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(54) **Title:** INTESTINAL AND FECAL BIOMARKERS FOR INTESTINAL HEALTH OF POULTRY

(57) **Abstract:** Growth performance, health and welfare of domesticated birds such as broilers heavily depends on a well-functioning intestinal tract. Consequently, there is a high need to find biomarkers which are specific for gut damage and which are easily applicable in the field. The present invention discloses a set of 20 specific proteins which can be quantified in fresh fecal droppings and/or intestinal content of said birds and which each specifically correlate with a damaged gut.

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Intestinal and fecal biomarkers for intestinal health of poultry

Technical field of invention

Growth performance, health and welfare of domesticated birds such as broilers heavily depends on a well-functioning intestinal tract. Consequently, there is a high need to find biomarkers which are specific for gut damage and which are easily applicable in the field. The present invention discloses a set of 20 specific proteins which can be quantified in fresh fecal droppings and intestinal content of said birds and which each specifically correlate with a damaged gut.

Background art

Poultry such as broiler chickens have the lowest feed conversion of all meat-producing animals, and are therefore considered to be a relative sustainable source of animal protein, of which the production and consumption are still rising worldwide (Scanes, 2007, Cowieson and Selle, 2012). A well-functioning intestinal tract is of key importance for digestion and nutrient absorption and consequently low feed conversion, and is also crucial for health and welfare of broilers (Bailey *et al*., 2010). Indeed, intestinal diseases and syndromes are rather common in broilers and constitute the most important cause for treatment (Casewell *et al.*, 2003). In poultry practice, coccidiosis is by far the most important intestinal disease (Yegani and Korver, 2008; Caly *et al*., 2015). Clinical diseases caused by bacterial pathogens are not common, but it is widely recognized that a variety of intestinal syndromes are affecting broiler performance, including subclinical necrotic enteritis and coccidiosis, viral enteritis, and various non-defined enteritis syndromes (Yegani and Korver, 2008). It is not evident to diagnose these subclinical entities and differentiate these from performance problems that have no infectious etiology, such as those caused by suboptimal formulated diets that not always cause intestinal damage.

The gut wall structure and morphology is a major determinant of intestinal health. Macroscopic observations of the intestinal wall at necropsy can easily be used to monitor for intestinal lesions caused by *Clostridium perfringens* and coccidia (Johnson and Reid, 1970), but are less clear for more subtle intestinal pathologies. Macroscopic alterations of the gut wall (such as gut wall tonus and thickness) and intestinal content (such as viscosity) can be used by experienced veterinarians as parameters for intestinal health but are to some extent

subjective (Teirlynck *et al*., 2011). Histopathological microscopic observations are giving an accurate picture of intestinal health as villus structure, epithelial cell defects and inflammation can be scored (Yamauchi, 2002). In any case, the above mentioned intestinal health monitoring systems should be performed post-mortem and do not have high predictive diagnostic value, although in flocks animals can be sacrificed and used to monitor for disease. Quantifiable easy-to-measure biomarkers for intestinal health are still not in use in broiler chickens in practice but would be of tremendous value as a tool to monitor for subclinical intestinal entities that cause performance problems and to evaluate control methods for intestinal health, independent of whether the triggers are derived from host, nutritional or microbial factors. Epithelial damage and epithelial permeability are likely the main drivers for intestinal health problems in broiler chickens, and damage to these cells and the gut mucosa is characterized by shortening of villi, lengthening of crypts and infiltration of inflammatory cells (Teirlynck *et al*., 2011; Adelman *et al*., 2018). It has been shown that an increase of villus length and villus-to-crypt ratio is associated with improvement of growth performance (Awad *et al*., 2009). While an inflammatory response in the gut is essential to control and contain infections, these responses should also have an accurate transition to an anti-inflammatory state as inflammation is costing energy that affects performance (Broom and Kogut, 2018).

Various systems have been developed to measure intestinal permeability, but are mainly used in experimental models (Gilani *et al.*, 2016; Gilani *et al*., 2017; Wang *et al*., 2015). Quantification in serum or plasma of molecules that are orally administered and, because of their difference in size, can either or not cross the epithelial layer without intestinal damage and increased permeability have been shown to have value. Examples are the ratio of concentrations of lactulose and mannitol (lactulose/mannitol ratio (LMR), Gilani *et al*., 2017) in plasma or fluorescein isothiocyanate-dextran (FITC-dextran) in serum (Gilani *et al*., 2018; Kuttappan *et al*., 2015), after oral delivery of these compounds to the animals, with increased LMR or FITC-dextran levels indicative of high intestinal permeability. Other biomarkers for gut health have been evaluated in serum, most of them being acute phase proteins (O'Reilly and Eckersall, 2014), but these are not specific for gut damage. In addition, all the blood markers need invasive sampling, what is not preferred as diagnostic test in poultry practice. While quite some markers have been identified using transcriptomic approaches on gut tissue (Hong *et al*., 2014), these are also not applicable in the field. There is thus clearly a need to find an intestinal and/or fecal biomarker for intestinal health of poultry which is present in fresh fecal

droppings, litter or an intestinal content sample so that it is applicable in the field. In humans, calprotectin has been used to evaluate inflammation in case of severe gut diseases, and has been shown to be reliable and specific (Canani *et al.*, 2008; Chang *et al.*, 2014). However, for poultry such as chickens, no intestinal protein biomarkers for gut health are known.

Brief description of figures

Figure 1| Mean body weight (g) (fig 1a), macroscopic gut appearance score (fig 1b) and coccidiosis score (fig 1c) per pen for control and challenge on day 26. * denotes statistical significance at $p < 0.05$ between control and challenged treatment.

Figure 2| Mean villus length (fig 2a), crypt depth (fig 2b), villus-to-crypt ratio (fig 2c) and T-lymphocyte infiltration (CD_3 area%) per pen for control and challenge (fig 2d) on day 26. *** denotes statistical significance at $p < 0.0001$ between control and challenge group.

Description of invention

The present invention relates to a reliable, rapid and non-invasive biomarker test to diagnose gut health of poultry. With the term 'poultry' are meant domesticated birds kept by humans for their eggs, their meat or their feathers. These birds are most typically members of the superorder Galloanserae, especially the order Galliformes which includes chickens, quails and turkeys. The present invention discloses the identification of biomarkers that are indicative of intestinal pathology. The present invention further describes a gut damage model in poultry wherein a set of intestinal and/or fecal biomarkers correlate with -for example- shortening of villi and CD_3 infiltration, the latter being markers for intestinal inflammation.

Hence, the present invention relates in first instance to a method to determine the intestinal health status of a domesticated bird comprising:

- Providing a fecal sample or an intestinal content sample obtained from said domesticated bird, and
- quantifying a protein, or a fragment thereof, in said fecal sample or intestinal content sample,

  wherein said protein is chosen from the group consisting of: myeloid protein 1, fibronectin, annexin A5, nucleophosmin, carbonic anhydrase 2, aminopeptidase

Ey, transthyretin, ovoinhibitor, apolipoprotein A-1, hemoglobin subunit beta, superoxide dismutase [Cu-Zn], alpha-actinin-4, angiotensin-converting enzyme, WD repeat-containing protein 1, mitochondrial aspartate aminotransferase, histone H2A-IV, immunoglobulin lambda chain C region, immunoglobulin lambda chain V-1 region, cathepsin D and retinol-binding protein 4.

More specifically, the present invention relates in first instance to a method to determine the intestinal health status of a domesticated bird comprising:

- obtaining a fecal sample or an intestinal content sample of said domesticated bird, and
- quantifying a protein, or a fragment thereof, in said fecal or intestinal content sample,

  wherein said protein is chosen from the group consisting of: myeloid protein 1, fibronectin, annexin A5, nucleophosmin, carbonic anhydrase 2, aminopeptidase Ey, transthyretin, ovoinhibitor and apolipoprotein A-1.

The term 'intestinal health status' relates in first instance to the status of the gut wall structure and morphology which can be affected by –for example- infectious agents or a non-infectious cause such as a suboptimal formulated diet. The latter term thus mainly relates to epithelial damage and epithelial permeability which is characterized by a shortening of villi, a lengthening of crypts and an infiltration of inflammatory cells. The latter damage and inflammation markers can also be associated with a 'severe' macroscopic appearance of the gut –compared to a 'normal' appearance- when evaluated using a scoring system such as the one described by Teirlynck *et al*. (2011).

The term 'obtaining a fecal or intestinal content sample' refers to any means to collect of a fresh fecal dropping from said birds or intestinal content at necropsy of said birds. The terms 'intestinal content at necropsy of birds' mean a sample taken from the content present in ileum or colon after said bird is euthanized.

The terms 'quantifying a protein, or a fragment thereof, in said fecal or intestinal content sample' refers to any method known to a skilled person to quantify said proteins or fragments of in said sample. Non-limiting examples of the latter means are mass spectrometric methods (e.g. discovery and targeted proteomics, multiple reaction monitoring (MRM) assay,

sequential window acquisition of all theoretical spectra assay (SWATH), ...) which require prior isolation of said proteins or fragments thereof from said sample. The latter isolation can be undertaken via protein extractions with different lysis buffers such as Sodium dodecyl sulfate (SDS)-based protein lysis buffer, Bacterial Protein extraction reagent (B-Per) or Urea-based lysis buffer with or without bead beating or other commonly used methods. Other non-limiting examples of means to quantify proteins or fragments thereof are ELISA and Western Blotting which can be performed without prior protein isolation from said sample. Instead said fecal or intestinal content sample can be diluted (10% m/v) in phosphate-buffered saline (PBS) or a 50mM Tris, 150 mM NaCl (pH 7,2) buffer with or without bead beating prior to quantification.

It should be clear that the quantification of a single protein might be sufficient to determine the intestinal health status but that also a combination of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more proteins can be used to determine the intestinal health status of said poultry.

The protein biomarkers of the present invention which are indicative of intestinal pathology in poultry are the following:

1. **Myeloid protein 1**

This protein has accession number P09840 in the UniProt database (see http://www.uniprot.org/uniprot/P08940). The protein is a granule protein present in secretory granules of heterophilic granulocytes.

This protein has the following amino acid sequence (i.e. SEQ ID N° 1; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MPALSLIALLSLVSTAFARQWEVHPPQQQGRHWAQICSGNPFNRIRGCDRYGCGNYGASR
QGKGEKHKGVDVICTDGSIVYAPFSGQLSGPIRFFHNGNAIDDGVQISGSGYCVKLVCIH
PIRYHGQIQKGQQLGRMLPMQKVFPGIVSHIHVENCDQSDPTHLLRPIPDISPPFPQQDA
HWAVVCAGNPTNEIRGCDKYGCGYFGAPRRNGKGEKHKGVDVICADGATVYAPFSGELSG
PVKFFHNGNAIDDGVQIRGSGFCVKLLCIHPIRYNGRISKGQVLGRMLPMQRVFPGIISH
IHVENCDRSDPTSNLERGKGESEMEV

2. Fibronectin

This protein has accession number P11722 in the UniProt database (see http://www.uniprot.org/uniprot/P11722). Fibronectin (Fn) is a high molecular weight glycoprotein that consists in a soluble form in plasma and in an insoluble form as extracellular matrix (ECM) component (Pankov and Yamada, 2002). It contributes to a variety of cellular activities including wound healing. Production of fibronectin is influenced by pro-inflammatory cytokines such as IL-1 -alpha, IL-6 and TNF-alpha.

This protein has the following amino acid sequence (i.e. SEQ ID N° 2; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

PLDQCQDSETRTFYQIGDSWEKYVHGVRYQCYCYGRGIGEWHCQPLQAYAPLSPPTNLRL
EPNPDTGILIVSWDRSTTPGISGYRVTTAPTNGQQGSTLEEVVGADQTSCTFENLNPGVE
YNVSVYAVKDDQESIPISKTITQEVPQLTDLSFVDITDSSIGLRWTPLNASTIIGYRITV
VAAGESVPIFEDFVDSSVGYYTVTGLEPGIDYDISVITLINGGESAPTTLTQQTAVPPPT
DLRFTNVGPDTMRVTWTAPTSIVLSSFLVRYSPVKKEEDVAELTISPSDNVVVLTNLLPG
TEYLVRVYSVAEQHESAPLSGIQKTGLDSPTGLDFSDITANSFTVHWIAPRATITGYKIR
HHPEHGVGRPKEDRVPPSRNSITLTNLLPGTEYVVSIIAVNGREESVPLVGQQTTVSDVP
RDLEVNPTSPTSLEISWDAPAVTVRYYRITYGETGGSSPVQEFTVPGTMSRATITGLKPG
VDYTITVYAVTGRGDSPASSKPVTVTYKTEIDTPSQMQVTDVQDNSISIRWLPSSSPVTG
YRVTAVPKKGHGPTKTKNVPPDQTQVTIEGLQPTVEYMVSVYAQNQNGESLPLVETAVTN
IDRPKGLTFTEVDVDSIKIAWESPQGQVTRYRVTYSSPEDGIHELLPAPGGEEDTAELHG
LRPGSEYTINIVAIYDDMESLPLTGTQSTAIPPPTNLKFTQVTPTSLTVNWNAPNVRLTG
YRVRVNPKEKTGPMKEINLSPDSTSAVVSGLMVATKYEVSVYALKDSLTSRPAQGVVTTL
ENVSPPRRARVTDATETTITITWRTKTETITGFQIDAIPAASGQNPIQRTISPDVRTYTI
TGLQPGNDYKIYLYTLNENARSSPVVIDASTAIDAPSNLRFLTTTTNSLLASWQPPRAKI
TGYIIRYDKPGSPAKELLPRPRPGTTEATITGLEPGTEYTIYIIAVKNNQKSEPLVGRKR
TDDLPTLITGPHPNQPDMLDVPSVDEGTPYLTNNRYDNGNGIQLPGTSGHPQTIGHQGQQ
VFFEEHGYRRPVPTTATPLRPGSRRQPPNVDEAIEIPGYQVPIIVVPSYPHSREPRRNDT
TGQEALSQTTISWRPLLESTEYIISCQPVSQDEDTLQFRVPGTSSSATLTGLTRGATYNI
IVEALKDHRRQKVLEEVVTVGNTVSEGLNQPADDTCYDTYTGSFYSIGEEWERLSETGFK

LWCQCLGFGSGHFRCDSSKWCHDNGVNYKIGEKWDRQGENGQMIDCTCLGNGKGEF

3. Annexin A5

This protein has accession number P17153 in the UniProt database (see http://www.uniprot.org/uniprot/P17153). This protein is an anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex, which is involved in the blood coagulation cascade.

