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(54) Title: ANIMAL FEED COMPOSITION AND USE THEREOF

(57) Abstract: The present invention provides a method for improving performance, immunity and/or gut health of animals comprising administering to the animals one or more microbial muramidase(s) and one or more organic acid(s).

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ANIMAL FEED COMPOSITION AND USE THEREOF

REFERENCE TO A SEQUENCE LISTING

This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to a method for improving performance, immunity and/or gut health of animals by using one or more microbial muramidase(s) and one or more oganic acid(s).

BACKGROUND OF THE INVENTION

Muramidase is an *O*-glycosyl hydrolase produced as a defensive mechanism against bacteria by many organisms. The enzyme causes the hydrolysis of bacterial cell walls by cleaving the glycosidic bonds of peptidoglycan, an important structural molecule in bacteria. After having their cell walls weakened by muramidase action, bacterial cells lyse as a result of umbalanced osmotic pressure.

Muramidase naturally occurs in many organisms such as viruses, plants, insects, birds, reptiles and mammals. In mammals, muramidase has been isolated from nasal secretions, saliva, tears, intestinal content, urine and milk. The enzyme cleaves the glycosidic bond between carbon number 1 of *N*-acetylmuramic acid and carbon number 4 of *N*-acetyl-D-glucosamine. In vivo, these two carbohydrates are polymerized to form the cell wall polysaccharide of many microorganisms.

Muramidase has been classified into five different glycoside hydrolase (GH) families (www.cazy.org): hen egg-white muramidase (GH22), goose egg-white muramidase (GH23), bacteriophage T4 muramidase (GH24), *Sphingomonas* flagellar protein (GH73) and *Chalaropsis* muramidases (GH25). Muramidase extracted from hen egg white (a GH22 muramidase) is the primary product available on the commercial market, and traditionally has just been referred to as muramidase even though nowadays there are many other known muramidases.

Organic acids are widely distributed in nature as normal constituents of plants or animal tissues. They are also formed through microbial fermentation of carbohydrates mainly in the large intestine. They are often used in swine and poultry production as a replacement of antibiotic growth promoters since they have a preventive effect on the intestinal problems like necrotic enteritis in chickens and *Escherichia coli* infection in young pigs.

Surprisingly, the inventors of the present invention discovered that fungal muramidases in combination of orgaic acids provides additional benefits in improving performance, immunity and gut health of animals.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a method for improving performance, immunity and/or gut health of animals comprising administering to the animals one or more microbial muramidase(s) and one or more organic acid(s).

The present invention also provides a feed composition, a feed additive and an animal feed comprising one or more microbial muramidase(s) and one or more organic acid(s) for improving performance, immunity and/or gut health of animals, and use thereof.

OVERVIEW OF SEQUENCE LISTING

SEQ ID NO: 1 is the mature amino acid sequence of a wild type GH25 muramidase from *Acremonium alcalophilum* with N-terminal SPIRR as described in WO 2013/076253.

SEQ ID NO: 2 is the mature amino acid sequence of a wild type GH24 muramidase from *Trichophaea saccata*.

SEQ ID NO: 3 is the mature amino acid sequence of a wild type GH25 muramidase from *Acremonium alcalophilum* as described in WO 2013/076253.

BRIEF DESCRIPTION OF FIGURES

Figure 1 is diagram indicating average fecal score of piglets over the whole period (day 0-28); and

Figure 2 is diagram indicating soluble peptidoglycan percentage in the ileum digesta.

DEFINITIONS

Animals: The term “animal” or “animals” refers to any animal except human. Examples of animals are monogastric animals, including but not limited to pigs or swine (including but not limited to, piglets, growing pigs and sows); poultry such as turkeys, ducks, quail, guinea fowl, geese, pigeons (including squabs) and chicken (including but not limited to broiler chickens (referred to herein as broiles), chicks, layer hens (referred to herein as layers)); pets such as cats and dogs; horses (including but not limited to hotbloods, coldbloods and warm bloods), crustaceans (including but not limited to shrimps and prawns) and fish (including but not limited to amberjack, arapaima, barb, bass, bluefish, bocachico, bream, bullhead, cachama, carp, catfish, catla, chanos, char, cichlid, cobia, cod, crappie, dorada, drum, eel, goby, goldfish, gourami, grouper, guapote, halibut, java, labeo, lai, loach, mackerel, milkfish, mojarra, mudfish, mullet, paco, pearlspot, pejerrey, perch, pike, pompano, roach, salmon, sampa, sauger, sea bass, seabream, shiner, sleeper, snakehead, snapper, snook, sole, spinefoot, sturgeon, sunfish, sweetfish, tench, terror, tilapia, trout, tuna, turbot, vendace, walleye and whitefish). Preferably, the animal is selected from the group consisting of pigs or swine (including, but not limited to, piglets, growing pigs, and sows); poultry such as turkeys, ducks, quail,

guinea fowl, geese, pigeons (including squabs) and chicken (including but not limited to broiler chickens (referred to herein as broiles), chicks, layer hens (referred to herein as layers)); pets such as cats and dogs; and horses (including but not limited to hotbloods, coldbloods and warm bloods).

Microbial muramidase: The term "microbial muramidase" means a polypeptide having muramidase activity which is obtained or obtainable from a microbial source. Examples of microbial sources are fungi; i.e. the muramidase is obtained or obtainable from the kingdom *Fungi*, wherein the term kingdom is the taxonomic rank. In particular, the the microbial muramidase is obtained or obtainable from the phylum *Ascomycota*, such as the sub-phylum *Pezizomycotina*, wherein the terms phylum and sub-phylum is the taxonomic ranks.

If the taxonomic rank of a polypeptide is not known, it can easily be determined by a person skilled in the art by performing a BLASTP search of the polypeptide (using e.g. the National Center for Biotechnology Information (NCIB) website http://www.ncbi.nlm.nih.gov/) and comparing it to the closest homologues. An unknown polypeptide which is a fragment of a known polypeptide is considered to be of the same taxonomic species. An unknown natural polypeptide or artificial variant which comprises a substitution, deletion and/or insertion in up to 10 positions is considered to be from the same taxonomic species as the known polypeptide.

