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(54) Title: A *PEDIOCOCCUS ACIDILACTICI* STRAIN CAPABLE OF INCREASING MILK YIELD

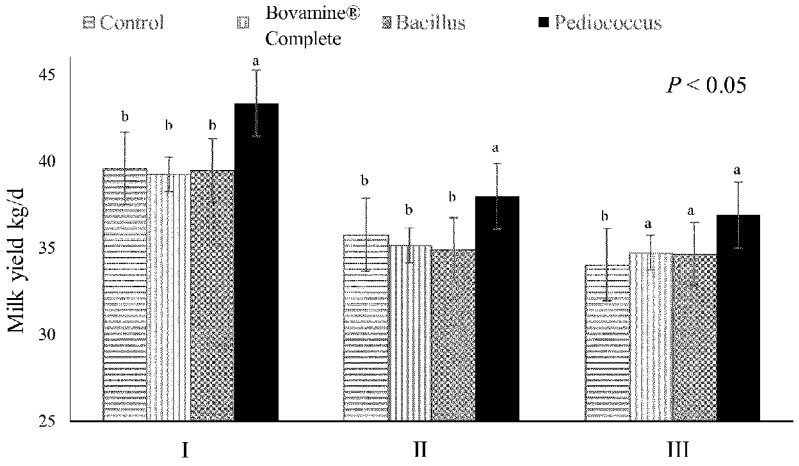


Figure 1

(57) Abstract: The present invention provides a composition comprising the *Pediococcus acidilactici* strain deposited as DSM 32500 or a mutant strain thereof, as well as to these strains. The present invention further relates to a method of increasing milk yield from a mammal, the method comprising feeding the mammal with feed, food, a dietary supplement or water to which a *Pediococcus acidilactici* strain of the invention has been added.

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TITLE

A *Pediococcus acidilactici* strain capable of increasing milk yield

FIELD OF THE INVENTION

The present invention relates to a composition comprising the *Pediococcus acidilactici* strain deposited as DSM 32500 or a mutant strain thereof, as well as to these strains.

The invention further relates to a method of increasing milk yield from a mammal, the method comprising oral administration of a *Pediococcus acidilactici* strain of the invention to the mammal, e.g. with feed, food, a dietary supplement or water to which the strain has been added.

BACKGROUND OF THE INVENTION

WO2016/046706 discloses use of *Pediococcus acidilactici* strain BaltBio01 MSCL P1480 and *Pediococcus pentosaceus* BaltBio02 MSCL P1481 separately or as mixtures as a probiotic (as the initial fermentative microorganism) in the fermented feed additives, supplements, premixtures, feed materials and compound feeds. Examples 1 and 2 assess the effects of the mixture of the two strains, whereas Example 3 relates to the *Pediococcus pentosaceus* additive. No results are provided for the *Pediococcus acidilactici* strain BaltBio01 MSCL P1480 separately.

US2106/0029666 describes a composition comprising a mix of probiotic bacteria *Bacillus subtilis, Pedicoccus acidilactici*, *Pedicoccus pentosaceus*, and *Lactobacillus plantarum*. Example 9 provides a dairy cattle feeding study wherein the composition of Example 1 was used which was prepared by combining premixes A, B, and C in equal proportion by weight and mixing to homogeneity then mixing in Premix D and a filler. A 1.9% increase in milk production was observed. However, the results are not reproducible as no strain numbers are provided.

Krungleviciute et al., 2017, "Applicability of *Pediococcus* strains for fermentation of cereal bran and its influence on the milk yield of dairy cattle", Zemdirbyste-Agriculture, vol. 104, No. 1, p. 1392-1396, describes a *Pediococcus acidilactici* strain BaltBio01 and a *Pediococcus pentosaceus* strain BaltBio02 for the production of fermented feed stock and the influence of the fermented feed stock on milk production and composition was determined. Significantly higher milk yield was obtained by using *Pediococcus* mix (*P. acidilactici* BaltBio01 x *P. pentosaceus* BaltBio02) cultivated in cereal by-products for

dairy cattle feeding but not when using *P. acidilactici* BaltBio01 compared to control (Table 6 Group A (control) vs Group B (*P. acidilactici*).

Different yeast products, such as live or dead yeast, yeast culture/hydrolysed yeast, yeast cell wall, and yeast extract or fractions (peels, yeast extracts, etc.) have been used for many years in the cattle business to improve animal performance (meat and milk production).

Direct Fed Microbials (DFM) are used for feeding animals in order to improve their overall health and nutrition *via* competitive exclusion and direct inhibition of enteric pathogens, modulation of the gut health and immune system increased digestibility of the fed. The effect of DFM on ruminants has been considered to be very low because of the high amounts of microorganisms in the rumen. The DFM need either to pass the rumen and have an effect in the small intestine or compete with millions of other microorganisms in the rumen.

While combinations of yeast and DFM for increasing milk production are known, the present invention relates to a method of increasing milk yield using a DFM only, i.e. without any yeast products.

SUMMARY OF THE INVENTION

The present invention provides a *Pediococcus acidilactici* strain which shows surprisingly good effect on milk yield. In an *in* vivo study comparing administration of *Pediococcus acidilactici* DSM 32500, *Bacillus subtilis* DSM 25841 and the commercial product Bovamine® Complete consisting of three *Enterococcus faecium* strains and yeast, it was surprisingly found that the milk yield increased only for the group of animals fed with *Pediococcus acidilactici* DSM 32500 as the only active component.

Thus, the present invention relates to a composition according to the invention for use in increasing the milk yield of a human as well as to a method for increasing milk yield from a non-human mammal, the method comprising oral administration of a composition comprising a *Pediococcus acidilactici* DSM 32500 or a mutant strain thereof to the non-human mammal.

