*Journal of Applied Animal Nutrition*, Vol. 2; e13; page 1 of 8

doi:10.1017/jan.2014.5

*Original Article*

# Bioefficacy of a mono-component protease in the diets of pigs and poultry: a meta-analysis of effect on ileal amino acid digestibility

Aaron J. Cowieson and Franz F. Roos
*DSM Nutritional Products, Kaiseraugst, Switzerland*

## Summary

A meta-analysis of the effect of a mono-component bacterial protease (RONOZYME® ProAct) on the apparent ileal digestibility of amino acids in poultry and swine diets was conducted to examine functional patterns, mean effects and variability of response. A total of 25 independently-conducted experiments were included comprising a total of 804 datapoints. The mean response to protease was +3.74% (SE 1.1%, $P < 0.001$) and this ranged from +5.6% for Thr (SE 1.2%, $P < 0.001$) to +2.7% for Glu (SE 1.2%, $P < 0.05$). For the most economically critical amino acids (Lys, Cys, Met and Thr) the mean response was 4.5%. The effect of protease was independent of geography, animal species and diet composition ($P > 0.05$). However, the inherent digestibility of amino acids in the control diet as a single explanatory term explained around 47% of the variance ($P < 0.001$) in effect. When the inherent digestibility of amino acids in the control diet was less than 70% protease addition improved amino acid digestibility in 90% of cases with a mean improvement of around 10%. When the inherent digestibility of amino acids in the control diet was more than 90% there was a protease-mediated improvement in digestibility in only 60% of cases with a mean improvement of around 2%. It can be concluded that the inherent digestibility of amino acids in the diet without protease supplementation is the primary explanatory term for the efficacy of this exogenous protease, demonstrating that it is highly effective in improving the digestibility of amino acids across a wide range of feed ingredients. Benchmarking diets or feed ingredients as to their relative nutritional value would enhance the ability of nutritionists to determine the likely return on investment on use of bacterial proteases in their operation.

**Keywords:** **protease: poultry: pigs: amino acid: nutrition**

*(Received 5 March 2014 – Accepted 11 May 2014)*

Journal of Applied Animal Nutrition

## Introduction

It has previously been established that the inherent digestibility of nutrients in the diet prior to enzyme intervention is a key explanatory term that predicts likely enzyme effect (Cowieson & Bedford, 2009; Cowieson, 2010). Though starch, and in most cases fat, are generally well recovered by the distal small intestine of pigs and poultry the apparent ileal digestibility of amino acids can be as low as 65–70%, particularly in diets that contain a substantial concentration of by-products (Ravindran *et al.*, 2005) or where anti-nutrients such as fibre or phytate are present at high levels (Angkanaporn *et al.*, 1994; Cowieson *et al.*, 2004). However, though on a macro-level there may be potential for more improvement in amino acid digestibility than is the case for starch, the opportunities are highly amino acid dependent. For example, the apparent ileal digestibility of Met is typically high (90-95%; Ravindran *et al.*, 2005; Cowieson, 2010) whereas the apparent ileal digestibility of Thr and Cys are often rather low (around 75–85%; Ravindran *et al.*, 2005; Cowieson, 2010). Thus, intuitively there is more opportunity for enzymes to mediate a beneficial effect for the digestibility of amino acids such as Cys, Thr, Ser, Gly, Pro and Asp than would be the case for Met, Glu, Arg or indeed Lys due to the ranking of inherent digestibility. It is likely, at least at an 'apparent' level, that the reason

**Corresponding author:** aaron.cowieson@dsm.com

that amino acid digestibility follows a moderately predictable pattern in terms of ranking of inherent digestibility is due to the amino acid profile of endogenous proteins such as mucoprotein, sloughed epithelial cells and digestive enzymes. Both basal and specific endogenous loss contribute to the apparent digestibility values reported in the literature and so though notionally the digestibility of Thr is almost always lower than Met the 'true' dietary digestibility of Thr and Met may not differ substantially, especially when transamination and synthetic amino acid use is considered. Nevertheless it is likely that protease will enhance the digestibility of Thr and Cys more consistently than Met or Arg and this has been demonstrated to be the case for carbohydrases and phytases (Cowieson, 2010). This dependency of enzymes on the quality of the diet to which they are added may explain some of the equivocal responses reported in the literature for the effect of protease on both digestibility and performance.

