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Scientific Expert Panel

Evaluation of the change in fermentation and metabolites profile on animal health and welfare

1. Introduction

This document is part of a set of documents developed by the IFIF with the support of the IFIF Scientific Expert Panel and adopted by 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 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 document will provide recommendations with a focus on a specific purpose, in relation with microbiome, gut function, exposure control, immunity, physiology, and others. The information provided in the document does not depend on a particular nutritional strategy and may be used to evaluate any nutritional strategy having an impact on the gastrointestinal fermentation patterns and metabolites.

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The present document is focusing on the evaluation in the change of carbohydrate fermentation metabolites (especially the concentration of short chain fatty acids (SCFA) in the gastrointestinal (GI) tract) and of fermentation metabolites (especially with regards to the concentration of ammonia and hydrogen sulphide in the GI tract).

2. Scope

The document relates to microbial fermentation end-products in the GI tract, with non-digested materials as substrates. Microbial fermentation end-products of non-digested carbohydrates to which the current guideline is related are SCFA, more particularly acetic, propionic, and butyric acid, and combinations and ratios thereof. Microbial fermentation end-products of non-digested protein to which the guidelines are related to are ammonia and hydrogen sulphide.

3. Descriptions of endpoints

Dietary substrates can either be digested by the host and the resulting nutrients thus absorbed by the intestinal epithelium or escape enzymatic digestion but be fermented by the microbiota in the hindgut of the animals (See IFIF document on impact of nutrient digestibility on pigs and poultry health and welfare).

Short-chain fatty acids (SCFAs)

Food carbohydrates can be digested by the host (starch and simple sugars), or reach distal intestinal compartments for microbial fermentation, i.e., dietary fibre reaching distal intestinal compartments and consisting of non-starch polysaccharides (e.g., pectin, cellulose, hemicellulose) and other non-digestible carbohydrates (such as resistant starch, fructans, and oligosaccharides). The fermentation of substrates depends on the diet and microbiota composition. Metabolic pathways, that cause SCFA production are multiple and may require a cascade of reactions carried out by a microbial community, and is highly complex (Koh et al., 2016). The end products are mainly acetic, propionic, and butyric acid (apart from lactic acid).

The physiological role of carbohydrate fermentation products is well described in the literature. Acetic and propionic acid are absorbed through the distal intestinal barrier and transported to the liver through the portal vein, while butyric acid is oxidized by the distal intestinal barrier epithelial cells. Acetic acid is used in lipid metabolism by muscles, while propionic acid is used in gluconeogenesis in the liver. Butyric acid has many health-related effects, either after binding to cell receptors and activation of intracellular signaling cascades, or after cellular uptake through transporters followed by butyric acid oxidation in the cells. The health-related effects include anti-inflammatory effects, epithelial barrier strengthening through effects on tight junctions, trophic effects on epithelial cells, regulation of cytokine expression, amongst others. There are numerous scientific papers that describe the effects of propionic and butyric acid on animal health and performance, both in poultry and pigs (Onrust et al., 2015; Liu et al., 2020, Dengler et al., 2021; Gomez-Osorio et al., 2021; Liu et al., 2021). In addition, the effects of feed materials and additives that promote propionic and butyric acid production in the gut, on health and performance, are published. It has been documented that butyrate producing microbiota are a key population in gut homeostasis (De Maesschalck et al., 2015).

In that regard, not only numerical increases in concentrations of these SCFAs (in mM for example) but also ratios of propionic acid and/or butyric acid relative to the total SCFA concentration can be relevant (e.g., % of butyric acid relative to total SCFAs). Molar ratios in normal conditions are in the following

range: approximately 70-80% for acetic acid vs 5 to 15% for butyric and propionic acid, mostly the lowest for propionic acid (Onrust et al., 2020; Ruckman et al., 2020; Kumar et al., 2022). To measure the concentration and molar ratios, the site for collecting samples ideally is distal intestinal tract content, which is main site of fermentation, but fecal material may also be used if taken soon after defecation to avoid the loss linked to the volatility of SCFAs. It is not possible to discriminate between endogenously produced butyrate and diet-supplemented butyrate, but it has been shown that dietary butyrate supplementation affects the microbiota composition, reducing opportunistic pathogens and increasing SCFA producing bacterial families (Onrust et al., 2020).