It is known from the literature that bile has negative influences on the survival and growth of bacterial cells in the gastrointestinal tract (GIT) of animals and humans. Therefore probiotic bacteria shall generally be able to survive and proliferate in the GIT of animals or humans. *Pediococcus acidilactici* has to be able to tolerate a low pH and to

be resistant to bile salt in order to be useful as probiotic and to be considered for addition to animal feed or for administration to a human. The examples provide useful *in vitro* tests in this regard. The test for sensitivity to low pH (simulating gastric conditions) focuses on the activity of bacterial cells at pH 4, as gastric pH may have pH values of up to 4 especially in feeding conditions making it relevant to test the sensitivity of the cells at pH 4. Selected strains should preferably be active at pH 4. The *Pediococcus acidilactici* strain deposited as DSM 32500 has been assayed for sensitivity of the vegetative cells at pH 4 and bile resistance to ensure that the strain is suitable.

The ability to adhere to mucosal surface is mandatory for pathogens to cause infection and for the probiotic organisms to exert their beneficial influence. The bacterial cell surface characteristics predominantly determine their ability to adhere to mucosal surfaces, autoaggregate and coaggregate. Adhesion to mucosal surface is another pivotal requirement for probiotic bacteria, since it confers to them the ability to resist the flux of ruminal and intestinal content and provides bacterial persistence in the GIT of the host. The examples provide useful *in vitro* tests in this regard using Caco-2 and HT29-MTX cells.

Autoaggregation helps LAB cells to adhere and colonize gastrointestinal surfaces, to form a barrier against colonization of mucosal surfaces by pathogens, to provide protection in the hostile environment of the GIT, and to influence the host's health by affecting the microbial homeostasis towards healthier composition.

In vitro assays demonstrate that *Pediococcus acidilactici* DSM 32500

(i) is sensitive to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin,

(ii) exhibits an OD_{610} value of at least 1.0 after 4 h of exposure to pH 4.0 or 6.0 followed by 8 h of exposure to 0.3% bile,

(iii) exhibits an OD_{600} value of at least 0.8 after 24 h of exposure to pH 4.0, pH 6.5 or 0.3% bile,

(iv) exhibits an adhesion to Caco-2 of at least 80% and to HT29-MTX cells of at least 67%, and

(v) exhibits an autoaggregation after 5 h of at least 11%.

Accordingly, the present invention relates to a *Pediococcus acidilactici* strain selected from the group consisting of the *Pediococcus acidilactici* strain deposited as DSM 32500, as well as functional mutant strains thereof.

Compositions comprising at least one *Pediococcus acidilactici* strain according to the

invention, e.g. as Direct Fed Microbial (DFM), an animal feed additive or premix, or an animal feed may be fed to an animal. The at least one *Pediococcus acidilactici* strain according to the invention may be added to the feed/premix during production, after production by the supplier or by the person feeding the animal, just prior to providing the feed to the animal.

The present invention further provides an animal feed, animal feed additive or premix comprising at least one *Pediococcus acidilactici* strain according to the invention, and further comprising one or more of concentrate(s), vitamin(s), mineral(s), enzyme(s), amino acid(s) and/or other feed ingredient(s). In one embodiment the animal feed, animal feed additive or premix comprises the *Pediococcus acidilactici* strain DSM 32500.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising", "having", "including" and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All steps of methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g. "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

In general, the terms and phrases used herein have their art-recognized meaning, which can be found by reference to standard texts, journal references, and context known to those skilled in the art. The following definitions are provided to clarify their specific use in context of the disclosure.

Animal feed: The term "animal feed" refers to any compound, preparation, or mixture suitable for, or intended for intake by an animal. Animal feed comprises concentrates as

well as for example vitamins, minerals, enzymes, amino acids and/or other feed ingredients (such as in a premix). The animal feed may further comprise forage.

Bacterial strain: A "bacterial strain" refers to a bacterium which remains genetically unchanged when grown or multiplied. The multiplicity of such identical bacteria is included when reference is made to a strain. The bacterial strains described herein are isolated, i.e. present in a form or environment which does not occur in nature.

Composition: The term "composition" refers to a composition comprising a carrier and at least one bacterial strain as described herein. The compositions described herein may be a Direct Fed Microbial (DFM), an animal feed additive or premix, or an animal feed. Compositions comprising at least one *Pediococcus acidilactici* strain according to the invention intended for human use will generally be provided as a dietary supplement, e.g. in the form of a powder, tablet or capsule, or incorporated in a food product.

Concentrate: The term "concentrate" means a feed with high protein and energy concentrations, such as fish meal, molasses, oligosaccharides, sorghum, seeds and grains (either whole or prepared by crushing, milling, etc. from, e.g. corn, oats, rye, barley, wheat), oilseed press cake (e.g. from cottonseed, safflower, sunflower, soybean (such as soybean meal), rapeseed/canola, peanut or groundnut), palm kernel cake, yeast derived material and distillers grains (such as wet distillers grains (WDS) and dried distillers grains with solubles (DDGS)).

Direct Fed Microbial: The term "direct fed microbial" or "DFM" means live micro-organisms which, when administered in adequate amounts, confer a benefit, such as improved digestion or health, on the host.

Effective amount/concentration/dosage: The terms "effective amount", "effective concentration", or "effective dosage" are defined as the amount, concentration, or dosage of the bacterial strain(s) sufficient to improve the digestion or yield of an animal or human. The actual effective dosage in absolute numbers depends on factors including the state of health of the animal or human in question and other ingredients present. The "effective amount", "effective concentration" or "effective dosage" of the bacterial strains may be determined by routine assays known to those skilled in the art.

Feeding: The terms "feeding" or "oral administration" mean that the composition of the present invention is administered orally to the animal or human in an effective amount. The oral administration may be repeated, e.g. one or more times daily over a specified time period such as several days, one week, several weeks, one months or several

months. Accordingly, the terms "feeding" or "fed" or "oral administration" mean any type of oral administration such as administration via an animal feed, a food product or as a dietary supplement or via drinking water.

Forage: The term "forage" as defined herein also includes roughage. Forage is fresh plant material such as hay and silage from forage plants, grass and other forage plants, seaweed, sprouted grains and legumes, or any combination thereof. Examples of forage plants are Alfalfa (lucerne), birdsfoot trefoil, brassica (e.g. kale, rapeseed (canola), rutabaga (swede), turnip), clover (e.g. alsike clover, red clover, subterranean clover, white clover), grass (e.g. Bermuda grass, brome, false oat grass, fescue, heath grass, meadow grasses, orchard grass, ryegrass, Timothy-grass), corn (maize), millet, barley, oats, rye, sorghum, soybeans and wheat and vegetables such as beets. Forage further includes crop residues from grain production (such as corn stover, straw from wheat, barley, oat, rye and other grains), residues from vegetables like beet tops, residues from oilseed production like stems and leaves from soy beans, rapeseed and other legumes, and fractions from the refining of grains for animal or human consumption or from fuel production or other industries.

