

DETECTING ANTIBIOTIC AND/OR CHEMICAL USE IN ANIMALS USING EPIGENETIC MEANS**FIELD OF THE INVENTION**

5 The present invention relates to a method for detecting the use and/or administration of at least one antibiotic and/or veterinary chemical to an animal using epigenetic means. In particular, the method is capable of detecting the use of at least one antibiotic or the administration of at least one antibiotic an/or veterinary chemical during the breeding or rearing of at least one terrestrial animal by determining the methylation status of a CpG site in a test animal and comparing the resultant methylation status with a
10 reference methylation status of a control animal which was bred without the use of or administration of an antibiotic and/or veterinary chemical.

BACKGROUND OF THE INVENTION

Antibiotics are used for the treatment of bacterial diseases in human and veterinary medicine. Antibiotics
15 have also been used prophylactically at low levels in feed or water to improve growth rates and mortality levels in livestock. However, overuse of antibiotics especially in agriculture, has been associated with an increase in antibiotic resistant bacteria, which will eventually impair the ability to treat bacterial diseases in humans and animals alike. Additionally, antibiotic usage has been associated with other unintended and undesirable physiological consequences like low birth weight in infants from antibiotic
20 treated mothers, increased disease susceptibility, and the like. Tools for assessing not only current, but historical antibiotic use would therefore be of high interest in livestock production for the purpose of guiding breeding, feeding, veterinary and/or management practices. Additionally, analysis of meat and meat products to assess historic antibiotic use in the individual animal prior to slaughter could be of high interest for auditing, certifying, and labelling of meat products.

25 To date antibiotic testing involves chemical assays to find residues of antibiotics present in the blood or tissues of animals. However, most antibiotics used for growth promotion are either added to the feed or water and most of these classes are poorly absorbed in the gastrointestinal system, so residue testing in blood or tissues may not capture usage of all antibiotic classes, particularly after the withdrawal period (where the animal is no longer given the antibiotics) commences. There is also currently no method by
30 which animal or meat products can be tested for previous antibiotic usage unless antibiotic residues remain in the blood or tissues.

Accordingly, there is a need in the art for a simple and accurate means of detection of current and past antibiotic and/or drug use in animals, particularly livestock.

Epigenetics is the study of inherited traits caused by mechanisms other than changes in the underlying
35 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.

Several studies have shown that antibiotic exposure can change epigenetic markers in eukaryotic cells. For example, in a study examining the relationship between antibiotic usage during pregnancy and birth weight outcomes, it was found that there were specific regions of the genome that were differentially methylated in response to antibiotic exposure (Vidal et al., 2013). A study conducted with cultured plant cells observed changes in global DNA methylation when the cells were exposed to the antibiotic kanamycin (Bardini et al., 2003). However, there is no mention in the art of epigenetic changes that may occur due to current and/or past antibiotic and/or drug use in animals, particularly livestock.

Accordingly, there is still a need in the art for a simple and accurate method for detection of current and/or past antibiotic and/or drug use in animals, particularly livestock.

DESCRIPTION OF THE INVENTION

The present invention solves the problems above by providing a means of using alterations in DNA methylation patterns to develop novel analytics for assessment of antibiotic or other chemical usage in terrestrial agricultural animal production, meat and/or meat products. In particular, the means comprises a method of detecting the use of antibiotics and/or veterinary chemicals in a test animal and/or a test animal from which a product is derived by comparing the methylation status of at least one CpG site in the test animal and the corresponding CpG site in a control animal and/or a control animal from which a product is derived, where no antibiotics and/or veterinary chemicals were used on the control animal and, wherein the presence of hypomethylation or hypermethylation at the CpG site in the test animal is indicative of the test animal having been treated with antibiotics and/or veterinary chemicals. The method according to any aspect of the present invention is a simple and accurate method for detection of current and/or past antibiotic and/or drug use in animals.

The present invention is based on the finding that contact with antibiotics and/or veterinary chemicals to an animal, results in stress in the internal environment of the animal and this can permanently change the genome of the animal through epigenetics. In particular, the capability to adapt to the environment (i.e., presence of antibiotics and/or veterinary chemicals) and maintain the adapted biological pattern depends on epigenetic mechanisms, including DNA methylation. In particular, the present invention is based on the finding that contact with antibiotics and/or veterinary chemicals may also result in changes in epigenetic mechanisms of the animal, including DNA methylation patterns and these patterns may also 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 the presence of antibiotics and/or veterinary chemicals of not just one animal but possibly all the animals that are brought into contact with at least one of antibiotic and/or veterinary chemical. For example, these 'epigenetic fingerprints' may be specific not only for the animal that was brought into contact with antibiotics and/or veterinary chemicals but also for any product that stems from the very animal (an individual). Based on these findings, the present invention provides means to detect use of antibiotics and/or veterinary chemicals in animals, in particular terrestrial animals, for example rearing animals also known as livestock and poultry from which an animal-derived product comes from. In this way, the method according to any aspect of the present invention may then be used to accurately and reliably determine if any test animal or product therefrom has been brought into contact or treated with at least one antibiotic and/or veterinary chemical. The method according to any aspect of the present invention may then use DNA methylation analysis to also differentiate usage of antibiotics prophylactically (growth promotion) from usage of antibiotics therapeutically. DNA methylation analysis may also be a step, in elucidating, whether an animal treated with veterinary chemicals and/or antibiotics was given a withdrawal period from the veterinary chemical and/or antibiotic prior to slaughter or differentiate usage of different classes of antibiotics. The method according to the present invention may also be used for assessment of current and historic antibiotic usage in livestock or meat to investigate epigenetic markers which can then subsequently be used for the creation of analytics to test antibiotic usage in terrestrial livestock species. Further, the method according to any aspect of the present invention may then be used to predict and avoid undesired traits contributed by use of antibiotics and/or veterinary chemicals to the animal welfare and to a more sustainable livestock production. In one example, the method according to any aspect of the present invention may also be used to differentiate the route of administration of antibiotics (i.e., water vs feed vs injection types).

According to one aspect of the present invention, there is provided a method of determining if a test animal and/or a test animal from which a product is derived has been treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical, the method comprising:

- (a) determining a test methylation profile from the genomic material contained in a biological sample obtained from the test animal and/or the animal-derived product; and
- (b) comparing the test methylation profile obtained from (a) with a reference methylation profile obtained from a control animal, where the control animal was not treated and/or is not currently undergoing treatment with at least one antibiotic and/or veterinary chemical,

wherein a difference in the test methylation profile of (a) compared to the reference methylation profile from the control animal, is indicative of the test animal having been treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical; and

wherein the test animal is a terrestrial animal and wherein the veterinary chemical is an anti-parasitic, an anti-viral, a feed additive, a water additive, a disinfectant, glutaraldehyde, and/ or formalin.

In one example, the veterinary chemical is at least one anti-microbial such as an anti-parasitic, an anti-viral or antibiotic.

According to another aspect of the present invention, there is provided a method of determining if a test animal and/or a test animal from which a product is derived has been treated and/or is currently undergoing treatment with at least one antibiotic the method comprising:

- 5 (a) determining a test methylation profile from the genomic material contained in a biological sample obtained from the test animal and/or the animal-derived product; and
- (b) comparing the test methylation profile obtained from (a) with a reference methylation profile obtained from a control animal, where the control animal was not treated and/or is not currently undergoing treatment with at least one antibiotic,

10 wherein a difference in the test methylation profile of (a) compared to the reference methylation profile from the control animal, is indicative of the test animal having been treated and/or is currently undergoing treatment with at least one antibiotic.

According to a further aspect of the present invention, there is provided a method of determining if a test animal and/or a test animal from which a product is derived has been treated and/or is currently undergoing treatment with at least one antibiotic or veterinary chemical or a combination thereof, the method comprising:

- 15 (a) determining the methylation status of at least one CpG site in genomic material contained in a biological sample obtained from the test animal,
- (b) comparing the methylation status of the CpG site from (a) with that of a control animal which was not treated and/or is not currently undergoing treatment with at least one antibiotic and/or veterinary chemical,

20 wherein a difference in the test methylation status of (a) in the test animal compared to the CpG site in the control animal is indicative of the test animal having been treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical; and

25 wherein the test animal is a terrestrial animal and the veterinary chemical is an anti-parasitic, an anti-viral, a feed additive, a water additive, a disinfectant, glutaraldehyde, and/ or formalin.

In one example, the veterinary chemical is at least one anti-microbial such as an anti-parasitic, an anti-viral or antibiotic.

30 The difference in methylation status according to any aspect of the present invention is hypomethylation or hypermethylation of the CpG site in the test animal and the hypomethylation or hypermethylation of the CpG site is indicative of the test animal having been treated and/or is currently undergoing treatment with at least one antibiotic and/or drug.

