

DNA-METHYLATION BASED EVALUATION OF ANIMAL FEED**FIELD OF THE INVENTION**

5 The present invention relates to a method of determining the effects of specific components of an animal feed on general health and/or performance of an animal that consumes the feed. In particular, the method uses DNA methylation profiling to determine if the specific component improves the general health and/or performance of the animal or in fact has detrimental effects. The general health and/or performance of the animal is depicted using specific panels of CpG
10 sites.

BACKGROUND OF THE INVENTION

By 2050 the global population is estimated to reach 9 billion people. In order to feed the growing
15 population, agricultural practices must become as efficient as possible to sustainably make use of the resources available. For livestock production, it is estimated that the cost of feed constitutes 60-70 % of the overhead costs, therefore making animal feed an obvious focus for innovations aimed at improving the efficiency and sustainability of production.

Additionally, the feed an animal consumes is directly related to their growth rates, feed efficiency
20 (amount of weight gained in ratio to feed consumed), pathogenic and metabolic disease resistance and overall physiological development. The feed industry is continually testing new nutritional concepts, raw ingredients, nutrient sources and feed additives. Generally, feeding trials all measure the animal variables directly related to return on investment, like final weights, growth rates and feed efficiency. However, these measurements are not always able to determine subtle changes in
25 a single trial, although these subtle changes might have significant cost benefits for the producer and/or health and welfare benefits for the animals over time.

For example, the addition of probiotic feed additives in diets has been shown to improve the development of digestive and immune systems of animals. In cases where diets are fed that are less digestible and/or where disease pressure is present, the effects of these probiotic supplements
30 can easily be observed phenotypically in the growth rates and disease resistance of the animals. However, in scenarios where high-quality feed ingredients are utilized and there is no pathogenic exposure, the benefits of feed additives like probiotics are very difficult to measure using only growth metrics and gross physiological health status. This makes the testing and benchmark comparison of feed additives difficult and expensive as not all experiments are able to show
35 significant effects of the feed additives with phenotypic measurements. More sensitive and reliable analytics are sought to reduce the number of trials and therefore costs required in feed additive research in various conditions.

Evaluating nutrient sources is another example of animal feed research which could be more sensitive and cost effective. For example, essential nutrients such as methionine or vitamins and/or
40 minerals are often available in various forms through various products on the market. When comparing two or more sources, very large dose response trials are required with a high number of animal replicates in order to mathematically model the optimal doses and sources of each nutrient based on the animal performance data (e.g. feed intake, growth rate, milk or egg production and

feed efficiency). If the dose range selected for such trials start slightly too low or slightly too high or does not contain enough levels, a large expensive trial may not result in the data points required for accurate modelling to define the optimal doses and sources based on the growth data alone. This wastes time, costs, efforts, and animal lives unnecessarily. Therefore, analytics with increased sensitivity to determine optimal outcomes with fewer animals and higher reliability would be a welcome addition to the assessment of nutrients sources for animal feed.

Epigenetics is the study of inherited traits caused by mechanisms other than changes in the underlying DNA sequence. In other words, epigenetic marks “orchestrate” our genes. Epigenetic marks can be either chemical (e.g. methylation), protein-based (e.g. histones) or a combination of the two. During development and cell differentiation, DNA methylation is dynamic, but some DNA methylation patterns may be retained as a form of epigenetic memory, accumulated and/or inherited to next generation. Those changes might be responsible for heritable changes in gene activity as DNA methylation events have been shown to be regulation mechanisms associated with gene silencing, expression, chromatin remodelling or imprinting. Epigenetics is attractive for animal breeding as it may identify causality and heritability of complex traits and diseases. DNA methylation patterns are modified along the life of an individual by environmental forces like diet, stress, drugs, or pollution among many others. Some environments are more likely to increase certain methylation patterns, and these patterns could contribute to the epigenetic and/or phenotypic variation between individuals.

Recent studies have shown that DNA methylation patterns of animals contain important information about their rearing conditions. For example, a comparison of genome-wide patterns of methylation and variation at the DNA level revealed that a highly significant proportion of epigenetic variation could be associated with fitness and environmental differences such as captivity in salmon (Le Luyer J et al. 2017 PNAS vol 114, no 49). Further, Whelan, R. et al., 2023, Front. Vet. Sci., Sec. Veterinary Experimental and Diagnostic Pathology: 9 discloses the use of epigenetic biomarkers for welfare monitoring. Koop et al. (Koop et al., 2021 International Journal of Legal Medicine 135:167–173) and Rhein et al. (Rhein et al., 2015 Frontiers in Genetics 6: 182) also separately confirmed that methylation patterns are maintained post-mortem and are consistent regardless of the level of decomposition of the sample. One of the only aspects that still affects the methylation pattern of the sample would be the DNA integrity and amount of DNA that is found in the sample. In view of the above, there is still an urgent need to provide a means possibly using epigenetics for the assessment of different nutrient sources, feed additives, raw ingredients, nutritional concepts and compound feeds.

DESCRIPTION OF THE INVENTION

The present invention attempts to solve the problems above by providing a method using DNA methylation patterns to distinguish the effect of one animal feed from another on an animal which consumes the animal feed. This method according to any aspect of the present invention is not only accurate and reliable but it also saves time, costs and effort needed to determine the effect of a particular animal feed or component thereof on the animal's general health or performance in the short or long term. In particular, the effect of the animal feed or component thereof on the growth rate and/ or gut health of the animal consuming the animal feed may be determined and/or

predicted for the long run using the method according to any aspect of the present invention, without having to monitor the animal or a group of animals for a long time. The method according to any aspect of the present invention provides for methods of predicting animal performance based on the DNA methylation profile of an animal. In addition, since the DNA methylation profiles may be altered for example by changing the animal's diet, the effect of components of animal feed on the performance of an animal may be determined using the method according to any aspect of the present invention. In particular, the method according to any aspect of the present invention also provides methods of monitoring the effect of a feed or feed additive regimen on the current performance or future performance of the animal. The method according to any aspect of the present invention further provides a means of managing an animal growing or processing operation by determining suitable animal feed and/or additives to feed the animal to achieve the best performance from the animal. Improved management can thereby optimize animal performance and the animal products produced therefrom.

The present invention is based on the finding that components of animal feed can change the epigenome of the animal through epigenetics. In particular, the capability to adapt to the environment and maintain the adapted biological pattern depends on epigenetic mechanisms, including DNA methylation. In particular, the present invention is based on the finding that animal feed may also result in changes in epigenetic mechanisms of the animal, including DNA methylation patterns and these patterns may be passed down to the different products that may derive from the animal.

The inventors have unexpectedly found that this property can be utilized to identify "epigenetic fingerprints" on the genome that are specific to a component of animal feed that may improve general health and/or performance of not just one animal being fed the feed but possibly all the animals that consume the same animal feed or components thereof. Based on these findings, the present invention provides means to identify the specific effect short term and in the long run of any component of an animal feed on the general health and/or performance of the animal consuming the feed. In particular, the method according to any aspect of the present invention may be used to determine if a specific component of any animal feed has a positive or negative effect on the general health and/or performance of the animal. For example, a component X in the animal feed may improve general health and/or performance of the animal consuming the feed in the short and/or long run resulting in the animal having relatively good general health and/or performance. In another example, a component Y in the animal feed may worsen the existing general health and/or performance of the animal consuming the feed resulting in the animal having relatively bad general health and/or performance. More in particular, the method according to any aspect of the present invention may be used to determine if a particular component of animal feed or the animal feed in itself has a positive or negative effect on the general health and/or performance of the animal. In this way, the method according to any aspect of the present invention may then be used to accurately, reliably and quickly determine the specific effect of a component in animal feed on the animal and based on these results, it can be decided if the component should be included in the regular diet of the animal or should be removed from the animal's diet.

According to one aspect of the present invention, there is provided a method of assessing the effect of at least one test component of animal feed on at least performance and/or general health of a test animal consuming the animal feed with the test component, the method comprising the steps of:

- 5 (a) determining a test methylation profile of one or more pre-selected methylation sites within the DNA of the test animal;
- (b) comparing the test methylation profile obtained from (a) with at least one control methylation profile from a control animal of the same biological taxon as the test animal consuming animal feed without the test component; and
- 10 wherein the results of (a) is used to determine the performance and/or general health of the test animal; and
- wherein a significant similarity in the test methylation profile of (a) compared to the control methylation profile, is indicative of the test component not having an effect on the performance and/or general health of the test animal; and
- 15 wherein a significant difference in the test methylation profile of (a) compared to the control methylation profile, is indicative of the test component having an effect on the performance and/or general health of the test animal; and
- wherein the test animal is selected from livestock or poultry.