An "increased milk yield" refers to an increase of the quantity of milk produced by a mammal to which a composition of the invention has been administered compared with a mammal to which a composition without said composition of the invention has been administered. Specifically, the increased milk yield is the increase of milk produced by the mammal over a specified time period such as a week. In one embodiment, the improvement in milk yield is of at least 5%, such as at least 6%, such as at least 7%, such as at least 8%, such as at least 9%, such as at least 10%.

Isolated: The term "isolated" means that the bacterial strains described herein are in a form or environment which does not occur in nature, i.e. the strain is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature.

Mammal: Examples of mammals are human, companion animals such as dog and cat and livestock animals such as cattle/bovine, sheep/ovine, goat/caprine, horse and donkey/equine, lama and camel/camelid, swine, and rabbit.

The term "premix" refers to a *Pediococcus acidilactici* strain added to a carrier to make a premix which is then added to an animal feed at a desired inclusion rate. Usually fat- and water-soluble vitamins, as well as trace minerals form part of the premix, whereas macro minerals are usually separately added to the feed.

Sensitive to antibiotics: The term "sensitive to antibiotics" means the phenotypic property of a bacterial strain, that growth of said bacterial strain is inhibited under conditions where the bacterial strain would otherwise grow. In this context sensitivity to antibiotics is tested after the CLSI guidelines (M07-A8 and M45-A2). A strain of *Pediococcus acidilactici* is considered sensitive if growth is only detected at or below the breakpoint concentration specified in Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, EFSA Journal 2012; 10(6):2740 for ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin.

Yeast: The term "yeast" includes live or dead yeast, yeast culture/hydrolysed yeast, yeast cell wall, and yeast extract or fractions (peels, yeast extracts etc.)

The present invention provides a *Pediococcus acidilactici* strain selected from the group consisting of

a) the *Pediococcus acidilactici* strain deposited as DSM 32500, and
b) a mutant strain thereof which
   (i) is sensitive to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin,
   (ii) exhibits an OD_{610} value of at least 0.8 after 4 h of exposure to pH 4.0 or 6.0 followed by 8 h of exposure to 0.3% bile,
   (iii) exhibits an OD_{600} value of at least 0.6 after 24 h of exposure to pH 4.0, pH 6.5 or 0.3% bile,
   (iv) exhibits an adhesion to Caco-2 cells of at least 70% and to HT29-MTX cells of at least 55%, and
   (v) exhibits an autoaggregation after 5 h of at least 9%.

The tests described in the examples can be used in order to measure whether a strain fulfils the above requirements.

A "mutant bacterium" or a "mutant strain" refers to a natural (spontaneous, naturally occurring) mutant bacterium or an induced mutant bacterium comprising one or more mutations in its genome (DNA) which are absent in the parent strain DNA. An "induced mutant" is a bacterium where the mutation was induced by human treatment, such as treatment with any conventionally used mutagenesis treatment including treatment with chemical mutagens, such as a chemical mutagen selected from (i) a mutagen that associates with or become incorporated into DNA such as a base analogue, e.g. 2-aminopurine or an interchelating agent such as ICR-191, (ii) a mutagen that reacts with

the DNA including alkylating agents such as nitrosoguanidine or hydroxylamine, or ethane methyl sulphonate (EMS) or N-methyl-N'-nitro-N-nitroguanidine (NTG), UV- or gamma radiation etc. In contrast, a "spontaneous mutant" or "naturally occurring mutant" has not been mutagenized by man.

A mutant may have been subjected to several mutagenesis treatments (a single treatment should be understood one mutagenesis step followed by a screening/selection step), but it is presently preferred that no more than 20, or no more than 10, or no more than 5 treatments (or screening/selection steps) are carried out. In a presently preferred mutant less than 1%, less than 0.1, less than 0.01, less than 0.001% or even less than 0.0001% of the nucleotides in the bacterial genome have been replaced with another nucleotide, or deleted, compared to the mother strain.

Mutant bacteria as described above are non-GMO, i.e. not modified by recombinant DNA technology. As an alternative to the above preferred method of providing the mutant by random mutagenesis, it is also possible to provide such a mutant by site-directed mutagenesis, e.g. by using appropriately designed cloning techniques.

When the mutant is provided as a spontaneously occurring mutant the strain is subjected to the selection step without any preceding mutagenesis treatment.

In one embodiment the *Pediococcus acidilactici* strain of the invention has at least 98% (such as at least 98.5%, such as at least 99%, such as at least 99.5%, such as at least 99.6%, such as at least 99.7%, such as at least 99.8%, such as at least 99.9%) sequence identity to the nucleotide sequence of DSM 32500.

In one embodiment the *Pediococcus acidilactici* strain of the invention has at least 98% (such as at least 98.5%, such as at least 99%, such as at least 99.5%, such as at least 99.6%, such as at least 99.7%, such as at least 99.8%, such as at least 99.9%) sequence identity to the amino acid sequence of DSM32324.

The relevant *Pediococcus acidilactici* strain or strains are provided in a commercially relevant form known to the skilled person. Accordingly, in an embodiment the *Pediococcus acidilactici* strain or strains of the composition are present in a dried (e.g. spray dried) or frozen form. The composition may be provided in any suitable form such is in the form of a liquid e.g. a gel, a slurry, a powder or a pellet.

In a preferred embodiment the *Pediococcus acidilactici* composition comprises from 10^5 to 10^{12} CFU/g, more preferably from 10^6 to 10^{12} CFU/g, and most preferably from 10^7 to 10^{12} CFU/g, such as from 10^8 to 10^{11} CFU/g, e.g. from 10^9 to 10^{10} CFU/g of each of the bacterial strains in the composition. The *Pediococcus acidilactici* composition comprises at least 5 x 10^4 CFU of each strain per gram of the composition which distinguishes a composition of the present invention from e.g. animal feed/premix with naturally occurring strains.

