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Scientific Expert Panel

Evaluation of the impact of nutrition on immune functions to support 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 of having 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 at supporting the development of adequate animal 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 to microbiome, gut function, exposure control, immunity, physiology, and others.

The present recommendation document is focusing on the evaluation of the role of nutrition on immune functions to support animal health and welfare. Immune functions depend on various

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activities, and their evaluation requires the measurements of various endpoints to verify the effect of a specific nutritional strategy on the immune functions. Furthermore, immune functions are important lines of defense for maintaining the resilience of animals to various stressors.

2. Scope

The document relates to measurement of various endpoints for the evaluation of immune functions in the animal. Measurements of endpoints will depend on the objective and expected effect of the nutritional strategy under assessment.

The effect of diet on immune function in animals includes (Smith et al., 2018; Williams et al., 2020; Mentout et al, 2021; Patil et al., 2021; Munteanu and Schwartz, 2022):

- Nutrients improve immunity by providing antioxidants, increasing immune cells, and improving immune cell function.
- Nutrition regulates immunological responses by providing adequate nutrients to immune cells.
- Modulatory effects may reduce harmful immunopathology during infections and enhance protective immune mechanisms.
- Nutritional strategies support optimal immune function in livestock.
- Vitamins and minerals play a role in immune function and inflammatory responses in various animal species.

In animal agriculture, the health and well-being of animals play a pivotal role in determining the overall productivity and sustainability of farming operations. Among the multitude of factors which influence livestock health, nutrition is pointedly a critical component, not only for growth and reproduction but also for supporting the immune system of animals. Nutrition plays an essential role in the regulation of optimal immunological response, by providing adequate nutrients in sufficient concentrations to immune cells. Several micronutrients, such as minerals, and vitamins, as well as macronutrients such as some amino acids and fatty acids exert a very important and specific impact on appropriate immune activity.

The immune system of livestock functions is a complex network of cells, tissues, and organs working together to defend against pathogens, including bacteria, viruses, and parasites. Immune defenses are broadly categorized into innate (non-specific) and acquired (specific) immunity with innate immunity serving as the first line of defense, offering immediate but generalized protection. Whereas, acquired immunity develops over time, shaping its response to specific pathogens encountered by the animal.

The interaction between animal nutrition and an animal's immune response has long been known and studied. Several factors can influence the efficacy of the immune system in livestock, including genetics, age, stress levels, and notably, nutrition. Nutritional deficiencies or imbalances can compromise immune function, rendering animals more susceptible to infections and diseases. Conversely, a well-formulated diet can enhance immune responsiveness, equipping livestock with a better capacity to fight off pathogens.

Klasing's (1998) formative paper on poultry, but applicable to any production animal, classified nutritional modulation of immune function into seven (7) physiological types: (1) immune system development, (2) immune cell substrates, (3) leukocyte regulatory activity, (4) reduction of immune pathology, (5) regulation of hormonal activity, (6) physiological actions on the intestinal tract, and (7)

limiting nutrient availability to pathogens during infections. The field of nutritional immunology has become a specialty within immunology over the past decade. There is a large body of evidence that increasingly recognized the impact of nutritional components on the animal gut and systemic immunity. Furthermore, the implications of nutrition and nutritional intervention on prevention of disease and even corrections of pathophysiological conditions have become established concepts in livestock production and aquaculture (Kogut, 2009; Sordillo, 2016; Rodriquez et al., 2022; Humphrey et al., 2019; Martin and Krol, 2017; Dawood, 2020).

a. Nutrition and Modulating Immunity

To use nutrition to modulate the immune health of livestock, several key dietary components must be considered, such as macronutrients (proteins, fats, carbohydrates), micronutrients (vitamins, minerals), and certain feed additives:

