Review

Morphological Assessment and Biomarkers of Low-Grade, Chronic Intestinal Inflammation in Production Animals

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Simple Summary: Production animals are continuously exposed to environmental and dietary factors that might induce a state of low-grade, chronic intestinal inflammation. This condition compromises the productive performance and well-fare of these animals, requiring studies to understand what causes it and to develop control strategies. An intestinal inflammatory process is generally associated with alterations in the structure and functionality of its wall, resulting in the release of cellular components into the blood and/or feces. These components can act as biomarkers, i.e., they are measured to identify and quantify an inflammatory process without requiring invasive methods. In this review we discuss the mechanisms of low-grade inflammation, its effects on animal production and sustainability, and the identification of biomarkers that could provide early diagnosis of this process and support studies of useful interventional strategies.

Abstract: The complex interaction between the intestinal mucosa, the gut microbiota, and the diet balances the host physiological homeostasis and is fundamental for the maximal genetic potential of production animals. However, factors such as chemical and physical characteristics of the diet and/or environmental stressors can continuously affect this balance, potentially inducing a state of chronic low-grade inflammation in the gut, where inflammatory parameters are present and demanding energy, but not in enough intensity to provoke clinical manifestations. It's vital to expand the understanding of inflammation dynamics and of how they compromise the function activity and microscopic morphology of the intestinal mucosa. These morphometric alterations are associated with the release of structural and functional cellular components into the feces and the blood stream creating measurable biomarkers to track this condition. Moreover, the identification of novel, immunometabolic biomarkers can provide dynamic and predictors of low-grade chronic inflammation, but also provide indicators of successful nutritional or feed additive intervention strategies. The objective of this paper is to review the mechanisms of low-grade inflammation, its effects on animal production and sustainability, and the biomarkers that could provide early diagnosis of this process and support studies of useful interventional strategies.

Keywords: low-grade inflammation; intestinal biomarkers; immunometabolism; gut integrity

1. Introduction

The gastrointestinal tract (GIT) is of great importance in animal production, not simply because of it is role in digestion and absorption of nutrients, but also due to its immunometabolic and neuroendocrinologic functions in regulating animal physiology [1–4]. The intestine of all animals is a complex, but hollow tube in which the external environment within the lumen is separated from the inside of the animal by a thin, yet remarkably efficient barrier system. The lumen side of the intestine harbors a diverse community of commensal microbes that have co-evolved with the host immune system which is predominately located in the internal side of the intestine. These microbes, tolerated and

anatomically contained by the mucosal immune system, execute functions that are critical for host physiology.

The immune system contributes to the intestinal homeostasis by diverse ways. Besides defending the organism against potential pathogens, the immune system prevents organisms of the microbiota from reaching the intestinal epithelium or deeper tissues and develops a state of tolerance to the commensal microorganisms of the lumen [5]. This constant vigilance of the microbiota involves physical, biochemical, and immunological barriers of the intestinal mucosa [5]. During intestinal homeostasis, the microbiota and the immune system operates in symbiosis through a bi-directional relationship, where both characters influence one another's development, composition, and functionality [6]. The tolerance required to maintain this state of homeostasis involves the activation of pattern recognition receptors (PRRs) present in certain cells of the innate immune system. These receptors are activated by their continuous interaction with structural and functional elements from the gut microbial community, including lipopolysaccharides (LPS) and peptidoglycans (PGNs) from the bacterial cell walls and other microbial-associated molecular patterns (MAMPs). Cells containing these receptors present PRR-mediated signaling pathways that, when activated, regulates the responsiveness of PRRs, induce the production of high levels of regulatory proteins, such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), and induce the differentiation of the T-regulatory subpopulation of T lymphocytes [5,7].

The 'intestinal ménage trois', composed by the gut microbiota, mucosal immune system, and diet, represent a multidirectional, chemical, and biological interactive complex. These elements work together, influencing each other at the cellular and molecular level to coordinate an intestinal immunometabolic homeostasis [8] that is translated as a 'healthy' gut. The nutritional components and physical form of an animals' diet, as well as its presence or not (e.g., starving, increased feed intakes and nutrient excesses) on the intestinal lumen have profound effects not only on gut health, but also on the total energy homeostasis and overall health of the animal [9,10]. In the context of this review, the microbiota transduces dietary nutrients into signals recognized by the PRRs of the innate immune system to promote or inhibit inflammation [10]. This process is mediated by the metabolic reprogramming of immune cells, merging immunological and metabolic processes into a process called immunometabolism and that is intimately associated with the gut and organismal health [11–15].

Historically, metabolism and inflammation have been viewed as two separate physiological processes with distinct but essential roles in animal biology: metabolism regulates nutrient uptake and usage while inflammation is a protective immune response involved in host defense and repair. Both systems react to a host's environmental stressors to restore homeostasis. The interplay between metabolic status and immune response (immunometabolism) plays an important role in maintaining homeostasis, understanding disease pathogenesis, and developing novel disease interventions in the context of the metabolic regulation of immune functionality.