The term "CFU/g" relates to the gram weight of the composition including carriers such as calcium carbonate, anti caking agents such as aluminium silicates and kieselgur (diatomaceous earth), and other components present in the composition.

Compositions of the present invention include at least one *Pediococcus acidilactici* strain of the invention and at least one carrier and/or other component that make the composition suitable for feeding to an animal or administration to a human or as an additive for drinking water.

Animal diets can be manufactured e.g. as mash feed (non-pelleted) or pelleted feed. Typically, the milled feed-stuffs are mixed and sufficient amounts of essential vitamins and minerals are added according to the specifications for the species in question. The bacteria cultures and optionally enzymes can be added as solid or liquid formulations. For example, for mash feed a solid or liquid culture formulation may be added before or during the ingredient mixing step. For pelleted feed the (liquid or solid) *Pediococcus acidilactici* composition may also be added before or during the feed ingredient step. Typically a liquid *Pediococcus acidilactici* composition of the invention comprises the bacterial strain(s) optionally with a polyol, such as glycerol, ethylene glycol or propylene glycol, and is added after the pelleting step, such as by spraying the liquid formulation onto the pellets. The bacteria may also be incorporated in an animal feed additive or premix.

In one embodiment, the forage and at least one *Pediococcus acidilactici* strain of the invention are mixed with a concentrate. In another embodiment, the forage and at least one *Pediococcus acidilactici* strain of the invention are mixed with a premix. In a further embodiment, the forage and at least one *Pediococcus acidilactici* strain of the invention are mixed with vitamins and/or minerals. In a further embodiment, the forage and at least one *Pediococcus acidilactici* strain of the invention are mixed with one or more enzymes. In a further embodiment, the forage and at least one *Pediococcus acidilactici* strain of the invention are mixed with other feed ingredients, such as colouring agents, stabilisers, growth improving additives and aroma compounds/flavourings,

polyunsaturated fatty acids (PUFAs); reactive oxygen generating species, anti-microbial peptides, anti-fungal polypeptides and amino acids.

In a particular embodiment the animal feed consists of or comprises milk (e.g. from cow sheep, goat and sow). In a particular embodiment the animal feed consists of or comprises milk replacement, e.g. for feeding of a calf.

The composition may include one or more vitamins, such as one or more fat-soluble vitamins and/or one or more water-soluble vitamins and optionally one or more minerals, such as one or more trace minerals and/or one or more macro minerals. Non-limiting examples of fat-soluble vitamins include vitamin A, vitamin D3, vitamin E, and vitamin K, e.g. vitamin K3. Non-limiting examples of water-soluble vitamins include vitamin B12, biotin and choline, vitamin B1, vitamin B2, vitamin B6, niacin, folic acid and panthothenate, e.g. Ca-D-panthothenate. Non-limiting examples of trace minerals include boron, cobalt, chloride, chromium, copper, fluoride, iodine, iron, manganese, molybdenum, selenium and zinc. Non-limiting examples of macro minerals include calcium, magnesium, potassium and sodium.

The animal feed, animal feed additive or premix further comprises a carrier. The carrier can comprise one or more of the following compounds: water, glycerol, ethylene glycol, 1,2-propylene glycol or 1,3-propylene glycol, sodium chloride, sodium benzoate, potassium sorbate, sodium sulfate, potassium sulfate, magnesium sulfate, sodium thiosulfate, calcium carbonate, sodium citrate, dextrin, maltodextrin, glucose, sucrose, sorbitol, lactose, whey, whey permeate, wheat flour, wheat bran, corn gluten meal, starch and cellulose.

In an embodiment, the composition, animal feed, animal feed additive or premix further comprises one or more additional microorganisms. In a particular embodiment, the composition, animal feed, animal feed additive or premix further comprises a bacterium from one or more of the following genera: *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Bacillus*, *Pediococcus*, *Enterococcus*, *Leuconostoc*, *Carnobacterium*, *Propionibacterium*, *Bifidobacterium*, *Clostridium* and *Megasphaera* or any combination thereof.

In a preferred embodiment, the composition, animal feed, animal feed additive or premix according to the invention does not comprise a *Pediococcus pentosaceus* strain.

If more than one strain is used, it is contemplated that the proportion of each strain in the composition will be 1 to 99%, such as 20 to 80%, e.g. 30 to 70%, more particularly 20%, 33%, 40% or 50% of the total amount of bacterial isolates calculated as CFU/g composition. The individual strains may be present in about equal numbers or in unequal numbers.

LEGEND TO FIGURES

Figure 1

Milk yield of dairy cows receiving daily probiotic supplements

I: From week 1 to 5

II: From week 6 to 10

III: From week 11 to 15

Figure 2

Diurnal pattern of rumen pH in dairy cows receiving dietary probiotic supplements

Figure 3

Continuous measuring of the ability of *Pediococcus acidilactici* to increase the electrical resistance across Caco-2 cell monolayers (trans-epithelial electrical resistance; TEER)

EXAMPLES

EXAMPLE 1

In vitro screening

Materials:

ISO-SENSITEST Broth (Oxoid CM0473)

LSM broth: 10% MRS and 90% ISO-SENSITEST Broth

MRS broth (Oxoid CM0359)

Simulated gastric fluid (SGF): 0.72 g/l NaCl, 0.05 g/l KCl, 0.37 g/l $NaHCO_3$, 0.3 g/l pepsin)(Sigma S7653, P9333, S5761, P7000)

Simulated intestinal fluid (SIF): 0.1% w/v pancreatin and 0.3% w/v Ox-gall bile salts

Pancreatin (Sigma P3292)

Ox-gall bile salts (Sigma 70168)

PBS (Sigma PBS1)

Caco-2 (DSMZ ACC169)

HT29-MTX (Sigma 1204041)

Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (Fisher Sci. 21885-025)

Fetal bovine serum (FBS, Fisher Sci. Gibco 10500064)

Non-essential amino acids (Fisher Sci. GE Hyclone SH30238.01)

Penicillin/Streptomycin/Amphotericin B (In vitro, Biological Industries 03-033-1B)

Methanol (Sigma 322415)

Gram staining kit (Sigma 77730)

Pediococcus acidilactici DSM 32500

Antibiotic susceptibility testing and MIC determinations

Pediococcus acidilactici DSM 32500 was subjected to screening for antibiotic susceptibility according to "Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance." EFSA Journal 2012;10(6):2740 as described below.