35 As used herein, the term "terrestrial animal" refers to any organism that lives entirely on land or that lives predominantly on land, especially compared with aquatic animals. In particular, the terrestrial animal according to any aspect of the present invention may be any animal in the animal kingdom. More in particular, the terrestrial animal according to any aspect of the present invention may be may a mammal or a bird. Even more in particular, the terrestrial animal according to any aspect of the present invention may be rearing animals selected from livestock or poultry. In particular, 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. More in particular, the terrestrial animal according to any aspect of the present invention
5 may be selected from the group consisting of cow, sheep, pig, goat, horse, camel, donkey, mule, rabbit, chicken, turkey, duck, goose, and quail.

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 undergo the method according to any aspect of the present invention. These products from animals may
10 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,
15 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. 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.

20 In one example, the test animal and/or test animal derived product may be selected from the group consisting of pig for fattening, cattle for fattening, piglet, turkey for fattening, chicken for fattening, veal calf, horse, sheep meat, rabbit for fattening, sheep dairy products, horse for fattening, lamb for fattening, sheep for fattening, cow dairy products, laying hen, sow with piglets, goat dairy products and the like.

The term "test" used in conjunction with the term subject and/ or animal in the present disclosure
25 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 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" or 'control' shall denote, mostly
30 predetermined, entities which are used for a comparison with the test entity. For example, the term 'reference animal' used interchangeably with 'control animal' refers to an animal of the same biological taxon 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
35 sample may be any (test) animal-derived product that may be subject to the method of the present invention to determine if the test animal has had or has contact with at least one antibiotic and/or veterinary chemical from which the test animal-derived product is obtained from. For example, an animal-derived product sample may be a piece of meat tested according to any aspect of the present invention to determine if the test animal has had contact with at least one antibiotic and/or veterinary
40 chemical from which the test animal-derived product is obtained from. In another example, a (test)

animal-derived product sample may be milk which is tested according to any aspect of the present invention to determine if the test animal currently still has contact with at least one antibiotic and/or veterinary chemical from which the test animal-derived product is obtained from. Blockchain may also be used to make the information easily available for the consumer.

5

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.

20

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 DNA segment may also include specific pre-selected methylation sites.

25

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 $\geq 5x$ were considered and for ≥ 5 qualified CpG sites. Additionally, genes that have an average 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-Dimensional Scaling.

35

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.

40

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
5 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
10 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
15 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
20 control DNA sample. In particular, control refers to an animal derived product or animal that has not had contact with antibiotics and/or veterinary chemicals.

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
25 control DNA sample. In particular, control refers to an animal derived product or animal that has not had contact with antibiotics and/or veterinary chemicals.

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.
30 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
35 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
40 "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.

5

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*. Some of these sites may be hypermethylated and some may be hypomethylated in an animal that was brought into contact with an antibiotic and/or veterinary chemical compared to a cell with no contact with an antibiotic and/or veterinary chemical.

10

A "CpG island" as used herein describes a segment of DNA sequence that comprises a functionally or structurally deviated CpG density. For example, Yamada et al. have described a set of standards for determining a CpG island: it must be at least 400 nucleotides in length, has a greater than 50% GC content, and an OCF/ECF ratio greater than 0.6 (Yamada et al., 2004, Genome Research, 14, 247-266). Others have defined a CpG island less stringently as a sequence at least 200 nucleotides in length, having a greater than 50% GC content, and an OCF/ECF ratio greater than 0.6 (Takai et al., 2002, Proc. Natl. Acad. Sci. USA, 99, 3740-3745). 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.

25

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.

30

As used herein, the term "genomic material" refers to nucleic acid molecules or fragments of the genome of the subject or group of subjects. In particular, such nucleic acid molecules or fragments are DNA or RNA or hybrids thereof, and most preferably are molecules of the DNA genome of a subject or group of subjects.

35

As used herein, the "DNA sample" refers to the DNA extracted from the terrestrial animal according to any aspect of the present invention using known methods in the art.

In particular, when there is differential methylation detected in a test animal, that is to say that the test animal and/or a product derived from the test animal displays hypermethylation or hypomethylation at, at least one CpG site in comparison to the control (i.e., an animal without contact with at least one antibiotic and/or veterinary chemical), then the test animal has or has had contact with at least one antibiotic and/or veterinary chemical. The difference in methylation according to any aspect of the present invention may be hypomethylation or hypermethylation.

A "biological sample" in context of the 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, etc. In particular, the biological sample useful for the present invention may comprise biological cells or fragments thereof. More in particular, the biological sample is selected from the group consisting of blood, brain, sperm, milk and any other tissue or sample that provides genomic DNA.

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.

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.

As used herein the term 'antibiotic' refers to any medicine that may be fed to the terrestrial animal for therapeutic and/or preventive purposes. The antibiotic may be administered by any method known in the art. The antibiotic may be fed orally to the terrestrial animal according to any aspect of the present invention in the animal feed, or water such that it is ingested. In another example, the antibiotic may be injected into the animal. In one example, the antibiotic may be introduced into the terrestrial animal via udder injections. A skilled person would understand the best way to provide the antibiotic to the animal based on the specific biological taxon of the animal, the type of antibiotic and the disease to be treated or prevented. In particular, the antibiotic according to any aspect of the present invention may be selected from the group of classes consisting of amphenicols, aminocyclitols, aminoglycosides, ansamycins, beta-lactams, carbaephem, carbapenems, cephalosporins, chloramphenicol, fluoroquinolones, glycopeptides, glycylicyclines, ketolides, lincosamides, lipopeptides, macrolides, monobactams, nitrofurans, nitroimidazoles, oxazolidinones, penicillins, phosphonic acid derivatives, pleuromutilins, polymyxins, polypeptides, quinolones, rifamycins, riminofenazines, steroid antibacterials, streptogramins, sulfonamides, tetracyclines, and trimethoprim. More in particular, the antibiotic may be selected from the group consisting of tetracycline hydrochloride, Amoxicillin and Colist.

The test animal according to any aspect of the present invention may be fed with at least one or more antibiotics mentioned above simultaneously or consecutively. The contact of antibiotics with the

terrestrial animal may bring about epigenetic changes, at least DNA methylation changes, that may then be determined using the method according to any aspect of the present invention. The concentration of antibiotics in each dose and/or the period of time the antibiotic has been given to the test animal may affect the extend of differential methylation in the test animal relative to the control animal. It is within
5 the knowledge of a skilled person to determine the concentration of each dose and the period of antibiotic exposure that the test animal requires depending on whether the antibiotic is given for preventive or therapeutic measures.

As used herein the term 'veterinary chemical' refers to drugs or medicines used to treat or prevent disease, injury, and pests in animals. In particular, 'veterinary chemical' may refer to an anti-parasitic,
10 an anti-viral, a feed additive, a water additive, a disinfectant, glutaraldehyde, formalin, mixtures thereof and the like. In one example, the feed additive may be a coccidiostat or ionophore. Water additive refers to chemicals that may be added to water lines rather than into feed of the terrestrial animals. The veterinary chemical may be administered by any method known in the art to the terrestrial animal. In particular, the veterinary chemical refers to at least one anti-microbial such as an anti-parasitic, an anti-
15 viral or antibiotic.

The test animal used in the method according to any aspect of the present invention may be brought into contact with both an antibiotic and a veterinary chemical simultaneously and/or consequently. The change in the internal environment of the test animal leads to an epigenetic change and this can be determined using the method according to any aspect of the present invention.

20 In particular, in the method according to any aspect of the present invention, in step (a) the methylation status of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 CpG sites are determined. A skilled person would be capable of determining the number of CpG sites that need to be used in step (a) according to any aspect of the present invention. Even more in particular, the methylation status of at least two CpG sites are determined in step (a) of
25 the method according to any aspect of the present invention.

The method according to any aspect of the present invention, further comprises the step of:

- (i) performing bisulfite modification to the DNA sample before step (a).

'Bisulfite treatment' of genomic DNA used interchangeably with the term 'bisulfite modification', refers to the treatment of the genomic DNA with a deaminating agent such as a bisulfite that may be used to
30 treat all DNA, methylated or not. In particular, the term "bisulfite" as used herein encompasses any suitable type of bisulfite, such as sodium bisulfite, or other chemical agents that are capable of chemically converting a cytosine (C) to an 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. As used herein, a reagent that "differentially modifies"
35 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.

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.

Accordingly, before step (a) according to any aspect of the present invention is carried out, the genomic DNA contained/ obtained or extracted from the cell, is first bisulfite treated. An alternative method available in the art may be used instead of bisulfite treatment. A skilled person will understand which other methods to use. In one example, TET-assisted pyridine borane sequencing (TAPS) may be used for detection of 5mC and 5hmC (Yibin Liu, et al., *Nature Biotechnology*, 37: 424–429 (2019)).

