Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3092519 A1 2019/09/06

(21) 3 092 519

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2019/02/28
(87) Date publication PCT/PCT Publication Date: 2019/09/06
(85) Entrée phase nationale/National Entry: 2020/08/28
(86) N° demande PCT/PCT Application No.: EP 2019/054947
(87) N° publication PCT/PCT Publication No.: 2019/166534
(30) Priorité/Priority: 2018/03/02 (EP18159632.1)

(51) Cl.Int./Int.Cl. *G01N 33/48* (2006.01), *G01N 33/543* (2006.01), *G01N 33/558* (2006.01)

(71) Demandeur/Applicant:
EVONIK OPERATIONS GMBH, DE

(72) Inventeurs/Inventors:
FLUGEL, MONIKA, DE;
PELZER, STEFAN, DE;
THIEMANN, FRANK, DE;
VAN IMMERSEEL, FILIP, BE;
DUCATELLE, RICHARD, BE;
GOOSSENS, EVY, BE;
DEVREESE, BART, BE;
DEBYSER, GRIET, BE

(74) Agent: ROBIC

(54) Titre : PROCEDE IN VITRO DE DETECTION D'UNE DEFAILLANCE DE LA BARRIERE INTESTINALE CHEZ DES ANIMAUX PAR DETERMINATION DE L'OVOTRANSFERRINE

(54) Title: IN VITRO METHOD FOR DETECTING INTESTINAL BARRIER FAILURE IN ANIMALS BY DETERMINING OVOTRANSFERRIN

(57) Abrégé/Abstract:
The present invention pertains to an in vitro method for detecting intestinal barrier failure in animals, the method comprising the following steps: a) collecting intestinal sample material of an individual animal or of an animal population; and b) determining the amount of at least one protein marker contained in said sample material; wherein the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof, and wherein an increased amount of said at least one protein marker contained in said sample versus a reference sample indicates intestinal barrier failure.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
06 September 2019 (06.09.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/166534 A1

(51) International Patent Classification:
G01N 33/68 (2006.01)

(21) International Application Number:
PCT/EP2019/054947

(22) International Filing Date:
28 February 2019 (28.02.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
18159632.1 02 March 2018 (02.03.2018) EP

(71) Applicant: **EVONIK DEGUSSA GMBH** [DE/DE]; Rellinghauser Straße 1-11, 45128 Essen (DE).

(72) Inventors: **FLÜGEL, Monika**; Brandenburger Str. 34, 33803 Steinhagen (DE). **PELZER, Stefan**; Ludwig-Uhland-Str. 33a, 33335 Gütersloh (DE). **THIEMANN, Frank**; Heller 38, 48031 Nottuln (DE). **VAN IMMERSEEL, Filip**; Eikenstraat 7, 9810 Eke (BE). **DUCATELLE, Richard**; Lozerstraat 1, 9790 Wortegem-Petegem (BE). **GOOSSENS, Evy**; Walderdonk 39a, 9185 Wachtebeke (BE). **DEVREESE, Bart**; Landegemstraat 21, 9850 Vosselare (BE). **DEBYSER, Griet**; Diksmuidseheerweg 105, 8200 Brügge (BE).

(74) Agent: **EVONIK PATENT ASSOCIATION**; c/o Evonik Industries AG, IP Management, Bau 1042A/PB 15, Paul-Baumann-Straße 1, 45772 Marl (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

Published:
— *with international search report (Art. 21(3))*

WO 2019/166534 A1

(54) **Title:** IN VITRO METHOD FOR DETECTING INTESTINAL BARRIER FAILURE IN ANIMALS BY DETERMINING OVOTRANSFERRIN

(57) **Abstract:** The present invention pertains to an in vitro method for detecting intestinal barrier failure in animals, the method comprising the following steps: a) collecting intestinal sample material of an individual animal or of an animal population; and b) determining the amount of at least one protein marker contained in said sample material; wherein the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof, and wherein an increased amount of said at least one protein marker contained in said sample versus a reference sample indicates intestinal barrier failure.

IN VITRO METHOD FOR DETECTING INTESTINAL BARRIER FAILURE IN ANIMALS BY DETERMINING OVOTRANSFERRIN

Field of the Invention

The present invention relates to an *in vitro* method for detecting intestinal barrier failure in animals. More specifically, the present invention pertains to an acute phase protein (APP)-based method for evaluating the gut health status of an individual animal and of an animal population, respectively.