- 20 According to another aspect of the present invention, there is provided a method of assessing the effect of at least one test component of animal feed on at least performance and/or general health of a test animal consuming the animal feed with the test component, the method comprising the steps of:

- (a) determining a test methylation profile of one or more pre-selected methylation sites within
25 the DNA of the test animal; and
- (c) comparing the test methylation profile obtained from (a) with
- (i) a first reference methylation profile obtained from a control animal of the same biological taxon as the test animal having good performance and/or general health; and/or
- (ii) a second reference methylation profile obtained from a control animal of the same
30 biological taxon as the test animal having poor performance and/or general health;
- and
- wherein a significant similarity in the test methylation profile of (a) to the first reference methylation profile and/or a difference in the test methylation profile of (a) to the second reference methylation profile is indicative of the test animal having good performance and/or general health as the control
35 cell; and/or
- wherein a difference in the test methylation profile of (a) compared to the first reference methylation profile and/or a significant similarity in the test methylation profile of (a) to the second reference methylation profile is indicative of the test animal having poor performance and/or general health as the control animal; and
- 40 wherein the test animal is selected from livestock or poultry.

The method according to this aspect of the present invention further comprising a step of:

- (b) comparing the test methylation profile obtained from (a) with at least one control methylation profile from a control animal of the same biological taxon as the test animal consuming animal feed without the test component; and
wherein the results of (a) is used to determine the performance and/or general health of the test animal; and
wherein a significant similarity in the test methylation profile of (a) compared to the control methylation profile, is indicative of the test component not having an effect on the performance and/or general health of the test animal; and
wherein a significant difference in the test methylation profile of (a) compared to the control methylation profile, is indicative of the test component having an effect on the performance and/or general health of the test animal.

In particular, the results of (a), namely the test methylation profile of one or more pre-selected methylation sites within the DNA of the test animal is used to determine the performance and/or general health of the test animal in general (with the test component). In particular, the test methylation profile from (a) can be used to determine if the test animal (having been fed the test component) has a good performance and/or general health or a poor performance and/or general health. The results of the comparisons carried out in (b) and (c) are then used to determine if the test component has an effect on the general health and/ or performance of the animal and if the effect is positive or negative.

In particular, the general health and/or performance of the animal is a measure of at least one metric of the animal and the metric is selected from the group consisting of bodyweight or carcass weight, growth rate, animal feed intake, feed conversion ratio (FCR), digestive function, gut inflammation status, medical costs, transition period, use of antibiotics, mortality and combinations thereof.

Improvement in one or more metrics of an animal (such as, without limitation, any of improved feed conversion ratio (FCR); improved weight gain; improved feed efficiency; improved carcass quality; and/or improved milk production) can be measured using any methods known in the art.

As used herein, the term "feed" used interchangeably with the term "animal feed" broadly refers to a material, liquid or solid, that is used for nourishing an animal, and for sustaining normal or accelerated growth of an animal including newborns or young and developing animals. The term includes a compound, preparation, mixture, or composition suitable for intake by an animal, particularly livestock. Animal feeds typically include a number of different components that may be present in forms such as concentrate(s), premix(es) co-product(s), or pellets. Examples of feeds and feed components include, but are not limited to, Total Mixed Ration (TMR), corn, soybean, forage(s), grain(s), distiller grain(s), sprouted grains, legumes, vitamins, amino acids, minerals, molasses, fiber(s), fodder(s), grass(es), hay, straw, silage, kernel(s), leaves, meal, soluble(s), and supplement(s). As used herein the term "selected component of animal feed(s)" refers to an animal feed selected for analysis using the methods according to any aspect of the present invention. In

one example, a feed or feed composition comprises a basal food composition and one or more feed additives or feed additive compositions. The term "feed additive" as used herein refers to components included for purposes of fortifying basic feed with additional components to promote feed intake, treat or prevent disease, or alter metabolism. Feed additives include pre-mixes.

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As used herein, the term "feed additive" refers to a substance which is added to a feed. "Animal feed and components thereof", refers to the bulk component that makes up the largest percentage of the animal feed and feed additives. Feed additives may be added to feed for a number of reasons. For instance, to enhance digestibility of the feed, to supplement the nutritional value of the feed, improve the immune defense of the recipient and/or to improve the shelf life of the feed. In some examples, the feed additive supplements the nutritional value of the feed and/or improves the immune defense of the recipient. Representative feed additives include one or more components such as medicated feed additives, enzymes, probiotic microbials, direct-fed microbials, antimicrobials, prebiotics, phytochemicals, immunomodulators, antibodies, plant extracts, essential oils, organic acids, antioxidants, amino acids, oligosaccharides, oleoresins, herbs, spices, saponins, and sea plants.

A "premix," as used herein, may be a composition composed of micro-ingredients such as, but not limited to, one or more of vitamins, minerals, chemical preservatives, antibiotics, fermentation products, and other essential ingredients. Premixes are usually compositions suitable for blending into commercial rations.

In particular, the component of the animal feed according to any aspect of the present invention may be selected from the group consisting of compound feeds, probiotics, vitamins, minerals, chemical preservatives, antibiotics, and fermentation products.

The term "performance" as used herein may be determined by the feed efficiency and/or weight gain of the animal and/or by the feed conversion ratio and/or by the digestibility of a nutrient in a feed (e.g., amino acid digestibility or phosphorus digestibility) and/or digestible energy or metabolizable energy in a feed and/or by nitrogen retention and/or by animals' ability to avoid the negative effects of diseases or by the immune response of the subject. Performance characteristics may include but are not limited to: body weight; weight gain; mass; body fat percentage; height; body fat distribution; growth; growth rate; milk production; nutrient absorption; nutrient excretion; mineral absorption; mineral excretion, mineral retention; bone density; bone strength; feed conversion rate (FCR); average daily feed intake (ADFI); Average daily gain (ADG) retention and/or a secretion of any one or more of copper, sodium, phosphorous, nitrogen and calcium; amino acid retention or absorption; mineralization, bone mineralization carcass yield and carcass quality. In particular, 'nutrient' may be fat, carbohydrate, protein, amino acids and the like.

By "improved animal performance" it is meant that there is increased feed efficiency, and/or increased weight gain and/or reduced feed conversion ratio and/or improved digestibility of nutrients or energy in a feed and/or by improved nitrogen retention and/or by improved ability to

avoid the negative effects of pathogenic enteric diseases such as for example, necrotic enteritis and/or by an improved immune response in the subject resulting from the use of feed comprising the feed additive composition described herein as compared to a feed which does not comprise said feed additive composition. In some embodiments, by "improved animal performance" it is
5 meant that there is increased feed efficiency and/or increased weight gain and/or reduced feed conversion ratio. The improvement in performance parameters may be in respect to a control in which the feed used does not comprise the component in question (i.e. selected component in the feed to be tested).

10 Similarly, the term 'general health' in reference to an animal as used herein refers to the wellbeing of an animal and may be determined at least by measuring presence of enteric disease and/or inflammation and/or increased resistance to any disease in the animal. The measurement may be carried out using any method known in the art. In one example, general health may be measured by measuring gut inflammation status. By "improved general health of the animal" it is meant that
15 there is reduced chances or instances of enteric disease and/or inflammation and increased resistance to any disease. In one example, improved general health of the animal" may be reduced chances of gut inflammation in the animal (i.e. positive gut inflammation status).

As used herein, the term "feed efficiency" refers to the amount of weight gain in an animal that
20 occurs when the animal is fed ad-libitum or a specified amount of food during a period of time. By "increased feed efficiency" it is meant that the use of a feed additive composition according to any aspect of the present invention in feed results in an increased weight gain per unit of feed intake compared with an animal fed without said feed additive composition being present.

