METHODS OF USE OF PROBIOTIC BIFIDOBACTERIA FOR COMPANION ANIMALS

VERFAHREN ZUR VERWENDUNG VON PROBIOTISCHEN BIFIDOBakterien FÜR HAUSTIERE

PROCEDE D’UTILISATION DE BIFIDOBACTERIES PROBIOTIQUES POUR DES ANIMAUX DE COMPAGNIE

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Remarks:
The file contains technical information submitted after the application was filed and not included in this specification

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FIELD OF THE INVENTION

[0001] The present invention relates to the field of probiotic Bifidobacteria, more specifically methods of use of probiotic Bifidobacteria in companion animals.

BACKGROUND OF THE INVENTION

[0002] The defense mechanisms to protect the mammalian gastrointestinal (GI) tract from colonisation by bacteria are highly complex. The GI tract of most mammals are colonised by native microflora, and invasive pathogenic microorganisms. In a healthy state, these competing microflora are in a state of equilibrium. Modification of the intestinal microflora equilibrium may lead to or prevent many GI disorders, both in humans, and other mammalian species, such as companion animals including cats, dogs and rabbits. The well being of companion animals is closely related to their feeding and GI health, and maintenance of the intestinal microflora equilibrium in these animals may result in healthier pets.

[0003] The number and composition of the intestinal microflora tend to be stable, although age and diet may modify it. Gastric acidity, bile, intestinal peristalsis and local immunity are factors thought to be important in the regulation of bacterial flora in the small intestine of human beings and various other mammals. Often pet GI disorders, including those found in canines and felines, are linked to bacterial overgrowth and the production of enterotoxins by pathogenic bacteria. These factors disrupt the intestinal microflora equilibrium and can promote inflammation and aberrant immune responses.

[0004] Recently, research has begun to highlight some valuable strains of bacteria obtainable by isolation from resected and washed gastrointestinal tract of mammals, such as humans and canines, and their potential use as probiotic agents. Probiotics are considered to be preparations of bacteria, either viable or dead, their constituents such as proteins or carbohydrates, or purified fractions of bacterial ferments that promote mammalian health by preserving the natural microflora in the GI tract, and reinforcing the normal controls on aberrant immune responses. It is believed by some that probiotic bacteria are more effective when derived from the species, or closely related species, intended to be treated. Whilst several strains of probiotic bacteria have been elucidated, methods of use of these strains and their therapeutic efficacy has been limited to modulation of gastro-intestinal disorders in humans. As yet, there has not been much investigation into the potential for these organisms to beneficially affect physiological systems other than the gastrointestinal tract in companion animals, such as canines and felines.

[0005] WO01/90311 relates to novel probiotics for pet food applications.

[0006] The paper by Colum dunne et al. in Antonie van Leeuwenhoek, Dordrecht, NL, Vol 76, no 1-4, july 1999, pages 279-292 relates to a study to demonstrate the functionality of probiotics in animal models of disease and in human clinical trials.

[0007] WO03/010297 relates to probiotic Bifidobacterium strains.


SUMMARY OF THE INVENTION

[0009] The present invention relates to a composition comprising a probiotic strain of bifidobacteria obtainable by isolation from resected and washed mammalian gastrointestinal tract for use in treatment of a companion animal selected from the group consisting of canines and felines, treatment being selected from regulating the immune system of a companion animal, maintaining or improving the health of the skin and/or coat system of a companion animal, ameliorating or reducing the effects of aging in a companion animal, preventing weight loss during and following infection in a companion animal, treating or preventing urinary tract ailments in companion animals, increasing fiber digestion in companion animals, preventing or treating infection of the gastrointestinal tract of a companion animal, improving digestion in companion animals, reducing stress levels in a companion animal or mixtures thereof, wherein said probiotic Bifidobacteria is selected from the group consisting of Bifidobacterium pseudolongum NCIMB 41199 and Bifidobacterium globosum NCIMB 41198 and mixtures thereof.

DETAILED DESCRIPTION OF THE INVENTION

Sequences

[0010] SEQ. ID NO. 1 - 16s-23s intergenic spacer nucleotide sequence from Bifidobacteria globosum AHCF (NCIMB 41198).
SEQ. ID NO. 2 - 16s-23s intergenic spacer nucleotide sequence from *Bifidobacteria pseudolongum* AHC7 (NCIMB 41199).

SEQ. ID NO. 3 - 16s-23s left PCR primer sequences for sequence analysis.

SEQ. ID NO. 4 - 16s-23s right PCR primer sequences for sequence analysis.

Bacterial Deposit Numbers

The table below describes the species, strain number and deposit number for probiotic *Bifidobacteria* obtainable by isolation from resected and washed gastrointestinal tract of mammals useful in the present invention. The bacterial strains have been deposited with the National Collections of Industrial Food and Marine Bacteria (NCIMB), Aberdeen, UK.

<table>
<thead>
<tr>
<th>Strain Reference</th>
<th>NCIMB Deposit Number</th>
<th>Sequence Listing</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH208</td>
<td>NCIMB 41050</td>
<td>-</td>
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<tr>
<td>AH209</td>
<td>NCIMB 41051</td>
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<td>AHC7</td>
<td>NCIMB 41199</td>
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</tr>
<tr>
<td>AHCF</td>
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</tbody>
</table>

All weights, measurements and concentrations herein are measured at 25°C on the composition in its entirety, unless otherwise specified.

Unless otherwise indicated, all percentages of compositions referred to herein are weight percentages and all ratios are weight ratios.

Unless otherwise indicated, all molecular weights are weight average molecular weights.

Unless otherwise indicated, the content of all literature sources referred to within this text are incorporated herein in full by reference.

Except where specific examples of actual measured values are presented, numerical values referred to herein should be considered to be qualified by the word "about".