The cell used according to any aspect of the present invention is obtained from a biological sample selected from the group consisting of blood, brain, sperm and any other tissue or sample that provides genomic DNA to be used in the method according to any aspect of the present invention. In particular, the biological sample may comprise any biological material obtained from the subject that contains DNA, and may be liquid, solid or both, may be tissue or bone, or a body fluid such as blood, lymph, etc. In particular, the biological sample useful for the present invention may comprise biological cells or fragments thereof.

According to a further aspect of the present invention, there is provided a method of determining if a test animal and/or a test animal from which a product is derived has been treated and/or is currently undergoing treatment with at least one antibiotic, and if so, determining the distinct class of antibiotics with which the test animal is being treated and/or is currently undergoing treatment, the method comprising:

- (a) determining a test methylation profile from the genomic material contained in a biological sample obtained from the test animal and/or the animal-derived product sample; and
- (b) comparing the test methylation profile obtained from (a) with one or more predetermined reference methylation profiles, wherein each of the predetermined reference methylation profiles is from a different animal of the same biological taxon as the test animal, and each of the different animals was treated with a different class of antibiotics,

wherein if the test methylation profile of (a) is significantly similar to one of the predetermined reference methylation profiles, the test animal is confirmed for being treated with the same class of antibiotics with which the animal with the predetermined reference profile is treated with and the test animal has been confirmed for having been treated and/or is currently undergoing treatment with the distinct class of antibiotics; and wherein the test animal is a terrestrial animal.

The distinct classes of antibiotics are amphenicols, aminocyclitols, aminoglycosides, ansamycins, beta-lactams, carbaephem, carbapenems, cephalosporins, chloramphenicol, fluoroquinolones, glycopeptides, glycylicyclines, ketolides, lincosamides, lipopeptides, macrolides, monobactams, nitrofurans, nitroimidazoles, oxazolidinones, penicillins, phosphonic acid derivatives, pleuromutins, polymyxins, polypeptides, quinolones, rifamycins, riminofenazines, steroid antibacterials, streptogramins, sulfonamides, tetracyclines, and trimethoprim.

In one example, a panel of pre-determined reference profiles may be prepared for different animals to be used as a control where each animal has been contacted with a different class of antibiotics and/or each part of the animal (i.e., tissue, muscle, blood, skin) has its own unique pre-determined methylation reference profile that also forms a part of the panel of pre-determined reference profiles. Different animals from the same biological taxon as the test animal, each being treated with a different class of antibiotic may have its own panel of pre-determined reference profiles for each part of the animal or animal-derived product that is used as the genomic material. For example, each panel may be specific for a single animal in contact with a first antibiotic and/or veterinary chemical where each reference profile may be distinct for a part of the animal from which the genomic material is extracted. There will thus be a compilation of panels of pre-determined reference profiles, each panel specific for one control animal of the same biological taxon as the test animal, the control animal being in contact with or is in contact with a first, second, third and the like antibiotic and/or veterinary chemical. When a test methylation profile from an unknown animal-derived product sample is obtained, this is then compared with the different panels of pre-determined reference profiles for the same animal taxon as the test animal to determine the distinct class of antibiotic and/or veterinary chemical the test animal is or was in contact with.

According to yet another aspect of the present invention refers to a method of determining if a test animal and/or a test animal from which a product is derived has been treated and/or is currently undergoing treatment with at least one antibiotic, and if so, determining if the antibiotic is used as a growth promotant or as a therapeutant, the method comprising:

- (a) determining a test methylation profile from the genomic material contained in a biological sample obtained from the test animal and/or the animal-derived product; and

- (b) comparing the test methylation profile obtained from (a) with one or more predetermined reference methylation profiles, wherein each of the predetermined reference methylation profiles is from a different control animal of the same biological taxon as the test animal, and each of the different control animals was treated with the antibiotic as a growth promotant or therapeutant,

5

wherein if the test methylation profile of (a) is significantly similar to one of the predetermined reference methylation profiles, the test animal is confirmed for being treated with the antibiotic in the same way that the control animal with the similar predetermined reference profile is treated and the test animal has been confirmed for having been treated and/or is currently undergoing treatment with the antibiotic as a growth promotant or therapeutant; and wherein the test animal is a terrestrial animal.

10

As used herein the term 'growth promotant' refers to the antibiotic being used to help increase the efficiency of animal production by increasing weight gain and product output. The antibiotic may be used as a growth promotant in contrast to it being used as a therapeutant (i.e., for treatment of a disease)

15

According to a further aspect of the present invention, there is provided a method of determining if a test animal from which a product is derived underwent a withdrawal period of no treatment with at least one antibiotic and/or veterinary chemical prior to the product being obtained, the method comprising:

- (a) determining a test methylation profile from the genomic material contained in a biological sample obtained from the animal-derived product; and
- (b) comparing the test methylation profile obtained from (a) with at least two predetermined reference methylation profiles, wherein at least one of the predetermined reference methylation profiles is from a control animal that underwent a withdrawal period and at the other one of the predetermined reference methylation profiles is from a control animal of the same biological taxon as the test animal, that did not undergo a withdrawal period before the product was obtained,

20

wherein if the test methylation profile of (a) is significantly similar to the predetermined reference methylation profile, the test animal is confirmed for having undergone a withdrawal period or not; and

25

wherein the test animal is a terrestrial animal.

30

As used herein, the term 'withdrawal period' refers to the period from the time point where the animal is no longer fed the antibiotic and/or veterinary chemical to the point where the remaining antibiotic is broken down in the body until it becomes a non-functional agent and is finally, eliminated from the body of the animal. Withdrawal periods of different antibiotics may vary from 1 or 2 days to couple of weeks. A "withdrawal" period is required from the time antibiotics are administered until it is legal to slaughter the animal or to derive products from the animal. The time it therefore takes the body to break down the antibiotic until it is no longer functional, or present is called the withdrawal time (or withdrawal period).

35

Once the withdrawal period has passed the antibiotic has been eliminated from the animal's system.

According to another aspect of the present invention, there is provided a use of DNA methylation profiling for determining if a test animal and/or a test animal from which a product is derived has been

treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical, wherein the test animal is a terrestrial animal and the veterinary chemical is an anti-parasitic, an anti-viral, a feed additive, a water additive, a disinfectant, glutaraldehyde, and/ or formalin..

In one example, the veterinary chemical is at least one anti-microbial such as an anti-parasitic, an anti-viral or antibiotic.

According to any aspect of the present invention, test methylation profile and reference methylation profile may be obtained by determining the methylation status of a pre-selected group of CpG sites in the genome of the test and reference animal respectively. The CpG site according to any aspect of the present invention may be selected from the Table A below. In particular, the CpG site according to any aspect of the present invention is at least one CpG site selected from the Table A below:

Table A. CpG sites of a test or reference animal that may be differentially methylated in the animal exposed to antibiotic and/or veterinary chemical.

No.	Chromosome	Position	No.	Chromosome	Position
1	20	10783159	17	3	3328494
2	24	4184075	18	4	1627649
3	25	1910205	19	6	11522056
4	4	1712424	20	6	23106717
5	4	50270218	21	8	5481923
6	5	57738909	22	33	716102
7	7	10956706	23	22	4604122
8	12	18699814	24	22	4617202
9	12	2415548	25	23	502108
10	17	4082979	26	24	5683979
11	18	9932606	27	27	3015432
12	2	29649123	28	3	10479779
13	23	4069359	29	19	5380287
14	25	1445548	30	15	12638203
15	28	3467160	31	10	20073849
16	3	10490126	32	5	55805858

EXAMPLES

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

An *in vitro* to investigate the effects of three different antibiotics on the DNA methylation quantity in a swine intestinal cell line.

An immortalised cell line from swine intestines (IPEC2-J2 ACC 701 from DSMZ cell culture) was utilized in the 7th passage. In a 12 well, flat-bottom plate, 1×10^4 cells/well were seeded with DPBS D8537 (Sigma-Aldrich) and initially incubated for 4 days. After incubation cells were treated with one of 4 treatments for up to an additional 4 days:

- 1) Incubation for 4 days without any antibiotics
- 2) Incubation for 1 day with antibiotics followed by incubation for 1- 3 days in antibiotic free media
- 3) Incubation for 2 days with antibiotics followed by incubation for 1-2 days in antibiotic free media
- 4) Incubation for 3 days with antibiotics followed by incubation for 1 day in antibiotic free media

Treatments 2, 3 and 4 were repeated with 3 different antibiotic treatments; tetracycline hydrochloride at 200mg/L, Amoxicillin at 100mg/L and Colistin at 480mg/L. Duplicate cells per treatment were harvested at each time point. Media was removed, cells were washed once with DPBS, and a cell scraper was used to disassociate the cells from the bottom of the well. Cell pellets were then stored at -80°C until DNA could be isolated using the NucleoSpin® DNA Stool Kit (Macherey and Nagel). Methylation grade of 100ng of isolated DNA was determined using the Imprint® Methylated DNA Quantification Kit (Sigma-Aldrich) which compared samples to a standard curve of known methylated DNA.