Recognizing the immunometabolic pathways and their morphological, cellular, and biochemical components will help to identify, characterize, and standardize specific biomarkers that are accurate indicators of normal or pathogenic processes in production animals.

2. Intestinal Inflammation: What Is It?

The intestinal inflammation participates in dysbiosis in poultry and swine [16,17], but more studies are necessaire for us to comprehend if the inflammation is the cause or the effect of this condition [18–21]. However, both acute and chronic inflammation are known to cause dysbiosis, irrespective if the process derives from infection or not ("sterile inflammation") [17]. The disruption of GIT homeostasis can jeopardize animals' health thus affecting performance and profitability. Along with the complexity of the organ, several external factors affect the intestinal homeostatic status and stimulates inflammation. These

factors include thermal and behavioral stress, nutritional challenges, animal density and pathogen exposure [18,22,23].

Inflammation is a normal and highly regulated physiological reaction that aims to recover and repair a harmed tissue. In response to the detection of infectious processes and damage to host structures, cells of the innate immunity produce signaling molecules that activate a network of immunological and physiological events for the purpose of restoring the homeostasis and functionality [24–26]. Varied factors can trigger an inflammatory response, shaping its phenotype, efficacy, duration, and consequences. The physiological inflammation, one of the possible phenotypes, is the balance between tolerance to the microbiota and reactivity to pathogens, as we discuss in our introduction to this review. The pathologic inflammation is usually an acute process that occurs in response to toxins and infection, commonly leading to collateral damage to the inflamed site and its surrounding, as well as to increased metabolic energy use. The metabolic inflammation is a chronic low-grade response induced by the excessive nutrient intake, where the consequent metabolic surplus activates the same signaling pathways that operate in immune responses to pathogens. (vi) Sterile inflammation, also a chronic low-grade inflammatory process, occurs in the absence of infection as a response to chemical, physical, and metabolic stimuli [24].

The physiological inflammation is an essential component of the intestinal homeostasis, but other inflammatory phenotypes must be limited due to their impact on the intestinal functionality and their energetic cost. Animals in the modern production industry are not only threatened by microbiologic challenges, but also by non-infectious factors that can lead to low-graded inflammation, such as increased feed intake/nutrient excess and components in ingredients of the diet that can activate receptors of the immune system. However, the impact of these nutritional triggers can be minimized by thoughtful dietary strategies. These practices include the use of enzymes (e.g., mannanase, proteases, xylanases) to reduce undigestible dietary components (e.g., mannans and other non-starch polysaccharides from the cell wall of vegetable ingredients) that may be recognized by PRRs or be fermented by the microbial community, resulting in the production of pro-inflammatory metabolites.

3. Inflammation Impact on Animal Production

Although not constituting a clinical disease, an excessive or chronically activated inflammatory response contributes to loss of tissue function and waste of metabolic energy, leading to poor performance and decreased animal welfare [25]. Gut inflammation promotes drastic alterations on intestinal architecture, resulting in increased intestinal permeability [27–29], decreased absorption and digestibility of nutrient, occurrence of fluid loss and diarrhea [23,28,29]. The increase in the permeability of the intestinal mucosa, also referred as "leaky gut", leads to translocation of gut bacteria, microbial compounds, and/or antigens to the blood stream, generating a systemic immune response [23,27–30].

Besides diverting nutrients and energy from functions that are essential to the animal productivity, the inflammatory process can also induce tissue catabolism [31–33]. As an example, proteins in the skeletal muscles undergo proteolysis and protein synthesis is limited to increase the availability of amino acids to fuel the inflammatory responses [34]. Lastly, the metabolism of lipids and sugars stores are increased to produce more energy for the immune system [31].

4. Chronic Inflammation

In his recent essay, Medzhitov [35] stated that "because infection and injury-induced inflammation are the most prominent and most studied forms of the response, we may have skewed our understanding of inflammation according to these extreme conditions". In commercial animal production, this reductionist view of inflammation and the use of antibiotic growth promoters (AGPs) in animal feeds limited our understanding of the complete physiological roles of inflammation in maintaining and monitoring tissue homeostasis [24,25]. Specifically, the removal of AGPs from animal feeds has provided evidence that the failure to remove the inflammatory trigger results in a chronic and low-

grade sterile inflammation. As mentioned above, this basal inflammatory response impairs the ability of the animal to reach 100% of its genetic potential given that nutrients are diverted away from growth to support the inflammatory response.

Currently, commercial swine and broiler operations are regularly exposed to several non-infectious, environmental factors that can act as inflammatory triggers, including animal density, poor quality of feed ingredients, nutrient dense diets (high protein or high non-starch polysaccharides), changes in feed formulation, mycotoxins, diets with high amounts of antinutritional factors, and heat stress. The exposure to any of these triggers for an extended period (days to weeks) can lead to the above-mentioned chronic intestinal inflammatory state, resulting in a disrupted digestive function, increased oxidative stress, microbiota dysbiosis, loss of barrier functionality, and immune dysfunctions, as we described in our study on the development of models to induce low-graded intestinal inflammation [36]. Additionally, some swine and broiler producers have relied on antibiotic growth promoters AGPs to compensate for potential poor husbandry and management issues. Therefore, AGPs were never truly used to control enteric infections, as stated by Dal Pont et al. [36] given that intestinal infections predominately promote acute but not chronic inflammation.