Within the following description, the abbreviation CFU ("colony-forming unit") designates the number of bacterial cells revealed by microbiological counts on agar plates, as will be commonly understood in the art.

As used herein, the term "mutants thereof" includes derived bacterial strains having at least 88% homology, preferably at least 90% homology, more preferably 95% homology to the 16s-23s intergenic spacer polynucleotide sequence of a referenced strain, but otherwise comprising DNA mutations in other DNA sequences in the bacterial genome.

As used herein, "DNA mutations" includes natural or induced mutations comprising at least single base alterations including deletions, insertions, transversions, and other DNA modifications known to those skilled in the art, including genetic modification introduced into a parent nucleotide or amino acid sequence whilst maintaining at least 50% homology to the parent sequence. Preferably, the sequence comprising the DNA mutation or mutations has at least 60%, more preferably at least 75%, more preferably still 85% homology with the parental sequence. As used herein, sequence "homology" can be determined using standard techniques known to those skilled in the art. For example, homology may be determined using the on-line homology algorithm "BLAST" program, publicly available at http://www.ncbi.nlm.nih.gov/BLAST/.

As used herein "genetic modification" includes the introduction of exogenous and/or endogenous DNA sequences into the genome of an organism either by insertion into the genome of said organism or by vectors including plasmid DNA or bacteriophage as known by one skilled in the art, said DNA sequence being at least two deoxyribonucleic acid bases in length.

As used herein, "companion animal" means a domestic animal. Preferably, "companion animal" means a domestic canine, feline, rabbit, ferret, horse, cow, or the like. More preferably, "companion animal" means a domestic canine or feline.

The strain of lactic acid bacteria of the genus *Bifidobacteria globosum* obtainable by isolation from resected...
and washed canine gastrointestinal tract can be used to deliver probiotic benefit following oral consumption in animals, preferably companion animals or humans. This probiotic benefit generally maintains and improves the overall health of the animal. Non-limiting elements of animal health and physiology that benefit, either in therapeutically relieving the symptoms of, or disease prevention by prophylaxis include inflammatory disorders, immunodeficiency, inflammatory bowel disease, irritable bowel syndrome, cancer (particularly those of the gastrointestinal and immune systems), diarrhoeal disease, antibiotic associated diarrhoea, appendicitis, autoimmune disorders, multiple sclerosis, Alzheimer’s disease, amyloidosis, rheumatoid arthritis, arthritis, joint mobility, diabetes mellitus, bacterial infections, viral infections, fungal infections, periodontal disease, urogenital disease, surgical associated trauma, surgical-induced metastatic disease, sepsis, weight loss, weight gain, excessive adipose tissue accumulation, anorexia, fever control, cachexia, wound healing, ulcers, gut barrier infection, allergy, asthma, respiratory disorders, circulatory disorders, coronary heart disease, anaemia, disorders of the blood coagulation system, renal disease, disorders of the central nervous system, hepatic disease, ischaemia, nutritional disorders, osteoporosis, endocrine disorders, and epidermal disorders. Preferred are treatment of the gastrointestinal tract, including treatment or prevention of diarrhoea; immune system regulation, preferably treating or preventing autoimmune disease and inflammation; maintaining or improving the health of the skin and/or coat system, preferably treating or preventing atopic disease of the skin; ameliorating or reducing the effects of aging, including mental awareness and activity levels; and preventing weight loss during and following infection.

The treatment of the disorders disclosed above may be measured using techniques known to those skilled in the art. For example, inflammatory disorders including autoimmune disease and inflammation may be detected and monitored using in vivo immune function tests such as lymphocyte blastogenesis, natural killer cell activity, antibody response to vaccines, delayed-type hypersensitivity, and mixtures thereof. Such methods are briefly described herein, but well known to those skilled in the art.

1. Lymphocyte blastogenesis: This assay measures the proliferative response in vitro of lymphocytes isolated from fresh whole blood of test and control animals to various mitogens and is a measure of overall T- and B-cell function. Briefly, peripheral blood mononucleocytes (PBMC) are isolated from whole blood by Ficoll-Hypaque density centrifugation methods known to those skilled in the art. The isolated PBMCs are washed twice in RPMI 1640 cell media supplemented with HEPES, L-glutamine and penicillin/streptomycin. The washed cells are resuspended in RPMI 1640, counted, and the cell density adjusted appropriately. The 2x10⁵ cells are exposed to a range of concentrations (0.1 μg/ml to 100 μg/ml) of various mitogens, some examples of which include pokeweed mitogen (Gibco), phytohaemagglutinin (Gibco) and concanavalin A (Sigma) in triplicate for 72 hours at 37°C and 5% CO₂ with 10% foetal bovine serum (Sigma). At 54 hours the cells are pulsed with 1 μCi³H-thymidine, and the cells harvested and scintillation counts read on a TopCount NXT at 72 hours.

2. Natural killer cell activity: As described in US6,310,090, this assay measures the in vitro effector activity of natural killer cells isolated from fresh whole blood of test and control animals. Natural killer cells are a component of the innate immune function of a mammal. Canine thyroid adenocarcinoma cells were used as target cells in assessing NK cell cytotoxic activity. This cell line was previously shown to be susceptible to killing by canine NK cell. Target cells were cultured in a T75 flask with 20 mL minimum essential medium (MEM; Sigma Chem. Co., St. Louis, Mo.) supplemented with 10% fetal calf serum (FCS), 100 U/mL of penicillin and 100 μg/mL of streptomycin. When confluent, target cells were trypsinized, washed 3 times and resuspended to 5x10⁵ cells/mL in complete medium (RPMI-1640+10% FCS+100 U/mL of penicillin+100 μg/mL of streptomycin). Triplicate 100 μL aliquots of the target cells were pipetted into 96-well U-bottom plates (Costar, Cambridge, Mass.) and incubated for 8 hours to allow cell adherence. Lymphocytes (effector cells; 100 μL) isolated by Ficoll-Hypaque separation (as described above) were then added to the target cells to provide an effector/target cell (E:T) ratio of 10:1. After 10 hours of incubation at 37°C, 20 μL of a substrate containing 5 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. The mixture was incubated for 4 hours at 37°C. After which the unmetabolized MTT was removed by aspiration. The formazan crystals were dissolved by adding 200 μL of 95% ethanol. Optical density was measured at 570 nm using a microplate reader. The percentage of NK cell-specific lysis was calculated as follows:

\[
\text{Specific Cytotoxicity (\%) = 100 \times \{1 - [(OD of target cells and effector cells - OD of effector cells)/(OD of target cells)]\}}
\]