Muramidase activity: The term "muramidase activity" means the enzymatic hydrolysis of the 1,4-beta-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in a peptidoglycan or between *N*-acetyl-D-glucosamine residues in chitodextrins, resulting in bacteriolysis due to osmotic pressure. Muramidase belongs to the enzyme class EC 3.2.1.17. Muramidase activity is typically measured by turbidimetric determination. The method is based on the changes in turbidity of a suspension of *Micrococcus luteus* ATCC 4698 induced by the lytic action of muramidase. In appropriate experimental conditions these changes are proportional to the amount of muramidase in the medium (c.f. INS 1105 of the Combined Compendium of Food Additive Specifications of the Food and Agriculture Organisation of the UN (www.fao.org)). For the purpose of the present invention, muramidase activity is determined according to the turbidity assay described in example 5 ("Determination of Muramidase Activity") of WO 2020/053274 A1.

The polypeptides of the present invention may have at least 20%, *e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 100% of the muramidase activity of SEQ ID NO: 1, 2 or 3.

Fragment: The term "fragment" means a polypeptide or a catalytic domain having one or more (*e.g.*, several) amino acids absent from the amino and/or carboxyl terminus of a mature polypeptide or domain, wherein the fragment has muramidase activity.

Mature polypeptide: The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc.

Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues × 100) / (Length of Alignment - Total Number of Gaps in Alignment)

Variant: The term "variant" means a polypeptide having muramidase activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion, of one or more (several) amino acid residues at one or more (*e.g.*, several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion means removal of the amino acid occupying a position; and an insertion means adding 1, 2, or 3 amino acids adjacent to and immediately following the amino acid occupying the position.

A muramidase variant according to the invention may comprise from 1 to 5; from 1 to 10; from 1 to 15; from 1 to 20; from 1 to 25; from 1 to 30; from 1 to 35; from 1 to 40; from 1 to 45; or from 1-50, i.e. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 alterations and have at least 20%, *e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 100% of the muramidase activity of the muramidase, such as SEQ ID NO: 1, 2 or 3.

Animal Feed: the term "animal feed" refers to any compound, preparation, or mixture suitable for or intended for intake by an animal and capable of maintaining life and/or promoting production of the animal without any additional substance being consumed except water.

Feed Addtive: the term "feed additive" refers to an ingredient or combination of ingredients added to the animal feed, usually used in micro quantities and requires careful handling and mixing. Such ingredient includes but is not limited to vitamins, amino acids, minerals, enzymes, eubiotics, colouring agents, growth improving additives and aroma compounds/flavourings, polyunsaturated fatty acids (PUFAs); reactive oxygen generating species, antioxidants, anti-microbial peptides, anti-fungal polypeptides and mycotoxin management compounds etc..

DETAILED DESCRIPTION OF THE INVENTION

Method for improving performance, immunity and/or gut health of animals

It has been surprisingly found that supplementing an animal feed with the combination of a microbial muramidase and an organic acid results in a significant benefit in improving growth and health

performance, immunity and gut health of animals, compared to an animal feed without the combination.

Accordingly, the invention relates to a method for improving performance, immunity and/or gut health of an animal comprising administering to the animal one or more microbial muramidase(s) and one or more organic acid(s).

The invention also relates to use of one or more microbial muramidase(s) and one or more organic acid(s) for improving performance, immunity and/or gut health of an animal.

In the present invention, the performance may be growth performance and/or health performance of animals. The growth performance of an animal may be charaterized by one or more of the following parameters: weight gain (WG), daily weight gain (DWG), feed intake (FI), daily feed intake (DFI), feed conversion ratio (FCR), European Production Efficiency Factor (EPEF) and digestibility of nutrients such as crude protein, nitrogen and energy. The health performance of an animal may be characterized by level of diarrhea and mortality etc..

In the present invention, the immunity of an animal may be represented by the ability of response to stress in the animal, and/or by antioxidant enzymes activity, such as catalase activity, SOD activity and GPx (Glutathione peroxidase) activity, Oxidative stress index (OSI) and IgG, in plasma and/or tissue lysates (ileum and jejunum) of the animal.

In the present invention, the gut health of an animal may be characterized by one or more of the following parameters: villi length, crypt depth, villi length/crypt depth ratio, lamina propria and epithelial thickness in intestines of the animal, and concentration of beneficial compounds such as benzoic acid, hippuric acid (HA) and soluble peptidoglycan in plasma or intestinal contents.

Particularly, the present invention provides a method or use for

- improving growth performance such as DWG, DFI and FCR; and/or
- improving nitrogen digestability; and/or
- reducing diarrhea; and/or
- improving antioxidant enzymes activity; and/or
- improving stress response; and/or
- improving gut health, for example, increasing villi length, crypth depth and/or epithelium thickness of intestines, and/or enhancing concentration of benzoic acid and/or hippuric acid and/or soluble peptidoglycan in intestines (such as ileum and jejunum),

of animals by using one or more microbial muramidase(s) and one or more organic acid(s).

In the present invention, the improvement is compared to an animal feed additive wherein the microbial muramidase and the organic acid are not included (herein referred to as the control). Preferably, one or more of the parameters on performance, immunity and/or gut health of animals is changed in a desired direction by at least 0.5%, such as by at least 0.6%, at least 0.7%, at least 0.8%, at least 0.9%, at least 1.0%, at least 1.2% or at least 1.4%, compared to the control.

In the present invention, the microbial muramidase may be of fungal origin. Preferably, the microbial muramidase is obtained or obtainable from the phylum *Ascomycota*, such as the sub-phylum *Pezizomycotina*. More preferably, the microbial muramidase is obtained or obtainable from *Acremonium alcalophilum* or *Trichophaea saccate.*

Preferably, the microbial muramidase comprises one or more domains selected from GH24 and GH25. More preferably, the microbial muramidase is GH24 muramidase or GH25 muramidase. An example of the microbial muramiase is Balancius® (DSM Nutritional Products, Switzerland).

In the present invention, the microbial muramidase may have at least 50%, e.g., at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1, 2 or 3.

In the present invention, the microbial muramidase may comprise or consist of the amino acid sequence of SEQ ID NO: 1 or an allelic variant thereof; or is a fragment thereof having muramidase activity, wherein the fragment comprises at least 170 amino acids, such as at least 175 amino acids, at least 177 amino acids, at least 180 amino acids, at least 185 amino acids, at least 190 amino acids, at least 195 amino acids or at least 200 amino acids. Preferably, the microbial muramidase comprises or consists of the amino acid sequence of SEQ ID NO: 1 or an allelic variant thereof and a N-terminal and/or C-terminal His-tag and/or HQ-tag. More preferably, the polypeptide comprises or consists of amino acids 1 to 213 of SEQ ID NO: 1.

