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Proteases and Intestinal Health in Non-ruminants: A Perspective

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Proteases and Intestinal Health in Non-Ruminants: A Perspective

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Abstract: Exogenous proteases are well established for hydrolysing proteins and improving the digestibility of amino acids, which, in animal production, enhances growth performance and diet formulation flexibility. Attention has now turned to the wider, or 'extra-proteinaceous', benefits of proteases. Both host and (gut) microbiome utilise amino acids for diverse functions beyond just cellular growth and proliferation. For example, the host produces a diverse array of proteinaceous secretions that seek to modulate the activity of the intestinal microbiome and opportunities to interact with host (e.g., epithelial) receptors. Reciprocally, the microbiome utilises various protein-based components to perform individual lifestyle traits, such as colonising gut niches, production of virulence traits, etc., which can be influenced by protease activity and amino acid availability. In addition, microbial fermentation of proteins and amino acids produces metabolites that may be considered undesirable (e.g., toxic) for the host's cells or, for example, can act as signalling molecules, shaping the secretion of gut hormones and function. Parallels may also exist between various antibiotic growth promoters and proteases, in that both can target/impact microbial proteins (synthesis and degradation, respectively). There are many aspects to consider, such as determining optimal intestinal levels and spectra of proteolytic activity under various scenarios, but great potential exists.

Keywords: protease; microbiome; intestine; pig; poultry; virulence

1. Introduction

Proteases are enzymes that hydrolyse proteins to polypeptides and free amino acids, which are more suitable forms for absorption by host enterocytes (Moran, 2016). Within animal production, initial interest in supplementing non-ruminant diets with exogenous proteases, typically of microbial (bacterial and fungal) origin, sought to target proteinaceous antinutrients and/or immunogenic components of the diet, such as trypsin inhibitors, to improve growth performance (Cowieson and Roos, 2016). Subsequently, there was a shift to focus more on potential improvements in protein digestibility, providing the opportunity for more flexibility in (least cost) feed formulation. Indeed, meta-analysis of 25 studies reported that the mean response in apparent ileal amino acid digestibility to exogenous protease supplementation was +3.7% (+2.7% to +5.6%) for pigs and poultry, with greater improvement in diets with lower inherent digestibility of amino acids (Cowieson and Roos, 2014). With improvements in ileal amino acid digestibility now well accepted, attention has increasingly turned to the potentially wider benefits of exogenous proteases on host nutrition and (gut) health, referred to as 'extra-proteinaceous' effects (Cowieson and Roos, 2016). Improving small intestine protein digestibility should increase amino acid availability to the host and help reduce fermentation of protein by bacteria (putrefaction), particularly in more distal sites, where microbial numbers and activity are higher (Apajalahti and Vienola, 2016). Protein fermentation leads to the production of potentially toxic metabolites (e.g., ammonia), which can be detrimental to intestinal (and host) health. In addition, metabolites of protein and amino acid putrefaction can also influence gut function (e.g., transit time) via interactions with the enteric nervous system, gut hormone secretion, etc., which in turn shapes intestinal microbiota composition and metabolic output (Toft et al., 2023). Moreover, proteases could also have direct effects on proteinaceous components of intestinal microbes or metabolites and/or modulate the dynamics of amino acid availability in the intestine, which can shape host-microbiome interactions.

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Microbes produce numerous proteins or protein-based components as part of, and for, their activity and functioning. Some of these proteinaceous components can be membrane-bound or secreted and thus exposed to the external milieu. Some of these microbial proteins can play essential roles in niche finding, colonisation, growth and/or dissemination (Chaban et al., 2015). Flagellin, a globular protein, is the primary component of bacterial flagella, which enables the bacterium's movement to, for example, favourable niches for colonisation. Moreover, once at a suitable colonisation site, flagellin has also been reported to act as an adherence molecule, allowing either free-living, single planktonic, or multicellular biofilm modes of growth (Chaban et al., 2015). Flagella occur widely across bacteria (Chaban et al., 2015) and biofilms are a common form of bacterial existence in the intestine (Motta et al., 2021). Biofilms form when a group of, typically polymicrobial, organisms adhere to, and colonise, a surface and produce protective, extracellular polymeric substances/matrix. Other proteinaceous structures, such as fimbriae and pili, also play important roles in adherence to host (or other microbial) surfaces. Biofilm formation is a significant virulence factor and mediate many infections, thus presenting a potential target for therapeutic intervention (Motta et al., 2021). Moreover, (e.g., disease-causing) microbes produce exotoxins (actively secreted), most of which are proteins that compromise intestinal cell, and thus host, health (Silbergleit et al., 2020). In contrast, endotoxins, generally cell wall components, are typically released upon cell division or death, and can be degraded by relevant enzymes (Frederiksen et al., 2021).