Thus, it was the purpose of the work presented herein to explore the relationship between inherent ileal digestibility of amino acids in the notional 'control' diet and the magnitude of the response to bacterial protease. Furthermore, functional patterns of response can give mechanistic insight where, for example, relationships exist between amino acid effect and the amino acid profile of endogenous proteins. These correlations have been shown previously for the amino acid effects of phytases and the relationship with the amino acid profile of pepsin and mucin (Cowieson *et al.*, 2008). Finally, an overview of the effect of protease on ileal digestibility of amino acids is useful to aid in least cost formulation and for consideration of alignment to the ideal protein concept.

## Materials and Methods

The focal enzyme for the present meta-analysis was RONOZYME® ProAct (DSM Nutritional Products, Kaiseraugst, Switzerland), a random acting serine endopeptidase from *Nocardiopsis prasina* and expressed in *Bacillus licheniformis*. A total of 25 independent experimental datasets were included in the meta-analysis. These experiments included published works (Iwaniuk *et al.* (2011); Angel *et al.* (2011); Viera *et al.* (2009); Messias *et al.*, (2010a) and (2010b); Carvalho *et al.* (2009); Bertechini *et al.* (2009a); Bertechini *et al.* (2009b); Guggenbuhl *et al.* (2011)) and internal research reports (DSM Nutritional Products, Kaiseraugst, Switzerland). The internal reports were generated during product development and registration trials as well as for regional marketing and research purposes and were mainly conducted in University experimental facilities. No experiments were systematically excluded from the analysis unless there was no appropriate control diet with which to contrast the protease treatment or where protease had been added only in concert with other enzyme activities. For the purposes of the present analysis only experiments where the digestibility of amino acids was reported were included i.e. performance effects etc were excluded. In most instances digestibility was reported as apparent ileal digestibility though in several experiments a N-free diet was also fed to correct apparent values to a standardised value. In order to keep the data as similar as possible apparent values were used in the analysis (as standardised values were not universally available).

Descriptive information on the scope of the database is presented in Table 1. Of the 25 datasets that were included in the analysis the vast majority (>70%) were obtained from work in broiler chickens and turkeys with the remaining 30% being fairly equally divided between pigs and laying hens. In most experiments more than one control diet was fed and as the digestibility of several amino acids are reported for each experiment a total of 804

**Table 1.** Database characteristics

| Characteristic | Number of observations (total = 804) |
|---|---|
| *Geography* | |
| Asia Pacific | 42 |
| Europe | 452 |
| Latin America | 101 |
| North America | 209 |
| *Species* | |
| Broiler | 537 |
| Laying hens | 40 |
| Pigs | 68 |
| Turkeys | 159 |
| *Ingredient profile* | |
| Corn | 330 |
| Full-fat soybean meal | 52 |
| Soybean meal | 185 |
| Bakery meal | 30 |
| Barley | 36 |
| Corn DDGS | 78 |
| Sorghum DDGS | 28 |
| Rape/Canola seed meal | 69 |
| Wheat | 37 |
| Corn/Wheat blend | 45 |
| Sunflower seed meal | 52 |
| Cottonseed meal | 18 |
| Animal protein meal | 46 |
| *Diet description* | |
| Complete, adequate | 149 |
| Complete, low protein | 175 |
| Semi-synthetic/single ingredients | 480 |

datapoints were used in the analysis. Diet characteristics varied from study to study and in many cases 'semi-synthetic' diets were fed which contained a starch, fat and mineral source as well as the test article e.g. soybean meal or bakery meal (Table 1). However, corn was the dominant cereal in the vast majority of studies and wheat was the focal cereal for only 37 out of 804 obervations (Table 1). Soybean meal was the dominant protein source in most cases, although distillers dried grains with solubles, meat and bone meal, rapeseed meal and canola were used in around 200 out of the 804 observations. Geographically 42 of the 804 datapoints were from trials run in Asia Pacific, 452 from Europe, 101 from Latin America and 209 from North America.