This protein has the following amino acid sequence (i.e. SEQ ID N° 3; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MAKYTRGTVTAFSPFDARADAEALRKAMKGMGTDEETILKILTSRNNAQRQEIASAFKTL
FGRDLVDDLKSELTGKFETLMVSLMRPARIFDAHALKHAIKGAGTNEKVLTEILASRTPA
EVQNIKQVYMQEYEANLEDKITGETSGHFQRLLVVLLQANRDPDGRVDEALVEKDAQVLF
RAGELKWGTDEETFITILGTRSVSHLRRVFDKYMTISGFQIEETIDRETSGDLEKLLLAV
VKCIRSVPAYFAETLYYSMKGAGTDDDTLIRVMVSRSEIDLLDIRHEFRKNFAKSLYQMI
QKDTSGDYRKALLLLCGGDDE

4. Nucleophosmin

This protein has accession number P16039 in the UniProt database (see http://www.uniprot.org/uniprot/P16039). Nucleophosmin is a DNA binding nuclear protein which has been described as a wound-associated protein (Mellgren, 2010).

This protein has the following amino acid sequence (i.e. SEQ ID N° 4; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MEDSAMDMESMGPLRPQTFLFGCELKAEKEYQFKVDDEENEHQLSLRTVTLGAGAKDELH
VVEAEALDYEGNPTKVVLASLKMSVQPTVSLGGFEITPPFVLRLKCGSGPVYVSGQHLVA
LEEEPESEDEEEDTKIGNASTKRPASGGGAKTPQKKPKLSEDDEDDDEDEDDDEDDEDDL

DDDEEEIKTPMKKPAREPAGKNMQKAKQNGKDSKPSTPASKTKTPDSKKDKSLTPKTPKV
PLSLEEIKAKMQASVDKGCSLPKLEPKFANYVKNCFRTEDQKVIQALWQWRQTL

5. Carbonic anhydrase 2

This protein has accession number P07630 in the UniProt database (see http://www.uniprot.org/uniprot/P07630). This enzyme causes the rapid interconversion of carbon dioxide and water to bicarbonate and protons (or vice versa), a reaction that is important for acid/base equilibrium. The enzyme is a marker for differentiation in epithelial cells.

This protein has the following amino acid sequence (i.e. SEQ ID N° 5; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):
MSHHWGYDSHNGPAHWHEHFPIANGERQSPIAISTKAARYDPALKPLSFSYDAGTAKAIV
NNGHSFNVEFDDSSDKSVLQGGALDGVYRLVQFHIHWGSCEGQGSEHTVDGVKYDAELHI
VHWNVKYGKFAEALKHPDGLAVVGIFMKVGNAKPEIQKVVDALNSIQTKGKQASFTNFDP
TGLLPPCRDYWTYPGSLTTPPLHECVIWHVLKEPITVSSEQMCKLRGLCFSAENEPVCRM
VDNWRPCQPLKSREVRASFQ

6. Aminopeptidase Ey

This protein has accession number O57579 in the UniProt database (see http://www.uniprot.org/uniprot/O57579). Aminopeptidase Ey (EC 3.4.11.20) from chicken (Gallus gallus domesticus) egg yolk is a homodimeric exopeptidase with a broad specificity for N-terminal amino acid residues at P1 position of the substrate (Midorikawa *et al*. 1998). Aminopeptidases are members of a membrane-bound metallopeptidase family that are expressed at a high level on the brush-border membrane of enterocytes (Gal-Garber and Uni, 2000).

This protein has the following amino acid sequence (i.e. SEQ ID N° 6; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent

non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MAAGFFISKSVGIVGIVLALGAVATIIALSVVYAQEKNKSSGGSGGSDTTSTTTASTTTT
STTTASTTAAPNNPWNRWRLPTALKPESYEVTLQPFLTPDDNNMYIFKGNSSVVFLCEEA
TDLILIHSNKLNYTLQGGFHASLHAVNGSTPPTISNTWLETNTQYLVLQLAGPLQQGQHY
RLFSIFTGELADDLAGFYRSEYTEGNVTKVVATTQMQAPDARKAFPCFDEPAMKAVFTVT
MIHPSDHTAISNMPVHSTYQLQMDGQSWNVTQFDPTPRMSTYLLAFIVSQFDYVENNTGK
VQIRIWGRPAAIAEGQGEYALEKTGPILSFFERHYNTAYPLPKSDQVGLPDFNAGAMENW
GLVTYRENSLLYDNAYSSIGNKERVVTVIAHELAHQWFGNLVTLRWWNDLWLNEGFASYV
EYLGADSAEPTWDIKDLMVLNELYTVMATDALTTSHPLTFREDEINTPAQISEVFDSIAY
SKGASVLRMLSDFLTEDVFKEGLQSYLHDFSYNNTVYTDLWDHLQEAVNKNSVPLPDSIG
AIMDRWTLQMGFPVVTVNTLTGSVQQSHFLLDSNSTVERPSVFNYTWIVPITWMTPSRTG
DRYWLVDVSATNSDFSVGSSTWLLLNLNVSGYFRVNYNQENWDQLLQQLSNNHQAIPVIN
RAQIIDDAFNLARAQQVSVTLALNTTRFLSGETAYMPWQAALNNLQYFQLMFDRSEVFGA
MTKYIQKQVTPLFEYYRTATNNWTAIPSALMDQYNEINAISTACSYGIAECQQLATALYQ
QWRQNVSNNPIAPNLRSAIYCSAVATGGEEVWDFIWERFLEAPVVSEADKLRTALTCSTE
TWILQRYLQYTIDPTKIRKQDATSTINSIASNVVGQPLAWDFIRSNWRTLFGQYGGGSFS
FSRLISAVTQRFNTEFELKQLEQFKADNQDIGFGSGTRALEQALERTRTNINWVKENKEV
VHAWFRAETASS

7. Transthyretin

This protein has accession number P27731 in the UniProt database (see http://www.uniprot.org/uniprot/P27731). Plasma transthyretin (TTR) is a plasma protein secreted by the liver that circulates bound to retinol-binding protein 4 (RBP4) and its retinol ligand. TTR is a highly conserved protein in animal species.

This protein has the following amino acid sequence (i.e. SEQ ID N° 7; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MAFHSTLLVFLAGLVFLSEAAPLVSHGSVDSKCPLMVKVLDAVRGSPAANVAVKVFKKAA
DGTWQDFATGKTTEFGEIHELTTEEQFVEGVYRVEFDTSSYWKGLGLSPFHEYADVVFTA
NDSGHRHYTIAALLSPFSYSTTAVVSDPQE

The following gray boxes indicate peptides obtained via a trypsin digest of ileal samples of SEQ ID N° 7 as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample:

MAFHSTLLVFLAGLVFLSEAAPLVSHGSVDSKCPLMVKVLDAVRGSPAANVAVKFKKAA
DGTWQDFATGKTTEFGEIHELTTEEQFVEGVYRVEFDTSSYWKGLGLSPFHEYADVVFTA
NDSGHRHYTIAALLSPFSYSTTAVVSDPQE

8. Ovoinhibitor

This protein has accession number P10184 in the UniProt database (see http://www.uniprot.org/uniprot/P10184). Ovoinhibitor is found in egg white and is a serine proteinase inhibitor that can reduce enzymatic digestion by trypsin and chymotrypsin.

This protein has the following amino acid sequence (i.e. SEQ ID N° 8; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MRTARQFVQVALALCCFADIAFGIEVNCSLYASGIGKDGTSWVACPRNLKPVCGTDGSTY
SNECGICLYNREHGANVEKEYDGECRPKHVMIDCSPYLQVVRDGNTMVACPRILKPVCGS
DSFTYDNECGICAYNAEHHTNISKLHDGECKLEIGSVDCSKYPSTVSKDGRTLVACPRIL
SPVCGTDGFTYDNECGICAHNAEQRTHVSKKHDGKCRQEIPEIDCDQYPTRKTTGGKLLV
RCPRILLPVCGTDGFTYDNECGICAHNAQHGTEVKKSHDGRCKERSTPLDCTQYLSNTQN
GEAITACPFILQEVCGTDGVTYSNDCSLCAHNIELGTSVAKKHDGRCREEVPELDCSKYK
TSTLKDGRQVVACTMIYDPVCATNGVTYASECTLCAHNLEQRTNLGKRKNGRCEEDITKE
HCREFQKVSPICTMEYVPHCGSDGVTYSNRCFFCNAYVQSNRTLNLVSMAAC

9. Apolipoprotein A-1

This protein has accession number P08250 in the UniProt database (see http://www.uniprot.org/uniprot/P08250). Apolipoprotein (apo) A-1 is a 28 kDa exchangeable apolipoprotein that plays a key role in lipoprotein metabolism.

This protein has the following amino acid sequence (i.e. SEQ ID N° 9; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MRGVLVTLAVLFLTGTQARSFWQHDEPQTPLDRIRDMVDVYLETVKASGKDAIAQFESSA
VGKQLDLKLADNLDTLSAAAAKLREDMAPYYKEVREMWLKDTEALRAELTKDLEEVKEKI
RPFLDQFSAKWTEELEQYRQRLTPVAQELKELTKQKVELMQAKLTPVAEEARDRLRGHVE
ELRKNLAPYSDELRQKLSQKLEEIREKGIPQASEYQAKVMEQLSNLREKMTPLVQEFRER
LTPYAENLKNRLISFLDELQKSVA

The following gray boxes indicate peptides obtained via a trypsin digest of ileal samples of SEQ ID N° 9 as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample:

MRGVLVTLAVLFLTGTQARSFWQHDEPQTPLDRIRDMVDVYLETVKASGKDAIAQFESSA
VGKQLDLKLADNLDTLSAAAAKLREDMAPYYKEVREMWLKDTEALRAELTKDLEEVKEKI
RPFLDQFSAKWTEELEQYRQRLTPVAQELKELTKQKVELMQAKLTPVAEEARDRLRGHVE
ELRKNLAPYSDELRQKLSQKLEEIREKGIPQASEYQAKVMEQLSNLREKMTPLVQEFRER
LTPYAENLKNRLISFLDELQKSVA

10. Hemoglobin subunit beta

This protein has accession number P02112 in the UniProt database (see http://www.uniprot.org/uniprot/P02112). The detection of hemoglobin subunit beta (HBB) in intestinal content indicates that the administered challenges induce gut leakage and endothelial damage allowing red blood cell leakage from the blood to the lumen.

This protein has the following amino acid sequence (i.e. SEQ ID N° 131; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MVHWTAEEKQLITGLWGKVNVAECGAEALARLLIVYPWTQRFFASFGNLSSPTAILGNPM
VRAHGKKVLTSFGDAVKNLDNIKNTFSQLSELHCDKLHVDPENFRLLGDILIIVLAAHFS
KDFTPECQAAWQKLVRVVAHALARKYH

11. Superoxide dismutase

This protein has accession number P80566 in the UniProt database (see http://www.uniprot.org/uniprot/P80566). Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals to hydrogen peroxide (H_2O_2) and oxygen and contributes to enhanced small intestinal preservation in feline (Sun et al., 1991).

This protein has the following amino acid sequence (i.e. SEQ ID N° 132; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MATLKAVCVMKGDAPVEGVIHFQQQGSGPVKVTGKITGLSDGDHGFHVHEFGDNTNGCTS
AGAHFNPEGKQHGGPKDADRHVGDLGNVTAKGGVAEVEIEDSVISLTGPHCIIGRTMVVH
AKSDDLGRGGDNESKLTGNAGPRLACGVIGIAKC

The following gray boxes indicate peptides obtained via a trypsin digest of ileal samples of SEQ ID N° 132 as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample:

MATLKAVCVMKGDAPVEGVIHFQQQGSGPVKVTGKITGLSDGDHGFHVHEFGDNTNGCTS
AGAHFNPEGKQHGGPKDADRHVGDLGNVTAKGGVAEVEIEDSVISLTGPHCIIGRTMVVH
AKSDDLGRGGDNESKLTGNAGPRLACGVIGIAKC

12. Alpha-actinin-4

This protein has accession number Q90734 in the UniProt database (see http://www.uniprot.org/uniprot/Q90734). By indirect immunofluorescence, alpha-actinin-4 (ACTN4) was shown to be localized in the apical part of chicken intestinal epithelial cells (Craig and Pardo, 1979), more specifically as a component of the tight junction (*zonula occludens*) (Chen *et al*., 2006) and/or belt desmosome (*zonula adherens*) (Milanini *et al*., 2017).