Alternatively, the microbial muramidase may comprise or consist of the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof; or is a fragment thereof having muramidase activity, wherein the fragment comprises at least 210 amino acids, such as at least 215 amino acids, at least 220 amino acids, at least 225 amino acids, at least 230 amino acids, at least 235 amino acids or at least 240 amino acids. Preferably, the microbial muramidase comprises or consists of the amino acid sequence of SEQ ID NO: 2 or an allelic variant thereof and a N-terminal and/or C-terminal His-tag and/or HQ-tag. More preferably, the polypeptide comprises or consists of amino acids 1 to 245 of SEQ ID NO: 2.

More alternatively, the microbial muramidase may comprise or consist of the amino acid sequence of SEQ ID NO: 3 or an allelic variant thereof; or is a fragment thereof having muramidase activity, wherein the fragment comprises at least 170 amino acids, such as at least 175 amino acids, at least 177 amino acids, at least 180 amino acids, at least 185 amino acids, at least 190 amino acids, at least 195 amino acids or at least 200 amino acids. Preferably, the microbial muramidase comprises or consists of the amino acid sequence of SEQ ID NO: 3 or an allelic variant thereof and a N-terminal and/or C-terminal His-tag and/or HQ-tag. More preferably, the polypeptide comprises or consists of amino acids 1 to 208 of SEQ ID NO: 3.

In the present invention, the microbial muramidase may be a variant of SEQ ID NO: 1, 2 or 3 wherein the variant has muramidase activity and comprises one or more substitutions, and/or one or more deletions, and/or one or more insertions or any combination thereof in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38,

39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 positions. Preferably, the number of positions comprising one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in SEQ ID NO: 1, 2 or 3 is between 1 and 45, such as 1-40, 1-35, 1-30, 1-25, 1-20, 1-15, 1-10 or 1-5 positions. More preferably, the number of positions comprising one or more amino acid substitutions, and/or one or more amino acid deletions, and/or one or more amino acid insertions or any combination thereof in SEQ ID NO: 1, 2 or 3 is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Further preferably, the number of substitutions, deletions, and/or insertions in SEQ ID NO: 1, 2 or 3 is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Futher preferably, the number of substitutions, preferably conservative substitutions, in SEQ ID NO: 1, 2 or 3 is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10. Further preferably, the number of conservative substitutions in SEQ ID NO: 1, 2 or 3 is not more than 10, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

Any person skilled in the art can understand, the polypeptide of the microbial muramidase may have amino acid changes. The amino acid changes may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the groups of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In*, *The Proteins*, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

Essential amino acids in a polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for muramidase activity to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide.

In the present invention, the microbial muramidase may be adminstered at a level of 100 to 1000 mg enzyme protein per kg animal feed, such as 200 to 900 mg, 300 to 800 mg, 400 to 700 mg, 500 to 600 mg enzyme protein per kg animal feed, or any combination of these intervals.

The organic acid for use according to the present invention is selected from the group consisting of short chain fatty acids (e.g. formic acid, acetic acid, propionic acid, butyric acid), medium chain fatty acids (e.g. caproic acid, caprylic acid, capric acid, lauric acid), di/tri-carboxylic acids (e.g. fumaric acid and succinic acid), hydroxy acids (e.g. lactic acid), aromatic acids (e.g. benzoic acid), citric acid, sorbic acid, malic acid, and tartaric acid, or their salt (typically sodium or potassium or ammonium salt such as potassium diformate or sodium butyrate or ammonium formate). Examples of commercial organic acid products are VevoVitall® (DSM Nutritional Products, Switzerland), Biotronic®, Amasil®, Luprisil®, Lupro-Grain®, Lupro-Cid®, Lupro-Mix® (BASF), n-Butyric Acid AF (OXEA) and Adimix Precision (Nutriad).

In the present invnetion, the organic acid may be adminstered at a level of from 0.001% to 10%, preferably from 0.01% to 5%, more preferably from 0.1% to 1% by weight of animal feed.

Feed composition and feed additive

The microbial muramidase and the organic acid of the present invention may be formulated as a feed composition or a feed additive (premix) for improving performance, immunity and/or gut health of animals, which is also the present invention intents to cover.

In the present invention, the feed composition, the feed additive and/or the components such as the microbial muramidase and the organic acid contained therein may be formulated as a liquid formulation or a solid formulation, and thus may contains one or more formulating agents.

The formulating agents may be selected from the group consisting of polyol such as glycerol, sorbitol, ethylene glycol, diethylene glycol, triethylene glycol, 1,2-propylene glycol, 1,3-propylene glycol, dipropylene glycol and polyethylene glycol (PEG); a salt such as organic or inorganic zinc, sodium, potassium, calcium or magnesium salts (for example, magnesium sulfate, calcium acetate, calcium benzoate, calcium carbonate, calcium chloride, calcium citrate, calcium sorbate, calcium sulfate, potassium acetate, potassium benzoate, potassium carbonate, potassium chloride, potassium citrate, potassium sorbate, potassium sulfate, sodium acetate, sodium benzoate, sodium carbonate, sodium chloride, sodium citrate, sodium sulfate, zinc acetate, zinc benzoate, zinc carbonate, zinc chloride, zinc citrate, zinc sorbate and zinc sulfate); and starch or a sugar or sugar derivative such as sucrose, dextrin, glucose, lactose and sorbitol; small organic molecules, flour, cellulose and minerals and clay minerals (also known as hydrous aluminum phyllosilicates such as kaolinite or kaolin).

The feed composition or the feed additive according to the present invention may also comprise one or more emulsifying agents. The emulsifying agents may be selected advantageously from the group consisting of polyglycerol esters of fatty acids such as esterified ricinoleic acid or propylene glycol esters of fatty acids, saccharo-esters or saccharo-glycerides, polyethylene glycol, lecithins, etc..

Animal Feed

The microbial muramidase and the organic acid of the present invention may also be formulated as an animal feed for improving performance, immunity and/or gut health of animals, which is also the present invention intents to cover.