5. Morphometric Assessment of Gut Health

Morphometric analysis is one of the possible methods to evaluate gut health in animal production and it becomes even more important during the occurrence of low-grade chronic inflammation, where lesions in the intestine are not visible to the naked eye. Dal Pont and colleagues clearly demonstrated this in their development of a diet-triggered low-grade, chronic inflammation model in broiler chickens, where no macroscopic lesions were observed throughout the 35-days growth cycle [36]. A robust characterization of the microscopic processes constituting this continuous basal inflammation becomes essential, as long as we understand that its negative impacts on animal performance starts long before macroscopic alterations are observed, if at all.

For many years, the microscopic analysis of gut health has been based on the measurement of intestinal villus height and crypt depth [37]. Briefly, the villi function as absorptive structures and their height has been indicative of the absorptive area for nutrients. Whereas, the crypts are the primary sites of epithelial progenitor cells, with their depth expressing the turnover rate of the intestinal epithelium [38]. Thus, a higher villus enhances the absorptive capacity of the lumen and deeper crypts indicate a rapid epithelial cell turnover because various types of special cells are present in the crypt, including absorptive, secretory, and regenerative cells [38]. Although these two morphological features may provide an indication of structural alterations in the intestines, they provide no real direct functional evaluations of them. In fact, the recognition of MAMPS and damage-associated molecular patterns (DAMPS) by PRRs releases a cascade of chemical mediators that will alter the morphology of the intestinal mucosa. Consequently, several microscopic alterations may be observed during this process, including increases in lamina propria thickness, epithelial thickness, enterocytes proliferation, inflammatory cell infiltration in the epithelium, inflammatory cell infiltration in the lamina propria, goblet cells proliferation, and congestion of villus by blood cells and could increase the surface area of the villi that is inverse to its functionality.

In broilers, the histological alterations that succeed the macroscopic lesions lead to the development of the I See Inside (ISI) methodology [39–43]. This tool has gained attention by translating microscopic changes in numbers that can be correlated to zootechnical performance and by characterizing the health and functionality of the intestinal mucosa based on specific parameters that are compromised under inflammation. Moreover, the ISI methodology has supported the characterization of sub-clinical inflammatory processes that might not cause gross lesions in all upper intestine and cecum. In studies applying the methodology, broilers fed with low levels of aflatoxin (50 to 250 ppb) in experimental conditions did not present any macroscopic lesions in targeted organs, although histopathological changes at the same ages were observed [39,44,45]. Applying a model of enteritis

elicited by *Clostridium perfringens* and *Eimeria* spp., Sanches et al. [43] did not observe gross lesions in the challenged broilers, but microscopic alterations were indeed verified in the intestinal mucosa these animals. Interestingly, the unchallenged birds presented a similar (but statistically lower) pattern in the evolution of the inflammatory parameters when compared to the infected group. This finding suggested that the experimental challenge just worsened an already ongoing low-grade inflammation in the gut mucosa, which could be linked to dietary, management, and/or environmental factors, as cited above. In fact, the ISI methodology allowed the researchers to differentially quantify the histological inflammatory lesions between groups and to statistically correlate them with zootechnical performance.

Correlations between the villus width and the ISI scores (data not published) suggest that the inflammatory process (expressed through the ISI methodology) turns the villus larger, although the expanded surface of these structures does not mean a better absorptive capacity. In fact, villi can present very close heights but with different levels of internal alterations related to inflammation (Figure 1). In broilers, the parameters measured by the ISI methodology have better characterized the losses of functionality in the whole intestine. The chronic inflammation characterized by the ISI scores at initial ages was statistically correlated with lower zootechnical performance at later periods [41].
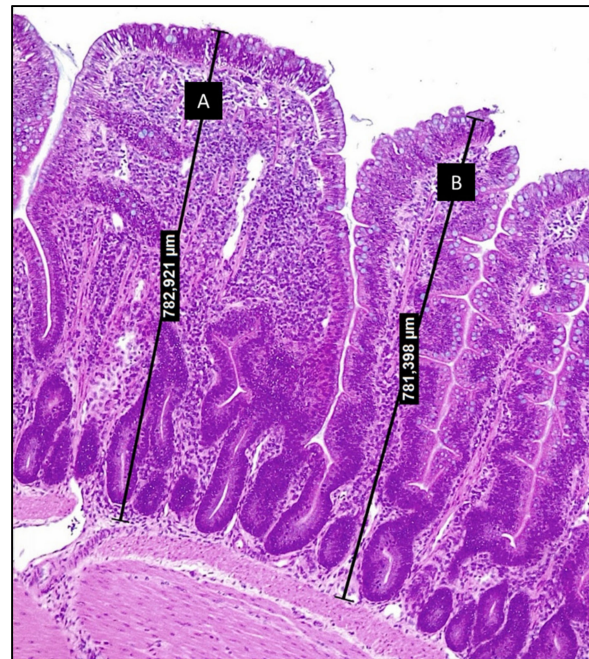

Figure 1. Photomicrography of chicken ileum section stained with hematoxylin and eosin (200×) (from authors archive). Villi A and B display very close heights, although villus A presents increased lamina propria thickness and increased infiltration by inflammatory cells. These alterations are comprised by the ISI methodology and allow to relate structural alterations to the loss of functionality.

