPORTING BACTERIA TO REDUCE INFANTILE COLIC

FIELD OF THE INVENTION

5 The invention herein provides certain strains of *Lactobacillus reuteri* bacteria selected for their ability of increasing melatonin levels for prophylaxis and/or treatment of colic (or other diseases associated with reduced levels of melatonin), including the ability to produce precursors and other important components for melatonin release, a method of selecting such strains, and products containing such strains. Moreover this invention relates to preparations
10 comprising the substrate component AMP being specifically chosen to enhance the efficacy of such strains.

BACKGROUND OF THE INVENTION

 Despite its salience in terms of both prevalence and distress the nature and causes of infantile colic have remained poorly understood. There are also different terms used to
15 describe the condition. These terms include, “infantile colic”, “evening colic” because the pain is mainly confined to evening, and “three months colic” under the pretext that it disappears after about three months after birth. Different authors have used different definitions. The definition of Wessels that colic is paroxysms of crying for three or more hours per days for three days or more per week during a period of at least three weeks has
20 been mostly accepted in the literature. In 2006, the Rome III criteria was published modifying this criteria to consider the diagnosis of “infantile colic” applicable to infants with paroxysms of irritability, fussing, or crying that start and stop without obvious cause, lasting 3 or more hours per day and occurring at least 3 days per week, but for at least 1 week (sometimes referred to as modified Wessel’s criteria). Further fine-tuned in the Rome IV criteria;
25 (Benninga et al; Gastroenterology 2016;150:1443–1455). Either of these definitions (Rome III or Rome IV) is relevant to the present invention.

 To date, the main discussed possible causative factors of infantile colic have been divided into: psychosocial, gastrointestinal, including microbial dysbiosis, and neuro-
developmental disorders.

30 Psychosocial factors include: variants of normal crying, behavioural effects of atypical parenting and manifestation of problems in parent-infant interaction.

Gastrointestinal disorders have been implicated in colic because of the infant's leg position and grimacing during a crying spell. The gastrointestinal factors are briefly reviewed below:

Improper feeding techniques like bottle feeding, feeding in a horizontal position and lack of burping post-feeding have been considered as causative factors. Breast-feeding in the first six months has been found to be protective factor. The risk of infantile colic is 1.86 times higher among non-breast fed infants (Saavedra MA, Dacosta JS, Garcias G, Horta BL, Tomasi E, Mendoca R. Infantile colic incidence and associated risk factors: a cohort study. *Pediatr (Rio J)* 2003; 79(2): 115-122).

At present there is no complete cure for colic. The current treatment paradigm for colic consists of either pharmacological and/or non-pharmacological methods, providing at best reduction of symptoms. Typical therapeutic interventions for colic offered to parents fall within four categories, including, dietary, physical, behavioral and pharmacological. Dietary manipulations include professional advice on various feeding techniques, or the use of hypoallergenic milk, soy or lactose free formulas, food supplements such as various probiotics and prebiotics and an early introduction to solids.

A non-prescription medication for treatment of colic has largely included the administration of simethicone or dimethylpolysiloxane, a non-absorbable, over-the-counter drug, which reduces the size of intestinal gas bubbles. Simethicone has a safe profile and is frequently recommended, despite several studies demonstrating that effectiveness of Simethicone on infantile colic is no better than placebo.

There have been some reports implicating an altered microbiota-pattern in colicky infants, making several researchers to perform and publish different randomized clinical controlled trials (RCTs) where the ability of for example *Lactobacillus reuteri* DSM 17938 to reduce crying time in these infants has been evaluated. For example, Savino et al (*Pediatrics* 2010;126:e526–33) conducted an RCT in 2010 to test the efficacy of this strain on infantile colic. Fifty exclusively breastfed colicky infants, diagnosed according to modified Wessel's criteria, were randomly assigned to receive either *L reuteri* DSM 17938 (10^8 colony-forming units) or placebo daily for 21 days. Parental questionnaires monitored daily crying time and adverse effects. Forty-six infants (*L reuteri* group: 25; placebo group: 21) completed the trial. Daily crying times in minutes/day (median) were 370 vs 300 on day 0 and 35.0 vs 90.0 ($p=.022$) on day 21.

In 2013 Szajewska et al (*J Pediatr* 2013;162:257–62) published with a similar design an RCT in 80 infants aged <5 months, identifying that the rate of responders to treatment was

significantly higher in the probiotic group compared with the placebo group. Minutes/day (median) were 180 vs 180 on day 7, 75 vs 128 on day 14 and 52 vs 120 ($p<0.05$) on day 21. There have also been several other studies showing the effect of *L. reuteri* DSM 17938 in colicky infants in significantly reducing crying-time in breastfed infants. There is however a need for even more effective interventions to reduce the crying at a higher rate in children with colic, including at a higher rate in formula-fed children.

Therefore, there currently is a need for new (and preferably improved) safe and effective compounds and compositions and techniques that prove useful for treating colic in infants. The compositions and methods of the present invention, respond to this need, providing products that can safely and effectively prevent and treat colic (or the symptoms associated with colic) in infants.

Other objects and advantages will be more fully apparent from the following disclosure.

SUMMARY OF THE INVENTION

New-borns hormone immaturity is implicated to be a major factor in early problems of the development of gut motility and thereby also causing pain and crying of the infant, such as in infantile colic. Melatonin is a hormone that has the ability to support biological rhythms and has important effects on many functions in humans and animals. A precursor to melatonin is serotonin, a neurotransmitter itself that can be derived from the amino acid tryptophan.

Melatonin is a neurotransmitter previously thought to be secreted predominantly by the pineal gland. There are however extra-pineal sites of melatonin production, such as the retina and the gastrointestinal tract.

Synchronization between production and the different circadian rhythms and the following release of serotonin and melatonin in young infants is potentially important to avoid gut motility disturbances and pain, such as infantile colic (IC).

Serotonin increases intestinal smooth muscle contraction, and melatonin relaxes intestinal smooth muscles. The serotonin concentration peaks can cause intestinal cramps if not balanced by other agents, whereof melatonin is an important one. Both serotonin and melatonin have circadian rhythms. While there is serotonin produced and serotonin circadian rhythms developed when an infant is born, the endogenous melatonin production and melatonin circadian rhythm is in many infants not ready to begin until after the 3rd month in life. This time coincides with the time of normal disappearance of infantile colic.

It can be said that infantile colic is a condition of melatonin-deficiency. It has therefore been suggested to give exogenous melatonin to infants with IC, but many physicians and parents are reluctant to give hormones to an infant.

Of all new-born children there is an estimated prevalence of 5% to 25% of IC which is in several ways burdensome and costly for society, the parents and the infant itself.

Thus, although some children appear able to produce and release needed melatonin from their food and/or their intestinal bacteria and are therefore less likely to develop infantile colic, there are many others who do not, and thus need added support to prevent or treat IC.

There is thus a need to find and develop products and methods to support melatonin production and release in children at risk of developing IC or having IC.

The present invention is based on the finding that bacteria, for example lactic acid bacteria, can be selected which are capable of producing adenosine. Lactic acid bacteria producing adenosine have not been reported in the art and such bacteria can for example be used to increase the production of arylalkylamine- N-acetyltransferase (AANAT), the rate-limiting enzyme in melatonin synthesis. Although the AANAT enzyme is found in the pineal gland it is also produced at the extra-pineal sites of melatonin production, such as in the gastrointestinal tract. Thus, an increase in AANAT production (for example via an increase in adenosine production) provides a means of increasing melatonin production or increasing melatonin levels in the gastrointestinal tract. Such bacteria can thus be used in the treatment or prevention of infantile colic or other diseases associated with or characterised by reduced, low or deficient melatonin production or synthesis.

Embodiments of the present invention are defined in the claims.

In one aspect, the present invention provides a bacterial strain, wherein said strain is *Lactobacillus reuteri* DSM 32846, DSM 32847, DSM 32849 or DSM 33198.

In another aspect, the present invention provides a bacterial strain of the invention for use in therapy.

In another aspect, the present invention provides a bacterial strain of the invention for use in the treatment or prevention of infantile colic in a subject.

In another aspect, the present invention provides a method for the selection of a lactic acid bacterial strain capable of producing adenosine, wherein said strain is *Lactobacillus reuteri* and wherein said method comprises:

- a) screening a lactic acid bacterial strain for the presence of a gene encoding a 5'-nucleotidase, wherein said strain is *Lactobacillus reuteri*; and/or

- b) screening a lactic acid bacterial strain, or its supernatant, for the presence of an active 5'-nucleotidase enzyme, wherein said strain is *Lactobacillus reuteri*; and
- c) selecting a *Lactobacillus reuteri* strain which produces adenosine at a level which is increased as compared to *Lactobacillus reuteri* DSM 17938; and optionally
- d) culturing or propagating such a *Lactobacillus reuteri* strain.

In another aspect, the present invention provides a combination product suitable for increasing the production of melatonin or adenosine in a subject comprising:

- (i) a first *Lactobacillus reuteri* strain capable of inducing the production of melatonin or producing adenosine, wherein said strain is a strain of the present invention; and
- (ii) a substrate component to increase or enhance the induction of production of melatonin and/or the production of adenosine by said *Lactobacillus reuteri* strain, wherein said substrate component is AMP.

Thus, the bacterial strains of the invention are capable of producing adenosine and may be used in the production of adenosine (e.g. increasing levels of adenosine or increasing or promoting the production of adenosine) in a subject, or in the production of melatonin (e.g. increasing levels of melatonin or increasing or promoting the production of melatonin) in a subject.

Adenosine or melatonin may be produced (e.g. levels of adenosine or melatonin may be increased, or the production of adenosine or melatonin may be increased or promoted) in a subject, by administering an effective amount of a bacterial strain of the invention, which is capable of producing adenosine, to said subject.

The bacterial strains of the invention are capable of inducing the production of melatonin. Said bacterial strains can thus be used for the production of melatonin, for example for increasing the production or levels of melatonin, in a subject.