2. Opportunities for proteases to influence intestinal (and host) health

As mentioned, there could be two important opportunities for proteases to shape intestinal health: 1) direct hydrolysis of microbial endogenous proteins important to their lifestyle, and 2) modulating the availability of proteins/amino acids to the gut microbiota and/or host.

2.1. Microbial proteins as targets

Components of intestinal contents can be highly immunogenic (e.g., microbe-associated molecular patterns (MAMPs)), such as lipopolysaccharide (LPS), lipoteichoic acid, peptidoglycan, and flagellin (Vijay-Kumar et al., 2023). While the gastrointestinal tract is organised such that mechanisms exist to try and shield (e.g., mucus, location, etc.,) gut epithelial receptors (e.g., toll-like receptors (TLRs)) from engagement by these MAMPs, their degree of immunogenicity is influenced by their structure, as well as the host species (d'Hennezel et al., 2017). In fact, the total gut-derived LPS (from 'healthy' humans) has been reported to silence TLR4 (LPS receptor) signalling to promote host tolerance of (commensal) microbes (d'Hennezel et al., 2017). More recently, bacterial flagellin (recognised by TLR5) was reported to be the primary component of rodent intestinal contents responsible for inducing strong innate immune responses in-vitro and in-vivo, making it a potentially interesting target to try and reduce the immunogenicity of gut contents (Vijay-Kumar et al., 2023). Flagellin is susceptible to proteolytic degradation, which significantly reduces its immunogenicity (Lu and Swartz, 2016; Vijay-Kumar et al., 2023).

Interactions between enterotoxigenic *E. coli* (ETEC) fimbrial adhesions and the intestinal mucosa are amongst the best studied and these dynamics can be disrupted by proteases (Mynott et al., 1991). Although encouraging results have been reported, the specific effect of the protease on decreased ETEC attachment and/or diarrhoea in the in-vivo models is not always clear and could be via degradation of the microorganisms extracellular proteinaceous appendages, exotoxins, or host receptors for either. Some studies have, however, pointed to reduced stability and activity of host mucosal receptors, with localised effects of endogenous proteases suggested to shape regional differences in the activity of these receptors (Chandler et al., 1994; Mynott et al., 1996).

The effects of proteases on the formation and stability of adhered microbial communities protected by an extracellular matrix (i.e., biofilms) have been relatively well studied. Proteins are considered to play important roles in biofilm formation and stability (Mitrofanova et al., 2017), with secreted proteases helping to remodel, mature and potentially disperse the biofilm (Ramírez-Larrota and Eckhard, 2022). Both vertebrate (e.g., trypsin) and microbial (e.g., proteinase K) proteases have been reported to disrupt or destroy biofilms (Banar et al., 2016; Mitrofanova et al., 2017), although

there have been contrasting results (Gilan and Sivan, 2013), so understanding the specifics of their efficacy (or otherwise) is important.

Proteases are key players at the (intestinal) host-microbe interface for both microbe and host (Caminero et al., 2023). Given the prominence of proteinaceous microbial components in these interactions, appropriate modulation of proteolytic activity offers opportunities for interventions to influence these dynamics.

2.2. Modulating proteins/amino acid availability

We have already outlined the benefit of seeking to increase amino acid availability to the host and reducing protein fermentation by intestinal microbes. Amino acids are clearly critical for host tissue growth, but also have important and diverse roles within the host, including in immune defence, as well as influencing the composition of the gut microbiota, intraluminal metabolism, gut barrier function, and microbial pathogenesis, with disrupted metabolism of some amino acids associated with various (e.g., gastrointestinal) disorders (Nüse et al., 2023). Moreover, certain physiological states (e.g., infection) can increase the host's requirement for amino acids.

Amino acids are critical for the growth, development, virulence, and/or reproduction of most, if not all, microbes (Dai et al., 2022). Therefore, competition for amino acids occurs between microbial and host cells where these entities interact. Sufficient amino acids help stabilise the gut microbiota and its functions (e.g., colonisation resistance), helping to maintain or restore homeostasis, while pathogens may seek to deprive the host of amino acids central to (intestinal) defence (Nüse et al., 2023). In addition, amino acids can directly influence microbial virulence and disease development (Gogoi et al., 2016).

