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(54) **Title**: A METHOD FOR IMPROVING THE NUTRITIONAL VALUE OF ANIMAL FEED

(57) **Abstract**: The invention relates to the use of at least one bacterial phytase in combination with one or more protease(s) in animal feed for improving weight gain and/or Feed Conversion Ratio (FCR), wherein the phytase is administered in one or more of the following amounts (dosage ranges): 1'000 FYT /kg feed, 2'000 FYT /kg feed 3'000 FYT /kg feed and wherein the proteaseis administered in one of the following amounts (dosage ranges): 10'000 units/kg feed, 11'000, 12'000, 13'000, 14'000, 15'000, 16'000, 17'000, 18'000, 19'000, 20'000 units/kg feed.

A METHOD FOR IMPROVING THE NUTRITIONAL VALUE OF ANIMAL FEED

Reference to a Sequence Listing

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

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method for improving the nutritional value of animal feed. More specifically, the invention relates to a method for improving feed conversion ratio (FCR) of animal feed, which method comprises treating the animal feed source with a high dose of at least one phytase in combination with a proteolytic enzyme.

The invention furthermore relates to feed additive compositions comprising at least one superdosed phytase together one or more proteolytic enzyme, i.e. protease.

SUMMARY OF THE INVENTION

The present invention relates to a method for increasing weight gain and/or improving Feed Conversion Ratio of farm animals, the method comprising the step of applying to the animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed and

b. the protease is administered in a dosage of between 10'000 units/kg feed and 30'000 units/kg feed.

The invention also relates to the use of one or more proteolytic enzymes in combination with at least one phytase in animal feed for increasing weight gain and/or improving Feed Conversion Ratio of farm animals, wherein:

a. the phytase is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed and

b. the protease is administered in a dosage of between 10'000 units/kg feed and 30'000 units/kg feed.

Overview of Sequence Listing

SEQ ID NO: 1 is the mature amino acid sequence of the AppA phytase from *E. Coli*.

SEQ ID NO: 2 is the mature amino acid sequence of the AppA2 phytase from *E. Coli*.

SEQ ID NO: 3 is the mature amino acid sequence of a phytase derived from *E. Coli*.

SEQ ID NO: 4 is the mature amino acid sequence of a phytase derived from *E. Coli*.

SEQ ID NO: 5 is the mature amino acid sequence of a phytase derived from *E. Coli*.

SEQ ID NO: 6 is the mature amino acid sequence of a phytase disclosed as SEQ ID NO: 1 of WO2008/017066.

SEQ ID NO: 7 is the mature amino acid sequence of a phytase disclosed as SEQ ID NO: 3 of WO2014/164442.

SEQ ID NO: 8 is the mature amino acid sequence of a phytase disclosed as SEQ ID NO: 6 of WO2014/164442.

SEQ ID NO: 9 is the mature amino acid sequence of a phytase disclosed as SEQ ID NO: 8 of WO2014/164442.

SEQ ID NO: 10 is the mature amino acid sequence of a phytase from *Citrobacter braakii* ATCC 51113.

SEQ ID NO: 11 is the mature amino acid sequence of a phytase from *Citrobacter gillenii*.

SEQ ID NO: 12 is the mature amino acid sequence of a phytase from *Citrobacter amalonaticus*.

SEQ ID NO: 13 is the mature amino acid sequence of a phytase from *Citrobacter braakii* YH-15.

SEQ ID NO: 14 is the mature amino acid sequence of a phytase from *Citrobacter freundii* P3-42.

SEQ ID NO: 15 is the mature amino acid sequence of a phytase from *Buttiauxella* sp P1-29.

SEQ ID NO: 16 is the mature amino acid sequence of a phytase from *Buttiauxella* sp P1-29.

SEQ ID NO: 17 is the mature amino acid sequence of a phytase disclosed as SEQ ID NO: 1 of WO2008/097619.

SEQ ID NO: 18 is the mature amino acid sequence of a phytase from *Buttiauxella gaviniae* DSM18930.

SEQ ID NO: 19 is the mature amino acid sequence of a phytase from *Buttiauxella agrestis* DSM18931.

SEQ ID NO: 20 is the mature amino acid sequence of a phytase from *Buttiauxella agrestis* DSM18932.

SEQ ID NO: 21 is the mature amino acid sequence of a phytase from *Peniophora lycii* CBS No. 686.96.

SEQ ID NO: 22 is the mature amino acid sequence of a phytase variant of *Peniophora lycii* CBS No. 686.96.

SEQ ID NO: 23 is the mature amino acid sequence of a phytase from *Hafnia alvei*.

SEQ ID NO: 24 is the mature amino acid sequence of a phytase from *Hafnia* sp. LU11047.

SEQ ID NO: 25 is the mature amino acid sequence of a fusion phytase disclosed as SEQ ID NO: 18 of WO2011/048046.

SEQ ID NO: 26 is the mature amino acid sequence of a fusion phytase variant disclosed as SEQ ID NO: 24 of WO2012/143862.

SEQ ID NO: 27 is the amino acid sequence of a protease from *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43235.

SEQ ID NO: 28 is the mature amino acid sequence of a protease from *Bacillus clausii*.

SEQ ID NO: 29 is the amino acid sequence of a protease from *Nocardiopsis* sp. DSM 16424.

SEQ ID NO: 30 is the amino acid sequence of a protease from *Nocardiopsis alba* DSM 15647.

SEQ ID NO: 31 is the amino acid sequence of a protease from *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43235.

SEQ ID NO: 32 is the mature amino acid sequence of a protease from *Nocardiopsis* sp. NRRL 18262.

SEQ ID NO: 33 is the amino acid sequence of a protease from *Nocardiopsis prasina* DSM 15648.

SEQ ID NO: 34 is the amino acid sequence of a protease from *Nocardiopsis prasina* DSM 15649.

SEQ ID NO: 35 is the amino acid sequence of a protease from *Nocardiopsis prasina* DSM 15649.

SEQ ID NO: 36 is the amino acid sequence of a protease from *Nocardiopsis prasina* DSM 14010.

SEQ ID NO: 37 is the amino acid sequence of a protease from *Nocardiopsis alkaliphila* DSM 44657.

SEQ ID NO: 38 is the amino acid sequence of a protease from *Nocardiopsis lucentensis* DSM 44048.

SEQ ID NO: 39 is the mature amino acid sequence of a protease from *Kribella solani*.

SEQ ID NO: 40 is the mature amino acid sequence of a protease from *Kribella aluminosa*.

SEQ ID NO: 41 is the mature amino acid sequence of a protease from *Saccharomonospora viridis*.

SEQ ID NO: 42 is the mature amino acid sequence of a protease from *Saccharothrix australiensis*.

SEQ ID NO: 43 is the mature amino acid sequence of a protease from *Saccharopolyspora erythraea*.

SEQ ID NO: 44 is the mature amino acid sequence of a protease from *Bacillus* sp NN019138.

SEQ ID NO: 45 is the mature amino acid sequence of a protease from *Saccharopolyspora erythraea*.

SEQ ID NO: 46 is the mature amino acid sequence of a protease from *Meripilus giganteus*.