3. Antibody response to vaccines: The test subjects are given an array (up to 5) of vaccines after at least 12 weeks of probiotic or control feeding. The vaccines may be a mixture of novel and redundant vaccines. Non-limiting examples of vaccine arrays that may be used include mixtures of vaccines prepared by Fort Dodge Animal Health. Non-limiting examples of vaccines suitable for use herein include Canine distemper, adenovirus, coronavirus, parainfluenza, and parvovirus. The test subject’s vaccine history will determine the vaccines to be used. The specific antibodies
to the vaccines given are measured in blood for 3 weeks and the length and strength of response in control and probiotic feeding groups compared.

4. Delayed-type hypersensitivity: An in vivo, non-invasive method of assessing immune system status. This test comprises an intradermal injection of the polyclonal mitogen Phytohemagglutinin (PHA) in combination with sheep red blood cells a multivalent vaccine, histamine (100μL of 0.0275 g/L Histamine Phosphate; Greer, Lenoir, NC), or PBS (100μL of Phosphate Buffered Saline, 8.5 g/L; Sigma). The immune response to the antigen is recorded as skinfold thickness using calipers at time intervals of 0, 24, 48 and 72 hours post-injection. An increase in skin fold thickness is indicative of a greater hypersensitivity response that should be decreased by treatment with the bacteria of the present invention.

[0027]  Additional methods for determining the effect of the Bifidobacteria bacteria of the present invention are described in US6,133,323 and US6,310,090.

[0028]  Furthermore, ameliorating the effects of age may be determined using dual x-ray absorptometry or CT scan for measuring body composition, including body fat mass, fat-free mass and bone mineral content. Similarly, this method may be used to determine anatomy changes such as weight loss or bone density in subjects following infection.

[0029]  The Bifidobacteria of the present invention may also be used in a method for reducing stress levels in companion animals. Concentrations of blood stress hormones including epinephrine, norepinephrine, dopamine, cortisol and C-reactive protein may be measured to determine stress levels and their reduction or maintenance. These hormones are recognized biomarkers of stress and can be readily measured using techniques known to those skilled in the art.

[0030]  Further still, maintenance or improvement of the health of the skin and/or coat system of companion animals, including atopic disease of the skin, may be measured using skin and coat assessments conducted by two trained individuals. Examples of criteria examined during such assessments include:

 a) Shedding index: A shedding index is assigned to each test subject by collecting hair produced during a standardized brushing session. The hair is retained and weighed, and control and test subjects compared.

 b) Subjective skin/coat evaluations: Trained panelists subjectively evaluate skin and coat condition by assessing shedding, dander, shine, uniformity, softness and density.

 c) Skin functional assessment: The barrier function of the skin may be assessed by wiping the skin surface with an acetone-soaked gauze. This technique effectively disrupts the skin barrier by removing single cell layers and associated lipid fractions of the stratum corneum. Barrier disruption is quantified by measuring the increase in transepidermal water loss (TEWL) and the degree of redness of the insulted site using methods known to those skilled in the art. Redness (erythema) scores are obtained using the previously described camera and lighting system. TEWL readings and redness scores are obtained immediately before and after disruption, and at five and 24-hour endpoints to assess the protective and healing properties of skin.

[0031]  The treatment or prevention of gastrointestinal infection, including diarrhoea, in companion animals may be measured using stool scores. Stools scores may be recorded daily according to the following guidelines and control and test groups compared before and after feeding with the bacteria according to the present invention.

 Score: 5 Extremely Dry

[0032]  This stool is hard and does not stick to surfaces. Stool will roll when pushed. No indentations are made when stool is picked up. Stool is often defecated in groups of individual stools instead of one complete unit. The stool maintains original shape after collection.

 Score: 4 Firm (Ideal stool)

[0033]  This stool is firm, well shaped, and cylindrical. This stool does not break apart easily when picked up. This stool may leave residue on surfaces and gloves. This stool is often defecated as one unit. The stool maintains original shape after collection.

 Score: 3 Soft, with shape

[0034]  This stool is soft, however there are definite shapes. This stool will break apart easily and will definitely leave residue on surfaces and gloves. The stool often loses original shape after collection. This stool is often present with another score but can comprise whole stool sample.
This stool is soft and will have no cylindrical shape. The shape often associated with a "2" is a "cow patty" shape. This stool will lose the original shape when collected and will definitely leave residue on surfaces and gloves. This stool score is often present with another score but can comprise the whole stool sample. This stool sample may spread over an area of several inches.

This stool score will always resemble liquid and there may or may not be particulate matter present. This stool will often be defecated in groups of piles instead of one complete unit. Mucous is often present with this stool sample. This stool sample is very difficult to collect and residue is always left on surfaces and gloves. This stool sample may spread over an area of several inches.

In addition, other observations are also recorded, including: blood in stool; foreign object in stool; or mucous in stool.