An animal feed according to the present invention may have a crude protein content of between 50 and 800 g/kg, and furthermore comprises one or more microbial muramidase(s) and one or more organic acid(s) as described herein.

The animal feed of the present invention may contain animal protein, such as meat and bone meal, feather meal, and/or fish meal, typically in an amount of 0-25%. The animal feed of the present invention may also comprise dried distillers grains with solubles (DDGS), typically in amounts of 0-30%.

Preferably, the animal feed of the present invention comprises vegetable proteins. In the present invention, the vegetable proteins may be derived from vegetable protein sources, such as legumes and cereals, for example, materials from plants of the families *Fabaceae* (*Leguminosae*), *Cruciferaceae*, *Chenopodiaceae*, and *Poaceae*, such as soybean meal, lupin meal, rapeseed meal, and combinations thereof. The protein content of the vegetable proteins is at least 10, 20, 30, 40, 50, 60, 70, 80, or 90% (w/w).

Preferaly, the animal feed of the present invention contains 0-80% maize; and/or 0-80% sorghum; and/or 0-70% wheat; and/or 0-70% Barley; and/or 0-30% oats; and/or 0-40% soybean meal; and/or 0-25% fish meal; and/or 0-25% meat and bone meal; and/or 0-20% whey.

In the animal feed of the present invention, the microbial muramidase may be contained at a level of 100 to 1000 mg enzyme protein per kg animal feed, such as 20 to 900 mg, 300 to 800 mg, 400 to 700 mg, 500 to 600 mg enzyme protein per kg animal feed, or any combination of these intervals.

In the animal feed of the present invention, the organic acid may be contained at a level of from 0.001% to 10%, preferably from 0.01% to 5%, more preferably from 0.1% to 1% by weight of animal feed.

Additional Enzymes

In the present invention, the feed composition, the feed additive or the animal feed described herein optionally include one or more enzymes. Examples of the enzymes include but are not limited to phytase (EC 3.1.3.8 or 3.1.3.26), xylanase (EC 3.2.1.8), galactanase (EC 3.2.1.89), alpha-galactosidase (EC 3.2.1.22), protease (EC 3.4), phospholipase A1 (EC 3.1.1.32), phospholipase A2 (EC 3.1.1.4), lysophospholipase (EC 3.1.1.5), phospholipase C (3.1.4.3), phospholipase D (EC 3.1.4.4), amylase such as alpha-amylase (EC 3.2.1.1), arabinofuranosidase (EC 3.2.1.55), beta-xylosidase (EC 3.2.1.37), acetyl xylan esterase (EC 3.1.1.72), feruloyl esterase (EC 3.1.1.73), cellulase (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), beta-glucosidase (EC 3.2.1.21), pullulanase (EC 3.2.1.41), alpha-

mannosidase (EC 3.2.1.24), mannanase (EC 3.2.1.25) and beta-glucanase (EC 3.2.1.4 or EC 3.2.1.6), or any combination thereof.

Examples of commercially available phytases include Bio-Feed™ Phytase (Novozymes), Ronozyme® P, Ronozyme® NP and Ronozyme® HiPhos (DSM Nutritional Products, Switzerland), Natuphos™ (BASF, Germany), Finase® and Quantum® Blue (AB Enzymes, Germany), OptiPhos® (Huvepharma, Bulgaria) Phyzyme® XP (DuPont, USA) and Axtra® PHY (DuPont, USA). Other preferred phytases include those described in e.g. WO 98/28408, WO 00/43503 and WO 03/066847.

Examples of commercially available xylanases include Ronozyme® WX and Ronozyme® G2 (DSM Nutritional Products, Switzerland), Econase® XT and Barley (AB Vista, GB), Xylathin® (Verenium, USA), Hostazym® X (Huvepharma, Bulgaria) and Axtra® XB (Xylanase/beta-glucanase, DuPont, USA). Examples of commercially available proteases include Ronozyme® ProAct (DSM Nutritional Products, Switerland).

Microbes

In the present invention, the feed composition, the feed additive or the animal feed may further comprise one or more additional microbes. For example, the feed composition, the feed additive or the animal feed 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.

Preferably, the feed composition, the feed additive or the animal feed of the present invention further comprises a bacterium from one or more of the following strains: *Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus cereus, Bacillus pumilus*, *Bacillus polymyxa*, *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus circulans*, *Enterococcus faecium, Enterococcus spp, and Pediococcus spp, Lactobacillus spp, Bifidobacterium spp, Lactobacillus acidophilus, Pediococsus acidilactici, Lactococcus lactis, Bifidobacterium bifidum, Propionibacterium thoenii, Lactobacillus farciminus, lactobacillus rhamnosus, Clostridium butyricum, Bifidobacterium animalis ssp. animalis, Lactobacillus reuteri, Lactobacillus salivarius ssp. salivarius, Megasphaera elsdenii,* and *Propionibacteria sp.*

More preferably, the feed composition, the feed additive or the animal feed of the present invention further comprises a bacterium from one or more of the following strains of *Bacillus subtilis:* 3A-P4 (PTA-6506), 15A-P4 (PTA-6507), 22C-P1 (PTA-6508), 2084 (NRRL B-500130), LSSA01 (NRRL-B-50104), BS27 (NRRL B-501 05), BS 18 (NRRL B-50633), BS 278 (NRRL B-50634), DSM 29870, DSM 29871, NRRL B-50136, NRRL B-50605, NRRL B-50606, NRRL B-50622 and PTA-7547.

More preferably, the feed composition, the feed additive or the animal feed of the present invention further comprises a bacterium from one or more of the following strains of *Bacillus pumilus*: NRRL B-50016, ATCC 700385, NRRL B-50885 and NRRL B-50886.

More preferably, the composition, the animal feed additive or the animal feed further comprises a bacterium from one or more of the following strains of *Bacillus lichenformis*: NRRL B 50015, NRRL B-50621 and NRRL B-50623.

More preferably, the feed composition, the feed additive or the animal feed of the present invention further comprises a bacterium from one or more of the following strains of *Bacillus amyloliquefaciens*: DSM 29869, DSM 29872, NRRL B 50607, PTA-7543, PTA-7549, NRRL B-50349, NRRL B-50606, NRRL B-50013, NRRL B-50151, NRRL B-50141, NRRL B-50147 and NRRL B-50888.