Table 1. The average amount of methylated DNA (ng/100ng isolated DNA) isolated from IPEC2 cells incubated in media without antibiotics or with either 200mg/L Tetracycline Hydrochloride (Tet), 100 mg/L Amoxicillin (Amx) or 480mg/L Colistin (Col) over 4 days.

Treatment	Day 0	Day 1	Day 2	Day 3	Day 4
4 Days without antibiotics	27.8 ± 2.4^1	27.7 ± 19.7	35.0 ± 11.6	16.1 ± 2.6	31.6 ± 1.4
1 Day Tet before washing	NA ²	-5.6 ± 2.9	12.0 ± 6.9	18.2 ± 16.0	25.8 ± 22.4
2 Day Tet before washing			0.1 ± 8.1	-8.6 ± 11.1	7.4 ± 9.7
3 Day Tet before washing				6.0 ± 13.2	21.7^3
1 Day Amx before washing		4.1 ± 8.9	12.3 ± 3.2	20.4 ± 8.3	19.0 ± 2.1
2 Day Amx before washing			20.9 ± 10.4	9.8 ± 4.7	21.3 ± 3.4
3 Day Amx before washing				26.0 ± 10.2	10.2 ± 9.4
1 Day Col before washing		26.1 ± 5.0	11.7 ± 6.8	19.6 ± 5.5	24.9 ± 0.8
2 Day Col before washing			29.5 ± 3.8	1.4 ± 7.9	19.0 ± 5.4
3 Day Col before washing				29.1 ± 18.0	28.5 ± 16.4

¹Represents the standard error of the mean for the reported average

²NA, Not Applicable

³Standard error of the mean could not be calculated as one of the two replicates had insufficient isolated DNA

The results indicate that all three antibiotics resulted in a reduction in overall DNA methylation within 24 hrs of treatment. This effect was largest in Tetracycline, followed by Amoxicillin and finally Colistin treated cells when compared with the antibiotic free control cells. The differential methylation was also partially reverted within 1-3 after cells were washed and antibiotic treated media was replaced with antibiotic-free media. The results signify that antibiotic treatment can alter the methylation status of eukaryotic cells, and that these effects may be specific to antibiotic class and duration of treatment.

Example 2

Impact of three classes of antibiotics administered as either growth promoters or therapeutically (either with or without withdrawal period) on the methylation of broiler chickens.

A study was conducted to determine whether methylation of CpG sites in the genome of chicken breast tissue and blood could be differentially affected by:

- 1) The administration of an antibiotic for growth promotion
- 2) The administration of antibiotics as a therapeutic
- 3) The administration of localized antibiotic classes
- 4) The administration of systemically absorbed antibiotic classes
- 5) The administration of therapeutic doses of antibiotics with or without a withdrawal period prior to slaughter

To create the treatments for the *in vivo* chicken trial, three different antibiotics were utilized (Table 2).

Table 2. Antibiotic classes, modes of action, absorption level and withdrawal period for the 3

Antibiotic (Class)	Antibiotic Mode of Action	Absorption	Recommended Withdrawal for Broilers
Bacitracin Methylene Disalicylate (Peptide)	Inhibits peptidoglycan synthesis Used as an AGP or therapeutically	Local	None required
Tylosin (Macrolide)	Inhibits protein synthesis Used therapeutically	Systemic	5 days
Amoxicillin (Beta Lactam)	Inhibits peptidoglycan synthesis Used therapeutically	Systemic	1-4 days

antibiotics utilized to create treatments in an *in vivo* chicken trial.

A chicken trial was conducted from day of hatch to 42 days to represent the average slaughter age of broiler chickens in Europe. A total of 870 Ross308 male broilers were separated into 58 floor pens containing 15 birds/pen. Corn-soybean meal-based broiler diets were formulated to meet all nutrient requirements for starter (day 0-14), grower (day 14-28) and finisher (day 28-42) feeding phases. Treatments were created by adding different quantities of antibiotics to the basal diet for different durations (Table 3).

Table 3. Description of control and antibiotic fed treatments in a 42-day broiler chicken trial.

Treatments	Pen Replicates	Antibiotic Dose, kg/MT feed
1. Negative control (no antibiotic)	10	-
2. BMD - AGP Dose	8	50g/MT feed day 1-42
3. BMD - Therapeutic Dose	8	200g/MT Feed Day 23-28
4. Tylosin - withdrawal	8	2kg/MT Feed Day 32-37
5. Tylosin - no withdrawal	8	2kg/MT Feed Day 32-42
6. Amoxicillin - withdrawal	8	1.86 kg/MT Feed Day 32-37
7. Amoxicillin - no withdrawal	8	1.86 kg/MT Feed Day 37-42

Two animals per pen were sacrificed for sampling on days 28 (for treatments 1-3) and 42 (all treatments) for extraction of blood and various tissues including breast muscle tissue (Pectoralis major). A total of 96 samples were then selected for DNA isolation with Invitrogen™ Purelink™ Genomic DNA Minikit (ThermoFisher Scientific, Waltham, MA, USA) and subsequent reduced bisulphite sequencing to compare methylation level of CpG sites in the genome:

- Day 28 breast and blood from 4 birds/treatment = (4 birds x 3 treatments) x (2 tissues/bird) = 24 samples
- Day 42 breast and blood from 5 birds/treatment = (5 birds x 7 treatments) x (2 tissues/bird) = 70 samples

- Day 42 additional blood sample from Treatment 1 and Treatment 3 = 2 samples

Total = 96 samples

Chicken samples for blood as well as for breast tissue were prepared with the Zymo RRBS protocol. The resulting libraries were sequenced and mapped to the *gallus gallus* 5.0 genome using the tool bsmmap 2.9.

From the resulting methylation ratios all CpGs were kept, which had a non-zero coverage in all individual samples for a certain tissue. This resulted in 1,078,713 CpGs for breast and 1,077,240 CpGs for blood.

To further analyze the ability to classify the samples based on the methylation data for treatments with different antibiotics, the R package randomForest was used and a random forest-based classification was set up, using the 100,000 most variable CpGs in each tissue.

First, the set of CpGs was split in chunks of 10,000 and each chunk was used to classify the samples into different categories, using a random forest. This was repeated 100 times. All CpGs having a positive score "MeanDecreaseAccuracy" in an individual classification were pooled and the top 10,000 CpGs, occurring most often, were kept. Then a second random forest was set up, using these selected 10,000 CpGs as features and a classification was performed. This was repeated 100 times and the average classification error, as well as the optimal classification error was evaluated.

The results showed no difference in growth (body weight gain, feed intake or feed conversion ratio) of broilers in the different antibiotic treated groups. This is likely due to the very clean conditions and optimal nutrition these birds received during the trial, as antibiotics were then not needed to improve growth performance or prevent illness. Therefore, bird weight or health status did not need to be corrected for when comparing the treatments for methylation of CpG sites.

The results of the random forest analysis of CpG methylation identified that the tylosin withdrawal and amoxicillin treatment groups were distinguishable from the control with error rates of < 0.05 when considering the best classification (Table 4). The BMD treatments and the Tylosin treatment without a withdrawal period were also distinguishable from the control group, although the best classification error rates were slightly higher between 0.056 – 0.111 in these treatments.

Table 4. Random forest analysis of CpG methylation based on the 100,000 most variable CpGs in breast tissue genomic DNA samples from antibiotic treated broiler chickens compared to controls.

Group 1	Group 2	Average Classification Error	Best Classification Error	Number of Features in Best Classification
1. Control	2. BMD - AGP Dose	0.314	0.111	38
1. Control	3. BMD - Therapeutic Dose	0.275	0.056	34
1. Control	4. Tylosin - Withdrawal	0.169	0.000	49
1. Control	5. Tylosin - No Withdrawal	0.189	0.071	26
1. Control	6. Amoxicillin - Withdrawal	0.161	0.000	41
1. Control	7. Amoxicillin - No Withdrawal	0.165	0.000	35

When random forest analysis was conducted on the 100,000 most variable CpGs in blood samples from the different treatments, the best classification error rates were under 0.100 for the differentiation of each antibiotic treatment from the control group (Table 5).

The location of differentially methylated cytosine residues in antibiotic treated compared to control chickens are listed for breast (Table 6) and blood (Table 7).

These results indicate that treatment of broiler chickens with antibiotics results in significant shifts to the CpG methylation profiles, which may therefore be used to differentiate antibiotic- free from antibiotic-treated poultry meat regardless of the antibiotic class, purpose or usage of a withdrawal period.

Table 5. Random forest analysis of CpG methylation based on the 100,000 most variable CpGs in blood genomic DNA samples from antibiotic treated broiler chickens compared to controls.