SEQ ID NO: 47 is the mature amino acid sequence of a protease from *Dactylosporangium variesporum*.

SEQ ID NO: 48 is the mature amino acid sequence of a protease variant from *Bacillus amyloliquefaciens*.

DETAILED DESCRIPTION OF THE INVENTION

It has been found surprisingly that the addition of at least one phytase as defined hereineafter to animal feed, results in a significant improvement of weight gain and/or FCR if the phytase is supplemented in high dosage and combined with a proteolytic enzyme.

Thus in one aspect, the invention relates to a method for increasing weight gain and/or improving Feed Conversion Ratio of farm animals, the method comprising the step of applying to the animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed and

b. the protease is administered in a dosage of between 10’000 units/kg feed and 30’000 units/kg feed.

The Feed Conversion Ratio (FCR) is indicative of how effectively a feed is utilized. The lower the FCR, the better the feed is utilized. The FCR may be determined on the basis of an animal trial comprising a first treatment in which the phytase and protease for use according to the invention are added to the animal feed in a desired concentration (e.g., 6 or 30 mg enzyme protein per kg feed), and a second treatment (control) with no addition of the enzymes to the animal feed. In particular embodiments, the FCR is improved (i.e., reduced) as compared to the control by at least 1.0%, preferably at least 1.5%, 1.6%, 1.7%, 1.8%, 1.9%, 2.0%, 2.1%, 2.2%, 2.3%, 2.4%, or at least 2.5%. In further particular embodiments, the FCR is improved (i.e. reduced) as compared to the control by at least 2.6%, 2.7%, 2.8%, 2.9%, or at least 3.0%. In still further particular embodiments, the FCR is improved (i.e., reduced) as compared to the control by at least 3.1%, 3.2%, 3.3%, 3.4%, 3.5%, 3.6%, 3.7%,3.8%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, 6.5%, 7.0% or at least 8.0%. In other particular embodiments, the FCR is improved (i.e. reduced) as compared to the control by between 1.0% and 15.0%, preferably between 1.5% and 12.0%, 2.0% and 11.0%, 2.5% and 11.0%, 3.0% and 10.5%, 4.0% and 10.5% or between 5.0% and 10.0%.

In another embodiment, the FCR is improved (i.e. reduced) as compared to using the phytase at the same dose alone by at least 1.0%, preferably at least 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5%, 5.0%, 5.5%, 6.0%, 6.5%, 7.0% or at least 8.0%. In other particular embodiments, the FCR is improved (i.e. reduced) as compared to using the phytase at the same dose alone by between 1.0% and 15.0%, preferably between 1.5% and 12.0%, 2.0% and 11.0%, 2.25% and 11.0%, 2.5% and 10.5%, 2.75% and 10.5% or between 3.0% and 10.0%.

An improved weight gain means an improved daily, weekly, bi-weekly, or monthly weight gain (in g or kg per the relevant time period), relative to a control without added phytase and protease.

Phytases

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8) are enzymes that hydrolyze phytate (*myo*-inositol hexakisphosphate) to myo-inositol and inorganic phosphate and are known to be valuable feed additives.

A variety of Phytases differing in pH optima, substrate specificity, and specificity of hydrolysis have been identified in plants and fungi. Acid Phytases from wheat bran and Aspergilli have been extensively studied and the stereo specificity of hydrolysis has been well established. Based on the specificity of initial hydrolysis, two classes of acid Phytases are recognized by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB, 1975), the 6- Phytase, found for example in plants, and the

3-Phytase, found in fungi. The 6-Phytase hydrolyses the phosphate ester at the L-6 (or D-4) position of phytic acid, and the 3-Phytase hydrolyses the phosphate ester at the D-3 position.

The ENZYME site at the internet (http://www.expasy.ch/enzyme/) is a repository of information relative to the nomenclature of enzymes. It is primarily based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUB-MB) and it describes each type of characterized enzyme for which an EC (Enzyme Commission) number has been provided (Bairoch A. The ENZYME database, 2000, Nucleic Acids Res 28:304-305). See also the handbook Enzyme Nomenclature from NC-IUBMB, 1992).

According to the ENZYME site, two different types of phytases are known: A so-called 3-phytase (myo-inositol hexaphosphate 3-phosphohydrolase, EC 3.1.3.8) and a so-called 6-phytase (myo-inositol hexaphosphate 6-phosphohydrolase, EC 3.1.3.26). For the purposes of the present invention, both types are included in the definition of phytase.

Examples of ascomycete phytases are those derived from a strain of Aspergillus, for example Aspergillus awamori PHYA (SWISSPROT P34753, Gene 133:55-62 (1993)), Aspergillus niger (ficuum) PHYA (SWISSPROT P34752, EP 420358, Gene 127:87-94 (1993)), Aspergillus awamori PHYB (SWISSPROT P34755, Gene 133:55-62 (1993)), Aspergillus niger PHYB (SWISSPROT P34754, Biochem. Biophys. Res. Commun. 195:53-57(1993)); or a strain of Emericella, for example Emericella nidulans PHYB (SWISSPROT O00093, Biochim. Biophys. Acta 1353:217-223 (1997)); or a strain of Thermomyces (Humicola), for example the Thermomyces lanuginosus phytase described in WO 97/35017. Other examples of ascomycete phytases are disclosed in EP 684313 (for example derived from strains of Aspergillus fumigatus, Aspergillus terreus, and Myceliophthora thermophila); JP 11000164 (a phytase derived from a strain of Penicillium.); US 6139902 (a phytase derived from a strain of Aspergillus), and WO 98/13480 (Monascus anka phytase).

Examples of basidiomycete phytases are the phytases derived from Paxillus involutus, Trametes pubescens, Agrocybe pediades and Peniophora lycii (see WO 98/28409).

In the present context, a preferred Phytase according to the invention is classified as belonging to the EC 3.1.3.26 group. The EC numbers refer to Enzyme Nomenclature 1992 from NC-IUBMB, Academic Press, San Diego, California, including supplements 1-5 published in Eur. J. Biochem. 1994, 223, 1-5; Eur. J. Biochem. 1995, 232, 1-6; Eur. J. Biochem. 1996, 237, 1-5; Eur. J. Biochem. 1997, 250, 1-6; and Eur. J. Biochem. 1999, 264, 610-650; respectively. The nomenclature is regularly supplemented and updated; see e.g. the World Wide Web at http://www.chem.qmw.ac.uk/iubmb/enzyme/index.html.

Examples of Phytases for use according to the present inventions are:

Phytases derived from strains of E coli, from strains of Buttiauxella, Ascomycete Phytases as disclosed in EP 684313 (for example derived from strains of Aspergillus fumigatus, Aspergillus terreus, and Myceliophthora thermophila); JP 11000164 (a Phytase derived from a strain of Penicillium.); US 6139902 (a Phytase derived from a strain of Aspergillus), WO 98/13480 (Monascus anka Phytase), WO 2008/116878 and WO 2010/034835 (Hafnia phytase).

A preferred phytase for use according to the invention is derived from the family *Enterobacteriaceae*, and more preferably is a species of *Escherichia*, *Citrobacter, Buttiauxella* or *Hafnia*.