This protein has the following amino acid sequence (i.e. SEQ ID N° 133; the gray boxes indicate peptides obtained via a trypsin digest of colon samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MVDYHSAGQPYPYGGNGPGPNGDYMAQEDDWDRDLLLDPAWEKQQRKTFTAWCNSHLRKA
GTQIENIDEDFRDGLKLMLLLEVISGERLPKPERGKMRVHKINNVNKALDFIASKGVNVV
SIGAEEIVDGNAKMTLGMIWTIILRFAIQDISVEETSAKEGLLLWCQRKTAPYKNVNVQN
FHISWKDGLAFNALIHRHRPELIEYDKLRKDDPVTNLNNAFEVAEKYLDIPKMLDAEDIV
NTARPDEKAIMTYVSSFYHAFSGAQKAETAANRICKVLAVNQENEHLMEDYEKLASDLLE
WIRRTIPWLEDRSPQKTIQEMQQKLEDFRDYRRVHKPPKVQEKCQLEINFNTLQTKLRLS
NRPAFMPSEGRMVSDINTGWQHLEQAEKGYEEWLLNEIRRLEPLDHLAEKFRQKASIHEA
WTEGKEAMLKQKDYETATLSDIKALIRKHEAFESDLAAHQDRVEQIAAIAQELNELDYYD
SPSVNARCQKICDQWDVLGSLTHSRREALEKTEKQLETIDELHLEYAKRAAPFNNWMESA
MEDLQDMFIVHTIEEIEGLIAAHDQFKATLPDADREREAILGIQREAQRIADLHSIKLSG
NNPYTSVTPQVINSKWERVQQLVPTRDRALQDEQSRQQCNERLRRQFAGQANIVGPWMQT
KMEEIGRISIEMHGTLEDQLQHLKHYEQSIVDYKPNLELLEHEHQLVEEALIFDNKHTNY
TMEHIRVGWEQLLTTIARTINEVENQILTRDAKGISQEQMQEFRASFNHFDKDHCGALGP
EEFKACLISLGYDVENDRQGDAEFNRIMSLVDPNGSGSVTFQAFIDFMSRETTDTDTADQ
VIASFKVLAGDKNYITAEELRRELPPEQAEYCIARMAPYRGPDAAPGALDYKSFSTALYG
ESDL

13. Angiotensin-converting enzyme

This protein has accession number Q10751 in the UniProt database (see http://www.uniprot.org/uniprot/Q10751). Angiotensin-converting enzyme (ACE) are localized in the intestinal brush border membrane and are involved as major functional enzymes in the final stadium of protein digestion in the small intestine (Yoshioka *et al*., 1987).

This protein has the following amino acid sequence (i.e. SEQ ID N° 134; the gray boxes indicate peptides obtained via a trypsin digest of ileal samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

AKELYGNIWSNFSDPQLKKIIGSIQTLGPSNLPLDKRQQYNTILSDMDKIYSTAKVCLDN
GTCWDLEPDISDIMATSRSYKKLLYAWEGWHNAAGNPLRAKYQEFVTLSNEAYQMDGFED
TGSYWRSWYDSTTFEDDLEHLYNQLEPLYLNLHAFVRRKLYDRYGPKYINLKGPIPAHLL
GNMWAQQWNNIYDLMVPYPDKPNLDVTNTMVNQGWNATHMFRVSEEFFTSLGLLEMPPEF
WEKSMLEKPADGREVVCHASAWDFYNRKDFRIKQCTTVTMEQLFTVHHEMGHVQYYLQYK
DQPVSFRGGANPGFHEAIGDVLSLSVSTPSHLQKIGLLSSAVEDEESNINYLLKMALEKI
AFLPFGYLIDQWRWNVFSGRTPPSRYNYDWWYLRTKYQGICAPVSRNESNFDPGAKYHIP
GNTPYIRYFVSFILQFQFHKALCQAANHTGPLHTCDIYMSKEAGAKLREVLKAGSSKSWQ
EILFNLTGTDKMDAGALLEYFSPVTTWLQEQNNKTNEVLGWPEFDWRSPIPEGYPEGIDK
IVDEAQAKEFLSEYNSTAEVVWNAYTEASWEYNTNITDHNKEVMLEKNLAMSKHTIEYGM
RARQFDPSDFQDETVTRILNKLSVLERAALPEDELKEYNTLLSDMETTYSVAKVCRENNT
FHPLDPDLTDILATSRDYNELLFAWKGWWDASGAKIKDKYKRYVELSNKAAVLNGYTDNG
AYWRSLYETPTFEEDLERLYLQLQPLYLNLHAYVRRALYNKYGAEHISLKGPIPAHLLGN
MWAQSWSNIFDLVMPFPDATKVDATPAMKQQGWTPKMMFEESDRFFTSLGLIPMPQEFWD
KSMIEKPADGREVVCHASAWDFYNRKDFRIKQCTVVNMDDLITVHHEMGHVQYFLQYMDQ
PISFRDGANPGFHEAIGDVMALSVSTPKHLHSINLLDQVTENEESDINYLMSIALDKIAF
LPFGYLMDQWRWKVFDGRIKEDEYNQQWWNLRLKYQGLCPPVPRSEDDFDPGAKFHIPAN
VPYIRYFVSFVIQFQFHQALCKAAGHTGPLHTCDIYQSKEAGKLLGDAMKLGFSKPWPEA
MQLITGQPNMSAEALMSYFEPLMTWLVKKNTENGEVLGWPEYSWTPYAVTEFHAATDTAD
FLGMSVGTKQATAGAWVLLALALVFLITSIFLGVKLFSSRRKAFKSSSEMELK

14. WD repeat-containing protein 1

This protein has accession number O93277 in the UniProt database (see http://www.uniprot.org/uniprot/O93277). WD (tryptophan-aspartate) repeat-containing protein 1 (WDR1), also called actin-interacting protein 1 (AIP1), acts as a cofactor of ADF-cofilin and facilitates actin turnover by disassembly of actin filaments (Fujibuchi *et al*., 2004).

This protein has the following amino acid sequence (i.e. SEQ ID N° 135; the gray boxes indicate peptides obtained via a trypsin digest of ileal samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MRMPYEIKKVFASLPQVERGVSKIIGGDPKGNNFLYTNGKCVVIRNIDNPAIADIYTEHA
HQVVVAKYAPSGFYIASGDVSGKLRIWDTTQKEHLLKYEYQPFAGKIKDLAWTEDSKRIA
VVGEGREKFGAVFLWDSGSSVGEITGHNKVINSVDIKQTRPYRLATGSDDNCAAFFEGPP
FKFKFTLSDHTRFVNCVRFSPDGNRFATASADGQIFIYDGKTGEKVCALGGGKAHDGGIY
AISWSPDSSQLLSASGDKTAKIWDVGANSVVSTFNMGSNVLDQQLGCLWQKDHLLSLSLS
GYINYLDKNNPDKPLRVIKGHSKSIQCLTVHKNGGKSYIYSGSNDGHINYWDSDTGENDG
FSGKGHTNQVSRMAVDEMDQLVTCSMDDTVRYTNLSKRDYSGQDAVKMDVQPKCLAVGPG
GYTVVLCIGQIVLMKDKKKCFAIDDLGYEPEAVAVHPGGGSVAVGGTDGNVRLYSIQGTS
LKSDDKTLEAKGPVTDLAYSHDGAFLAVCDANKVVTVFSVPDGYVEHNVFYGHHAKVVCI
AWSPDNEHFASGGMDMMVYVWTVSDPETRIKIPDAHRLHHVSGLAWLDEHTLVTTSHDAS
VKEWSISYN

15. Aspartate aminotransferase, mitochondrial

This protein has accession number P00508 in the UniProt database (see http://www.uniprot.org/uniprot/P00508). Aspartate aminotransferase, mitochondrial (AATM), formerly known as glutamic-oxaloacetic transaminase, catalyzes the reaction of L-aspartate and 2-oxoglutatarate to oxaloacetate and glutamate. This mitochondrial isotype is present predominantly in liver (Kaneko *et al*., 2008).

This protein has the following amino acid sequence (i.e. SEQ ID N° 136; the gray boxes indicate peptides obtained via a trypsin digest of ileal samples as is described further and represent

non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MALLQSRLLLSAPRRAAATARASSWWSHVEMGPPDPILGVTEAFKRDTNSKKMNLGVGAY
RDDNGKSYVLNCVRKAEAMIAAKKMDKEYLPIAGLADFTRASAELALGENSEAFKSGRYV
TVQGISGTGSLRVGANFLQRFFKFSRDVYLPKPSWGNHTPIFRDAGLQLQAYRYYDPKTC
SLDFTGAMEDISKIPEKSIILLHACAHNPTGVDPRQEQWKELASVVKKRNLLAYFDMAYQ
GFASGDINRDAWALRHFIEQGIDVVLSQSYAKNMGLYGERAGAFTVICRDAEEAKRVESQ
LKILIRPMYSNPPMNGARIASLILNTPELRKEWLVEVKGMADRIISMRTQLVSNLKKEGS
SHNWQHITDQIGMFCFTGLKPEQVERLTKEFSIYMTKDGRISVAGVASSNVGYLAHAIHQ
VTK

16. Histone H2A-IV

This protein has accession number P02263 in the UniProt database (see http://www.uniprot.org/uniprot/P02263). H2A4 is a core component of nucleosomes that wrap and compact DNA into chromatin.

This protein has the following amino acid sequence (i.e. SEQ ID N° 137; the gray boxes indicate peptides obtained via a trypsin digest of ileal samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MSGRGKQGGKARAKAKSRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYLAAVLEYLT
AEILELAGNAARDNKKTRIIPRHLQLAIRNDEELNKLLGKVTIAQGGVLPNIQAVLLPKK
TDSHKAKAK

17. Ig lambda chain C region

This protein has accession number P20763 in the UniProt database (see http://www.uniprot.org/uniprot/P20763). Antibodies consist of two heavy and light chains whereby birds only have one isotope of light chain, namely lambda (λ). The light chain is made up by a constant, Ig lambda chain C-region (LAC), and a variable region, Ig lambda chain V1-region (LV1) (Benčina *et al*., 2014).

This protein has the following amino acid sequence (i.e. SEQ ID N° 138; the gray boxes indicate peptides obtained via a trypsin digest of ileal samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

QPKVAPTITLFPPSKEELNEATKATLVCLINDFYPSPVTVDWVIDGSTRSGETTAPQRQS

NSQYMASSYLSLSASDWSSHETYTCRVTHNGTSITKTLKRSEC

18. Ig lambda chain V1 region

This protein has accession number P04210 in the UniProt database (see http://www.uniprot.org/uniprot/P04210). Antibodies consist of two heavy and light chains whereby birds only have one isotope of light chain, namely lambda (λ). The light chain is made up by a constant, Ig lambda chain C-region (LAC), and a variable region, Ig lambda chain V1-region (LV1) (Benčina *et al*., 2014).

This protein has the following amino acid sequence (i.e. SEQ ID N° 139; the gray boxes indicate peptides obtained via a trypsin digest of ileal samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MAWAPLLLAVLAHTSGSLVQAALTQPSSVSANPGETVKITCSGDRSYYGWYQQKAPGSAP

VTLIYDNTNRPSNIPSRFSGSKSGSTATLTITGVQADDEAVYYCGSADSSSTA

19. Cathepsin D

This protein has accession number Q05744 in the UniProt database (see http://www.uniprot.org/uniprot/Q05744). Cathepsin D (CATD), an aspartic proteinase, is optimally active against denatured proteins at acidic pH. CATD is expressed in lysozomes, but also exists bound to some intracellular membranes which has been detected in several different cell types (Fusek and Vetvicka, 1995).

This protein has the following amino acid sequence (i.e. SEQ ID N° 140; the gray boxes indicate peptides obtained via a trypsin digest of ileal samples as is described further and represent

non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MAPRGLLVLLLLALVGPCAALIRIPLTKFTSTRRMLTEVGSEIPDMNAITQFLKFKLGFA
DLAEPTPEILKNYMDAQYYGEIGIGTPPQKFTVVFDTGSSNLWVPSVHCHLLDIACLLHH
KYDASKSSTYVENGTEFAIHYGTGSLSGFLSQDTVTLGNLKIKNQIFGEAVKQPGITFIA
AKFDGILGMAFPRISVDKVTPFFDNVMQQKLIEKNIFSFYLNRDPTAQPGGELLLGGTDP
KYYSGDFSWVNVTRKAYWQVHMDSVDVANGLTLCKGGCEAIVDTGTSLITGPTKEVKELQ
TAIGAKPLIKGQYVISCDKISSLPVVTLMLGGKPYQLTGEQYVFKVSAQGETICLSGFSG
LDVPPPGGPLWILGDVFIGPYYTVFDRDNDSVGFAKCV

20. Retinol-binding protein 4

This protein has accession number P41263 in the UniProt database (see http://www.uniprot.org/uniprot/P41263). Transthyretin (TTR) is a highly conserved protein in animal species that is involved in transport of thyroid hormones and retinol bound to retinol-binding protein 4 (RET4) in the bloodstream (Ingenbleek & Bernstein, 2015). Retinol (vitamin A) is known to be essential for differentiation and proliferation of epithelial cells (Thomas *et al*., 2004).