The data were analysed using the statistical software R, version 2.15.2 (R Core Team, 2012). A mixed model was applied using the function lme() in the library nlme (Pinheiro *et al.*, 2012). To estimate the benefit of protease overall and by individual amino acids, random intercepts were included for each of the 25 studies nested in 4 world regions. The same model was applied to estimate the control digestibilities *per se*. To model the response of protease as a function of digestibility in the control group, the digestibility of the control group and the square of the digestibility of the control group were used as fixed effects. The random effects included a random intercept for each of the 25 studies nested in 4 world regions, and random slopes of control digestibility. An approximated $r^2$ for the mixed model was calculated based on Xu (2003). Point estimates and the corresponding 95% confidence intervals were calculated and plotted for the entire range of observed control digestibilities. In all cases the response to protease is expressed as a relative change compared with the appropriate control datapoint. Absolute percentage changes were also considered and though there is an obvious scaling effect the conclusions were unchanged (data not shown).

## Results

The mean digestibility of amino acids in the control diets across all 25 experiments was 79.7 ± 1.5 % (Table 2). This ranged from 72.7% for Cys to 85.4% for Glu. Amino acids that had relatively high digestibility (>82%) included Glu, Met, Arg, Phe and Leu, and those that had low digestibility (<75%) included Cys, Gly and Thr.

The mean improvement in amino acid digestibility with protease was 3.74% with a standard error of 1.10 (Table 2). This ranged from 5.6% for Thr ($P < 0.001$) to 2.7% for Glu ($P < 0.05$). The effect of protease was greater than the mean for Thr, Cys, Gly, Pro, Ala and Val and below the mean effect for Arg, Asp, Glu, His, Ile, Leu, Lys, Met, Phe, Ser, Trp and Tyr. The digestibility of all amino acids was significantly improved relative to the control diet with the exception of Trp where the number of observations was low which contributed to this effect.

Variance in protease effect on amino acid digestibility was not explained by geography, diet ingredient mix or species ($P > 0.05$). However, as noted earlier, the database is heavily biased in favour of broilers, which makes separation of genuine species effects difficult. It is possible that were more data available for other species different effects would be observed. The digestibility of

**Table 2.** Inherent ileal amino acid digestibility and the effect of exogenous protease in poultry and pigs

| Amino Acid | Control Digestibility (%) | S.E. | Protease effect over control* (%) | N | S.E. | P < |
|---|---|---|---|---|---|---|
| Ala | 78.0 | *1.82* | 3.99 | 34 | *1.232* | *<0.01* |
| Arg | 83.4 | *1.72* | 3.55 | 51 | *1.200* | *<0.01* |
| Asp | 77.4 | *1.71* | 3.68 | 54 | *1.193* | *<0.01* |
| Cys | 72.7 | *1.70* | 5.36 | 53 | *1.195* | *<0.001* |
| Glu | 85.4 | *1.80* | 2.68 | 40 | *1.222* | *<0.05* |
| Gly | 73.7 | *1.82* | 4.35 | 38 | *1.228* | *<0.001* |
| His | 81.7 | *1.73* | 3.17 | 46 | *1.202* | *<0.01* |
| Ile | 80.7 | *1.71* | 3.20 | 54 | *1.193* | *<0.01* |
| Leu | 82.3 | *1.80* | 3.31 | 40 | *1.222* | *<0.01* |
| Lys | 80.1 | *1.70* | 3.77 | 55 | *1.191* | *<0.01* |
| Met | 84.2 | *1.71* | 3.25 | 54 | *1.193* | *<0.01* |
| Phe | 82.9 | *1.80* | 2.90 | 40 | *1.222* | *<0.05* |
| Pro | 79.3 | *1.82* | 4.09 | 38 | *1.228* | *<0.001* |
| Ser | 79.3 | *1.71* | 3.71 | 53 | *1.195* | *<0.01* |
| Thr | 75.0 | *1.71* | 5.64 | 54 | *1.193* | *<0.001* |
| Trp | 80.2 | *2.47* | 2.76 | 11 | *1.458* | *NS* |
| Tyr | 81.4 | *1.84* | 3.28 | 35 | *1.237* | *<0.01* |
| Val | 79.5 | *1.71* | 3.87 | 54 | *1.193* | *<0.01* |

