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Relevance of amino acid digestibility for the protein utilization efficiency in poultry

Habilitation thesis
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Abbreviations

In addition to abbreviations for the units defined by The International System of Units the following abbreviations were used:

AA	Amino acid
ADL	Acid detergent lignin
Adj. R^2	Adjusted coefficient of determination
Ala	Alanine
$aNDF_{om}$	Neutral detergent fiber determined without residual ash and after treatment with α-amylase
Arg	Arginine
Asx	Aspartic acid/asparagine
Ca	Calcium
CA	Crude ash
CF	Crude fiber
CL	Crude fat
CP	Crude protein
CV	Coefficient of variation
Cys	Cysteine
δPUE	Difference in protein utilization efficiency
Eq.	Equation
FTU	Phytase units
GE	Gross energy
Glx	Glutamic acid/glutamine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
ME_N	Nitrogen-corrected metabolizable energy
Met	Methionine
N	Nitrogen
NIRS	Near infrared reflectance spectroscopy
NSP	Non-starch polysaccharide
P	Phosphorus
pc	Prececal/prececally
Phe	Phenylalanine
pp	Percentage point
Pro	Proline
PUE	Protein utilization efficiency
R^2	Coefficient of determination
SD	Standard deviation
Ser	Serine
Thr	Threonine
TPUE	Total protein utilization efficiency
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

1 Extended introduction

1.1 General introduction

Poultry meat and eggs currently are the most common animal-based foods worldwide, followed by pork, beef, and sheep meat. Increasing meat consumption is expected for all of the mentioned meat types in the next decades, with poultry meat expected to increase more relative to other meat types (OECD/FAO 2021). Production of animal-based food requires land area and water, mainly through the production of feed crops (Mottet and Tempio 2017). Hence, arable land and water for feed cropping is expected to become an increasingly scarce resource. Another aspect is the environmental impact of animal farming, which has led to growing concerns over the past decades. Feed production and emissions from excreta represent the major impacts of animal farming (Gerber et al. 2013). Major environmentally relevant impacts of feed production include energy consumption of crop farming and transport, emissions from the fields, and the consequences of land-use change when crops are cultivated on converted forests or grasslands. Regarding excreta, the major environmentally relevant impacts include nitrogenous emissions (ammonia, nitrates, nitrous oxide), phosphorus (**P**) emissions, or fine particles. These emissions contribute to climate change, acidification, eutrophication, and air and water pollution. Those emissions occur in the barn, during manure storage, and during and after application of the manure on the fields (Cappelaere et al. 2021).

The aims of farm animal nutrition research include to minimize the negative effects of farm animal husbandry on the environment, to ensure animal health and well-being as well as to contribute to global food security. Investigations identifying nutrient and energy requirements and evaluating feed ingredients and feed additives contribute to these aims (Gesellschaft für Ernährungsphysiologie 2017b). Such investigations have enabled a considerable reduction in protein concentrations in poultry feed without performance loss. This change has reduced nitrogen (**N**) excretion substantially (Siegert and Rodehutscord 2019; Cappelaere et al. 2021). Hence, animal nutrition research allows to reduce the needed amount of feed crops and the environmental impact of poultry farming. A key figure to assess the efficiency of the production of animal-based food is protein utilization efficiency (**PUE**).

Formulating diets to match digestible amino acid (**AA**) concentrations with the requirement for AA is one way to increase PUE. This thesis evaluates the current state of knowledge on the

relevance of AA digestibility on PUE and puts the relevance of AA digestibility into the context of other strategies to increase PUE.

1.2 Definition and relevance of the protein utilization efficiency

There are numerous concepts of PUE depending on the subject of interest. Within the field of animal nutrition research, PUE usually is regarded as protein that is not excreted via feces and urine relative to the ingested protein (Lewis et al. 1976). Protein thereby commonly is measured as crude protein (**CP**), which is defined as N multiplied by 6.25. Hence, PUE in poultry nutrition research usually is calculated as:

$$\text{PUE (\%)} = \frac{\text{N accreted in body weight } \left(^{g}/_{d}\right) + \text{N accreted in eggs } \left(^{g}/_{d}\right)}{\text{ingested N } \left(^{g}/_{d}\right)} \times 100 \qquad \text{[Eq. 1]}$$

This definition is used in this thesis unless stated otherwise. Another definition on the efficiency of protein utilization, herein named total protein utilization efficiency (**TPUE**), combines the protein utilization of animals with the fertilizer value of the excreta. TPUE considers N losses occurring after excretion until manure is applied, as e.g. quantified by Portejoie et al. (2004) for pigs. High PUE is favorable for several reasons:

1) The demand for animal-based food worldwide has been increasing while the arable land for cropping has been decreasing for decades (Figure 1). These developments are prognosticated to continue in the future (FAO Agricultural Development Economics Division 2012). Hence, a shortage of feed ingredients is to be expected alongside increasing competition between the use

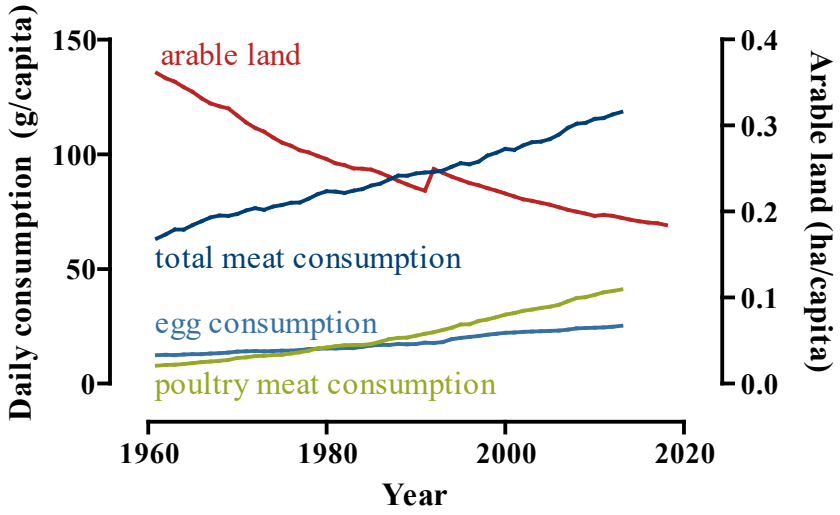

Figure 1. Global consumption of selected animal-based food (FAOSTAT 2021) and arable land per person (World Bank 2021). Note that the data on arable land are only available after 1990 for some regions.

of crops as food or feed. The constrained supply impacts prices. Increased prices have been shown to affect the affordability of food especially in developing countries (Fazeni and Steinmüller 2011). Higher PUE would mean that the same amount of animal protein-based food can be supplied using less feed protein. Thus, PUE is one important efficiency measure that needs to be pursued to moderate the consequences of constrained supply. Utilization efficiency measures of other constituents of animal feed, like energy, are also relevant but will not be dealt with herein.