So many of the key components secreted by the host to help manage interactions with their associated microbiome are proteinaceous in nature, including antimicrobial peptides, acute phase proteins, chemokines, cytokines, mucins, secretory IgA, tight junction proteins, whereby increased expression would enhance the requirement for constituent amino acids. For example, arginine (nitric oxide) and cystine, glutamine, glycine-serine, proline, and threonine (mucins) are amongst the best considered in the context of gastrointestinal physiology (Moran, 2016). Nitric oxide can be produced in large quantities by immune cells, particularly activated macrophages, from arginine by inducible nitric oxide synthases (Bogdan, 2001). Similarly, there is considerable production of mucins by goblet cells, with basal production estimated at ~4 kg per kg of intake for growing (55 kg) pigs, and insufficient supply of amino acids critical for mucin synthesis can impair their production (Moran and Bedford, 2023).

Both the host and microbiota have requirements for amino acids and the availability of individual amino acids can influence their interactions. Changes in amino acid availability will affect host-microbiome interactions and, therefore, exogenous proteases can impact these dynamics.

3. Parallels with antibiotic growth promoters?

Targeting proteinaceous components of microbes has potential similarities to antibiotic growth promoters (AGPs). Various antibiotics inhibit bacterial protein synthesis, including those that were/are used for growth promotion in livestock (Broom, 2017). Although often fed at concentrations below relevant minimum inhibitory concentrations (MICs, defined as preventing the visible growth of target bacteria (i.e., bacteriostatic effect)), such antibiotic concentrations still impair the growth characteristics and/or function of susceptible bacteria (Broom, 2017). Effects include suppressing or altering protein and virulence factor expression/formation, thus affecting the ability of bacterial cells to adhere to surfaces, form biofilms, produce toxins, etc.), rendering them less of a (potential) threat to, or more 'easily' managed by, the host. It is interesting that the expression of flagellin, which we've highlighted here as a potential immunogen of particular interest, can be suppressed by sub-inhibitory concentrations of various antibiotics (Kawamura-Sato, 2000). In fact, sub-inhibitory antimicrobial concentrations can result in mis-assembled, -anchored, -shapen, and/or -sized bacterial flagella, impairing motility, the ability to reach surfaces for attachment, and the initial stages of biofilm

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formation (Giacomucci et al., 2019). It is interesting that, via somewhat different mechanisms, AGPs and proteases have the potential to interfere with protein-dependent bacterial lifestyle traits.

4. Conclusions

Proteins and amino acids are required by both host and gut microbiota, and thus play central roles in their dynamic relationship. Proteinaceous components are important for microbial lifestyles (e.g., intestinal colonisation), while amino acids modulate their growth and potential virulence, and help support the stability of the microbiome. Similarly, the host uses amino acids for growth of tissues, but also for mechanisms (e.g., gut secretions) to help manage their interactions with these microbes. Proteases clearly offer the opportunity to influence these dynamics. Exogenous proteases have, in general, been used in livestock to improve the availability of amino acids to the host, thereby enhancing growth, and/or more economical production, performance. This increased capture of amino acids by the host helps to reduce protein/amino acid fermentation by the microbiome, which can benefit gut health, but the benefits of proteases likely extend beyond the recent interest, as they have the potential to target important, immunogenic microbial proteins within the gut, alter host receptor activity, and/or modulate amino acid-mediated host-microbiome interactions.

Many of the exogenous proteases currently on the market have been developed with complementarity to endogenous proteases (and the host) in my mind. Getting the balance right is important when developing and applying exogenous proteases to maximise desired efficacy, while seeking to minimise any potential collateral damage (e.g., interfering with the dynamics of host protein digestion or degrading host tissues or secretions). It is worth remembering that proteases are key virulence factors deployed by various microbes to drive their pathogenesis (Frees et al., 2013). Moreover, host and/or microbiota requirements for amino acids can change in response to shifts in host-microbiome interactions and disrupted amino acid metabolism has been associated with various disorders. Due to this intricate balance, it has been suggested that an appropriate strategy should seek to modulate amino acid availability at the appropriate site, without (unwanted) knock-on effects that could be undesirable (Nüse et al., 2023), which represents a challenge. In addition, protein and amino acid-related kinetics in the intestine are complex and dynamic, and exogenous protease efficacy will be influenced by various factors, including dose, amino acid concentrations, and dietary ingredients (Cowieson and Roos, 2016).

There are various questions to consider and address in the context of this paper. For example, there is a need to better understand the general accessibility for, and susceptibility to, proteases of these microbe-associated proteinaceous components (e.g., protective flagella sheath?)? Are host endogenous proteases often lacking in desired activity, specificity, etc., against these components, which could be suitably (and economically) complemented by exogenous protease supplementation? Given proteases are both necessary and potentially harmful to both host and microbiome, what is the optimal level and spectrum of proteolytic activity in the intestine and how can this be determined in practice?

We see this area as a significant opportunity, requiring further studies to fully harness, and hope to publish more on this topic in the near future.

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