25 As used herein, "feed conversion ratio" refers to a measure of a subject's efficiency in converting feed mass into increases of a desired output and is calculated by dividing the mass of the food eaten by the output for a specified period. For example, if an animal is raised for meat (e.g., chicken), the output may be the mass gained by the animal. If an animal is raised for another intended purpose (e.g., milk or egg production), the output will be different. The term "feed
30 conversion ratio" may be used interchangeably herein with the terms "feed conversion rate" or "feed conversion efficiency". By "lower feed conversion ratio" or "improved feed conversion ratio" it is meant that the use of a specific feed additive composition in feed results in a lower amount of feed being required to be fed to an animal to increase the weight of the animal by a specified amount compared to the amount of feed required to increase the weight of the animal by the same
35 amount when the feed does not comprise the specific feed additive composition.

The term "test" used in conjunction with the term subject and/ or animal herein refers to an entity that is subjected to the method according to any aspect of the present invention and is the basis for an analysis application of the present invention. An "(individual) test subject", an "(individual) group
40 of test subjects" or a "test profile" or an 'test animal derived product' is therefore a (individual) subject or group of subjects being tested according to the invention or a profile being obtained or generated in this context. Conversely, the term "reference" shall denote, mostly predetermined,

entities which are used for a comparison with the test entity. For example, the term 'reference animal' refers to an animal used for comparison or as a control in reference to the 'test animal'. Similarly, the term 'sample' and/or 'test animal-derived product sample' used in accordance with any aspect of the present invention refers to an entity that may be subject to the method of the present invention. In particular, a sample may be any DNA sample obtained from a test animal that may be subject to the method of the present invention to determine the effect of a selected component of animal feed on the general health and/or performance of the animal by first determining the DNA methylation profile and then comparing this test methylation profile with a control (reference methylation profiles from control animals showing good or bad general health and/or performance).

The livestock according to any aspect of the present invention includes terrestrial and aquatic livestock. In particular, livestock may be rearing animals selected from terrestrial and aquatic livestock or poultry. In particular, terrestrial livestock may include cattle, sheep, pigs, goats, horses, camels, donkeys, mules, rabbits and the like and poultry may include chickens, turkeys and other gallinaceous birds, ducks, geese, quail, and the like. As used herein, the term 'livestock' may also include poultry and refer to any farm animal or animal that may be used in agriculture.

As used herein, the term "aquatic livestock" refers to any organism that is reared entirely in water or that lives predominantly in water, especially compared with terrestrial animals. These aquatic livestock may live in different water forms, such as seas, oceans, rivers, lakes, ponds, etc. More in particular, the aquatic livestock according to any aspect of the present invention may be any fish, cephalopod, aquatic molluscs, or aquatic crustaceans, at all life stages, including eggs, sperm and gametes. Even more in particular, the 'aquatic animal' means animals of the following species: (i) fish belonging to the superclass *Agnatha* and to the classes *Chondrichthyes*, *Sarcopterygii* and *Actinopterygii*; (ii) aquatic molluscs belonging to the phylum *Mollusca*; and (iii) aquatic crustaceans belonging to the subphylum *Crustacea*. Even more in particular, the aquatic livestock according to any aspect of the present invention may be aquatic livestock used in aquaculture. Some non-limiting examples of aquatic animals according to any aspect of the present invention include barramundi, carp, catfish, halibut, marbled crayfish, marine and brackish fishes, marine shrimp, mitten crabs, mussels, oysters, pangasius, rainbow trout, salmonids, scallops, sea bass, sea bream, soft-shelled crabs, soft-shelled turtles, tiger prawns, tilapia, turbot, white-leg prawn, shrimp, octopus, squid and other decapod crustaceans, bivalves and gastropods.

As used herein, the term "comprising" is to be construed as encompassing both "including" and "consisting of", both meanings being specifically intended, and hence individually disclosed aspects of the present invention. Where used herein, "and/or" is to be taken as specific disclosure of each of the two specified features or components with or without the other. For example, "A and/or B" is to be taken as specific disclosure of each of (i) A, (ii) B and (iii) A and B, just as if each is set out individually herein. In the context of the present invention, the terms "about" and "approximately" denote an interval of accuracy that the person skilled in the art will understand to still ensure the technical effect of the feature in question. The term typically indicates deviation from the indicated

numerical value by $\pm 20\%$, $\pm 15\%$, $\pm 10\%$, and for example $\pm 5\%$. As will be appreciated by the person of ordinary skill, the specific deviation for a numerical value for a given technical effect will depend on the nature of the technical effect. For example, a natural or biological technical effect may generally have a larger such deviation than one for a man-made or engineering technical effect. Where an indefinite or definite article is used when referring to a singular noun, e.g. "a", "an" or "the", this includes a plural of that noun unless something else is specifically stated.

In context of the present invention, the terms "methylation profile", "methylation pattern", "methylation state" or "methylation status," are used herein to describe the state, situation or condition of methylation of a genomic sequence, and such terms refer to the characteristics of a DNA segment at a particular genomic locus in relation to methylation. Such characteristics include, but are not limited to, whether any of the cytosine (C) residues within this DNA sequence are methylated, location of methylated C residue(s), percentage of methylated C at any particular stretch of residues, and allelic differences in methylation due to, e.g., difference in the origin of the alleles.

The term "methylation status" refers to the status of a specific methylation site (i.e. methylated vs. non-methylated) which means a residue or methylation site is methylated or not methylated. Then, based on the methylation status of one or more methylation sites, a methylation profile may be determined. Accordingly, the term "methylation profile" or also "methylation pattern" refers to the relative or absolute concentration of methylated C residues or unmethylated C residues at any particular stretch of residues in the genomic material of a biological sample. For example, if cytosine (C) residue(s) not typically methylated within a DNA sequence are methylated, it may be referred to as "hypermethylated"; whereas if cytosine (C) residue(s) typically methylated within a DNA sequence are not methylated, it may be referred to as "hypomethylated". Likewise, if the cytosine (C) residue(s) within a DNA sequence (e.g., the DNA from a sample nucleic acid from a test subject) are methylated as compared to another sequence from a different region or from a different individual (e.g., relative to normal nucleic acid or to the standard nucleic acid of the reference sequence), that sequence is considered hypermethylated compared to the other sequence. Alternatively, if the cytosine (C) residue(s) within a DNA sequence are not methylated as compared to another sequence from a different region or from a different individual, that sequence is considered hypomethylated compared to the other sequence. These sequences are said to be "differentially methylated". Measurement of the levels of differential methylation may be done by a variety of ways known to those skilled in the art. One method is to measure the methylation level of individual interrogated CpG sites determined by the bisulfite sequencing method, as a non-limiting example.

The term "hypermethylation" refers to the average methylation state corresponding to an increased presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample. In particular, control refers to an animal known to have good performance and/or general health or an animal with bad performance and/or general health.

The term "hypomethylation" refers to the average methylation state corresponding to a decreased presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample. In particular, control refers to an animal known to have good performance and/or general health or an animal with bad performance and/or general health.

As used herein, a "methylated nucleotide" or a "methylated nucleotide base" refers to the presence of a methyl moiety on a nucleotide base, where the methyl moiety is usually not present in a recognized typical nucleotide base. For example, cytosine in its usual form does not contain a methyl moiety on its pyrimidine ring, but 5-methylcytosine contains a methyl moiety at position 5 of its pyrimidine ring. Therefore, cytosine in its usual form may not be considered a methylated nucleotide and 5-methylcytosine may be considered a methylated nucleotide. In another example, thymine may contain a methyl moiety at position 5 of its pyrimidine ring, however, for purposes herein, thymine may not be considered a methylated nucleotide when present in DNA. Typical nucleotide bases for DNA are thymine, adenine, cytosine and guanine. Typical bases for RNA are uracil, adenine, cytosine and guanine. Correspondingly a "methylation site" is the location in the target gene nucleic acid region where methylation has the possibility of occurring. For example, a location containing CpG is a methylation site wherein the cytosine may or may not be methylated. In particular, the term "methylated nucleotide" refers to nucleotides that carry a methyl group attached to a position of a nucleotide that is accessible for methylation. These methylated nucleotides are usually found in nature and to date, methylated cytosine that occurs mostly in the context of the dinucleotide CpG, but also in the context of CpNpG- and CpNpN-sequences may be considered the most common. In principle, other naturally occurring nucleotides may also be methylated but they will not be taken into consideration with regard to any aspect of the present invention.