Background of the Invention

Intestinal health is critically important for the welfare and performance of livestock animals. Enteric diseases that affect the structural integrity of the gastrointestinal tract (GIT) lead to high economic losses due to reduced weight gain, poor feed conversion efficiency, increased mortality rates and greater medication costs (M'Sadeq, S.A., Wu, S., Swick, R.A. & Choct, M. (2015). Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. *Animal Nutrition,* 1, 1-11; Timbermont, L., Haesebrouck, F., Ducatelle, R. & Van Immerseel, F. (2011). Necrotic enteritis in broilers: an updated review on the pathogenesis. *Avian Pathol,* 40, 341-347).

An intact intestinal barrier provides a number of physiological and functional features, including nutrient digestion and absorption, host metabolism and energy generation, a stable microbiome, mucus layer development, barrier function, and mucosal immune responses (Kogut, M. H. and R. J. Arsenault (2016). Editorial: Gut health: The new paradigm in food animal production. Frontiers in Veterinary Science **3** (AUG)). As the largest organ in the body, the gut serves as a selective barrier to take up nutrients and fluids into the body, while excluding undesirable molecules and pathogens. Therefore, proper gut barrier function is essential to maintain optimal health and balance throughout the body, and represents a key line of defense against foreign antigens from the environment.

Coccidiosis and necrotic enteritis (NE) probably are the most common enteric diseases of poultry (Dalloul, R.A. & Lillehoj, H.S. (2006). Poultry coccidiosis: recent advancements in control measures and vaccine development. *Expert Rev Vaccines,* 5, 143-163; Williams, R.B. (2005). Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. *Avian Pathol,* 34, 159-180). In poultry, coccidiosis can be caused by multiple species belonging to the genus *Eimeria*, from which *Eimeria acervulina, E. maxima* and *E. tenella* are the most common species in intensively reared broilers. Depending on the species, the lesions can range from a limited malabsorptive enteritis (*E. acervulina*) to more severe inflammation of the intestinal wall (*E. maxima*) and even extensive caecal haemorrhage and death (*E. tenella*) (Chapman, H.D. (2014). Milestones in avian coccidiosis research: a review. *Poult Sci,* 93, 501-511). Furthermore, the presence of *Eimeria* species can also exacerbate the outcome of co-infection with bacterial pathogens such as *Clostridium perfringens* (Moore, R.J. (2016). Necrotic

enteritis predisposing factors in broiler chickens. *Avian Pathol,* 45, 275-281). Indeed, the mucosal damage caused by these coccidial pathogens is an important predisposing factor for necrotic enteritis. NE is the most common clostridial enteric disease in poultry, which typically occurs in broiler chickens. The disease is caused by *C. perfringens* and can occur either as an acute clinical or as a mild subclinical form. Acute NE typically leads to a massive increase in flock mortality. The more common subclinical form is characterized by multifocal necrosis and inflammation of the small intestine with a significant decline in growth performance. The reduction in performance is not only associated with impaired growth rate and feed conversion during production, but also with increased condemnation rates in broilers due to hepatitis at processing (Paiva, D. & McElroy, A. (2014). Necrotic enteritis: Applications for the poultry industry. *Journal of Applied Poultry Research,* 23, 557-566). Both coccidiosis and necrotic enteritis can be present in a flock without showing clinical signs. Therefore, multiple birds have to be sacrificed for macroscopic examination of the intestine to diagnose the disease.

Similar considerations apply for other enteric diseases or conditions in livestock animals which are leading to mucosal damage, such as severe bacterial overgrowth in the small intestine, all forms of excessive gut inflammation, exposure to mycotoxins, and every condition which leads to intestinal barrier failure. In addition, intestinal barrier failure might enable normal inhabitants of the GIT, like *Enterococcus caecorum* to invade the systemic circulation. This can lead to further diseases like arthritis and osteomyelitis and finally lead to lower performance of the animals or the animal flock, respectively.

A marker, or a set of markers, that can accurately detect intestinal barrier failure or intestinal inflammation and concomitant perturbation of the intestinal integrity at an early stage would thus be highly desirable.

Recently, there has been increased interest in research on intestinal permeability in chickens, resulting in different strategies to measure intestinal inflammation and concomitant intestinal barrier failure. However, none of the proposed strategies represent a good marker for the broiler industry as they are either not applicable under field conditions (e.g. oral administration of a marker that can be measured in the blood on a later timepoint (Gilani, S., Howarth, G.S., Kitessa, S.M., Tran, C.D., Forder, R.E.A. & Hughes, R.J. (2017). New biomarkers for increased intestinal permeability induced by dextran sodium sulphate and fasting in chickens. *J Anim Physiol Anim Nutr (Berl),* 101, e237-e245; Vicuna, E.A., Kuttappan, V.A., Tellez, G., Hernandez-Velasco, X., Seeber-Galarza, R., Latorre, J.D., et al. (2015). Dose titration of FITC-D for optimal measurement of enteric inflammation in broiler chicks. *Poult Sci,* 94, 1353-1359) or non-specific for intestinal barrier failure, such as serum markers that can be elevated by non-gastrointestinal conditions as well (Chen, J., Tellez, G., Richards, J.D. & Escobar, J. (2015). Identification of Potential Biomarkers for Gut Barrier Failure in Broiler Chickens. *Front Vet Sci,* 2, 14; O'Reilly, E.L. & Eckersall, P.D. (2014). Acute phase proteins: a review of their function, behaviour and measurement in chickens. *Worlds Poultry*