In the present invention, the one or more bacterial strains may be present in the form of a stable spore.

Amino Acids

The feed composition, the feed additive or the animal feed of the present invention may further comprise one or more amino acids. Examples of the amino acids include but are not limited to lysine, alanine, beta-alanine, threonine, methionine and tryptophan.

Vitamins and Minerals

The feed composition, the feed additive or the animal feed of the present invention may include one or more vitamins, such as one or more fat-soluble vitamins and/or one or more water-soluble vitamins. Optionally, the feed composition, the feed additive or the animal feed of the present invention include one or more minerals, such as one or more trace minerals and/or one or more macro minerals. Usually fat- and water-soluble vitamins, as well as trace minerals form part of a so-called premix intended for addition to the feed, whereas macro minerals are usually separately added to the feed.

Non-limiting examples of fat-soluble vitamins include vitamin A, vitamin D_3, vitamin E, and vitamin K, *e.g.*, vitamin K_3.

Non-limiting examples of water-soluble vitamins include vitamin B_{12}, biotin, choline, vitamin B_1, vitamin B_2, vitamin B_6, 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.

Preferably, the feed composition, the feed additive or the animal feed of the invention comprises at least one of the below vitamins, to provide an in-feed-concentration within the ranges specified in the below Table 1 (for piglet and broiler diets, respectively).

Table 1: Typical vitamin recommendations

Vitamin	Piglet diet	Broiler diet
Vitamin A	10,000-15,000 IU/kg feed	8-12,500 IU/kg feed
Vitamin D_3	1800-2000 IU/kg feed	3000-5000 IU/kg feed
Vitamin E	60-100 mg/kg feed	150-240 mg/kg feed
Vitamin K_3	2-4 mg/kg feed	2-4 mg/kg feed
Vitamin B_1	2-4 mg/kg feed	2-3 mg/kg feed
Vitamin B_2	6-10 mg/kg feed	7-9 mg/kg feed
Vitamin B_6	4-8 mg/kg feed	3-6 mg/kg feed
Vitamin B_{12}	0.03-0.05 mg/kg feed	0.015-0.04 mg/kg feed
Niacin (Vitamin B_3)	30-50 mg/kg feed	50-80 mg/kg feed
Pantothenic acid	20-40 mg/kg feed	10-18 mg/kg feed
Folic acid	1-2 mg/kg feed	1-2 mg/kg feed
Biotin	0.15-0.4 mg/kg feed	0.15-0.3 mg/kg feed
Choline chloride	200-400 mg/kg feed	300-600 mg/kg feed

Other feed ingredients

The feed composition, the feed additive or the animal feed of the present invention may further comprise colouring agents, stabilisers, growth improving additives and aroma compounds/flavourings, polyunsaturated fatty acids (PUFAs), reactive oxygen generating species, anti-microbial peptides and anti-fungal polypeptides.

Examples of the colouring agents are carotenoids such as beta-carotene, astaxanthin, and lutein. Examples of the stabilizing agents (e.g. acidifiers) are organic acids. Examples of these are benzoic acid (VevoVitall®, DSM Nutritional Products, Switzerland), formic acid, butyric acid, fumaric acid and propionic acid.

Examples of the aroma compounds/flavourings are creosol, anethol, deca-, undeca-and/or dodeca-lactones, ionones, irone, gingerol, piperidine, propylidene phatalide, butylidene phatalide, capsaicin and tannin.

Examples of the polyunsaturated fatty acids are C_{18}, C_{20} and C_{22} polyunsaturated fatty acids, such as arachidonic acid, docosohexaenoic acid, eicosapentaenoic acid and gamma-linoleic acid.

Examples of the reactive oxygen generating species are chemicals such as perborate, persulphate, or percarbonate; and enzymes such as an oxidase, an oxygenase or a syntethase.

Examples of the antimicrobial peptides (AMP's) are CAP18, Leucocin A, Tritrpticin, Protegrin-1, Thanatin, Defensin, Lactoferrin, Lactoferricin, and Ovispirin such as Novispirin (Robert Lehrer, 2000),

Plectasins, and Statins, including the compounds and polypeptides disclosed in WO 03/044049 and WO 03/048148, as well as variants or fragments of the above that retain antimicrobial activity.

Examples of the antifungal polypeptides (AFP's) are the *Aspergillus giganteus*, and *Aspergillus niger* peptides, as well as variants and fragments thereof which retain antifungal activity, as disclosed in WO 94/01459 and WO 02/090384.

EXAMPLES

Example 1: In vivo trial 1

1. Animal housing

Forty castrated male crossbreds (Redon x Large-White) weaned piglets at 28 days of age were used in a 28-day experiment. At the start of the trial, the animals had an initial body weight of 9.0 ± 1.0 kg. They were allocated randomly in 20 cages of 2 animals. They were housed in flat-deck cages in an environmentally controlled room. Each cage had a plastic-coated welded wire floor and was equipped with two water nipples and two stainless-steel individualized feeders. Room temperature was initially 28°C and was lowered weekly by about 2°C until 21-22° C. Humidity percentage was 50 %.

2. Feeding and treatement

Piglets were fed *ad libitum*, throughout a 28-day experimental period, two diets based on wheat/soybean meal and barley (see Table 2). A Pre-starter diet formulated to have 210 g crude protein and 13.4 MJ/kg metabolizable energy was distributed during 14 days in mash form. The last two weeks of the experiment, a starter diet containing 190 g crude protein and 13.2 MJ/kg metabolizable energy was provided in pellet form.

Table 2: Diet composition

Ingredients (%)	**Pre-starter**	**Starter**
Wheat	62.60	49.70
Barley	13.40	21.30
Soybean meal	20.00	24.70
Soy oil	1.00	1.00
Premix 3145	3.00	3.00
HiPhos 10,000 (GT)	0.01	0.01
TiO2	0.00	0.30
Calculated energy & nutrient contents		
Crude protein (%)	21.0	19.0
Metabolizable energy (MJ/kg)	13.4	13.2

Four dietary treatments were involved and consisted of a negative control (NC) without product supplementation, and the negative control supplemented with either Vevovitall® ("VV", 90-100 % benzoic acid) or Balancius® ("BAL",100 000 LSU/g) alone or in combination as follows (Table 3).