As used herein, a "CpG site" or "methylation site" is a nucleotide within a nucleic acid (DNA or RNA) that is susceptible to methylation either by natural occurring events *in vivo* or by an event instituted to chemically methylate the nucleotide *in vitro*.

The term "methylation marker", or "CpG site" as used herein refers to a CpG position that is potentially methylated. Methylation typically occurs in a CpG containing nucleic acid. The CpG containing nucleic acid may be present in, e.g. a CpG island, a CpG doublet, a promoter, an intron, or an exon of a gene. For instance, the potential methylation sites may encompass the promoter/enhancer regions of the indicated genes. The "set of specific CpG sites in the DNA" refers to the CpG sites showing the best correlations with performance and/or growth rate of an animal.

As used herein, a "methylated nucleic acid molecule" refers to a nucleic acid molecule that contains one or more nucleotides that is/are methylated.

The term 'epigenetic change' as used herein refers to a chemical (e.g., methylation) change or protein (e.g., histones) change that takes place to a gene body or a promoter thereof. Through epigenetic changes, environmental factors like diet, stress and prenatal nutrition can make an imprint on genes passed from one generation to the next.

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The term "bisulfite" as used herein encompasses any suitable type of bisulfite, such as sodium bisulfite, or another chemical agent that is capable of chemically converting a cytosine (C) to a uracil (U) without chemically modifying a methylated cytosine and therefore can be used to differentially modify a DNA sequence based on the methylation status of the DNA, e.g., U.S. Pat. Pub. US 2010/0112595 (Menchen et al.). As used herein, a reagent that "differentially modifies" methylated or non-methylated DNA encompasses any reagent that modifies methylated and/or unmethylated DNA in a process through which distinguishable products result from methylated and non-methylated DNA, thereby allowing the identification of the DNA methylation status. Such processes may include, but are not limited to, chemical reactions (such as a C to U conversion by bisulfite) and enzymatic treatment (such as cleavage by a methylation-dependent endonuclease). Thus, an enzyme that preferentially cleaves or digests methylated DNA is one capable of cleaving or digesting a DNA molecule at a much higher efficiency when the DNA is methylated, whereas an enzyme that preferentially cleaves or digests unmethylated DNA exhibits a significantly higher efficiency when the DNA is not methylated.

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In context of the present invention also any "non-bisulfite-based method" and "non-bisulfite-based quantitative method" are comprised to test for a methylation status at any given methylation site to be tested. Such terms refer to any method for quantifying methylated or non-methylated nucleic acid that does not require the use of bisulfite. The terms also refer to methods for preparing a nucleic acid to be quantified that do not require bisulfite treatment. Examples of non-bisulfite-based methods include, but are not limited to, methods for digesting nucleic acid using one or more methylation sensitive enzymes and methods for separating nucleic acid using agents that bind nucleic acid based on methylation status. The terms "methyl-sensitive enzymes" and "methylation sensitive restriction enzymes" are DNA restriction endonucleases that are dependent on the methylation state of their DNA recognition site for activity. For example, there are methyl-sensitive enzymes that cleave or digest at their DNA recognition sequence only if it is not methylated. Thus, an unmethylated DNA sample will be cut into smaller fragments than a methylated DNA sample. Similarly, a hypermethylated DNA sample will not be cleaved. In contrast, there are methyl-sensitive enzymes that cleave at their DNA recognition sequence only if it is methylated. As used herein, the terms "cleave", "cut" and "digest" are used interchangeably.

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A "biological sample" according to any aspect of the present invention may comprise any biological material obtained from the subject or group of subjects that contains genomic material, and may be liquid, solid or both, may be tissue or bone, or a body fluid such as blood, lymph, saliva, semen etc. In particular, the biological sample useful for the present invention may comprise biological cells or fragments thereof.

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As used herein, the term "pre-selected methylation sites" refers to methylation sites that were selected from genes or regions that showed the highest degree of methylation variation during the training of the method and fulfils certain quality criteria such as a minimum sequencing coverage of $\geq 5\times$ were considered and for ≥ 5 qualified CpG sites. Additionally, genes that have an average

5 methylation level < 0.1 or an average methylation level > 0.9 can be excluded due to their limited dynamic range.

"Reference methylation profiles" may be defined on the basis of multiple training samples using multivariate statistical methods, such as such as Principal Component analysis or Multi-

10 Dimensional Scaling.

The term "pre-determined reference profile" used herein refers to a typical or standard methylation profile of the genomic material of a type of reference animal that is confirmed or shown to have good performance and/or good health in the industry. In one example, the pre-determined

15 reference profile may be used in the context of a control animal, where the control animal has exhibited good performance traits (i.e. the control animal exhibits what is considered to be good values in at least one metric selected from the group consisting of bodyweight or carcass weight, growth rate, animal feed intake, feed conversion ratio (FCR), digestive function, gut inflammation status, medical costs, transition period, use of antibiotics, mortality and combinations thereof of the

20 animal). In particular, the term "pre-determined reference profile" herein may be used in the context of a control animal, where the control animal has good performance and/or general health wherein the control animal has improved bodyweight or carcass weight, growth rate, animal feed intake, feed conversion ratio (FCR), digestive function, gut inflammation status, reduced medical costs, transition period, use of antibiotics, mortality, reproductive measurements and combinations of

25 these traits thereof compared to baseline values of an animal of the same taxon as the control animal. Examples of reproductive measurements may include percentage of progeny born alive, litter size and the like.

As used herein, the term "baseline" used relative to performance or performance traits refers to

30 various aspects of an animal when the animal is fed animal feed without one or more optional feed supplements. Examples of baseline performance include an animal's meat/ milk production and/or meat/ milk production efficiency. A panel of pre-determined reference profiles for control animals may also include profiles from different samples that have been obtained from different parts of control animals (animals with different levels of performance and/or general health). For example,

35 the panel of pre-determined reference profiles may include at least one profile for egg, at least one profile for meat (muscle, tissue, organs etc.), at least one profile for milk and the like. Each of these samples may have its own unique pre-determined methylation reference profile that also forms a part of the panel of pre-determined reference profiles. The panel may also include a pre-determined reference profile for each of these animal derived products and animals thereof specific

40 for each merit of an animal related to general health and/or performance of the animal.

A panel of pre-determined reference profiles may be prepared for different samples that are from animals of the same taxon as the test animal that have been confirmed in the industry to exhibit good performance and/ or good health. Again here, there may be a panel of pre-determined reference profiles for each product that is derived from the animal which exhibits at least one merit showing good performance and/or good health. For example, the panel of pre-determined reference profiles for cow may include at least one profile for meat (muscle, tissue, organs etc.), at least one profile for milk and at least a second panel of pre-determined reference profiles where one profile is for the sperm, tissue, blood, etc. of a living cow and each profile is related one merit showing good performance and/or good health. For example, one of the pre-determined reference profiles in the panel, is for tissue sample of a cow with improved bodyweight or carcass weight, another of the pre-determined reference profiles is for tissue sample of a cow with high growth rate, and another for tissue sample of a cow with good animal feed intake, and another with high feed conversion ratio (FCR), and another with good digestive function, and another profile is for tissue sample of a cow with mild gut inflammation and another for tissue sample of a cow with no gut inflammation and another of the pre-determined reference profiles is for tissue sample of a cow with a combination of merits that a cow with good performance/ good general health exhibit. Each of these samples may have its own unique pre-determined methylation reference profile that also forms a part of the panel of pre-determined reference profiles. A third panel of pre-determined reference profiles for cow may include at least one profile for meat (muscle, tissue, organs etc.), at least one profile for milk and at least a fourth panel of pre-determined reference profiles where one profile is for the sperm, tissue, blood, etc. of a living cow and each profile is related one merit showing poor performance and/or poor health. For example, one of the pre-determined reference profiles in the panel, is for tissue sample of a cow with low bodyweight or carcass weight, another of the pre-determined reference profiles is for tissue sample of a cow with low growth rate, and another for tissue sample of a cow with poor animal feed intake, and another with low feed conversion ratio (FCR), and another with poor digestive function, and another profile is for tissue sample of a cow with severe gut inflammation and another of the pre-determined reference profiles is for tissue sample of a cow with a combination of merits that a cow with poor performance/ bad general health exhibit. Each of these samples may have its own unique pre-determined methylation reference profile that also forms a part of the panel of pre-determined reference profiles. In yet another example, the panel may be based on the animal of a different taxon. In a further example, the panel may be based on a timeline that is to say there are different DNA methylation profiles from a control animal at different time points each specific for a control animal with good performance and/or general health or poor performance and/or general health.