**effect expressed as relative, not absolute, percentage change* amino acids in the control diet was found to be a highly significant explanatory variable for the effect of protease on amino acid digestibility (approximated $r^2 = 0.472$; $P < 0.001$, Xu (2003)). The following quadratic model captures this relationship:

$$\textit{Protease effect (\% change over control)} = 61.318 + C^{*}(-1.23448) + C^{2*}0.00629$$

where, C is the digestibility of amino acids in the control diet in percent. This quadratic model explained around 47% of the variance in the effect of protease in amino acid digestibility where an increase of around 5.5%, on average, is predicted when control digestibility is 70% and an increase of around 1.3%, on average, when control digestibility is 90%. Thus, the magnitude of protease effect decreases rapidly as control digestibility increases from 70 to 80% and more moderately from 80 to 90%. A graphical representation of this model is described in Fig. 1 and a more complete representation is illustrated in Fig. 2.

The effect of protease on amino acid digestibility indexed against Lys (100%) is presented in Fig. 3 in order to allow contrast to be made with the ideal protein concept (discussed later). The amino acids that diverge from Lys the most substantially include Thr (index 150), Cys (index 142) and Glu (index 71).

Finally, a correlation between the amino acid profile of intestinal mucin and the percentage change in amino acid digestibility with protease addition is presented in Fig. 4 ($P = 0.012$; $r^2 = 0.35$). Correlations between protease effect and the amino acid profile of amylase, trypsin, pepsin, maltase, lipase, isomaltase and a wide range of acute phase proteins proved entirely non-significant with $r^2$ values between 0 and 0.1.

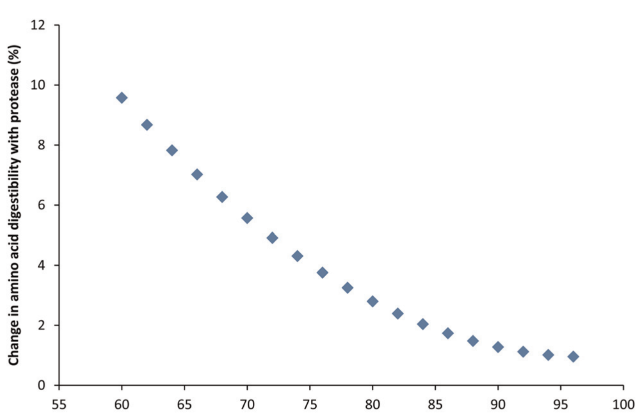


**Figure 1.** Simple graphical representation of the best fit model for the effect of protease addition based on the relative digestibility of amino acids in the control diet ($r^2 = 0.47$; $P < 0.001$).

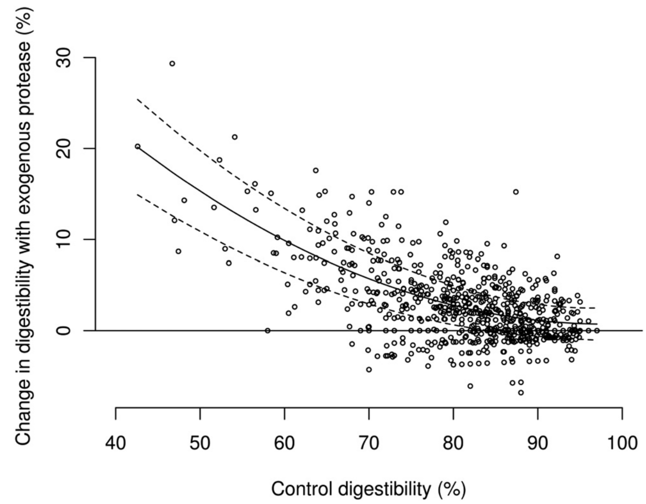


**Figure 2.** Correlation between inherent amino acid digestibility in the control diet (%) and the effect of exogenous protease (% change relative to control diet). Solid quadratic line indicates the best fit model. Dotted lines are 95% confidence intervals. Solid horizontal line indicates zero effect of protease.

