v. 20 March 2024

Scientific Expert Panel

Evaluation of the impact of the maintenance of a proper gut structure on animal health and welfare

1. Introduction

This recommendation document is part of a set of recommendation documents developed by the IFIF Scientific Expert Panel and the IFIF Working Group (WG) on' Nutritional Innovation to Promote Animal Health'.

The IFIF WG was launched in 2017 with the objective to have '*animal nutrition solutions contributing to animal health and animal wellbeing* ***scientifically recognized, clearly understood,*** *and benefit from a proper regulatory* ***framework*** *to be valorized and implemented*'.

Nutritional solutions, now called nutritional strategies are aimed to support the development of animal adequate nutrition.

Adequate nutrition is defined as '*the oral intake of animals of adequate levels of nutrients, substances, microorganisms, and other feed constituents, considering their combination and presentation, necessary to fulfill functions related to their physiological states, including the expression of most normal behavior, and their resilience capabilities to cope with stressors of various type encountered in appropriate husbandry conditions.'* Furthermore, the way to achieve adequate nutrition is described as follows:

- *Optimization of feed composition, manufacturing, presentation, and delivery to animals,*
- *Minimization of the exposure of animals to stressors in feeds,*
- *Coverage of the animal's requirements for maintenance, activity, growth, production, and reproduction,*
- *Support of digestion and physiological functions, body systems, and behavioral expression.*

The purposes of these recommendation documents are to provide:

- The developers of nutritional strategies with information on the way to evaluate the effectiveness of their strategy for a given purpose.
- The evaluation bodies in the different jurisdictions with an approach for the evaluation of the effectiveness of nutritional strategies for a given purpose.

Each recommendation document will focus on a specific purpose, in relation with microbiome, gut function, exposure control, immunity, physiology, and others.

The present recommendation document is focusing on the evaluation of the role of nutrition to maintain a proper gut structure, related to animal health and welfare. A variety of strategies can be used to evaluate gut structure, from macroscopic and microscopic evaluation to determination of gut

Suggested Citation : van Immerseel F., Choct M., Smidt H., IFIF Working Group 'Nutritional Innovation', 2024. Evaluation of the impact of the maintenance of a proper gut structure on animal health and welfare, 7 pages

leakage markers that are administered orally and measured in the blood, to protein biomarkers related to villus structure, intestinal permeability, and inflammation. All have advantages and disadvantages. Most data are available for broiler chickens, and to a lesser extent piglets, but in theory these gut health evaluation methods can be species-independent, including calves.

2. Scope

The document relates to measurement of gut structure, and the components that drive gut structure, i.e., gut inflammation, leakage, and epithelial cell death. These all relate to gut structure, with villus morphology as the most important characteristic that associates with animal performance. The document thus relates to macroscopic evaluation of the appearance of the mucosal surface of the intestinal tract, microscopical evaluation of villus structure (e.g., villus length and crypt depth), and (bio-)markers associated with gut permeability and intestinal inflammation.

3. Descriptions of endpoints

Gut structure can be directly or indirectly evaluated using a variety of measures, with histology being the golden standard. Indirect measures include macroscopic evaluation of the mucosal surface and intestinal content, and the use of biomarkers that are associated with loss of (or maintenance of) gut structure. Below these are classified into 1) Macroscopic evaluation methods; 2) Microscopic evaluation methods; 3) Blood biomarkers and 4) Intestinal and faecal biomarkers.

- Macroscopic evaluation of gut structure

The macroscopic gut wall appearance can give information on the gut structure to some extent. In case of diseases that cause lesions in the intestinal tract, such as necrotic enteritis and coccidiosis in poultry, evaluation of the serosal or mucosal surface of the intestinal tract is an easy way to see gut structure losses, and lesion scoring systems are used (Williams and Andrews, 2001; Keyburn et al., 2013). High lesions scores have been associated with decreases in villus length, inflammation, and thus performance losses. Often however severe lesions are not observed but alterations in coloration of the mucosal surface (often hyperemia of blood vessels), appearance of the excreta (e.g. scoring systems for litter quality), and abnormalities in the gut wall as compared to the healthy gut can be used to evaluate the gut structure.. Alterations of the macroscopic appearance have been associated with poor villus structure and scoring systems have been used in the field (Teirlynck et al., 2011; Caekebeke et al., 2020; Ringenier et al., 2021; Dal Pont et al., 2021; Prentza et al., 2023). The major drawback of macroscopical evaluation methods is that they require euthanasia and should be carried out by experienced persons (often veterinarians) and are thus subjective in nature, as they depend on the experience of the evaluators. These methods are also not quantitative.