Group 1	Group 2	Average Classification Error	Best Classification Error	Number of Features in Best Classification
1. Control	2. BMD - AGP Dose	0.245	0.000	42
1. Control	3. BMD - Therapeutic Dose	0.256	0.062	38
1. Control	4. Tylosin - Withdrawal	0.184	0.000	29
1. Control	5. Tylosin - No Withdrawal	0.205	0.071	32
1. Control	6. Amoxicillin - Withdrawal	0.147	0.000	33
1. Control	7. Amoxicillin - No Withdrawal	0.194	0.000	34

Table 6. Genome sites of the CpG cytosine residues differentially methylated in blood extracted DNA from the control group compared to each individual antibiotic treatment group.

Internal ID	Chromosome_Methylated C	Internal ID	Chromosome_Methylated C	Internal ID	Chromosome_Methylated C
Abx_Brs_1v3_02	chr1_101775337	Abx_Brs_1v6_17	chr20_10175049	Abx_Brs_1v3_20	chr3_104916057
Abx_Brs_1v5_01	chr1_109886119	Abx_Brs_1v5_13	chr20_10299706	Abx_Brs_1v4_34	chr3_105060547
Abx_Brs_1v5_02	chr1_168710537	Abx_Brs_1v4_16	chr20_10685149	Abx_Brs_1v3_21	chr3_105641615
Abx_Brs_1v7_01	chr1_194449380	Abx_Brs_1v2_17	chr20_10691918	Abx_Brs_1v5_18	chr3_110149140
Abx_Brs_1v2_01	chr1_29662475	Abx_Brs_1v6_18	chr20_10715960	Abx_Brs_1v6_33	chr3_110317373
Abx_Brs_1v3_01	chr1_56888987	Abx_Brs_1v3_09	chr20_10754367	Abx_Brs_1v3_22	chr3_110439865
Abx_Brs_1v4_01	chr1_88691204	Abx_Brs_1v4_17	chr20_10783158	Abx_Brs_1v4_30	chr3_18608799
Abx_Brs_1v6_01	chr10_1009688	Abx_Brs_1v3_10	chr20_11750109	Abx_Brs_1v2_30	chr3_20355838
Abx_Brs_1v7_02	chr10_20042571	Abx_Brs_1v2_14	chr20_4769487	Abx_Brs_1v7_27	chr3_3328494
Abx_Brs_1v6_03	chr10_20068846	Abx_Brs_1v2_13	chr20_491396	Abx_Brs_1v6_32	chr3_4360629
Abx_Brs_1v5_03	chr10_20073849	Abx_Brs_1v2_15	chr20_5034399	Abx_Brs_1v7_28	chr3_4365085
Abx_Brs_1v6_04	chr10_20074592	Abx_Brs_1v4_14	chr20_573931	Abx_Brs_1v4_28	chr3_4450147
Abx_Brs_1v5_04	chr10_20387798	Abx_Brs_1v4_15	chr20_8224955	Abx_Brs_1v4_31	chr3_55069595
Abx_Brs_1v6_02	chr10_2642098	Abx_Brs_1v2_16	chr20_9547652	Abx_Brs_1v4_32	chr3_84847235
Abx_Brs_1v2_02	chr10_2704683	Abx_Brs_1v2_18	chr21_1227836	Abx_Brs_1v4_33	chr3_96903509
Abx_Brs_1v4_02	chr10_8631840	Abx_Brs_1v6_19	chr21_3027896	Abx_Brs_1v3_25	chr33_1012846
Abx_Brs_1v7_03	chr12_11443637	Abx_Brs_1v6_20	chr21_3198224	Abx_Brs_1v3_26	chr33_1033386
Abx_Brs_1v3_04	chr12_14666603	Abx_Brs_1v7_15	chr21_4009474	Abx_Brs_1v3_23	chr33_527740
Abx_Brs_1v3_05	chr12_18618473	Abx_Brs_1v3_11	chr21_545937	Abx_Brs_1v3_24	chr33_571089
Abx_Brs_1v6_07	chr12_18699814	Abx_Brs_1v2_19	chr21_6631083	Abx_Brs_1v6_34	chr33_716102
Abx_Brs_1v4_06	chr12_19604396	Abx_Brs_1v4_18	chr22_2091053	Abx_Brs_1v2_31	chr4_1071433
Abx_Brs_1v4_03	chr12_2404196	Abx_Brs_1v4_19	chr22_2817639	Abx_Brs_1v7_29	chr4_1627649
Abx_Brs_1v3_03	chr12_2563862	Abx_Brs_1v7_17	chr22_2817746	Abx_Brs_1v5_19	chr4_1637731
Abx_Brs_1v5_05	chr12_2631784	Abx_Brs_1v4_20	chr22_4722610	Abx_Brs_1v6_35	chr4_1712423
Abx_Brs_1v4_04	chr12_2917439	Abx_Brs_1v7_16	chr22_584861	Abx_Brs_1v6_36	chr4_2164691
Abx_Brs_1v4_05	chr12_3128179	Abx_Brs_1v3_12	chr23_2232689	Abx_Brs_1v7_30	chr4_29468173
Abx_Brs_1v6_05	chr12_8715912	Abx_Brs_1v5_14	chr23_2479273	Abx_Brs_1v5_20	chr4_32158109
Abx_Brs_1v6_06	chr12_8863536	Abx_Brs_1v3_13	chr23_4069359	Abx_Brs_1v2_32	chr4_39300003
Abx_Brs_1v5_06	chr12_9026657	Abx_Brs_1v7_18	chr23_5272414	Abx_Brs_1v4_36	chr4_39300039
Abx_Brs_1v5_08	chr13_1014353	Abx_Brs_1v6_21	chr23_5401469	Abx_Brs_1v4_35	chr4_4575867
Abx_Brs_1v6_08	chr13_10165614	Abx_Brs_1v3_14	chr23_5497533	Abx_Brs_1v3_27	chr4_5215030
Abx_Brs_1v6_09	chr13_10171884	Abx_Brs_1v4_21	chr24_1539954	Abx_Brs_1v3_28	chr4_5359917
Abx_Brs_1v6_10	chr13_10259925	Abx_Brs_1v7_19	chr24_288436	Abx_Brs_1v4_38	chr5_14142074
Abx_Brs_1v7_06	chr13_12996512	Abx_Brs_1v6_22	chr24_320580	Abx_Brs_1v4_39	chr5_16902835
Abx_Brs_1v7_07	chr13_13586246	Abx_Brs_1v3_16	chr24_3486236	Abx_Brs_1v3_31	chr5_17949276
Abx_Brs_1v6_11	chr13_15902163	Abx_Brs_1v2_20	chr24_433801	Abx_Brs_1v2_34	chr5_23142051
Abx_Brs_1v2_03	chr13_1651193	Abx_Brs_1v5_15	chr24_4445719	Abx_Brs_1v4_37	chr5_26025
Abx_Brs_1v3_06	chr13_2362190	Abx_Brs_1v6_23	chr24_4988184	Abx_Brs_1v5_21	chr5_267088
Abx_Brs_1v7_04	chr13_2439445	Abx_Brs_1v5_16	chr24_5365357	Abx_Brs_1v3_29	chr5_28895
Abx_Brs_1v4_07	chr13_3224847	Abx_Brs_1v7_20	chr24_5683979	Abx_Brs_1v4_40	chr5_38160498
Abx_Brs_1v5_07	chr13_879182	Abx_Brs_1v3_15	chr24_902556	Abx_Brs_1v6_37	chr5_3933111
Abx_Brs_1v7_05	chr13_9207110	Abx_Brs_1v2_21	chr24_985579	Abx_Brs_1v3_30	chr5_398964
Abx_Brs_1v7_09	chr14_12290697	Abx_Brs_1v7_21	chr25_1386837	Abx_Brs_1v5_22	chr5_47834959
Abx_Brs_1v3_08	chr14_12748323	Abx_Brs_1v2_22	chr25_1445548	Abx_Brs_1v2_33	chr5_5214204
Abx_Brs_1v7_10	chr14_13118754	Abx_Brs_1v6_24	chr25_1543129	Abx_Brs_1v5_23	chr5_54481629
Abx_Brs_1v4_09	chr14_14042992	Abx_Brs_1v6_25	chr25_1739406	Abx_Brs_1v7_31	chr5_54512733
Abx_Brs_1v6_12	chr14_2402355	Abx_Brs_1v3_17	chr25_1830314	Abx_Brs_1v3_32	chr5_55805858
Abx_Brs_1v7_08	chr14_2952896	Abx_Brs_1v7_22	chr25_1910204	Abx_Brs_1v4_41	chr5_56948077
Abx_Brs_1v4_08	chr14_3368850	Abx_Brs_1v3_18	chr25_2567594	Abx_Brs_1v6_38	chr5_57738908
Abx_Brs_1v3_07	chr14_4037141	Abx_Brs_1v6_26	chr25_2588121	Abx_Brs_1v2_35	chr5_58258604
Abx_Brs_1v2_04	chr14_6772062	Abx_Brs_1v7_23	chr26_1523957	Abx_Brs_1v3_33	chr6_23106717
Abx_Brs_1v5_09	chr17_4082979	Abx_Brs_1v6_27	chr26_3668517	Abx_Brs_1v7_32	chr6_34218231
Abx_Brs_1v7_11	chr17_5425184	Abx_Brs_1v4_22	chr26_5033269	Abx_Brs_1v6_39	chr7_10956706
Abx_Brs_1v2_05	chr17_6713697	Abx_Brs_1v3_19	chr26_546306	Abx_Brs_1v2_36	chr7_16342057
Abx_Brs_1v2_06	chr17_9085844	Abx_Brs_1v4_23	chr27_1521644	Abx_Brs_1v5_24	chr7_22221784
Abx_Brs_1v4_10	chr17_9178805	Abx_Brs_1v6_28	chr27_3117998	Abx_Brs_1v6_40	chr7_22317969
Abx_Brs_1v7_12	chr17_9218402	Abx_Brs_1v2_23	chr27_4008176	Abx_Brs_1v5_25	chr7_22769202
Abx_Brs_1v4_11	chr17_9934986	Abx_Brs_1v2_24	chr27_4220838	Abx_Brs_1v4_42	chr7_3551750
Abx_Brs_1v7_13	chr18_3866724	Abx_Brs_1v6_29	chr27_4500127	Abx_Brs_1v2_37	chr7_36230809
Abx_Brs_1v6_13	chr18_8259871	Abx_Brs_1v4_24	chr27_4738796	Abx_Brs_1v6_41	chr7_36500218
Abx_Brs_1v2_07	chr18_8720811	Abx_Brs_1v6_30	chr27_4813851	Abx_Brs_1v4_43	chr7_5682303
Abx_Brs_1v5_10	chr18_8976506	Abx_Brs_1v2_25	chr27_4814530	Abx_Brs_1v4_44	chr7_9315550
Abx_Brs_1v2_08	chr18_9807966	Abx_Brs_1v2_26	chr27_5159755	Abx_Brs_1v4_46	chr8_12053510
Abx_Brs_1v6_14	chr19_45656	Abx_Brs_1v2_27	chr27_5190614	Abx_Brs_1v4_45	chr8_5481923
Abx_Brs_1v4_12	chr19_5380287	Abx_Brs_1v7_24	chr27_5387338	Abx_Brs_1v7_33	chr8_5838854
Abx_Brs_1v5_11	chr19_5832099	Abx_Brs_1v6_31	chr28_1172540	Abx_Brs_1v7_34	chr9_15008106
Abx_Brs_1v2_10	chr19_5913067	Abx_Brs_1v5_17	chr28_2723178	Abx_Brs_1v7_35	chrW_3664770
Abx_Brs_1v5_12	chr19_5932076	Abx_Brs_1v4_25	chr28_3055975	Abx_Brs_1v5_26	chrW_4279843
Abx_Brs_1v2_09	chr19_666115	Abx_Brs_1v7_25	chr28_3080430	Abx_Brs_1v4_47	chrW_4279851
Abx_Brs_1v7_14	chr19_8863974	Abx_Brs_1v7_26	chr28_3404973	Abx_Brs_1v4_49	chrZ_50685017
Abx_Brs_1v2_11	chr2_22862997	Abx_Brs_1v4_26	chr28_3467160	Abx_Brs_1v2_38	chrZ_67986966
Abx_Brs_1v6_15	chr2_29649123	Abx_Brs_1v4_27	chr28_3886908	Abx_Brs_1v3_34	chrZ_74050782
Abx_Brs_1v4_13	chr2_32645930	Abx_Brs_1v2_28	chr3_10072585	Abx_Brs_1v4_48	chrZ_9203114
Abx_Brs_1v6_16	chr2_32664023	Abx_Brs_1v4_29	chr3_10479779		
Abx_Brs_1v2_12	chr2_56841223	Abx_Brs_1v2_29	chr3_10490126		