2) The excretion of nitrogenous compounds by animals has negative effects on the environment and the health of animals and humans. N emissions into the water, soil, and air are inevitable. Consideration of nutrient cycling includes that manure is used to fertilize plants, but this practice risks N leakage into the groundwater when too high amounts are distributed (Verstegen and Jongbloed 2003). Part of the N in manure is volatilized as ammonia. The volatilized N reduces TPUE and, hence, represents a loss in efficiency that would not occur when feed ingredients are used instead as human food. Ammonia volatilization occurs in the animal house, during manure storage, and with application of the manure on the ground (Portejoie et al. 2004). The environmentally damaging effects of ammonia include soil acidification, eutrophication, formation of fine particulates, and secondary emissions from nitrous oxide (Martínez-Lagos et al. 2013). Ammonia exposure also represents a health risk for animals and humans and impairs the acceptance of animal husbandry by the public due to the unpleasant odor (Aneja et al. 2009). Ammonia volatilization is highly variable. The influences include stable ventilation (Knížatová et al. 2010), the manure application technique (Huijsmans et al. 2001), weather conditions such as ambient temperature and wind speed (Huijsmans et al. 2001), and the characteristics of the manure. Relevant manure characteristics include pH and moisture, but excretion of urinary N by animals is the most relevant determinant (Groot Koerkamp 1994, 1998).

3) Increased PUE is associated with less water consumption. This factor is of immediate importance in arid areas and probably will become more momentous throughout the world due to climate change. Further, water excretion by animals causes wet excreta, which promote ammonia formation (Groot Koerkamp 1994) and health issues, including footpad lesions (Lemme et al. 2019; van Harn et al. 2019). Reduced water consumption with increasing PUE is caused by several mechanisms. First, when PUE is increased by decreasing dietary CP, proportions of soybean meal in diets usually decrease. As soybean meal is rich in potassium, the animals need water to excrete excess potassium renally. Second, when PUE is increased by adjusting the AA supply to the requirement of the animals, less heat is produced by metabolic processes. Hence, less water is needed to maintain body temperature, particularly when animals

are kept in high ambient temperatures (Alleman and Leclercq 1997). Third, water is needed to excrete surplus N renally. This effect is less relevant in uricotelic species, like poultry, compared with ureotelic species, like pigs or cattle.

There are no benefits of aiming for low PUE in farm animal husbandry including poultry. Diminished PUE may be accepted when high PUE conflicts with other aims, such as economic, legal, or management constraints.

1.3 Nutritional influences on protein utilization efficiency

Increasing animal performance such as growth rate or egg production raises the PUE. The total AA requirement of animals is split into requirements for maintenance, representing the AA requirement to maintain body functions, and requirements for performance, like protein accretion in body weight or eggs (Gesellschaft für Ernährungsphysiologie 1999). The N supplied to fulfill the AA requirement for maintenance is excreted completely. Increasing performance raises the total AA requirement but decreases the ratio between the AA requirement for maintenance and the total AA requirement. Hence, increasing performance reduces the N excreted per unit of animal product. This effect is described extensively in the literature (e.g., Flachowsky and Lebzien 2007). Recently, studies determined an increasing PUE when performance of broiler chickens was reduced as a consequence of nonessential AA deficiency (Hofmann et al. 2019, 2020b). This outcome probably can be explained by maximized AA utilization efficiency (increment in AA accretion per increment in AA intake) at an AA intake level below what is needed for maximized AA accretion. This phenomenon was found for methionine (**Met**) (Fatufe and Rodehutscord 2005) and lysine (**Lys**) (Fatufe et al. 2004) in broiler chickens and for eight AA in the rainbow trout (Rodehutscord et al. 1995, 1997). Potential physiological explanations for these results include a lower muscle protein turnover in the state of AA deficiency (Tesseraud et al. 1992; Urdaneta-Rincon and Leeson 2004) and, specifically for poultry, that urinary N was reabsorbed in the hindgut and used for the synthesis of nonessential AA (Karasawa 1989, 1999). Increased PUE at an AA supply that does not allow for maximum performance results in a conflict between maximized growth and maximized PUE. At present, high growth performance commonly is considered most important for diet formulation, and reduced nutrient excretion is the next highest priority. However, expected future economic and ecological constraints (see Section 1.2) may lead to priority changes in the industry. Submaximal growth might be accepted when maximizing utilization of protein sources emerges as more economically beneficial than maximizing growth. Reasons

for that may include higher prices of protein sources and increasingly strict fertilizer legislation that which determines the animal production volume of a farm.

Recommended AA concentrations in diets decrease considerably throughout a production period of laying and growing poultry, mainly because of the changing N-corrected metabolizable energy (**ME**$_N$) requirement (Gesellschaft für Ernährungsphysiologie 1999). Phase feeding is an established strategy where several diets with AA concentrations adjusted to the concentrations recommended for specific growth or performance period are offered. The alignment of recommended dietary AA concentrations and actual AA concentrations in diets is closer the more phases are implemented (Jeroch 2020). This approach reduces surpluses in AA supply and, hence, increases PUE.

The requirements of animals for some single nitrogenous nutrients and the digestibility of the nitrogenous nutrients are often not exactly known. In compound feeding, feed formulators usually counter these uncertainties by formulating the feed with higher concentrations of nitrogenous nutrients than what could be utilized by the animals. Such safety margins offer the opportunity to increase PUE by two nutrition-related strategies. The first is to increase knowledge of the requirement of animals for single nitrogenous nutrients. This approach offers the possibility to reduce the amount of dietary CP that usually is included to account for uncertainties in requirements. Proteinogenic AA thereby represent the biggest part of relevant nitrogenous nutrients. In the past, research had focused almost exclusively on essential AA. Within the past decade, researchers have increasingly focused on requirements for nonessential AA, which resulted in a considerable potential to increase PUE (reviewed by Siegert and Rodehutscord 2019).

The second nutrition-related strategy to reduce safety margins in supply with nitrogenous nutrients is to increase knowledge of AA digestibility. The term ‘digestibility’ usually refers to the proportion of an ingested nutrient that has disappeared in the entire digestive tract or up to a certain section of the digestive tract (Simon 2020). It is a measure for the proportion of dietary AA that can be utilized by the animals. Knowledge of AA digestibility enables formulating diets according to the requirement of the animals for digestible AA. Knowledge of AA digestibility is a prerequisite to avoid excess or deficient supply of digestible AA (Figure 2). A deficient supply leads to decreased PUE due to of performance loss, degradation of other AA because they cannot be used for protein synthesis due to AA deficiency (Simon 2020), and health issues in certain situations (Alagawany et al. 2020). An excess supply causes decreased PUE because AA that cannot be used for protein synthesis are deaminated by the animal (Simon