Science Journal, 70, 27-43; Xie, H., Newberry, L., Clark, F.D., Huff, W.E., Huff, G.R., Balog, J.M., et al. (2002). Changes in serum ovotransferrin levels in chickens with experimentally induced inflammation and diseases. *Avian Dis,* 46, 122-131).

It was thus a remaining need to provide a fast and reliable, ideally non-invasive *ante mortem* method for determining whether or not an individual animal or an animal population suffers from intestinal barrier failure that can be performed under field conditions at low cost and with minimal effort.

Summary of the Invention

Accordingly, one objective of the present invention is to provide an *in vitro* method for detecting intestinal barrier failure in animals, the method comprising the following steps:

a) collecting intestinal sample material of an individual animal or of an animal population; and

b) determining the amount of at least one protein marker contained in said sample material;

wherein

the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof,

and wherein

an increased amount of said at least one protein marker contained in said sample versus a reference sample indicates intestinal barrier failure.

An additional aspect of the present application is the use of ovotransferrin or of functional fragments thereof as intestinal markers for detecting intestinal barrier failure in an animal subject or in an animal population.

A further objective of the present invention is the provision of an *in vitro* method for detecting the extent of intestinal barrier failure in animals, the method comprising the following steps:

a) collecting intestinal sample material of a specific animal or of an animal population; and

b) determining the amount of at least one protein marker contained in the sample material;

wherein

the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof,

and wherein

the amount of said at least one protein marker contained in the sample indicates the extent of the intestinal barrier failure.

Finally, the present invention provides an *in vitro* method for monitoring the status of the intestinal barrier in animals, the method comprising the following steps:

a) collecting intestinal sample material of a specific animal or of an animal population at consecutive points in time;

b) determining the amount of at least one protein marker contained in the samples obtained in step a); and

c) determining deviations in the amounts of said at least one protein marker contained in the samples obtained in step a);

wherein

the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof.

In the following, the crucial aspects of the present invention are described in detail.

Detailed Description of the Invention

The present inventors have unexpectedly found that the amount of acute phase protein (APP)-based markers contained in sample material of animal origin correlates with the manifestation of intestinal barrier failure. In particular, it was found that the amount of ovotransferrin, or functional fragments thereof, in intestinal sample material of animal origin correlates with intestinal barrier failure.

More specifically, the inventors have found that an increase in the amount of ovotransferrin, or functional fragments thereof, contained in intestinal sample material of animal origin versus a reference sample indicates intestinal barrier failure.

Accordingly, the present invention pertains to an *in vitro* method for detecting intestinal barrier failure in animals, the method comprising the following steps:

a) collecting intestinal sample material of an individual animal or of an animal population; and

b) determining the amount of at least one protein marker contained in said sample material;

wherein

the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof,

and wherein

an increased amount of said at least one protein marker contained in said sample versus a reference sample indicates intestinal barrier failure.

As used in the context of the present invention, the term “intestinal barrier failure” refers to conditions in which the intestinal barrier function is significantly impaired (e.g. due to oxidative stress, poorly digestible protein, coccidiosis, etc.); and comprises conditions of intestinal barrier dysfunction, intestinal leakages/permeability and conditions caused by histopathologic injuries.

Intestinal barrier failure is often associated with inflammatory processes. The terms “intestinal barrier failure” and “gut barrier failure” may be used interchangeably.

The reference sample is a species-specific control representing an intact intestinal barrier or gut barrier. Suitable reference samples are samples obtained from an individual animal or from an animal population of the same animal species or sub-species, whereby said animal or said animal population has a proven intact intestinal barrier.

As an example, a reference sample may be taken within an animal trial from an animal of a non-treated control, which was checked via pathology, histopathology and/or other measures to have no signs of intestinal barrier failure.

The ovotransferrin markers may be detected and quantified using the commonly known, conventional techniques, such as immunoassays like ELISA (Enzyme-linked Immunosorbent Assay), lateral flow assays, mass spectrometry (MS) analyses, or any method enabling the detection of proteins or functional fragments thereof.

In one specific embodiment, the ovotransferrin, or functional fragments thereof, is detected and quantified via ELISA.

The use of monoclonal antibodies enables a specific detection in the complex sample matrix used for the analysis.