Preferred examples of Escherichia species are Escherichia coli such as those disclosed in WO 2000/71728, WO 2001/90333, WO 2002/095003, WO 2002/095003, WO 2006/028684, WO 2006/028684, WO 1999/08539 and WO 2003/037102 or variants thereof, such as SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8 and SEQ ID NO: 9. Preferred examples of Citrobacter are Citrobacter amalonaticus, C. farmer, C. freundii, C. gillenii, C. intermedius, C. koseri and C. rodentium, such as those disclosed in WO 2004/085638, WO 2006/037327, WO 2006/037328, WO 2006/038062, WO 2006/038128 and WO 2007/112739 or variants thereof, such as SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14. Preferred examples of Hafnia are Hafnia alvei and H. paralvei such as those disclosed in WO 2008/116878, WO 2010/034835, WO 2011/048046, WO 2012/143861 and WO 2012/143862 or variants thereof, such as SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26. Preferred examples of Buttiauxella are Buttiauxella agrestis, B. brennerae, B. ferragutiae, B. gaviniae, B. izardii, B. noackiae and B. warmboldiae such as those disclosed in WO WO 2006/043178, 2008/092901, WO 2008/097619, WO 2008/097620, WO 2009/129489 or variants thereof, such as SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19 and SEQ ID NO: 20.

In another preferred embodiment, the phytase for use according to the invention is derived from the family Peniophoraceae and more preferably is a species of Peniophora, such as those disclosed in WO 1998/028408, WO 1998/028409 and WO 2003/066847 or variants thereof, such as SEQ ID NO: 21 and SEQ ID NO: 22.

Examples of Peniophora species are: Peniophora aurantiaca, P. cinerea, P. decorticans, P. duplex, P. ericsonii, P. incamate, P. lycii, P. meridionalis, P. nuda, P. piceae, P. pini, P. pithya, P. polygonia, P. proxima, P. pseudo-pini, P. rufa, P. versicolor, and species simply classified as Peniophora sp. A preferred species is Peniophora lycii. A preferred strain is Peniophora lycii CBS 686.96.

In a preferred embodiment, the amino acid sequence of the phytase has at least 70% sequence identity to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 or SEQ ID NO: 26. In a more preferred embodiment, the amino acid sequence of the phytase has at least 80% sequence identity to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 or SEQ ID NO: 26. In an even more preferred embodiment, the amino acid sequence of the phytase has at least 90% sequence identity to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 or SEQ ID NO: 26. In an even more preferred embodiment, the amino acid sequence of the phytase has at least 95%, such as at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 or SEQ ID NO: 26.

For purposes of the present invention, preferred phytases are the phytases contained in the following commercial products: Ronozyme®HiPhos, Ronozyme®NP and Ronozyme® P (DSM Nutritional Products AG), NatuphosTM (BASF), Finase® and Quantum® Blue (AB Enzymes), OptiPhos® (Huvepharma) Phyzyme® XP (Verenium/DuPont) and Axtra® PHY (DuPont).

For the purpose of the present invention, phytase activity is determined by the libertation of inorganic phosphate from Na-phytate solution, wherein one phytase activity unit is the amount of enzyme which liberates 1 µmol inorganic phosphate per min from a 0.0051 M Na-phytate solution in 0.25 M Na-acetate, pH 5.5 and at 37 ˚C (Engelen, A. J., *et al.*, 1994, "Simple and rapid determination of phytase activity", *J. AOAC Int.* 77:760-764). Examples of activity unit names are: FYT, FTU and U. Phytase activity may be determined using the assay

as described in Example 1 ("Determination of phytase activity"). In one aspect, the polypeptides of the present invention 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 phytase activity of SEQ ID NO: 10.

Specific activity is measured on highly purified samples (an SDS poly acryl amide gel should show the presence of only one component). The enzyme protein concentration may be determined by amino acid analysis, and the phytase activity in the units of FYT. Specific activity is a characteristic of the specific phytase variant in question, and it is calculated as the phytase activity measured in FYT units per mg phytase enzyme protein.

For determining mg Phytase protein per kg feed or feed additive, the enzyme is purified from the feed composition or the feed additive, and the specific activity of the purified enzyme is determined using a relevant assay. The Phytase activity of the feed composition or the feed additive is also determined using the same assay, and on the basis of these two determinations, the dosage in mg Phytase protein per kg feed is calculated.

According to the invention, the phytase should of course be applied in an effective amount, i.e. in an amount adequate for improving nutritional value of feed if it is used in combination with a proteolytic enzyme [obtaining the desired effect, e.g. improving FCR]. It is at present contemplated that the phytase is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed. In particular embodiments, the specific activity is at least 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900 or at least 3000 FYT/kg feed. In another particular embodiment, the specific activity is between 1200 and 3900 FYT/kg feed, preferably between 1400 and 3800 FYT/kg feed, between 1600 and 3700 FYT/kg feed, between 1800 and 3600 FYT/kg feed, between 1900 and 3500 FYT/kg feed, between 2000 and 3500 FYT/kg feed, between 2200 and 3500 FYT/kg feed, between 2400 and 3500 FYT/kg feed, between 2500 and 3500 FYT/kg feed, between 2600 and 3400 FYT/kg feed, between 2700 and 3300 FYT/kg feed and between 2800 and 3200 FYT/kg feed.

In a preferred embodiment, the phytase is applied at between 2 and 4 times standard commercial dose for that phytase.

Proteases

Proteolytic enzymes or proteases, or peptidases, catabolize peptide bonds in proteins breaking them down into fragments of amino acid chains, or peptides.

Proteases are classified on the basis of their catalytic mechanism into the following groups: serine proteases (S), cysteine proteases (C), aspartic proteases (A), metalloproteases (M), and unknown, or as yet unclassified, proteases (U), see Handbook of Proteolytic

Enzymes, A. J. Barrett, N. D. Rawlings, J. F. Woessner (eds), Academic Press (1998), in particular the general introduction part.

Proteases for use according to the invention are acid stable proteases, preferably acid stable serine proteases. In a further preferred embodiment, the acid stable serine proteases are S1 serine proteases. Acid stability may be determined using the kinetic Suc-AAPF-pNA assay as described in example 2 of WO 01/58276 and a protease is considered to be acid stable if there is >50% residual activity at pH 3 compared to the activity to samples which were kept at stable conditions (5°C and the optimal pH for that protease).

In a particular embodiment, the protease for use according to the invention is a microbial protease, the term microbial indicating that the protease is derived from, or originates from a microorganism, or is an analogue, a fragment, a variant, a mutant, or a synthetic protease derived from a microorganism. It may be produced or expressed in the original wild-type microbial strain, in another microbial strain, or in a plant; i. e. the term covers the expression of wild-type, naturally occurring proteases, as well as expression in any host of recombinant, genetically engineered or synthetic proteases.

Examples of microorganisms are bacteria, e. g. bacteria of the phylum *Actinobacteria*, e. g. of the class *Actinobacteria*, e.g. of the order *Streptosporangiales*, e.g. of the family *Nocardiopsaceae*, e.g. of the genus *Nocardiopsis*, e. g. *Nocardiopsis* sp. NRRL 18262, and *Nocardiopsis alba*; e.g. of the species *Bacillus* or mutants or variants thereof exhibiting protease activity.