- Proteins: Adequate protein intake is essential for the production and development of antibodies and immune cells. Diets deficient in protein can lead to impaired immune responses. Essential amino acids, such as lysine and methionine, are particularly important for maintaining the health of the immune system ((Bunchasak, Poosuwan, & Nukraew, 2005) (Kelly & Pearce, 2020) (Li, Yin, Li, Kim, & Wu, 2007) (van Heugten, Spears, & Coffey, 1994)).
- Fats: Polyunsaturated fatty acids (PUFAs) are involved in the regulation of inflammatory responses. Omega-3 fatty acids, found in fish oils and flaxseed, can modulate the production of immune-related signaling molecules, reducing the risk of chronic inflammation ((Miles, Childs, & Calder, 2021) (Al-Khalaifah, 2020)).
- Carbohydrates: Primarily considered as an energy source, specific carbohydrates have prebiotic effects and promote the growth of beneficial gut bacteria which are linked to improved immune function.
- Vitamins: Vitamins such as A, D, E, and C support function of different aspects of the immune system. Vitamin A is pivotal for maintaining the integrity of skin and mucosal cells (physical barriers to infection), vitamin D regulates the expression of immune response genes, Vitamin E acts as an antioxidant, protecting cells and tissues from oxidative damage, and vitamin C supports the production and function of white blood cells.
- Minerals: Minerals like zinc, selenium, and copper are critical for various aspects of immune function. Zinc is necessary for the development and function of innate and adaptive immune cells. Selenium acts as an antioxidant, protecting against oxidative stress, and copper is involved in the development and maintenance of immune cells.
- Feed Additives: Some probiotics can help balance gut microbiota, improving gut health and immune response, prebiotics support the growth of beneficial bacteria, phytochemicals can provide bioactive compounds with antimicrobial and immunomodulatory properties, and exogenous enzymes can break down large immunogenic dietary components that are no longer recognized by the innate immune system.

Prioritizing nutrition as a key factor in livestock health, farmers and producers can not only enhance the disease resistance of their animals but also contribute to the sustainability and productivity of their operations. As research on the cellular and molecular mechanisms between diet and immunity continues to evolve, new insights and strategies will likely emerge, offering further opportunities to support the well-being of livestock through informed nutritional practices.

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b. Immunometabolism

Immunometabolism refers to the links between metabolism and function in immune cells (Amiel and Perona-Wright, 2020). It plays a central role in sustaining immune system functionality and preserving physiological homeostasis within the organism (Xu et al., 2024). Immunometabolism involves metabolic reprogramming during differentiation and activation of immune cells (Hu et al., 2024). The interplay between metabolism-associated environmental cues and immune receptors is also important in this context (Chi, 2022). Immunometabolism describes the changes that occur in intracellular metabolic pathways in immune cells (T lymphocytes and macrophages) during activation.

Immunometabolism explores the interplay between immunological and metabolic processes in production animals (Palladino and Colombatto, 2022). It helps animal scientists understand the link between production and health and is crucial for managing nutrient resources between growth and survival (Davis, 2022). Basically, immunometabolism describes the immune system not only as a host defense mechanism but also as a tool for maintaining homeostasis (Brestoff and Artis, 2015) since immune defenses are a reaction to a perturbation in the host's physiological steady state (Brestoff and Artis, 2015). Thus, immune system activation brings an energy cost to the animal, which redirects nutrients from growth and reproduction purposes to those mechanisms that help the organism to survive (Davis, 2022; Niu et al., 2022).

The field of immunometabolism has begun to chart out the mechanisms in production animals whereby metabolic changes induced by environmental cues, infectious and non-infectious, can regulate transcriptional and post-transcriptional events, leading to modulation of the immune and inflammatory responses (Arsenault et al., 2016; Kogut and Arsenault, 2017; Lee et al., 2021; Aylward et al., 2022; Niu et al., 2022; Loor and Elolimy, 2022). The immune system regulates the functioning of cellular metabolism within organs by sensing and adapting to fluctuations in the microenvironment's nutrients, thereby reshaping metabolic pathways that greatly impact a pro- or anti-inflammatory immunophenotype. Although the immune system relies on adequate nutritional status to function properly, we are only just starting to understand how the supply of single or combined nutrients (immunonutrients) can direct immune cells towards a less inflamed, tolerogenic immunophenotype.

3. Descriptions of endpoints

Main endpoints for the evaluation of the role of nutrition on the immunological state of the animals refer to the cellular metabolism of immune cells.