This protein has the following amino acid sequence (i.e. SEQ ID N° 141; the gray boxes indicate peptides obtained via a trypsin digest of ileal samples as is described further and represent non-limiting examples of protein fragments which can be quantified in a fecal or an intestinal content sample):

MAYTWRALLLLALAFLGSSMAERDCRVSSFKVKENFDKNRYSGTWYAMAKKDPEGLFLQD
NVVAQFTVDENGQMSATAKGRVRLFNNWDVCADMIGSFTDTEDPAKFKMKYWGVASFLQK
GNDDHWVVDTDYDTYALHYSCRELNEDGTCADSYSFVFSRDPKGLPPEAQKIVRQRQIDL
CLDRKYRVIVHNGFCS

The present invention further relates to a method as described above wherein an increased level of a protein chosen from the group consisting of myeloid protein 1, fibronectin, annexin A5, nucleophosmin, carbonic anhydrase 2, transthyretin, ovoinhibitor,

apolipoprotein A-1, hemoglobin subunit beta, alpha-actinin-4, histone H2A-IV and retinol-binding protein 4 present in said fecal or intestinal content sample, when compared to the level found in fecal and/or intestinal content samples of healthy control animals, is an indicator of poor intestinal health. The terms “increased levels of protein compared to the level found in healthy control animals” means at least a 2 fold increase such as a 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold … increase compared to the level found in healthy control animals.

The present invention further relates to a method as described above wherein a decreased level of the protein aminopeptidase Ey, superoxide dismutase [Cu-Zn], angiotensin-converting enzyme, WD repeat-containing protein 1, mitochondrial aspartate aminotransferase, immunoglobulin lambda chain C region, immunoglobulin lambda chain V-1 region and cathepsin D present in said fecal or an intestinal content sample, when compared to the level found in fecal and/or an intestinal content samples of healthy control animals, is an indicator of poor intestinal health. The terms “decreased levels of protein compared to the level found in healthy control animals” means at least an 2 fold decrease such as a 2 fold, 2.5 fold, 3 fold, 3.5 fold, 4 fold, 4.5 fold … decrease compared to the level found in healthy control animals.

The present invention thus relates to a method as described above wherein said protein abundance is significantly differential between healthy and challenged group and/or level of protein correlates with the intestinal health as determined by measuring villus length in the duodenum of said birds, and/or, by measuring villus-to-crypt ratio in the duodenum of said birds, and/or by measuring T-lymphocyte infiltration is said villi, and/or, by scoring the macroscopic gut appearance of said birds. It is clear that said intestinal health is a measure for/correlates with performance parameters of the birds such as body weight and feed conversion ratio.

The present invention further relates to a method as described above wherein said intestinal content sample is a colonic content sample and wherein said protein is chosen from the group consisting of: myeloid protein 1, fibronectin, annexin A5, nucleophosmin, carbonic anhydrase 2, aminopeptidase Ey, transthyretin, ovoinhibitor, apolipoprotein A-1, hemoglobin subunit beta, superoxide dismutase [Cu-Zn] and alpha-actinin-4.

The present invention further relates to a method as described above wherein said intestinal content sample is an ileal content sample and wherein said protein is chosen from the group consisting of: aminopeptidase Ey, transthyretin, apolipoprotein A-1, superoxide dismutase [Cu-Zn], angiotensin-converting enzyme, WD repeat-containing protein 1, mitochondrial aspartate aminotransferase, histone H2A-IV, immunoglobulin lambda chain C region, immunoglobulin lambda chain V-1 region, cathepsin D and retinol-binding protein 4.

More specifically, the present invention relates to a method as described above wherein said fragment of myeloid protein 1 comprises at least one of the following amino acid sequences: APFSGELSGPVK (SEQ ID N° 10), APFSGQLSGPIR (SEQ ID N° 11), FSGELSGPVK (SEQ ID N° 12), HGQIQK (SEQ ID N° 13), SDPTSNLER (SEQ ID N° 14), SGELSGPVK (SEQ ID N° 15), SGQLSGPIR (SEQ ID N° 16), VFPGIISHI (SEQ ID N° 17), VFPGIVSH (SEQ ID N° 18), VFPGIVSHI (SEQ ID N°19); wherein said fragment of fibronectin comprises at least one of the following amino acid sequences: ATITGYK (SEQ ID N° 20), DDQESIPISK (SEQ ID N° 21) ; wherein said fragment of annexin A5 comprises at least one of the following amino acid sequences: KAMKGMGTDEETILK (SEQ ID N° 22), LLLAVVK (SEQ ID N° 23), VDEALVEK (SEQ ID N° 24); wherein said fragment of nucleophosmin comprises at least one of the following amino acid sequences: IGNASTK (SEQ ID N° 25) , TPDSK (SEQ ID N° 26), TVTLGAGAK (SEQ ID N° 27), VVLASLK (SEQ ID N° 28); wherein said fragment of carbonic anhydrase 2 comprises at least one of the following amino acid sequences: VGNAKPEIQK (SEQ ID N° 29), VVDALNSIQTK (SEQ ID N° 30); wherein said fragment of aminopeptidase Ey comprises at least one of the following amino acid sequences: ADNQDIGFGSGTR (SEQ ID N° 31), AIAEGQGEYALEK (SEQ ID N° 32), APVVSEADK (SEQ ID N° 33), AQIIDDAFNLAR (SEQ ID N° 34), AVFTVTMIHPS (SEQ ID N° 35), AWDFIR (SEQ ID N° 36), DFIWER (SEQ ID N° 37), DFLTEDVFK (SEQ ID N° 38), DHLQEAVNK (SEQ ID N° 39), DLWDHLQEAVNK (SEQ ID N° 40), DNAYSSIGNK (SEQ ID N° 41), EAPVVSEADK (SEQ ID N° 42), EGQGEYALEK (SEQ ID N° 43), ENSLLYDNAYSSIGNK (SEQ ID N° 44), EQALER (SEQ ID N° 45), FLEAPVVSEADK (SEQ ID N° 46), FLEAPVVSEADKLR (SEQ ID N° 47), FNTEFELK (SEQ ID N° 48), GADSAETWDIK (SEQ ID N° 49), HYNTAYPLPK (SEQ ID N° 50), IAEGQGEYALEK (SEQ ID N° 51), ILSFFER (SEQ ID N° 52), IWGRPAAIAE (SEQ ID N° 53), IWGRPAAIAEGQGEY (SEQ ID N° 54), IWGRPAAIAEGQGEYALEK (SEQ ID N° 55), KQDATSTIN (SEQ ID N° 56) KQDATSTINSIASNVVGQPL (SEQ ID N° 57), KQDATSINSIASNVVGQPLA (SEQ ID N° 58), LAGPLQQGQHYR (SEQ ID N° 59),

LEAPVVSEADK (SEQ ID N° 60), LPTALKPESYEVTLQPF (SEQ ID N° 61), MLSDFLTEDVFK (SEQ ID N° 62), NSVPLPDSIGAIMDR (SEQ ID N° 63), PAAIAEGQGEYALEK (SEQ ID N° 64), QAIPVINR (SEQ ID N° 65), QDATSTINSIASNVVGQPL (SEQ ID N° 66), QNVSNNPIAPNLR (SEQ ID N° 67), SDFLTEDVFK (SEQ ID N° 68), SDQVGLPDFNAGAMENWG (SEQ ID N° 69), SEVFDSIAYSK (SEQ ID N° 70), SLLYDNAYSSIGNK (SEQ ID N° 71), SNNHQAIPVINR (SEQ ID N° 72), SVPLPDSIGAIMDR (SEQ ID N° 73), TDLWDHLQEAVNK (SEQ ID N° 74), TGELADDLAGFYR (SEQ ID N° 75), TGPILSFFER (SEQ ID N° 76), TIDPTK (SEQ ID N° 77), TLFGQYGGGSFSFSR (SEQ ID N° 78), TNINWVK (SEQ ID N° 79), VNYNQENWDQLL (SEQ ID N° 80), VNYQENWDQLLQ (SEQ ID N° 81), VNYQENWDQLLQQ (SEQ ID N° 82), VVATTQMQAPDAR (SEQ ID N° 83), WRLPTAL (SEQ ID N° 84), WRLPTALKPES (SEQ ID N° 85), WRLPTALKPESYEVTLQPF (SEQ ID N° 86), YDNAYSSIGNK (SEQ ID N° 87), YLQYTIDPTK (SEQ ID N° 88), YPLPK (SEQ ID N° 89); wherein said fragment of transthyretin comprises at least one of the following amino acid sequences: AADGTWQDFATGK (SEQ ID N° 90), CPLMVK (SEQ ID N° 91), DGTWQDFATGK (SEQ ID N° 92), DVVFTANDSGHR (SEQ ID N° 93), GLGLSPFH (SEQ ID N° 94), GLGLSPFHEY (SEQ ID N° 95), GLGLSPFHEYA (SEQ ID N° 96), GLGLSPFHEYADVVF (SEQ ID N° 97), GLGLSPFHEYADVVFTANDSGHR (SEQ ID N° 98), GSPAANVAVK (SEQ ID N° 99), GSPAANVAVKV (SEQ ID N° 100), GTWQDFATGK (SEQ ID N° 101), HYTIAALL (SEQ ID N° 102), HYTIAALLSPF (SEQ ID N° 103), HYTIAALLSPFS (SEQ ID N° 104), TTEEQFVEGVYR (SEQ ID N° 105), TTEFGEIHEL (SEQ ID N° 106), TTEFGEIHELTTEEQ (SEQ ID N° 107), TTEFGEIHELTTEEQFVEGV (SEQ ID N° 108), TTEFGEIHELTTEEQFVEGVYR (SEQ ID N° 109), TTEFGEIHELTTEEQFVEGVYRVEFDTSSYWK (SEQ ID N° 110), VEFDTSSYWK (SEQ ID N° 111), VLDAVR (SEQ ID N° 112); wherein said fragment of ovoinhibitor comprises at least one of the following amino acid sequences: EHGANVEK (SEQ ID N° 113), TLNLVSMAAC (SEQ ID N° 114), TLVACPR (SEQ ID N° 115); wherein said fragment of apolipoprotein A-1 comprises at least one of the following amino acid sequences: DLEEVKEK (SEQ ID N° 116), EMWLK (SEQ ID N° 117), IRDMVDV (SEQ ID N° 118), IRPFLDQF (SEQ ID N° 119), IRPFLDQFSAK (SEQ ID N° 120), LADNLDTLSAAAAK (SEQ ID N° 121), LISFLDELQK (SEQ ID N° 122), LSQKLEEI (SEQ ID N° 123), LTPVAEEAR (SEQ ID N° 124), LTPVAQELK (SEQ ID N° 125), LTPYAENLK (SEQ ID N° 126), MTPLVQEFR (SEQ ID N° 127), QKLSQK (SEQ ID N° 128), QLDLK (SEQ ID N° 129), YKEVR (SEQ ID N° 130); wherein said fragment of hemoglobin subunit beta comprises at least one of the following amino acid sequences: KVLTSFGDAV (SEQ ID N° 142), LHVDPENF (SEQ ID N° 143), LLIVYPWTQR (SEQ ID N° 144), NLDNIK (SEQ ID N° 145), VLTSFGDAVK (SEQ ID N° 146); wherein said fragment of superoxide dismutase [Cu-Zn] comprises at least one of the following amino