The method of the present invention may be used for determining whether or not an individual animal suffers from intestinal barrier failure. In that case, the intestinal sample material originates from an individual animal.

The individual animal may for example be a pet or domestic animal, a farm animal as occurring in live stocks, a wild-living animal or a zoo animal. Further, animal individuals being transported for slaughter or for re-location may be examined using the above method.

In one embodiment, the individual animal is an avian subject.

The avian subject to be tested is preferably poultry. Preferred poultry according to the invention are chickens, turkeys, ducks and geese. The poultry can be optimized for producing young stock. This type of poultry is also referred to as parent and grandparent animals. Preferred parent and grandparent animals are, accordingly, (grand)parent broilers, (grand)parent ducks, (grand)parent turkeys and (grand)parent geese.

The poultry according to the invention can also be selected from fancy poultry and wild fowl. Preferred fancy poultry or wild fowl are peacocks, pheasants, partridges, guinea fowl, quails, capercailzies, goose, pigeons and swans. Further preferred poultry according to the invention are ostriches and parrots. Most preferred poultry according to the invention are broilers.

The intestinal sample material obtained from an individual animal may be selected from the group consisting of gut content samples, samples of bodily excrements and solutions or suspensions thereof; and from materials being contaminated with bodily excrements. The term “gut content” is to be understood as the content of the small intestine, the content of the large intestine and/or the content of the caecum. Methods for taking such gut content samples are known in the art.

As used in the context of the present invention, bodily excrements are fecal or cecal excrements. Materials being contaminated with bodily excrements are, for example, dust samples, swab samples, litter samples, liquid manure samples, fur samples, feather samples and skin samples.

In general, the term “litter” is to be understood as a mixture of animal excrements with the bedding material.

As used in the context of this embodiment, the term “litter samples” refers to excremental droppings from an individual animal. Further, in the context this embodiment, the term “liquid manure samples” refers to an excremental sample containing feces and urine from an individual animal.

Samples from individual animals can be taken either directly from the animal, e.g. with swabs. Alternatively and especially in case of single-housed animals, the sample material can be collected from the floor of the pen, cage or slat. The sample material has to be assignable to the investigated animal.

In one embodiment, the intestinal sample material used for determining whether or not an individual animal suffers from intestinal barrier failure is feces.

For specific applications, it is also useful to analyze gut content samples, e.g. samples from the small intestine, samples from the large intestine and/or samples from the caecum.

Suitable sample volumes are, for example, 0.05 ml to 20 ml or 0.1 to 20 ml, in particular 0.2 to 10 ml, preferably 0.5 to 5 ml. Suitable sample masses are, for example 0.05 g to 20 g or 0.1 to 20 g, in particular 0.2 to 10 g, preferably 0.5 to 5 g

In an alternative embodiment, the method is used for determining whether or not an animal population suffers from intestinal barrier failure. In that case, the sample material originates from the group of animals to be tested.

As used herein, the term “animal population” refers to a group of animal individuals belonging to the same species. The animal population may for example be a group of pets or domestic animals as occurring in animal breeding, a group of farm animals as occurring in livestock production or in livestock breeding, or a group of wild-living animals or zoo animals.

In one embodiment, the animal population is an animal flock as occurring in livestock production processes. For example, the animal population or the animal flock can be an avian flock; a flock of sheep, goat or cattle, a flock of horses or a flock of pigs.

In one specific embodiment, the animal population is an avian population.

The animal population preferably is an avian flock. The avian flock according to the invention is preferably poultry. Preferred poultry according to the invention are chickens, turkeys, ducks and geese. The poultry can be optimized for producing young stock. This type of poultry is also referred to as parent and grandparent animals. Preferred parent and grandparent animals are, accordingly, (grand)parent broilers, (grand)parent ducks, (grand)parent turkeys and (grand)parent geese.

The poultry according to the invention can also be selected from fancy poultry and wild fowl. Preferred fancy poultry or wild fowl are peacocks, pheasants, partridges, guinea fowl, quails, capercailzies, goose, pigeons and swans. Further preferred poultry according to the invention are ostriches and parrots. Most preferred poultry according to the invention are broilers.

The method of the present invention is particularly suitable for determining the health status of an animal population via bulk testing. As used herein, the term “bulk testing” refers to a test method, wherein the sample material is a pooled sample of an animal population. A “pooled sample” in the context of this embodiment is to be understood as a composite sample from randomly selected separate samples, one sample taken with one or several moistened fabric swabs or pooled samples made up of separate samples of fresh samples taken at random from a number of sites in the house or space in which the animal population or the animal flock is kept. It may be necessary that the sample material is homogenized prior to sample analysis. Suitable homogenization techniques are known in the art.

The pooled samples reflect the amount of ovotransferrin present in the animal population.