- Dendritic cells and macrophages provide new insights on the impact on the phenotype and functioning of these critical controllers of innate and adaptive immunity (O'Neill and Pearce, 2016). Both cell types undergo profound metabolic reprogramming in response to environmental cues, such as nutrient alterations, but importantly also in response to danger signals and cytokines. Metabolites such as succinate and citrate have a direct impact on the functioning of macrophages. Immunogenicity and tolerogenicity of dendritic cells are also determined by anabolic and catabolic processes, respectively.
- T cells are the central components of the acquired immune system and undergo a series of metabolic changes that correlate with their maturation, activation, and differentiation states (Pearce et al, 2013; Buck et al., 2015). These metabolic changes are accompanied by the activation of key receptor signaling pathways, transcriptional and posttranslational modulations, as well as epigenetic remodeling (Pearce et al, 2013; Buck et al., 2015). Thus,

dynamic reprogramming in metabolism not only provides the necessary energy and biosynthetic precursors in response to environmental cues and nutrient license but also actively regulates immune responses of T cells (Geltink et al., 2020; Chapman et al., 2020; Powell and Delgaffe, 2010).

4. Parameters for the evaluation of the endpoints

Practical non-specific indicators of immune response

Category	Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site: Invasive [I] Non-invasive [NI]	Method: Measurement requires materials which are frequently used in laboratories and/or are related to rapid test analysis and be easily applied by laboratories.	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
Non-specific immune status	Cellular response	Whiter blood cell (WBC) response	Differential cell counts	Blood [I] Milk [NI]	Blood smear, microscopic cell counts	Diagnostic: provides insights into the types and proportions of WBC. Indicates health conditions or effectiveness of nutrition in response to infection, inflammation, stress
	Humoral response	Total immunoglobulin (IgG, IgM, IgA, IgY (poultry)	General indication of antibody production and capacity to mount a humoral immune response	Serum [I] Eggs [NI] Feces [NI}	Commercial ELISA kits, Radial immunodiffusion, Hemagglutination	Diagnostic and monitoring the health of the animal. Assess the ability of diet to enhance ability to fight infection and/or general immune deficiency. Fecal IgA (secretory IgA) – can be used to assess immune status related to gut health and enteric pathogen challenges. Egg IgY – well established to assess hen immune response and effectiveness of therapeutics’

Category	Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site: Invasive [I] Non-invasive [NI]	Method: Measurement requires materials which are frequently used in laboratories and/or are related to rapid test analysis and be easily applied by laboratories.	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
	Acute phase proteins (APPs)	Proteins whose concentrations change rapidly in response to injury, inflammation, or infection.	APPs have antimicrobial activity (activate complement), blood clotting, formation of matrix for tissue repair, regulate iron homeostasis	Serum ([I] Plasma [I] Milk [NI]	Commercial ELISA kits for c-reactive protein, haptoglobin, serum amyloid A, fibrinogen, ferritin, hepcidin	Elevated levels indicate ongoing, but non-specific immune response, indicators of inflammation and infection.

Category	Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site: Invasive [I] Non-invasive [NI]	Method: Measurement requires materials which are frequently used in laboratories and/or are related to rapid test analysis and be easily applied by laboratories.	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
	Complement components	Proteins to assess immune competence and response to vaccination and susceptibility to infection	Disease diagnosis, immune monitoring, assessing impact of stress on immune function	Serum ([I] Plasma [I] Milk [NI]	Commercial ELISA kits: hemolytic assays to measure classical (sheep RBC) and alternate (rabbit RBC) complement pathways	Assess immune function and health status; levels and activity indicative of disease state

Practical assays for evaluation of innate immune response

Category Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site Invasive [I] Non-invasive [NI]	Method: Measurement requires materials which are frequently used in laboratories and/or are related to rapid test analysis and be easily applied by laboratories.	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
Immune response Innate immunity	WBC function	Host defense	Whole blood [I]: Stimulating heparinized blood with compounds (LPS; PMA + ionomycin) to trigger the release of cytokines	Level of cytokine release quantified using ELISA	Assesses the impact of diet on the innate immune system.
	Phagocytosis	Host defense: macrophages, neutrophils, heterophils (poultry)	Blood [I]	Light and fluorescent microscopy to quantify uptake of fluorescently labeled bacteria or beads	Measure the ability of phagocytes to engulf and eliminate pathogens or foreign particles. Increased phagocytosis indicated activated innate immunity
	Oxidative burst	Host defense: macrophages, neutrophils, heterophils (poultry)	Blood [I]	Chemiluminescence Assay: Phagocytes are incubated with a chemiluminescent probe (luminol); stimulated with a reagent that triggers an oxidative burst (zymosan, phorbol myristate acetate (PMA)). The emitted light is measured using luminometer, the intensity of the light is directly proportional to the amount of ROS produced	Relatively simple, sensitive, and can be used to assess the overall bactericidal capacity of phagocytes