- Microscopic evaluation of gut structure

Gut structure can ideally be evaluated using histology and thus measurement of villus length, crypt depth, villus to crypt ratio or other structural and functional characteristics (thickness of the wall, inflammatory cell infiltration (e.g. $CD3^+$ T-cells, macrophages), detection of tight junctions, amongst others). While these characteristics have been associated with diseases and performance losses using infectious and non-infectious triggers in experimental models in poultry and pigs (Zhang et al., 2021; Zhang et al., 2023; Rysman et al., 2023b), only recently it was shown that villus length at a specific time point is associated with performance characteristics (of broiler flocks) in the field (Rysman et al., 2023a). The technique is considered to be the golden standard for determination of the gut structure

but requires time-consuming methodologies and euthanasia. Although measurements give quantitative values, multiple microscopic fields and animals per flock or herd need to be taken for a good estimate.

- Blood biomarkers

Blood biomarkers for gut structure can be classified in 2 categories, i.e., those that are derived from biological material (e.g., cells, bacteria) and those that are orally administered and in case of increased intestinal permeability, can pass to the blood. The latter are typically large molecular size carbohydrates, such as lactulose, mannitol or rhamnose, that are not digested in the intestinal tract, and of which their ratio in the blood can be used as a measure for intestinal permeability. Alternatives are FITC-dextran and iohexol, the first a large molecular size fluorescent molecule, the latter a radiographic molecule. Papers have shown that all systems can be used to detect intestinal damage in models using infectious and non-infectious triggers in poultry and pigs (Gilani et al., 2017; Zhang et al., 2021; Dal Pont et al., 2021; Mortensen et al., 2023; Rysman et al., 2023b). Application in the field is cumbersome because of the oral administration of the molecules and blood analysis, including specific expertise and equipment for quantification. Blood biomarkers from biological origin are microbial molecules and metabolites (e.g., LPS and D-lactate) and host cell proteins, originating from intestinal epithelial cells, such as intestinal fatty acid binding protein (He et al. 2021), or related to inflammation (e.g. alpha-1-acid glycoprotein, Serum Amyloid A, transaminase, and proinflammatory cytokines from IL-1 & 6 families) (Ceciliani et Lecchi, 2019; Broom, 2019). The inflammation-related proteins might not be specific for intestinal damage. Drawbacks for the microbial molecules are the likely differences in concentration in the intestinal tract between animals, because of microbiota composition differences. For all these biological blood markers, data on the use to evaluate gut structure in field samples are lacking.

- Intestinal and faecal biomarkers

Intestinal and fecal protein biomarkers related to gut structure have been quantified in experimental models, either by detection of specific proteins, or after proteomic analysis and untargeted detection of proteins with different abundance in diseased vs healthy animals (De Meyer et al., 2019; Barekatain et al., 2020). Examples are published but often only validated in experimental models. Ovotransferrin has been described as an intestinal biomarker that is increased under experimental coccidiosis and necrotic enteritis challenges in broilers and is associated with animal performance in field samples (Goossens et al., 2018; Rysman et al., 2023c). Ovotransferrin is an acute phase protein produced in the liver, so used as intestinal permeability marker as it measures leakage from blood to intestinal content. Calprotectin and lipocalin-2, inflammation-related proteins, have been described as intestinal biomarkers in non-starch polysaccharide induced challenge models in broilers and in porcine colitis (Barbosa et al., 2021; Dal Pont et al., 2021; Song et al., 2022). Other examples have been proposed in the literature but mostly by extrapolation from rodent studies. Also, quantitative analysis of gene transcripts in gut tissue has been used, quantifying tight junction markers or cytokines, indirectly assessing gut structure, in poultry and pigs (examples: Rychlik et al., 2014; Fasina et al., 2019; Fries-Craft et al., 2021; Zhang et al., 2021; Dal Pont et al., 2023; Zhang et al., 2023).

