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Gut health in poultry and considerations of additives as alternatives to antibiotics

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Abstract

'Gut health' is currently a hot topic in animal production but lacks precise definition or definitive assessment. Antibiotics have been used for many years to manage gut health issues but are under pressure in many parts of the world to help protect their long-term effectiveness. The intestine is composed of numerous components, including a microbiome, nutrients and host factors (e.g. cells, secretions, mediators, etc.) that are continually interacting. The gut microbiome governs the development and functionality of the immune system and strongly influences 'gut health'. Features of the innate gut immune system are largely present and functionally mature at hatch and may represent a particular focus for exogenous interventions. Inflammation is a key innate response and, although often viewed only as a negative response, evidence indicates an enhanced or less regulated acute inflammatory response capability is beneficial. Unresolved sterile or metabolic inflammation, resulting from innate immune system stimulation by non-infectious cellular components and metabolites, are, however, generally recognised as undesirable and are a focus for intervention. Antibiotic growth promoters (AGPs) were effective at preventing and/or controlling intestinal disorders and although most evidence suggests benefits from microbiome modification there is still debate over precise mode of action. The clear protective effects of the gut microbiome and influences on immunity makes for potential interventions that have largely focused on the application of various microbiome (e.g. probiotics) and/or immune (e.g. cytokines) modulators. Better understanding of key microbiome-immune interactions, and thus how these can be appropriately modulated, is essential for significant progress in promoting gut and animal health.

Keywords: gut health, microbiome, inflammation, probiotic, prebiotic, mucosal immunity

‘Gut’ or ‘intestinal health’ is a hot topic in animal production, even though clear definition(s) and definitive assessment are lacking. ‘Gut’ or ‘intestine’ clearly relate to the gastrointestinal tract (GIT) but clearly defining ‘health’ in this context is challenging. Gut health has, however, recently been defined as the ability of the gut to perform normal physiological functions and to maintain homeostasis, thereby supporting its ability to withstand infections and non-infectious stressors [1].

For many years, antibiotics were used at low, below therapeutic, concentrations to improve the growth performance of farm animals (AGPs) and various hypotheses suggest that their mode of action relates to effects on intestinal bacteria and ultimately the functioning of the gut and/or reduction of nutrient losses. The proposed mechanisms include 1) reducing total microbial density in the GIT, 2) promoting a more favourable GIT microbial balance and/or reducing sub-clinical infections, 3) reducing the production of potentially toxic bacterial metabolites and 4) better absorption of nutrients through a thinner intestinal epithelium [2]. A more recent suggestion has been that AGPs work by directly inhibiting the negative effects of intestinal inflammatory cells [3] but unravelling potentially direct or indirect (via microbiome modification) effects of AGPs on inflammatory responses or cells is challenging. Microbiota-independent proposals may have arisen from the misconception that providing antibiotics at concentrations below minimum inhibitory concentrations (MIC) have no effect on bacteria. However, MIC is defined as the lowest concentration of an antimicrobial that prevents the *visible* growth of the target bacteria (bacteriostatic effect) and thus non-*visible* effects are possible. Various studies have provided clear evidence that antibiotics at sub-MIC can affect bacterial growth characteristics, protein and virulence factor expression or formation (e.g. cell adhesion capability, toxin production, biofilm formation, etc.) and susceptibility to host immune responses [4]. Thus, even when below relevant MICs, AGPs can still impede bacterial growth (rate) and production of various virulence factors, and can therefore impact, any or all

of, GIT microbial populations, concentrations of harmful metabolites, initiation and clearance of (subclinical) infections, intestinal structure and dimensions, and stimulation, response and efficacy of host defence mechanisms.

Whilst understanding the mode of action of AGPs is of interest, there has been a global push to eliminate the use of AGPs [5,6] and, where possible, to develop strategies that can appropriately reduce therapeutic use of antibiotics. A consequence of AGP removal is an increase in GIT-related disorders, such as coccidiosis and necrotic enteritis (NE) [7], which are prominent and costly diseases in poultry [8]. This is placing renewed interest on understanding these diseases and their control, the gut microbiome, immune responses and factors that can influence these interactions (e.g. exogenous compounds or immunosuppressive diseases) and optimise bird health and productivity.