Category Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site Invasive [I] Non-invasive [NI]	Method: Measurement requires materials which are frequently used in laboratories and/or are related to rapid test analysis and be easily applied by laboratories.	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
	Oxidative burst	Host defense: macrophages, neutrophils, heterophils (poultry)	Blood [I]	Spectrophotometric Assays: rely on measuring absorbance or absorbance changes of a solution containing a substrate that reacts with ROS; Cytochrome c assay: Cytochrome c is reduced by superoxide anions, and the absorbance change at a specific wavelength can be measured: Nitroblue tetrazolium (NBT) assay: NBT is reduced by superoxide anions to form a colored formazan product that can be measured spectrophotometrically	Relatively simple, inexpensive, and can be used to assess the overall bactericidal capacity of phagocytes
	Degranulation	Host defense: Neutrophils, eosinophils (mammals), heterophils (poultry)	Blood [I] Fecal [NI]	Degranulation assays: study the immune response of granulocyte, understand how these cells respond to various stimuli and how they contribute to disease resistance. Assays involve stimulating granulocytes with various agents (e.g., PMA, bacteria, pathogens) and measuring the release of specific granule contents using markers like myeloperoxidase (MPO) (swine, cattle), lactoferrin, β-glucuronidase. All can be assessed using colorimetric, fluorescent, or ELISA assays.	Granulocytes play a crucial role in the innate immune system, releasing antimicrobial substances and inflammatory mediators upon activation. Excessive release in response can cause tissue damage. It can be used to assess immune response, inflammation, impact of stress, and evaluate effects of treatments (nutritional/dietary).

Category Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site Invasive [I] Non-invasive [NI]	Method: Measurement requires materials which are frequently used in laboratories and/or are related to rapid test analysis and be easily applied by laboratories.	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
	Cytokine production	Host defense: Monocytes, macrophages, neutrophils, heterophils, T lymphocytes	Blood [I]: serum	The assay closely reflects the natural environment of immune cells, providing a more accurate representation of immune responses compared to assays using isolated cells. Whole blood assays require less manipulation of the blood sample, reducing the risk of altering immune cell function. Whole blood is collected from the production animals in heparinized tubes to prevent clotting. blood sample is then incubated with a stimulus of interest (bacterial components such as LPS, antigens, or other immune cell activators), after incubation, the blood is centrifuged to separate the plasma (liquid portion of the blood), the plasma supernatant is then analyzed for the presence and concentration of various cytokines using commercial ELISA kits specific for production animal species. The measured cytokine levels are then analyzed to assess the immune response to the stimulus. Changes in cytokine production can indicate the activation of specific immune pathways and the overall immune status of the animal.	Valuable for assessing immune responses to different stimuli, including pathogens vaccines, and used to assess the impact of different nutritional interventions on immune function. Changes in cytokine production can indicate the activation of specific immune pathways and the overall immune status of the animal.

Practical assays for evaluation of acquired (adaptive) immune response.

Category Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site: Invasive [I] Non-invasive [NI]	Method: measurement requires materials, which are frequently used in laboratories and/or are related to rapid test analyze and be easily applied by laboratories	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
Immune response Acquired immunity	Humoral immunity: Antigen specific immunoglobulin IgM, IgG, IgA IgY (poultry)	Host defense: B lymphocytes	Blood [I] Milk Fecal (IgA) Eggs (IgY)	ELISA kits detect antibodies against specific pathogens in blood or other biological fluids. ELISA is highly sensitive and specific.	Produced in response to specific antigen(s): crucial to understanding immune response to vaccination or infection. In neonates, evaluates passive immunity
	Cell-mediated immunity	Host defense: T lymphocytes	Blood [I]	Whole blood is collected with an anticoagulant, to prevent clotting. The blood is stimulated with antigens that are relevant to the immune response being studied (pathogens, vaccines, or other relevant stimuli). After stimulation, the blood is processed to measure the levels of cytokines produced by the activated immune cells. Cytokine detection using commercial ELISA kits.	Whole blood assay for measuring cell-mediated immunity involves stimulating whole blood samples with specific antigens and then measuring the release of cytokines (interferon-gamma (IFN-γ), which are produced by activated T cells. These assays can help assess the effects of different dietary interventions on immune function.