Two novel models to induce low-grade gut inflammation were applied by Dal Pont et al. [36] in broilers chickens with the ISI methodology as a "gold test" of microscopic villi changes. The increased ISI scores of histologic alterations in the duodenal, jejunal, and ileal mucosa were joined by a significant increase in the leak of calprotectin and lipocalin through the feces, important inflammatory biomarkers. In fact, the ISI methodology was able to prove the occurrence of a low-grade inflammation and demonstrated that the chicken intestinal inflammation evolves in a spatial and temporal pattern through the small intestine.

6. Structural and Inflammatory Biomarkers

Production animal industries require reliable non-invasive biomarkers of gut health, inflammation, and disease. Two recent reviews have documented a number of potential biomarkers in poultry [3,46]. However, most of the potential biomarkers of animal gut health been inconsistent in documenting a healthy or diseased intestine. Here, we outline a number of potential novel biomarkers that have recently been identified as viable tools to evaluate production animal gut health, but probably will require more samples under different conditions to validate their value to the production animal industry. In addition to the descriptions, Figure 2 illustrates how these components reach the blood and/or feces in a state of inflammation.
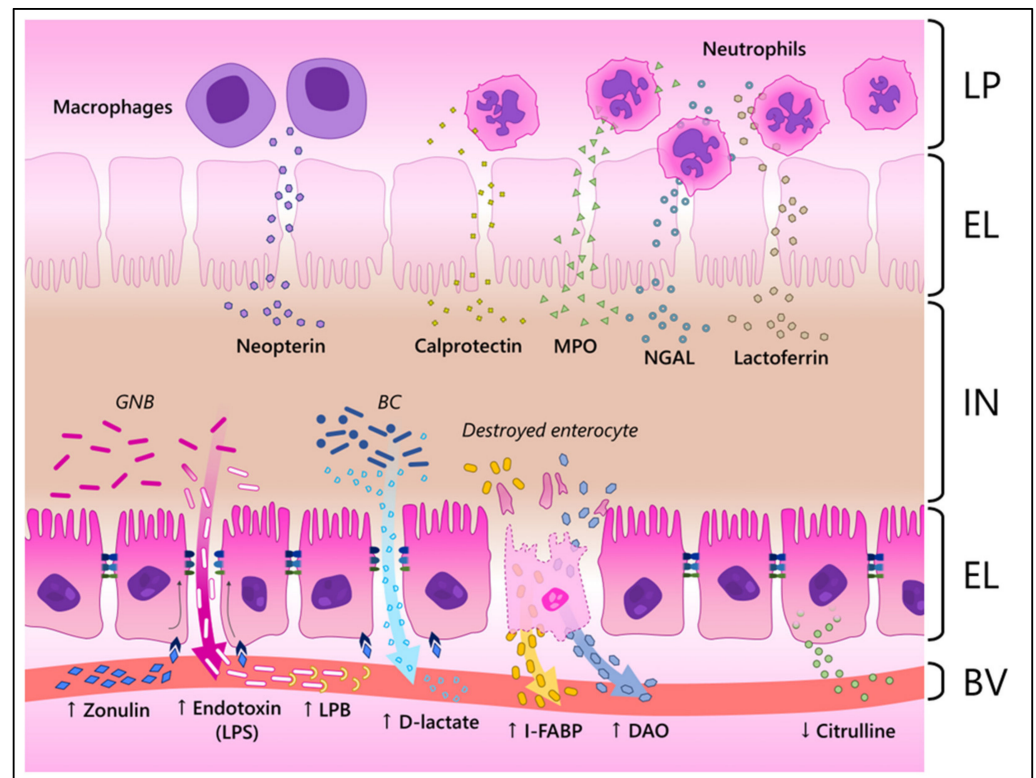

Figure 2. Illustration of how different biomarkers reach the blood and/or the feces during inflammation in the intestinal mucosa. LP, lamina propria; EL, epithelial layer; IN, intestinal lumen; BV, blood vessel; GNB, Gram-negative bacteria; BC, bacterial community; MPO, myeloperoxidase; NGAL, neutrophil-gelatinase associated lipocalin; LPS, lipopolysaccharide; LPB, LPS-binding protein; I-FABP, intestinal fatty acid binding protein; DAO, diamine oxidase. ↑, increased; ↓, decreased levels during inflammation. In the illustration, zonulin interacts with its receptors in enterocytes, changing the structure of the tight junctions and, consequently, increasing the paracellular influx. This increased permeability allows bacterial components and metabolites, such as endotoxins and D-lactate, to cross the epithelial barrier and reach the blood stream. Destroyed enterocytes resultant of the inflammatory process release their internal components, such as I-FABP and DAO, in the circulatory system. Healthy enterocytes produce citrulline and release it into the blood stream. Therefore, its levels are reduced when enterocytes are damaged. Neutrophils and macrophages that reach the mucosa release antimicrobial components such as neopterin, calprotectin, MPO, NGAL and lactoferrin, which reach the IN. It must be noted that the described biomarkers might reach both the IN and BV.