Table 7. Genome sites of the CpG cytosine residues differentially methylated in blood extracted DNA from the control group compared to each individual antibiotic treatment group.

Internal ID	Chromosome_Methylated C	Internal ID	Chromosome_Methylated C	Internal ID	Chromosome_Methylated C
Abx_Bld_1v3_03	chr1_105181389	Abx_Bld_1v4_07	chr19_4086429	Abx_Bld_1v4_19	chr3_16322261
Abx_Bld_1v3_04	chr1_112363445	Abx_Bld_1v4_08	chr19_5491370	Abx_Bld_1v4_20	chr3_17158125
Abx_Bld_1v2_01	chr1_112396072	Abx_Bld_1v6_12	chr19_5536508	Abx_Bld_1v4_21	chr3_25893764
Abx_Bld_1v6_03	chr1_128573573	Abx_Bld_1v5_10	chr19_8144962	Abx_Bld_1v7_21	chr3_3150548
Abx_Bld_1v7_02	chr1_144303500	Abx_Bld_1v2_16	chr19_8989012	Abx_Bld_1v7_22	chr3_3831201
Abx_Bld_1v3_05	chr1_154756172	Abx_Bld_1v6_14	chr2_115635929	Abx_Bld_1v4_22	chr3_66735580
Abx_Bld_1v5_01	chr1_156676	Abx_Bld_1v5_13	chr2_129424345	Abx_Bld_1v6_18	chr3_76106431
Abx_Bld_1v3_06	chr1_178417326	Abx_Bld_1v3_21	chr2_136077087	Abx_Bld_1v7_23	chr30_21732
Abx_Bld_1v6_04	chr1_194856444	Abx_Bld_1v3_20	chr2_18062865	Abx_Bld_1v5_26	chr4_11856003
Abx_Bld_1v6_01	chr1_1962010	Abx_Bld_1v5_11	chr2_22523723	Abx_Bld_1v2_30	chr4_43673227
Abx_Bld_1v3_01	chr1_50122971	Abx_Bld_1v6_13	chr2_315910	Abx_Bld_1v3_31	chr4_4578012
Abx_Bld_1v6_02	chr1_51169599	Abx_Bld_1v5_12	chr2_76061542	Abx_Bld_1v3_32	chr4_47166665
Abx_Bld_1v7_01	chr1_67153637	Abx_Bld_1v6_15	chr20_10090542	Abx_Bld_1v6_20	chr4_5380207
Abx_Bld_1v3_02	chr1_98606031	Abx_Bld_1v7_11	chr20_10096111	Abx_Bld_1v3_33	chr4_68799893
Abx_Bld_1v5_04	chr10_18811046	Abx_Bld_1v3_23	chr20_10100558	Abx_Bld_1v5_27	chr4_69408805
Abx_Bld_1v3_08	chr10_19473861	Abx_Bld_1v5_16	chr20_10870977	Abx_Bld_1v3_34	chr4_72510975
Abx_Bld_1v2_02	chr10_20061379	Abx_Bld_1v4_09	chr20_12980419	Abx_Bld_1v4_23	chr5_15848298
Abx_Bld_1v4_01	chr10_20160145	Abx_Bld_1v5_15	chr20_4046143	Abx_Bld_1v2_32	chr5_16029341
Abx_Bld_1v5_03	chr10_2654646	Abx_Bld_1v3_22	chr20_4835413	Abx_Bld_1v6_22	chr5_16968682
Abx_Bld_1v5_02	chr10_845224	Abx_Bld_1v2_17	chr20_488444	Abx_Bld_1v2_33	chr5_24963086
Abx_Bld_1v3_07	chr10_8640927	Abx_Bld_1v5_14	chr20_902666	Abx_Bld_1v2_34	chr5_26187561
Abx_Bld_1v4_02	chr11_1547400	Abx_Bld_1v5_17	chr21_1123800	Abx_Bld_1v7_24	chr5_27550747
Abx_Bld_1v6_05	chr11_17628332	Abx_Bld_1v2_18	chr21_2857891	Abx_Bld_1v4_24	chr5_30587252
Abx_Bld_1v5_05	chr11_17901197	Abx_Bld_1v2_19	chr21_3932464	Abx_Bld_1v2_35	chr5_32283491
Abx_Bld_1v3_10	chr11_19107727	Abx_Bld_1v7_12	chr22_2486087	Abx_Bld_1v6_21	chr5_370898
Abx_Bld_1v3_09	chr11_8438570	Abx_Bld_1v4_10	chr23_1877366	Abx_Bld_1v7_25	chr5_37861456
Abx_Bld_1v3_11	chr12_1097339	Abx_Bld_1v3_24	chr23_191817	Abx_Bld_1v2_31	chr5_399337
Abx_Bld_1v4_03	chr12_1126979	Abx_Bld_1v7_13	chr23_2040288	Abx_Bld_1v3_35	chr5_54483355
Abx_Bld_1v3_12	chr12_1306284	Abx_Bld_1v5_18	chr23_4262739	Abx_Bld_1v6_23	chr5_58816074
Abx_Bld_1v2_05	chr12_13166753	Abx_Bld_1v4_11	chr23_4632788	Abx_Bld_1v2_36	chr6_10306539
Abx_Bld_1v2_03	chr12_1328470	Abx_Bld_1v6_16	chr23_4958640	Abx_Bld_1v7_27	chr6_22248119
Abx_Bld_1v7_03	chr12_2397978	Abx_Bld_1v2_20	chr23_5304481	Abx_Bld_1v7_28	chr6_22301186
Abx_Bld_1v2_04	chr12_2418533	Abx_Bld_1v6_17	chr23_5357470	Abx_Bld_1v5_28	chr6_22875585
Abx_Bld_1v7_04	chr12_3220416	Abx_Bld_1v4_12	chr23_5497714	Abx_Bld_1v3_36	chr6_34299915
Abx_Bld_1v6_06	chr12_9058585	Abx_Bld_1v2_21	chr23_5507020	Abx_Bld_1v5_29	chr6_34299915
Abx_Bld_1v3_14	chr13_10139895	Abx_Bld_1v3_25	chr24_1676702	Abx_Bld_1v7_29	chr6_34911376
Abx_Bld_1v2_06	chr13_1032633	Abx_Bld_1v7_14	chr24_2238427	Abx_Bld_1v7_26	chr6_9225335
Abx_Bld_1v5_07	chr13_10441903	Abx_Bld_1v2_22	chr24_287622	Abx_Bld_1v6_24	chr7_22164945
Abx_Bld_1v5_08	chr13_12948949	Abx_Bld_1v3_26	chr24_5290703	Abx_Bld_1v7_30	chr7_22176711
Abx_Bld_1v4_06	chr13_13347784	Abx_Bld_1v5_19	chr24_5373054	Abx_Bld_1v2_37	chr7_22480250
Abx_Bld_1v7_05	chr13_15703345	Abx_Bld_1v7_15	chr25_1710811	Abx_Bld_1v6_26	chr8_14105687
Abx_Bld_1v3_13	chr13_1921148	Abx_Bld_1v5_20	chr25_1805702	Abx_Bld_1v2_39	chr8_21102815
Abx_Bld_1v4_04	chr13_4682096	Abx_Bld_1v5_21	chr25_1823943	Abx_Bld_1v7_31	chr8_23866489
Abx_Bld_1v5_06	chr13_9178753	Abx_Bld_1v3_27	chr25_2397267	Abx_Bld_1v6_27	chr8_27355783
Abx_Bld_1v4_05	chr13_9208825	Abx_Bld_1v5_22	chr25_2485917	Abx_Bld_1v2_40	chr8_29884082
Abx_Bld_1v3_15	chr14_12747204	Abx_Bld_1v2_23	chr25_2529119	Abx_Bld_1v2_38	chr8_5350190
Abx_Bld_1v2_08	chr14_13821711	Abx_Bld_1v7_17	chr26_1322363	Abx_Bld_1v6_25	chr8_9903290