The sample material obtained from an individual animal may be selected from the group consisting of gut content samples, samples of bodily excrements and solutions or suspensions thereof; and

from materials being contaminated with bodily excrements. Materials being contaminated with bodily excrements are, for example, dust samples, swab samples, litter samples, liquid manure samples, fur samples, feather samples and skin samples.

As used in the context of this embodiment, the term “litter samples” refers to mixed excremental droppings in the pen, cage or slat. Further, in the context this embodiment, the term “liquid manure samples” refers to mixed excremental samples containing feces and urine.

These litter samples can, for example, be collected from an animal population using the overshoe method or using litter grabs at different places in the pen.

Boot swabs being sufficiently absorptive to soak up moisture are particularly suitable for collecting pooled animal samples. Tube gauze socks are also acceptable.

In case the animal population is kept in cages or slats, the excremental samples may be collected by a conveying belt.

In one embodiment, the sample material used for determining whether or not an animal population suffers from intestinal barrier failure is feces. Preferably, the sample material is a pooled fecal sample deriving from an avian flock.

For specific applications, it is also useful to analyze pooled gut content samples, e.g. pooled samples from the small intestine, pooled samples from the large intestine and/or pooled samples from the caecum.

Suitable sample volumes are, for example, 0.1 to 20 ml, in particular 0.2 to 10 ml, preferably 0.5 to 5 ml. Suitable sample masses are, for example 0.1 to 20 g, in particular 0.2 to 10 g, preferably 0.5 to 5 g.

Depending on the sample material and storage conditions, it may be helpful to stabilize the samples taken in order to avoid enzymatic degradation of the ovotransferrin contained in the samples, for example by treating the samples with protease inhibitors. Preferably, the stabilizing agent is added to the sample immediately after sample collection.

In accordance with the above, one specific embodiment of the present application pertains to an *in vitro* method for detecting intestinal barrier failure in an avian flock, the method comprising the following steps:

a) collecting and pooling fecal sample material deriving from said avian flock;
b) optionally stabilizing the pooled sample material; and
c) determining the amount of ovotransferrin contained in said pooled sample material;

wherein
an increased amount of ovotransferrin contained in the sample versus a reference sample indicates intestinal barrier failure.

In addition to the above, the inventors have unexpectedly found that the amount of ovotransferrin contained in intestinal sample material deriving from an individual animal or from an animal population correlates with the extent of intestinal barrier failure. Accordingly, the present invention provides an *in vitro* method for detecting the extent of intestinal barrier failure in animals, the method comprising the following steps:

a) collecting intestinal sample material of a specific animal or of an animal population; and

b) determining the amount of at least one protein marker contained in the sample material;

wherein
the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof,
and wherein
the amount of said at least one protein marker contained in the sample indicates the extent of the intestinal barrier failure.

Suitable sample materials testing parameters and –conditions are as defined above.

In a particularly preferred embodiment, the intestinal sample material is a pooled fecal sample deriving from an avian flock and the at least one protein marker is ovotransferrin or a functional fragment thereof.

The present invention provides the abovementioned methods for detecting intestinal barrier failure and for determining the extent thereof, respectively. This enables the farmer to make a qualified decision on whether or not measures for improving intestinal health are to be taken.

Measures against the development and/or against the progression of intestinal barrier failure involve feeding or administering health-promoting substances, such as zootechnical feed additives, or therapeutic agents. The term “administering” or related terms includes oral administration. Oral administration may be via drinking water, oral gavage, aerosol spray or animal feed. The term “zootechnical feed additive” refers to any additive used to affect favorably the performance of animals in good health or used to affect favorably the environment. Examples for zootechnical feed additives are digestibility enhancers, i.e. substances which, when fed to animals, increase the digestibility of the diet, through action on target feed materials; gut flora stabilizers; micro-organisms or other chemically defined substances, which, when fed to animals, have a positive effect on the gut flora; or substances which favorably affect the environment. Preferably, the health-promoting substances are selected from the group consisting of probiotic agents, praebiotic agents,

botanicals, organic/fatty acids, zeolithes, bacteriophages and bacteriolytic enzymes or any combinations thereof.

The inventors have found that the testing procedures underlying the present invention may also be used for monitoring the intestinal health status in animals.

As used in the context of this embodiment, the term “intestinal health status” refers to status of the intestinal barrier.

By the above method, the development or the progression of an intestinal barrier failure may be detected. On the other hand, the effectiveness of measures taken against the development and/or against the progression of intestinal barrier failure may be controlled.