## Discussion

The importance of the inherent digestibility of focal nutrients in the control diet as a predictor of enzyme effect has been reported previously (Cowieson & Bedford, 2009; Cowieson, 2010). However, these previous reports have focused almost exclusively on phytase and carbohydrase (largely xylanase, glucanase and amylase). To the authors knowledge, the data reported herein are the first that have been reported for a mono-component protease that shows that the relationships previously observed for other enzyme classes also hold for protease. Though this is of significance for

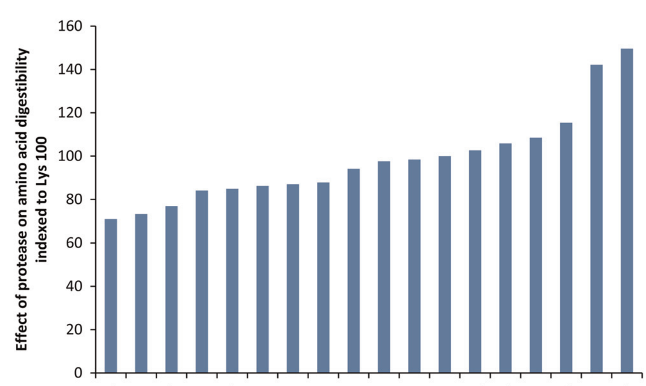


**Figure 3.** Effect of protease on ileal amino acid digestibility in pigs and poultry index Lys 100.

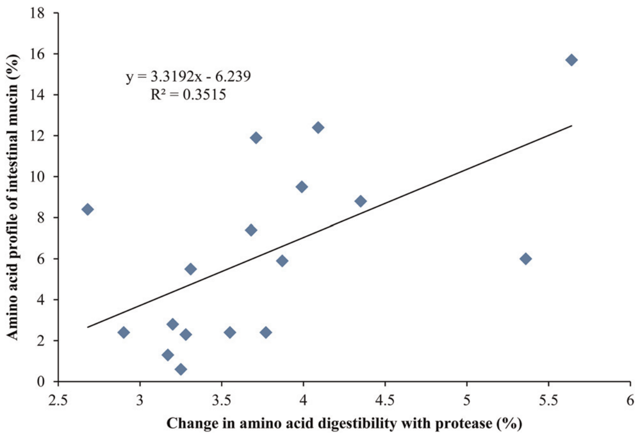


**Figure 4.** Correlation between the amino acid profile of intestinal mucin and the effect of protease on ileal amino acid digestibility ($P = 0.012$).

optimisation of use of protease at an end user level and to help explain equivocal responses to protease it is of substantial importance at a macro-level for understanding enzyme responses *per se* and also how enzymes may be used simultaneously to best effect.

There has been interest in supplementation of poultry and swine diets with exogenous proteases for several decades in order to augment endogenous peptidase production that may be insufficient in the neonate. Krogdahl and Sell (1989) reported that intestinal and pancreatic protease production increased throughout the first 21d of rearing of turkey poults suggesting that endogenous production of digestive enzymes may be inadequate in the initial post-hatch period. There are a handful of relatively early publications that showed promising responses to exogenous protease (Simbaya *et al.*, 1996; Rooke *et al.*, 1998; Ghazi *et al.*, 2002; Ghazi *et al.*, 2003) and these responses have been confirmed more recently (Angel *et al.*, 2011; Freitas *et al.*, 2011; Fru-Nji *et al.*, 2011). Furthermore, in a recent study where dose response of protease was investigated, a quadratic improvement in FCR was observed which is supportive of the data presented herein (Freitas *et al.*, 2011; Figs. 1 & 2).