Preferred proteases according to the invention are acid stable serine proteases obtained or obtainable from the class *Actinobacteria*, such as those derived from *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43235 (A1918L1), *Nocardiopsis prasina* DSM 15649 (NN018335L1), *Nocardiopsis prasina* (previously *alba*) DSM 14010 (NN18140L1), *Nocardiopsis* sp. DSM 16424 (NN018704L2), *Nocardiopsis* alkaliphila DSM 44657 (NN019340L2) and *Nocardiopsis lucentensis* DSM 44048 (NN019002L2), as well as homologous proteases. Other preferred proteases are those described in WO 2001/058276, WO 2004/111220, WO 2004/111221, WO 2004/072221, WO 2005/123911, WO 2013/026796, WO 2013/098185, WO 2013/110766, WO 2013/189972, WO 2014/096259, WO 2014/122161 and WO 2014/037438.

The term serine protease refers to serine peptidases and their clans as defined in the above Handbook. In the 1998 version of this handbook, serine peptidases and their clans are dealt with in chapters 1-175. Serine proteases may be defined as peptidases in which the catalytic mechanism depends upon the hydroxyl group of a serine residue acting as the nucleophile that attacks the peptide bond. Examples of serine proteases for use according to

the invention are proteases of Clan SA, e. g. Family S2 (Streptogrisin), e. g. Sub-family S2A (alpha-lytic protease), as defined in the above Handbook.

Protease activity can be measured using any assay, in which a substrate is employed, that includes peptide bonds relevant for the specificity of the protease in question. Examples of protease substrates are casein, and pNA-substrates, such as Suc-AAPF-pNA (available e.g. from Sigma S-7388). Another example is Protazyme AK (azurine dyed crosslinked casein prepared as tablets by Megazyme T-PRAK). Example 2 of WO 01/58276 describes suitable protease assays. A preferred assay is the Protazyme assay of Example 2D (the pH and temperature should be adjusted to the protease in question as generally described previously).

There are no limitations on the origin of the acid stable serine protease for use according to the invention. Thus, the term protease includes not only natural or wild-type proteases, but also any mutants, variants, fragments etc. thereof exhibiting protease activity, as well as synthetic proteases, such as shuffled proteases, and consensus proteases. Such genetically engineered proteases can be prepared as is generally known in the art, e. g. by Site-directed Mutagenesis, by PCR (using a PCR fragment containing the desired mutation as one of the primers in the PCR reactions), or by Random Mutagenesis. The preparation of consensus proteins is described in e. g. EP 0 897 985.

In a preferred embodiment, the amino acid sequence of the phytase has at least 70% sequence identity to the polypeptide of SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47 or SEQ ID NO: 48. In a more preferred embodiment, the amino acid sequence of the phytase has at least 80% sequence identity to the polypeptide of SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47 or SEQ ID NO: 48. In an even more preferred embodiment, the amino acid sequence of the phytase has at least 90% sequence identity to the polypeptide of SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47 or SEQ ID NO: 48. In an even more preferred embodiment, the amino acid sequence of the phytase has at least 95%, such as at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the polypeptide of SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ

ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47 or SEQ ID NO: 48.

For calculating percentage identity, any computer program known in the art can be used. Examples of such computer programs are the Clustal V algorithm (Higgins, D. G., and Sharp, P. M. (1989), Gene (Amsterdam), 73, 237-244 ; and the GAP program provided in the GCG version 8 program package (Program Manual for the Wisconsin Package, Version 8, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S. B. and Wunsch, C. D., (1970), Journal of Molecular Biology, 48, 443-453.

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 x 100)/(Length of Alignment – Total Number of Gaps in Alignment).

In another particular embodiment, the protease for use according to the invention, besides being acid-stable, is also thermostable.

The term thermostable means for proteases one or more of the following: That the temperature optimum is at least 50 °C, 52 °C, 54 °C, 56 °C, 58 °C, 60 °C, 62 °C, 64 °C, 66 °C, °68 C, or at least °70 C.

A commercially available serine proteases derived from *Nocardiopsis* is Ronozyme®ProAct® (DSM Nutritional Products AG).

In the use according to the invention it is at present contemplated that the protease is administered in a dosage of between 5’000 units/kg feed and 30’000 units/kg feed, preferably between 7’000 units/kg feed and 28’000 units/kg feed, between 8’000 units/kg feed and 26’000 units/kg feed, between 9’000 units/kg feed and 24’000 units/kg feed, between 10’000 units/kg feed and 22’000 units/kg feed, between 11’000 units/kg feed and 20’000 units/kg feed, between 12’000 units/kg feed and 18’000 units/kg feed, or between 13’000 units/kg feed and 17’000 units/kg feed, or for example in one of the following amounts (dosage ranges): 5’000 units/kg feed, 7’000, 8’000, 9’000, 10’000, 11’000, 12’000, 13’000, 14’000, 15’000, 16’000, 17’000, 18’000, 19’000, 20’000,22’000, 24’000, 26’000, 28’000, 30’000 units/kg feed.

One protease unit (PROT) is the amount of enzyme that releases 1 µmol of p-nitroaniline from 1 mM substrate (Suc-Ala-Ala-Pro-Phe-pnA) per minute at pH 9.0 and 37°C.

Phytase and Protease Combinations

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed and

b. the protease is administered in a dosage of between 10'000 units/kg feed and 30'000 units/kg feed.

In an embodiment, the monogastric animal is selected from the group consisting of pigs, swine (including, but not limited to, piglets, growing pigs, and sows); poultry, turkeys, ducks, quail, guinea fowl, geese, pigeons (including squabs) and chicken (broilers, chicks, layers). In a preferred embodiment the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

In a preferred embodiment, the phytase is applied at between 2 and 4 times standard commercial dose for that phytase.

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is derived from the family *Enterobacteriaceae* and is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed and

b. the protease is an acid stable serine protease derived from the class *Actinobacteria* and is administered in a dosage of between 10'000 units/kg feed and 30'000 units/kg feed.

In a preferred embodiment, the phytase is applied at between 2 and 4 times standard commercial dose for that phytase.

In an embodiment, the monogastric animal is selected from the group consisting of pigs, swine (including, but not limited to, piglets, growing pigs, and sows); poultry, turkeys, ducks, quail, guinea fowl, geese, pigeons (including squabs) and chicken (broilers, chicks,

layers). In a preferred embodiment the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

In an embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is derived from the family *Nocardiopsaceae*, preferably the genus *Nocardiopsis.* In an embodiment, the phytase is derived from the genus *Escherichia* and the acid stable serine protease is derived from the genus *Nocardiopsis.* In an embodiment, the phytase is derived from the genus *Citrobacter* and the acid stable serine protease is derived from the genus *Nocardiopsis.* In an embodiment, the phytase is derived from the genus *Buttiauxella* and the acid stable serine protease is derived from the genus *Nocardiopsis.* In an embodiment, the phytase is derived from the genus *Hafnia* and the acid stable serine protease is derived from the genus *Nocardiopsis.*

In another embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is derived from the family *Pseudonocardiaceae.* In another embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is derived from the family *Micromonosporaceae.* In another embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is derived from the family *Nocardioidaceae.* In another embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is derived from the family *Bacillaceae*, preferably the genus *Bacillus.*

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is derived from the family *Peniophoraceae* and is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed and

b. the protease is an acid stable serine protease derived from the class *Actinobacteria* and is administered in a dosage of between 10'000 units/kg feed and 30'000 units/kg feed.