Furthermore, the treatment of gastrointestinal infection in companion animals may comprise improving microbial ecology of companion animals. Improving the microbial ecology of companion animals preferably comprises reducing the levels of pathogenic bacteria in the faeces of companion animals. The levels of pathogenic bacteria present in the faeces of companion animals may be enumerated using the standard plate count method known to those skilled in the art. More preferably, the pathogenic bacteria are selected from the group consisting of Clostridia, Escherichia, Salmonella, bacteriodes and mixtures thereof. Non-limiting examples of suitable strains of pathogenic bacteria include C. perfringens, C. difficile, Eschericia coli, Salmonella typhimium and mixtures thereof.

The method of use of the bacteria of the present invention may also include the treatment, either prophylactic or therapeutic of the urinary tract of mammals, preferably companion animals. Non-limiting examples of urinary tract treatment include treatment or prevention of urinary tract infections, treatment or prevention of kidney disease, including kidney stones, treatment or prevention of bladder infections and the like. Without being bound by theory, it is believed that the Bifidobacteria bacteria of the present invention are useful in preventing these ailments as a result of their ability to degrade oxalic acid, as demonstrated in vitro. Oxalic acid is a by-product of urinary metabolism that can form insoluble precipitates that result in kidney, bladder and other urinary tract infections. By degrading oxalic acid, and therefore potentially preventing its precipitation and build up in the urinary tract, the bacteria of the present invention may treat and prevent infections and other ailments of the urinary tract. Oxalic acid degradation may be measured in vitro using the Oxalic acid test kit cat # 755699 commercially available from Boehringer Mannheim/R-Biopharm.

The Bifidobacteria pseudolongum of the present invention may be used in a method for improving or maintaining the health of companion animals comprising improving fiber digestion. Improving fiber digestion is desirable as it promotes the growth of said probiotic bacteria, as well as beneficial endogenous microflora, which aid in the suppression of some potentially pathogenic bacteria. In addition, a decrease in the amount of toxic metabolites and detrimental enzymes that result from colonic fermentation has been documented in humans (Tomomatsu, H. "Health effects of oligosaccharides", (1994) Food Technol, 48, 61-65). Fiber digestion may be determined using the method described in Vickers et al. (2001), "Comparison of fermentation of selected fructooligosaccharides and other fiber substrates by canine colonic microflora", Am. J. Vel. Res. 61 (4), 609-615, with the exception that instead of inoculating using diluted fecal samples each experiment used pure cultures of bacterial strains of interest.

Preferably, the methods of the present invention comprise administration of Bifidobacteria selected from the species comprising Bifidobacteria longum, Bifidobacteria pseudolongum, Bifidobacteria infantis or Bifidobacteria glo- bosum.

Non-limiting examples of probiotic Bifidobacteria obtainable by isolation from resected and washed mammalian GI tract useful in the present invention are described in more detail in WO 00/42168 and WO 03/010297.

WO 00/42168 describes probiotic Bifidobacteria isolated from resected and washed human GI tract. These bacteria are deposited at the NCIMB under deposit numbers 41050, 41051, 41052, 41053, 41099, and 41100.

WO 03/010297 describes a further example of probiotic Bifidobacteria isolated from resected and washed human GI tract. This bacterium is deposited at the NCIMB under the deposit number 41003.

Further examples include strains of Bifidobacteria globosum obtainable by isolation from resected and washed canine gastrointestinal tract having probiotic activity in animals. It has been found that strains of Bifidobacteria obtainable by isolation directly from resected and washed GI tract of mammals are adherent to the GI tract following feeding of viable bacterial cells, and are also significantly immunomodulatory when fed to animals in viable, non-viable or fractionated form. Without being bound by theory, it is believed that the Bifidobacteria obtainable by isolation from resected and washed GI tract closely associate with the gut mucosal tissues. Without further being bound by theory, this is believed to result in the probiotic Bifidobacteria generating alternative host responses that result in its probiotic action. It has been found that probiotic bacteria obtainable by isolation from resected and washed GI tract can modulate the host's immune
system via direct interaction with the mucosal epithelium, and the host's immune cells. This immunomodulation, in conjunction with the traditional mechanism of action associated with probiotic bacteria, i.e. the prevention of pathogen adherence to the gut by occlusion and competition for nutrients, results in the *Bifidobacteria* being highly efficacious as a probiotic organism.

[0046] The *Bifidobacteria* useful in the present invention, obtainable by isolation from resected and washed mammalian GI tract, have *in vitro* anti-microbial activity against a number of pathogenic bacterial strains/species. Without being bound by theory, it is believed that this *in vitro* anti-microbial activity is indicative of potential probiotic activity *in vivo* in animals, preferably companion animals such as canines and felines. The lactic acid bacteria of the present invention preferably have *in vitro* anti-microbial activity against *Salmonella typhimurium*, *Listeria monocytogenes*, *Listeria innocua* or *Eschericia coli*, more preferably a mixture of these strains, more preferably still, all of these strains.

[0047] Without being bound by theory, it is believed that the anti-microbial activity of the *Bifidobacteria* bacteria may be the result of a number of different actions by the *Bifidobacteria* bacteria. It has previously been suggested in the art that several strains of bacteria isolated from faecal samples exert their probiotic effect in the GI tract following oral consumption by preventing the attachment of pathogenic organisms to the gut mucosa by occlusion. This requires oral consumption of "live" or viable bacterial cells in order for a colony of bacteria to be established in the gut. However, it is believed that the *Bifidobacteria* useful in the present invention, obtainable by isolation from resected and washed mammalian GI tract, whilst exerting some probiotic effect due to occlusion if given in a viable form, may deliver a substantial probiotic effect in either the viable or non-viable form due to the production during fermentation *in vitro* of a substance or substances that either inhibit the growth of or kill pathogenic micro-organisms, and/or alter the host animal's immune competence. This form of probiotic activity is desirable, as the bacteria of the present invention can be given as either viable or non-viable cultures or purified fermentation products and still deliver a beneficial therapeutic effect to the host animal.