acid sequences: AVCVMK (SEQ ID N° 147), FQQQGSGPVK (SEQ ID N° 148), GDAPVEGVIHFQQQGSGPVK (SEQ ID N° 149), GGVAEVEI (SEQ ID N° 150), GGVAEVEIEDSVISLTGPH (SEQ ID N° 151), GVIGIAK (SEQ ID N° 152), HVGDLGNVTA (SEQ ID N° 153), HVGDLGNVTAK (SEQ ID N° 154), ITGLSDGDHGFHVH (SEQ ID N° 155), LACGVIGIAK (SEQ ID N° 156), LTGNAGPR (SEQ ID N° 157), SDDLGR (SEQ ID N° 158), SDDLGRGGDNESK (SEQ ID N° 159), TMVVHA (SEQ ID N° 160); wherein said fragment of alpha-actinin-4 comprises at least one of the following amino acid sequences: DAEDIVNTARDPEK (SEQ ID N° 161), TIPWLEDR (SEQ ID N° 162); wherein said fragment of angiotensin-converting enzyme comprises at least one of the following amino acid sequences: AALPEDELKEYNTLLSDMETTYSVAK (SEQ ID N° 163), ALYNK (SEQ ID N° 164), DGANPGFHEAIGDV (SEQ ID N° 165), DGANPGFHEAIGDVMA (SEQ ID N° 166), DGANPGFHEAIGDVMAL (SEQ ID N° 167), DYNELLFAWK (SEQ ID N° 168), ETPTFEEDLER (SEQ ID N° 169), EVMLEK (SEQ ID N° 170), FEESDR (SEQ ID N° 171), FFTSLGLIPMPQEFWDK (SEQ ID N° 172), GGANPGFHEAIGDVLS (SEQ ID N° 173), GLIPMPQEFWDK (SEQ ID N° 174), GLLEMPPEFWEK (SEQ ID N° 175), GPIPAHL (SEQ ID N° 176), GPIPAHLLGNMW (SEQ ID N° 177), GPIPAHLLGNMWAQQ (SEQ ID N° 178), GPIPAHLLGNMWAQS (SEQ ID N° 179), GYLIDQWR (SEQ ID N° 180), IIGSIQTLGPSNLPLDK (SEQ ID N° 181), IIGSIQTLGPSNLPLDKR (SEQ ID N° 182), IKEDEYNQQWWNL (SEQ ID N° 183), IYSTAK (SEQ ID N° 184), KIIGSIQTLGPSNLPLDK (SEQ ID N° 185), LLGDAMK (SEQ ID N° 186), LLYAWEGWHNAAGNPLR (SEQ ID N° 187), LSVLER (SEQ ID N° 188), MSIALDK (SEQ ID N° 189), NTILSDMDK (SEQ ID N° 190), QCTVVNMDDLITVH (SEQ ID N° 191), QFDPSDFQDETVTR (SEQ ID N° 192), QQGWTPK (SEQ ID N° 193), QQYNTILSDMDK (SEQ ID N° 194), RYVELSNK (SEQ ID N° 195), SLGLIPMPQEFWDK (SEQ ID N° 196), SLSVSTPSHLQK (SEQ ID N° 197), SLYETPTFEEDLER (SEQ ID N° 198), SMIEKPADGR (SEQ ID N° 199), SNIFDLVMPFPDATK (SEQ ID N° 200), SVSTPK (SEQ ID N° 201), SVSTPSHLQK (SEQ ID N° 202), TLGPSNLPLDK (SEQ ID N° 203), TNEVLGWPEFDWRSPIPEGYPEGIDK (SEQ ID N° 204), TSLGLIPMPQEFWDK (SEQ ID N° 205), TSLGLLEMPPEFWEK (SEQ ID N° 206), VDATPAMK (SEQ ID N° 207), VELSNK (SEQ ID N° 208), YGAEHISLK (SEQ ID N° 209), YHIPGNTPY (SEQ ID N° 210), YINLK (SEQ ID N° 211), YNELLFAWK (SEQ ID N° 212), YQGLCPPVPR (SEQ ID N° 213), YVELSNK (SEQ ID N° 214); wherein said fragment of WD repeat-containing protein 1 comprises at least one of the following amino acid sequences: IIGGDPK (SEQ ID N° 215), KVFASLPQVERGVSK (SEQ ID N° 216), VINSVDIK (SEQ ID N° 217); wherein said fragment of mitochondrial aspartate aminotransferase comprises at least one of the following amino acid sequences: GPPDPILGVTEAFK (SEQ ID N° 218), LLLSAPR (SEQ ID N° 219), MDKEYLPI (SEQ ID N° 220),

MGLYGER (SEQ ID N° 221), NPTGVDPR (SEQ ID N° 222), TQLVSNLK (SEQ ID N° 223); wherein said fragment of histone H2A-IV comprises at least one of the following amino acid sequences: NDEELNK (SEQ ID N° 224), VTIAQGGVLPNIQAAVLLPK (SEQ ID N° 225); wherein said fragment of immunoglobulin lambda chain C region comprises at least one of the following amino acid sequences: DFYPSPVTVDWVIDGSTR (SEQ ID N° 226), ITLFPPSK (SEQ ID N° 227), NDFYPSPVTVDWVIDGSTR (SEQ ID N° 228), SGETTAPQR (SEQ ID N° 229), THNGTSITK (SEQ ID N° 230), TVDWVIDGSTR (SEQ ID N° 231), VAPTITLFPPSK (SEQ ID N° 232), VAPTITLFPPSKEELN (SEQ ID N° 233), VAPTITLFPPSKEELNEAT (SEQ ID N° 234), VAPTITLFPPSKEELNEATK (SEQ ID N° 235), VTHNGTSITK (SEQ ID N° 236); wherein said fragment of immunoglobulin lambda chain V1 region comprises at least one of the following amino acid sequences: ALTQPSSVSANPGETVK (SEQ ID N° 237), APGSAPVTLIYDNTNRPSNIPSR (SEQ ID N° 238), GSAPVTLIYDNTNRPSNIPSR (SEQ ID N° 239), ITCSGDR (SEQ ID N° 240), NPGETVK (SEQ ID N° 241), PSNIPSR (SEQ ID N° 242), RPSNIPSR (SEQ ID N° 243), SANPGETVK (SEQ ID N° 244), SVSANPGETVK (SEQ ID N° 245), YGWYQQK (SEQ ID N° 246); wherein said fragment of cathepsin D comprises at least one of the following amino acid sequences: DPTAQPGGELLLGGTDPK (SEQ ID N° 247), ELQTAIGAKPL (SEQ ID N° 248), ELQTAIGAKPLI (SEQ ID N° 249), FDGILGMAFPR (SEQ ID N° 250), IPLTK (SEQ ID N° 251), QPGGELLLGGTDPK (SEQ ID N° 252), VTPFFDNVMQQK (SEQ ID N° 253); wherein said fragment of retinol-binding protein 4 comprises at least one of the following amino acid sequences: QIDLCLDR (SEQ ID N° 254) , TVDENGQMSATAK (SEQ ID N° 255).

Furthermore, the present invention relates to a method as described above wherein said domesticated bird is a broiler. The term 'broiler' refers to any chicken (Gallus gallus domesticus) that is bred and raised specifically for meat production.

Moreover, the present invention relates to a method as described above wherein said proteins or fragments thereof are quantified by using antibodies which specifically bind to said proteins or fragments thereof.

The phrase "specifically (or selectively) binds (or detects)" a protein or peptide, when referring to an antibody, refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two

times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g., Harlow & Lane, Antibodies, A Laboratory Manual (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background. "Immunoassay" is an assay that uses an antibody to specifically bind an antigen (e.g. a marker). The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

Furthermore, the present invention relates to a method as described above wherein said antibodies are part of an Enzyme-Linked Immuno Sorbent Assay.

The present invention will be further illustrated by the following, non-limiting examples.

Examples

List of abbreviations

BHI	**Brain Heart Infusion**
BW	Body weight
CD	Crypt depth
CD_3	Cluster of differentiation 3
CFU	Colony forming units
DAB	Di-amino-benzidine
DDA	Data Dependent Acquisition
DFI	Daily feed intake
DTT	Dithiothreitol
DWG	Daily weight gain
FCR	Feed conversion ratio
HPLC-MS	High performance liquid chromatography-mass spectrometry
HRP	Horseradish peroxidase
LB	Lysogeny Broth
MGAS	Macroscopic Gut Appearance Score
MMTS	Methyl methanethiosulfonate
MPDS 2	Mass prep digestion standard 2
MRS	Man-Rogosa-Sharpe
MWCO	Molecular weight cut-off
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline

psi	Pound per square inch
TEABC	Triethylammonium bicarbonate
VL	Villus length
VL/CD	Villus-to-crypt ratio

Materials and methods

Study design

A total of 360 day-old broilers (Ross 308) were obtained from a local hatchery and housed in floor pens on wooden shavings. Throughout the study, feed and drinking water were provided *ad libitum.* The broilers were randomly assigned to two treatment groups, a control and challenge group (9 pens per treatment and 20 broilers per pen). All animals were fed a commercial feed till day 12 and the feed was switched to a wheat (57.5%) based diet supplemented with 5% rye (Table 1). From day 12 to 18, all animals from the challenge group received 10 mg florfenicol and 10 mg enrofloxacin per kg body weight via the drinking water daily, to induce substantial changes in the gut microbial community. After the antibiotic treatment, 1ml of a bacterial cocktail consisting of *Escherichia coli* (G.78.71), *Enterococcus faecalis* (G.78.62), *Lactobacillus salivarius* (LMG22873), *Lactobacillus crispatus* (LMG49479), *Clostridium perfringens* (netB-) (D.39.61) and *Ruminococcus gnavus* (LMG27713) was given daily by oral gavage from day 19 till 21. Cultures of the bacteria were prepared as follows. Lysogeny Broth (LB, Oxoid,) was used for growing *E. coli*. *Enterococcus faecalis* and *C. perfringens* were grown in Brain Heart Infusion (BHI, Sigma) broth. Man-Rogosa-Sharpe (MRS, Oxoid) medium was used for the growth of *L. crispatus* and *L. salivarius*. For the growth of *R. gnavus*, anaerobic M2GSC medium (pH 6) as described by Miyazaki *et al.* (1997) was used but with 15% clarified rumen fluid instead of 30% and addition of 1 mg/ml cysteine HCl and 4 mg/ml $NaHCO_3$ after autoclaving. *E. coli* and *Enterococcus faecalis* was cultured in aerobic conditions at 37°C. In an aerobic (5% O_2) incubator, *Lactobacillus spp.* were cultured. *C. perfringens* and *R. gnavus* were cultured an anaerobic chamber (gas mixture 84% N_2, 8% O_2 en 8% H_2, GP[concept], Jacomex, France) at 37°C. The bacterial cells were collected by ultracentrifugation (10 000 rpm, 10 min, 20°C) and each pellet was resuspended in 100 ml anaerobic phosphate buffered saline (PBS, 1 mg/ml cysteine HCl, pH 6). The resuspended pellets were mixed and diluted with anaerobic PBS to a total volume of 1.5 l to reach a final concentration of about 10^9 colony forming units (CFU)/ml for each bacterial strain (Table 2).

On day 20, the animals were administered a coccidial challenge consisting of different *Eimeria sp.*, namely 60.000 oocysts of *E. acervulina* and 30.000 oocysts *E. maxima.*

At day 26, the birds were weighed and 3 birds per pen were euthanized. The duodenal loop was sampled for histological examination and content from ileum and colon was collected for protein extraction.

Table 1| Composition and nutrient content of the wheat/ rye based broiler diet. Starter diet was given till day 11. Grower was given from day 12 to day 34.

	Starter	Grower		Starter	Grower
Feedstuff	%	%	**Calculated nutrient composition (%)**	%	%
Wheat	55.13	57.87	Dry matter	88.45	88.38
Rye	0.00	5.00	Ash	5.11	4.79
Soy meal, crude fiber content < 50	22.86	22.86	Crude protein	20.85	18.98
Full fat soybeans	7.50	2.50	Crude fat	10.83	9.90
Animal fat	7.20	7.20	Crude fiber	2.93	2.49
Soybean oil	1.00	1.00	Carbohydrates	48.46	51.95
Premix	0.50	0.50	Starch	34.62	38.46
Lime fine	1.11	1.11	Sugars	4.77	4.53
Monocalciumphosphate	0.83	0.83	NDF	10.52	9.93
Salt	0.18	0.18	ADF	4.18	3.52
$NaHCO_3$	0.25	0.25	Calcium	0.69	0.66
L-lysine HCl	0.30	0.30	Phosphorus, total	0.57	0.54
DL-methionine	0.30	0.30	Calcium/dP poultry	0.22	0.22
L-threonine	0.10	0.10	Magnesium	0.16	0.14
Rapeseed meal < 380	2.74	0.00	Potassium	0.88	0.79
			Sodium	0.15	0.15
			Chloride	0.20	0.20
			Base-excess (mEq/kg)	23.39	20.97

Linolic acid	2.38	1.92

Table 2| Broilers were orally inoculated with 1 ml of a bacterial cocktail on day 19, 20 and 21 with 10^{6}-10^{10} CFU of *Escherichia coli, Enterococcus faealis, Lactobacillus salivarius, Lactobacillus crispatus, Clostridium perfringens* (netB-) *and Ruminococcus gnavus*

Bacterial strain	Day 19 (CFU/ml)	Day 20 (CFU/ml)	Day 21 CFU/ml)
E. coli	$2.11x10^{9}$	$1.22x10^{9}$	$2.28x10^{9}$
Enterococcus faecalis	$3.44x10^{9}$	$2.28x10^{10}$	$3.56x10^{9}$
Lactobacillus salivarius	$4.78x10^{7}$	$1.16x10^{7}$	$2.39x10^{7}$
Lactobacillus crispatus	$1.89x10^{9}$	$7.78x10^{7}$	$7.22x10^{6}$
Clostridium perfringens	$1x10^{7}$	$1.06x10^{7}$	$2.78x10^{8}$
Ruminococcus gnavus	$2.89x10^{8}$	$2.78x10^{8}$	$3.17x10^{8}$

Macroscopic scoring system

The macroscopic appearance of the gut was evaluated using a previously described scoring system (Teirlynck *et al*., 2011), in which in total 10 parameters were assessed and assigned 0 (absent) or 1 (present), which resulted in a total score between 0 and 10. A total score of 0 represents a normal appearance of the intestinal tract while 10 points to severe deviations from the normal appearance. The parameters are (1) 'ballooning' of the gut; (2) inflammation, cranial to the Meckel's diverticulum; (3) macroscopically visible and tangible fragile small intestine cranial to the Meckel's diverticulum; (4) loss of tonus in longitudinal cutting of the intestine cranial to the Meckel's diverticulum within the 3 seconds after incision; (5) abnormal occurrence of the intestinal content (excess mucus, orange content, gas) cranial to the Meckel's diverticulum; (6,7,8,9) are identical to (2,3,4,5) but caudal to the Meckel's diverticulum and (10) presence of undigested particles in the colon. A coccidiosis scoring was performed as described in Johnson & Reid (1970) which the animals were given a score for typical lesions associated with *Eimeria acervulina, E. maxima* and *E. tenell*a. For each, a score was given between 0 (absent) and 4 (severe). A total coccidiosis score was calculated as the sum of the scores given for lesions caused by each individual *Eimeria* species.