Melatonin may be produced (e.g. levels of melatonin may be increased or the production of melatonin may be increased or promoted) in a subject, by administering an effective amount of a lactic acid bacterial strain of the invention which is capable of inducing the production of melatonin to said subject.

As discussed above, said strains can be used in the treatment or prevention of infantile colic, or other diseases associated with or characterised by reduced, low or deficient melatonin production or synthesis or levels.

Thus the lactic acid bacterial strains of the invention which are capable of inducing the production of melatonin may be used in the treatment or prevention of infantile colic, or other

diseases associated with or characterised by reduced, low or deficient melatonin production or synthesis or levels in a subject.

Infantile colic, or other disease associated with or characterised by reduced, low or deficient melatonin production or synthesis or levels, in a subject, may be treated or prevented by administering an effective amount of a lactic acid bacterial strain of the invention which is capable of inducing the production of melatonin to said subject.

Said strains of the invention are capable of producing adenosine and thereby inducing the production (for example downstream production) of melatonin as described elsewhere herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic showing the melatonin production pathway.

Figure 2 is a schematic showing how a bacteria capable of producing adenosine of the invention affects the melatonin production pathway.

Figure 3 shows the measurement of 5'-nucleotidase activity in the supernatant of *L. reuteri* bacterial cells.

Figure 4 shows the melatonin production in an intestinal enteroid model by the addition of supernatant of *L. reuteri* bacterial cells.

Figure 5A shows the measurement of 5'-nucleotidase activity in the supernatant of *L. reuteri* DSM 33198 bacterial cells as compared to DSM 17938 bacterial cells. **Figure 5B** shows the measurement of 5'-nucleotidase activity in the supernatant of various *L. reuteri* bacterial cells as compared to DSM 17938 bacterial cells.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS THEREOF

Thus, as outlined above, the present invention provides bacterial strains which are capable of stimulating or inducing or supporting the production of melatonin, for example to increase levels of melatonin, particularly in the gastrointestinal (GI) tract.

Said strains can indirectly result in the production of melatonin, for example by producing or stimulating or inducing the production of an upstream component of the melatonin synthesis pathway (or a precursor of melatonin) and thereby supporting or

increasing melatonin production. A preferred example would be the production or induction of arylalkylamine-N-acetyl transferase (AANAT), which is the rate limiting enzyme in the conversion of serotonin to N-acetyl serotonin, which in turn gets converted into melatonin.

As outlined above, a lactic acid bacterial strain of the invention is one which can produce adenosine in a subject, particularly in the gastrointestinal (GI) tract. Such adenosine can act on appropriate cell surface adenosine receptors (for example A2A receptors, A2B receptors, A3 or A4 receptors, preferably A2A receptors) and result in the production of elevated levels of intracellular cAMP. A2A and other adenosine receptors are known to be present in cells found in the GI tract. Intracellular cAMP in turn is involved in the production of AANAT (see Figure 2). Thus, increased intracellular cAMP levels can result in increased AANAT levels and therefore increased melatonin levels. The adenosine should thus preferably be provided extracellularly in order to be able to bind to cell surface adenosine receptors. To this end, the adenosine may be produced within the bacterial cells and be transported extracellularly (or secreted). Alternatively the adenosine may be produced extracellularly, for example on the bacterial cell surface or in the supernatant. Such extracellular production can for example conveniently take place by way of the presence of a cell wall (or cell surface) anchored 5'-nucleotidase enzyme (or ecto 5'-nucleotidase enzyme), which can convert appropriate substrate to adenosine. Such extracellular production can equally take place by way of the presence of a 5'-nucleotidase enzyme (or ecto 5'-nucleotidase enzyme) in the cell supernatant or extracellular space. As shown herein, bacterial strains can provide or produce or release such a 5'-nucleotidase enzyme (or ecto 5'-nucleotidase enzyme) into the cell supernatant or extracellular space where it can then convert appropriate substrate to adenosine. Appropriate substrates include AMP.

Thus, when bacterial strains are referred to herein as being capable of producing adenosine, this includes the direct production of adenosine by the bacterial cells themselves and also includes the production of adenosine by the bacterial cells by way of the cells having an active 5'-nucleotidase enzyme (for example present on the bacterial cell surface or released into the cell supernatant or extracellular space) and thereby converting or being able to convert appropriate substrate, e.g. AMP, to adenosine. Such substrate can be naturally present, e.g. endogenously in the environment, or can be provided to the bacteria, e.g. by exogenous means.

Said strains can thus result in increased levels of melatonin, for example in comparison with the levels where no such strains are present.

Melatonin is known to be produced in the GI tract and thus production or increased

production in this region should be physiologically effective. Preferably, the amount of melatonin produced is clinically or therapeutically significant. Thus, the strains of the invention can be used to treat any disease or condition associated with (or characterised by) reduced or decreased levels of melatonin, or associated with (or characterised by) melatonin deficiency, or can be used to treat any disease or condition which would benefit from increased levels of melatonin. Such diseases or conditions (and indeed subjects suffering from such diseases or conditions) would be readily recognised by those skilled in the art and would for example include diseases, conditions or subjects which have low, reduced e.g. significantly reduced (or abnormal) levels of melatonin or melatonin deficiency, e.g. compared to levels in a normal or healthy subject, for example levels in a normal or healthy subject of the same, equivalent or comparable age. A preferred example of such a disease is infantile colic.

Thus, a lactic acid bacterial strain of the invention is capable of inducing the production of melatonin, for example by virtue of said strain being capable of producing adenosine. A lactic acid bacterial strain of the invention is capable of producing adenosine. Therapeutic uses of said strains are also provided. Said strains have a gene encoding a 5'-nucleotidase and have an active 5'-nucleotidase enzyme, for example to convert AMP (or other appropriate substrate) to adenosine. Exemplary levels of adenosine production or levels of 5'-nucleotidase activity or melatonin production are described elsewhere herein.

The strains of the present invention are *L. reuteri* strains DSM 32846, DSM 32847, DSM 32849 and/or DSM 33198 (the depositary details of which are provided elsewhere herein), and said strains, e.g. isolated strains or biologically pure cultures or preparations of said strains, form aspects of the invention, as do compositions (e.g. pharmaceutical or nutritional, e.g. food supplements, or probiotic, compositions, e.g. with pharmaceutically or nutritionally acceptable diluents and/or excipients) comprising said strains, or the therapeutic use of such strains, for example as described elsewhere herein, in particular for the treatment or prevention of infantile colic (or other diseases as described elsewhere herein). Thus, the present invention provides the *Lactobacillus reuteri* strains DSM 32846, DSM 32847, DSM 32849 and/or DSM 33198 for use in therapy, for example for the treatment or prevention of diseases as described elsewhere herein. The present invention further provides the *Lactobacillus reuteri* strains DSM 32846, DSM 32847, DSM 32849 and/or DSM33198 for use in the treatment or prevention of infantile colic.

Infantile colic, or other diseases as described elsewhere herein, in a subject, may be treated or prevented by administering an effective amount of the *Lactobacillus reuteri* strain

DSM 32846, DSM 32847, DSM 32849 and/or DSM 33198, to said subject.

Alternative and preferred embodiments and features of the invention as described elsewhere herein apply equally to the uses of the invention described here and elsewhere
5 herein.

The strains *L. reuteri* DSM 32846, DSM 32847, DSM 32849 and/or DSM 33198 have been selected for their capability to produce adenosine, for example by having a gene encoding a 5'-nucleotidase, and an active 5'-nucleotidase enzyme which can convert AMP substrate to adenosine, and are suitable not only to increase levels of adenosine (e.g. in
10 comparison to levels where the strains are not present) but also to increase levels of melatonin, for example by increasing levels of AANAT as described elsewhere herein. The strains comprise an active cell wall anchored 5'-nucleotidase enzyme and also show 5'-nucleotidase enzyme activity in their respective supernatants (see Figure 3 and Figure 5 A/B).

These strains of *L. reuteri* have been developed (in other words are modified or
15 adapted or evolved) from naturally occurring strains and have also been selected for one or more other improved properties such as increased resistance to bile or increased adherence to mucosal surfaces, e.g. surfaces of the GI tract. Thus, DSM 32846 and DSM 32847 have been evolved to be more tolerant to bile acids and thereby for example survive in larger numbers in the GI-tract. DSM 32849 has been evolved to adhere better to mucus, with the aim to colonize
20 better in the GI-tract and thereby function better for example according to the present invention. The *L. reuteri* strain DSM 33198 has also been modified in a multi-step selection process including a repeated freeze-drying procedure to allow it to be more tolerant and give a higher survival in the production process than its native isolate (parent strain). Thus, such strains do not correspond to strains occurring in nature and have been forced to evolve and are
25 non-native strains. All strains *Lactobacillus reuteri* DSM 32846, DSM 32847, DSM 32849 and DSM 33198 are selected. Preferred strains are DSM 32846, DSM 32847 or DSM 33198.

In one embodiment strains *Lactobacillus reuteri* DSM 32846, DSM 32847, and/or DSM 32849 are selected or used. More preferred strains are DSM 32846 or DSM 32847, for example DSM 32846 is preferred in some embodiments. In some embodiments, strain DSM
30 33198 is selected or used.

Said strains of the invention can be used in the treatment or prevention of infantile colic or other diseases as described elsewhere herein, for example irritable bowel syndrome (IBS), sleep disorders, or neurodegenerative diseases or disorders, for example Alzheimer's disease or other types of dementia, for example other types of senile dementia. Such diseases

are also examples of diseases associated with (or characterised by) a deficiency or reduction in melatonin, or which would for example benefit from elevated or increased levels of melatonin.

5 Gastrointestinal motility means the stretching and contractions of the muscles in the gastrointestinal tract to transfer foods being digested. The synchronized contraction of these muscles is called peristalsis.