There may be a compilation of several pre-determined reference profiles and comparing the methylation profile of the test sample with the pre-determined reference profiles in the compilation may enable identifying the specific pre-determined reference profile that is (significantly) similar to the methylation profile of the test sample and then the test animal from which the test sample is derived may be confirmed to have good performance and/or good general health now or is expected to have good performance and/or good general health in the future. In one example, the pre-determined reference profiles may include methylation profiles of different parts of meat (i.e.

breast, thigh, kidney, liver, shoulder, ribs, intestines, etc.) from an animal (chicken, goat, cow, lamb, sheep etc.) with good performance and/or good general health.

5 In particular, the panel of predetermined reference methylation profiles according to any aspect of the present invention is distinct for different test animal-derived products or animal parts from which the DNA sample is derived from. That is to say, each predetermined reference methylation profile is distinct for a single animal-derived product or animal part. The panel of predetermined reference methylation profiles may thus include many different predetermined reference methylation profiles from different parts of an animal or several animals of the same biological taxon as the test animal.

10 There will also be different panels of predetermined reference methylation profiles for different animal taxon, and the relevant panel of predetermined reference methylation profiles unique for an animal taxon will depend on the animal taxon of the test animal.

The term "significantly similar" in context of the present disclosure, and in particular in context with the comparison of methylation profiles (such as the comparison between test profiles (from test subject(s) (i.e. sample X) and reference profiles) shall mean a similarity observed by statistical means (i.e. by using bioinformatics) and/or also by observation using the eye. A significant similarity is observed for example if a test profile overlaps with a reference profile that is defined by multiple training samples through multivariate statistical methods, such as Principal Component analysis or Multi-Dimensional Scaling. In particular, a test profile is significantly similar to the pre-determined reference profile if more than 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99% of the methylation pattern/ profile overlaps with that of the reference profile. A similarity of a test profile to more than one, such as two, three or even all reference profile reduces the significance of the similarity. Of course, the similarity of the test methylation profile to the reference methylation profile takes into consideration the experimental error that occurs in all methods.

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The animal-derived product sample may be a single type of meat, different types of meat, a single part of a type of meat, different parts of a single type of meat or different parts of different types of meat. The sample may be from any biological entity having a DNA genome and DNA genome methylation. In particular, the methylation site is a CpG site. The biological entity may be any animal excluding a pig. In particular, the animal may be selected from the group consisting of chicken, lamb, camel, cow, goat, sheep, horse, donkey, turkey, duck, goose, quail, rabbit and mule. More in particular, the animal may be selected from the group consisting of cow, sheep, goat, camel, chicken, goose, duck and turkey. The term 'meat' herein may thus be understood to include chicken, lamb, beef, mutton, goat, camel, chicken, goose, duck, turkey and mixtures thereof.

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The one or more pre-selected methylation sites in (a) are methylation sites associated with tissue specific gene expression, preferably wherein the pre-selected methylation sites are associated with gene expression of one distinct tissue.

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The tissue may be selected from

(i) metabolic tissue such as gut tissue, said gut tissue preferably being ileum or jejunum,

- (ii) muscular tissue,
- (iii) skin tissue, and
- (iv) organ tissue, said organ tissue preferably being hepatic and / or pancreatic tissue.

- 5 In particular, the difference according to any aspect of the present invention refer to a difference in methylation and is hypomethylation or hypermethylation.

According to another aspect of the present invention, there is provided an *in vitro* method for establishing the effect of at least one test component of animal feed on performance and/or general

- 10 health of a test animal consuming the animal feed, the method comprising the steps of:

(a) determining the test methylation level of a set of specific CpG sites in test DNA obtained from the test animal before consuming of the animal feed, T0,

(b) determining the methylation level of the same set of specific CpG sites in (a) in test DNA obtained from the test animal at a specific time point after consuming of the animal
15 feed, T1, and

(c) comparing the methylation levels of the same set of specific CpG sites from (a) and (b) with the methylation level of the same CpG sites from a reference that correlates DNA methylation patterns to performance and/or general health of the animal,

- 20 thereby establishing the effect of the test component of animal feed on the performance and/or general health of the test animal,
wherein the test animal is selected from livestock or poultry.

- In particular, the method according to this aspect of the present invention may be used to predict the effect of at least one test component of animal feed on performance and/or general health of a
25 test animal consuming the animal feed at the next time point T2.

According to one aspect of the present invention, there is provided an *in vitro* method for establishing the effect of at least one test component of animal feed on performance and/or general health of a test animal consuming the animal feed, the method comprising the steps of:

- 30 (a) determining the test methylation level of a set of specific CpG sites in test DNA obtained from the test animal consuming of the animal feed, ,

(b) determining the methylation level of the same set of specific CpG sites in (a) in DNA obtained from a control animal not consuming of the animal feed, and

- 35 (c) comparing the methylation levels of the same set of specific CpG sites from (a) and (b) with the methylation level of the same CpG sites from a reference that correlates DNA methylation patterns to performance and/or general health of the animal,

thereby establishing the effect of the test component of animal feed on the performance and/or general health of the test animal,

wherein the test animal is selected from livestock or poultry.

According to yet another aspect of the present invention, there is provided a method of assessing the effect of at least one component of animal feed on gut inflammation status of a meat producing and/or milk producing test animal consuming the animal feed, the method comprising the steps of:

5 (a) determining a test methylation profile of one or more pre-selected methylation sites within the genomic material obtained from the test animal; and

(b) comparing the test methylation profile obtained from (a) with at least one first reference methylation profile obtained from a control animal of the same biological taxon as the test animal

10 having positive gut inflammation status and/or with at least one second reference methylation profile obtained from a control animal of the same biological taxon as the test animal having negative gut inflammation status; and

wherein a significant similarity in the test methylation profile of (a) compared to the first or second reference methylation profile of the control animal, is indicative of the test animal having positive or

15 negative gut inflammation status as the control animal respectively;

and

wherein a difference in the test methylation profile of (a) compared to the first or second reference methylation profile of the control animal, is indicative of the test animal having negative or positive gut inflammation status as the control animal respectively.

20 The term "negative gut inflammation status" refers to gut material free of any signs of inflammatory processes. As used herein the term "gut inflammation" and "intestinal inflammation" are used interchangeably and have identical meanings. The expression "classifying the gut inflammation status" refers to both, the classification whether or not there are inflammatory processes ongoing (YES/NO), as well as to the classification into different inflammation grades (e.g. severe, moderate

25 or weak inflammations). In particular, the positive gut inflammation status is further classified into classes of severely inflamed, moderately inflamed, or weakly inflamed based on the average methylation level. Positive gut inflammation status is further classified into classes of severely inflamed, moderately inflamed, or weakly inflamed based on the average methylation level. The

30 genomic material used according to any aspect of the present invention may be obtained from a gut test sample and the gut test sample is gut tissue, preferably the gut tissue is ileum or jejunum.