In a preferred embodiment, the phytase is applied at between 2 and 4 times standard commercial dose for that phytase.

In an embodiment, the monogastric animal is selected from the group consisting of pigs, swine (including, but not limited to, piglets, growing pigs, and sows); poultry, turkeys,

ducks, quail, guinea fowl, geese, pigeons (including squabs) and chicken (broilers, chicks, layers). In a preferred embodiment the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

In an embodiment, the phytase is derived from the genus *Peniophora* and the acid stable serine protease is derived from the family *Nocardiopsaceae*, preferably the genus *Nocardiopsis.* In another embodiment, the phytase is derived from the family *Peniophoraceae* and the acid stable serine protease is derived from the family *Peniophoraceae.* In another embodiment, the phytase is derived from the family *Peniophoraceae* and the acid stable serine protease is derived from the family *Micromonosporaceae.* In another embodiment, the phytase is derived from the family *Peniophoraceae* and the acid stable serine protease is derived from the family *Nocardioidaceae.* In another embodiment, the phytase is derived from the family *Peniophoraceae* and the acid stable serine protease is derived from the family *Bacillaceae*, preferably the genus *Bacillus.*

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is derived from the family *Enterobacteriaceae* and is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed;

b. the protease is an acid stable serine protease derived from the class *Actinobacteria* and is administered in a dosage of between 10'000 units/kg feed and 20'000 units/kg feed; and

c. the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

In a preferred embodiment, the phytase is applied at between 2 and 4 times standard commercial dose for that phytase.

In an embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is an S1 protease derived from the family *Nocardiopsaceae*, preferably the genus *Nocardiopsis.* In an embodiment, the phytase is derived from the genus *Escherichia* and the acid stable serine protease is an S1 protease derived from the genus *Nocardiopsis.* In an embodiment, the phytase is derived from the genus *Citrobacter* and the acid stable serine protease is an S1 protease derived from the genus *Nocardiopsis.* In an

embodiment, the phytase is derived from the genus *Buttiauxella* and the acid stable serine protease is an S1 protease derived from the genus *Nocardiopsis.* In an embodiment, the phytase is derived from the genus *Hafnia* and the acid stable serine protease is an S1 protease derived from the genus *Nocardiopsis.*

In another embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is an S8 protease derived from the family *Pseudonocardiaceae.* In another embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is an S8 protease derived from the family *Micromonosporaceae.* In another embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is an S8 protease derived from the family *Nocardioidaceae.* In another embodiment, the phytase is derived from the family *Enterobacteriaceae* and the acid stable serine protease is derived from the family *Bacillaceae*, preferably the genus *Bacillus.*

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is derived from the family *Peniophoraceae* and is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed;

b. the protease is an acid stable serine protease derived from the class *Actinobacteria* and is administered in a dosage of between 10'000 units/kg feed and 20'000 units/kg feed; and

c. the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

In a preferred embodiment, the phytase is applied at between 2 and 4 times standard commercial dose for that phytase.

In an embodiment, the phytase is derived from the genus *Peniophora* and the acid stable serine protease is an S1 protease derived from the family *Nocardiopsaceae*, preferably the genus *Nocardiopsis.* In another embodiment, the phytase is derived from the family *Peniophoraceae* and the acid stable serine protease is an S1 protease derived from the family *Peniophoraceae.* In another embodiment, the phytase is derived from the family *Peniophoraceae* and the acid stable serine protease is an S1 protease derived from the family

Micromonosporaceae. In another embodiment, the phytase is derived from the family *Peniophoraceae* and the acid stable serine protease is an S1 protease derived from the family *Nocardioidaceae.* In another embodiment, the phytase is derived from the family *Peniophoraceae* and the acid stable serine protease is an S8 protease derived from the family *Bacillaceae*, preferably the genus *Bacillus.*

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is derived from the genus *Citrobacter* and is administered in such amounts that the specific activity in the final feed is between 1500 FYT/kg feed and 3500 FYT/kg feed;

b. the protease is an acid stable S1 serine protease derived from the genus *Nocardiopsis* and is administered in a dosage of between 10'000 units/kg feed and 20'000 units/kg feed; and

c. the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is derived from the genus *Buttiauxella* and is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 2000 FYT/kg feed;

b. the protease is an acid stable S1 serine protease derived from the genus *Nocardiopsis* and is administered in a dosage of between 10'000 units/kg feed and 20'000 units/kg feed; and

c. the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is derived from the genus *Buttiauxella* and is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 2000 FYT/kg feed;

b. the protease is an acid stable S8 serine protease derived from the genus *Bacillus* and is administered in a dosage of between 10'000 units/kg feed and 20'000 units/kg feed; and

c. the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is derived from the genus *Escherichia* and is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 2000 FYT/kg feed;

b. the protease is an acid stable S8 serine protease derived from the genus *Bacillus* and is administered in a dosage of between 10'000 units/kg feed and 20'000 units/kg feed; and

c. the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

In a particular embodiment, the invention relates to a method for improving the Feed Conversion Ratio of monogastric animals, the method comprising the step of applying to the monogastric animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:

a. the phytase is derived from the genus *Escherichia* and is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 2000 FYT/kg feed;

b. the protease is an acid stable S8 serine protease derived from the genus *Bacillus* and is administered in a dosage of between 10'000 units/kg feed and 20'000 units/kg feed; and

c. the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

Animal Feed

In a particular embodiment, the phytase and the protease, in the form in which they are added to the feed, or when being included in a feed additive, are well-defined. Well-defined means, that the enzyme preparation is at least 50% pure on a protein-basis. In other particular embodiments the enzyme preparation is at least 60, 70, 80, 85, 88, 90, 92, 94, or at least 95% pure. Purity may be determined by any method known in the art, e.g. by SDS-PAGE, or by Size-exclusion chromatography (see Example 12 of WO 01/58275).

A well-defined enzyme preparation is advantageous. For instance, it is much easier to dose correctly to the feed an enzyme that is essentially free from interfering or contaminating other enzymes. The term dose correctly refers in particular to the objective of obtaining consistent and constant results, and the capability of optimising dosage based upon the desired effect.

For the present purposes, the term animal includes all animals, including human beings. In a particular embodiment, the phytase variants and compositions of the invention can be used as a feed additive for non-human animals. Examples of animals are non-ruminants, and ruminants, such as cows, sheep and horses. In a particular embodiment, the animal is a non-ruminant animal. Non-ruminant animals include mono-gastric animals, e.g. 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)); 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). In an embodiment, the monogastric animal is selected from the group consisting of pigs, swine (including, but not limited to, piglets, growing pigs, and sows); poultry, turkeys, ducks, quail, guinea fowl, geese, pigeons (including squabs) and chicken (broilers, chicks, layers). In a preferred embodiment the monogastric animal is selected from the group consisting of chicken, broilers, chicks and layers.