Components of the gut

The intestine is composed of digesta (exo- and endogenous-derived nutrients of varying digestibility), microbiota and their metabolites (such as short-chain fatty acids (SCFAs)), mucus layers and host-derived antimicrobial compounds (e.g. host defence peptides (HDPs), IgA). These components primarily seek to create a hostile environment to undesirable microbes and function to trap and inhibit invading microbes and facilitate their removal from the intestine with assistance from peristalsis [9]. Below these features are a single layer of epithelial cells and the underlying lamina propria, with its associated immune cells, and muscularis layers. The epithelial cells are broadly differentiated into 4 major types - goblet cells (mucin production), Paneth cells (HDP secretion), endocrine cells and absorptive enterocytes – which are linked by tight junction (TJ) (multi-protein) complexes, including claudins and occludins, which regulate paracellular permeability [10]. The underlying tissues contain various innate (e.g. dendritic cells (DC), heterophils, macrophages, and natural killer (NK) cells) and adaptive

(e.g. T, B and plasma cells) immune cells, with some of these cells within the epithelium itself (intraepithelial lymphocyte (IEL)). There is a plethora of interactions between immune, as well as with non-immune, cells that help provide intestinal protection, tolerance and homeostasis.

Ideally, these components work in a coordinated way to prevent disease, maintain homeostasis and maximise the acquisition of dietary nutrients. Indeed, the gastrointestinal tract is the largest surface area of the body in constant contact with the environment and is home to more than 70% of all the host's immune cells [11].

Gut microbiome and immune system

The GIT is naturally colonised by microbes upon exposure to the eternal environment (e.g. at hatch). Each region of the GIT, having different functions, provides differing levels of nutrients, pH, oxygen, antimicrobial compounds, etc., which thus determines the establishment of specific microbiomes. Pre-caecal GIT regions host up to $>10^9$ microbial cells per g of digesta dominated by *Lactobacillus* spp. (up to 99 per cent) [12]. Microbial diversity and cell numbers increase distally. In the caeca, which are particularly well studied due to their microbial and nutritional significance, cell numbers in excess of 10^{11} per g of digesta are possible. Here, Firmicutes and Bacteroidetes are the dominant phyla, with lesser contributions from other phyla (notably Proteobacteria and Actinobacteria), while *Ruminococcus*, *Clostridium*, *Eubacterium* and *Bacteroides* are prominent genera [13]. A challenge in providing an overview of the gut microbiome is that differences in study conditions and analytical methodology can compromise the accuracy and validity of comparisons or consolidation made across studies [14].

Short-chain fatty acids (SCFAs), primarily acetate, propionate and butyrate, are a major class of bacterial metabolites arising from microbial fermentation of dietary fibres. As well as having strong antimicrobial activity, SCFAs can serve as an energy source and can influence host

cellular function by inhibiting histone deacetylase activity, and thus gene expression regulation, or through binding to, and activation of, G-protein coupled receptors (GPRs), which are sensors of metabolites [15]. Through these interactions, SCFAs are considered to contribute positively to intestinal homeostasis through, at least in part, regulation of inflammasomes (e.g. NLRP3), which are multiprotein complexes expressed by various cell subsets (dendritic cells (DC), macrophages, epithelial cells, T cells, etc.) and are regarded as key signalling pathways in inflammation [16]. However, there remain uncertainties regarding the relationship between intestinal concentrations of SCFAs and optimal physiological effects. In addition, protein fermentation can also contribute to the intestinal SCFA pool through generation of branched-chain fatty acids (e.g. isobutyrate, 2-methylbutyrate, and isovalerate) but protein fermentation is not considered beneficial for gut health.