According to a further aspect of the present invention, there is provided a method of assessing the effect of at least one test component of animal feed on growth rate and/ or feed conversion ratio

35 (FCR) of a meat and/or a milk producing test animal consuming the animal feed with the test component, the method comprising the steps of:

(a) determining a test methylation profile of one or more pre-selected methylation sites within the DNA obtained from the test animal; and

(b) comparing the test methylation profile obtained from (a) with at least one control methylation

40 profile from a control animal of the same biological taxon as the test animal consuming animal feed without the test component; and

- wherein the results of (a) is used to determine the growth rate and/ or feed conversion ratio (FCR) of the test animal; and
- wherein a significant similarity in the test methylation profile of (a) compared to the control methylation profile, is indicative of the test component not having an effect on the growth rate and/
- 5 or FCR of the test animal; and
- wherein a significant difference in the test methylation profile of (a) compared to the control methylation profile, is indicative of the test component having an effect on the growth rate and/ or FCR of the test animal.
- 10 According to this aspect of the present invention, there is provided a further step of
- (c) comparing the test methylation profile obtained from (a) with
- (i) a first reference methylation profile obtained from a control animal of the same biological taxon as the test animal having fast growth rate and/or FCR; and/or
- (ii) a second reference methylation profile obtained from a control animal of the same biological
- 15 taxon as the test animal having slow growth rate and/or FCR; and
- wherein a significant similarity in the test methylation profile of (a) to the first reference methylation profile and/or a difference in the test methylation profile of (a) to the second reference methylation profile is indicative of the test animal having fast growth rate and/or FCR; and/or
- wherein a difference in the test methylation profile of (a) compared to the first reference methylation
- 20 profile and/or a significant similarity in the test methylation profile of (a) to the second reference methylation profile is indicative of the test animal having slow growth rate and/or FCR.
- According to a further aspect of the present invention, there is provided a method of assessing the effect of at least one component of animal feed on growth rate of a meat, egg and/or a milk
- 25 producing test animal consuming the animal feed, the method comprising the steps of:
- (a) determining a test methylation profile of one or more pre-selected methylation sites within the genomic material obtained from the test animal; and
- (b) comparing the test methylation profile obtained from (a) with at least one first reference methylation profile obtained from a control animal of the same biological taxon as the test
- 30 animal having slow growth rate and/or with at least one second reference methylation profile obtained from a control animal of the same biological taxon as the test animal having fast growth rate; and
- wherein a significant similarity in the test methylation profile of (a) compared to the first or second reference methylation profile of the control animal, is indicative of the test animal having slow or
- 35 fast growth rate as the control animal respectively; and
- wherein a difference in the test methylation profile of (a) compared to the first or second reference methylation profile of the control animal, is indicative of the test animal having fast or slow growth rate as the control animal respectively.
- 40 As used herein, the term 'animal-derived product' refers to products that originate from animals. In particular, the term 'test animal-derived product' refers to the sample or subject in question that is to be introduced to the array according to any aspect of the present invention. These products from

animals may include meat and meat products, also including fat, flesh, blood, processed meat, and lesser-known products, such as isinglass and rennet, poultry products (meat and eggs), dairy products (milk and cheese), and non-food products such as fibre (wool, mohair, cashmere, leather, and the like). Animal-derived products may also include products that can be made using animal products (e.g., fat) such as soap, creams, and such. In one example, the animal-derived product is meat, eggs, blood, brain, sperm/ semen, milk and any other tissue or sample that provides genomic DNA. In particular, the animal-derived product is meat. In one example, the animal-derived product sample may be a single type of meat, different types of meat, a single part of a type of meat, different parts of a single type of meat or different parts of different types of meat. In the event the animal is an aquatic animal, these products from animals may include meat and meat products, also including eggs, fat, flesh, blood, rumen, processed meat and lesser-known products, and non-food products such as fibre (shells, scales and the like). Animal-derived products may also include products that can be made using animal products (e.g. fish oil) such as tablets, powder and such. In one example, the animal-derived product is meat, eggs, blood, brain, shell, scale, skin, tissue, abdominal muscle tissue or any other tissue or sample that provides genomic DNA.

In particular, the animal-derived product is meat, skin, blood, trimmings or any organ from the aquatic animal. In particular, trimmings are used as biproducts for fish meal/oil which end up in the animal feed industry or pets. The sample may be from any biological entity having a DNA genome and DNA genome methylation. In particular, the methylation site is a CpG site.

The test methylation profile of step (a) according to any aspect of the present invention may be determined by contacting the genomic material of the test animal with a DNA methylation-based array.

The term "array" as used herein refers to an intentionally created collection of probe molecules which can be prepared either synthetically or biosynthetically. The probe molecules in the array can be identical or different from each other. The array can assume a variety of formats, for example, libraries of soluble molecules; libraries of compounds tethered to resin beads, silica chips, or other solid supports.

In particular, a DNA methylation-based array provides a convenient platform for simultaneous analysis of large numbers of CpG sites, for example, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 50, 100, 500, 1000, 5000, 10,000, 100,000 or more sites or loci. In particular, the array comprises a plurality of different probe molecules that can be attached to a substrate or otherwise spatially distinguished in an array. Examples of arrays that may be used according to any aspect of the present invention include slide arrays, silicon wafer arrays, liquid arrays, bead-based arrays and the like. In one example, array technology used according to any aspect of the present invention combines a miniaturized array platform, a high level of assay multiplexing, and scalable automation for sample handling and data processing.

In particular, the array according to any aspect of the present invention may be an array of arrays, also referred to as a composite array, having a plurality of individual arrays that is configured to allow processing of multiple samples simultaneously. Examples of composite arrays and the technology behind them are disclosed at least in US 6,429,027 and US 2002/0102578. A substrate of a composite array may include a plurality of individual array locations, each having a plurality of probes, and each physically separated from other assay locations on the same substrate such that a fluid contacting one array location is prevented from contacting another array location. Each array location can have a plurality of different probe molecules that are directly attached to the substrate or that are attached to the substrate via rigid particles in wells (also referred to herein as beads in wells).

In one example, an array substrate can be a fibre optical bundle or array of bundles as described in US6,023,540, US6,200,737 and/or US6,327,410. An optical fibre bundle or array of bundles can have probes attached directly to the fibres or via beads. A skilled person would be able to easily determine which substrate will be most suitable for the array according to any aspect of the present invention. WO2004110246 further discloses other substrates and methods of attaching beads to the substrates that may be used in the array according to any aspect of the present invention.

In one example, a surface of the substrate may have physical alterations to enable the attachment of probes or produce array locations. For example, the surface of a substrate can be modified to contain chemically modified sites that are useful for attaching, either-covalently or non-covalently, probe molecules or particles having attached probe molecules. Probes may be attached using any of a variety of methods known in the art including, an ink-jet printing method, a spotting technique, a photolithographic synthesis method, or printing method utilizing a mask. WO2004110246 discloses these techniques in more detail.

In one example, the DNA methylation-based array according to any aspect of the present invention may be a bead-based array, where the beads are associated with a solid support such as those commercially available from Illumina, Inc. (San Diego, Calif.). An array of beads useful according to any aspect of the present invention can also be in a fluid format such as a fluid stream of a flow cytometer or similar device. Commercially available fluid formats for distinguishing beads include, for example, those used in XMAP(TM) technologies from Luminex or MPSS(TM) methods from Lynx Therapeutics.

The term "solid support", "support", and "substrate" as used herein are used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many examples, at least one surface of the solid support will be substantially flat, although in some examples it may be desirable to physically separate synthesis regions for different compounds with, for example, wells, raised regions, pins, etched trenches, or the like.

The DNA methylation array according to any aspect of the present invention may be a very high-density array, for example, those having from about 10,000,000 probes/cm² to about 2,000,000,000 probes/cm² or from about 100,000,000 probes/cm² to about 1,000,000,000

probes/cm². High density arrays are especially useful according to any aspect of the present invention for including the multitude of CpG sites from the different species on the array.

5 The DNA methylation array according to any aspect of the present invention may be used to analyse or evaluate such pluralities of loci simultaneously or sequentially as desired. In one example, a plurality of different probe molecules can be attached to a substrate or otherwise spatially distinguished in an array. Each probe is typically specific for a particular locus and can be used to distinguish methylation state of the locus.

10 The term "probe molecules" as used herein refers to a surface-immobilized molecule that can be recognized by a particular target. Probes used in the array can be specific for the methylated allele of a CpG site, the non-methylated allele of the CpG site or both.

The term "target" as used herein refers to a molecule that has an affinity for a given probe
15 molecule. Targets may be naturally occurring or man-made molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Targets may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of targets which can be employed according to any aspect of the present invention are methylated and non-methylated CpG sites. Targets are sometimes referred to in the art as anti-probes. As the
20 term targets is used herein, no difference in meaning is intended.

In particular, the probe molecule according to any aspect of the present invention comprises a nucleic acid sequence that is complementary to a distinct CpG site. The array according to any aspect of the present invention thus comprises several distinct or unique locations, wherein each
25 location comprises a specific probe molecule that is complementary to a distinct CpG site of the animal. The array thus comprises a plurality of locations, each location with a specific probe molecule that is complementary to a distinct CpG site of the animal.