[0048] Preferably, the *Bifidobacteria* bacteria are able to maintain viability following transit through the GI tract. This is desirable in order for live cultures of the bacteria to be taken orally, and for colonisation to occur in the intestines and bowel following transit through the oesophagus and stomach. Colonisation of the intestine and bowel by the lactic acid bacteria of the present invention is desirable for long-term probiotic benefits to be delivered to the host. Oral dosing of non-viable cells or purified isolates thereof induces temporary benefits, but as the bacteria are not viable, they are not able to grow, and continuously deliver a probiotic effect *in situ*. As a result this may require the host to be dosed regularly in order to maintain the health benefits. In contrast, viable cells that are able to survive gastric transit in the viable form, and subsequently colonise by adhering to and proliferating on the gut mucosa are able to deliver probiotic effects continuously *in situ*.

[0049] Therefore, it is preferable that the lactic acid bacteria of the present invention maintain viability after suspension in a media having a pH of 2.5 for 1 hour. As used herein, "maintain viability" means that at least 25% of the bacteria initially suspended in the test media are viable using the plate count method known to those skilled in the art. Preferably, "maintain viability" means that at least 50% of the bacteria initially suspended are viable. It is desirable for the lactic acid bacteria of the present invention to maintain viability following exposure to low pH as this mimics the exposure to gastric juices in the stomach and upper intestine *in vivo* following oral consumption in companion animals.

[0050] Furthermore, it is preferable that the lactic acid bacteria of the present invention have a growth of at least 33% when in the presence of at least 0.5% porcine bile salts. Growth, as used herein is described in further detail in example 3. More preferably, the bacteria of the present invention have a growth of at least 33% when in the presence of at least 1% porcine bile salts. Without being bound by theory it is believed that the lactic acid bacteria of the present invention, capable of maintaining viability in the presence of at least 0.5% porcine bile salts, are able to survive the conditions present in the intestine. This is thought to be a result of the addition of porcine bile to the culture medium mimicking the conditions of the intestine.

[0051] Further still, it is preferable that the *Bifidobacteria* bacteria useful in the present invention have significant adhesion to gut epithelial cells *in vitro*. As used herein, "significant adhesion" means at least 4% of the total number of lactic acid bacteria co-incubated with the epithelial cells *in vitro* adhere to the epithelial cells. More preferably, at least 6% of bacterial cells co-incubated adhere to epithelial cells *in vitro*. Without being bound by theory, it is believed that gut epithelial cell adherence *in vitro* is indicative of the lactic acid bacteria’s ability to colonise the GI tract of an animal *in vivo*.

[0052] The 16S-23s intergenic polynucleotide sequence is known to those skilled in the art as the sequence of DNA in the bacterial genome that can be used in order to identify different species and strains of bacteria. This intergenic polynucleotide sequence can be determined by the method detailed below.

[0053] *Bifidobacteria globosum* colonies were picked from an Agar plate and resuspended in IX PCR buffer, heated at 96°C for 5 minutes, frozen at -70°C for 5-10 minutes, thawed and an aliquot was added to a PCR eppendorf tube. PCR was performed using the intergenic spacer (IGS) primers, IGS L: 5’-GCTGGATCACCTCCTTTT-3’ and IGS R: 5’-CTGGTGCCAAGGCATCCA-3’. The cycling conditions were 96°C for 1 min (1cycle), 94°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec (28 cycles). The PCR reaction contained 5 μl of DNA, PCR buffer (Bioline, UK), 0.2 mM dNTPs (Roche,
The present invention is Bifidobacteria globosum strain NCIMB 41198 (AHCF), or a mutant thereof. More preferably still, the strain of lactic acid bacteria according to the present invention is Bifidobacterium pseudolongum strain NCIMB 41199 (AHC7), or a mutant thereof.

In a preferred embodiment of the present invention, the strain of Bifidobacteria globosum has a 16s-23s polynucleotide sequence according to SEQ. ID NO. 1. More preferably still, the strain of lactic acid bacteria according to the present invention is Bifidobacteria globosum strain NCIMB 41198 (AHCF), or a mutant thereof.

In another preferred embodiment of the present invention, the strain of Bifidobacteria pseudolongum, has a 16s-23s polynucleotide sequence according to SEQ. ID NO. 2. More preferably still, the strain of lactic acid bacteria according to the present invention is Bifidobacterium pseudolongum strain NCIMB 41199 (AHC7), or a mutant thereof.

The method of use of the Bifidobacteria bacteria of the present invention typically involves oral consumption by the animal. Oral consumption may take place as part of the normal dietary intake, or as a supplement thereto. The oral consumption typically occurs at least once a month, preferably at least once a week, more preferably at least once per day. The Bifidobacteria may be given to the companion animal in a therapeutically effective amount to maintain or improve the health of the animal, preferably a companion animal. As used herein, the term “therapeutically effective amount” with reference to the lactic acid bacteria, means that amount of the bacteria sufficient to provide the desired effect or benefit to a host animal in need of treatment, yet low enough to avoid adverse effects such as toxicity, irritation, or allergic response, commensurate with a reasonable benefit/risk ratio when used in the manner of the present invention. The specific “therapeutically effective amount” will vary with such factors as the particular condition being treated, the physical condition of the user, the duration of the treatment, the nature of concurrent therapy (if any), the specific dosage form to be used, the carrier employed, the solubility of the dose form, and the particular dosing regimen.

Preferably, the lactic acid bacteria are given to the companion animal at a dose of from 1.0E+04 to 1.0E+14 CFU per day, more preferably from 1.0E+06 to 1.0E+12 CFU per day. The composition preferably may contain at least 0.001% of from 1.0E+04 to 1.0E+12 CFU/g of the Bifidobacteria obtainable by isolation from resected and washed mammalian GI tract. The Bifidobacteria bacteria can be given to the animal in either viable form, or as killed cells, or distillates, isolates or other fractions of the fermentation products of the lactic acid bacteria of the present invention, or any mixture thereof.