Accordingly, the present invention also pertains to an *in vitro* method for monitoring the status of the intestinal barrier in animals, the method comprising the following steps:

a) collecting intestinal sample material of a specific animal or of an animal population at consecutive points in time;

b) determining the amount of at least one protein marker contained in the samples obtained in step a); and

c) determining deviations in the amounts of said at least one protein marker contained in the samples obtained in step a);

wherein

the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof.

Therein, an increase in the amount of ovotransferrin over time indicates the development or progression of intestinal barrier failure. Conversely, a decrease in the amount of ovotransferrin over time indicates improvements in the intestinal health situation which may be caused by natural healing processes or by specific measures being taken against the development or progression of intestinal barrier failure.

An “increase” or a “decrease” in the amount of ovotransferrin typically refers to a statistically relevant amount.

Suitable sample materials and testing parameters and –conditions are as defined above. In a specific embodiment, the intestinal sample material is a pooled sample deriving from an avian flock and the at least one protein marker is ovotransferrin or a functional fragment thereof.

As an example, after initial determination of the amount of ovotransferrin in an intestinal sample, the amount of ovotransferrin may be monitored in test samples collected and analyzed in a weekly,

daily our hourly manner. In one embodiment, excremental samples are collected and analyzed at consecutive days. The excremental test samples may be taken and analyzed on a daily basis from birth to slaughter.

In a specific embodiment for poultry, a first test sample is preferably taken and analyzed during the initial growth phase (starter phase, day 5 to day 10), a second test sample is taken and analyzed during the enhanced growth phase (day 11 to day 18) and, optionally, a third test sample is taken and analyzed on a later stage.

In an alternative embodiment, a first test sample is taken and analyzed in the initial growth phase and further test samples are taken and analyzed for example on a daily basis during the enhanced growth phase, optionally until slaughter.

A further aspect of the present invention is the use of ovotransferrin, or functional fragments thereof, as intestinal markers for detecting intestinal barrier failure in an animal subject or in an animal population. A specific embodiment of the present invention is the use of ovotransferrin as a fecal marker for detecting intestinal barrier failure in an avian subject or in an avian population.

Applications of the methods according to the invention are for example (i) aiding in the diagnosis and/or prognosis of intestinal barrier failure caused by enteric diseases; (ii) monitoring the progress or reoccurrence of intestinal barrier failure or (iii) aiding in the evaluation of treatment efficacy for an animal population undergoing or contemplating treatment.

Applications of the invention in particular help to avoid loss in animal performance like weight gain and feed conversion.

In the following, the invention is illustrated by non-limiting examples and exemplifying embodiments.

Examples

Coccidiosis and necrotic enteritis in broiler chickens were used as models for intestinal barrier failure. Ovotransferrin serves as protein marker.

Necrotic enteritis trials – sample collection

Groups of 27 one-day-old Ross 308 broiler chickens were fed a diet rich in proteins and non-starch polysaccharides which predispose to the development of necrotic enteritis. The detailed diet composition was described by Gholamiandehkordi et al. (Gholamiandehkordi, A.R., Timbermont, L., Lanckriet, A., Van Den Broeck, W., Pedersen, K., Dewulf, J., et al. (2007). Quantification of gut lesions in a subclinical necrotic enteritis model. *Avian Pathol,* 36, 375-382). Other predisposing factors consist of the administration of Gumboro vaccine to induce mild immunosuppression and a

ten-fold dose of coccidiosis vaccine (either Paracox-8 or Hipracox, depending on the trial) to induce predisposing intestinal damage. To induce necrotic lesions, animals were challenged with approximately 4.10^8 CFU of the netB-positive *C. perfringens* strain CP56 on three consecutive days, after which the animals were euthanized. At necropsy, lesion scoring in the small intestine (duodenum, jejunum and ileum) was performed as described by Keyburn *et al.* (Keyburn, A.L., Sheedy, S.A., Ford, M.E., Williamson, M.M., Awad, M.M., Rood, J.I., et al. (2006). Alpha-toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens. *Infect Immun,* 74, 6496-6500) as follows: score 0 = no lesions, score 1 = congested intestinal mucosa, score 2 = focal necrosis or ulcerations (1-5 foci), score 3 = focal necrosis or ulcerations (6-15 foci), score 4 = focal necrosis or ulcerations (≥ 16 foci), score 5 = patches of necrosis of 2-3 cm long, score 6 = diffuse necrosis. Birds with a lesion score of 2 or more are classified as necrotic enteritis positive. Fresh cloacal samples were collected from all birds and frozen at -70°C. In addition, mixed litter was collected from each pen and frozen at -70°C.

After lesion scoring, the samples were grouped according to the disease severity of the animal, leading to the following disease severity groups: birds that received all predisposing factors but were not challenged with *C. perfringens*: negative control; birds challenged with *C. perfringens* but no necrosis: score 0 or challenged with *C. perfringens* and various severity degrees: score 2 (mild), score 3-4 (moderate) or score 5-6 (severe).