The strain was analysed for antibiotic susceptibility by measuring the minimum inhibitory concentration (MIC) for ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin. The method used was a broth microdilution method as outlined by the standard of CLSI (Clinical and Laboratory Standards Institute M07-A8 and M45-A2).

A suspension of an overnight culture of the strain was inoculated in LSM broth in microtitre plates at an approximate concentration of 10^5 CFU/ml (colony-forming units/ml) in two-fold serial dilutions of the antibiotic to be tested (total volume 100 µl/well) and incubated aerobically for 20-24 h at 30°C. The results were recorded after 20 h of incubation as the lowest concentration of the antibiotic to inhibit visible growth. The test was performed twice as two independent biological replicates.

Tolerance to low pH and high bile levels

The strain has been tested for tolerance to low pH and high bile levels. The strain was grown in a 96-well microtitre plate containing MRS broth and incubated anaerobically for 48 h at 37°C. 20 µl culture (10% inoculum) was inoculated into 180µl MRS broth with pH of 2.0, 4.0 and 6.0. The plate was incubated anaerobically for 4 h at 37°C and then 10 µl of the 4 h culture (5% inoculum) was inoculated into a fresh microtitre plate with MRS broth containing 0.3% Ox bile with further 8 h of anaerobic incubation at 37°C before reading the OD_{610}. Fresh MRS broth inoculated with 10% inoculum and incubated for 12 h was used as control. The data were expressed as means of three observations derived from three independent experiments.

Viability during simulated gastrointestinal transit

The strain was tested for viability during simulated gastrointestinal transit and intestinal fluid transit. A single aliquot of bacteria from 48 h pure culture (3 x 10^8 CFU/ml) was exposed to simulated gastric fluid (SGF) and adjusted to pH 2.0 with HCl 1 M and to pH 7.0 with NaOH 1 M as the control condition for 0 and 90 min. After 90 min of incubation in SGF at pH 2.0 and in control condition (pH 7.0), the strain was exposed to simulated intestinal fluid (SIF, pH 8.0) for 0 and 180 min. The OD_{600} of the suspensions was

measured. The data were expressed as means of three observations derived from three independent experiments.

Adhesion to intestinal epithelial cells

The assay was performed by employing the Caco-2 and HT29-MTX cell lines. The Caco-2 cells were grown on Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), L-glutamine (2 mmol l^{-1}), 1% non-essential amino acids, 100 µg streptomycin ml^{-1}, 100 U penicillin ml^{-1}, and 0.25 µg amphotericin B ml^{-1} at 37 °C in a 5 % CO2 atmosphere. For the HT29-MTX cells, 10% FBS was used and the non-essential amino acids were omitted from the DMEM medium. For the adhesion assay, cells were seeded in 6-well plates with cover slip inserts at a concentration of 1 x 10^6 cells/well and incubated until confluence (21 days for Caco-2, 14 days for HT29-MTX) prior to the assay. The cell culture medium was changed on alternate days with preheated fresh medium, and the cells were washed with DMEM without antibiotics prior to use.

The adherence assay was performed by adding 4 ml of bacterial suspension (approximately 10^8 CFU/ml) diluted in DMEM without antibiotics at pH 7.0. After incubating for 1 h at 37 °C, 5% CO2, monolayers were washed twice with sterile preheated PBS, fixed with methanol for 10min, stained with Gram and air dried at 37°C for 1 h. The slides were then examined microscopically at 100× magnification under oil immersion. The mean ± standard deviation (STDEV) of adherent bacteria was determined on 10 random microscopic fields. The data were expressed as means of three observations derived from three independent experiments.

Autoaggregation

Autoaggregation assay was performed using overnight cultures in MRS broth. Cultures were washed with 2 x 200 µl PBS, the washed cell suspension (2 ml, 1 x 10^8 CFU/ml) was vortexed for 10 s, and incubated at 37°C. An aliquot of 0.1 ml collected from the upper surface at regular time interval was mixed with 0.9 ml PBS and its A_{600} was recorded. The autoaggregation (%) is calculated as [(A0−At)/A0]×100, where A0 is A_{600} at 0 h and At represents the A_{600} of cell suspension at different time intervals (2, 5 and 24 h).

Results of *in vitro* screening

Antibiotic susceptibility testing and MIC determinations

Pediococcus acidilactici DSM 32500 was sensitive to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, vancomycin as no growth was observed at breakpoint level given by EFSA (EFSA, 2012).

Tolerance to low pH and high bile concentration

Viability and survival of probiotic bacteria during passage through the stomach is a considerable parameter to reach the intestine and provide potentially beneficial effect. Additionally, it is important that the probiotic bacteria can survive the bile concentrations in the small intestine. *Pediococcus acidilactici* DSM 32500 had the ability to survive exposure to 0.3% bile and was further exposed to various pH values.

Pediococcus acidilactici DSM 32500 expressed good acid and bile tolerance after a successive 4 h and 8 h exposure. In particular, *Pediococcus acidilactici* DSM 32500 has excellent abilities to survive at pH levels of 4.0 and 6.0 followed by exposure to 0.3% bile. These conditions correspond to the physiological conditions present in the stomach as well as the small intestine.

Table 2

The OD_{610} values after 4 h exposure of *Pediococcus acidilactici* DSM 32500 to different pH values followed by 8 h exposure to 0.3% bile

Tolerance to various pH values and bile	pH 4.0 and bile	pH 6.0 and bile	pH and bile control
Pediococcus acidilactici DSM 32500	1.0	1.1	1.1

Viability during simulated gastrointestinal transit

The pH in stomach ranges from 1.0, during fasting, to 4.5, after a meal. *Pediococcus acidilactici* DSM 32500 shows the ability to tolerate pH 4.0 after 4 h of exposure. Moreover, the strain also retains its viability after 24 h exposure to pH 4.0 and 6.5 (Table 3). The cell viability was also retained after 24 h exposure to 0.3% bile.