The term "complementary" as used herein refers to the hybridization or base pairing between
30 nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single stranded nucleic acid to be sequenced or amplified. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate
35 nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Perfectly complementary refers to 100% complementarity over the length of a sequence. For example, a 25-base probe is perfectly complementary to a target when all 25 bases of the probe are complementary to a contiguous 25 base sequence of the target with no mismatches between the
40 probe and the target over the length of the probe.

According to one aspect of the present invention, there is provided a method of establishing a reference that correlates DNA methylation patterns to performance and/or general health of at least one animal, the method comprising:

- 5 (a) determining the methylation ratio and the read coverage of genomic CpG sites of a performance and/or general health -correlated training sample of a specific animal tissue;
 - (b) defining a set of CpG sites having methylation ratios in all training samples of step (a) using a read coverage cutoff value; and
 - (c) performing a penalized regression model using the methylation ratios of step (b) as
10 input and the performance and/or general health correlated to the training sample as dependent variable, by applying the penalized regression model;
- thereby obtaining a set of CpG sites with corresponding weighting factors and intercept of the penalized regression model as parameters defining the reference that correlates DNA methylation patterns to performance and/or general health of the animal,
wherein the test animal is selected from livestock or poultry.

15 According to this aspect of the present invention, the performance and/or general health of the animal should be a quantitative value. For example, a metric of performance and/or general health.

The method according to any aspect of the present invention, involves the identification of a
20 number of CpG (Cytosine-phosphate Guanine) sites in an animal genome for which the level of DNA methylation is both tissue-specifically and tissue-independently correlated with the performance and/or growth rate of the animal. That is, measuring DNA methylation at these locations (CpG sites) enables making accurate predictions of the performance and/or growth rate of the animal.

25 The term "methylation ratio" refers to number of methylated cytosine(s) divided by the total number of cytosine(s) covered at the specific site(s).

The term "read coverage of the CpG site" is to be understood as the number of reads that align
30 with the known CpG site in the reference sequence.

The methylation ratio and the read coverage of the genomic CpG sites may be determined in step (a) using bisulfite sequencing.

35 The performance and/or growth rate-correlated training sample of a specific tissue used in step (a) may, for example, be gut tissue, muscle tissue, organ tissue or skin tissue.

In particular, the coverage cutoff value defined in step (b) may be at least 3. More in particular, the coverage cutoff value defined in step (b) may be 3 and above.

40 The method according to at least this aspect of the present invention may further comprise a step of (d) optimizing the fit of the penalized regression model such that the number of CpG sites is

reduced to below 100. For optimizing the fit of the penalized regression model in step (d), the algorithm preferably applies ridge regression in combination with lasso regression.

Advantageously, the methods of ridge regression and lasso regression are balanced using an alpha value of between 0 and 1. The penalized regression model is a linear model.

5

According to a further aspect of the present invention, there is provided a use of DNA methylation profiling for assessing the effect of at least one component of animal feed on performance and/or general health of a test animal consuming the animal feed.

- 10 According to yet another aspect of the present invention, there is provided a DNA methylation-based array for carrying out the method according to any aspect of the present invention.

EXAMPLES

- 15 The foregoing describes preferred embodiments, which, as will be understood by those skilled in the art, may be subject to variations or modifications in design, construction or operation without departing from the scope of the claims. These variations, for instance, are intended to be covered by the scope of the claims.

Example 1

- 20 *Assessment of nutrient digestibility in pigs*

Nutrient digestibility in pigs can be measured in several ways currently but there are drawbacks to each (Zhang and Adeola, 2017, *Animal Nutrition* 3:344e352). Indigestible markers can be added to the feed and then nutrients in the feed vs feces measured in comparison to the marker to assess the amount digested by the animal. However, hindgut fermentation and endogenous losses (e.g. host intestinal cell sloughing) cannot be accounted for and create inaccuracies in the calculated digestibility. Animals can be cannulated at the ileum so that digesta samples can be taken at one of the final points of digestive small intestine before hindgut fermentation can occur, which makes for more accurate digestibility calculations. However, animals need to undergo surgical procedures prior to the digestibility study to place the canula which raises both ethical concerns and questions regarding how canulation itself might affect the physiology and therefore digestibility of the animal.

- Epigenetic signatures of 'good' vs 'bad' digestibility are therefore established from the blood epigenome of pigs, to create a less invasive but still accurate method of determining nutrient digestibility. Blood is collected from grower pigs between 9 and 14 weeks of age. The piglets are from various trials testing digestibility of nutrients in response to different feed ingredients or feed additives (e.g. probiotics or enzymes). The blood is sorted into categories of 'good' and 'poor' digestibility based on the various methods utilised in the studies including faeces marker digestibility measurements and digesta measurements from cannulas. At least 100 replicate samples from each category are extracted for DNA. DNA is bisulphite converted and sent for whole genome bisulphite sequencing as per the methods outlined below. Significantly differentially methylated CpGs between the 'good' and 'poor' gut health categories are utilised in random forest

analyses to define distinct epigenetic signatures differentiating good from poor gut health. The CpG sites for these profiles are used to create probes for a livestock methylation bead-based array.

5 In a future digestibility feeding trial one group of 9 week old pigs is fed enzymes and another is untreated as a control for 4 weeks. A sample of 5ml of blood is taken for analysis from each pig every week. DNA is extracted and bisulphite converted before being analysed on the bead-based array. The results show that the nutrient digestibility of pigs fed enzymes is significantly higher than observed in the control group.

10 *DNA Extraction*

DNA is extracted using the PureLink Genomic DNA Isolation Minikit kit (Invitrogen), including RNAase treatment following the manufacturer's instructions. DNA quantity is measured by PicoGreen assay and DNA quality is assessed via NanoDrop (Thermo Scientific) to ensure the A260/280 ratio is ≤ 1.8 . A small amount of sample is analysed on an agarose gel to ensure each
15 sample contains high molecular weight DNA.

Bisulfite Conversion and BeadChip Analysis

The genomic DNA samples are subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research). The methylation levels are then quantified using Methylation EPIC
20 BeadChip kits (Illumina) which can analyze over 50,000 methylation sites quantitatively across the genome at single-nucleotide resolution.

Data processing

Sequenced reads are trimmed and mapped with BSMAP1 version 2.5 using the assembly version
25 5.0 of the chicken (Gallus gallus) genome as reference sequence. After deduplication using picard2, the methylation ratios are determined using a Python script (methratio.py) distributed with the BSMAP package. To analyse the ability to classify the samples based on the methylation data for treatments with different antibiotics, the R package random Forest is used and a random forest-based classification is set up, using the most variable CpGs in each group.

30

The customized chip array data processing is performed in R version 4.1.2 using sesame version 1.14.2. DNA methylation level for each site is calculated as methylation β -value. Beta values are defined as methylated signal/(methylated signal + unmethylated signal). It is computed using getBetas function. Quality check on samples is performed to remove probes that have a detection
35 P-value < 0.05 in all samples. The SeSAMe pipeline (Zhou et al. 2018, Nucleic Acids Res; 46(20):e123.) is used to generate normalized β -values and for quality control. Low intensity- based detection calling and making (based on p-value) is done with pOOBAH. Background subtraction based on normal-exponential deconvolution using out-of-band probes noob (Triche et al. 2013, Nucleic Acids Res.; 41(7): e90.) and optionally with extra bleed-through subtraction also
40 implemented.

The methylation profile of sample is then input into the built random forest classifier to identify the accurate feed group.

Results elucidate a methylation profile consistent with positive attributes of nutrient digestibility in piglets fed enzymes, while control piglets not fed enzymes align more with a poor digestibility profile.

5

Example 2

Assessment of feed efficiency in broiler chickens in response to different nutrient source supplementation in feed.

- 10 Feed conversion ratio (FCR; Amount of feed ingested in kg / amount of live weight gained in kg) is a major determinant of profitability for chicken producers. To date FCR is generally measured by weighing out the feed intake of an entire pen or barn of birds and dividing by the total weight of the animals in that pen or barn. This requires many animals and a duration of time in which to measure the changes. In order to measure feed efficiency of broiler chicks in real time with fewer animals an
- 15 epigenetic signature of FCR is created. Blood and breast meat from 1000 broiler chickens from various trials is collected, with known and individually measured FCR for each bird. DNA is extracted from the blood and breast muscle tissue of each of the broilers, bisulphite converted and sent for whole genome bisulphite sequencing. CpG positions in the broiler bird population sampled is correlated to the range of FCRs measured to create a penalized regression models that allows
- 20 for the prediction of individual broiler FCR from DNA-methylation profiles. The relevant CpG sites are then used to create a custom methylation bead-based array for determination of individual broiler FCR in real time.