The immune system consists of innate and acquired components that are fully integrated. Innate factors include mucus layers, antimicrobial secretions and cells such as dendritic (DC), macrophages, heterophils and natural killer cells residing in the lamina propria and/or within the epithelium. These components are able to provide the first response to microbial invasion. Innate cells can recognise microbe-associated and host-derived danger-associated molecular patterns (MAMPs and DAMPs, respectively) via their limited diversity cell surface and cytoplasmic pattern recognition receptors (PRRs). There are various classes of PRRs, including TLRs and NODs, which, when activated by their cognate ligand, leads to the induction of intracellular signalling pathways and extracellular processes that promote the recruitment of effector cells (inflammatory response) and phagocytosis of the invader through various cytokines, chemokines, and adhesion and co-stimulatory molecules. These processes lead to the activation of adaptive immunity, which provides more antigen-specific responses with memory. Adaptive immunity is orchestrated by T and B cells that express near infinite antigen-specific receptor diversity via processes known as gene rearrangement or conversion [17]. T

cells recognise major histocompatibility complex(MHC)–peptide antigen complexes displayed on the surface of nucleated (MHC class I (intracellular peptides)) or antigen-presenting cells (e.g. DC; MHC class II (extracellular peptides)). Cytotoxic, CD8 expressing, T cells recognise MHC class I–peptide antigen complexes and helper, CD4 expressing, T cells recognise MHC class II–peptide antigen complexes, with different subsets of CD4 T cells (Th1, Th2, Th17 and T regulatory (reg) cells). CD4 T cells promote cytotoxic or phagocytotic activity against intracellular bacteria, viruses and protozoa (Th1), antibody responses against extracellular pathogens, including helminths (Th2), activities targeting extracellular pathogens, including most fungi (Th17) or immune response regulation (Treg) [18]. Importantly, the paradigm of polarised adaptive type 1 and 2 immune responses appears to hold true for chickens [19]. B cells can be activated (plasma (antibody production) or memory cells) by specific antigen recognition by B cell surface receptors (BCR) and through cytokine-driven stimulation by Th cells.

GIT Innate immune components, including macrophages, heterophils and HDPs, appear to be largely present at hatch, or soon after, and appear to be fully functional, with cell numbers increasing with age. Avian β-defensins (AvBDs) 1-10 are present at hatch and duodenal mucosal scrapings have pronounced bacterial killing capability [20]. B and T cells only become prominent in the gut from 7-14 days of age (doa), with endogenous intestinal IgA detected from 7 to 14 doa. The gut microbiome plays a clear and prominent role in the development of the GIT immune system, particularly adaptive responses [21]. In germ-free birds, B and T cells, and IgA, are not detected through to 28 doa [22]. Equally, HDPs and IgA, collectively, play a major role in shaping the gut microbiota and interactions with the intestinal mucosa, while specific components of the gut microbiota help promote different T cell subsets, for example, segmented filamentous bacteria (SFB) promote Th17 cells in the intestine and polysaccharide A from specific commensal *Bacteroides fragilis* strains enhance Treg differentiation [23].

Gut environment interactions

As mentioned, the GIT is the largest body surface in constant contact with a complex microbiome. There is significant interplay between the gut microbiome and the host, which drives induction of tolerance, protection from pathogens and intestinal homeostasis [21]. Microbes, such as *Eimeria* and *C. perfringens*, can obviously, individually or collectively, disrupt intestinal homeostasis and cause the economically important poultry diseases coccidiosis and/or NE. *Eimeria* replicate in GIT epithelial cells, while *C. perfringens* produces toxin(s) that damages epithelial cells. Both cause GIT lesions, which compromise intestinal function. Detection of invading microorganisms and/or resultant host damage by PRRs results in an inflammatory response by the immune system. Inflammation is a critical innate immune process that seeks to contain an infection, activate adaptive immunity, repair damaged tissue and return to a homeostatic state [24], but is often incorrectly only considered as a dispensable or unwanted response due to, for example, associated nutrient costs. Various studies have shown that an enhanced inflammatory response increases resistance to key poultry diseases and that overregulation of the response (e.g. based on greater IL-10 expression) may not be beneficial [25]. It is, however, recognised that an excessive or prolonged inflammatory response can contribute to tissue pathology [25]. The evidence, therefore, suggests that the capacity to mount a rapid, acute, inflammatory response to contain the infection, followed by a timely, IL-10 and TGFβ led, transition, once the infection is controlled, to dampen this response and promote tissue repair processes is the optimum scenario to maintain an animal's resistance to infection from a range of pathogens and minimise non-productive nutrient losses [26].