We should point out that all the potential biomarkers reviewed herein (particularly the metabolic markers discussed in the next topic) are projected as functional quantitative measurements of intestinal inflammation and are not disease specific. Fecal biomarkers would be more definitive of intestinal inflammation, whereas plasma biomarkers would be indicators of more systemic conditions. Overall, these biomarkers can be predictive and

have the potential to serve as a diagnostic screening tests. Each biomarker will require the development of a functional range to determine a 'threshold' level that is an indicator of chronic intestinal inflammation. The main applications of these tests would be to rapidly differentiate healthy animals from diseased.

Lipopolysaccharide-binding protein (LPB) is produced by hepatocytes and released to the blood stream as part of the acute phase response to LPS, upregulated by IL-1, IL-6, and tumor necrosis factor α [47]. While a N-terminal domain of LPB binds to the LPS lipid A with high affinity, its C-terminal continues the endotoxin processing by delivering it to (i) CD14 proteins, thus forming a LPS-CD14 complex that is recognized by Toll-like receptors 4 and stimulates the immune response, or (ii) to circulating lipoproteins, which take them inside hepatocytes for metabolizing and excretion on the bile, where it's deactivated by bile salts [48–52]. Plasmatic/serum level of LPB have been an indirect biomarker of endotoxin leakage from the gut, applied to track factors that interact with the gut inflammation and integrity [53,54] as infections [55,56], heat or/and feed restriction stress [57–64], diet, and bacterial gut composition [65–70].

Calprotectin is a calcium-bound protein complex expressed and released by neutrophils in inflamed sites, but also by monocytes, activated macrophages, and dendritic cells [71–74]. As part of the innate immune response, this protein sequestrates zinc and manganese thus disrupting bacterial and fungal metabolic pathways and exposing these microorganisms to stress [75–77]. Other calprotectin functions include chemotaxis and endothelial adhesion of neutrophils, and the upregulation of certain cytokines, as IL-1β, IL-6 and TNF-α [73]. Fecal and circulating levels of calprotectin have been used as a biomarker to track the gut inflammatory activity in human [78–83] and canine [74,84–86] patients with chronic enteropathies. In swine, the biomarker has been applied to evaluate the intestinal disruption by pathogenic enterobacteria and their toxins [87–92], but also to evaluate the positive effects of dietary supplementations on the intestinal inflammation and integrity [92–94]. Studies in human health show that the blood levels of calprotectin increase in response to diverse conditions involving tissue damage and inflammation, not exclusively in the intestine [72,95]. Consequently, measuring the calprotectin levels in the feces should provide safer inferences between the biomarker and an intestinal inflammatory process [96].

Lipocalin-2 or Neutrophil-gelatinase associated lipocalin (NGAL) is a glycoprotein highly abundant in the cytoplasm of neutrophils, where it is stored in granules. The NGAL antibacterial activity relies on binding to bacterial siderophores, thus preventing microorganisms from capturing necessary iron for their metabolism [97–99]. While increased NGAL levels in the blood and urine have been associated with acute kidney injury [100,101], the upregulation of NGAL expression during intestinal injury and inflammation [102,103] has made the NGAL concentration in the feces a potential biomarker of intestinal impairment in IBD patients [104–109]. The same concept has been applied in swine, where fecal lipocalin has been targeted to evaluate the intestinal integrity response to variables such as dietary supplementation [110–112], pregnancy, lactation, and obesity in sows [113,114].

Lactoferrin is a glycoprotein secreted by neutrophils and epithelial cells and with affinity for iron, a feature that allows lactoferrin to limit the bacterial growth by reducing the Fe^{3+} availability for their metabolism. Besides its bacteriostatic potential, lactoferrin also displays direct antibacterial effects and can modulate the immune response [115,116]. Lactoferrin is an important secretion of neutrophils in inflamed sites, thus allowing its fecal concentration to be applied as a biomarker of intestinal inflammatory activity in human patients with chronic enteropathies [117–119]. We did not find any report of fecal or circulating lactoferrin as a biomarker in swine, [NO_PRINTED_FORM] thus requiring preliminary studies to understand its applicability in pigs.

Myeloperoxidase (MPO) is an enzyme abundantly found in cytoplasmatic granules of myeloid cells, as neutrophils. In an inflamed site, elicited leucocytes (i) degranulate the enzyme in the extracellular microenvironment and (ii) phagocytizes invading elements, fusing the phagosome with MPO-filled granules. The enzyme reacts with hydrogen peroxide

to form hypochlorous acid, a potent bactericidal agent that contributes to the elimination of pathogens [120–122]. Molecules of MPO secreted in the intestinal lumen and inside leucocytes debris during inflammatory episodes can be quantified in the feces, thus unveiling an ongoing intestine inflammation. This principle is applied to track enteropathies in humans and dogs [123–125], but little was found in swine, where the biomarker did not respond to a chemical challenge to induce enteritis [126]. Studies have related MPO expression and activity to the development of cardiovascular and other inflammatory diseases [127–129]. Therefore, the fecal measurement of the biomarker would be recommended to track the intestinal impairment specifically.