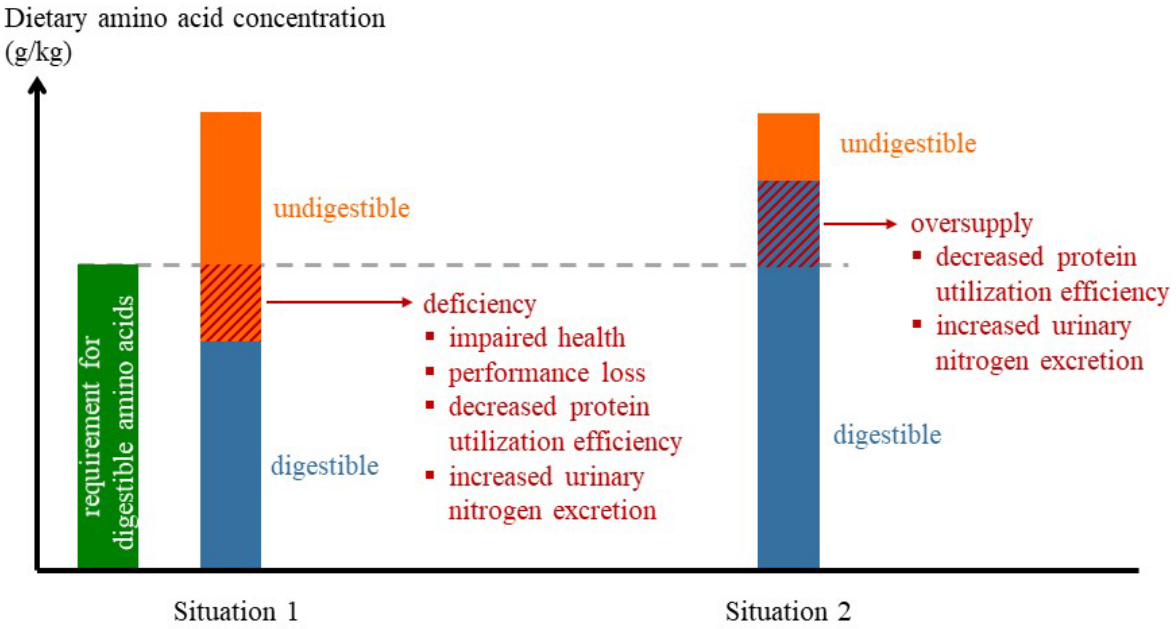

Figure 2. Scheme of consequences of a low (situation 1) and high (situation 2) amino acid digestibility of diets with identical analyzed amino acid concentrations resulting in a lower and higher supply with digestible amino acids relative to the requirement, respectively. See the text for references.

2020). In both cases, more N relative to the intake is excreted via the kidneys (Simon 2020), which is the fraction of excreted N of particular environmental relevance (see Section 1.2).

The advantage of formulating diets on the basis of digestible instead of total AA concentrations meanwhile is commonly accepted. A wealth of studies revealed that this practice increases predictability of performance, particularly when dietary ingredients with a wide range of AA digestibility were used (Fernandez et al. 1995; Rostagno et al. 1995; Khaksar and Golian 2009). By contrast, studies investigating the effects of formulating diets based on digestible instead of total AA on PUE are not available. However, the previously mentioned effects of performance on PUE suggest that PUE also was increased along with performance in the studies of Fernandez et al. (1995), Rostagno et al. (1995), and Khaksar and Golian (2009).

1.4 Determination of amino acid digestibility

1.4.1 Sampling sites and methods

Irrespective of the animal species, AA digestibility represents the proportion of an ingested nutrient that has disappeared in the entire digestive tract or up to a certain section of the digestive tract. Therefore, samples of the content of the digestive tract need to be obtained. This endeavor is not complicated if the total tract digestibility is measured because collection of excreted feces is relatively simple. In nonruminants like poultry or pigs, however, total tract

AA digestibility is little meaningful. Reasons are that AA absorption is completed at the end of the ileum (Webb 1990) and microbial fermentation in the post-ileal digestive tract can change the amount of protein contained in the digesta and its AA composition (Thornburn and Willcox 1965). Post-ileal changes in the amount of protein in the digesta and its AA composition on determined AA digestibility is substantial (Ravindran et al. 1999b). As a consequence, AA digestibility excluding post-ileal fermentation effects has been established as the most precise way to characterize a diet or dietary component regarding its digestibility. AA digestibility not excluding post-ileal fermentation meanwhile is generally accepted as unsuitable (Ravindran et al. 2017).

Three methods – each with advantages and disadvantages – are commonly used to obtain digesta, with an aim to determine AA digestibility excluding post-ileal fermentation in poultry and pigs:

1) Sampling digesta using a t-cannula that is surgically implanted at the end of the ileum is a method commonly used in pigs (Gesellschaft für Ernährungsphysiologie 2005; Bach Knudsen et al. 2006) and has been applied in some studies on adult roosters (Raharjo and Farrell 1984; Gurnsey et al. 1985) and laying hens (Kamisoyama et al. 2011). The major advantage of this method is the possibility for repeated measurements. The disadvantages include the effort for post-surgical animal care, the difficulty of implanting the small cannulas in poultry, a possible rejection of the cannula, altered peristalsis and flow of digesta through the cannula (Ravindran and Bryden 1999; Bach Knudsen et al. 2006), and an undigestible marker is needed to determine digestibility. The high growth rate and short production period of broiler chickens make digesta sampling via t-cannulas impractical in this group of animals.

2) A method commonly applied in studies on small animals, like broiler chickens, and some pig studies is to obtain digesta from the terminal small intestine after slaughtering the animals. This method requires little effort to sample digesta from any section of the digestive tract and surgical interventions and associated animal care are not needed. The disadvantages include that small sample amounts are obtained from one point in time, no repeated measurements with the same animals are possible (Gesellschaft für Ernährungsphysiologie 2005; Bach Knudsen et al. 2006), and it is necessary to include an undigestible marker. The results are highly variable among individual animals. To a large extent, this variation probably is due to sampling at one point in time. Digesta from several animals are often pooled into one sample to compensate for differences between individual animals and to increase the sample size. A possible source of error for accurate sampling is post-mortal peristalsis, but Kluth and Rodehutscord (2009b)

concluded in their review that the consequences of post-mortal peristalsis seem to be of minor relevance.