Interpretation of the endpoints

It is difficult to set thresholds and cut-off values for the above-mentioned endpoints that would be linked to health-related effects in practice. It is therefore recommended to compare the scores and quantitative values obtained with the nutritional strategy used to improve gut health, with a baseline on the farm, in which the nutritional strategy is tested (or in experimental studies in which a treatment group is compared to a control). The baseline shall correspond to practical conditions on farms. Ideally, follow-up is done longitudinally at specific time points of each production round. As no systems are commercially available and no big datasets exist until now showing values over many farms worldwide, it is not yet relevant to compare farms with each other. In general, a decrease in macroscopic gut evaluation score, high villus to crypt ratio, and a low concentration of intestinal permeability and leakage markers, is considered favorable to maintaining a good health status of the animals. It should be noted that specific dietary additives, such as immunomodulating substances, might act more subtle and promote gut health without affecting gut structure directly.

4. Parameters for the evaluation of the endpoints

Parameters for the evaluation of the endpoints vary according to the type of method used for gut structure evaluation. For the macroscopic evaluation of the appearance of the gut wall, typically scoring systems are used based on presence or absence of specific characteristics, leading to a final total macroscopic gut health score. For microscopical analysis, typically quantitative values in µm for villus length and crypt depth are the measured parameters, or the villus length to crypt depth ratio. Other characteristics are sometimes also measured, including gut length intestinal wall thickness or immune cell infiltration (sometimes as counts per surface, or percentage of covered area). For measurement of the blood and intestinal/fecal biomarkers, absolute concentrations are measured based on a standard curve. Often thresholds are lacking, as these are continuous variables, and as such increases or decreases relative to control conditions are used, mostly to compare treatment groups in experimental settings.

5. Methods to measure the parameters.

For macroscopical evaluation of the gut structure, experienced and trained persons are required that were educated in lesion and gut health scorings. Systems are published and use a set of macroscopic parameters that are scored (Teirlynck et al., 2011; Dal Pont et al., 2021). Microscopical analysis requires histopathology, including methods for fixation and paraffin embedding of intestinal segments, cutting sections, and staining techniques, such as hematoxylin/eosin stains (Zhang et al., 2021, Rysman et al., 2023a). Also, immunohistochemical antibody-based detection techniques are required when specific cell populations (e.g., specific types of immune cells, e.g. $CD3^+$ T-cells, macrophages) need to be visualized (Rysman et al., 2023a). For analysis of gut morphometry and quantification of immune cell infiltrates, microscopes connected to image-based analysis software need to be available. For blood biomarkers chromatographic and spectrophotometric (FITC-dextran) methods are to be used (Gilani et al., 2017; Zhang et al., 2021; Dal Pont et al., 2021; Rysman et al., 2023b). As an example, ultra-high performance liquid chromatography–tandem mass spectrometry **(**UHPLC-MS/MS) has been used to quantify iohexol (Rysman et al., 2023b). Intestinal and faecal biomarkers of proteinaceous nature can be quantified using antibody-based methods, such as ELISA (Goossens et al., 2018). Gene transcripts in gut tissue can be quantified using QPCR. A major point is the site of sampling (intestinal segment), sample collection, preservation, and handling but this applies to all systems described above. While fecal sampling for protein markers is noninvasive, these might be instable, and intestinal sampling might be needed. This needs to be determined for each marker individually.

6. Conclusions

There is an obvious association between some of the above-mentioned parameters and gut structure, especially morphometric analytical parameters (villus length and inflammatory status). Also, macroscopical evaluation of the mucosal surface of the gut and blood and intestinal biomarkers have merit, as some of these associate with villus length and even with animal performance parameters. Although the association between some of the parameters and the gut structure is clear, it will be difficult to compare different flocks/farms with each other, as many factors (e.g., diet composition, microbiota) play a role in the basal levels of the quantified variables. Despite this, the endpoints can be used when longitudinal follow-up in a cohort of animals is done, such as for example is the case with farm-specific interventions or changes in diets or application of dietary additives.

7. Abbreviations

ELISA: Enzyme-linked Immuno-Sorbent Assay; FITC: Fluorescein isothiocyanate; QPCR: quantitative Polymerase Chain Reaction ; UHPLC-MS/MS : Ultra High Performance Liquid Chromatography / Mass Spectrometry

8. References

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9. Glossary of Terms

Endpoints: The measurable impact of a nutritional strategy on the animal, its physiology, or its microbiome.

Health: The state of normally functioning animal, especially the state of being sound, free from physical disease, pain or (symptom of) stress.

Welfare: The physical and mental state of an animal in relation to the conditions in which it lives and dies.