10 There are a great number of locally released factors including hormones in the gastrointestinal tract that modulate motility and secretion. The movements of the digestive system, and the transit of the contents within it define the motility. When nerves and/or muscles in any portion of the digestive tract, the gastrointestinal motor function, do not function with normal strength and coordination, a person develops symptoms related to motility disturbances.

It has been described that disturbances of motor function produce a variety of symptoms in various gut regions such as in a subject with infantile colic.

15 Infantile colic is the term used to describe infants who cry excessively for no apparent reason during the first three months of life. Colic is one of the most distressing problems of infancy. It is distressing for the infant, the parents, and for the health care provider. The cause of colic is not fully understood.

20 New-borns hormone immaturity is implicated to be a major factor in early problems of the development of gut motility and thereby also causing pain and crying of the infant, such as in infantile colic.

25 Melatonin is a hormone that has the ability to support biological rhythms and has important effects on many functions in humans and animals. The hormone influences gastrointestinal motility by selective receptors (melatonin receptors) expressed on the smooth muscles and myenteric plexus cells of the gastrointestinal tract. The basic mechanism underlying the gastrointestinal smooth muscle contraction is the cyclic generation of electrical current. The myoelectric activity of gastrointestinal tract consists of two kinds of electrical potentials: slow wave and spike activity organized in the myoelectric migrating complex.

30 A precursor to melatonin is serotonin, a neurotransmitter itself that can be derived from the amino acid tryptophan.

It has been shown that serotonin induced decrease of the food transit is in part blocked by melatonin. It is a counterbalancing system of melatonin and serotonin in regulation of gut activity.

Further the functional and balanced melatonin-serotonin system affect appetite and digestive processes by endocrine as well as paracrine effects in both the brain and the GI tract.

The action of the two hormones seems to have the contrary effect on the motility and the end effects are dose dependent. Melatonin and serotonin are also known to be involved in the hypersensitivity processes and pain conduction.

Melatonin, also known as N-acetyl-5-methoxy tryptamine, is a hormone and a neurotransmitter earlier thought to be secreted predominantly by the pineal gland. But there are important extra-pineal sites of melatonin production, such as the retina and the gastrointestinal tract. There are now reports showing at least 400 times more melatonin in the gastrointestinal tract than in the pineal gland. Circadian variation of gastrointestinal melatonin does not appear to be controlled by light exposure (like the pineal gland), but by eating and food composition.

Synchronization between production and the different circadian rhythms and the following release of serotonin and melatonin in young infants is important to avoid gut motility disturbances and pain, such as infantile colic (IC).

Serotonin increases intestinal smooth muscle contraction, and melatonin relaxes intestinal smooth muscles. The serotonin concentration peaks can cause intestinal cramps if not balanced by other agents, whereof melatonin is an important one. Both serotonin and melatonin have circadian rhythms. While there is serotonin produced and serotonin circadian rhythms developed when an infant is born, the endogenous melatonin production and melatonin circadian rhythm is in many infants not ready to begin until after the 3rd month in life. This time coincides with the time of normal disappearance of infantile colic. It has been shown that melatonin is low in the mornings early in life for the infants who have colic, whereas it is high in infant who does not have colic (Cengiz et al 2015, Turkish journal of family medicine and primary care; 9 (1)/10-15). Such infants (for example infants with forms of infantile colic associated with deficiencies in melatonin or melatonin balance, for example deficiencies in melatonin levels in the mornings, for example at or before 6 AM, 8 AM, 10 AM or 12 noon) are examples of subjects which can be treated using the present invention.

Thus, although some children appear able to produce and release needed melatonin endogenously, from their food and/or their intestinal bacteria and are therefore less likely to develop infantile colic, there are many others who do not, and thus need added support to prevent or treat IC. Such infants (for example infants with deficiencies in melatonin caused for example by a not fully developed intestinal microbiome or infants suffering from nutrition

problems or feeding problems) are also examples of subjects which can be treated using the present invention.

It can be said that infantile colic is a condition of melatonin-deficiency. This is especially important for children in stressful situations, or who are less able to cope with stress, such as for children born before full gestational time or not fully functional food metabolism etc. Such infants (for example infants with deficiencies in melatonin born before full gestational time, or by cesarean section, or without fully functional food metabolism) are also examples of subjects which can be treated using the present invention.

Another example of a condition that relates to a balance of melatonin function is irritable bowel syndrome (IBS) which is a common disorder characterized by recurrent abdominal pain or discomfort, in combination with disturbed bowel habits in the absence of identifiable causes. Melatonin is suggested to show a positive effect on pain perception and bowel habits (Siah KTH et al, 2014, World J Gastroenterol; 20(10): 2492-2498). Thus, subjects having IBS, in particular forms of IBS associated with deficiencies in melatonin or melatonin balance, are also examples of subjects which can be treated using the present invention.

Melatonin deficiency has been mainly investigated in the pineal gland, circulation, saliva, cerebrospinal fluid, and, by measuring the metabolite 6-sulfatoxymelatonin, in urine.

There are more than 20 types of enteroendocrine cells in the intestine, which makes it the largest endocrine organ in the human body. Enterochromaffin (EC) cells are a type of enteroendocrine and neuroendocrine cell. They reside alongside the epithelium lining the lumen of the digestive tract and play a crucial role in gastrointestinal regulation, particularly intestinal motility and secretion. EC cells modulate neuron signalling in the enteric nervous system (ENS) via the secretion of the neurotransmitter serotonin and other peptides, they further express adenosine receptors and have an active synthesis of melatonin.

A common symptom of insufficient melatonin signalling is sleep disturbances and melatonin is one of the most popular natural health supplements of our time. Melatonin is often referred to as “the hormone of darkness” because its synthesis and secretion is in some locations of the body enhanced by darkness and inhibited by light. Sleep disturbances are highly prevalent in children and, without appropriate treatment, can become chronic and last for many years (Esposito et al. 2019, J Transl Med, 17:77). Even the elderly population is reported to experience sleep disturbances. The night-time melatonin peak is regularly observed to decrease during aging. Thus, subjects having or suffering from sleep disturbances or sleep disorders, in particular forms of sleep disturbances or sleep disorders associated with

deficiencies in melatonin or melatonin balance, are also examples of subjects which can be treated using the present invention.

Decreased levels of melatonin have also been repeatedly described in neurodegenerative disorders, especially in Alzheimer's disease and other types of senile dementia (Blumenbach Johann Friedrich, 2012, The Scientific World Journal Volume 2012, Article ID 640389). Thus, subjects having such neurodegenerative disorders, in particular forms of such neurodegenerative disorders associated with deficiencies in melatonin or melatonin balance, are also examples of subjects which can be treated using the present invention.

The rate of melatonin formation depends on the activity of the enzyme arylalkylamine- N-acetyltransferase (AANAT) (Fig 1 and Fig 2) in the pineal gland but also at the extra-pineal sites of melatonin production, such as in the gastrointestinal tract.

Adenosine increases intracellular cAMP levels via adenosine receptor, which increases the production of AANAT, the rate limiting enzyme in melatonin synthesis.

It is an object of the present invention to use adenosine producing lactic acid strains of the invention to prevent and/or treat infantile colic.

It is another object of the present invention to use adenosine producing lactic acid strains of the invention to prevent and/or treat IBS.

It is another object of the present invention to use adenosine producing lactic acid strains of the invention to prevent and/or treat sleep disorders.

It is another object of the present invention to use adenosine producing lactic acid strains of the invention to prevent and/or treat neurodegenerative disorders, e.g. Alzheimer's disease and other types of dementia, e.g. other types of senile dementia.

This invention also relates to a method for the selection of specific lactic acid bacterial strains which are *Lactobacillus reuteri*, which are capable of producing adenosine.

One important metabolic process in the human body is purine metabolism, wherein purines are metabolized and broken down by specific enzymes. One example of those enzymes are ecto-5'-nucleotidase (CD73), which is considered to be a key enzyme in the generation of adenosine.

The inventors surprisingly found that some specific probiotic bacterial strains were capable of producing adenosine.

The present invention thus includes a new method as defined elsewhere herein of selecting specific strains of lactic acid bacteria which are *Lactobacillus reuteri*, that are effective in producing adenosine. The purpose of selecting specific bacterial strains is to use

them for treating certain disorders such as infantile colic (or other diseases as described elsewhere herein).

The present invention provides a method as defined elsewhere herein for selecting lactic acid bacterial strains which are *Lactobacillus reuteri*, which are useful as probiotics and in therapy, e.g. as pharmaceuticals or as food supplements.

One aspect of the present invention thus provides a method for the selection of a lactic acid bacterial strain capable of producing adenosine, wherein said strain is *Lactobacillus reuteri* and wherein said method comprises:

- a) screening a lactic acid bacterial strain for the presence of a gene encoding a 5'-nucleotidase, e.g. a cell wall anchored 5'-nucleotidase, wherein said strain is *Lactobacillus reuteri*; and/or
- b) screening a lactic acid bacterial strain, or its supernatant, for the presence of an active 5'-nucleotidase enzyme, e.g. a cell wall anchored 5'-nucleotidase enzyme, wherein said strain is *Lactobacillus reuteri*; and
- c) selecting a *Lactobacillus reuteri* strain which produces adenosine at a level which is increased as compared to *Lactobacillus reuteri* DSM 17938; and optionally
- d) culturing or propagating such a *Lactobacillus reuteri* strain.

Such 5'-nucleotidase (5'NT) enzymes can also be referred to as ecto-5' nucleotidase enzymes or CD73 (cluster of differentiation 73). Strains which are capable of producing adenosine can then be selected.

Once an appropriate strain has been selected using the method of the present invention it can then be used for the production, e.g. the local production, of adenosine in a subject.

Products or compositions, e.g. pharmaceutical compositions, probiotic compositions, or dietary/nutraceutical compositions, comprising the bacterial strains (for example comprising one or more of the bacterial strains) of the invention and therapeutic uses of said products or compositions as described herein form yet further aspects of the invention.

The Food and Agricultural Organization of the United Nations define probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Nowadays, a number of different bacteria are used as probiotics for example, lactic-acid producing bacteria such as strains of *Lactobacillus* and *Bifidobacterium*.