Table 3: treatmens used in this study

Treatment	Code	Product	Inclusion level (mg/Kg)
A	Control (NC)	-	
B	NC + BAL	BAL	500
C	NC + VV	VV	5000
D	NC + BAL + VV	BAL + VV	500 + 5000

3. Specific treatments or sampling

The health status of the animals was controlled daily, with special attention to fecal consistency. Body weight of the individual animal and feed consumption per pen as recorded on days 1, 14 and 28 of the study. Performance, average daily weight gain (DWG), average daily feed intake (DFI) and feed conversion ratio (FCR)) were calculated for phases 1 (day 0-14) and 2 (day 14-28), and for the whole experimental period.

In order to evaluate the effect of products supplementation (VV and BAL) on the immune response of piglets and on growth performance, the animals were subjected to acute stress which consists of removing their feeder trough for a period of 16 hours.

Blood was collected by jugular puncture at day 21 (pre-stress), 22 (post-stress) and at day 28 from all the pigs. 6 ml was collected at day 21, 22 and 28 in heparin tube for immune response analysis (SOD/CAT/GPx/OSI and IgG).

Ileum and jejunum tissues were also sampled for histology measurement and for the determination of antioxidant parameters.

4. Analysis

Fecal score (presence or absence of diarrhea) was determined visually by a trained person in the animal facilities. The scores were record each day by pen according to the following scale:

0 = Normal, formed faeces (sausage-shaped firm feaces + clean piglets)

1 = Without form, pasty faeces (log-shaped moist and soft feaces + clean piglets)

2 = No consistency observed, liquid faeces (mild diarrhea; texturized, no shape faeces + dirty piglets)

3 = No consistency observed, liquid faeces (severe diarrhea; liquid faeces + dirty and wet piglets)

The concentration of the marker in feed and digesta together with the content of the nutrients and amino acids in the feed and digesta were used to calculate the apparent fecal digestibility coefficient (AFD) of nutrient according to the following equation:

$$AFD\ (\%) = 100 - [(CMf/CMe) \times (CNe/CNf)] \times 100$$

CMf =concentration of marker in feed; CMe =concentration of marker in excreta;
CNf = concentration of nutrient in feed; CNe =concentration of nutrient in excreta

The activity of catalase (CAT) in plasma and tissue lysates (ileum and jejunum) was determined using the Amplex® Red Catalase assay kit (Molecular Probe, A22180), following the supplier's instructions. The activity of SOD in plasma and tissue lysates (ileum and jejunum) was determined using SOD assay kit (Merck, ref 19160), following the supplier's instruction.

The activity of the Glutathione peroxidase was determined in plasma and tissue lysates (ileum and jejunum) using the GPx determination Kit (Abcam ab102530), following the supplier's instructions.

An oxidative stress index (OSI) was calculated as the ratio of reactive oxygen metabolites (ROM) and Plasma antioxidant capability (PAT) determined in plasma samples. ROM and PAT were analyzed using commercially available methods (d-ROM fast test and PAT test) on FRAS5 instrument (Innovatics Laboratory. Philadelphia. USA).

Small parts of the ileum and jejunum were collected in 4% formaldehyde. Twenty-four hours after collection these samples were transferred into 50% ethanol and within a week were processed. The samples were embedded in wax and cut into 5µm sections. Sections were stained with Alcian Blue (mucus staining). Pictures were taken with a Zeiss Axio Observer A1 microscope combined with an Axiocam 705 C camera (Zeiss) and the ZEN 3.1 Pro (Zeiss) software. Villi length, crypt depth and Epithelial thickness was measured using the ZEN 3.1 Pro software and goblet cells were analysed using Image J.

Benzoic acid and hippuric acid in the digesta were measured by LCMS/MS system and muramic acid in the digesta was measured by UPLC-MS system.

5. Statistical Analysis

Statistical analyses were performed using the StatGraphics Centurion XVI statistical software package (Manugistics, Rockville, MD). One-factorial ANOVA and Tukey multiple comparison test was used to assess differences among means in treatment groups. Variability in the data was expressed as the pooled standard error. In all instances, differences were reported as significant at $P < 0.05$.

6. Results and Discussion

6.1 Growth performance

Results of the growth performance and apparent fecal digestibility (AFD) of nitrogen are summarized in Table 4. During the starter period (day 0-14), the addition of the combined products led to an improvement of the daily weight gain (DWG) by 3 % and of the feed conversion ratio (FCR) by 2% compared to the negative control group (NC). Although no beneficial effect of addition of the products alone or in combination were record during the grower period (day 14-28), the PCR was improved by feeding the products alone and in combination over the whole period (day 0-28). In addition, AFD of nitrogen in the piglets receiving the combination provided additional improvement compared to other treatments.

Table 4: Growth performance of piglets in this study

Treatment		Day 0-14			Day 14-28			Day 0-28			Nitrogen
		DWG (g/s/d)	DFI (g/s/d)	FCR	DWG (g/s/d)	DFI (g/s/d)	FCR	DWG (g/s/d)	DFI (g/s/d)	FCR	AFD
NC	Mean	383	534	1.401	795	1176	1.550	584	914	1.585	80.3
BAL		376	535	1.419	766	1117	1.461	571	826	1.446	79.0
VV		353	518	1.483	739	1043	1.413	546	780	1.432	81.6
BAL+VV		395	540	1.378	730	1051	1.448	563	796	1.420	82.3

g/s/d=grams/swine/day

6.2 Health performance

Fecal consistency scores are presented in Figure 1. As shown, the average fecal score recorded with the addition of the combined products (VV + BAL) was significantly lower when compared to the control.

6.3 Immunity

The determination of GPx activity and Oxidative stress index (OSI) in plasma showed difference between treatments (see Table 5).

At d21 (prior to stress), the activity of Glutathione peroxidase (GPx) in plasma increased in pigs receiving BAL compared to control (17.6 vs. 15.0 U/g prot; P=0.05). When combining VV and BAL, the GPx activity increased just after stress compared to the day prior and the week after (17.2 vs. 16.6 vs. 17.0 U/g prot; P<0.05), and then returned to a similar level prior to stress, suggesting that these products can help the pigs in protecting tissues during times of stress.