The major conclusion of the data reported in the present paper is that proteases are more effective in situations where the digestibility of amino acids in the diet is low. Indeed, for every 10% decrease in ileal amino acid digestibility the efficacy of protease doubles (Fig. 1). Though this relationship explains only around 50% of the variance in the effect of protease, it is of considerable significance as a single explanatory. Thus, whilst the efficacy of protease at an end user level is likely to be influenced by a range of interacting terms, an appreciation for the 'starting point' is critical as is an understanding of other management, nutrition, health and husbandry factors that may influence the digestibility of amino acids with a concomitant effect on protease value. Such factors may include animal species, age, diet or ingredient quality, nutrient balance, use of competitive micro-ingredients, environment, disease status, whole grain feeding and feed processing conditions.

Huang *et al.*, (2005) report the effect of broiler age (d14 vs. d42) on apparent ileal amino acid digestibility of a range of common feed ingredients. Though it may be intuitively expected that amino acid digestibility would increase with age (for reasons suggested above), in fact for wheat specifically, and to some extent also for canola meal and meat and bone meal, amino acid digestibility declines from d14 to d42. Based on data presented by Huang *et al.* (2005), a theoretical blend of 50% wheat and 50% canola meal would have a mean amino acid digestibility on d14 of 78.7% and on d42 of 75.8%, representing an age-dependent decrease in AA digestibility of −2.7%. However, a similar blend of maize and soybean meal would have a mean amino acid digestibility on d14 of 81.6% and on d42 of 85.5% representing an age-dependent increase in AA digestibility of +4.7%. This is of significance *per se* but particularly in the context of the likely responsiveness to exogenous protease as the inherent digestibility is such a pivotal explanatory of effect (Fig. 1). Indeed, based on the meta-analysis presented herein and the results of Huang *et al.* (2005) the predicted response to protease for a wheat/canola-based diet would increase from around 3% on d14 to 4% on d42 whereas for a corn/soy-based diet the effect of protease would decrease from 2.2% on d14 to 1.8% on d42. These age x diet base interactions may explain why there have been equivocal reports in the literature (Wallis & Balnave, 1984; Ten Doeschate *et al.*, 1993; Huang *et al.*, 2005) on the effect of animal age on amino acid digestibility and also explain some of the age-related changes in protease effect.

In addition to growth stage, there are a variety of other factors that modulate the digestibility of amino acids in the diets of pigs and poultry and so will invariably influence protease efficacy. These factors include dietary fat concentration (Li & Sauer, 1994; Cowieson *et al.*, 2010), xylanase and phytase (Hew *et al.*, 1998; Cowieson & Bedford, 2009), dietary calcium or limestone inclusion concentration (Wilkinson *et al.*, 2013), supplemental amino acids and amino acid density (Fan *et al.*, 1994; Selle *et al.*, 2007), ingredient type and quality (Lemme *et al.*, 2004; Liu *et al.*, 2013a), anti-nutrients (Angkanaporn *et al.*, 1994; Mosenthin *et al.*, 1994; Cowieson *et al.*, 2004; Clarke & Wiseman, 2005) and hydrothermal conditioning (Liu *et al.*, 2013b). It is beyond the scope of the present paper to detail these various effects (which will almost certainly overlap in the way they influence nutritional value) but it is consistent with the results presented in Fig. 1 to suggest that the efficacy of exogenous protease will be influenced by factors that leverage the inherent digestibility of amino acids in the diet.

As presented in Table 2 the improvement in ileal amino acid digestibility with protease was not the same for all amino acids examined. Responses ranged from >5% for Thr and Cys to <3% for Trp and Glu. It is interesting that the pattern of response to protease is similar to that of xylanase (Cowieson & Bedford, 2009) and phytase (Selle & Ravindran, 2007). In fact, there is a highly significant correlation between the effect of protease and the effect of xylanase ($P < 0.001$; $r^2 = 0.46$) and phytase ($P < 0.001$; $r^2 = 0.36$) on ileal amino acid digestibility (data not shown). This is indicative of a shared reliance on the inherent digestibility of amino acids in the diet prior to enzyme intervention. However, though 'room for improvement' is an explanatory of effect for various feed enzymes, the substrates that these enzymes target are clearly very different and there is unlikely to be substantial overlap in effect. Furthermore the effect of phytase on amino acid digestibility is equivocal, having been determined clearly in only around 50% of studies (Selle & Ravindran, 2007) so though pattern of effect between phytase and protease may be similar in some instances, the consistency of effect is not.