Alternative and preferred embodiments and features of the invention as described elsewhere herein apply equally to the uses and products of the invention.

As described above, the present invention relates to the selection and use of bacterial strains that are capable of producing adenosine.

Said strains which are effective in producing adenosine can be used for local production of adenosine in a subject, e.g. a mammal, preferably a human.

Thus, as outlined above, the present invention provides a method for the selection or screening of *Lactobacillus reuteri* bacterial strains capable of producing adenosine.

5 Some of the methods of the invention comprise a step (step a)) of screening for the presence of a gene encoding a 5'-nucleotidase, e.g. a cell wall anchored 5'-nucleotidase. Such screening can be carried out using any appropriate method and strains positive for a 5'-nucleotidase gene are selected. Such methods will conveniently be carried out *in vitro*, for example will be genetic or nucleic-acid based methods to detect the presence of a gene
10 encoding a 5'-nucleotidase, e.g. a cell wall anchored 5'-nucleotidase (or an identificatory fragment thereof) the sequences of which are known in the art. For example, a PCR protocol (or other nucleic acid based technique) can be readily designed to do this based on the known nucleic acid sequences encoding the enzymes, or as an alternative, genome sequences of candidate strains could be scrutinized with the aim to identify the said gene encoding a
15 5'-nucleotidase, e.g. a cell wall anchored 5'-nucleotidase, for example, based on homology to known 5'-nucleotidase sequences, including for example the presence of an LPXTG-motif (SEQ ID NO:1) as discussed below.

An exemplary gene to be detected in the methods of the invention is the cell wall anchored (ecto) 5'-nucleotidase gene from *L. reuteri* (e.g. GenBank accession number:
20 AEI56270.1, LPXTG-motif cell wall anchor domain protein [*Lactobacillus reuteri* SD2112]), or an appropriate homologue/5'-nucleotidase from other species of bacteria, e.g. other lactic acid bacteria. An exemplary technique is described in the experimental Examples.

Some methods of the invention comprise a step (step b)) of screening for the presence of an active (functional) 5'-nucleotidase enzyme, e.g. a cell wall anchored 5'-nucleotidase
25 enzyme. Such a step can be carried out using any appropriate method, for example, using an enzymatic assay. The 5'-nucleotidase enzyme catalyzes the following reaction: $\text{AMP} + \text{H}_2\text{O} \rightleftharpoons \text{adenosine} + \text{phosphate}$ (AMP is adenosine monophosphate) and an assay to measure this reaction can readily be used to determine the presence of an active 5'-nucleotidase enzyme (or 5'-nucleotidase activity). In other words the reference herein to an active or functional 5'-
30 nucleotidase enzyme is one which is capable of catalyzing this reaction under suitable conditions, e.g. when supplied with appropriate substrate such as AMP. The activity of the 5'-nucleotidase enzyme can, if desired, be quantified, e.g. in such an enzymatic assay, for example by measuring the amount or concentration of phosphate or adenosine or other appropriate downstream product of adenosine which is generated in the reaction and can be

measured or quantitated. The activity can conveniently be measured on a sample of the bacterial cells, or the supernatant from the bacterial cells.

Methods for carrying out such an assay would be well-known to a person skilled in the art. For example, appropriate kits are commercially available, such as the Crystal Chem 5'-Nucleotidase Assay Kit (Crystal Chem, catalogue # 80229, Downers Grove, IL, USA) in which 5'-nucleotidase enzyme activity is measured by way of the production of a dye (a quinone dye) which is formed as a downstream product of the conversion of AMP to adenosine (i.e. as a result of the production of adenosine). AMP is provided as a substrate. An exemplary technique is described in the Examples section. Appropriate methodology is also readily available from other reagent suppliers, e.g. Sigma, in conjunction with their 5'-nucleotidase enzyme reagent.

The strains selected by the method of selection of the invention produce adenosine at a level which is increased as compared to *Lactobacillus reuteri* DSM 17938. Strains with high or significant production levels of 5'-nucleotidase enzyme (or 5'-nucleotidase activity) are preferred, for example strains that have a 5'-nucleotidase activity of at least or greater than 8 units/L (units per litre) and/or are capable of producing adenosine at a level of at least or greater than 8 $\mu\text{mol L}^{-1} \text{ min}^{-1}$ ($\mu\text{mol per litre per minute}$). Thus, in preferred embodiments, a strain is selected for having 5'-nucleotidase activity at a level of at least, or greater than, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 or 150 units/L, and/or is capable of producing adenosine at a level of at least, or greater than, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 or 150 $\mu\text{mol L}^{-1} \text{ min}^{-1}$. In some embodiments such values can represent upper limits. Such values generally refer to values of 5'-nucleotidase activity measured on a sample of the bacterial cells, e.g. on the surface of the bacterial cells, or in the supernatant of the bacterial cells in culture, preferably in the supernatant. Such values generally refer to adenosine levels (preferably extracellular adenosine levels) measured in a sample of the bacterial cells, e.g. in the supernatant of the bacterial cells in culture. Such values generally refer to values of 5'-nucleotidase activity (or adenosine levels) when measured with a concentration of 10^9 bacteria/ml, or the supernatant from such a culture. One unit is defined as the amount of enzyme needed for producing 1 $\mu\text{mol product/min}$ (e.g. 1 $\mu\text{mol product/litre/min}$). The concentration in units/liter will then correspond to the enzyme concentration needed for increasing the concentration with 1 $\mu\text{M/min}$ (e.g. 1 $\mu\text{M/litre/min}$). An appropriate and exemplified assay for measuring this activity is shown in the Examples using the 5'-nucleotidase kit (#80229) from Crystal Chem Inc. Thus, in preferred embodiments the above units and values refer to units and values when

measured using this kit and/or the conditions set out in the Examples, in particular with a concentration of 10^9 bacteria/ml, or the supernatant from such a culture, or an equivalent assay. Thus, such methods will conveniently be carried out *in vitro*.

Methods comprising at least step b) will be preferred, as the presence of a gene encoding a 5'-nucleotidase is not always indicative of the presence of an active 5'-nucleotidase enzyme. In the methods, uses and lactic acid bacterial strains of the invention, adenosine production or adenosine activity should take place extracellularly, i.e. outside or on the surface of the lactic acid bacteria, so that it can for example be present in the supernatant or other extracellular fluid produced by the lactic acid bacteria. Thus, the presence of an active 5'-nucleotidase enzyme on the cell surface, for example in the form of a cell wall anchored 5'-nucleotidase, or extracellular from the bacterial cell, for example in the supernatant, can be a useful feature such that the reaction to produce adenosine takes place outside the cell, for example on the surface of the bacterial cell.

Thus, the production of good levels of adenosine (e.g. extracellular adenosine produced by the 5'-nucleotidase enzyme) can also be an indicator of the presence of a 5'-nucleotidase gene or the presence of an active 5'-nucleotidase enzyme. Thus, the selection method of the invention can also involve the step of selecting a strain which is producing adenosine. Strains with high or significant production levels of adenosine, e.g. extracellular adenosine, are preferred, for example strains that produce adenosine at a level which is therapeutically effective in a subject, or at a level such that increased (and preferably therapeutically effective) levels of melatonin are observed. Such values generally refer to values of adenosine measured in the supernatant of strains in culture or on the cell surface of strains in culture (in vitro) and some exemplary specific values are provided above.

However, in some embodiments it is appropriate to refer to values, levels or amounts of adenosine produced in a subject, for example locally, e.g. at the site of administration, e.g. in the gastrointestinal tract. For example, preferred strains can enable increased production of adenosine, for example increased in vivo production, e.g. local production (e.g. in the GI tract), of adenosine, for example when compared to a relevant control as described elsewhere herein, such as the levels of adenosine when no strains are administered, or the base or natural level of adenosine in a particular subject.

In all aspects of the invention described herein, local production can refer to production or levels of adenosine at the site of administration, for example production or levels of adenosine in the gastrointestinal tract (e.g. when administration is oral). In one aspect the local production of adenosine can have systemic downstream effects and may

increase levels of adenosine or melatonin in the circulatory system, for example in blood or plasma, or other places outside the gastrointestinal tract.

Strains which can give rise to increased local levels of adenosine (or melatonin or 5'nucleotidase activity), for example in the GI tract of a subject, are thus preferred, in particular where such an effect is observed when the strains are administered orally. Preferably said increases are measurable or significant increases, for example are statistically or clinically significant. By way of example, strains which can give rise to increases of at least, or up to, 1.5 fold, 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold, 5 fold, 6 fold, 7 fold, 8 fold, 9 fold or 10 fold, in levels of adenosine (or melatonin or 5'nucleotidase activity), for example local levels of adenosine (or melatonin or 5'nucleotidase activity), are preferred. Any appropriate comparison can be used, for example an increase when compared to the levels observed when no strain is administered, or the levels when a control formulation, e.g. a control formulation not containing the relevant strain, is administered.

Appropriate methods of measuring levels of adenosine (or melatonin or 5'nucleotidase activity) production would be well known to a person skilled in the art. Thus, in some embodiments of the invention, the selection method will involve the step of detecting or determining the amount or level (e.g. the concentration) of adenosine produced by a candidate strain.

The levels of adenosine production can conveniently be compared to DSM 17938 as a positive control strain or to a negative control strain. DSM 17938 is an appropriate positive control strain which has been shown in the Examples to produce significant levels of adenosine/ 5'nucleotidase activity, for example in the supernatant of bacterial cells. Strains selected by the method of the invention as defined elsewhere herein will produce higher levels, sometimes significantly higher levels, of adenosine/ 5'nucleotidase activity than DSM 17938, for example in the supernatant of bacterial cells. Strains selected by the method of the invention are capable of producing higher (increased) levels, or significantly higher (increased) levels, of 5'nucleotidase activity than DSM 17938, for example in the supernatant of bacterial cells, for example when assessed *in vitro*. Exemplary strains are the strains of the invention, i.e. DSM 32846, DSM 32847, DSM 32849 and DSM 33198 (see Figure 3 and Figure 5 A/B). Viewed alternatively, strains selected by the method of the invention are capable of producing higher (increased) levels, or significantly higher (increased) levels, of adenosine than DSM 17938, for example in the supernatant of bacterial cells, for example when assessed *in vitro*. For example, strains selected by the method of the invention may be capable of producing or inducing production of higher (increased) levels, or significantly

higher (increased) levels of adenosine in a subject, for example higher etc., local levels of adenosine in a subject than DSM 17938, for example when assessed *in vivo*, in particular when the strains are administered orally.