3) Using birds with surgically removed ceca, that is, cecectomized birds, is an alternative method suitable especially for non-growing poultry, like laying hens and roosters. Cecectomy markedly decreases the impact of microbial fermentation in the post-ileal digestive tract because the ceca are the major location of microbial fermentation in birds (Parsons 1985). Cecectomy is not a suitable alternative for pig and growing poultry studies because the microbial fermentation in the large intestine of pigs is considerable (Gargallo and Zimmerman 1981) and the post-surgical recovery time is too long compared with the short production period in most types of growing poultry. In pigs, the effects of microbial fermentation in the large intestine can be excluded by ileo-rectal anastomosis (Laplace et al. 1994). Once recovered from surgery, cecectomized adult birds do not require different care compared with intact animals. This surgery allows to conduct experiments with few experimental animals and large sample sizes based on total excreta collection that are obtained with the same animals within a short period of time. The quantitative sample collection eliminates the disadvantages of spot-sampling and renders the inclusion of undigestible markers, which are needed for the aforementioned methods, unnecessary. This eliminates the sources of error associated with markers, including representative flow through the digestive tract and analytical errors (Rezvani et al. 2007; Adedokun et al. 2009). The CP digestibility cannot be determined using this method because the collected excreta include urinary N, which is excluded in true protein analyses. Hence, true protein digestibility is more informative than CP digestibility in cecectomized birds. Determining glycine (**Gly**) digestibility using this method is inaccurate because a part of the Gly measured in the collected excreta can originate from uric acid hydrolysis during sample preparation (Dalgliesh and Neuberger 1954). These drawbacks could be avoided by separating the excretion of feces and urine by surgically establishing a stoma, that is colostomized animals, (Ivy et al. 1968) but care for such animals is intense. Determining AA digestibility based on quantitative excreta collection of cecectomized hens yielded digestibility values similar to those determined after collection of digesta from the terminal small intestine of slaughtered hens (Rezvani et al. 2008a). Variation between replicates in the study by Rezvani et al. (2008a) was substantially smaller when excreta were collected quantitatively. The authors concluded that more precise measurements of AA digestibility determined by quantitative excreta collection compared with spot-sampling from the terminal small intestine increase the likelihood of detecting differences in digestibility between AA sources.

Concerning terminology, ‘prececal (**pc**) digestibility’ or ‘ileal digestibility’ is often used to distinguish digestibility excluding post-ileal fermentation from total tract digestibility (Simon 2020). This thesis will only use ‘pc digestibility’ because this term is used consistently by the Committee on Nutrient Requirement Standards of the Society of Nutrition Physiology (Gesellschaft für Ernährungsphysiologie 2008, 2017a) and Simon (2020) argued that ‘ileal digestibility’ might be understood as the digestibility only occurring within the ileum. However, the term ‘pc digestibility’ is only suitable when samples are taken from the digestive tract proximal to the ceca, that is, cannulas or the slaughter technique, was used but not when samples of cecectomized birds are collected quantitatively because no pc measurements were performed. In the absence of a concise term, digestibility obtained in studies on cecectomized birds usually is simply termed ‘digestibility’ (e.g., Rezvani et al. 2007), although the downside is potential confusion with total tract digestibility that includes post-ileal fermentation.

1.4.2 Relevance of endogenous amino acid secretions and their determination

AA in the digesta consist of unabsorbed AA from the diet and endogenous AA. Endogenous AA originate from abraded epithelial cells and mucins as well as secreted proteins from saliva and secretions from the stomach, pancreas, and small intestine (Adedokun et al. 2009). Endogenous AA can be separated into basal endogenous AA, which are independent of the diet composition, and specific endogenous AA, which originate from particular characteristics of the diet (Butts et al. 1993; Donkoh and Moughan 1999; Rodehutscord et al. 2004). Feed intake commonly is regarded as the biggest determinant of basal endogenous AA present in the digesta (Butts et al. 1993; Adeola et al. 2016).

In studies focusing on the supply of animals with digestible AA, both determining AA digestibility without exclusion of endogenous AA or AA digestibility excluding basal endogenous AA are considered advantageous depending on the aim. AA digestibility excluding both basal and specific endogenous AA is only interesting for a few special research questions focusing on the potential to absorb AA or the amount of endogenous AA, rather than the supply of digestible AA (e.g., Angkanaporn et al. 1997). Determining AA digestibility without exclusion of endogenous AA, often designated ‘apparent AA digestibility’, is easy to determine because any effort to quantify endogenous AA is omitted (Lemme et al. 2004). This represents a suitable measure to determine differences between dietary treatments provided that basal endogenous AA are hardly affected. Determining AA digestibility excluding the basal endogenous AA but including specific endogenous AA is preferable when the supply with digestible AA of one feed ingredient is studied. Excluding basal endogenous AA from AA

digestibility has been shown to increase additivity of digestible AA concentrations in mixed diets – meaning that the amount of digestible AA in a mixed diet is closer to the sum of digestible AA obtained from each dietary ingredient – in broiler chickens (Angkanaporn et al. 1996; Kong and Adeola 2013a), Pekin ducks (Hong et al. 2001; Kong and Adeola 2013a), turkeys (Osho et al. 2019), and pigs (Furuya and Kaji 1991; Xue et al. 2014).

Methods of determining basal and specific endogenous AA have been reviewed extensively in the literature (Ravindran and Bryden 1999; Lemme et al. 2004; Ravindran 2021), so only methods relevant for this thesis are outlined herein. The most commonly used method is to measure apparent AA digestibility and basal endogenous AA separately and to subtract the basal endogenous AA losses from the AA determined in the digesta. The resulting digestibility estimate commonly is designated 'standardized AA digestibility'. Using this method, two experimental diets are needed to determine the standardized AA digestibility of a feed ingredient. In one diet, the digestibility of a feed ingredient without consideration of basal endogenous AA is measured using a diet that contains the feed ingredient under study as the only source of AA. Basal endogenous AA are measured by using another diet devoid of N. This approach assumes that feeding a N-free diet causes secretion of basal endogenous AA identical to that of a diet with AA concentrations that fulfill the requirement of the animals. However, the absence of protein in the N-free diet and the unbalanced nutrient composition of the diet containing the feed ingredient under study considerably affect the physiological state of an animal, leading to the conclusion that the basic assumption of this approach cannot hold true. Another aspect is that the determined basal endogenous AA depend on the composition of the N-free diet (Kluth and Rodehutscord 2009a; Kong and Adeola 2013c; Adedokun et al. 2017) or age (Barua et al. 2021a). Irrespective of the downsides of this approach, excluding basal endogenous AA determined using N-free diets from AA digestibility increases the additivity of AA digestibility because any of the studies on additivity of AA digestibility cited in the previous paragraph have used this method.

An alternative is to determine AA digestibility excluding basal endogenous AA of a feed ingredient using a regression approach (Figure 3), which was first proposed by Rodehutscord et al. (2004). This approach is based on the linear relationship between the amount of ingested and digested AA (Rodehutscord et al. 2004; Rezvani et al. 2008b; Kluth and Rodehutscord 2010). Two or three experimental diets are needed using this approach: a basal diet containing a feed ingredient devoid of AA (usually cornstarch), a diet containing the test ingredient at the expense of the feed ingredient devoid of AA, and, if needed, a diet containing the test ingredient and the feed ingredient devoid of AA in equal shares. All diets are formulated identically apart