Neopterin is a substance produced by activated monocytes/macrophages, with IFN-γ as its major inducer. Neopterin acts in the cellular redox system of leucocytes, promoting the synthesis of oxygen and nitrogen reactive species and boosting their antimicrobial effect [130–132]. Serum, urinary and fecal neopterin concentrations have satisfactorily related to chronic enteropathies in humans, with increased values in patients with Crohn's disease and ulcerative colitis due to its synthesis in inflamed sites [133,134]. Studies in swine have targeted serum neopterin to assess the immune activation during surgeries [135,136] and kidney injury [137], but no study was found applying it as a marker of intestinal injury. Although blood levels of neopterin have been associated with intestinal inflammation, as mentioned above, studies demonstrate that increased circulating concentrations of neopterin can be also related to cardiovascular diseases, cancer, and sepsis in humans [138–142]. This indicates that feces would be the proper material to measure the biomarker when aiming to assess intestinal health.

Intestinal Fatty Acid Binding Protein (I-FABP) is present in the cytoplasm of mature enterocytes, where it binds non-covalent and reversibly to fatty acids thus enhancing their solubility and improving their transportation throughout the cytosol [143,144]. Variants are expressed in many organs and, as intracellular proteins, their presence in the blood stream indicates tissue damage with the leak of protein from harmed cells [145–147]. Factors inducing intestinal inflammation and injury as sepsis, ischemia, infection, and chemical challenge have increased the I-FABP serum concentration in varied species, including swine [126,148–155].

Diamine oxidase (DAO) is an intracellular enzyme highly expressed in mature enterocytes (thus present in the tip of intestinal villi) and that catalyzes the oxidation of polyamines, as histamine [156–158]. Injuries to the enteric mucosa are related to higher circulating levels of DAO, which leak from the damaged enterocytes into the blood stream [159,160]. Factors compromising the gut integrity as heat stress [161], bacterial [162–165], viral [166,167] or chemical [126] enteric challenge, ischemia [160,168], and exposure to mycotoxins [163,169] have increased the DAO circulating concentrations, thus confirming its potential to express the mucosa impairment.

Citrulline is an amino acid produced and exclusively released by enterocytes, mainly in the small intestine, through the metabolization of dietary glutamine and its derivatives [170,171]. Decreased circulating levels of citrulline may imply that enterocytes are being damaged, thus compromising the amino acid synthesis and reducing its release in the blood stream. Such a relation is explored in human health to assess the cytotoxic effects of chemo- and radiotherapy on the gut mucosa, which result in mucositis [172–175]. Citrulline circulating levels have also responded to intestinal cytotoxicity in dogs and rats, but this is still controversial in these species [176–179]. In swine, the concentration in plasma was reduced in animals with chemically induced enteritis [126], but no other report was found to insure the reliability of citrulline as a biomarker of intestinal damage in pigs.

Zonulin is an important modulator of the tight junctions (TJ), increasing the paracellular permeability to, theoretically, enhance the water outflux and "wash" the luminal surface from harmful elements [180,181]. Its expression in the gut mucosa was shown to be upregulated by bacterial infections [182] and exposure to gluten [183], thus being associated with enteric inflammation. The reaction cascade evoked by zonulin culminates in the displacement of certain TJ proteins, including the zonula occludens-1 (ZO-1). As an

evident target of zonulin, ZO-1 is found rearranged in the cytoplasm instead of near the cell membrane, where it mediates the bridge between the external claudin and occludin to the internal cytoskeleton [182,184]. In human health, fecal zonulin is a potential biomarker of Crohn's Disease activity [185,186], being also applied in studies with dogs to evaluate gut integrity [187–190]. Serum levels of zonulin have been targeted for the same purpose in swine [111,169,191], although the zonulin expression by other tissues may not allow a secure relation to gut health [192].

Endotoxin is an alternative to host-based biomarkers, being originated from the enteric bacterial community. The term "endotoxin" refers to Lipid A, a glucosamine-based phospholipid that constitutes the hydrophobic portion of LPS from the outer membrane of gram-negative bacteria [193,194]. Given that these bacterial structures are not supposed to reach the blood stream in the absence of factors increasing the intestinal permeability, researchers have already targeted the plasma/serum levels of endotoxin to track the effects of intestinal infection, obesity, gestation, lactation, heat stress and exposure to mycotoxins, and treatments on the inflammatory status of the intestine in varied animal species, including swine [163,169,191,195–199].