Said strains have one or more improved (e.g. significantly improved) properties when compared to DSM17938.

Preferably said increases (in adenosine production or 5' nucleotidase activity) are measurable or significant increases, for example are statistically or clinically significant. By way of example, strains which can give rise to increases of at least, or up to 10 %, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, 550%, or higher, in levels of adenosine, for example local or *in vitro* levels of adenosine, or in levels of 5' nucleotidase activity (for example when assessed *in vitro*), compared to the levels with DSM 17938 are preferred. Viewed alternatively, strains which can give rise to increases of at least, or up to, 1.5 fold, 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold, 5 fold, 6 fold, 7 fold, 8 fold, 9 fold, or 10 fold, in levels of adenosine, for example local or *in vitro* levels of adenosine, or in levels of 5' nucleotidase activity, compared to the levels with DSM 17938 are preferred. *In vitro* levels can conveniently be measured as described elsewhere herein, for example in bacterial cultures. Preferably, such increases are increases in extracellular levels of adenosine or 5' nucleotidase activity, for example as measured *in vitro*, for example in the supernatant of bacterial cultures.

An appropriate negative control strain might be a strain that does not contain a gene encoding a 5' nucleotidase or does not contain an active 5' nucleotidase enzyme (or does not have 5' nucleotidase activity as described elsewhere herein).

Because of the downstream uses of the strains which are selected by the methods of the invention, after adenosine producing *Lactobacillus reuteri* strains are selected or isolated, other embodiments will involve the further steps of culturing or propagating or producing such strains, and optionally formulating said cultured or propagated or produced strains into a composition comprising said strain, e.g. a pharmaceutical or nutritional composition, e.g. as described elsewhere herein, or possibly storing such strains for future uses, for example through lyophilisation or freeze drying. In one embodiment the bacterial strains are provided in a freeze-dried form.

The selection steps of the methods of the invention (and indeed the therapeutic uses as described herein) will generally need to be carried out in an appropriate culture medium (or *in vivo* environment), which supports adenosine production. Preferred culture media (or *in vivo* environment) will contain an appropriate carbon source, which will support the production of

adenosine by said strain, preferably together with an appropriate substrate for adenosine production by the 5' nucleotidase enzyme, e.g. AMP.

Although such assays can conveniently be carried out *in vitro*, another option would be to assess the strains in an appropriate *ex vivo* assay, e.g. using an appropriate intestinal enteroid model (see for example the enteroid model and assays described in Example 5, where for example levels of melatonin can be assessed).

Thus, although any appropriate *in vitro* or *ex vivo* models for assessment of the strains, for example for assessment of the induction of adenosine or melatonin production by the strains, preferred *in vitro* or *ex vivo* models for assessment of the strains are intestinal enteroid models, preferably human intestinal enteroid models, which can for example be used to assess melatonin levels. Such models preferably comprise enteroendocrine cells, for example as described in Example 5.

Preferred strains of the invention are capable of producing or inducing the production of at least or up to 10, 20, 30, 40, 50, 60 or 70 pg/ml melatonin. In some embodiments such values can represent upper limits. Such values generally refer to levels of melatonin production induced by said strains when measured in an appropriate *in vitro* or *ex vivo* assay, for example refer to levels of melatonin produced by enteroendocrine cells (preferably human cells), or produced in an intestinal enteroid model comprising enteroendocrine cells (for example with %s of enteroendocrine cells as described elsewhere herein), preferably a model comprising human cells, when said cells are brought into contact with the strains of the invention or supernatant taken from said strains. An appropriate and exemplified assay for measuring this activity is shown in the Examples using a human intestinal enteroid model comprising enteroendocrine cells, e.g. at least 20% or 30%, for example 20-40%, 30-40% or up to 40%, enteroendocrine cells. Thus, in preferred embodiments the above values refer to values when measured using this assay (or an equivalent assay, for example with the above %s of enteroendocrine cells) and/or the conditions set out in the Examples.

Some strains will be capable of inducing higher levels, sometimes significantly higher levels, of melatonin than DSM 17938, for example from enteroendocrine cells or in intestinal enteroid models as described above. Exemplary strains can give rise to increases of at least, or up to, 1.5 fold, 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold, 5 fold, 5.5 fold, 6 fold, 6.5 fold, 7 fold, 7.5 fold, 8 fold, 8.5 fold, 9 fold, 9.5 fold or 10 fold, in levels of melatonin production, compared to the levels with DSM 17938, and are preferred.

The screening methods of the invention can thus optionally comprise a further step of selecting a strain which is capable of inducing the production of melatonin, for example using

methodology as described above or any other appropriate methodology. Selected strains can preferably induce the production of melatonin at levels as described above.

In embodiments where more than one bacterial strain is screened using the methods of the invention, the amount of generated adenosine can optionally be quantified and the
5 *Lactobacillus reuteri* bacterial strain which produces the highest amount or level of adenosine or melatonin, or a sufficiently high amount or level of adenosine, with amounts or levels higher (preferably significantly higher) than DSM17938 can be selected.

Methods comprising certain steps as described herein also include, where appropriate, methods consisting of these steps.

10 Any appropriate lactic acid bacterial strain which is *Lactobacillus reuteri*, e.g. a probiotic lactic acid bacterial strain which is *Lactobacillus reuteri*, can be subjected to the selection methods of the invention.

The bacterial strains of the invention are *Lactobacillus reuteri* DSM 32846, DSM 32847, and DSM 32849 that were deposited under the Budapest Treaty at the DSMZ-
15 Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Mascheroder Weg 1b, Inhoffenstr. 7B, D-38124 Braunschweig) on July 4, 2018 and *Lactobacillus reuteri* DSM 33198 that was deposited under the Budapest Treaty at the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Mascheroder Weg 1b, Inhoffenstr. 7B, D-38124 Braunschweig) on July 9, 2019. These bacterial strains or isolated bacterial strains form
20 aspects of the invention and can for example be used to treat the diseases or conditions as described elsewhere herein or for any other uses as described herein.

Thus, a yet further aspect of the invention provides the use of said strains to treat or prevent one or more of the diseases as described herein.

Maximizing, increasing or improving the activity of the 5'-nucleotidase of the selected
25 *Lactobacillus reuteri* strains will preferably lead to increased or improved production of adenosine, to improve the use and effect of the treatment of specific disorders as described herein. This can be achieved by culturing the bacteria in specific growth conditions to allow a high yield of adenosine (or a higher, e.g. significantly higher, yield of adenosine, e.g. compared to the yield observed in the absence of the specific growth condition). The specific
30 growth condition can be e.g. culturing the *Lactobacillus reuteri* bacteria in normal growth medium supplemented with an appropriate substrate for adenosine production by the 5'-nucleotidase enzyme, e.g. AMP, or appropriate upstream components of AMP such as ADP and/or ATP.

To maximize the activity of the 5'-nucleotidase, a higher concentration of bacteria of

the invention, can be administered. An exemplary concentration might be 2 to 10 times higher than a conventional dose.

As described above, the strains of the invention, or strains selected by, produced, obtained, or obtainable by the methods of the invention have uses in therapy. The adenosine producing strains of the invention, or strains which are selected, obtained or obtainable using the selection methods of the invention, may be used in the production, e.g. local production, of adenosine, and thereby preferably melatonin in a subject. Said production of adenosine, etc., and preferably melatonin may be used for the treatment of infantile colic (and other diseases as described herein) which will benefit from production or increased production, e.g. local production, of adenosine and thereby melatonin.

The administration of the bacterial strains in the uses of the invention is carried out in pharmaceutically, therapeutically, or physiologically effective amounts, to subjects (animals/mammals) in need of treatment.

Treatment of disease or conditions in accordance with the present invention (for example treatment of pre-existing disease) includes cure of said disease or conditions, or any reduction or alleviation of disease (e.g. reduction in disease severity) or symptoms of disease.

The uses of the present invention are suitable for prevention of diseases or conditions as well as treatment of diseases or conditions. Thus, prophylactic treatment is also encompassed by the invention. For this reason in the uses of the present invention, treatment or therapy also includes prophylaxis or prevention where appropriate.

Such preventative (or protective) aspects can conveniently be carried out on healthy or normal subjects as described herein, and can include both complete prevention and significant prevention. Similarly, significant prevention can include the scenario where severity of disease or symptoms of disease is reduced (e.g. measurably or significantly reduced) compared to the severity or symptoms which would be expected if no treatment is given. A yet further aspect of the invention provides a strain or product for the therapeutic uses as defined elsewhere herein, wherein said use further comprises the administration of at least one further agent, e.g. a further therapeutic or nutritional agent. Exemplary agents might be substrate components which will increase or enhance the production of adenosine (e.g. a component which can increase or enhance the production of adenosine), or a source of such components.

Thus, in a yet further embodiment of the invention, where an adenosine producing bacteria of the invention is concerned, the administration of said strains or products further comprises the administration of a substrate component, e.g. AMP and/or a material or agent

that produces this component. A preferred substrate would be AMP, or a source of AMP.

In such embodiments, the substrate component, or other additional component, could be added to the bacterial preparation directly before administration to the subject.

Alternatively, it could be added to the bacterial preparation at the end of the manufacturing process, e.g. at the end of fermentation, after which the bacteria can be stored for future use, e.g. by lyophilisation or freeze-drying. Alternatively it can be administered separately as described below.