Table 3

The OD_{600} values of *Pediococcus acidilactici* DSM 32500 after 24 h growth at different pH values, 0.3% bile and simulated gastrointestinal conditions

Simulated growth conditions	pH=4	pH=6.5	0.3% oxbile
Pediococcus acidilactici DSM 32500	1.0	0.8	1.0

* SGF – simulated gastric fluid

** SIF – simulated intestinal fluid

The resistance to extended exposure to acid pH and high bile concentrations in the selected LAB strain might confer the ability to better survive and colonize during intestinal transit by enhancing their intestinal growth advantages *in vivo*.

In vitro adhesion to intestinal epithelial cells and autoaggregation

The ability to adhere *in vitro* to intestinal epithelial cells was investigated. *Pediococcus acidilactici* DSM 32500 is adhesive to both Caco-2 (80.4%) and mucus secreting HT29-MTX cells (67.0%) (Table 4).

Pediococcus acidilactici DSM 32500 exhibited a good ability to form autoaggregates. The % autoaggregation after 5 h was 11.0 (Table 4).

Table 4

In vitro adhesion (%) of *Pediococcus acidilactici* DSM 32500 to Caco-2 and HT29-MTX epithelial cells and autoaggregation

In vitro assays	*In vitro* adherence (%)		Autoaggregation (%)
	Caco-2 cells	HT-29 MTX cells	
Pediococcus acidilactici DSM 32500	80.4	67.0	11.0

EXAMPLE 2

Dairy Cattle Trial

The objective of the trial was to evaluate the effect of probiotics supplementation in the diet of lactating dairy cows on milk production and ruminal pH. The working hypothesis was that the two probiotics would have the same positive effect on dairy production measured as milk yield as the commercially available product Bovamine® Complete which was expected to increase production by 2-3% over the control treatment.

The trial was performed at Iowa State University. Forty-eight multiparous Holstein cows (121 ± 22 DIM) were blocked by milk yield in a randomized complete block design. Cows were randomly assigned to four groups (12 cows per group):

1) Control (CON) with no probiotic
2) Bovamine® Complete (BOV) (three *Enterococcus faecium* strains and yeast)
3) Bacillus (BAC) (*Bacillus subtilis* DSM 25841)
4) Pediococcus (PED) (*Pediococcus acidilactici* DSM 32500)

Cows were housed in a free stall barn with individual feeding gates, milked thrice a day and fed twice daily for 105 d; daily feed intake and milk yield data were averaged weekly. Cows in the Control group received only a conventional dairy diet without any probiotic supplementation (ingredient as % of DM:33.7% corn silage, 19.9% wet corn gluten feed, 15.5 % alfalfa hay and 30.9% grain mix concentrate) whereas the treatment groups (BOV, BAC and PED) received a conventional dairy diet top-dressed with the corresponding probiotic. The daily inclusion dose of each product was 1 x 10^{10} CFU/day. The trial lasted for approximately 98 days (14 weeks; 3.5 months).

Eight cows out of the 48 were surgically fitted with a 4-inch rumen cannula 3 weeks prior to introduction to the individual feeding system. The eight rumen fistulated cows, two per treatment, were used for rumen pH measurements every two hours during a 24 h period on d 105.

Results:

Data were analysed using a mixed model with week, treatment and their interaction as fixed effects with pre-trial milk yield as a covariate; block and cow were considered random effects. Primary response variables were feed intake, milk yield, body weight and body condition score. Additional evaluations included rumen pH.

Table 5

Effect of dietary addition of probiotics on the performance of dairy cows

	CON	BOV	BAC	PED	SEM[1]	*P*-Value[2]
DMI, kg/d	24.1	23.7	23.9	25.6	0.75	0.20
Body weight, kg	678	655	667	704	16.8	0.14
Body condition score[3]	2.9	2.8	2.9	2.8	0.06	0.65

[1] Highest standard error of treatment mean is shown
[2] Main effect of treatment
[3] Body condition score 1-5 scale

Dry matter intake was similar (P = 0.20) across treatments averaging 24.3±0.76 kg/d. Cows consuming CON and PED tended to be heavier compared to the BOV and BAC whereas the Body condition score was similar.

The results provided in Figure 1 demonstrated that supplementation with *Pediococcus acidilactici* DSM 32500 compared to control increased the milk yield with 6% (period II), 9% (period III) and 10% (period I). None of the other two products showed any increase in milk yield.

It is further noteworthy that the *Pediococcus acidilactici* DSM 32500 does not have negative effects on rumen pH as supplementation with either probiotic resulted in similar mean rumen pH compared with the control treatment (P = 0.29; Table 6) and Figure 2.

Table 6

Diurnal values (minimum, maximum and average) of rumen pH in dairy cows receiving probiotic supplements

	Treatment				SEM	P-value
	CON	BOV	BAC	PED		
Rumen pH,						
Minimum	5.37	5.29	5.34	5.21	0.05	0.26
Maximum	6.65	6.85	6.57	6.49	0.18	0.59
Mean	5.75	5.75	5.65	5.61	0.08	0.29

EXAMPLE 3

Effect of *Pediococcus acidilactici* on intestinal barrier integrity

Intestinal barrier integrity was measured by the strain's ability to increase the electrical resistance across Caco-2 cell monolayers (trans-epithelial electrical resistance; TEER).

Culturing of Caco-2 cells

The mammalian intestinal epithelial Caco-2 cell line (DSMZ ACC 169) was cultured in Caco-2 cell culture medium (DMEM (Gibco) supplemented with 20% heat inactivated fetal bovine serum (Gibco), 1X Non-essential amino acids (Thermo Scientific), and 1X Pen Strep (Biological industries)) at 37°C, 5% CO2 in an incubator. Caco-2 cells were used from passage 15 to 25. Cells were trypsinized when 60-80% confluent. A cell suspension of 10^5 cells/ml was prepared in DMEM and 500 µl was seeded in the apical compartment of 12 mm, 0.4 µm pore size Transwell® polyester membrane inserts (Corning, USA), while 1.5 ml of medium was added to the basolateral compartment. Cells were cultured on the inserts for 21 days with change of medium twice a week. On day 21, the Transwell inserts were moved to a cellZscope2® (nanoAnalytics, Germany). Culture medium was changed to antibiotics free medium, and accordingly 760 µl and 1.65 ml DMEM without antibiotics was added to the apical and basolateral compartment, respectively. The CellZscope was placed in the incubator and TEER was monitored every hour for 20-23 hours with automatic data collection.