Preferably, the Bifidobacteria bacteria, or a purified or isolated fraction thereof, are used to prepare a composition intended to maintain or improve the health of an animal. As indicated above, the composition may be part of the normal dietary intake, or a supplement. Where the composition comprises part of the normal dietary intake, the composition may be in the form of a dried animal food such as biscuits or kibbles, a processed grain feed, a wet animal food, yogurts, gravies, chews, treats and the like.

Such compositions may comprise further components. Other components are beneficial for inclusion in the compositions used herein, and are optional for purposes of the invention. For example, food compositions are preferably nutritionally balanced. In one embodiment, the food compositions may comprise, on a dry matter basis, from 20% to 50% crude protein, preferably from 22% to 40% crude protein, by weight of the food composition. The crude protein material may comprise any material having a protein content of at least 15% by weight, non-limiting examples of which include vegetable proteins such as soybean, cotton seed, and peanut, animal proteins such as casein, albumin, and meat tissue. Non-limiting examples of meat tissue useful herein include fresh meat, and dried or rendered meals such as fish meal, poultry meal, meat meal, bone meal and the like. Other types of suitable crude protein sources include wheat gluten or corn gluten, and proteins extracted from microbial sources such as yeast.

Furthermore, the food compositions may comprise, on a dry matter basis, from 5% to 35% fat, preferably from 10% to 30% fat, by weight of the food composition. Further still, food compositions comprising the lactic acid bacteria of the present invention may also comprise from 4% to 25% total dietary fiber. The compositions may also comprise a multiple starch source as described in WO99/51108.

The compositions of the present invention may further comprise a source of carbohydrate. Grains or cereals such as rice, corn, milo, sorghum, barley, alfalfa, wheat, and the like are illustrative sources. In addition, the compositions may also contain other materials such as dried whey and other dairy by products.

The compositions comprising the bacteria of the present invention may also comprise a prebiotic. “Prebiotic” includes substances or compounds that are fermented by the intestinal flora of the pet and hence promote the growth or development of lactic acid bacteria in the gastro-intestinal tract of the pet at the expense of pathogenic bacteria. The result of this fermentation is a release of fatty acids, in particular short-chain fatty acids in the colon. This has the effect
of reducing the pH value in the colon. Non-limiting examples of suitable prebiotics include oligosaccharides, such as inulin and its hydrolysis products commonly known as fructooligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides or oligo derivatives of starch. The prebiotics may be provided in any suitable form. For example, the prebiotic may be provided in the form of plant material which contains the fiber. Suitable plant materials include asparagus, artichokes, onions, wheat or chicory, or residues of these plant materials. Alternatively, the prebiotic fiber may be provided as an inulin extract, for example extracts from chicory are suitable. Suitable inulin extracts may be obtained from Orafti SA of Tirlemont 3300, Belgium under the trade mark “Raftiline”. For example, the inulin may be provided in the form of Raftilose (g) ST which is a fine white powder which contains 90 to 94% by weight of inulin, up to about 4% by weight of glucose and fructose, and 4 to 9% by weight of sucrose. Alternatively, the fiber may be in the form of a fructooligosaccharide such as obtained from Orafti SA of Tirlemont 3300, Belgium under the trade mark “Raftilose”. For example, the inulin may be provided in the form of Raftilose (g) P95. Otherwise, the fructooligosaccharides may be obtained by hydrolyzing inulin, by enzymatic methods, or by using micro-organisms.

[0064] For dried pet foods a suitable process is extrusion cooking, although baking and other suitable processes may be used. When extrusion cooked, the dried pet food is usually provided in the form of a kibble. If a prebiotic is used, the prebiotic may be admixed with the other ingredients of the dried pet food prior to processing. A suitable process is described in European patent application No 0850569. If a probiotic micro-organism is used, the organism is best coated onto or filled into the dried pet food. A suitable process is described in European patent application Number EP 0 862 863.

[0065] For wet foods, the processes described in US patents 4,781,939 and 5,132,137 may be used to produce simulated meat products. Other procedures for producing chunk type products may also be used; for example cooking in a steam oven. Alternatively, loaf type products may be produced by emulsifying a suitable meat material to produce a meat emulsion, adding a suitable gelling agent, and heating the meat emulsion prior to filling into cans or other containers. Typical wet food compositions may comprise from 5% to 15% protein, from 1% to 10% fat, and from 1% to 7% fiber. Non-limiting ingredients that may be used in wet food compositions include chicken, turkey, beef, whitefish, chicken broth, turkey broth, beef broth, chicken liver, brewers rice, corn grits, fish meal, egg, beet pulp, chloride, flax meal, lamb, beef by-products, chicken by-products and mixtures thereof.

[0066] In another embodiment, supplement compositions such as biscuits, chews, and other treats may comprise, on a dry matter basis, from 20% to 60% protein, or from 22% to 40% protein, by weight of the supplement composition. As another example, the supplement compositions may comprise, on a dry matter basis, from 5% to 35% fat, or from 10% to 30% fat, by weight of the supplement composition. Food and supplement compositions intended for use by canines or felines are commonly known in the art.

[0067] The pet foods may contain other active agents such as long chain fatty acids and zinc. Suitable long chain fatty acids include alpha-linoleic acid, gamma linolenic acid, linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid. Fish oils are a suitable source of eicosapentaenoic acids and docosahexaenoic acid.

[0068] Borage oil, blackcurrent seed oil and evening primrose oil are suitable sources of gamma linolenic acid. Safflower oils, sunflower oils, corn oils and soy bean oils are suitable sources of linoleic acid. These oils may also be used in the coating substrates referred to above. Zinc may be provided in various suitable forms, for example as zinc sulfate or zinc oxide. Further, many ingredients commonly used in pet foods are sources of fatty acids and zinc. It has been observed that the combination of chicory, as a source of prebiotic, with a linoleic-acid rich oil, such as soy bean oil, provides unexpected benefits, suggestive of a synergistic effect.