In such embodiments, the further therapeutic agent can be any further agent which is useful in the treatment of the disease in question.

Said further agents can be administered together with the strains or products of the invention (e.g. as a combined preparation) or can be administered separately. In addition, said further agents can be administered at the same time as the strains or products of the invention or at different time points, e.g. sequentially. Suitable administration regimes and timings can readily be determined by the skilled person depending on the further agent in question.

The invention also provides a combination product suitable for increasing the production of melatonin or adenosine in a subject comprising:

- (i) a first *Lactobacillus reuteri* strain capable of inducing the production of melatonin or producing adenosine, wherein said strain is a strain of the invention; and
- (ii) a substrate component to increase or enhance the induction of melatonin and/or the production of adenosine by said *Lactobacillus reuteri* strain, wherein said substrate component is AMP.

In the combination products of the invention, components (i) and (ii) are generally provided in separate compartments or vessels or as separate components of the kit or product. The kits may comprise further components, which may also be in separate compartment or vessels. The kits (or combination products) may be used in the uses of the present invention as described herein, for example in the treatment or prevention of other diseases described herein, in particular infantile colic. Instructions for use of the kits or products in the methods and uses of the invention may optionally be provided.

The term “subject” as used herein includes mammals, in particular humans, especially infants (for example where the disease to be prevented or treated is colic). In preferred embodiments of the invention, the selected strain of the invention is administered to a human. For diseases other than colic, any subject, e.g. human subject, e.g. adult or child or infant subject, suffering from said disease or suspected to be suffering from said disease or at risk from said disease is appropriate.

A preferred subject in accordance with the present invention is an infant of less than or up to 12 months old, preferably less than or up to 5 or 6 months old, preferably less than or up to 3 or 4 months old. Such subjects are particularly appropriate when the disease to be treated or prevented is infantile colic. Subjects can thus be treated from birth, or within the first few weeks (e.g. within the first 1, 2, 3 or 4 weeks) or within the first few months (e.g. within the first 1, 2, 3 or 4 months) of life.

As described elsewhere herein, preferred subjects are those suffering from infantile colic (or one of the other diseases as described herein) or believed or suspected to be suffering from infantile colic (or one of the other diseases as described herein). As the therapeutic uses of the invention can also be used to prevent disease, appropriate subjects include those at risk of suffering from infantile colic (or one of the other diseases as described herein).

Exemplary subjects will include exclusively (solely) breastfed infants and exclusively (solely) formula fed infants (non-breast fed infants) or infants which have been both breastfed and formula fed.

Other exemplary subjects include infants, in particular infants with colic, suspected of having colic, or at risk of developing colic, that do not yet produce or release melatonin or are otherwise unable to produce or release melatonin, for example infants in which the melatonin circadian cycle has not yet developed or started, or infants which are unable to produce or release the required melatonin from their food and/or their intestinal bacteria, or include infants which have low, reduced e.g. significantly reduced (or abnormal) levels of melatonin or melatonin deficiency, e.g. compared to levels in a normal or healthy subject, for example levels in a normal or healthy subject of the same, equivalent or comparable age.

For diseases other than colic, exemplary subjects include subjects which have low, reduced e.g. significantly reduced (or abnormal) levels of melatonin or melatonin deficiency, e.g. compared to levels in a normal or healthy subject, for example levels in a normal or healthy subject of the same, equivalent or comparable age.

Other exemplary subjects include infants that have some kind of nutritional or dietary issue or problem, for example which results in low or deficient levels of melatonin. Such subjects might include infants born before full gestational time, for example premature infants or infants born before 36 weeks of gestation, or infants that lack fully functional food metabolism, or infants which have problems feeding or nutrition problems.

Other exemplary subjects include those, preferably infants, with hormone immaturity (in other words suboptimal, subnormal, undeveloped, deficient, low or immature hormone production) for example compared to a normal infant or subject of the same age, for example

melatonin immaturity, in particular those subjects which have deficiencies in gut motility as a result of said hormone immaturity. Such hormone immaturity, e.g. melatonin immaturity or melatonin reduction or deficiency, may for example be associated with some infants suffering with infantile colic. Other preferred diseases (and hence subjects) to be treated are diseases or conditions associated with melatonin deficiency (or subjects suffering from said diseases).

In all such subjects, if appropriate or necessary, levels of melatonin in said subject can readily be measured using techniques readily available and known in the art. For example, levels of melatonin can readily be measured in serum/blood or saliva samples. Commercial kits for such measurements are also available.

Any appropriate mode of administration can be used. Conveniently said administration is oral, rectal or by tube-feeding. Thus, the bacteria can be administered to the GI tract, or oral cavity, as desired or appropriate. The bacteria can be included in a baby formula or a food supplement or oil drops, as well as in a pharmaceutical formulation.

Appropriate doses of the strains, products and compositions of the invention as defined herein can readily be chosen depending on the disease (or condition) to be treated, the mode of administration and the formulation concerned.

For example, regarding the bacterial strains for use of the invention, a dosage and administration regime is chosen such that the bacteria of the invention administered to the subject can result in an increased production, e.g. local production, of adenosine (or melatonin) and to give rise to the desired therapeutic effects or health benefits in infantile colic (or other disease to be treated). Thus, preferably said dosage is a therapeutically effective dosage which is appropriate for the type of mammal and condition being treated and is for example administered to a subject in need thereof. For example, daily doses, e.g. a single daily dose, of 10^4 to 10^{12} , for example 10^5 to 10^9 , or 10^6 to 10^8 , or 10^8 to 10^{10} , or 10^{10} to 10^{12} total CFUs of bacteria may be used.

In one embodiment the daily dose (e.g. 10^5 to 10^9 or 10^6 to 10^8 or 10^8 to 10^{10} or 10^{10} to 10^{12} of CFUs) is divided into several administrations e.g. 2-8 administrations (such as 3-5 administrations) or in association with every feeding (breast and/or formula) of the subject. In one embodiment, a higher dose, such as 2 to 10 or 10 to 100 times the dose after the starting period, and/or more frequently administered dose is administered as a starting dose for 3-14 days (e.g. 5-10 days, such as 7 days) for faster relief in a subject.

The term "increase" or "enhance" or "higher" (or equivalent terms) as described herein includes any measurable increase or elevation when compared with an appropriate control. Appropriate controls would readily be identified by a person skilled in the art and

might include levels when strains are not present, or levels in non-treated or placebo-treated subjects, or levels in a healthy or normal subject, e.g. an age-matched subject, or the same subject before treatment. Preferably the increase will be significant, for example clinically or statistically significant, for example with a probability value of ≤ 0.05 .

5 Preferably such increases (and indeed other increases, improvements or positive effects as mentioned elsewhere herein) are measurable increases, etc., (as appropriate), more preferably they are significant increases, preferably clinically significant or statistically significant increases, for example with a probability value of ≤ 0.05 , when compared to an appropriate control level or value (e.g. compared to an untreated or placebo treated subject or
10 compared to a healthy or normal subject, or the same subject before treatment).

 The term "decrease" or "reduce" (or equivalent terms) as described herein includes any measurable decrease or reduction when compared with an appropriate control. Appropriate controls would readily be identified by a person skilled in the art and might include levels when strains are not present, or levels in non-treated or placebo-treated subjects, or levels in a
15 healthy or normal subject, e.g. an age-matched subject, or the same subject before treatment. Preferably the decrease or reduction will be significant, for example clinically or statistically significant, for example with a probability value of ≤ 0.05 .

 Thus, preferably such decreases (and indeed other decreases, reductions or negative effects as mentioned elsewhere herein) are measurable decreases, etc., (as appropriate), more preferably they are significant decreases, preferably clinically significant or statistically
20 significant decreases, for example with a probability value of ≤ 0.05 , when compared to an appropriate control level or value (e.g. compared to an untreated or placebo treated subject or compared to a healthy or normal subject, or the same subject before treatment).

 As used throughout the entire application, the terms "a" and "an" are used in the sense
25 that they mean "at least one", "at least a first", "one or more" or "a plurality" of the referenced components or steps, except in instances wherein an upper limit is thereafter specifically stated.

 In addition, where the terms "comprise", "comprises", "has" or "having", or other equivalent terms are used herein, then in some more specific embodiments these terms
30 include the term "consists of" or "consists essentially of", or other equivalent terms. Lists "consisting of" various components and features as discussed herein can also refer to lists "comprising" the various components and features.

 Other objects and advantages will be more fully apparent from the following non-limiting examples and appended claims.

EXAMPLES

EXAMPLE 1

A method for identification of strains having a gene encoding a cell surface located 5'-nucleotidase

Cultivate the bacteria on MRS plates for 16 h at 37°C in anaerobic atmosphere. Collect
5 10 bacterial colonies with a sterile plastic loop and suspended in 100 microlitres of sterile water (PCR quality). (Alternative: Prepare DNA from the bacterial culture using a suitable method).

Presence of the 5'-nucleotidase gene can be examined by PCR, e.g. by using PuReTaq Ready
10 To Go PCR beads (GE HealthCare) and any of the primer pairs described below (0.4 mM of each). Bacterial suspension or DNA preparation (0,5 microliter) should be added to the PCR mix and the PCR reaction should be performed by running the program 95°C, 5 min; 30x (95°C, 30 s; 55°C, 30 s; 72°C, 30 s); 72°, 10 min. The PCR products could be separated and visualised by using standard agarose gel electrophoresis and the sequence determined by
15 standard Sanger sequencing (using the forward primer used for the PCR).