Preparation of *Pediococcus acidilactici*

Pediococcus acidilactici was cultured overnight aerobically in Man Rogosa Sharp (MRS) broth (Oxoid A/S, Denmark). From this culture 10-fold dilutions down to 10^{-8} were made in MRS and incubated overnight aerobically. On the 3rd day, late exponential phase cultures were harvested by centrifugation, washed twice in Hank's Balanced Salt solution (HBSS) and resuspended in DMEM. Bacteria were again harvested by centrifugation and resuspended in culture medium without antibiotics. The bacteria were then OD_{600} normalized to 3.8.

Stimulation of Caco-2 cells

To stimulate Caco-2 cells with *Pediococcus acidilactici* 100 µl of DMEM was gently removed from the apical part of the Transwell inserts and replaced by 100 µl of bacterial suspension to give a final OD_{600} of 0.5. Then the CellZscope was transferred back into the incubator and TEER was recorded every hour for 23 hours. DMEM without bacterial supplementation was used as control, i.e. unstimulated Caco-2 monolayers. All treatments were done in triplicates (3 independent Transwells). Changes in TEER during

bacterial stimulation were calculated relative to the latest value recorded prior to stimulation (set to 100%).

Results:

Continuous measuring of TEER showed that after 12 hours of stimulation *Pediococcus acidilactici* caused a 30% increase in TEER (compared to the control) which was maintained for the rest of the experiment (Figure 3). These results point to a beneficial effect of *Pediococcus acidilactici* on the integrity of the epithelial barrier.

DEPOSIT AND EXPERT SOLUTION

The *Pediococcus acidilactici* strain DSM 32500 has been deposited at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstrasse 7B, D-38124 Braunschweig) on May 9th, 2017 by Chr. Hansen A/S, Denmark. The deposit has been made under the conditions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

The *Bacillus subtilis* strain DSM25841 has been deposited at DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstrasse 7B, D-38124 Braunschweig) on April 3, 2012 by Chr. Hansen A/S, Denmark. The deposit has been made under the conditions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure.

For all of the above-identified deposited microorganisms, the following additional indications apply:

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn

CLAIMS

1. A *Pediococcus acidilactici* strain selected from the group consisting of

   a) the *Pediococcus acidilactici* strain deposited as DSM 32500, and
   b) a mutant strain thereof which
      i) is sensitive to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin,
      ii) exhibits an OD_{610} value of at least 0.8 after 4 h of exposure to pH 4.0 or 6.0 followed by 8 h of exposure to 0.3% bile,
      iii) exhibits an OD_{600} value of at least 0.6 after 24 h of exposure to pH 4.0, pH 6.5 or 0.3% bile,
      iv) exhibits an adhesion to Caco-2 cells of at least 70% and to HT29-MTX cells of at least 55%, and
      v) exhibits an autoaggregation after 5 hours of at least 9%.

2. A composition comprising a *Pediococcus acidilactici* strain according to claim 1.

3. A composition according to claim 2 which does not comprise yeast.

4. A composition according to any one of claim 2 or 3 which does not comprise a *Pediococcus pentosaceus* strain.

5. A composition according to any one of claims 2 to 4 which comprises the *Pediococcus acidilactici* strain deposited as DSM 32500.

6. A composition according to any one of claims 2 to 5 which is a Direct Fed Microbial (DFM), a premix, an animal feed additive, or an animal feed.

7. A composition according to claim 6 which is a Direct Fed Microbial.

8. A composition according to any one of claims 2 to 6 for use in increasing the milk yield of a mammal.

9. A method of increasing milk yield from a mammal, the method comprising administering a composition according to any one of claims 2 to 6 to the mammal.

10. The method according to claim 7 which comprises administering a composition according to claim 3.

11. The method according to claim 7 which comprises administering a composition according to claim 4.

12. The method according to claim 7 which comprises administering a composition according to claim 5.

13. The method according to any one of claims 7 to 9 wherein the mammal is an animal selected from the group consisting of non-human mammals such as companion animals and livestock animals.

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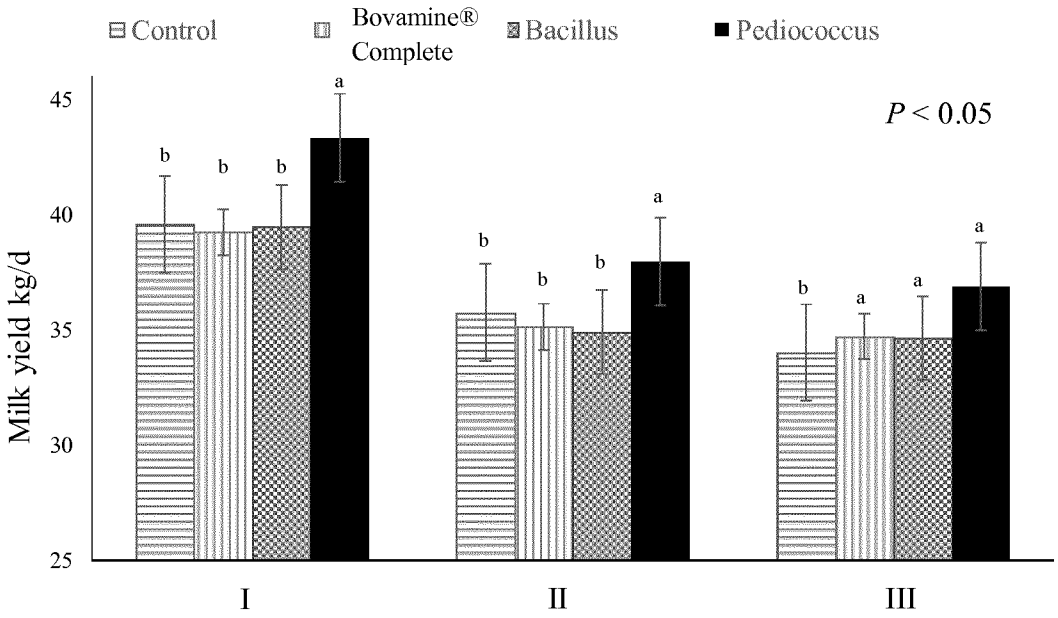