Metabolites produced by gut microbes can also be detected by PRR and thus the host can monitor and respond to the intestinal microenvironment and microbial activity [27]. Whilst some of these metabolites may be beneficial (e.g. SCFAs, taurine, indole, etc.) and are reported

194 to suppress pro-inflammatory cytokine expression by various cells and/or to promote barrier
195 function, including (as mentioned previously) by influencing the activity of inflammasomes
196 [27], some metabolites (or in excess) may not be. Commercial poultry are exposed to a myriad
197 of (infectious and non-infectious) inflammatory triggers. Sterile and metabolic inflammation
198 are, typically, chronic, low-grade inflammatory states resulting from innate immune system
199 stimulation by non-infectious cellular components and metabolites [28]. Components of the
200 diet, for example β-mannans, maybe recognised by PRRs and lead to feed-induced
201 inflammation and a wasteful, non-productive expenditure of nutrients on immune responses.
202 Equally, persistent nutrient excess (fatty acids and carbohydrates) can result in overloads of
203 metabolites that act as DAMPs that can be sensed by PRRs of the innate immune system.
204 Moreover, there is growing interest in the changes that occur in immune cell intracellular
205 metabolic pathways during activation (immunometabolism) and how these are or can be
206 influenced by metabolites or small molecules and thus alter the phenotypes of immune cells
207 [29, 30].

208 Features of modern animal production (e.g. increased feed intakes and nutrient excesses) are
209 likely to be contributing to increasing the incidence of (chronic) inflammatory triggers or states.
210 The key to optimum gut health is minimising exposure to the number and/or degree of immune
211 triggers, as well as the induction of tolerance to innocuous stimuli to prevent unnecessary
212 responses.

213 **Learnings from AGPs**

214 AGPs were reported to be effective most of the time that they were used, with typical growth
215 and feed conversion efficiency improvements of 3-5% [31, 32]. This led to AGPs becoming
216 recognised as the ‘gold standard’ of performance-enhancing feed additives, particularly in
217 ‘dirty’ or more *challenged* environments [e.g. 33]. Gut microbiota modification seems to be a

relatively constant feature reported by AGP studies [4] and include (although not always consistent) reduction of certain bacterial species (e.g. species of *Lactobacillus* and *Streptococcus*) and overall community diversity, increased abundance of lactobacilli in proximal GIT locations with a differing effect (no change or decreased) in distal regions, predominantly altering the ileal microbiota (lactobacilli dominant and *Enterobacteriaceae* reduced) and promoting (seemingly non-pathogenic) Clostridia. Most of the commonly used AGPs are primarily active against Gram-positive bacteria and so effects on bacteria within this grouping are not unexpected. Moreover, studies with AGPs have utilised quite different methodologies to investigate the microbiota (culture-dependent and culture-independent), which may affect consistency of results and/or interpretation.

The effects of AGPs on key features of the gut barrier have recently been reviewed [34]. Increases in certain bacterial families (e.g. *Lachnospiraceae*), genera (e.g. *Faecalibacterium*, *Propionibacterium* and *Ruminococcus*) or species (e.g. *F. prausnitzii*, *B. fragilis*, some *Lactobacillus* spp.) and effects on mucus-related parameters (e.g. goblet cell size, density, mucin mRNA expression) have been reported with AGP use, but data investigating effects on gut immune parameters (e.g. cell populations, cytokines and chemokines) are more limited. The clearest and most consistent effects reported to date seem to be enhanced epithelial structure and function (e.g. nutrient digestibility). As mentioned previously, AGPs may mediate these effects through modification of the gut microbiota and/or its activity or, potentially, via more direct mechanisms.