<u>*Coccidiosis trials – sample collection*</u>

Fifteen-day-old Ross 308 broiler chicks were orally challenged with *E. acervulina* and *E. tenella*. One mixed litter sample from each pen and cloacal samples from all birds were collected 7 days after challenge, when the chickens were euthanized for lesion scoring using the method of Johnson and Reid (Johnson, J. & Reid, W.M. (1970). Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. *Exp Parasitol,* 28, 30-36). On the same day, mixed litter and cloacal content samples were collected from their age-matched controls. All challenged birds showed macroscopically visible lesions of *Eimeria* infection, with a mean coccidiosis score of 5.11 ± 0.51, whereas only one out of then birds in the unchallenged control group was coccidiosis positive (coccidiosis score = 1). All samples were stored at -70°C.

<u>*Ovotransferrin detection by Enyzme-linked Immunosorbent Assay (ELISA)*</u>

Eight samples from the negative control birds (not challenged with *C. perfringens*) and eight sample per necrosis score group from challenged birds were selected. Also litter samples collected at the day of necropsy were included (one litter samples per pen, with in total 3 samples from pens with non-challenged birds and 3 samples from pens with challenged birds). Additionally, the ovotransferrin concentration was determined in both cloacal samples and litter samples from the coccidiosis trial. Therefore, 1 litter sample per pen was used, with in total 5 samples from pens with non-challenged birds and 6 samples from pens with *Eimeria*-challenged birds. Additionally, 20

cloacal samples from either *Eimeria*-challenged birds (*n* = 10) or their non-challenged controls (*n* = 10) were selected.

Unprocessed cloacal material or homogenized litter material was thawed at room temperature. 150 mg cloacal content or litter material was diluted in 1500 µl TBS (50mM Tris, 150mM NaCl, pH = 7.2) with protease inhibitor cocktail (P2714, Sigma-Aldrich). The samples were mixed by vortex (2x 1 min). Proteins (supernatants) were collected after centrifugation (13.000 x g, 10', 4°C) and were used in duplicate (1/50 dilution) in the ELISA (Chicken Ovotransferrin ELISA, KT-530, Kamiya Biomedical Company, Tukwila, USA). The ELISA was performed according to the instructions of the manufacturer.

Statistical analysis

Normality of the data was tested with the D'Agostino-Pearson normality test.

Differences in ovotransferrin levels between necrotic enteritis severity groups (as measured by ELISA) were calculated using an a Kruskal-Wallis test, followed by a Dunn's post test.

Differences in ovotransferrin levels between the *Eimeria*-challenged and the non-challenged control group were calculated using a Mann-Whitney test.

The Spearman rank correlation was used to assess the relationship between the ovotransferrin concentration in the cloacal samples and either the necrotic enteritis lesion score or the coccidiosis score. Results were reported as means and standard error of the means (SEM).

Correlation of fecal ovotransferrin concentration with severity of necrotic enteritis

Most samples from birds suffering from either mild necrotic enteritis (score 2) or without intestinal lesions (both challenged and negative control animals) showed a low signal. In samples from birds with more severe necrotic enteritis (necrosis score ≥ 3), significantly more ovotransferrin was detected than in samples from challenged birds who did not show intestinal disease (score 0), see Table 1. Furthermore, there was a positive correlation between the necrotic enteritis disease severity and the ovotransferrin concentration in the fecal samples from the NE *in vivo* trial (*p* = 0.0004).

	neg. ctr (BHI) [µg/g]	Score 0 [µg/g]	Score 2 [µg/g]	Score 3-4 [µg/g]	Score 5-6 [µg/g]
Mean	1,80	1,01	1,16	3,59	5,87
Std. Error	0,4353	0,2605	0,1921	1,034	1,504

Table 1 represents the ovotransferrin concentration (mean ± standard error of the means) in faeces from birds that received all predisposing factors but were not challenged with *C. perfringens* (neg. ctr; *n* = 8) or from birds challenged with *C. perfringens* resulting in varying degrees of necrotic enteritis: no necrotic lesions (score 0; *n* = 8); mild intestinal necrosis (score 2; *n* = 8); moderate necrotic enteritis (score 3-4; *n* = 8) or severe necrosis (score 5-6; *n* = 8). * $p < 0.05$.