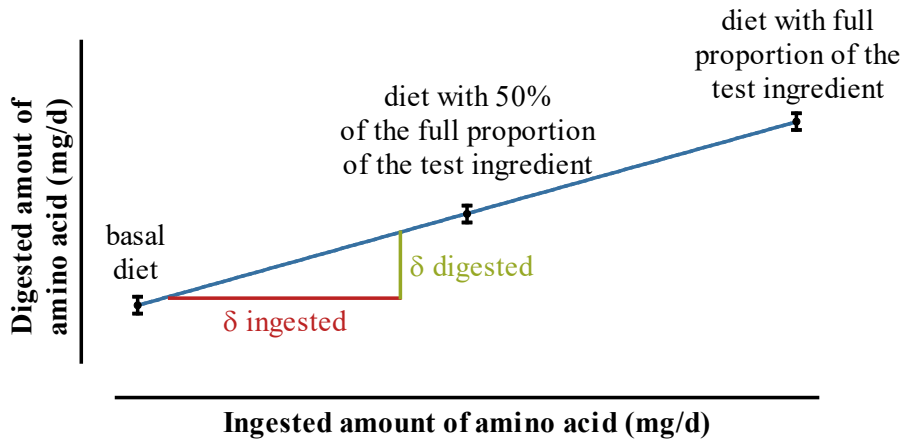

Figure 3. Scheme of the regression approach to determine the amino acid digestibility of a test ingredient.

from the test ingredient and the feed ingredient devoid of AA. Whether two or three experimental diets are needed depends on the accuracy of single measurements. Two experimental diets (e.g., Study 8; Zuber et al. 2016) have emerged as sufficient in studies on cecectomized laying hens because the quantitative excreta collection enables highly precise measurements (Rezvani et al. 2008a). Three experimental diets (e.g., Study 12; Bormann 2019) are advantageous in studies when digesta are collected from the terminal small intestine to increase the number of degrees of freedom to counteract the lower precision of single measurements (Rezvani et al. 2008a). Ideal concentrations of the feed ingredient devoid of AA and the test ingredient are discussed in Section 3.1.1. The AA digestibility of the test ingredient represents the slope of a linear regression between the amount of ingested and digested AA because the difference in ingested and digested amounts (δ ingested and δ digested in Figure 3, respectively) are caused by the test ingredient only. The amounts of undigested AA, specific endogenous AA, and basal endogenous AA remain unknown. AA digestibility estimates determined with this regression approach inherently include unabsorbed AA originating from the test ingredient and specific endogenous AA secreted due to the presence of the test ingredient but exclude basal endogenous AA.

The major advantage of this regression approach is that no separate measurements of basal endogenous AA are needed and that all experimental diets can be formulated to fulfill the nutrient requirements. Hence, measurements on animals in unphysiological conditions, like N-free diets, are avoided. This regression approach has been recommended in a common communication of recognized experts in the field of AA digestibility research when highest possible accuracy is targeted (Ravindran et al. 2017). A point of criticism on this approach is a higher number of experimental diets compared with the method using a N-free diet (Ravindran

2021). This is a valid point when a diet containing the test ingredient and the feed ingredient devoid of AA in equal shares is tested because then, one more experimental diet per test ingredient is needed.

Concerning terminology, the different methodology of determining AA digestibility by excluding basal endogenous AA using the regression approach renders the term 'standardized AA digestibility' inappropriate. Such digestibility estimates usually are designated 'AA digestibility' of a test ingredient, although the downside is potential confusion with apparent AA digestibility.

1.5 Background of this thesis

Work on CP digestibility (Desikachar and De 1950) and AA digestibility (Waring and Shannon 1969) in nonruminants has been published for decades. The collected findings over the years have led to the current situation that diets for pigs and poultry are formulated based on tabulated AA digestibility values of feed ingredients (e.g., National Research Council 1994; Evonik 2016; INRAE 2021). However, AA digestibility of feed ingredients varies considerably (Bryden and Li 2010; Frikha et al. 2012; Zuber et al. 2016). Tabulated AA digestibility values do not consider the variation in AA digestibility within one feed ingredient. Hence, AA digestibility values of the ingredients feed formulators use to formulate compound feed most likely deviate from the actual AA digestibility in the ingredient batch. In most cases, animals probably are oversupplied because nutritionists meet the uncertainty with safety margins. In other cases, animals are supplied deficiently, particularly when an unrecognized low digestibility exceeds safety margins. Altogether, it is highly improbable that animals are exactly supplied with digestible AA to meet the requirements.

Predictability of AA digestibility is a prerequisite for adjusting digestible AA concentrations in diets closer to the requirements and, hence, to increase PUE. The scientific publications amalgamated in this thesis have the overarching objective of making AA digestibility more predictable. Several aspects of AA digestibility research need to be considered to achieve the goal of a more predictable AA digestibility:

- Methods to determine AA digestibility need to be improved to standardize the assays. This requires knowledge to avoid factors that lead to erroneous AA digestibility estimates and knowledge on factors influencing AA digestibility by changing the digestive capacity.
- Effects of feed ingredients on the AA digestibility of other ingredients or of the complete diet need to be investigated.

- Variation in AA digestibility within feed ingredients needs to be investigated with highly accurate assays to gain insight regarding the extent and reasons of variation. Such investigations are also needed to predict AA digestibility of an ingredient in routine feed formulation based on rapid and easy techniques.

The brief summaries of the publications in Section 2 are assigned to these key points based on the primary objective of the respective study, whereby most studies are assignable to more than one of these key points.

2 Summaries of studies

This section gives summaries of the publication amalgamated in this thesis. The publications are enclosed in the Publications Section and the title of the publications are linked to the homepages of the respective journals. The publication titles are shown in British or American English as published in the respective journals. The summaries are written in American English only to be compliant with other sections of this thesis.

2.1 Methodological studies on amino acid digestibility

Study 1

Siegert W, Ganzer C, Kluth H, Rodehutscord M. 2019. Effect of amino acid deficiency on precaecal amino acid digestibility in broiler chickens. Journal of Animal Physiology and Animal Nutrition 103: 723–737, doi 10.1111/JPN.13066

This study determined whether deficient dietary AA concentrations in the basal diet influence pc AA digestibility in broiler chickens using the regression approach. Rapeseed cake and full-fat soybeans were evaluated as test ingredients with two basal diets, which were deficient or adequate in AA concentrations. Estimates of pc AA digestibility of rapeseed cake and full-fat soybeans differed considerably between the basal diets. Feed intake was reduced considerably in clearly AA-deficient diets compared with diets adequate in AA concentrations. Feed intake was hardly different among most diets adequate in AA concentrations, except for the diet containing a high proportion of rapeseed cake. An explanation for different pc AA digestibility estimates between the basal diets probably is that the affected feed intake alters the content of basal endogenous AA in the digesta, making the exclusion of basal endogenous AA from pc AA digestibility estimates inaccurate. It was concluded that diet formulation should make differences in feed intake improbable by formulating basal diets with adequate AA concentrations and by adequate maximum proportions of test ingredients in the assay diets.

Study 2

Siegert W, Ganzer C, Kluth H, Rodehutscord M. 2018. Influence of feed provisioning prior to digesta sampling on the precaecal amino acid digestibility in broiler chickens. Archives of Animal Nutrition 72: 190–204, doi 10.1080/1745039X.2018.1446810

This study investigated whether feed deprivation prior to digesta sampling affects the determined pc AA digestibility of soybean meal in broiler chickens using the regression approach. Feed deprivation for predefined periods of time prior to digesta sampling has often been applied in studies aiming to standardize the potential influence of the interval between the

last meal and digesta sampling, or to maximize gut lumen fill. Birds were either fed *ad libitum* prior to digesta sampling, or feed was provided for 30 min after a withdrawal period of 12 h, and digesta were sampled 1, 2, 4, or 6 h after feeding commenced. The pc AA digestibility values for soybean meal were influenced considerably by feeding protocols and ranked 6 h ≈ 4 h > *ad libitum* > 2 h > 1 h. The variance in pc digestibility ranked 1 h > *ad libitum* > 2 h > 6 h > 4 h for all AA. The magnitude of the effects makes the feed-provisioning protocol prior to digesta sampling a highly relevant aspect in pc AA digestibility experiments.