The term feed or feed composition means any compound, preparation, mixture, or composition suitable for, or intended for intake by an animal. The feed can be fed to the animal before, after, or simultaneously with the diet. The latter is preferred.

The composition of the invention, when intended for addition to animal feed, may be designated an animal feed additive. Such additive always comprises the enzymes in question, preferably in the form of stabilized liquid or dry compositions. The additive may comprise other components or ingredients of animal feed. The so-called pre-mixes for animal feed are particular examples of such animal feed additives. Pre-mixes may contain the enzyme(s) in question, and in addition at least one vitamin and/or at least one mineral.

In a preferred example, the phytase and the protease, which are added to the feed via a feed additive composition, are dosed such that the final feed has the following dosages:

Phytase: at least 2000 FYT /kg feed and Protease: 15'000 units/kg feed, or

Phytase: 3000 FYT /kg feed and Protease: 15'000 units/kg feed.

In another preferred example, the phytase and the protease, which are added to the feed via a feed additive composition, are dosed such that the final feed has the following dosages:

Phytase: 1700 to 2300 FYT /kg feed and Protease: 12'000 to 18'000 units/kg feed, or

Phytase: 2700 to 3300 FYT /kg feed and Protease: 12'000 to 18'000 units/kg feed.

Accordingly, in a particular embodiment, in addition to the component polypeptides, the composition of the invention may comprise or contain at least one fat-soluble vitamin, and/or at least one water-soluble vitamin, and/or at least one trace mineral. Also at least one macro mineral may be included.

Examples of fat-soluble vitamins are vitamin A, D3, E, and vitamin K, e.g. vitamin K3.

Examples of water-soluble vitamins are vitamin B12, biotin and choline, vitamin B1, vitamin B2, vitamin B6, niacin, folic acid and panthothenate, e.g. Ca-D-panthothenate.

Examples of trace minerals are manganese, zinc, iron, copper, iodine, selenium, and cobalt.

Examples of macro minerals are calcium, phosphorus and sodium.

Further, optional, feed-additive ingredients are colouring agents, aroma compounds, stabilizers, additional enzymes, and antimicrobial peptides.

Additional enzyme components of the composition of the invention include at least one polypeptide having xylanase activity; and/or at least one polypeptide having endoglucanase activity; and/or at least one polypeptide having endo-1,3(4)-beta-glucanase activity.

Xylanase activity can be measured using any assay, in which a substrate is employed, that includes 1,4-beta-D-xylosidic endo-linkages in xylans. Different types of substrates are available for the determination of xylanase activity e.g. Xylazyme cross-linked arabinoxylan tablets (from MegaZyme), or insoluble powder dispersions and solutions of azo-dyed arabinoxylan.

Endoglucanase activity can be determined using any endoglucanase assay known in the art. For example, various cellulose- or beta-glucan-containing substrates can be applied. An endoglucanase assay may use AZCL-Barley beta-Glucan, or preferably (1) AZCL-HE-Cellulose, or (2) Azo-CM-cellulose as a substrate. In both cases, the degradation of the substrate is followed spectrophotometrically at OD595 (see the Megazyme method for AZCL-polysaccharides for the assay of endo-hydrolases at http://www.megazyme.com/booklets/AZCLPOL.pdf .

Endo-1,3(4)-beta-glucanase activity can be determined using any endo-1,3(4)-beta-glucanase assay known in the art. A preferred substrate for endo-1,3(4)-beta-glucanase activity measurements is a cross-linked azo-coloured beta-glucan Barley substrate, wherein the measurements are based on spectrophotometric determination principles.

For assaying xylanase, endoglucanase, beta-1,3(4)-glucanase and protease activity the assay-pH and the assay-temperature are to be adapted to the enzyme in question (preferably a pH close to the optimum pH, and a temperature close to the optimum temperature). A preferred assay pH is in the range of 2-10, preferably 3-9, more preferably pH 3 or 4 or 5 or 6 or 7 or 8, for example pH 3 or pH 7. A preferred assay temperature is in the range of 20-80°C, preferably 30-80°C, more preferably 40-75°C, even more preferably 40-60°C, preferably 40 or 45 or 50°C. The enzyme activity is defined by reference to appropriate blinds, e.g. a buffer blind.

Examples of antimicrobial peptides (AMP's) are CAP18, Leucocin A, Tritrpticin, Protegrin-1, Thanatin, Lactoferrin, Lactoferricin, and Ovispirin such as Novispirin (Robert Lehrer, 2000), and variants or fragments thereof which retain antimicrobial activity. Other examples are anti-fungal polypeptides (AFP's) such as those derived from Aspergillus giganteus, and Aspergillus niger, as well as variants and fragments thereof which retain antifungal activity, as disclosed in WO 94/01459 and PCT/DK02/00289.

In a particular embodiment, the animal feed additive of the invention is intended for being included (or prescribed as having to be included) in animal diets or feed at levels of 0.0010-12.0%, or 0.0050-11.0%, or 0.0100-10.0%; more particularly 0.05-5.0%; or 0.2-1.0% (% meaning g additive per 100 g feed). This is so in particular for premixes.

Accordingly, the concentrations of the individual components of the animal feed additive, e.g. the premix, can be found by multiplying the final in-feed concentration of the same component by, respectively, 10-10000; 20-2000; or 100-500 (referring to the above three percentage inclusion intervals).

The final in-feed concentrations of important feed components may reflect the nutritional requirements of the animal, which are generally known by the skilled nutritionist, and presented in publications such as the following: NRC, Nutrient requirements in swine, ninth

revised edition 1988, subcommittee on swine nutrition, committee on animal nutrition, board of agriculture, national research council. National Academy Press, Washington, D.C. 1988; and NRC, Nutrient requirements of poultry, ninth revised edition 1994, subcommittee on poultry nutrition, committee on animal nutrition, board of agriculture, national research council, National Academy Press, Washington, D.C., 1994.

The composition of the invention can be prepared according to methods known in the art, e.g. by mixing the phytase and the protease with the additional ingredients, if any.

Animal feed compositions or diets have a relatively high content of protein. An animal feed composition according to the invention has a crude protein content of 50-800, or 75-700, or 100-600, or 110-500, or 120-490 g/kg, and furthermore comprises a composition of the invention.

Furthermore, or in the alternative (to the crude protein content indicated above), the animal feed composition of the invention has a content of metabolisable energy of 10-30, or 11-28, or 11-26, or 12-25 MJ/kg; and/or a content of calcium of 0.1-200, or 0.5-150, or 1-100, or 4-50 g/kg; and/or a content of available phosphorus of 0.1-200, or 0.5-150, or 1-100, or 1-50, or 1-25 g/kg; and/or a content of methionine of 0.1-100, or 0.5-75, or 1-50, or 1-30 g/kg; and/or a content of methionine plus cysteine of 0.1-150, or 0.5-125, or 1-80 g/kg; and/or a content of lysine of 0.5-50, or 0.5-40, or 1-30 g/kg.