Correlation of fecal ovotransferrin concentration with severity of coccidiose

Birds challenged with *E. acervulina* and *E. tenella* were used as a second model for intestinal barrier failure. The ovotransferrin levels in samples from coccidiosis-positive birds were elevated as compared to the unchallenged controls ($p = 0.0029$). Furthermore, there was a positive correlation between the coccidiosis score and the ovotransferrin concentration in the faeces ($p = 0.0082$). This difference in ovotransferrin levels was also reflected in the litter samples, where significantly higher ovotransferrin levels were detected in litter from *Eimeria*-challenged birds than in litter samples from non-challenged control groups ($p = 0.0043$), see Table 2.

	neg. ctr		**coccidiosis**	
	Feces [µg/g]	**Litter [µg/g]**	**Feces [µg/g]**	**Litter [µg/g]**
Mean	4,49	1,51	24,76	24,46
Std. Error	1,48	0,33	9,56	6,49

Table 2 represents the ovotransferrin concentration (mean ± standard error of the means) in faeces (grey) or mixed litter (white) from experimental coccidiosis-infected birds (coccidiosis; individual faeces samples: *n* = 10 or mixed litter samples: *n* = 6) or non-challenged control birds (neg. ctr; individual faeces: *n* = 10 or mixed litter samples: *n* = 5). Significant differences between the coccidiosis-positive group and the non-challenged control group are indicated with ** $p < 0.01$.

Results

As shown in the above, elevated fecal ovotransferrin levels were measured in birds with either experimental coccidiosis or necrotic enteritis, which both cause intestinal barrier failure, using different approaches. ELISA analysis samples from different NE *in vivo* trials revealed that ovotransferrin was more abundant in samples from birds suffering from necrotic enteritis as compared to unchallenged birds. Additionally, elevated ovotransferrin concentrations were measured in samples from coccidiosis-positive birds as compared to their unchallenged controls.

Fecal ovotransferrin levels were significantly correlated with the severity of intestinal barrier failure caused by either coccidiosis or necrotic enteritis.

The degree of gut barrier failure might be classified depending on the severity of the symptom on the affected sites (e.g. necrosis due to *C. perfringens*-induced necrotic enteritis), and the extent of the affected surface area. The degree of gut barrier failure is more severe with NE as this is associated with necrosis, the extent (in terms of surface area) is higher with coccidiosis.

As shown by the above experiments, the measurement of an specific APP (ovotransferrin) is a valuable tool to measure inflammation and concomitant intestinal barrier failure, as it can provide information on specific biological disease processes and is a useful tool to assess efficacy of molecules that reduce gastrointestinal disturbances.

Claims

1.) *In vitro* method for detecting intestinal barrier failure in animals, the method comprising the following steps:

a) collecting intestinal sample material of an individual animal or of an animal population; and

b) determining the amount of at least one protein marker contained in said sample material;

wherein

the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof,

and wherein

an increased amount of said at least one protein marker contained in said sample versus a reference sample indicates intestinal barrier failure.

2.) The method according to claim 1, wherein the reference sample is a species-specific control representing an intact intestinal barrier.

3.) The method according to any one of the preceding claims, wherein the acute phase protein is detected and quantified via Enyzme-linked Immunosorbent Assay (ELISA).

4.) The method according to any one of the preceding claims, wherein the acute phase protein is detected and quantified via lateral flow assay.

5.) The method according to any one of the preceding claims, 1 to 4, wherein the individual animal is an avian subject.

6.) The method according to any one of claims 1 to 4, wherein the animal population is an avian population.

7.) The method according to any one of the preceding claims, wherein the intestinal sample material is a pooled sample.

8.) The method according to any one of the preceding claims, wherein the intestinal sample material is selected from the group consisting of gut content samples; samples of bodily excrements and solutions or suspensions thereof; and materials being contaminated with bodily excrements.

9.) The method according to any one of the preceding claims, wherein the intestinal sample material is feces.

10.) The method according to any one of the preceding claims, wherein the intestinal sample material is a pooled fecal sample deriving from an avian population.

11.) The method according to any one of the preceding claims, wherein the intestinal sample material is stabilized immediately after sample collection.

12.) Use of ovotransferrin or of functional fragments thereof as intestinal markers for detecting intestinal barrier failure in an animal subject or in an animal population.

13.) *In vitro* method for detecting the extent of intestinal barrier failure in animals, the method comprising the following steps:

a) collecting intestinal sample material of a specific animal or of an animal population; and

b) determining the amount of at least one protein marker contained in the sample material;

wherein

the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof,

and wherein

the amount of said at least one protein marker contained in the sample indicates the extent of the intestinal barrier failure.

14.) In *vitro* method for monitoring the status of the intestinal barrier in animals, the method comprising the following steps:

a) collecting intestinal sample material of a specific animal or of an animal population at consecutive points in time;

b) determining the amount of at least one protein marker contained in the samples obtained in step a); and

c) determining deviations in the amounts of said at least one protein marker contained in the samples obtained in step a);

wherein

the at least one protein marker comprises or consists of ovotransferrin or a functional fragment thereof.