Study 3

Siegert W, Ganzer C, Kluth H, Rodehutscord M. 2018. Effect of particle size of maize and soybean meal on the precaecal amino acid digestibility of broiler chickens. British Poultry Science 59: 68–75, doi 10.1080/00071668.2017.1380295

The effect of grinding the test ingredients corn and soybean meal through 2- and 3-mm grids on pc AA digestibility in broiler chickens was investigated using the regression approach. The reduction in the grid size from 3 to 2 mm reduced the average particle size of both corn and soybean meal, mainly by reducing the proportion of coarse particles. Reducing the grid size significantly increased the pc AA digestibility of soybean meal. In corn, reducing the grid size caused a considerable but not significant decrease in pc AA digestibility. The reasons for opposing consequences of reducing the grid size between corn and soybean meal remain unclear. It was concluded that future investigations on pc AA digestibility should specify the particle size distribution and should investigate test ingredients ground similarly to practical applications.

Study 4

Ganzer C, Siegert W, Kluth H, Bennewitz J, Rodehutscord M. 2017. Comparison of prececal amino acid digestibility of soybean cake in fast- and slow-growing broiler chickens. Poultry Science 96: 2804–2810, doi 10.3382/ps/pex090

The pc AA digestibility of soybean cake was studied using the regression approach in ISA J-275 and Ross 308 broiler chickens, which represent commonly used slow- and fast-growing broiler strains, respectively. Differences between apparent pc digestibility of some AA of the experimental diets were found while the pc AA digestibility excluding basal endogenous AA of the soybean cake did not differ significantly between strains and was numerically almost identical. The results indicate that experimentally determined pc AA digestibility estimates of feed ingredients based on using the regression approach can be applied for both types of broiler strains.

2.2 Influence of feed composition

Study 5

Borda-Molina D, Zuber T, Siegert W, Camarinha-Silva A, Feuerstein D, Rodehutscord M. 2019. Effects of protease and phytase supplements on small intestinal microbiota and amino acid digestibility in broiler chickens. Poultry Science 98: 2906–2918, doi 10.3382/ps/pez038

The effect of supplementing three exogenous proteases in two levels each and one exogenous phytase on the pc AA digestibility of the experimental diets and composition of the microbiota in the terminal small intestine was investigated in broiler chickens. The pc AA digestibility was not influenced by protease supplementation. Another protease decreased the pc AA digestibility at the low supplementation level, while there was no difference between the high supplementation level and the unsupplemented diet. The third protease decreased and increased pc AA digestibility at the low and high supplementation level, respectively. Supplementing phytase increased pc AA digestibility to about the same level as the high supplementation level of the third protease. The microbiota composition and interactions between microbial groups were different between dietary treatments. However, there was no clear relationship between pc AA digestibility and relative abundances of microorganisms.

Study 6

Siegert W, Zuber T, Sommerfeld V, Krieg J, Feuerstein D, Kurrle U, Rodehutscord M. 2019. Effects of phytase and protease supplementation on prececal amino acid digestibility and phytate degradation in broiler chickens fed diets with different oilseed meals. Poultry Science 98: 5700–5713, doi 10.3382/ps/pez355

This study investigated the influences of three oilseed meals as the main protein sources in diets on the effects of supplemented exogenous phytase and protease on pc AA digestibility in broiler chickens. The effects of monocalcium phosphate on supplemented phytase was also investigated for one oilseed meal. No significant interactions were determined between the main protein sources, enzyme supplementation, or monocalcium phosphate addition for the pc digestibility of all AA except for cysteine (**Cys**). Phytase was supplemented at two levels. Supplementation at the lower phytase level increased pc AA digestibility; no further increase was observed for the higher phytase level. Protease supplementation increased pc AA digestibility to a lower magnitude than the lower phytase level. Monocalcium phosphate addition increased pc AA digestibility. This study provided no evidence that diet composition can explain the inconsistent effects of enzyme supplementation on pc AA digestibility described in the literature. The effects of enzyme supplementation pointed to conflicts of interest between consequences on pc AA digestibility and other traits such as phytate degradation and ME_N.

Study 7

Siegert W, Krieg J, Sommerfeld V, Borda-Molina D, Feuerstein D, Camarinha-Silva A, Rodehutscord M. 2021. Dietary calcium concentrations but not calcium source or formic acid influence phytase supplementation effects on amino acid digestibility in broiler chickens. Current Developments in Nutrition 5: nzab103, doi 10.1093/cdn/nzab103

This study investigated whether decreasing the dietary acid-binding capacity diets interacts with dietary calcium (**Ca**) and supplemented phytase on pc AA digestibility and whether changes in microbial functionality are associated with alterations in pc AA digestibility. Dietary acid-binding capacity was decreased by replacing Ca carbonate with Ca-formate or by adding formic acid to Ca carbonate-containing diets. Decreasing the acid-binding capacity increased pc AA digestibility without interactions with Ca concentration or phytase supplementation. The interaction between the Ca concentration and phytase supplementation was significant: Without phytase supplementation, increasing the dietary Ca concentration decreased pc AA digestibility. Phytase supplementation increased pc AA digestibility to a higher extent at the high Ca concentration than the low Ca concentration, thus causing a similar level of pc AA digestibility at both Ca concentrations. Functional predictions made from microbiota data point toward an influence of the microbiota in crop and ileum content on AA concentrations, as indicated by different relative abundances of predicted genes related to AA biosynthesis, degradation, and metabolism. The consequence of the functionality of the microbiota on AA concentrations and, hence, AA digestibility, remained unknown.

2.3 Variation and prediction of amino acid digestibility

Study 8

Siegert W, Boguhn J, Maurer HP, Weiss J, Zuber T, Möhring J, Rodehutscord M. 2017. Effect of nitrogen fertilisation on amino acid digestibility of different triticale genotypes in caecectomized laying hens. Journal of the Science of Food and Agriculture 97: 144–150, doi 10.1002/jsfa.7701

The effect of fertilizing 80 kg N/ha at the end of the heading stage on the AA digestibility of three triticale genotypes was investigated in cecectomized laying hens using the regression approach. N fertilization increased concentrations of all AA in triticale, but concentrations of first-limiting AA relative to CP decreased. The digestibility of most AA decreased upon N fertilization, and the level of digestibility differed between triticale genotypes. Concerning the first-limiting AA, digestible AA concentrations of triticale were mostly unaffected by N fertilization, while concentrations of some digestible nonessential AA increased upon N fertilization. The results imply that the consideration of fertilization and genotype-specific

digestibility data in feed formulation might contribute to make AA digestibility more predictable.