Figure 1

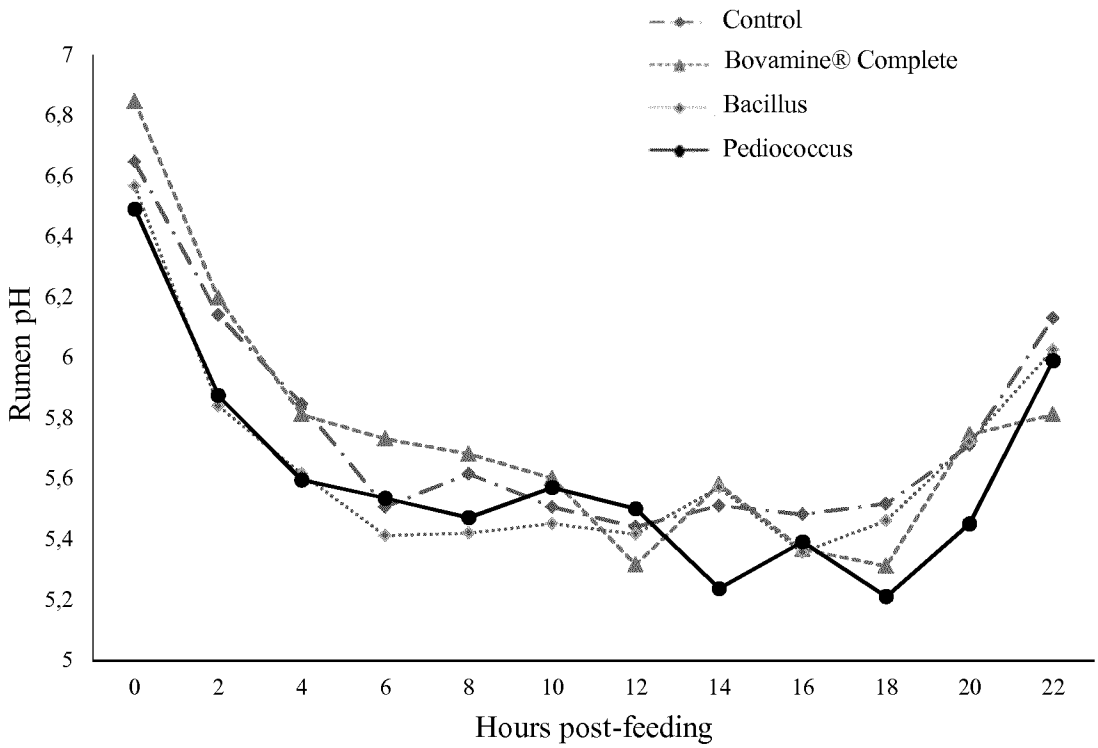


Figure 2

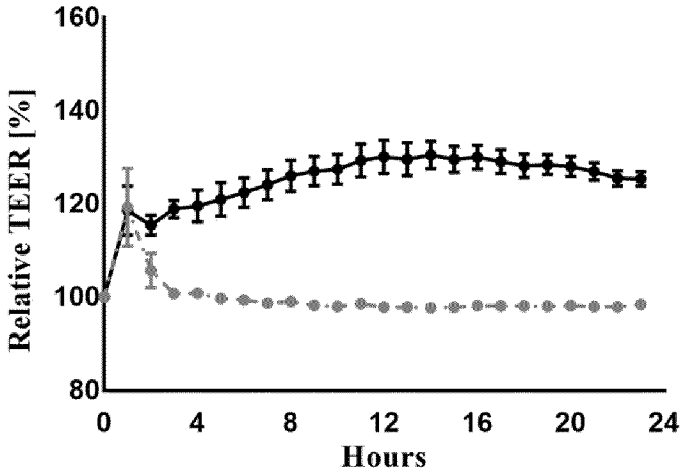


Figure 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/064570

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/74 C12R1/225 A23K50/10
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12R A23K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, 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	WO 2016/046706 A1 (UAB BALTIJOS BIOTECHNOLOGIJOS [LT]) 31 March 2016 (2016-03-31) page 2, paragraph 5 page 10, paragraph 2 - paragraph 5 example 2 claims 1, 4	1-13
X	US 2016/029666 A1 (CARPENTER RICHARD S [US] ET AL) 4 February 2016 (2016-02-04) paragraphs [0023], [0058] - [0067], [0094] - [0095] examples 1, 9	1-13

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[X] Further documents are listed in the continuation of Box C.

[X] 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
20 July 2018	10/08/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Surdej, Patrick

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/064570

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VITA KRUNGLEVICIUTE ET AL: "Applicability of Pediococcus strains for fermentation of cereal bran and its influence on the milk yield of dairy cattle", ZEMDIRBYSTE-AGRICULTURE, vol. 104, no. 1, 13 February 2017 (2017-02-13), pages 63-70, XP055398966, ISSN: 1392-3196, DOI: 10.13080/z-a.2017.104.009 abstract page 68, left-hand column, paragraph 2 - right-hand column page 69, last paragraph tables 5, 6 -----	1-13

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/064570

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016046706 A1	31-03-2016	EP 3198040 A1	02-08-2017
		LT 6270 B	10-05-2016
		WO 2016046706 A1	31-03-2016
US 2016029666 A1	04-02-2016	AU 2015296538 A1	19-01-2017
		CA 2954422 A1	04-02-2016
		CN 107072247 A	18-08-2017
		EP 3174405 A1	07-06-2017
		KR 20170033433 A	24-03-2017
		US 2016029666 A1	04-02-2016
		WO 2016019017 A1	04-02-2016