[0069] Where the composition is in the form of a gravy, the composition preferably comprises at least 10% of a broth, or stock, non-limiting examples of which include vegetable beef, chicken or ham stock. Typical gravy compositions may comprise from 0.5% to 5% crude protein, from 2% to 5% crude fat, and from 1% to 5% fiber.

[0070] Further non-limiting examples of supplements suitable for use herein include powders, oil suspensions, milk-based suspensions cheeses, and pills or capsules. Where the composition is in the form of a pill, suitable binding agents are required to maintain the pill in a solid, pressed form. Non-limiting examples of suitable binding agents include the natural gums such as xanthan gum, pectins, lecithins, alginites and others known to those skilled in the art. Where the composition is in the form of a capsule, the composition is preferably encapsulated using technologies known to those skilled in the art. Non-limiting examples of suitable encapsulation materials include polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), alginites, and gelatin. Yogurt-based compositions may comprise from 1% to 5% protein, from 10% to 20% carbohydrate, from 1% to 5% fiber, from 1% to 5% fat and from 50% to 90% liquid carrier such as milk.

Examples

[0071] Examples I to 4 are examples of dried kibble compositions comprising the probiotic Bifidobacteria globosum utilized in accordance with the present invention.
Examples 5 to 7 are examples of wet pet food compositions comprising the probiotic *Bifidobacteria globosum* utilized in accordance with the present invention.

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<th>Ingredient</th>
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<td>Probiotic (1 x 10^{10} cfu/g NCIMB 41199 in sunflower oil)</td>
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Examples 8 to 10 are examples of yogurt supplement compositions comprising the probiotic *Bifidobacteria globosum* utilized in accordance with the present invention.

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While particular embodiments of the present invention have been illustrated and described, it would be obvious to those skilled in the art that various other changes and modifications can be made without departing from the scope of the invention. It is therefore intended to cover in the appended claims all such changes and modifications that are within the scope of this invention.

SEQUENCE LISTING

<110> The IAMS Company
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O’Mahony, Liam
Sunvold, Greg
Tetrick, Mark
Vickers, Robert

<120> Methods of use of Probiotic Bifidobacteria for companion Animals

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1. Composition comprising a probiotic strain of *Bifidobacteria* obtainable by isolation from resected and washed mammalian gastrointestinal tract for use in treatment of a companion animal selected from the group consisting of canines and felines, treatment being selected from regulating the immune system of a companion animal, maintaining or improving the health of the skin and/or coat system of a companion animal, ameliorating or reducing the effects of aging in companion animals, preventing weight loss during and following infection in a companion animal, treating or preventing urinary tract ailments in companion animals, increasing fiber digestion in a companion animal, preventing or treating infection of the gastrointestinal tract of a companion animal, improving digestion in companion animals, reducing stress levels in a companion animal, or mixtures thereof, wherein said probiotic Bifidobacteria is selected from the group consisting of *Bifidobacterium pseudolongum* NCIMB 41199 and *Bifidobacteriuin globusum* NCIMB 41198 and mixtures thereof.

2. Composition according to Claim 1 wherein said probiotic strain of *Bifidobacteria* is isolated from resected and washed gastrointestinal tissue obtained from humans, canines or felines.

3. Composition according to any one of Claims 1 to 2 wherein said probiotic *Bifidobacteria* is selected for the ability to survive and colonise the gastrointestinal tract of companion animals.
4. Composition according to any one of the Claims 1 to 3 wherein said probiotic *Bifidobacteria* has at least 33% growth when cultured in the presence of 0.5% bile salts.

5. Composition according to any one of the Claims 1 to 4 wherein said probiotic *Bifidobacteria* is able to maintain viability following 1 hour at pH 2.5.

6. Composition according to any one of the Claims 1 to 5 for regulating the immune system of a companion animal, said regulation comprising treating or preventing autoimmune disease in a companion animal, treating or preventing inflammation in a companion animal or mixtures thereof.

7. Composition according to any one of the Claims 1 to 5 for maintaining or improving the health of the skin and/or coat system of a companion animal comprising treating or preventing atopic disease of the skin in companion animals.

8. Composition according to any of the Claims 1 to 5 for treating or preventing urinary tract ailments in companion animals wherein said urinary tract ailment comprises urinary tract infection, kidney stones or mixtures thereof.

9. Composition according to Claims 8, wherein said probiotic *Bifidobacteria* increase the degradation of oxalic acid in vitro.

10. Composition according to any one of the Claims 1 - 5 for the prevention or treatment of infection of the gastrointestinal tract, said prevention or treatment comprising improving the microbial ecology of said companion animal, reducing the number of pathogenic bacteria found in the faeces of said companion animal or mixtures thereof.

11. Composition according to Claim 10 wherein said pathogenic bacteria are selected from the group consisting of *Clostridia*, *Escherichia*, *Salmonella*, and mixtures thereof.

12. Composition according to any of the Claims 1 - 5 for reducing stress levels in a companion animal wherein said stress levels are measured by determining the level in the blood of said companion animal of stress hormones selected from the group consisting of epinephrine, norepinephrine, dopamine, cortisol, C-reactive protein and mixtures thereof.

**Patentansprüche**


2. Zusammensetzung nach Anspruch 1, wobei der probiotische *Bifidobakterien-Stamm* aus reseziertem und gewaschenem Gastrointestinalgewebe isoliert ist, das von Menschen, Hunden oder Katzen stammt.

3. Zusammensetzung nach einem der Ansprüche 1 bis 2, wobei die probiotischen *Bifidobakterien* aufgrund der Fähigkeit ausgewählt sind, dass sie im Gastrointestinaltrakt von Haustieren überleben und diesen besiedeln.