Oxidative stress index (OSI) can be used to show the antioxidant potential in the tissue of the animals. The piglets receiving VV+BAL provided higher OSI on d21 (pre-stress), d22(post-stress) and d28 (7 days after stress) compared to the treatment group of NC, VV alone and BAL alone.

Table 5: GPx activities and oxidative stress index (OSI) in plasma

Day	Treatment	GPx(U/g Prot)	OSI
		Mean	Mean
Day 21 (pre-stress)	NC	15.0^{b}	0.256^{b}
	NC + BAL	17.6^{a}	0.243^{b}
	NC + VV	16.6^{ab}	0.284^{ab}
	NC + BAL + VV	16.68^{ab}	0.312^{a}
Day 22 (post-stress)	NC	16.4	0.276
	NC + BAL	16.6	0.247
	NC + VV	16.5	0.288
	NC + BAL + VV	17.2	0.291
Day 28 (7 days after stress)	NC	17.6	0.268
	NC + BAL	16.9	0.243
	NC + VV	17.8	0.269
	NC + BAL + VV	17.0	0.291

The analysis of antioxidant enzyme activities in the ileum and jejunum tissue are shown in Table 6 below. The determination of SOD activity in ileum tissue lysates decreased in the piglets receiving

BAL alone and VV alone but increased in the piglets receiving BLA + VV, compared to the Control. The CAT activity in jejunum tissue lysates also increased in piglets receiving BAL alone and VV alone, and further increased in the piglets receiving BAL+ AA, compared to the Control. Thess increasings indicate more antioxidant capacity piglets offered in their diet.

Table 6: Antiooxidant enzymes activity in the ileum and jejunum

Treatment	SOD (U/g prot) in ileum Mean	CAT (U/g prot) in jejunum Mean
NC	7376b	3677
BAL	7218b	3713
VV	7291b	3725
BAL+VV	8680a	3943

6.4 Gut health

Supplementation with both BAL+VV increased the villi length, crypt depth and epithelium thickness in jejunum when compared to the control group and the other two treatment groups (see Table 7).

Table 7: Histology analysis in jejunum

Treatment	Villi length (µm) Mean	Crypt depth (µm) Mean	epithelium thickness (µm) Mean
NC	351	147	30.1
BAL	383	162	34.3
VV	379	159	30.1
BAL+VV	391	169	36.5

Benzoic acid and hippuric acid were measured in the jejunal digesta collected from the pigs at the end of the study. The results are shown in Table 8. As indicated, benzoic acid concentrations were higher in the jejunum of piglets offered diets containing VV, and the combination of VV+BAL resulted in the highest concentration. Hippuric acid followed a similar pattern in the jejunum too.

Table 8: Concentrations of benzoic acid and hippuric acid in the jejunum

Treatment	Benzoic acid (ng/g digesta)	Hippuric acid (ng/g digesta)
NC	2000^{b}	689^{c}
BAL	2000^{b}	606^{c}
VV	12006^{ab}	7767^{b}
BAL+VV	21961^{a}	10039^{a}

Muramic acid was measured in the ileum digesta collected from the piglets at the end of the study. The results are shown in Figure 2. As indicated, there are improvement in peptidoglycan solubility with pigs which were offered BAL+VV.

7. Conclusion

The results of this study strongly suggest that supplementing diets with one or more muramidase(s) in combination with one or more organi acid(s) can improve performance including growth performance (DWG, daily FI and FCR, and apparent facel digestibility of nitrogen) and health

performance (reduced diarrhea), immunity (antioxidant enzyames activity and osicative stress index in plasma and/or in ileum and jejunum), and gut health (increased villi length, crypth depth and epithelium thickness of intestines, enhanced concentration of benzoic acid and hippuric acid in jejunum and concentration of soluble peptidoglycan in ileum) of animals.

CLAIMS

1. A method for improving performance, immunity and/or gut health of an animal comprising administering to the animal one or more microbial muramidase(s) and one or more organic acid(s).

2. A method for improving growth performance, improving nitrogen digestibility, reducing diarrhea, improving antioxidant enzyme activity, improving stress response, and/or improving gut health of an animal, comprising administering to the animal one or more microbial muramidase(s) and one or more organic acid(s).

3. The method of claim 1 or 2, wherein the microbial muramidase is obtained or obtainable from the phylum *Ascomycota*, or the subphylum *Pezizomycotina*, preferably *Acremonium alcalophilum* or *Trichophaea saccate.*

4. The method of any of claims 1 to 2, wherein the microbial muramidase comprises one or more domains selected from GH24 and GH25.

5. The method of any of claims 1 to 4, wherein the microbial muramidase is selected from the group consisting of amino acids 1 to 213 of SEQ ID NO: 1, amino acids 1 to 245 of SEQ ID NO: 2 and amino acids 1 to 208 of SEQ ID NO: 3.

6. The method of any of claims 1 to 5, wherein the orgaic acid(s) is selected from the group consisting of short chain fatty acids (e.g. formic acid, acetic acid, propionic acid, butyric acid), medium chain fatty acids (e.g. caproic acid, caprylic acid, capric acid, lauric acid), di/tri-carboxylic acids (e.g. fumaric acid and succinic acid), hydroxy acids (e.g. lactic acid), aromatic acids (e.g. benzoic acid), citric acid, sorbic acid, malic acid, and tartaric acid, or their salt (typically sodium or potassium or ammonium salt such as potassium diformate or sodium butyrate or ammonium formate).

7. The method of any of claims 1 to 6, wherein the microbial muramidase is adminstered at a level of 100 to 1000 mg enzyme protein per kg animal feed, such as 200 to 900 mg, 300 to 800 mg, 400 to 700 mg, 500 to 600 mg enzyme protein per kg animal feed, or any combination of these intervals.

8. The method of any of claims 1 to 7, wherein the organic acid is adminstered at a level of from 0.001% to 10%, preferably from 0.01% to 5%, more preferably from 0.1% to 1% by weight of animal feed.

9. Use of one or more microbial muramidase(s) and one or more organic acid(s) in the preparation of a feed composition, a feed additive or an animal feed for improving performance, immunity and/or gut health of an animal.

10. Use of one or more microbial muramidase(s) and one or more organic acid(s) in the preparation of a feed composition, a feed additive or an animal feed for improving growth performance, improving nitrogen digestibility, reducing diarrhea, improving antioxidant enzyme activity, improving stress response, and/or improving gut health of an animal.