Crude protein is calculated as nitrogen (N) multiplied by a factor 6.25, i.e. Crude protein (g/kg)= N (g/kg) x 6.25 as stated in Animal Nutrition, 4th edition, Chapter 13 (Eds. P. McDonald, R. A. Edwards and J. F. D. Greenhalgh, Longman Scientific and Technical, 1988, ISBN 0-582-40903-9). The nitrogen content can be determined by the Kjeldahl method (A.O.A.C., 1984, Official Methods of Analysis 14th ed., Association of Official Analytical Chemists, Washington DC). But also other methods can be used, such as the so-called Dumas method in which the sample is combusted in oxygen and the amount of nitrous gasses formed are analysed and recalculated as nitrogen.

Metabolisable energy can be calculated on the basis of the NRC publication Nutrient Requirements of Swine (1988) pp. 2-6, and the European Table of Energy Values for Poultry Feed-stuffs, Spelderholt centre for poultry research and extension, 7361 DA Beekbergen, The Netherlands. Grafisch bedrijf Ponsen & looijen bv, Wageningen. ISBN 90-71463-12-5.

In a particular embodiment, the animal feed composition of the invention contains at least one vegetable protein or protein source. Examples of vegetable proteins or protein sources are soybean, peas and rape seed from leguminosae and brassica families, and the cereals such as barley, maize (corn), oat, rice, rye, sorghum and wheat.

Animal diets can e.g. be manufactured as mash feed (non-pelleted) or pelleted feed.

Typically, the milled feed-stuffs are mixed and sufficient amounts of essential vitamins and minerals are added according to the specifications for the species in question.

The phytase and protease of the invention can be added in the form of a solid or liquid enzyme formulation, or in the form of a feed additive, such as a pre-mix. A solid composition is typically added before or during the mixing step; and a liquid composition is typically added after the pelleting step.

The phytase and protease of the invention when added to animal feed leads to an improved nutritional value of the feed, e.g. the growth rate and/or the weight gain and/or the feed conversion (i.e. the weight of ingested feed relative to weight gain) of the animal is/are improved.

In particular embodiments the weight gain is at least 101, 102, 103, 104, 105, 106, 107, 108, 109, or at least 110% of the control (no enzyme addition).

In further particular embodiments the feed conversion is at most (or not more than) 99, 98, 97, 96, 95, 94, 93, 92, 91 or at most 90%, as compared to the control (no enzyme addition).

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

Example 1: Specific Activity of Phytases

The specific activity of phytases can be determined on highly purified samples dialysed against 20 mM sodium acetate, pH 5.5. The purity can be checked beforehand on an SDS poly acryl amide gel showing the presence of only one component.

The protein concentration can be determined by amino acid analysis as follows: An aliquot of the sample is hydrolyzed in 6N HCl, 0.1% phenol for 16 h at 110 C in an evacuated glass tube. The resulting amino acids are quantified using an Applied Biosystems 420A amino acid analysis system operated according to the manufacturer's instructions. From the amounts

of the amino acids the total mass - and thus also the concentration - of protein in the hydrolyzed aliquot can be calculated.

The phytase activity is determined in the units of FYT, and the specific activity is calculated as the phytase activity measured in FYT units per mg phytase variant enzyme protein. Phytase activity can be determined using the assay below.

Determination of phytase activity

75 microliter phytase-containing enzyme solution, appropriately diluted in 0.25M sodium acetate, 0.005% (w/v) Tween-20, pH5.5, is dispensed in a microtiter plate well, e. g. NUNC 269620, and 75 microliter substrate is added (prepared by dissolving 100mg sodium phytate from rice (Aldrich Cat.No. 274321) in 10ml 0.25M sodium acetate buffer, pH5.5). The plate is sealed and incubated 15min. shaken with 750rpm at 37°C. After incubation, 75 microliter stop reagent is added (the stop reagent being prepared by mixing 10 ml molybdate solution (10% (w/v) ammonium hepta-molybdate in 0.25% (w/v) ammonia solution), 10ml ammonium vanadate (0.24% commercial product from Bie&Berntsen, Cat.No. LAB17650), and 20ml 21.7% (w/v) nitric acid), and the absorbance at 405nm is measured in a microtiter plate spectrophotometer. The phytase activity is expressed in the unit of FYT, one FYT being the amount of enzyme that liberates 1 micromole inorganic ortho-phosphate per minute under the conditions above. An absolute value for the measured phytase activity may be obtained by reference to a standard curve prepared from appropriate dilutions of inorganic phosphate, or by reference to a standard curve made from dilutions of a phytase enzyme preparation with known activity (such standard enzyme preparation with a known activity is available on request from Novozymes A/S, Krogshoejvej 36, DK-2880 Bagsvaerd).

Example 2: In vivo broiler trial 1

The effect on the growth performance of broilers using feed with different amounts and combinations of enzymes (Ronozyme®ProAct & Ronozyme®HiPhos) was investigated. The trial ran 21 days and had 7 treatments with 6 replicate cages of 6 birds per cage.

The diet (NC-Diet) as used in the trial was a diet with low avP/Ca and moderate phytate concentrations (table 1). The experimental conditions are shown in table 2 and the results are presented in table 3.

Table 1: Diet

Ingredients (%)	**Amount**
Wheat	59.40
Soybean meal	23.49
Corn oil	5.21
Rapeseed solv ext	5.00
Wheat bran	4.00
NaCl	0.15
NaHCO3	0.31
DL-methionine	0.20
Lysine HCl	0.24
Threonine	0.06
Limestone	0.80
Dicalcium phosphate	0.60
Choline chloride	0.05
Vitamin Premix	0.50
Analyzed content	
Crude protein (%)	21.00
Metabolizable energy (MJ/kg)	3058.8
Ca %	0.70
P%	0.57
Available P%	0.28
Fat	6.83
Fibre	3.14
Phytate P%	0.22
Lysine (%)	1.20
Cysteine + Methionine (%)	0.90

Table 2: Experimental Treatments (T)

Treatment	Conditions
T1	NC + 15,000 units protease (Ronozyme®ProAct)
T2	NC + 1,000 FYT phytase (Ronozyme®HiPhos)
T3	NC + 1,000 FYT phytase + 15,000 units protease
T4	NC + 2,000 FYT phytase (Ronozyme®HiPhos)
T5	NC + 2,000 FYT phytase + 15,000 units protease
T6	NC + 3,000 FYT phytase
T7	NC + 3,000 FYT phytase + 15,000 units protease

Table 3: Results

Treatment	Weight Gain (g/bird)	FCR
T1	785	1.670
T2	905	1.451
T3	900	1.452
T4	905	1.475
T5	912	1.461
T6	870	1.542
T7	866	1.391
SEM	30.4318	0.0432
Significance (P=)	1.120	0.004

In particular the FCR results show that the protease benefits increasingly from higher and higher phytase dosing. With respect to the combination of the enzyme products Ronozyme®ProAct & Ronozyme®HiPhos as exemplified herein above, the really strong protease effect was surprisingly seen at 3000 FYT/kg phytase activity.

Example 3: In vivo broiler trial 2

The effect on the growth performance of broilers using feed with different amounts and combinations of phytase and protease (Ronozyme®ProAct & Axtra®PHY) was investigated. The individual treatments are given in table 4. The trial ran 36 days and each treatment had 6 replicate cages of 18 birds per cage.