D-lactate is the d-isomer of 2-hydroxypropanoate. Differently from its L-isomer, D-lactate is only produced in nanomolar concentrations by mammalian cells, being predominantly originated from the microbial fermentation in the colon [200,201]. Serum d-lactate levels have been purposed as a biomarker of gut injury induced by ischemia, including in swine, since this bacterial byproduct leaks from the gut lumen when the mucosa is compromised [60,155,202–204]. Moreover, the biomarker has also been associated with the expression of improvements in the gut health of unchallenged pigs treated with phytobiotic products [110,191] or prebiotic [205]. Challenging factor such as infection by enterotoxigenic *E. coli* [196,206], exposure to mycotoxin [207], weaning period [208], and septic shock [152] had their gut damaging effect represented by increased circulating levels of D-lactate in swine.

The measurement of fecal cytokines is an alternative to gene expression assays that require invasive samplings, besides allowing to target their production specifically in the gut mucosa. Studies have reported increased fecal concentrations of cytokines such as IL-6 and IL-8 in humans and dogs with intestinal inflammation [209–211], but we did not identify fecal assess of cytokines in swine. Additionally, we do not approach the quantification of cytokines in blood in this review since their circulating levels are not specific for intestinal inflammation. Therefore, inferences about gut health based on the blood measurement of cytokines as a biomarker would be limited without an additional tool to diagnose an enteric inflammatory process.

7. Metabolic Biomarkers of Low-Grade, Chronic Intestinal Inflammation

The process of inflammation promotes metabolic reprogramming in a process now known as immunometabolism [212]. Immunometabolism can be further separate into two categories: cellular and tissue. Cellular immunometabolism is the reprogramming of immune cells that regulates cell phenotype and function and environmental conditions and Tissue immunometabolism which is the study of the influence of the immune cells and their metabolic reprogramming on organ metabolism [212]. The main functional feature of metabolic reprogramming under inflammation is the (a) shift in energy production from oxidative phosphorylation to aerobic glycolysis (Warburg effect) and (b) the alteration in epigenetic programming through DNA accessibility and chromatin structure (enhanced histone acetylation and suppressed DNA methylation) [213,214]. What stands out is that immunometabolism provides not only energy and intermediate precursors of macromolecule biosynthesis for cellular and tissue housekeeping functions, but also delivers immune metabolites that serve as essential signaling molecules involved in the immunoregulatory processes that drive immune and inflammatory activation [215–217]. The reprogramming of metabolic pathways such as glycolysis, the Krebs cycle, mitochondrial respiration (oxidative phosphorylation) and the pentose phosphate pathway, during immune cell activation

all result in the production of these immune metabolites [217] or metabolic regulators [218]. Functionally, immunometabolites to act as signaling effector molecules that modulate transcription factors, alter protein function, epigenetic and posttranslational modifiers, and inhibitors/activators of specific enzyme pathways [217,219–224].

7.1. Transcription Factors as Metabolic Biomarkers

Hypoxia-inducible factor-1α (HIF1α) is essential to the cellular metabolism and adaptation to cellular stress caused by hypoxia and fundamental reprogrammer of inflammatory cell metabolism that promotes inflammatory gene expression. A set of "signature" anabolic genes/proteins involved in the control of cellular metabolism have their expression regulated by HIF-1α. These proteins include all enzymes in the glycolysis pathway, glucose transporters, transferrin receptor, and the induction of pro-inflammatory genes [225,226]. Further, HIF-1α play a role in effector functions of macrophages and T cells during inflammation.

Peroxisome proliferator-activated receptor gamma (PPAR-γ) is involved sustaining fatty acid metabolism by regulating fatty acid storage and glucose metabolism. The receptor is an endogenous regulator of intestinal inflammation whose levels are suppressed during chronic inflammation [227,228].

Nuclear factor-κ–light-chain-enhancer of activated B cells (NF-κB) regulates multiple components of innate and adaptive immune functions and functions as a focal facilitator of inflammatory responses including inducing the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines. Further, NF-κB is a regulator of energy metabolism networks by controlling the balance between the utilization of glycolysis and mitochondrial respiration [229,230].

7.2. Metabolic Intermediates as Metabolic Biomarkers

Succinate is an immunometabolite intermediate of the Kreb's cycle that plays a crucial role in adenosine triphosphate (ATP) generation in mitochondria but has been found to play a regulatory role as a signaling molecule during intestinal inflammation [231,232]. Specifically, succinate stabilizes HIF-1α in activated macrophages, activates dendritic cells, and post-translationally modifies pro-inflammatory cytokines.

Citrate, like succinate, is an intermediate of the Kreb's cycle which regulates carbohydrate and lipid metabolism and can have both pro- and anti-inflammatory functions in macrophages and dendritic cells depending on the cellular local of the immunometabolite [233]. Excess citrate, during the Kreb's cycle, is transported out of the mitochondria into the cytosol of macrophages where it is involved in the production of prostaglandins and nitric oxide, and via the generation of malonyl-CoA and acetyl-CoA impact cytokine production. Citrate can also generate itaconate via aconitate, which promotes an anti-inflammatory response.