Morphological parameters

The duodenal loop was fixated in 4% formaldehyde for 24 hours, dehydrated in xylene and embedded in paraffin. Sections of 4 µm were cut using a microtome (Microme HM360, Thermo Scientific) and were processed as described by De Maesschalck *et al*. (2015). Morphological parameters were determined using standard light microscopy. Villus length and crypt depth in the duodenum were measured by random measurement of twelve villi per intestinal segment using Leica DM LB2 Digital and a computer based image analysis program, LAS V4.1 (Leica Application Suite V4, Germany). Also the villus-to-crypt ratio was calculated.

Immunohistochemical examination

Antigen retrieval was performed on 4 µm sections with a pressure cooker in citrate buffer (10 mM, pH 6). Slides were rinsed with washing buffer (Dako kit, K4011) and blocked with peroxidase reagent (Dako, S2023) for 5 minutes. Slides were rinsed with aqua destillata and Dako washing buffer before incubation with anti- CD_3 primary antibodies (Dako CD_3, A0452) for 30 minutes at room temperature diluted 1:100 in antibody diluent (Dako, S3022). After rinsing again with washing buffer, slides were incubated with labelled polymer-HRP anti-rabbit (Envision$^+$ System-HRP, K4011) for 30 minutes at room temperature. Before adding di-amino-benzidine (DAB$^+$) substrate and DAB$^+$ chromogen (Dako kit, K4011) for 5 minutes, slides were rinsed 2 times with washing buffer. To stop the staining, the slides were rinsed with Aquadest, dehydrated using the Shandon Varistain-Gemini Automated Slide Stainer and counterstained with hematoxylin for 10 seconds. The slides were analyzed with Leica DM LB2 Digital and a computer based image analysis program LAS V4.1 (Leica Application Suite V4, Germany) to measure CD_3 positive area on a total area of 3 mm^2 which represents T-lymphocyte infiltration in approximately 10 villi per section.

Discovery proteomics

Sample preparation

Individual colon and ileal content samples were collected and stored at -20°C before use. 500 mg was solubilized in 10 ml 2M urea, 50mM ammonium bicarbonate and homogenized by vortexing. After centrifugation (20.000xg, 15min, 4°C), the supernatant was filtered through a 0,22 µm filter unit (Merck, Germany) directly in a Vivaspin 20 with a 5 kDa MWCO filter (Sartorius, Germany) and centrifuged for 1h at 4000xg. The filter was washed 3 times with 1 ml 2M urea, 50mM ammonium bicarbonate followed by centrifugation (4000xg, 10min, 4°C). The samples were washed 3 times with 1 ml 500mM triethylammonium bicarbonate (TEABC, Sigma) to remove the urea. Subsequently, the samples were concentrated to a volume of ± 500 µl. To determine the protein concentration, a Bradford assay was performed where OD was measured at 595 nm. Approximately 50µg of proteins were reduced with 1mM dithiothreitol (DTT) and incubated at 60°C for 30min, followed by alkylation for 10 min at room temperature with 10mM methyl methanethiosulfonate (MMTS). Hereafter, calcium chloride and acetonitrile were added to a final concentration of 1 mM and 5% (v/v) respectively. Finally trypsin was added in a 1:20 (trypsin:protein) ratio for overnight digestion at 37°C. The samples were vacuum dried and analyzed with high performance liquid chromatography-mass spectrometry (HPLC-MS).

HPLC-MS

Peptides were dissolved in 0.1% formic acid in HPLC-grade water (buffer A) to a final concentration of 1µg/µL. 100 fmol of mass prep digestion standard 2 (MPDS 2) was spiked into each sample. Data Dependent Acquisition MS analysis was performed on a TripleTOF 5600 (Sciex) fitted with a DuoSpray ion source in positive ion mode, coupled to an Eksigent NanoLC 400 HPLC system (Sciex). Peptides were separated on a microLC YMC Triart C18 column (id 300 µm, length 15 cm, particle size 3 µm) at a flow rate of 5 µL/min by means of trap-elute injection (YMC Triart C18 guard column, id 500 µm, length 5 mm, particle size 3 µm). Elution was performed using a gradient of 4–40% buffer B (0.1% formic acid, 5% DMSO in 80% ACN) over 90 min. Ion source parameters were set to 5.5 kV for the ion spray voltage, 30 psi for the curtain gas, 13 psi for the nebulizer gas and 80°C as temperature.

For DDA, a 2.25 s instrument cycle was repeated in high sensitivity mode throughout the whole gradient, consisting of a full scan MS spectrum (300–1250 m/z) with an accumulation

time of 0.2 s, followed by 20 MS/MS experiments (50–1800 m/z) with 0.2 s accumulation time each, on MS precursors with charge state 2 to 5+ exceeding a 500 cps threshold. Rolling collision energy was used as suggested by the manufacturer and former target ions were excluded for 10 s.

Database searching

The *.wiff files generated during LC-MS/MS analysis were imported into the Progenesis QI for Proteomics software (Non-linear Dynamics). The different samples were aligned based on retention time and m/z of reoccurring features to enable relative quantification. After subsequent peak picking, a merged *.mgf file was exported from the software and searched for identifications with MASCOT Daemon (Matrix Science, version 2.5.1) against a chicken database (reviewed protein database downloaded from Swissprot, January 2016) supplemented with the cRAP database (laboratory proteins and dust/contact proteins http://www.thegpm.org/crap/) and the internal standard. Maximum peptide mass tolerance and fragment mass tolerance were set to 10 ppm and 0.1 Da respectively. Additionally, methylthio on cysteine was set as a fixed modification and deamidation of asparagine and/or glutamine and oxidation of methionine were set as variable modifications. Enzyme specificity was set to trypsin with a maximum of one missed cleavage. The identifications were exported from MASCOT Daemon with a 5% false discovery rate (*.xml format) and imported into Progenesis QI for Proteomics.

Statistical analysis

Statistical analysis was performed with Graphpad Prism (v.5). To evaluate whether the data is normally distributed, a Kolmogorov-Smirnov test was performed. In case of a normal distribution, comparison of the data was performed with an independent samples t-test. Otherwise, the non-parametric Mann-Whitney test was performed. A p-value of <0.05 was considered statistically significant. The statistical coherence between different parameters was evaluated via correlation analysis.

Results

Performance parameters

Body weight (BW), daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR) were measured during different time periods. Significant differences between treatment and control groups were seen at day 26, 35 and 41, but not at the age of 12 days (Table 3).

Table 3| Mean ± standard deviation of body weight (BW), daily weight gain (DWG), daily feed intake (DFI) and feed conversion ratio (FCR) measured during different time periods for the control and challenge group. Significant differences ($p < 0.05$) are shown in bold.

		Control	Challenge	
Time period	**parameters**	**Mean ± SD**	**Mean ± SD**	***p*-value**
D1-D12	BW (g)	290 ± 13	295 ± 11	0.485
	DWG (g)	19,6 ± 1	20,2 ± 1	0.342
	DFI (g)	24 ± 1	25 ± 1	0.614
	FCR	1.25 ± 0.06	1.23 ± 0.05	0.321
D12-D26	BW (g)	1375 ± 58	1195 ± 46	**< 0.001**
	DWG (g)	78 ± 4	64 ± 4	**< 0.001**
	DFI (g)	116 ± 6	108 ± 7	**0.014**
	FCR	1.50 ± 0.07	1.69 ± 0.18	**0.004**
D26-D35	BW (g)	2345 ± 174	2153 ± 129	**0.003**
	DWG (g)	106 ± 10	105 ± 6	0.857
	DFI (g)	185 ± 10	203 ± 15	**0.016**
	FCR	1.76 ± 0.14	1.94 ± 0.20	**0.040**
D35-D41	BW (g)	3095 ± 164	2850 ± 176	**0.001**
	DWG (g)	121 ± 17	114 ± 21	0.276
	DFI (g)	161 ± 14	166 ± 10	0.427

	FCR	1.35 ± 0.14	1.53 ± 0.45	**0.041**
D12-D35	DWG (g)	86 ± 5	76 ± 4	**< 0.001**
	DFI (g)	136 ± 6	135 ± 8	0.811
	FCR	1.59 ± 0.08	1.79 ± 0.18	**0.002**
D12-D41	DWG (g)	91 ± 4	82 ± 5	**< 0.001**
	DFI (g)	139 ± 7	140 ± 7	0.864
	FCR	1.52 ± 0.04	1.72 ± 0.13	**< 0.001**

Macroscopic scoring

The appearance of the gut and a coccidiosis score were given to 27 birds per treatment. A lower body weight was observed at day 26 (p = 0.0001) for broilers which received challenge treatment. The score for the macroscopic appearance of the gut and the total coccidiosis score were higher in the challenged group at day 26 (p < 0.001) (Figure 1, Table 4).

Table 4| Mean ± standard deviation of body weight (BW), macroscopic gut appearance score (MGAS) and coccidiosis score (CS) for the control (n = 27) and challenge group (n = 27) at day 26. Significant differences (p < 0.05) are shown in bold.

		Control	Challenge	
Timepoint	**parameters**	**Mean ± SD**	**Mean ± SD**	***p*-value**
D26	BW (g)	1375 ± 157.4	1187 ± 170.1	***p* = 0.0001**
	MGAS	0.9 ± 0.7	3.1 ± 1.1	***p* < 0.001**
	CS	0.8 ± 0.7	3.5 ± 1.7	***p* < 0.001**

Intestinal morphology and immunohistochemistry

A significant shorter villus length, an increased crypt depth, a lower villus-to-crypt ratio and a higher inflammation level in duodenal sections on day 26 ($p < 0.0001$) were detected in the gut of animals from the treatment group as compared to the control group (Table 5).

Table 5| Mean ± standard deviation of villus length (VL), crypt depth (CD), villus-to-crypt ratio (VL/CD) and T-lymphocyte infiltration (CD_3 area%) on day 26 for control (n = 27) and challenge group (n = 27). For all evaluated parameters, statistical significance was $p < 0.0001$.

		Control	Challenge
Timepoint	**parameters**	**Mean ± SD**	**Mean ± SD**
D26	VL (µm)	2035.7 ± 134.6	1369.9 ± 158.7
	CD (µm)	190.1 ± 15.43	365.7 ± 31.41
	VL/CD	11.03 ± 1.03	3.85 ± 0.63
	CD_3 area%	7.88 ± 1.35	9.54 ± 2.71

Correlations

Pearson r has a value between -1 (total negative correlation) and +1 (total positive correlation). In case of a positive correlation, one parameter increases as the other parameter increases and vice versa. When one parameter decreases and the other one increases, there is a negative correlation. On day 26, all macroscopic (macroscopic gut appearance score, coccidiosis score and body weight) and histological parameters (villus length, crypt depth, villus-to-crypt ratio and T-lymphocyte infiltration) correlate with one another (Table 6).

Table 6| Pearson correlation coefficient between macroscopic gut appearance score (MGAS), coccidiosis score (CS), body weight (BW), villus length (VL), crypt depth (CD), villus-to-crypt ratio (VL/CD) and T-lymphocyte infiltration (CD_3 area%). Correlation coefficients with a statistical significance of $p < 0.05$ are shown.

	Day 26						
	MGAS	CS	BW	VL	CD	VL/CD	CD_3 area%

MGAS	-						
CS	0.0905	-					
BW	-0.6836	-0.5974	-				
VL	-0.8400	-0.8572	0.7400	-			
CD	0.8220	0.8045	-0.7546	-0.9073	-		
VL/CD	-0.8507	-0.8599	0.7303	0.9570	-0.9773	-	
CD_3 area%	0.8559	0.8177	-0.770	-0.9028	0.8959	-0.8979	-

Discovery proteomics 1.

Using MASCOT Daemon (Matrix Science, version 2.5.1) against a chicken database (reviewed protein database downloaded from Swissprot, January 2016) supplemented with the cRAP database (laboratory proteins and dust/contact proteins http://www.thegpm.org/crap/), 157 proteins were identified for colon. In theory, a good gut health biomarker should relate with one or more histological parameters since measurement of villus length and inflammation level are used as standard measurements in the evaluation of intestinal health. Also correlation with the macroscopic gut appearance score was evaluated. It is noted that proteins of which the colonic concentration has a negative correlation with the villus length, inverse correlation was seen with crypt depth, CD_3 area% and macroscopic gut appearance score. Only correlations with a statistical significance of $p < 0.1$ are shown (Table 7).

Table 7| Pearson correlation coefficient between the abundance of the protein in colon content and villus length (VL), crypt depth (CD), villus-to-crypt ratio (VL/CD) and T-lymphocyte infiltration (CD_3 area%) and macroscopic gut appearance score (MGAS) on day 26. Correlation coefficients with a statistical significance of $p < 0.1$ are shown.