The gene could be detected using any of the following primers:

Primer pair 1 (product size 233 bp)

LrNuc1f: GGAAC TTTGGGAAACCATGA (SEQ ID NO:2)

20 LrNuc1r: CGGGCAACTTTACCATCACT (SEQ ID NO:3)

Primer pair 2 (product size 212 bp)

LrNuc2f: TACTCGTGAAAATGCCGTTG (SEQ ID NO:4)

LrNuc2r: GTGCCCCTGTCATTTCAACT (SEQ ID NO:5)

25

Primer pair 3 (product size 232 bp)

LrNuc3f: AGCTTTACCAAATTGACCCTGA (SEQ ID NO:6)

LrNuc3r: TTGATATTAGGCGCATCCTTTT (SEQ ID NO:7)

30 **Sequences**

Gene encoding Cell surface anchored 5'-nucleotidase, GenBank accession number:

AEI56270.1, LPXTG-motif cell wall anchor domain protein [Lactobacillus reuteri SD2112]

(SEQ ID NO:8)

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5  ATGAAGAATAATAGTTCAAAATATTGTTTATTGTTAGGGACAGCGCTGTTAGGAC
   TATATTTCCAAGCTAATAGTGTTTCATGCGGATGCGACTGGTATCACAGCTAATGG
   AGAAACTACCCATAGCAATGTTACTCCAATGGTTCAGACTAATAAGGATGAGGCA
   AGTACACCGCAAACAACACTACTGATTGGTCTGACCCGGCCAAATATCAAAGTGACA
   TTCCAGTTCAGATTTTAGGAATCAATGACTTGCATGGTGGGTTAGAAACGACTGG
10  ATCAGCTACGATTGGAGATAAGACTTATTCGAATGCCGGAACAGTTGCACGCCTA
   GCTGGTAACCTTGATGCGGCGGAGGAAAGTTTTAAGAACGCTAATCCGACGGGA
   AGCTCAATTCGGGTAGAAGCCGGAGATATGGTTGGGGCTTCTCCAGCAAATTCTG
   CTCTTCTCCAAGACGAATCAACCATGCATGCTTTAGACGCAATGCATTTTGAAAT
   AGGAACTTTGGGAAACCATGAGTTCGATGAAGGTTTAGCTGAGTATATGCGGATT
15  GTTAATGGTGGTGAACCTACTAAACAATATAATGAAGCTGAGATGGCCTATCCTC
   ATGTGAAAACAGGGATTAATATCATTACTGCCAATGTTGTAAATAAATCTGATGG
   TCAAATCCCATTTGGAATGCAACCATACTTGATTAAAGAAATTCATACTAGTGAT
   GGTAAGTTGCCCGGATCGGATTTATTGGGATTGAAACTACTTCCCTACCAATTTT
   AACCTTATACGATAATTACAAAGATTATGATGTTTTAGACGAGGCTGAAACAATT
20  GCAAAATATGATCAAATTTTACGCAAAAAAGGTGTTAACGCAATTGTAGTTCTTG
   CCCATACAGGGGTTTCAACTGATAAAGATGGCAGCACTAAAGGTAATGCTGTTGA
   TATCATTAAGAAGCTTTACCAAATTGACCCTGATAATTCTGTGCGACCTTTATATTG
   CTGGTCACTCCCACCAATATGCTAATGCTACTGTTGGAAGTGTAATAATTAGTGCA
   AGCCATTTACACGGGTAAAGCTTACGATGATATTATCGGTTACATCGATCCAACA
25  ACTAATGATTTTGCGCCCAATAGTCTCGTTTCACATGTCTTTCCGGTACTATCTGA
   AAAGGATGCGCCTAATATCAAAACGGATGCAAATGTTACAGCAATTGTTGAAGA
   TGCGAACAACCGAGTAGCACCGATTATTAACAAGAAAATAGGGGAAGCTGCTAC
   AACAGGCGATATTCTTGGACGACTTCATAATACTCCTACTCGTGAAAATGCCGTT
   GGTGAATTAGTTGTGCGATGGTCAATTATATGCCGCTCATAAAGTAGGCTTACCAG
30  CTGATTTTTCGATGACTAATACAGGGGGCGTTCGTGCGAGATCTGCATGTTAATCC
   TGATCGTTCCATTACATGGGGGAGTGCCCAAGCAGTTCAACCATTTGGTAATATT
   TTGCGGGTAGTTGAAATGACAGGGGACAAATCGTTGAAGCCTTGAATCAACAAT
   ACGACGAAGATCAAGCTTACTACTTACAGATTTCCGGGCTACATTATACTTATAC
   TGACCAAAACGATCCTAACCAACCATATAAGGTCGTTCAAGTTTATGACCAACAT
35  AATCAACCGCTTGATATGAATAAGACTTACAATGTTGTTATTAATGACTTTTTAGC
   AGGTGGCGGAGATGGCTTTTCTGCATTTAAGGGTACTAAAGTTGTCGGGATTGTT
   GGTCAAGATACAGACGCGTTTATTGACTATATTACTGATATGACTAATGATGGTA
   AACCAATTACTGCGCCAACAATGAACCGTAAGATTTACTTGACTGCTGAACAAGT
   AGCGAAGGCTGACTCAGATTCACAGTTACAAACAGGAACTAATCAGAACACTCA
40  AAACGATGCTAATTCCCAGACTGAAGGAAATCAGCTTCAAGAAGTTCCGAGCCA
   ACCGGTATCTCCAACAGTAACCTTGCCAACAACAGCTGGTCAACCCGCCGAAACT
   GTTACACTACATGCTCAATCTAAGCAACAAACCGTAGCTGCTAATAATCAATTAA
   TTAATTTGACGCCTACATCAATTAATGGCCAAAAACAAAAAGCAGCTGACCAGCA
   AGCAGCTTTACCACAACTAGTAACGATGAAGATCTTGCATTACTTCTTCTCGGA
45  AGTTCATTAATGGCAGCAACCGGATTGACAATTATTGATCGCAAGCGTAAACATG
   CTTAA
```

Cell surface anchored 5'-nucleotidase protein, GenBank accession number: AEI56270.1, LPXTG-motif cell wall anchor domain protein [*Lactobacillus reuteri* SD2112] (SEQ ID NO:9)

5 MKNNSSKYCLLLGTALLGLYFQANSVHADATGITANGETTHSNVTPMVQTNKDEAS
TPQTTTDSWSDPAKYQSDIPVQILGINDLHGGLTTGSATIGDKTYSNAGTVARLAGNL
DAAEESFKNANPTGSSIRVEAGDMVGASPANSALLQDESTMHALDAMHFEIGTLGN
HEFDEGLAEYMRIVNGGEPTKQYNEAEMAYPHVKTGINIITANVVNKSDGQIPFGMQ
10 PYLIKEIHTSDGKVARIGFIGIETTSPLILTYDNYKDYDVLDEAETIAKYDQILRKKGV
NAIVVLAHTGVSTDKDGSTKGNVAVDIKKLYQIDPDNSVDLYIAGHSHQYANATVGS
VKLVQAIYTGKAYDDIIGYIDPTTNDFAVNSLVSHVFPVLSEKDAPNIKTANVTAIVE
DANNRVAPIINKKIGEAAATTGDILGRLHNTPTRENAVVELVVDGQLYAAHKVGLPAD
FAMTNTGGVRADLHVNPDRSITWGSAAQAVQPFGNILRVVEMTGAQIVEALNQQYDE
15 DQAYYLQISGLHYTYTDQNDPNQPYKVQVVDQHNQPLDMNKTYNVVINDFLAGG
GDGFSAFKGTKVVGIVGQDTDAFIDYITDMTNDGKPITAPTMNRKIYLTAEQVAKAD
SDSQLQTGTNQNTQNDANSQTEGNQLQEVSPQVSPVTLPTTAGQPAETVTLHAQS
KQQTVAANNQLINLTPTSINGQKQKAADQQAALPQTSNDEDLALLLLGSSLMAATGL
TIIDRKRKHA

20 **EXAMPLE 2**

Procedure for analysing 5'-nucleotidase activity

The Crystal Chem 5'-Nucleotidase Assay Kit (Crystal Chem, Elk Grove Village, IL, USA) was used for determining the 5'-nucleotidase activity of the bacterial cells and the
25 fermentation supernatants. In short, the procedure was as follows.

In two steps, reagents CC1 and CC2 was added to the samples containing the bacteria or the supernatants. Reagent 1 contains AMP that was converted to adenosine by the 5'-nucleotidase enzyme from the bacteria. Adenosine was further hydrolysed into inosine and hypoxanthine by components in reagent 1. In the second step, hypoxanthine was converted to uric acid and
30 hydrogen peroxide, which was used to generate a quinone dye that was measured kinetically at 550 nm in a spectrophotometer. The activity was determined by calculating the change in absorbance between 3 and 5 minutes and comparing with the value from a calibrator sample.

EXAMPLE 3

35 **5'-nucleotidase activity in *Lactobacillus reuteri* strains**

Experimental data showing 5'-nucleotidase activity in *Lactobacillus reuteri* DSM 32846, DSM 32847, DSM 32848 and DSM 32849 (all deposited under the Budapest Treaty at the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Mascheroder

Weg 1b, Inhoffenstr. 7B, D-38124 Braunschweig) on July 4, 2018) as well as in *Lactobacillus reuteri* DSM 17938 (deposited under the Budapest Treaty at the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Mascheroder Weg 1b, D-38124 Braunschweig) on 30 January 2006) was generated using the method described in Example 2 above. The results are shown in Figure 3.

EXAMPLE 4

Selection of strains

All new strains in Example 3 above, i.e. *Lactobacillus reuteri* DSM 32846, DSM 32847, DSM 32848 and DSM 32849 show 5'-nucleotidase activity in the bacterial supernatant. Analysis was carried out on the supernatant of bacterial cultures with the concentration of 10^9 bacteria per ml.

Lactobacillus reuteri DSM 32846, DSM 32847, DSM 32848 and DSM 32849 have been developed/evolved for improved properties. DSM 32846 and DSM 32847 have been made to evolve to be more tolerant to bile acids and thereby survive in larger numbers in the GI-tract. DSM 32848 and DSM 32849 have evolved to adhere better to mucus, with the aim to colonize better in the GI-tract and thereby function better according to the present invention.

All strains *Lactobacillus reuteri* DSM 32846, DSM 32847, DSM 32848 and DSM 32849 are selected. Preferred strains are DSM 32846, DSM 32847 and DSM 32849.

EXAMPLE 5

Effects of probiotic bacterial strains on melatonin production in a human intestinal enteroid model

Background

Human intestinal enteroids (HIEs) that are derived from intestinal crypt stem cells are biologically relevant in an in vitro model of the intestinal epithelium. HIEs contain all intestinal epithelial cell types; however, similar to the human intestine, HIEs normally produce Enteroendocrine cells (EECs) in low amounts (~1%), which limits their study. To increase the number of EECs in HIEs, a lentivirus transduction was used to stably engineer jejunal HIEs with doxycycline-inducible expression of neurogenin-3 (NGN3), a transcription

factor that drives EEC differentiation (tetNGN3- HIEs). In this genetically engineered cell line the number of enteroendocrine cells present in the culture increases from $\leq 1\%$ to $\sim 40\%$.

Material and methods

5

Human intestinal enteroids

HIE cultures were generated from crypts isolated from the jejunal tissues of adult patients undergoing bariatric surgery. These established cultures were obtained at Baylor College of Medicine through the Texas Medical Center Digestive Diseases Center Study Design and
10 Clinical Research Core. Three-dimensional HIE cultures were prepared from the tissue samples and maintained in culture. To increase the number of EECs in HIEs, a lentivirus transduction was used to stably engineer jejunal HIEs with doxycycline-inducible expression of neurogenin-3 (NGN3), a transcription factor that drives EEC differentiation (tetNGN3- HIEs). In this genetically engineered cell line the number of enteroendocrine cells present in