- A broiler trial is conducted to compare 2 different sources of supplemental methionine, as an
- 25 essential and often limiting amino acid in broiler diets. A total of eleven dietary treatments are used, including a diet without any methionine supplementation that is insufficient in methionine as well as 5 titrated doses of both supplemental methionine sources, ranging from insufficient, to sufficient to over supplementation. Each dietary treatment is fed to only 20 broilers each for a 1 week period. At the end of this period blood is taken from each of the broilers for DNA extraction. DNA from each
- 30 sample is bisulphite converted and analysed with the custom bead-based array for FCR determination. Results show that the lowest FCR (i.e. the most efficient utilisation of nutrients for broiler bird growth) is observed in the 4th supplementation level and with the first methionine source compared to the second. A recommendation for nutrient supplementation level and source is therefore possible with only 220 broilers, compared to standard trials that rely on weighing of
- 35 animals and feed that could require 10 pens of 20 birds each per treatment and therefore 2200 broilers.

Data analysis

- All CpGs that were associated with sex chromosomes are removed. All CpGs that are listed as
- 40 SNPs for the Gallus gallus genome in the dbSNP database (<https://www.ncbi.nlm.nih.gov/snp/>) are filtered out. The analysis is restricted to CpGs that show a strand specific coverage of greater

than 10 in every of the sequenced samples. To establish a chicken methylation clock for FCR, a penalized regression model (implemented in the R-package glmnet⁴⁷) is applied to regress the FCR of the animals on the normalized methylation values of the CpG probes. This approach computationally assigns weights to the set of CpG probes and thus selects an optimized set of
5 markers.

The customized chip array data processing is performed in R version 4.1.2 using sesame version 1.14.2. DNA methylation level for each site was calculated as methylation β -value. Beta values are defined as methylated signal/(methylated signal + unmethylated signal). It can be computed using
10 getBetas function. Quality check on samples is performed to remove probes that have a detection P-value < 0.05 in all samples. The SeSAmE pipeline (Zhou et al. 2018, Nucleic Acids Res; 46(20):e123.) is used to generate normalized β -values and for quality control. Low intensity- based detection calling and making (based on p-value) is done with pOOBAH. Background subtraction based on normal-exponential deconvolution using out-of-band probes noob (Triche et al. 2013,
15 Nucleic Acids Res.; 41(7): e90.) and optionally with extra bleed-through subtraction are also implemented.

The FCR is computed based on the methylation profile of individual broiler using the FCR methylation clock.

CLAIMS

1. A method of assessing the effect of at least one test component of animal feed on at least performance and/or general health of a test animal consuming the animal feed with the test component, the method comprising the steps of:
- 5 (a) determining a test methylation profile of one or more pre-selected methylation sites within the DNA of the test animal;
- (b) comparing the test methylation profile obtained from (a) with at least one control methylation profile from a control animal of the same biological taxon as the test animal
- 10 consuming animal feed without the test component; and
- (c) comparing the test methylation profile obtained from (a) with
- (i) a first reference methylation profile obtained from a control animal of the same biological taxon as the test animal having good performance and/or general health; and/or
- 15 (ii) a second reference methylation profile obtained from a control animal of the same biological taxon as the test animal having poor performance and/or general health; and
- wherein the results of (a) is used to determine the performance and/or general health of the test animal; and
- wherein a significant similarity in the test methylation profile of (a) compared to the control methylation profile, is indicative of the test component not having an effect on the
- 20 performance and/or general health of the test animal; and
- wherein a significant difference in the test methylation profile of (a) compared to the control methylation profile, is indicative of the test component having an effect on the performance and/or general health of the test animal; and
- 25 wherein a significant similarity in the test methylation profile of (a) to the first reference methylation profile and/or a difference in the test methylation profile of (a) to the second reference methylation profile is indicative of the test animal having good performance and/or general health as the control cell; and/or
- wherein a difference in the test methylation profile of (a) compared to the first reference methylation profile and/or a significant similarity in the test methylation profile of (a) to the
- 30 second reference methylation profile is indicative of the test animal having poor performance and/or general health as the control animal;
- and
- wherein the test animal is selected from livestock or poultry.
- 35
2. The method according to claim 1, wherein the general health of the animal is a measure of at least one metric of the animal and the metric is selected from the group consisting of bodyweight or carcass weight, growth rate, animal feed intake, feed conversion ratio (FCR), digestive function, gut inflammation status, medical costs, transition period, use of
- 40 antibiotics, mortality and reproductive measurements.

3. An *in vitro* method for establishing the effect of at least one test component of animal feed on performance and/or general health of a test animal consuming the animal feed, the method comprising the steps of:
- 5 (a) determining the test methylation level of a set of specific CpG sites in test DNA obtained from the test animal before consuming of the animal feed, T0,
- (b) determining the methylation level of the same set of specific CpG sites in (a) in test DNA obtained from the test animal at a specific time point after consuming of the animal feed, T1, and
- 10 (c) comparing the methylation levels of the same set of specific CpG sites from (a) and (b) with the methylation level of the same CpG sites from a reference that correlates DNA methylation patterns to performance and/or general health of the animal, thereby establishing the effect of the test component of animal feed on the performance and/or general health of the test animal, and wherein the test animal is selected from livestock or poultry.
- 15 4. The method according to any one of the preceding claims, wherein the livestock is aquatic livestock and is selected from the group consisting of carps, salmonids, tilapias, catfish, marine and brackish fishes, soft-shelled turtles, barramundi, marine shrimp, mitten crabs, marbled crayfish and other decapod crustaceans, bivalves, gastropods, or the livestock is
- 20 terrestrial livestock and is selected from the group consisting of cow, goat, sheep, pig, horse, donkey, rabbit and mule and/or the livestock is poultry and is selected from the group consisting of chicken, turkey, duck, goose, and quail.
5. The method according to any one of the preceding claims, for assessing the effect of the test component of animal feed on growth rate and/ or feed conversion ratio (FCR) of a meat and/or a milk producing test animal consuming the animal feed with the test component.
- 25 6. The method according to any one of the preceding claims, wherein the component of the animal feed is selected from the group consisting of compound feeds, medicated feed additives, enzymes, probiotic microbials, direct-fed microbials, antimicrobials, prebiotics,
- 30 phytochemicals, immunomodulators, antibodies, plant extracts, essential oils, organic acids, antioxidants, amino acids, oligosaccharides, oleoresins, herbs, spices, saponins, sea plants, vitamins, minerals, chemical preservatives, antibiotics, and fermentation products.
- 35 7. The method according to any one of the preceding claims, wherein the one or more pre-selected methylation sites in (a) are methylation sites associated with tissue specific gene expression, preferably wherein the pre-selected methylation sites are associated with gene expression of one distinct tissue.
- 40 8. The method according to any one of the preceding claims, wherein the test methylation profile of step (a) is determined by contacting the genomic material of the test animal with a DNA methylation-based array.

9. The method according to claim 8, wherein the DNA methylation-based array is a bead-based array.
- 5 10. A method of establishing a reference for use in the method according to any one of the preceding claims, the method comprising:
- 10 (a) determining the methylation ratio and the read coverage of genomic CpG sites of a performance and/or general health -correlated training sample of a specific animal DNA;
- (b) defining a set of CpG sites having methylation ratios in all training samples of step (a) using a read coverage cutoff value; and
- (c) performing a penalized regression model using the methylation ratios of step (b) as input and the performance and/or general health correlated to the training sample as dependent variable, by applying the penalized regression model;
- 15 thereby obtaining a set of CpG sites with corresponding weighting factors and intercept of the penalized regression model as parameters defining the reference that correlates DNA methylation patterns to performance and/or general health of the animal, wherein the test animal is selected from livestock or poultry.
- 20 11. The method according to claim 10, wherein the methylation ratio and the read coverage of the genomic CpG sites are determined in step (a) using bisulfite sequencing.