4. Zusammensetzung nach einem der Ansprüche 1 bis 3, wobei die probiotischen *Bifidobakterien* ein Wachstum von mindestens 33 % haben, wenn sie unter Vorhandensein von 0,5 % Gallensalzen kultiviert werden.

5. Zusammensetzung nach einem der Ansprüche 1 bis 4, wobei die probiotischen *Bifidobakterien* ihre Lebensfähigkeit nach 1 Stunde bei einem pH-Wert von 2,5 bewahren können.
6. Zusammensetzung nach einem der Ansprüche 1 bis 5 zur Regulierung des Immunsystems eines Haustiers, wobei die Regulierung die Behandlung oder Verhütung von Autoimmunkrankheit bei einem Haustier, Behandlung oder Verhütung von Entzündung bei einem Haustier oder Mischungen davon umfasst.


8. Zusammensetzung nach einem der Ansprüche 1 bis 5 zur Behandlung oder Verhütung von Harnwegsbeschwerden bei Haustieren, wobei die Harnwegsbeschwerden Harnwegsinfektion, Nierensteine oder Mischungen davon umfassen.


10. Zusammensetzung nach einem der Ansprüche 1 bis 5 zur Verhütung oder Behandlung von Infektionen des Gastrointestinaltraktes, wobei die Verhütung oder Behandlung die Verbesserung der mikrobiellen Ökologie des Haustiers, Verringerung der Anzahl pathogener Bakterien, die in den Fäkalien des Haustiers zu finden sind, oder Mischungen davon umfasst.


12. Zusammensetzung nach einem der Ansprüche 1 bis 5 zur Verringerung des Stressniveaus bei einem Haustier, wobei das Stressniveau durch Bestimmen der Menge von Stresshormonen, die ausgewählt sind aus der Gruppe, bestehend aus Epinephrin, Norepinephrin, Dopamin, Cortisol, C-reaktivem Protein und Mischungen davon, im Blut des Haustiers gemessen wird.

**Revendications**

1. Composition comprenant une souche probiotique de Bifidobacteria pouvant être obtenue par isolement d'un tube digestif mammalien réséqué et lavé pour une utilisation dans le traitement d’un animal de compagnie choisi dans le groupe constitué de canidés et félin, le traitement étant choisi parmi la régulation du système immunitaire d’un animal de compagnie, le maintien ou l’amélioration de la santé de la peau et/ou du système de pelage d’un animal de compagnie, l’amélioration ou la réduction des effets du vieillissement chez des animaux de compagnie, la prévention de la perte de poids pendant et après une infection chez un animal de compagnie, le traitement ou la prévention d’affections du système urinaire chez des animaux de compagnie, l’augmentation de la digestion des fibres chez un animal de compagnie, la prévention ou le traitement d’une infection du tube digestif chez un animal de compagnie, l’amélioration de la digestion chez des animaux de compagnie, la réduction des degrés de stress chez un animal de compagnie, ou leurs mélanges, dans laquelle ledit probiotique Bifidobacteria est choisi dans le groupe constitué de Bifidobacterium pseudolongum NCIMB 41199, et Bifidobacterium globusum NCIMB 41198 et leurs mélanges.

2. Composition selon la revendication 1, dans laquelle ladite souche probiotique de Bifidobacteria est isolée à partir d’un tissu gastro-intestinal réséqué et lavé obtenu auprès d’humains, de canidés ou de félin.

3. Composition selon l’une quelconque des revendications 1 à 2, dans laquelle ledit probiotique Bifidobacteria est choisi pour la capacité à survivre et à coloniser le tube digestif d’animaux de compagnie.

4. Composition selon l’une quelconque des revendications 1 à 3, dans laquelle ledit probiotique Bifidobacteria a une croissance d’au moins 33 % lorsqu’il est cultivé en présence de 0,5 % de sels de bile.

5. Composition selon l’une quelconque des revendications 1 à 4, dans laquelle ledit probiotique Bifidobacteria est capable de maintenir une viabilité après 1 heure à pH 2,5.

6. Composition selon l’une quelconque des revendications 1 à 5, destinée à réguler le système immunitaire d’un animal de compagnie, ladite régulation comprenant le traitement ou la prévention d’une maladie auto-immune chez un
animal de compagnie, le traitement ou la prévention de l’inflammation chez un animal de compagnie ou leurs mélanges.

7. Composition selon l’une quelconque des revendications 1 à 5, destinée à maintenir ou améliorer la santé de la peau et/ou du système de pelage d’un animal de compagnie comprenant le traitement ou la prévention d’une maladie atopique de la peau chez des animaux de compagnie.

8. Composition selon l’une quelconque des revendications 1 à 5 destinée au traitement ou la prévention d’affections du système urinaire chez des animaux de compagnie, dans laquelle ladite affection du système urinaire comprend une infection du système urinaire, des calculs rénaux ou leurs mélanges.


10. Composition selon l’une quelconque des revendications 1 à 5 pour la prévention ou le traitement d’une infection du tube digestif, ladite prévention ou ledit traitement comprenant une amélioration de l’écologie microbienne dudit animal de compagnie, une réduction du nombre de bactéries pathogènes trouvées dans les fèces dudit animal de compagnie ou leurs mélanges.

11. Composition selon la revendication 10, dans laquelle lesdites bactéries pathogènes sont choisies dans le groupe constitué de Clostridia, Escherichia, Salmonella, et leurs mélanges.

12. Composition selon l’une quelconque des revendications 1 à 5 destinée à réduire les degrés de stress chez un animal de compagnie, dans laquelle lesdits degrés de stress sont mesurés en déterminant le taux dans le sang dudit animal de compagnie d’hormones de stress choisies dans le groupe constitué de l’adrénaline, la noradrénaline, la dopamine, le cortisol, la protéine C-réactive et leurs mélanges.
REFERENCES CITED IN THE DESCRIPTION

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