Abx_Bld_1v2_09	chr14_14041698	Abx_Bld_1v3_28	chr26_3762706	Abx_Bld_1v2_41	chr9_14909691
Abx_Bld_1v2_07	chr14_2406601	Abx_Bld_1v5_23	chr26_404051	Abx_Bld_1v6_28	chr9_15840183
Abx_Bld_1v6_07	chr14_4945378	Abx_Bld_1v2_25	chr26_4164386	Abx_Bld_1v6_29	chr9_23157197
Abx_Bld_1v3_16	chr15_10852865	Abx_Bld_1v2_26	chr26_4812686	Abx_Bld_1v5_31	chr9_23470216
Abx_Bld_1v3_17	chr15_11248102	Abx_Bld_1v4_13	chr26_4812695	Abx_Bld_1v4_27	chr9_23509606
Abx_Bld_1v6_10	chr15_12394073	Abx_Bld_1v2_24	chr26_506540	Abx_Bld_1v4_25	chr9_4421109
Abx_Bld_1v7_06	chr15_7187787	Abx_Bld_1v7_16	chr26_627901	Abx_Bld_1v3_37	chr9_4853630
Abx_Bld_1v6_09	chr15_7376358	Abx_Bld_1v2_27	chr27_4283811	Abx_Bld_1v5_30	chr9_6531492
Abx_Bld_1v2_10	chr15_920285	Abx_Bld_1v4_14	chr27_445811	Abx_Bld_1v4_26	chr9_9246049
Abx_Bld_1v6_08	chr15_926910	Abx_Bld_1v4_15	chr27_4600438	Abx_Bld_1v5_32	chrZ_2038891
Abx_Bld_1v6_11	chr17_1959009	Abx_Bld_1v4_16	chr27_4813852	Abx_Bld_1v3_38	chrZ_21909666
Abx_Bld_1v3_18	chr17_5428705	Abx_Bld_1v2_28	chr27_4858045	Abx_Bld_1v6_30	chrZ_27238659
Abx_Bld_1v3_19	chr17_7760918	Abx_Bld_1v4_17	chr27_5078966	Abx_Bld_1v7_32	chrZ_27284938
Abx_Bld_1v7_07	chr17_9246565	Abx_Bld_1v3_30	chr28_1296205	Abx_Bld_1v4_29	chrZ_27627877
Abx_Bld_1v5_09	chr18_10428107	Abx_Bld_1v7_18	chr28_1399026	Abx_Bld_1v7_33	chrZ_39756286
Abx_Bld_1v7_09	chr18_10429145	Abx_Bld_1v5_24	chr28_1400149	Abx_Bld_1v2_42	chrZ_62115017
Abx_Bld_1v7_10	chr18_11039474	Abx_Bld_1v7_19	chr28_1411844	Abx_Bld_1v6_31	chrZ_68082819
Abx_Bld_1v2_12	chr18_7756868	Abx_Bld_1v7_20	chr28_3072184	Abx_Bld_1v7_34	chrZ_77245944
Abx_Bld_1v7_08	chr18_9116930	Abx_Bld_1v4_18	chr28_3600671	Abx_Bld_1v6_32	chrZ_78734434
Abx_Bld_1v2_13	chr18_9721568	Abx_Bld_1v3_29	chr28_863074	Abx_Bld_1v6_33	chrZ_81241584
Abx_Bld_1v2_11	chr18_988524	Abx_Bld_1v2_29	chr28_965371	Abx_Bld_1v4_28	chrZ_8328963
Abx_Bld_1v2_14	chr18_9889858	Abx_Bld_1v5_25	chr3_105678992		
Abx_Bld_1v2_15	chr19_3856575	Abx_Bld_1v6_19	chr3_110316257		

Example 3

Impact of three classes of coccidiostats on the methylome of broiler chickens.

Modern broiler chickens are ubiquitously affected by protozoan parasites of the *Eimeria spp.* which results in an intestinal disease known as coccidiosis. Anticoccidial agents are therefore commonly utilized in broiler chicken management to prevent and manage coccidiosis.

A study is conducted to determine whether methylation of CpG sites in the genome of chicken breast tissue and blood is differentially affected by the administration of different types of anticoccidial agents and if the resultant epigenetic signatures can be used to create an analytic tool to measure anticoccidial usage in broilers from meat products.

A chicken trial was conducted from day of hatch to 42 days to represent the average slaughter age of broiler chickens in Europe. A total of 400 Ross308 male broilers are separated into 40 floor pens containing 10 birds/pen. Corn-soybean meal-based broiler diets are formulated to meet all nutrient requirements for starter (day 0-14), grower (day 14-28) and finisher (day 28-42) feeding phases. Treatments are created by adding treating the broilers with different anticoccidial agents (Table 7).

Table 7. Description of control and anticoccidial treatments in a 42 day broiler chicken trial.

Treatments	Anticoccidial Agent	Pen Replicates	Dose and Mode of Administration
1. Control	None	10	Not Applicable
2. Ionophore	Monteban®, narasin (Elanco)	10	90g/ton, in the feed
3. Chemical Anticoccidial	Clinacox®, diclazuril (Elanco)	10	182g/ton, in the feed
4. Anti-Eimeria Vaccine	Coccivac B, live vaccine (Merk Animal Health)	10	Spray vaccination on Day 2 of broiler chick age

Two animals per pen are sacrificed for sampling on days 28 and 42 for extraction of blood and various tissues including breast muscle tissue (Pectoralis major). A total of 160 samples are then utilized for DNA isolation with Invitrogen™ Purelink™ Genomic DNA Minikit (ThermoFisher Scientific, Waltham, MA, USA) and subsequent reduced bisulphite sequencing to compare methylation level of CpG sites in the genome.

Chicken samples for blood as well as for breast tissue are prepared with the Zymo RRBS protocol. The resulting libraries are sequenced and mapped to the gallus gallus 5.0 genome using the tool bsmmap 2.9. From the resulting methylation ratios all CpGs are kept, which had a non-zero coverage in all individual samples for a certain tissue.