As noted in Fig. 4 there is a significant correlation between the amino acid profile of intestinal mucin and the effect of exogenous protease on amino acid digestibility. This relationship has been previously determined for both xylanase and phytase (Cowieson *et al.*, 2008; Cowieson, 2010). It is possible then that part of the beneficial effect of exogenous protease is mediated via a reduction in the loss of mucoprotein from the intestine. This may be associated with a reduction in mucin secretion or an increase in the autolytic recovery of mucin, or both. The mechanism by which this may occur is not clear but it is possible that exogenous protease reduces the secretion of HCl and pepsin in the gastric phase of digestion, reducing the need for mucin as a protective agent in the intestine. A similar mechanism has been proposed for the effect of phytase on protein solubility and digestion (Cowieson *et al.*, 2009). An alternative hypothesis is that exogenous protease influences the rate of recovery of amino acids in the intestine. Liu *et al.*, (2013c) observed a significant increase in the rate of amino acid recovery in the small intestine of broilers fed a sorghum-based diet when the diet was supplemented with bacterial protease. Kalmendal & Tauson (2012) show that supplementation of a wheat-based diet with protease reduced the relative length of the ileum in broiler chickens and that this was associated with improved FCR. These effects are indicative of protease-mediated changes in the dynamics of nutrient recovery and these may be of more importance than ileal digestibility changes *per se*. If protease increases the rate of recovery of amino acids and in doing so mediates a shortening of the small intestine then these effects would be detected only in FCR or other 'net' metrics and not necessarily in ileal digestibility coefficients. The effect of exogenous protease on endogenous secretion, particularly mucin, and on gut health and morphology, warrants further attention.

A final consideration for the appropriate use of exogenous protease is maintenance of amino acid balance. Baker & Han (1994) suggested that amino acids should be provided to broiler chicks in particular ratios to Lys and this so-called 'ideal amino acid' or 'ideal protein' concept is relatively well accepted by nutritionists and has been more recently expanded to include Val, Ile and Arg (Lemme *et al.*, 2003). Broadly, with Lys indexed at 100, the chicks' requirement for Met, SAA, Ile, Thr, Trp, Val and Arg are indexed at 44, 75, 70, 65, 16, 80 and 105 respectively. Therefore though some interventions (such as those detailed above) may increase amino acid digestibility coefficients it is possible that the pattern of such a response will deviate from the ideal protein concept. Thus, 'ideally' for every 1 unit of digestible Lys yield from an exogenous protease there should be 0.75 units of digestible SAA and 0.70 units of digestible Ile (for example). The effect of protease on amino acid digestibility indexed against Lys is presented in Fig. 3. In some instances the non-Lys amino acid yield by protease is well balanced relative to the yield for Lys. For instance, protease yields around 0.8 units of Ile for every 1 unit of Lys (against an ideal protein index of 0.7 to 1). However, in other instances, such as Thr, the yield relative to Lys is around 140 against an ideal protein concept of only 65, possibly resulting in an amino acid imbalance unless formulation strategies can overcome this risk. Relative to Lys the amino acids where protease addition may cause nutritional excess include SAA, Thr and Val whereas Arg may be undersupplied by protease relative to Lys. These qualitative effects will depend on the health status of the bird or pig, growth stage and also the interaction with quantitative amino acid supply.

It can be concluded that exogenous protease has potential to increase the amino acid digestibility of the diets of pigs and poultry. Equivocal responses in the literature may be partially explained by the leveraging effect of control diet digestibility. Prior to application of exogenous protease at an end user level consideration should be given to the inherent digestibility of amino acids in the diet and to the balance of amino acids relative to Lys. Protease may have considerable potential to improve gut health via sparing effects on mucoprotein and intestinal maintenance and further work is required on the interaction between exogenous protease and intestinal health, mucin integrity and enteric disease.

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