Practical assays using immunometabolic pathways as indicators of immune function.

Category Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site: Invasive [I] Non-invasive [NI]	Method: measurement requires materials, which are frequently used in laboratories and/or are related to rapid test analyze and be easily applied by laboratories	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
Immune response Inflammation	Immunometabolism	Immunometabolism describes the interplay between the immune system and metabolic processes, encompassing how metabolism influences immune cell functions and how immune responses, in turn, shape cellular metabolism. Immune cells undergo significant metabolic reprogramming during activation, shifting between pathways like glycolysis and oxidative phosphorylation (OXPHOS) to meet their energy and biosynthetic needs for proliferation, cytokine production, and effector functions. This metabolic flexibility is essential for an effective immune response.	Blood [I]: plasma, serum Urine [NI] Feces [NI] Tissue samples [I]: liver, spleen, intestine	Commercial ELISA kits: evaluate the activity of key metabolic pathways like glycolysis, fatty acid oxidation, and oxidative phosphorylation and enzyme activity (TCA cycle, glycolysis) involved in metabolic pathways in immune cells to understand how dietary interventions affect their energy production and biosynthesis and the activity of enzymes involved in the metabolic state of immune cells. Blood: reflects systemic metabolic changes induced by dietary or nutritional changes. Tissue homogenates: localized impacts into metabolite and immune response in specific organs affected by dietary changes. Fecal: assessment of how dietary/nutritional changes affect microbiota metabolites and their potential influences on the host.	A robust and adaptable immunometabolism allows immune cells to meet the diverse demands of different immune responses. This metabolic flexibility is a hallmark of good immune resilience. Metabolic Signatures and Immune Cell Function: Different immune cell subsets rely on distinct metabolic pathways to carry out their functions. A healthy immunometabolism ensures that the appropriate metabolic pathways are engaged to support the specific needs of each cell type, contributing to optimal immune function and resilience.

Practical assays for evaluating intestinal health

Category Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site: Invasive [I] Non-invasive [NI]	Method: measurement requires materials, which are frequently used in laboratories and/or are related to rapid test analyze and be easily applied by laboratories	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
Immune function Intestinal health	Intestinal barrier function	In vivo Permeability assays: administering non-digestible markers (FITC-dextran) orally and measuring their concentration in blood or urine. An increase in marker level indicates compromised barrier function. Typically provides a single permeability value, limiting the ability to localize the affected region of the gut. Fecal biomarkers: Measure specific substances in feces, can offer a non-invasive indication of gut barrier integrity.	Blood [I] Feces [NI] Tissues [I]	FITC-dextran passage (FITC-d)	Measure passage from gut to bloodstream
				Intestinal fatty acid binding protein (I-FABP)	Protein found in gut
				Intestinal alkaline phosphatase (IAP)	Enzyme produced in gut
				Ovotransferrin	Iron-binding protein in gut. Antimicrobial activity
				Tight junction proteins (Claudin, Zonulin)	Bind intestinal epithelial cells together
	Inflammation	A specialized pathological response activated in response to an infectious, toxic, or environmental stressor disturbance of an animal's homeostasis. Accurately and practically measuring intestinal inflammation is vital for optimizing gut health and overall performance	Blood [I] Feces [NI]	Lipocalin	Acute phase protein released by intestinal epithelial cells and heterophils
				Lactoferrin	Iron-binding protein released by heterophils
				Cytokines (IL1β, IL6, IL10, TNF-α, etc.)	Proteins produced are indicators of inflammation
				Succinate, citrate, Isocitrate	Metabolic indicators of inflammation
				Hypoxia inducible factor-α	Transcription factor that regulates metabolic switch from OxPhos to glycolysis (immunometabolic marker of inflammation)