15 the culture increases from $\leq 1\%$ to $\sim 40\%$. All enteroids were propagated and differentiated in 96-well monolayer form per standard protocols. More information on this enteroid line, how it is cultured and general methodology for the assay are described in Chang-Graham *et al* (Chang-Graham et al., Cellular and Molecular Gastroenterology and Hepatology, 2019, S2352-345X(19)30049-9, in press, online publication available).

20

Bacterial strains and preparations

In this experiment two different *Lactobacillus reuteri* strains (DSM 17938 and DSM 32846) were studied in regards to their capability of increasing the melatonin production in the intestine.

25 Conditioned medium (supernatant) from *Lactobacillus reuteri* DSM 17938 and *Lactobacillus reuteri* DSM 32846 grown in Lactobacillus Defined Media 4 (LDM4) media until they reach stationary phase was used in this study, see more details below.

A single colony of the bacterial strain was picked from an MRS agar plate and used to
30 inoculate 10 mL of MRS broth and incubated in a tightly closed conical tube in a 37°C water bath. After eight hours a 1:10 dilution of the culture was made and the optical density at 600 nm was read by spectrophotometer, then a 25 mL of pre-warmed LDM4 was inoculated at a starting OD of 0.1 (calculation below) and placed into the water bath to incubate for 16 hours.

- $OD = 0.307$. 1:10 Dilution = OD of 3.07

$$25\text{mL} * (0.1 \text{ OD}) = 3.07x \quad x = 0.814 \text{ mL}$$

- 814 uL was combined with 24.19 mL of LDM4

5

After 16 hours the culture was taken out. The final OD was 2.6. The cells were pelleted by centrifugation at 4700 rpm and the supernatant was transferred to a new 50 mL conical tube. The supernatants were stored at -20°C overnight.

10 *Chemicals used*

AMP = Adenosine 5'-monophosphate, 99% (Acros Organics) Code: 102790259, Lot: A0395274.

The 10 mL of stock solution (Stock solution of 2 mM = 6.9 mg/ml (FW=347.224)) was made in MiliQ water and filter sterilized through a 0.22 uM filter.

15

Experimental setup

Control treatments on induced enteroids:

- LDM4 (culture medium),
- LDM4 + 20 uM AMP,
- 20 ▪ LDM4 + 200 uM AMP

Experimental treatments of DSM 32846 and DSM 17938 supernatant on induced enteroids:

- DSM 32846 supernatant alone
- DSM 32846 supernatant + 20 uM AMP
- 25 ▪ DSM 32846 supernatant + 200 uM AMP
- DSM 17938 supernatant alone
- DSM 17938 supernatant + 20 uM AMP
- DSM 17938 supernatant + 200 uM AMP

30

Before the supernatant or the other additions was ready to be applied onto the enteroids, pH was neutralized and the supernatant was filter sterilized. The pH was adjusted to 7.0 using 10 – 30 uL of 10M Sodium Hydroxide solution and the pH was measured by applying 2 uL of the supernatant onto pH Paper (Fisher Brand – Cat. 13-640-510 range 6.0 – 8.0). The

supernatant was filter sterilized using a 0,22uM filter (Thermo Scientific Nalgene Rapid Flow 0.22 uM filters Code: 00158)

100 uL (total volume) of treatment was placed on the enteroids. Incubation was 1 hour on the enteroids at 37°C, 5% CO₂ tissue culture incubator. Following incubation, the supernatant was removed and stored at -20°C until downstream analysis.

Melatonin was detected by ELISA (Eagle Biosciences Inc, Amherst, NH, USA). The only step that was done prior to following the standard protocol was that the supernatants were thawed, and allowed to reach room temperature. (approximately 20 – 21 degrees Celsius).

Results

The human intestinal enteroids (HIEs) is biologically relevant as an in vitro model of the intestinal epithelium. As shown in Figure 4 DSM 32846 alone has no significant effect, but when supplemented with AMP it shows a pronounced effect on melatonin production from the HIEs. A higher concentration of AMP gave an even higher melatonin production. DSM 17938 has an increase in melatonin production compared to the control but smaller than DSM 32846. DSM 32846 is therefore a good candidate strain for use in the production of melatonin in the intestine.

EXAMPLE 6 **5'-nucleotidase activity in *Lactobacillus reuteri* strains**

Experimental data showing 5'-nucleotidase activity in *Lactobacillus reuteri* DSM 33198 was generated using the method described in Example 2 above. The results are shown in Figure 5A where they have been normalized in relation to the 5'-nucleotidase activity of DSM17938 (DSM17938 activity=1) from the same experiment. The same type of normalization in relation to the 5'-nucleotidase activity of DSM17938 (DSM17938 activity=1) has also been done on the results from Figure 3 and this is presented in Figure 5B, making it easy to compare the fold-change in 5'-nucleotidase activity in relation to DSM17938 for the different bacterial strains.

EXAMPLE 7 **Selection of strains**

The new strain in Example 6 above, i.e. *Lactobacillus reuteri* DSM 33198 show high 5'-nucleotidase activity in the bacterial supernatant. Analysis was carried out on the supernatant of the bacterial culture with the concentration of 10^9 bacteria per ml.

- 5 *Lactobacillus reuteri* DSM 33198 has been developed for improved properties. The *L. reuteri* strain DSM 33198 has been modified in a multi-step selection process including a repeated freeze-drying procedure to allow it to be more tolerant and give a higher survival in the production process than its native isolate.
- 10 *Lactobacillus reuteri* DSM 33198 is selected.

Claims:

1. A bacterial strain, wherein said strain is *Lactobacillus reuteri* DSM 32846, DSM 32847, DSM 32849 or DSM 33198.
- 5 2. The bacterial strain of claim 1, wherein said strain is *Lactobacillus reuteri* DSM 32846.
3. The bacterial strain of claim 1, wherein said strain is *Lactobacillus reuteri* DSM 32847.
- 10 4. The bacterial strain of claim 1, wherein said strain is *Lactobacillus reuteri* DSM 32849.
- 15 5. The bacterial strain of claim 1, wherein said strain is *Lactobacillus reuteri* DSM 33198.
6. The bacterial strain of any one of claims 1 to 5 for use in therapy.
- 20 7. The bacterial strain of any one of claims 1 to 5 for use in the treatment or prevention of infantile colic in a subject.
- 25 8. The strain for use of claim 7, wherein the subject is an infant of up to 12 months old, preferably up to 6 or 5 months old, or preferably up to 3 or 4 months old; and/or wherein the subject is breastfed or fed with formula milk, or a combination thereof.
9. The strain for use of claim 7 or claim 8, wherein said use further comprises the administration of at least one further agent, wherein said further agent is AMP.
- 30 10. A method for the selection of a lactic acid bacterial strain capable of producing adenosine, wherein said strain is *Lactobacillus reuteri* and wherein said method comprises:
 - a) screening a lactic acid bacterial strain for the presence of a gene encoding a 5'-nucleotidase, wherein said strain is *Lactobacillus reuteri*; and/or

- b) screening a lactic acid bacterial strain, or its supernatant, for the presence of an active 5'-nucleotidase enzyme, wherein said strain is *Lactobacillus reuteri*; and
- c) selecting a *Lactobacillus reuteri* strain which produces adenosine at a level which is increased as compared to *Lactobacillus reuteri* DSM 17938; and optionally
- d) culturing or propagating such a *Lactobacillus reuteri* strain.

11. The method of claim 10, wherein said strain is selected for:

- (i) having a 5'-nucleotidase activity of greater than 8 units/L and/or producing adenosine at a level of greater than 8 $\mu\text{mol L}^{-1} \text{ min}^{-1}$; and/or
- (ii) giving rise to increases of up to 5 fold in 5'-nucleotidase activity and/or levels of adenosine, compared with the levels with DSM 17938; or increases of at least 1.5 fold, preferably increases of at least 2 fold, more preferably increases of at least 3, 4 or 5 fold, in 5'-nucleotidase activity and/or levels of adenosine, compared with the levels with DSM 17938.

12. A combination product suitable for increasing the production of melatonin or adenosine in a subject comprising:

- (i) a first *Lactobacillus reuteri* strain capable of inducing the production of melatonin or producing adenosine, wherein said strain is the strain of any one of claims 1 to 5; and
- (ii) a substrate component to increase or enhance the induction of production of melatonin and/or the production of adenosine by said *Lactobacillus reuteri* strain, wherein said substrate component is AMP.