Considerations and optimising current and future ‘gut health’ additives

Available evidence clearly supports the importance of the gut microbiome for maintenance of intestinal health and it has been demonstrated that transferring the microbiome from healthy, adult hens to young chicks profoundly increases their colonisation resistance to *Salmonella*

[35]. These observations helped form the basis of the competitive exclusion concept and the application of probiotics. It is also apparent that the innate (gut) immune system is better developed and functionally more mature in young animals than adaptive immunity, and that these features are strongly influenced, particularly adaptive components, by the presence of a gut microbiome and its composition. In conjunction, there is good evidence that more rapid, enhanced, and/or less regulated, early inflammatory responses improve resistance to various pathogens. As an innate response, this may be particularly relevant for young animals due to developmental and functional differences between the two arms of the immune system outlined above. Challenges do, however, remain in fully understanding the individual and collective contributions of microbiome components in supporting optimum immune system development and functionality, and overall gut health.

Whilst the gut microbiome temporally retains a degree of plasticity, it is widely considered that early intervention probably provides the best opportunity to steer the microbiome, immunity and health, which has led to early life, including *in-ovo,* approaches. Studies have shown that the *in-ovo* (embryonic d18) supplementation of a commercial probiotic-based product (Primalac), or at hatch (PoultryStar) in conjunction with a hatchery gel droplet coccidiosis vaccine (Immunocox), reduce intestinal lesion scores and improve performance following a mixed *Eimeria* challenge [36, 37]. There are legitimate concerns that the more artificial practices (e.g. parental separation, high biosecurity, etc.) of contemporary animal production contribute to suboptimal microbiome and immune system development, and attempts have been made to seed hatchlings with the caecal microbiome from high or low performing chickens, by inoculating the surface of eggs, but results so far have not been overwhelming [38]. Probiotic use has, of course, become fairly commonplace for farm animals after birth, as have prebiotics (or their combination (synbiotics)), which, by providing substrates for microbes deemed beneficial, can help establish a more favourable gut microbiome [39, 40]. Recent

reviews have considered the effects of probiotics and/or prebiotics on poultry performance and immune responses [41, 42].

Various factors can influence microbiome and immune system development, and thus short- and long-term gut health. This includes antimicrobials, rearing environment, passive immunity, stressors (including feed and water deprivation), genetics, etc., which can all influence the multiple facets of gut health and function and have been considered in a recent review [43]. There are also numerous compounds or agents available that seek to modify the gut microbiome, immune responses, disease susceptibility and/or performance of farm animals, such as organic acids, phytogenics, bacteriophages, HDP, antibodies, enzymes, metals, clays, etc., or influence immune responses directly through the (oral) administration of, for example, TLR agonists, cytokines, vaccine antigens, adjuvants, etc. [42, 44]. It is not the intention of this review to cover these aspects in detail and so the reader is referred to the respective review(s).

Conclusion

The research community has taken great strides in better understanding components of 'gut health' and their interactions. However, the, perhaps even subtle, intricacies of these relationships are not completely understood and, currently, impedes our ability to clearly define 'gut health' (or similar terms), measure it, or confidently steer it appropriately. The host's contribution to the gut health axis primarily revolves around the immune system and the plethora of cellular pathways and interactions that are activated and regulated in response to the gut microbiome. Some of these immune processes maybe relatively redundant but nutrient costly, while others may have relatively minimal nutrient cost but be particularly advantageous. As we unravel these aspects, through the application of current, and development of new, methodologies we will be better placed to more effectively apply this knowledge through exogenous manipulation of the gut microbiome and host interactions. There is currently a

multitude of additives available that are reported to be effective at improving gut health and/or resistance to challenge. However, at the same time, many of the so called ‘AGP alternatives’ are considered to have inconsistent effects. Bringing together our developing knowledge of what we truly mean by ‘gut health’ will help drive, and inform, the more appropriate application of the current panel of AGP alternatives, as well as the development of novel products and/or strategies.

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