11. The use of claim 9 or 10, wherein the microbial muramidase is obtained or obtainable from the phylum *Ascomycota*, or the subphylum *Pezizomycotina*, preferably *Acremonium alcalophilum* or *Trichophaea saccate.*

12. The use of any one of claims 9 to 11 wherein the microbial muramidase comprises one or more domains selected from the list consisting of GH24 and GH25.

13. The use of any of claims 9 to 12, wherein the microbial muramidase is selected from the group consisting of amino acids 1 to 213 of SEQ ID NO: 1, amino acids 1 to 245 of SEQ ID NO: 2 and amino acids 1 to 208 of SEQ ID NO: 3.

Figure 1

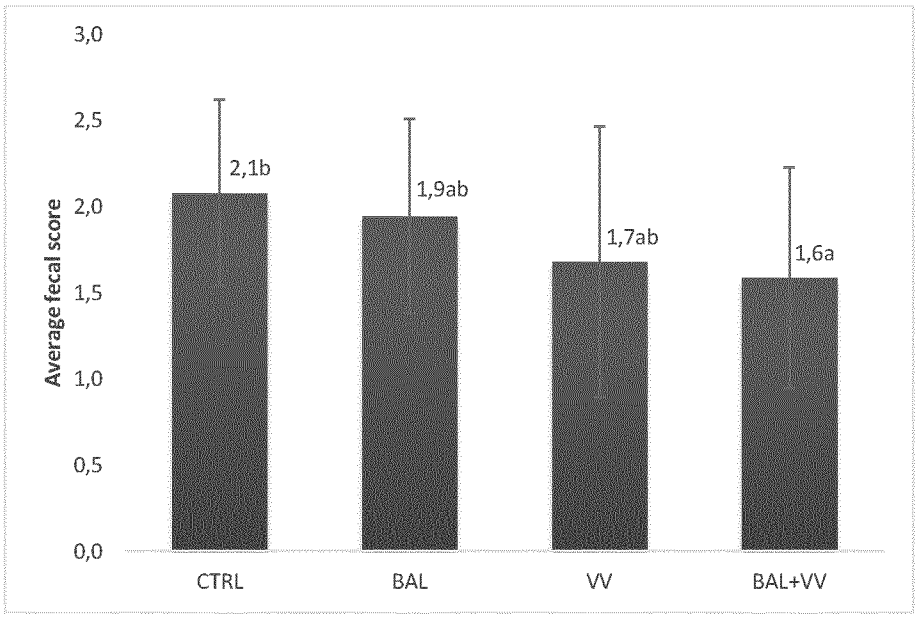
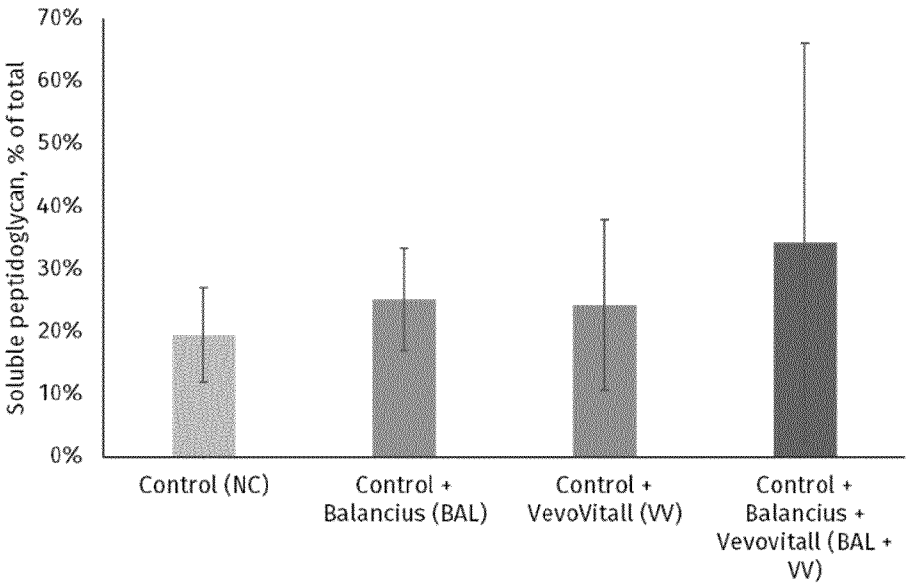


Figure 2



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/050137

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23K20/111 A23K20/158 A23K20/189 A23K50/30 A23K50/60
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)
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, FSTA, 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	US 2021/289818 A1 (CARDOSO BITTENCOURT LETICIA [CH] ET AL) 23 September 2021 (2021-09-23) claims 2-3,7-10,14-15,19 paragraphs [0198] - [0200], [0278], [0292] - [0293] -----	1-13
X	WO 2020/053274 A1 (DSM IP ASSETS BV [NL]; NOVOZYMES AS [DK]) 19 March 2020 (2020-03-19) cited in the application claims 1-16 page 2, lines 20-26 page 3, lines 2-17 page 13, lines 1-32 page 22, lines 1-3 ----- -/--	1-13

[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

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"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
6 April 2023	19/04/2023

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	Heirbaut, Marc

3

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	F Goodarzi Boroojeni ET AL: "Evaluation of a microbial muramidase supplementation on growth performance, apparent ileal digestibility, and intestinal histology of broiler chickens", Poultry science, 19 December 2018 (2018-12-19), pages 1-7, XP055558379, England DOI: 10.3382/ps/pey556 Retrieved from the Internet: URL:https://watermark.silverchair.com/pey556.pdf?token=AQECAHi208BE49Ooan9kkhW_Ercy7Dm3ZL_9Cf3qfKAc485ysgAAAk0wggJJBgkqhkiG9w0BBwagggI6MIICNgIBADCCAi8GCSqGSIb3DQEHATAeBglghkgBZQMEAS4wEQQMEHsMRvE7OHZkEZDfAgEQgIICAAd2bjtNfXdXNLmKEf9_wggWmnWUVIaTUxXYCZa0vJhrpIQiUEgyM18DQNM0J5kQ77TlfygsxcekVSPHUwHaqBUIwn0he the whole document	1-13

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/050137

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/050137

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 3853359 A1	28-07-2021
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