The diet (NC-Diet) as used in the trial is a diet with low avP/Ca (see table 5 and table 6).

Table 4: Experimental Treatments (T)

Treatment	Conditions
T1	NC + 500 U/kg Phytase Axtra®PHY
T2	NC + 500 U/kg Phytase Axtra®PHY + 15,000 units protease
T3	NC + 1500 U/kg Phytase Axtra®PHY
T4	NC + 1500 U/kg Phytase Axtra®PHY + 15,000 units protease

Table 5: Nutrient Composition of Diet

Diet	**Crude protein (%)**	**ME (MJ/kg)**	**Lysine (%)**	**Cysteine + Methionine (%)**	**P total (%)**	**P disponible (%)**	**Ca total (%)**
Starter	21.1	12.8	1.28	0.959	0.623	0.323	0.762
Grower	19.2	13.0	1.115	0.844	0.580	0.304	0.754

Table 6: Diet

Ingredients (%)	**Starter**	**Grower**
Wheat, 12.5% CP	55.50	61.00
SBM, 48% CP	28.00	22.36
RSM	5.00	5.00
Wheat bran	3.82	4.00
Soja oil	4.20	4.20
NaCl	0.20	0.20
DL Methionine	0.26	0.20
L-Lysine	0.24	0.22
L-Threonine	0.12	0.11
$CaCO_3$ (%)	0.40	0.50
DCP (%)	1.25	1.05
Premix (%)	1.00	1.00
Lasalocid (Avatec)(%)	0.06	0.06
Titanium dioxide	-	0.10

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Table 7: Results

Treatment	Weight Gain (g/bird)	FCR
T1	2690	1.54
T2	2517	1.59
T3	2612	1.62
T4	2549	1.54

The results show that the phytase in combination with protease benefits from higher phytase dosing. The combination of the commercial dose of Axtra®PHY together with Ronozyme®ProAct did not result in any performance improvement over the commercial dose of Axtra®PHY alone. However, the combination of 3 times the amount of the commercial dose of Axtra®PHY together with Ronozyme®ProAct surprisingly resulted in a strong FCR improvement over 3 times the amount of the commercial dose of Axtra®PHY alone.

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Claims

1. A method for increasing weight gain and/or improving Feed Conversion Ratio of farm animals, the method comprising the step of applying to the animal a feed with an efficient amount of one or more proteolytic enzymes in combination with at least one phytase wherein:
    a. the phytase is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed and
    b. the protease is administered in a dosage of between 10'000 units/kg feed and 30'000 units/kg feed.

2. The method of claim 1, wherein:
    a. the phytase is administered in one or more of the following amounts: 1000, 2000 or 3000 FYT/kg feed and
    b. the protease is administered in one of the following amounts: 10'000 units/kg feed, 11'000, 12'000, 13'000, 14'000, 15'000, 16'000, 17'000, 18'000, 19'000, 20'000 units/kg feed.

3. The method according to claim 2, wherein the enzymes are administered as follows:
    a. Phytase: 3000 FYT /kg feed and
    b. Protease: 15'000 units/kg feed.

4. The method according to any of claims 1 to 3, wherein the phytase is classified as belonging to the EC 3.1.3.26 group.

5. The method according to any of claims 1 to 4, wherein the phytase is derived from the family *Enterobacteriaceae*.

6. The method according to any of claims 1 to 5, wherein the protease is an acid stable serine protease obtained or obtainable from the class *Actinobacteria*.

7. The method according to claim 6, wherein the protease is an acid stable serine protease derived from *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43235 (A1918L1), *Nocardiopsis prasina* DSM 15649 (NN018335L1), *Nocardiopsis prasina* (previously *alba*) DSM 14010 (NN18140L1), *Nocardiopsis* sp. DSM 16424 (NN018704L2), *Nocardiopsis*

alkaliphila DSM 44657 (NN019340L2) and *Nocardiopsis lucentensis* DSM 44048 (NN019002L2), as well as homologous proteases.

8. Use of one or more proteolytic enzymes in combination with at least one phytase in animal feed for increasing weight gain and/or improving Feed Conversion Ratio of farm animals, wherein:
    a. the phytase is administered in such amounts that the specific activity in the final feed is between 1000 FYT/kg feed and 4000 FYT/kg feed and
    b. the protease is administered in a dosage of between 10'000 units/kg feed and 30'000 units/kg feed.

9. Use according to claim 8, wherein:
    a. the phytase is administered in one or more of the following amounts: 1000, 2000 or 3000 FYT/kg feed and
    b. the protease is administered in one of the following amounts: 10'000 units/kg feed, 11'000, 12'000, 13'000, 14'000, 15'000, 16'000, 17'000, 18'000, 19'000, 20'000 units/kg feed.

10. Use according to claim 9, wherein the enzymes are administered as follows:
    a. Phytase: 3000 FYT /kg feed and
    b. Protease: 15'000 units/kg feed.

11. Use according to any of claims 8 to 10, wherein the phytase is classified as belonging to the EC 3.1.3.26 group.

12. Use according to any of claims 8 to 11, wherein the phytase is derived from the family *Enterobacteriaceae*.

13. Use according to any of claims 8 to 12, wherein the protease is an acid stable serine protease obtained or obtainable from the class *Actinobacteria*.

14. The use according to claim 13, wherein the protease is an acid stable serine protease derived from *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43235 (A1918L1), *Nocardiopsis prasina* DSM 15649 (NN018335L1), *Nocardiopsis prasina* (previously *alba*) DSM 14010 (NN18140L1), *Nocardiopsis* sp. DSM 16424 (NN018704L2), *Nocardiopsis*

alkaliphila DSM 44657 (NN019340L2) and *Nocardiopsis lucentensis* DSM 44048 (NN019002L2), as well as homologous proteases.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/064739

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23K1/165
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 A23L

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A J COWIESON ET AL: "Carbohydrases, Protease, and Phytase Have an Additive Beneficial Effect in Nutritionally Marginal Diets for Broiler Chicks", POULTRY SCIENCE, vol. 84, 1 January 2005 (2005-01-01), pages 1860-1867, XP055174156,	1-5,8-12
Y	abstract page 1861, left-hand column, line 7 - line 48; tables 3, 4	6,7,13, 14

Y	US 2012/321747 A1 (LASSEN SOREN FLENSTED [DK] ET AL) 20 December 2012 (2012-12-20) abstract paragraph [0184]; claims 13,14,16,21	6,7,13, 14

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

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 September 2015	29/09/2015

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/064739

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 756 457 A1 (NOVO NORDISK AS [DK] NOVOZYMES AS [DK]) 5 February 1997 (1997-02-05) paragraph [0029] - paragraph [0049] paragraph [0054] - paragraph [0075]; claims 11-15, 19-21; example 1 -----	1-14
X	WO 2012/110777 A2 (DUPONT NUTRITION BIOSCI APS [DK]; MILLAN LUIS FERNANDO ROMERO [GB]) 23 August 2012 (2012-08-23) paragraph [0101] - paragraph [0134] paragraph [0228] - paragraph [0239] paragraph [0291] - paragraph [0299]; claims 1,8-13,15,16 -----	1-14

INTERNATIONAL SEARCH REPORT

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