Study 9

Zuber T, Maurer HP, Möhring J, Siegert W, Rosenfelder P, Rodehutscord M. 2016. Variability in amino acid digestibility of triticale grain from diverse genotypes as studied in cecectomized laying hens. Poultry Science 95: 2861–2870, doi 10.3382/ps/pew174

The AA digestibility of 20 triticale genotypes grown under identical conditions was investigated in cecectomized laying hens using the regression approach. The triticale genotypes were characterized comprehensively with chemical and physical measurements aiming to derive correlations and multiple regressions to enable predictions of AA digestibility based on grain analyses. The AA digestibility values varied considerably among triticale genotypes. A number of significant correlations between AA digestibility and chemical and physical characteristics of the grain were determined, but a consistent pattern among AA was not apparent. The explanatory power of multiple regressions was low. Hence, it was not possible to explain the determined variation in AA digestibility by chemical and physical characteristics of the grain.

Study 10

Zuber T, Siegert W, Salehi H, Hummel F, Rodehutscord M. 2019. Variability of amino acid digestibility of lupin and pea grains in caecectomised laying hens. British Poultry Science 60: 299–240, doi 10.1080/00071668.2018.1556389

Twelve variants each of peas and lupins were investigated in cecectomized laying hens using the regression approach to determine the variation in AA digestibility. The pea and lupin variants were analyzed comprehensively, with the aim to predict AA digestibility based on the analyzed characteristics. Variation in AA digestibility in the lupin variants was low, which probably contributed to the absence of a consistent pattern of significant correlations between the analyzed characteristics among AA. In peas, variation in AA digestibility was higher compared with lupins. Tannin concentrations in the white flower pea variants were not a relevant cause for the variations in AA digestibility, but might have contributed to the low AA digestibility of the one colored flower variant under study. Trypsin inhibitor activity seemed to be a determinant of AA digestibility in white flower pea variants. However, using the analyzed characteristics of peas and lupins, prediction of AA digestibility with sufficient accuracy was not possible.

Study 11

Siegert W, Ibrahim A, Link W, Lux G, Schmidtke K, Hartung J, Nautscher N, Rodehutscord M. 2021. Amino acid digestibility and metabolisable energy of spring and winter faba beans grown on two sites and effects of dehulling in caecectomised laying hens. Journal of the Science of Food and Agriculture, online available, doi 10.1002/jsfa.11424

The variation in AA digestibility in four spring and four winter faba bean genotypes differing in vicine/convicine concentrations grown on two sites was investigated in cecectomized laying hens using the regression approach. The effects of dehulling one faba bean genotype were also examined. The results indicate a genotype × environment interaction because lower digestibility of some AA was determined for the winter genotypes grown on one site compared with the other variants. The corresponding variants featured higher phytate concentrations compared with the others and probably caused significant correlations between phytate concentrations and the digestibility of the respective AA. Correlations between AA digestibility and the further analyzed characteristics were not significant. Dehulling increased the digestibility of some AA, while the digestibility of most AA was hardly influenced.

Study 12

Siegert W, Rodehutscord M. 2020. Precaecal crude protein and amino acid digestibility of guar meal in broiler chickens. European Poultry Science 84, doi 10.1399/eps.2020.297

The pc digestibility of guar meal was investigated in broiler chickens. The pc AA digestibility was very low, with an arithmetic mean of the pc digestibility of all AA of 51%. High proportions of soluble polysaccharides, predominantly galactomannans, in the guar meal probably contributed to that outcome by increasing the viscosity in the digestive tract of the animals.

3 General discussion

This thesis puts studies on AA digestibility in poultry into the context of increasing PUE, which is one tool for improved sustainability of farm animal husbandry in terms of long-term food security and the environmental impact. The overarching goal was to make AA digestibility sufficiently predictable to enable formulating diets that precisely meet the requirement of animals for digestible AA. This goal so far has not been achieved, but the studies compiled in this thesis together with other literature generate awareness and understanding of the influences on AA digestibility and how to conduct AA digestibility measurements. This general discussion will amalgamate the outcomes of the publications, point out pending issues, and present perspectives.

3.1 Methodological influences in amino acid digestibility determination

3.1.1 Relevance of feed intake

Modes of action

Feed intake seems to represent a crucial determinant on apparent AA digestibility. Two interconnected modes of action were identified. Feed intake usually is considered the predominant determinant of basal endogenous AA (see Section 1.4.2). Hence, feed intake represents a direct influence on apparent AA digestibility. Another probable mode of action is the influence of feed intake on lumen fill, which determines the passage rate of the digesta through the digestive tract. Lentle and de Loubens (2015) summarized that there is evidence that contractile activity disperses the digesta along the small intestine, and therefore, there is less digesta per unit length of the small intestine in consequence of lower feed intake. This facilitates mixing of the digesta by kneading and folding. The consequences of reduced feed intake on AA digestibility are ambiguous (discussed in Study 1): Apparent AA digestibility might be increased by increased access of digestive enzymes to the substrate, and increased transfer of absorbable AA (di- and tripeptides as well as free AA) to enterocytes. Alternatively, apparent AA digestibility might be decreased due to more endogenous AA in the digesta because of abrasion of the epithelial cells and disruption of the unstirred water layer of the intestine. The different passage rate through the digestive tract possibly contributed to variable basal endogenous AA losses between studies and depending on the composition of the N-free

diets that were used (Kluth and Rodehutscord 2009a; Kong and Adeola 2013c; Adeola et al. 2016; Adedokun et al. 2017).

Different feed intake among dietary treatments impairs the accuracy of estimates when AA digestibility of test ingredients is measured based on complete diets with the test ingredients as the only source of protein (Siegert et al. 2017). Differing feed intake among dietary treatments most likely also impairs the accuracy of estimates when basal endogenous AA are excluded from AA digestibility by basal endogenous AA determined using separate diets. Different feed intake possibly contributes to the variation in basal endogenous AA losses that has been described in the literature. Ravindran (2021) summarized that basal endogenous Met, Cys, and Lys ranged from 0.05–0.36, 0.03–0.55, and 0.16–1.24 g/kg dry matter intake, respectively, in 21 studies (coefficient of variation 30%, 33%, and 38%, respectively). Unfortunately, feed intake is stated in only a few of the numerous studies on AA digestibility of test ingredients excluding basal endogenous AA. The assumption that basal endogenous AA linearly depend exclusively on feed intake resulted in the common specification of basal endogenous AA as a linear function of feed intake on a dry matter basis (Adedokun et al. 2011; Adeola et al. 2016). However, feed intake was shown to represent a major but not exclusive determinant of basal endogenous AA, and the relationship between feed intake and basal endogenous AA is not linear for all AA (Butts et al. 1993). These uncertainties, together with highly variable basal endogenous AA measured under unphysiological conditions (see Section 1.4.2), may have contributed to the observation that AA digestibility values were higher than 100% after correction of basal endogenous AA losses in some studies on poultry (e.g., Adedokun et al. 2008) and pigs (e.g., Strang et al. 2017).