Ammonia and hydrogen sulphide

Food proteins are normally broken down to polypeptides in the stomach (low pH, pepsin activity), then further to peptides by pancreatic enzymes (trypsin, chymotrypsin, carboxypeptidases). These peptides can be further broken down by brush border enzymes (aminopeptidases, carboxypeptidases) to dipeptides or amino acids that can be taken up by epithelial cells. This process is highly efficient. Residual proteins that are not digested proceed to the distal intestinal tract where they can act as a substrate for microbial fermentation, yielding a variety of end products (Davila et al., 2013). Deamination of amino acids and ureolysis can yield ammonia. Hydrogen sulphide is produced from inorganic sulphate and from S-containing amino acids (e.g., cysteine, methionine) (Carbonero et al., 2012). The factors that drive ammonia and hydrogen sulphide production in the gut are thus diet constituents and the microbiota composition.

The physiological role of protein fermentation end products is concentration-dependent and often derived from *in vitro* studies. While ammonia has been associated with air quality problems (emissions, but also conjunctivitis and respiratory problems), high ammonia concentrations have also been shown to be associated with dose-dependent inhibition of mitochondrial respiration and inhibition of oxidation of SCFAs in epithelial cells (Cremin et al., 2003; Andriamihaja et al., 2010; Gilbert et al., 2018). Hydrogen sulphide has also been linked with serious air quality problems as well as with a concentration-dependent inhibition of mitochondrial respiration and increase in inflammation in the gut. Concentrations that have been described in *in vitro* studies to have these harmful effects are starting from 0,1 mM but data are scarce (Babidge et al., 1998; Leschelle et al., 2005; Beaumont et al., 2016; Gilbert et al., 2018). Hydrogen sulphide producing bacteria have a known role in maintaining inflammation in the gut (Dordevic et al., 2020).

Both ammonia and hydrogen sulphide can thus be toxic at higher concentrations, and the measured range is between 0 and 2.5mM. To measure the concentration of ammonia and/or hydrogen sulphide, the sample ideally is distal intestinal tract content (i.e., the site of fermentation), but might be fecal material, if taken soon after defecation (due to the volatile nature of these gases).

Interpretation of the endpoints

It is not possible to define definitive values for the above-mentioned endpoints that would be linked to health-related effects in all the cases in practice. It is therefore recommended to compare the concentrations of SCFAs, ammonia and hydrogen sulphide obtained with the nutritional strategy to a baseline on the farm in which the nutritional strategy is tested. The baseline shall correspond to practical conditions on farms.

In general, an increase in the concentration and molar ratio of butyric acid is considered favorable to maintaining a good health status of the animals, while a decrease of the concentration of ammonia and hydrogen sulphide is considered favorable for maintaining a good health status of the animals.

4. Parameters for the evaluation of the endpoints

Parameters for endpoint evaluation are either concentrations of individual or multiple fermentation end products, ratios of fermentation end products (e.g., butyric, and propionic acids vs acetic acid), or relative concentrations (e.g., butyric acid vs total SCFAs, or % butyric acid in total SCFAs).

5. Methods to measure the parameters

The quantitative determination of SCFAs in intestinal and faecal samples or faecal cultures can be done using multiple chromatographical methods coupled to a detection system (listed in Eberhardt et al., 2021). An example is gas chromatography (GC) in combination with flame-ionization detection or mass spectrometric detection. Also, high-performance liquid chromatography (HPLC) is commonly used, for example combined with a UV-detector (HPLC-UV) or coupled to mass spectrometry (HPLC-MS) (De Baere et al., 2013). Ultra-high-performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS) has also been used in the literature (Chen et al., 2021). Extraction methods, chromatographic columns, derivatization steps, chromatographic conditions need to be evaluated carefully to ensure repeatability, and standards need to be used.

Potential limitations of the measurements are related to the volatile nature of the compounds that are measured, and as such samples taken should be accompanied with methods to preserve or stabilize the volatiles. Also, there might be a difference in volatility between compounds, again reinforcing the relevance of proper and fast sampling and handling of samples. Another limitation is the need for experienced persons and an equipped laboratory, as the methods are requiring expert skills.

6. Conclusions

There is a clear link between the end products of fermentation and the substrates remaining undigested in the hindgut of monogastric animals. Fermentation characteristics indicate the potential health and welfare condition of the animal. Although the association between fermentation end products and health 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 fermentation end products. 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. Relative concentrations of the fermentation end products are more important than absolute concentrations of the individual molecules.

7. Abbreviations

GC: gas chromatography

GI: gastro-intestinal

MS: mass spectrometry

HPLC: high-performance liquid chromatography

UPLC-HRMS: Ultra high-performance liquid chromatography-high resolution mass spectrometry

UV: ultraviolet

SCFA: short-chain fatty acids

mM: millimolar

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.