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Category Sub-category	Evaluation	Endpoint: measurement is properly standardized (existing Standard Operating Procedures), of high scientific quality, and validated	Sampling site: Invasive [I] Non-invasive [NI]	Method: measurement requires materials, which are frequently used in laboratories and/or are related to rapid test analyze and be easily applied by laboratories	Criteria: enables the prediction and measurement of productivity, efficiency, health status (resilience), or maintenance of homeostasis.
Immune function Intestinal health	Oxidative stress	Oxidative stress in the intestinal tract of production animals has a significant impact on animal health and productivity. Induced when there is an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses, leading to cellular damage and inflammation within the gut. This imbalance can be caused by stress, disease, and dietary issues, and can result in reduced growth, impaired nutrient absorption, and increased susceptibility to illness. Identifying animals experiencing oxidative stress can then be used to implement appropriate management strategies, such as dietary modifications, antioxidant supplementation, or environmental adjustments, to improve animal health, welfare, and productivity	Blood [I] Tissues [I]	Thiobarbituric acid reactive substances (TBARS)	Marker of lipid peroxidation by reactive oxygen species
				Total antioxidant capacity (TAC)	Measure of overall ability to neutralize oxygen free radicals
				Superoxide dismutase (SOD) Glutathione peroxidase (GPx)	Enzymes to assess ability to neutralize free radicals

5. Methods to measure the parameters.

The following methods can be used to measure the various parameters.

- The Agilent Seahorse XF platform measures the two main metabolic pathways, mitochondrial respiration and glycolysis, for live cells in real time, to provide functional kinetic measurements of cellular bioenergetics.
    - To accurately investigate the bioenergetics of T cells, microphages, and dendritic cells function with an extracellular flux analyzer (Seahorse Bioscience, Inc, USA).
    - Real time metabolic parameters by measuring oxygen consumption rate (OCR) and Extracellular Acidification Rate (ECAR) as a surrogate marker for glycolysis using a Seahorse XFe 96-well extracellular flux analyzer.
- 96-well-plate-based immunometabolic profiling:

Methods	Assay principle	Advantages/ disadvantages	Equipment
extracellular glucose/lactate assay	estimate of glycolysis based on cumulative glucose consumption/lactate secretion	+ simple, fast, cheap	absorbance (glucose) or fluorescent (lactate) plate reader
NO/arginase assay	estimates L-arginine metabolism via iNOS/arginase	+ simple, fast, cheap	absorbance plate reader

- Basal assays of immunity (non-invasive) (numbers or concentrations in blood, plasma, milk, feces):

Category	Endpoint	Sample source	Assay
Cells	Differential cell counts	Blood (leukocytes)	Blood smears/ flow cytometry
	Based lymphocyte subsets	Blood (leukocytes)	Flow cytometry & microscopy: CD4, CD8, T cells, B cells:
	Expression of activation markers	Blood (leukocytes)	CD25, CD69
Mediators	Acute phase proteins	Plasma	
	Complement components	Plasma	
	Antigen specific immunoglobulins	Plasma, milk, eggs	IgG, IgM, IgY
	Cytokines, chemokines (protein production)	Plasma, milk, feces	IL-2, IL-10, IL-6, IFNγ, IL-12, IL-17, IL-16, IL-22
	Cytokine profiles	Plasma, milk	Th1/Th2/Th17; pro-/anti-inflammatory
	Adipokines	Plasma	Leptin, insulin growth factor
Inflammatory markers		Plasma, feces	Calprotectin, lipocalin, lactoferrin, hypoxia inducible factor-γ
		Plasma, feces	IgA, secretory IgA

- Systemic immune functional products:

Category	Endpoint	Sample source	Assay
Ex vivo innate	Host defense	Blood (monocytes/macrophages/DCs/ neutrophils/heterophils)	Phagocytosis
	Host defense	Blood (monocytes/macrophages/DCs)	Oxidative burst
	Host defense	Blood (monocytes/macrophages/DCs/ neutrophils/heterophils)	Cell migration
	Host defense	Blood (monocytes/macrophages/DCs)	Nitric oxide
	Host defense	Blood (neutrophils/heterophils)	Degranulation (β-glucuronidase)
	Host defense	Blood (monocytes/macrophages/DCs/ neutrophils/heterophils)	Cytokine production
	Host defense	Blood (monocytes/macrophages/DCs/ neutrophils/heterophils)	Eicosanoid production
Ex vivo acquired		Blood (T lymphocytes)	Proliferation/differentiation
		Blood (T lymphocytes)	Cytokine production
		Blood (T lymphocytes)	Expression of CD markers
		Blood (B lymphocytes)	Immunoglobulin production (total and Ag specific)

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7. 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.

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