To further analyze the ability to classify the samples based on the methylation data for treatments with different anticoccidial agents, the R package randomForest is used and a random forest-based classification set up, using the 100,000 most variable CpGs in each tissue

First, the set of CpGs is split in chunks of 10,000 and each chunk is used to classify the samples into different categories, using a random forest. This is repeated 100 times. All CpGs having a positive score

"MeanDecreaseAccuracy" in an individual classification are pooled and the top 10,000 CpGs, occurring most often, are kept. Then a second random forest is set up, using these selected 10,000 CpGs as features and a classification is performed. This is repeated 100 times and the average classification error, as well as the optimal classification error is evaluated.

5

The results show results indicate that treatment of broiler chickens with anticoccidial agents results in significant shifts to the CpG methylation profiles, which may therefore be used to differentiate anticoccidial - free from anticoccidial-treated poultry meat. The results also indicate that epigenetic signatures in the tissues of broiler chickens can be used to differentiate which anticoccidial type is

10

utilized.

CLAIMS

1. A method of determining if a test animal and/or a test animal from which a product is derived has been treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical, the method comprising:
- 5 (a) determining a test methylation profile from the genomic material contained in a biological sample obtained from the test animal and/or the animal-derived product; and
- (b) comparing the test methylation profile obtained from (a) with a reference methylation profile obtained from a control animal of the same biological taxon of the test animal, where the control animal was not treated and/or is not currently undergoing treatment with at least one antibiotic and/or veterinary chemical,
- 10 wherein a difference in the test methylation profile of (a) compared to the reference methylation profile from the control animal, is indicative of the test animal having been treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical; and
- 15 wherein the test animal is a terrestrial animal; and
- wherein the veterinary chemical is an anti-parasitic, an anti-viral, a feed additive, a water additive, a disinfectant, glutaraldehyde, and/ or formalin.
2. The method according to claim 1, wherein the difference is hypomethylation or hypermethylation.
- 20 3. A method of determining if a test animal and/or a test animal from which a product is derived has been treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical, the method comprising:
- (a) determining the methylation status of at least one CpG site in genomic material contained in a biological sample obtained from the test animal,
- 25 (b) comparing the methylation status of the CpG site from (a) with that of a control animal of the same biological taxon as the test animal, which was not treated and/or is not currently undergoing treatment with at least one antibiotic and/or veterinary chemical,
- wherein a difference in the test methylation status of (a) in the test animal compared to the CpG site in the control animal is indicative of the test animal having been treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical; and
- 30 wherein the test animal is a terrestrial animal and
- wherein the veterinary chemical is an anti-parasitic, an anti-viral, a feed additive, a water additive, a disinfectant, glutaraldehyde, and/ or formalin.
- 35 4. The method according to claim 3, wherein the difference in methylation status is hypomethylation or hypermethylation of the CpG site in the test animal and the hypomethylation

or hypermethylation of the CpG site is indicative of the test animal having been treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical.

5. The method according to either claim 3 or 4, wherein the CpG site is at least one CpG site selected from the Table below:

No.	Chromosome	Position	No.	Chromosome	Position
1	20	10783159	17	3	3328494
2	24	4184075	18	4	1627649
3	25	1910205	19	6	11522056
4	4	1712424	20	6	23106717
5	4	50270218	21	8	5481923
6	5	57738909	22	33	716102
7	7	10956706	23	22	4604122
8	12	18699814	24	22	4617202
9	12	2415548	25	23	502108
10	17	4082979	26	24	5683979
11	18	9932606	27	27	3015432
12	2	29649123	28	3	10479779
13	23	4069359	29	19	5380287
14	25	1445548	30	15	12638203
15	28	3467160	31	10	20073849
16	3	10490126	32	5	55805858

6. The method according to any one of the preceding claims, wherein the animal-derived product is meat, processed meat, milk, collagen, feather, dairy products, blood and/or bone.
- 10 7. The method according to any one of the preceding claims, wherein the animal derived product is from the terrestrial animal selected from the group consisting of chicken, lamb, cow, goat, sheep, pig, horse, donkey, turkey, duck, goose, quail, rabbit and mule.
- 15 8. The method according to any one of the preceding claims, wherein the biological sample is selected from the group consisting of blood, brain, sperm, milk and any other tissue or sample that provides genomic DNA.
- 20 9. The method according to any one of the preceding claims, wherein the antibiotic is selected from the group of classes consisting of amphenicols, aminocyclitols, aminoglycosides, ansamycins, beta-lactams, carbaephem, carbapenems, cephalosporins, chloramphenicol, fluoroquinolones, glycopeptides, glycylicyclines, ketolides, lincosamides, lipopeptides, macrolides, monobactams, nitrofurans, nitroimidazoles, oxazolidinones, penicillins, phosphonic acid derivatives, pleuromutilins, polymyxins, polypeptides, quinolones, rifamycins, riminofenazines, steroid antibacterials, streptogramins, sulfonamides, tetracyclines, and trimethoprim.
- 25

10. The method according any one of the preceding claims, wherein the feed additive is a coccidiostat or ionophore.
- 5 11. A method of determining if a test animal and/or a test animal from which a product is derived has been treated and/or is currently undergoing treatment with at least one antibiotic, and if so, determining the distinct class of antibiotics with which the test animal is being treated and/or is currently undergoing treatment, the method comprising:
- 10 (a) determining a test methylation profile from the genomic material contained in a biological sample obtained from the test animal and/or the animal-derived product sample; and
- (b) comparing the test methylation profile obtained from (a) with one or more predetermined reference methylation profiles, wherein each of the predetermined reference methylation profiles is from a different animal of the same biological taxon as the test animal, and each of the different animals was treated with a different class of antibiotics,
- 15 wherein if the test methylation profile of (a) is significantly similar to one of the predetermined reference methylation profiles, the test animal is confirmed for being treated with the same class of antibiotics with which the animal with the predetermined reference profile is treated with and the test animal has been confirmed for having been treated and/or is currently undergoing treatment with the distinct class of antibiotics; and
- 20 wherein the test animal is a terrestrial animal.
12. The method according to claim 10, wherein the distinct classes of antibiotics are amphenicols, aminocyclitols, aminoglycosides, ansamycins, beta-lactams, carbaephem, carbapenems, cephalosporins, chloramphenicol, fluoroquinolones, glycopeptides, glycyclines, ketolides, lincosamides, lipopeptides, macrolides, monobactams, nitrofurans, nitroimidazoles,
- 25 oxazolidinones, penicillins, phosphonic acid derivatives, pleuromutilins, polymyxins, polypeptides, quinolones, rifamycins, riminofenazines, steroid antibacterials, streptogramins, sulfonamides, tetracyclines, and trimethoprim.
13. A method of determining if a test animal and/or a test animal from which a product is derived
- 30 has been treated and/or is currently undergoing treatment with at least one antibiotic, and if so, determining if the antibiotic is used as a growth promotant or as a therapeutant, the method comprising:
- (a) determining a test methylation profile from the genomic material contained in a biological sample obtained from the test animal and/or the animal-derived product; and
- 35 (b) comparing the test methylation profile obtained from (a) with one or more predetermined reference methylation profiles, wherein each of the predetermined reference methylation profiles is from a different control animal of the same biological taxon as the test animal, and each of the different control animals was treated with the antibiotic as a growth promotant or therapeutant,

wherein if the test methylation profile of (a) is significantly similar to one of the predetermined reference methylation profiles, the test animal is confirmed for being treated with the antibiotic in the same way that the control animal with the similar predetermined reference profile is treated and the test animal has been confirmed for having been treated and/or is currently undergoing treatment with the antibiotic as a growth promotant or therapeutant; and

wherein the test animal is a terrestrial animal.

14. A method of determining if a test animal from which a product is derived underwent a withdrawal period of no treatment with at least one antibiotic and/or veterinary chemical prior to the product being obtained, the method comprising:

- (a) determining a test methylation profile from the genomic material contained in a biological sample obtained from the animal-derived product; and
- (b) comparing the test methylation profile obtained from (a) with at least two predetermined reference methylation profiles, wherein at least one of the predetermined reference methylation profiles is from a control animal that underwent a withdrawal period and at the other one of the predetermined reference methylation profiles is from a control animal of the same biological taxon as the test animal, that did not undergo a withdrawal period before the product was obtained,

wherein if the test methylation profile of (a) is significantly similar to the predetermined reference methylation profile, the test animal is confirmed for having undergone a withdrawal period or not; and

wherein the test animal is a terrestrial animal and

wherein the veterinary chemical is an anti-parasitic, an anti-viral, a feed additive, a water additive, a disinfectant, glutaraldehyde, and/ or formalin.

15. Use of DNA methylation profiling for determining if a test animal and/or a test animal from which a product is derived has been treated and/or is currently undergoing treatment with at least one antibiotic and/or veterinary chemical, wherein the test animal is a terrestrial animal and the veterinary chemical is an anti-parasitic, an anti-viral, a feed additive, a water additive, a disinfectant, glutaraldehyde, and/ or formalin.