Nicotinamide adenine dinucleotide (NAD+) is a critical metabolic intermediate that participates as enzymatic cofactor in redox reactions and as a co-substrate for some enzymes such as sirtuins, adenosine diphosphate ribose transferases and synthases. With these roles, NAD+ metabolism regulates a broad spectrum of cellular functions such as energy metabolism, DNA repair, regulation of the epigenetic landscape and inflammation [234].

Nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the rate-limiting step of the NAD salvage pathway [235] and is ubiquitously detected in all tissues. Extracellular NAMPT, also known as visfatin or pre-B cell colony-enhancing factor (PBEF), has been considered to be a circulating hormone/cytokine released by several tissues has been shown to play a key role metabolism and inflammation [236]. Sirtuins (SIRT) are NAD+-dependent epigenetic and metabolic regulators [237]. Members of the sirtuin family protect tissue homeostasis by sensing bioenergy demands and react by making alterations in the cell nutrients. Sirtuins play a critical role in homeostasis by stimulating anabolic glycolysis during inflammation and, alternatively, reprogram metabolism by inducing lipolysis/fatty acid oxidation after the removal of the inflammatory trigger.

7.3. Post-Translational Modifiers as Metabolic Biomarkers

The kinases AMPK and mTOR mediate signaling pathways that play crucial and opposing functions in immunometabolism of inflammation [238].

AMP-activated protein kinase (AMPK) is a serine threonine kinase that is highly conserved through evolution. This protein is a sensor of cellular energy status found in eukaryotic cells that is activated under conditions of low intracellular ATP. The AMPK is a vital metabolic energy regulator in avian species [239] whose main role is monitoring the ratio of AMP:ATP and to adjust metabolic processes accordingly. Furthermore, AMPK can receive signals of cellular energy state and respond, via phosphorylation, that then influences glycolysis/gluconeogenesis, protein synthesis, fatty acid synthesis, and fatty acid oxidation. Numerous studies have shown that the phosphorylation of AMPK hampers inflammatory responses, whereas dephosphorylation of AMPK is associated with increased inflammation [240–242]. The reprogramming of cellular metabolism is imperative for immune cells responding to environmental cues and AMPK directs the metabolic adaptation of immune cells upon nutrient limitation and contributes to the immune response in vivo [242]. Lastly, AMPK modifies inflammation by antagonizing NF-κB, transcription of pro-inflammatory cytokines [240]. Therefore, AMPK would be identified is a negative immunometabolic regulator of inflammation.

Mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that also plays a role in cell growth and metabolism by sensing environmental cues. This kinase is phosphorylated when nutrients are in abundance especially when immune cells are in metabolically demanding situations, such as stimulation with growth factors, nutrient availability, and immune regulatory signals [243,244]. Phosphorylated mTOR senses cues from the immune microenvironment and elevates immune cell growth and proliferation [245]. The mTOR pathway aids in a cell's ability to meet high metabolic demands by promoting anabolic process, such as lipid and protein synthesis and repressing catabolic processes [246]. Moreover, mTOR is considered a positive immunometabolic regulator of inflammation.

8. Conclusions

Maintenance of intestinal health is critical to a successful animal production industry. For decades the swine and poultry industry has relied upon the inclusion of AGPs in the feed to achieve this goal and by extension improve growth performance. With the removal of AGPs, the emergence of chronic, low-level gut inflammation has come to the forefront of concern. Although the mechanisms by which AGPs promote intestinal health and suppress the development of inflammation are not well understood, it is generally believed that it occurs as a result of stabilization of the microbial communities within the gut as well as suppression of pathogen colonization.

With the removal of AGPs from feed, there is now a critical need for examining new strategies that the animal industry can utilize to control the development of chronic low-grade inflammation. The intestine of swine is constantly exposed to a number of environmental triggers including weaning period, intestinal pathogens, poor quality of feed ingredients, high energy diets, changes in feed formulation, and intrinsic chemical and physical characteristics of dietary ingredients that stimulate inflammation and lead to a reduction in performance. Additionally, other conditions such as pregnancy, lactation and obesity have also been associated with alterations in the intestinal homeostasis.

The chronic low-grade inflammatory response that occur in the intestine results in impairments to the digestive function and, consequently, decreased growth rate. Besides capturing resources from functions that are essential to the animal productivity, pro-inflammatory mediators promote muscle catabolism to supply amino acids and energy substrates for the inflammatory process. In addition to the detrimental effects on performance, the compromising of carcass traits can be related to chronic inflammation and the consequent shifts in the systemic metabolism.

Intestinal low-grade, chronic inflammation is a key contributor to complications affecting production animal performance. To support novel approaches to these complications,

it is vital to expand our understanding of the inflammatory dynamics and to monitor them. Here we summarize potential biomarkers reflecting intestinal-specific inflammatory dynamics for use in swine and poultry production worldwide. Immune responses have been linked to energy metabolism (immunometabolism); thus, the integration of these systems and their cooperation in responding to fluctuations in the energy and nutritional environment would be beneficial. The identification of immunometabolic biomarkers should provide dynamic and definitive predictors and biomarkers of low, grade chronic inflammation, but also provide indicators of successful nutritional or feed additive intervention strategies.

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