		Day 26		
Accession number		VL	CD_3 area%	MGAS
P08940	Myeloid protein 1	-0,7283	0,5466	0,5668
P11722	Fibronectin	-0,8114	0,6515	0,6519
P17153	Annexin A5	-0,5746		

P16039	Nucleophosmin	-0,5263	0,7196	
P07630	Carbonic anhydrase 2	-0,5899		0,5726
O57579	Aminopeptidase Ey	0,5462		-0,7177
P27731	Transthyretin	-0,5778	0,6065	
P10184	Ovoinhibitor	-0.4852		
P08250	Apolipoprotein A-1	-0,5066		

Discovery proteomics 2

Using MASCOT Daemon (Matrix Science, version 2.5.1) against a chicken database (reviewed protein database downloaded from Swissprot, January 2016) supplemented with the cRAP database (laboratory proteins and dust/contact proteins http://www.thegpm.org/crap/), 157 and 181 proteins were identified for colon and ileum respectively whereby significant differential proteins between control and challenged birds were selected ($p < 0.05$). In broilers from the challenge group, the following proteins showed a significantly higher abundance compared to control animals in colonic content ($p < 0.05$): alpha-actinin-4 (ACTN4), annexin A5 (ANXA5), apolipoprotein A-1 (APOA1), fibronectin (FINC), hemoglobin subunit beta (HBB), myeloid protein 1 (MIM1), nucleophosmin (NPM), ovoinhibitor (IOV7) and transthyretin (TTR). Both in colonic and ileal content, superoxide dismutase [Cu-Zn] (SOD) showed a decreased abundance compared to control animals ($p < 0.05$). Angiotensin-converting enzyme (ACE), mitochondrial aspartate aminotransferase (AATM), cathepsin D (CATD), Ig lambda chain C region (LAC), Ig lambda chain V-1 region (LV1), TTR and WD repeat-containing protein 1 (WDR1) showed a lower abundance in challenged birds ($p < 0.05$) in ileal samples. Following proteins were more abundant ($p < 0.05$): APOA1, histone H2A-IV (H2A4) and retinol-binding protein 4 (RET4) (Table 8 and Table 9).

Table *8*: Significantly different proteins between control and challenge group in colonic content.

Accession number	Protein name	Abbreviation	*P*-value	Highest mean
Q90734	Alpha-actinin 4	ACTN4	0.0385	Challenge
P17153	Annexin A5	ANXA5	0.0266	Challenge
P08250	Apolipoprotein A-1	APOA1	0.0277	Challenge
P11722	Fibronectin	FINC	0.0106	Challenge
P02112	Hemoglobin subunit beta	HBB	0.0158	Challenge
P08940	Myeloid protein 1	MIM1	0.0008	Challenge
P16039	Nucleophosmin	NPM	0.0071	Challenge
P10184	Ovoinhibitor	IOV7	0.0254	Challenge
P80566	Superoxide dismutase [Cu-Zn]	SOD	0.0287	Control
P27731	Transthyretin	TTR	0.0317	Challenge

Proteomics using high performance liquid chromatography-mass spectrometry (HPLC-MS) was performed on colonic content of animals from the control (n = 9) and challenged (n = 9) group at day 26. This resulted in significant differential proteins ($p < 0.05$) with a higher normalized abundance of 9 proteins and a decrease of superoxide dismutase [Cu-Zn] in challenged birds.

Table *9*: Significantly different proteins between control and challenge group in ileal content.

Accession number	Protein name	Abbreviation	*P*-value	Highest mean
O57579	Aminopeptidase Ey	AMPN	0.0012	Control
Q10751	Angiotensin-converting enzyme	ACE	0.0006	Control
P08250	Apolipoprotein A-1	APOA1	0.0364	Challenge
P00508	Aspartate aminotransferase, mitochondrial	AATM	0.0067	Control
Q05744	Cathepsin D	CATD	0.0203	Control

P02263	Histone H2A-IV	H2A4	0.0079	Challenge
P20763	Ig lambda chain C region	LAC	0.0155	Control
P04210	Ig lambda chain V-1 region	LV1	0.0370	Control
P41263	Retinol-binding protein 4	RET4	0.0399	Challenge
P80566	Superoxide dismutase [Cu-Zn]	SOD	0.0004	Control
P27731	Transthyretin	TTR	0.0091	Control
O93277	WD repeat-containing protein 1	WDR1	0.0027	Control

Proteomics using high performance liquid chromatography-mass spectrometry (HPLC-MS) was performed on ileal content of animals from the control (n = 9) and challenged (n = 9) group at day 26. This resulted in 12 significant differential proteins ($p < 0.05$) with a higher normalized abundance of apolipoprotein A-1 (APOA1), histone H2A-IV (H2A4) and retinol-binding protein 4 (RET4) and decrease in normalized abundance for the other 9 proteins in challenged birds.

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Claims

1. A method to determine the intestinal health status of a domesticated bird comprising:
   - providing a fecal sample or an intestinal content sample obtained from said domesticated bird, and
   - quantifying a protein, or a fragment thereof, in said sample,
   wherein said protein is chosen from the group consisting of: myeloid protein 1, fibronectin, annexin A5, nucleophosmin, carbonic anhydrase 2, aminopeptidase Ey, transthyretin, ovoinhibitor, apolipoprotein A-1, hemoglobin subunit beta, superoxide dismutase [Cu-Zn], alpha-actinin-4, angiotensin-converting enzyme, WD repeat-containing protein 1, mitochondrial aspartate aminotransferase, histone H2A-IV, immunoglobulin lambda chain C region, immunoglobulin lambda chain V-1 region, cathepsin D and retinol-binding protein 4.
2. A method to determine the intestinal health status of a domesticated bird comprising:
   - providing a fecal sample or an intestinal content sample obtained from said domesticated bird, and
   - quantifying a protein, or a fragment thereof, in said sample,
   wherein said protein is chosen from the group consisting of: myeloid protein 1, fibronectin, annexin A5, nucleophosmin, carbonic anhydrase 2, aminopeptidase Ey, transthyretin, ovoinhibitor and apolipoprotein A-1.
3. A method according to claim 1 wherein an increased level of a protein chosen from the group consisting of myeloid protein 1, fibronectin, annexin A5, nucleophosmin, carbonic anhydrase 2, transthyretin, ovoinhibitor, apolipoprotein A-1, hemoglobin subunit beta, alpha-actinin-4, histone H2A-IV and retinol-binding protein 4 present in said fecal or intestinal content sample, when compared to the level found in fecal or intestinal content samples of healthy control animals, is an indicator of poor intestinal health.
4. A method according to claim 1 wherein a decreased level of the protein aminopeptidase Ey, superoxide dismutase [Cu-Zn], angiotensin-converting enzyme, WD repeat-containing protein 1, mitochondrial aspartate aminotransferase, immunoglobulin lambda chain C region, immunoglobulin lambda chain V-1 region and cathepsin D present in said fecal or intestinal content sample, when compared to the

level found in fecal or intestinal content samples of healthy control animals, is an indicator of poor intestinal health.

5. A method according to claim 2 wherein said level of protein correlates with the intestinal health as determined by measuring villus length in the duodenum of said birds, and/or, by measuring villus-to-crypt ratio in the duodenum of said birds, and/or by measuring T-lymphocyte infiltration in said villi, and/or, by scoring the macroscopic gut appearance of said birds.

6. A method according to claims 1 or 2 wherein said intestinal content sample is a colonic content sample and wherein said protein is chosen from the group consisting of: myeloid protein 1, fibronectin, annexin A5, nucleophosmin, carbonic anhydrase 2, aminopeptidase Ey, transthyretin, ovoinhibitor, apolipoprotein A-1, hemoglobin subunit beta, superoxide dismutase [Cu-Zn] and alpha-actinin-4.

7. A method according to claim 1 wherein said intestinal content sample is an ileal content sample and wherein said protein is chosen from the group consisting of: aminopeptidase Ey, transthyretin, apolipoprotein A-1, superoxide dismutase [Cu-Zn], angiotensin-converting enzyme, WD repeat-containing protein 1, mitochondrial aspartate aminotransferase, histone H2A-IV, immunoglobulin lambda chain C region, immunoglobulin lambda chain V-1 region, cathepsin D and retinol-binding protein 4.

8. A method according to any of claims 1-7 wherein said fragment of myeloid protein 1 comprises at least one of the following amino acid sequences: APFSGELSGPVK, APFSGQLSGPIR, FSGELSGPVK, HGQIQK, SDPTSNLER, SGELSGPVK, SGQLSGPIR, VFPGIISHI, VFPGIVSH, VFPGIVSHI; wherein said fragment of fibronectin comprises at least one of the following amino acid sequences: ATITGYK, DDQESIPISK; wherein said fragment of annexin A5 comprises at least one of the following amino acid sequences: KAMKGMGTDEETILK, LLLAVVK, VDEALVEK; wherein said fragment of nucleophosmin comprises at least one of the following amino acid sequences: IGNASKTK, TPDSK, TVTLGAGAK, VVLASLK; wherein said fragment of carbonic anhydrase 2 comprises at least one of the following amino acid sequences: VGNAKPEIQK, VVDALNSIQTK; wherein said fragment of aminopeptidase Ey comprises at least one of the following amino acid sequences: ADNQDIGFGSGTR, AIAEGQGEYALEK, APVVSEADK, AQIIDDAFNLAR, AVFTVTMIHPS, AWDFIR, DFIWER, DFLTEDVFK, DHLQEAVNK, DLWDHLQEAVNK, DNAYSSIGNK, EAPVVSEADK, EGQGEYALEK, ENSLLYDNAYSSIGNK,

EQALER, FLEAPVVSEADK, FLEAPVVSEADKLR, FNTEFELK, GADSAEPTWDIK, HYNTAYPLPK, IAEGQGEYALEK, ILSFFER, IWGRPAAIAE, IWGRPAAIAEGQGEY, IWGRPAAIAEGQGEYALEK, KQDATSTIN, KQDATSTINSIASNVVGQPL, KQDATSTINSIASNVVGQPLA, LAGPLQQGQHYR, LEAPVVSEADK, LPTALKPESYEVTLQPF, MLSDFLTEDVFK, NSVPLPDSIGAIMDR, PAAIAEGQGEYALEK, QAIPVINR, QDATSTINSIASNVVGQPL, QNVSNNPIAPNLR, SDFLTEDVFK, SDQVGLPDFNAGAMENWG, SEVFDSIAYSK, SLLYDNAYSSIGNK, SNNHQAIPVINR, SVPLPDSIGAIMDR, TDLWDHLQEAVNK, TGELADDLAGFYR, TGPILSFFER, TIDPTK, TLFGQYGGGSFSFSR, TNINWVK, VNYNQENWDQLL, VNYNQENWDQLLQ, VNYNQENWDQLLQQ, VVATTQMQAPDAR, WRLPTAL, WRLPTALKPES, WRLPTALKPESYEVTLQPF, YDNAYSSIGNK, YLQYTIDPTK, YPLPK; wherein said fragment of transthyretin comprises at least one of the following amino acid sequences: AADGTWQDFATGK, CPLMVK, DGTWQDFATGK, DVVFTANDSGHR, GLGLSPFH, GLGLSPFHEY, GLGLSPFHEYA, GLGLSPFHEYADVVF, GLGLSPFHEYADVVFTANDSGHR, GSPAANVAVK, GSPAANVAVKV, GTWQDFATGK, HYTIAALL, HYTIAALLSPF, HYTIAALLSPFS, TTEEQFVEGVYR, TTEFGEIHEL, TTEFGEIHELTTEEQ, TTEFGEIHELTTEEQFVEGV, TTEFGEIHELTTEEQFVEGVYR, TTEFGEIHELTTEEQFVEGVYRVEFDTSSYWK, VEFDTSSYWK, VLDAVR; wherein said fragment of ovoinhibitor comprises at least one of the following amino acid sequences: EHGANVEK, TLNLVSMAAC, TLVACPR; wherein said fragment of apolipoprotein A-1 comprises at least one of the following amino acid sequences: DLEEVKEK, EMWLK, IRDMVDV, IRPFLDQF, IRPFLDQFSAK, LADNLDTLSAAAAK, LISFLDELQK, LSQKLEEI, LTPVAEEAR, LTPVAQELK, LTPYAENLK, MTPLVQEFR, QKLSQK, QLDLK, YKEVR; wherein said fragment of hemoglobin subunit beta comprises at least one of the following amino acid sequences: KVLTSFGDAV, LHVDPENF, LLIVYPWTQR, NLDNIK, VLTSFGDAVK; wherein said fragment of superoxide dismutase comprises at least one of the following amino acid sequences: AVCVMK, FQQQGSGPVK, GDAPVEGVIHFQQQGSGPVK, GGVAEVEI, GGVAEVEIEDSVISLTGPH, GVIGIAK, HVGDLGNVTA, HVGDLGNVTAK, ITGLSDGDHGFHVH, LACGVIGIAK, LTGNAGPR, SDDLGR, SDDLGRGGDNESK, TMVVHA; wherein said fragment of alpha-actinin-4 comprises at least one of the following amino acid sequences: DAEDIVNTARDPEK, TIPWLEDR; wherein said fragment of angiotensin-converting enzyme comprises at least one of the following amino acid sequences:

9. AALPEDELKEYNTLLSDMETTYSVAK, ALYNK, DGANPGFHEAIGDV, DGANPGFHEAIGDVMA, DGANPGFHEAIGDVMAL, DYNELLFAWK, ETPTFEEDLER, EVMLEK, FEESDR, FFTSLGLIPMPQEFWDK, GGANPGFHEAIGDVLS, GLIPMPQEFWDK, GLLEMPPEFWEK, GPIPAHL, GPIPAHLLGNMW, GPIPAHLLGNMWAQQ, GPIPAHLLGNMWAQS, GYLIDQWR, IIGSIQTLGPSNLPLDK, IIGSIQTLGPSNLPLDKR, IKEDEYNQQWWNL, IYSTAK, KIIGSIQTLGPSNLPLDK, LLGDAMK, LLYAWEGWHNAAGNPLR, LSVLER, MSIALDK, NTILSDMDK, QCTVVNMDDLITVH, QFDPSDFQDETVTR, QQGWTPK, QQYNTILSDMDK, RYVELSNK, SLGLIPMPQEFWDK, SLSVSTPSHLQK, SLYETPTFEEDLER, SMIEKPADGR, SNIFDLVMPFPDATK, SVSTPK, SVSTPSHLQK, TLGPSNLPLDK, TNEVLGWPEFDWRSPIPEGYPEGIDK, TSLGLIPMPQEFWDK, TSLGLLEMPPEFWEK, VDATPAMK, VELSNK, YGAEHISLK, YHIPGNTPY, YINLK, YNELLFAWK, YQGLCPPVPR, YVELSNK; wherein said fragment of WD repeat-containing protein 1 comprises at least one of the following amino acid sequences: IIGGDPK, KVFASLPQVERGVSK, VINSVDIK,; wherein said fragment of mitochondrial aspartate aminotransferase comprises at least one of the following amino acid sequences: GPPDPILGVTEAFK, LLLSAPR, MDKEYLPI, MGLYGER, NPTGVDPR, TQLVSNLK; wherein said fragment of histone H2A-IV comprises at least one of the following amino acid sequences: NDEELNK, VTIAQGGVLPNIQAAVLLPK; wherein said fragment of immunoglobulin lambda chain C region comprises at least one of the following amino acid sequences: DFYPSPVTVDWVIDGSTR, ITLFPPSK, NDFYPSPVTVDWVIDGSTR, SGETTAPQR, THNGTSITK, TVDWVIDGSTR, VAPTITLFPPSK, VAPTITLFPPSKEELN, VAPTITLFPPSKEELNEAT, VAPTITLFPPSKEELNEATK, VTHNGTSITK; wherein said fragment of immunoglobulin lambda chain V1 region comprises at least one of the following amino acid sequences: ALTQPSSVSANPGETVK, APGSAPVTLIYDNTNRPSNIPSR, GSAPVTLIYDNTNRPSNIPSR, ITCSGDR, NPGETVK, PSNIPSR, RPSNIPSR, SANPGETVK, SVSANPGETVK, YGWYQQK; wherein said fragment of cathepsin D comprises at least one of the following amino acid sequences: DPTAQPGGELLLGGTDPK, ELQTAIGAKPL, ELQTAIGAKPLI, FDGILGMAFPR, IPLTK, QPGGELLLGGTDPK, VTPFFDNVMQQK; wherein said fragment of retinol-binding protein 4 comprises at least one of the following amino acid sequences: QIDLCLDR, TVDENGQMSATAK.

10. A method according to any of claims 1-8 wherein said domesticated bird is a broiler.

11. A method according to any of claims 1-9 wherein said proteins or fragments thereof are quantified by using antibodies which specifically bind to said proteins or fragments thereof.
12. A method according to claim said 10 wherein said antibodies are part of an Enzyme-Linked Immuno Sorbent Assay.

Figures

Fig 1a

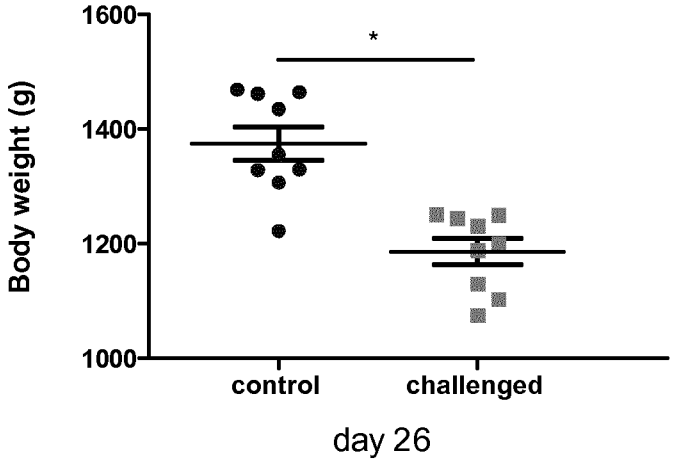


Fig 1b

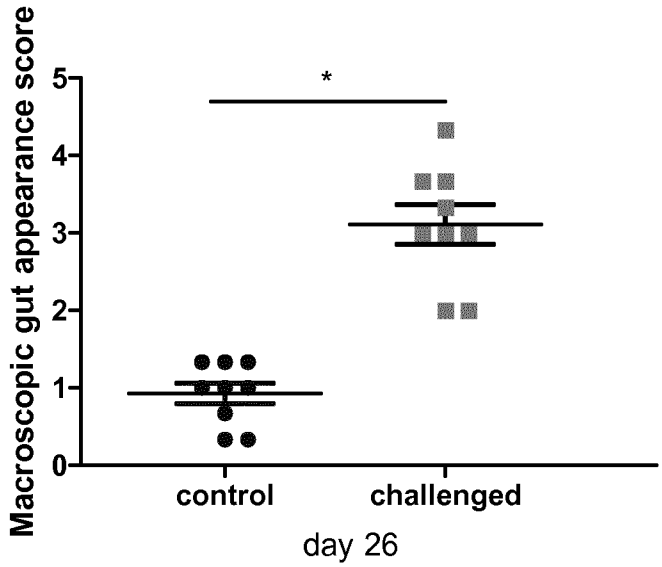

Macroscopic gut appearance score
5
4
3
2
1
0
*
control
challenged
day 26

Fig 1c

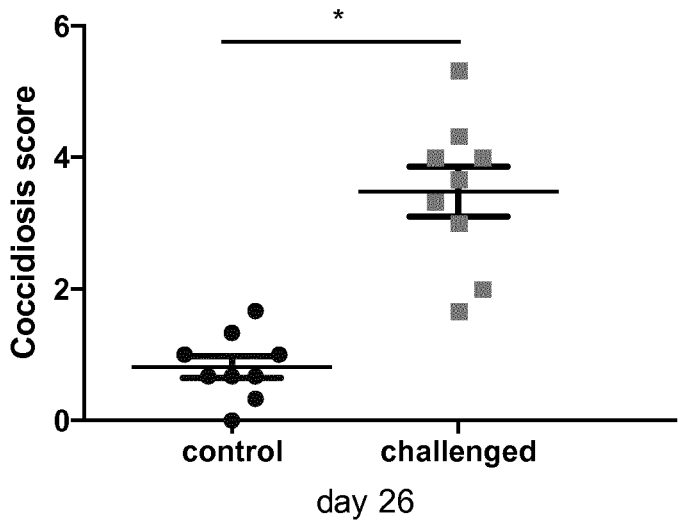


Fig 2a

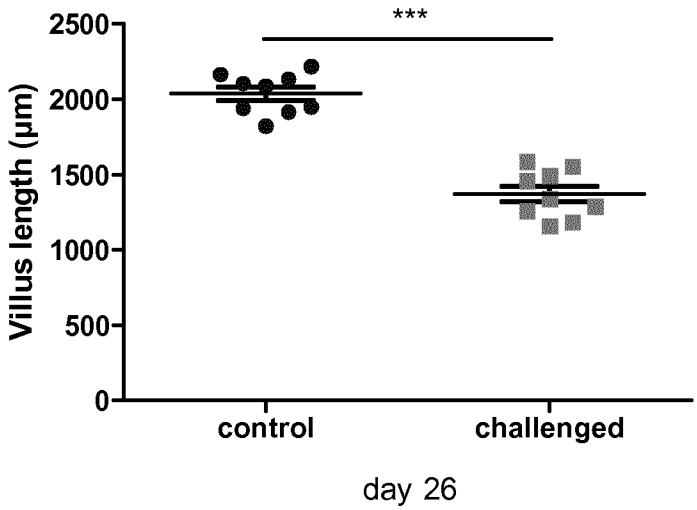

***
2500
2000
1500
1000
500
0
Villus length (µm)
control
challenged
day 26

Fig 2b

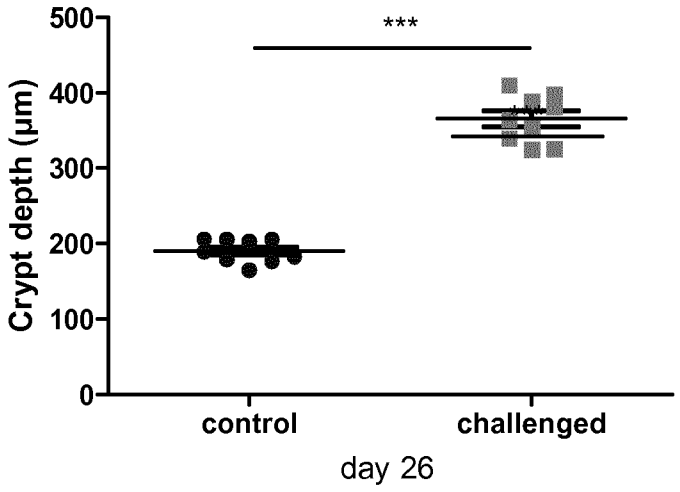

***
500
400
300
200
100
0
Crypt depth (µm)
control
challenged
day 26

Fig 2c

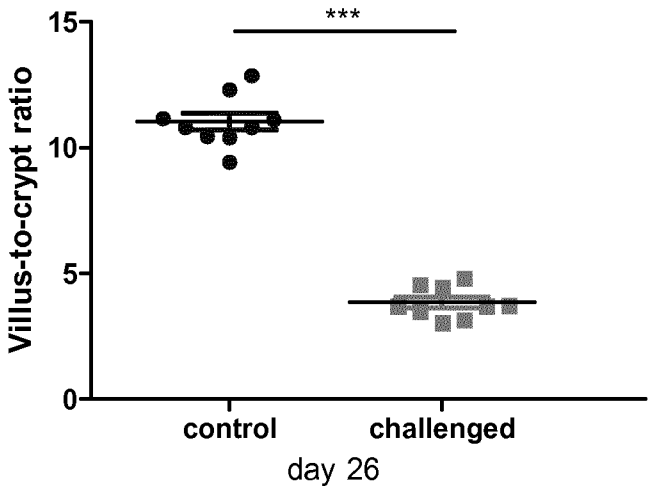
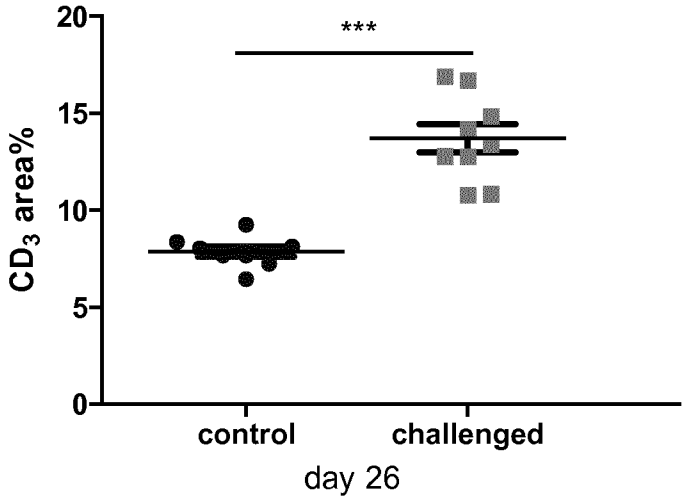


Fig 2d



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/058324

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	D A LIMA ET AL: "Faecal virome of healthy chickens reveals a large diversity of the eukaryote viral community, including novel circular ssDNA viruses", JOURNAL OF GENERAL VIROLOGY, vol. 98, no. 4, 1 April 2017 (2017-04-01), pages 690-703, XP055496137, DOI: 10.1099/jgv.0.000711 abstract, figure 1.	1-12

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

[X] See patent family annex.

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2019	05/06/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Weber, Peter

1

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J CHEN ET AL: "Identification of Potential Biomarkers for Gut Barrier Failure in Broiler Chickens", FRONTIERS IN VETERINARY SCIENCE, vol. 2, 26 May 2015 (2015-05-26), XP055465808, DOI: 10.3389/fvets.2015.00014 abstract, page 2, right-hand column, last paragraph - page 5, left-hand column.	1-12
A	WO 2017/066777 A1 (KEMIN IND INC [US]) 20 April 2017 (2017-04-20) abstract, paragraphs [0007]-[00010], [00032], [00037] and [00049], Table 5.	1-12
A	Theo Niewold: "Gut Health, Intestinal Innate Immunity and Performance", , 29 August 2014 (2014-08-29), pages 1-10, XP002783445, Retrieved from the Internet: URL:https://en.engormix.com/poultry-industry/articles/gut-health-intestinal-innate-t36261.htm [retrieved on 2018-07-27] the whole document, especially pages 4-6.	1-12
A,P	E GOOSSENS ET AL: "Elevated faecal ovotransferrin concentrations are indicative for intestinal barrier failure in broiler chickens", VETERINARY RESEARCH, vol. 49, no. 1, 20 June 2018 (2018-06-20), XP055496145, DOI: 10.1186/s13567-018-0548-4 the whole document	1-12
A,P	R DUCATELLE ET AL: "Biomarkers for monitoring intestinal health in poultry: present status and future perspectives", VETERINARY RESEARCH, vol. 49, no. 1, 8 May 2018 (2018-05-08), XP055586800, DOI: 10.1186/s13567-018-0538-6 the whole document	1-12

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/058324

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2017066777 A1	20-04-2017	US 2017106027 A1	20-04-2017
		WO 2017066777 A1	20-04-2017