The effect of feed intake and the level of basal endogenous AA is shown in a model calculation in Figure 4 with the digestibility of Lys as an example. This model calculation is based on measurements obtained in an unpublished experiment on cecectomized laying hens and values for basal endogenous Lys obtained from laying hens in the literature (Adedokun et al. 2018; An et al. 2020). The higher values for basal endogenous Lys led to unrealistically high levels of Lys digestibility (excluding basal endogenous Lys), with values close to or above 100%. The model calculation further demonstrates that the relevance of feed intake is more pronounced the higher the value of basal endogenous AA is. In case of the examples in Figure 4, the Lys digestibility increased by 0.04, 0.13, and 0.19 percentage points (**pp**) per g additional dry matter intake for basal endogenous Lys values of 1.13, 2.56, and 3.77 g/kg feed intake. However, the level of basal endogenous Lys had a greater effect on Lys digestibility excluding basal endogenous Lys than feed intake. This finding emphasizes the relevance of accurate basal

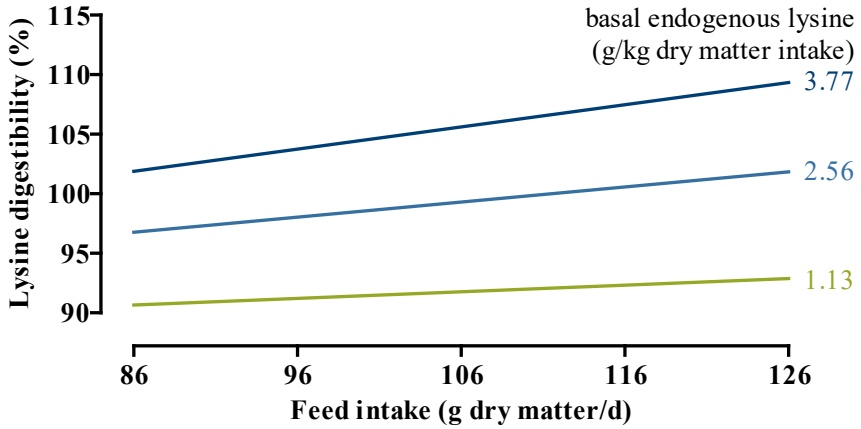

Figure 4. Model calculation of the influence of basal endogenous amino acids and feed intake on the amino acid digestibility excluding basal endogenous amino acids in laying hens. The lysine intake of 2.02 g/d and excreted lysine amount of 0.29 g/d are measured data of an unpublished experiment. The excreted lysine amount excluding basal endogenous lysine was calculated as: excreted lysine amount (g/d) – [basal endogenous lysine (g/kg dry matter intake) × feed intake (g dry matter/d) / 1000]. Basal endogenous lysine values were taken from the literature (Adedokun et al. 2018; An et al. 2020).

endogenous AA values if apparent AA digestibility of a test ingredient and basal endogenous AA are measured separately to determine AA digestibility excluding basal endogenous AA.

Varying feed intake among experimental diets also impairs the accuracy of AA digestibility estimates when the regression approach is applied. An equal flow of basal endogenous AA among experimental diets is a prerequisite for an accurate exclusion of basal endogenous AA. If feed intake varies among treatments, differences in AA concentrations in the digesta are not only caused by undigested AA of the test ingredient and specific endogenous AA due to the test ingredient, but also by basal endogenous AA. This effect is demonstrated in the model calculation in Figure 5. This model calculation assumed 1) that the feed intake of the diets with 0% inclusion of the test ingredient (left dots) is 90% that of the diets with 50% and 100% inclusion of the test ingredient (middle and right dots) and 2) that the proportion of undigested protein from the test ingredient and specific endogenous AA are unaffected by feed intake. AA digestibility excluding basal endogenous AA using the 50% and 100% inclusion of the test ingredient (blue regressions) was determined accurately because feed intake was the same. If feed intake among the three treatments was the same, the difference in ingested AA amount between the 50% and 0% inclusion level and between the 100% and 50% inclusion level of the test ingredient would have been the same. However, as feed intake at the 0% inclusion of the test ingredient was lower, the difference in the ingested AA amount between the 50% and 0% inclusion level was more pronounced than between the 100% and 50% inclusion level of the test ingredient. The slopes between the ingested and digested AA amount determined for the 0% and 50% inclusion level of the test ingredient (red regressions) was steeper than that

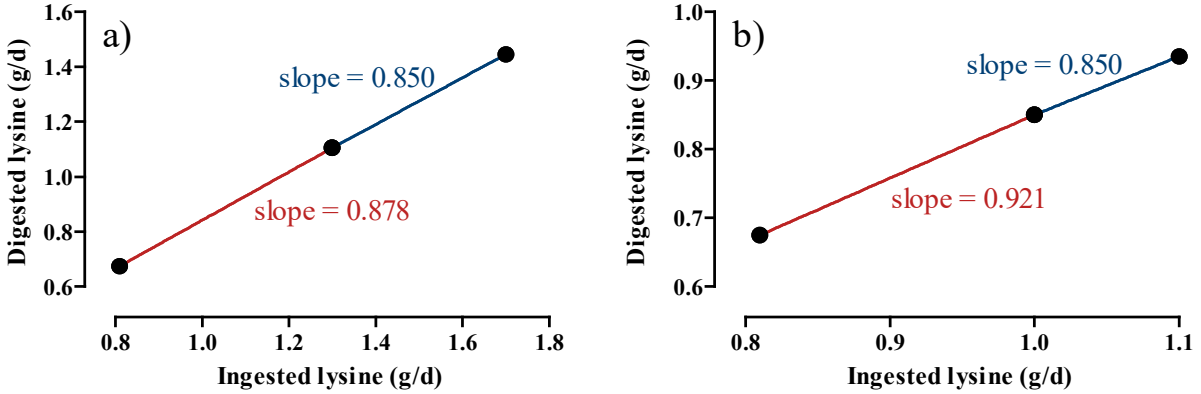

Figure 5. Model calculation of the influence of basal endogenous lysine on the determined lysine digestibility when the regression approach is applied. The three dots in each panel represent the lysine intake at a 0%, 50%, and 100% inclusion level of the test ingredient. Panels a) and b) show a difference in the ingested lysine amount between the 50% and 100% inclusion level of the test ingredient of 0.4 mg/d and 0.1 mg/d, which is equivalent to half of the difference in ingested lysine amount between the 0% and 100% inclusion levels in studies on soybean meal (unpublished data) and corn (Zuber and Rodehutscord 2017), respectively. At the 0% inclusion of the test ingredient, feed intake was assumed at 90% of the other inclusion levels of the test ingredient. Basal endogenous lysine was assumed at 1.125 g/kg dry matter intake (An et al. 2020). Additional assumptions are described in the text.

determined for the 100% and 50% inclusion level of the test ingredient because the basal endogenous AA differed. Basal endogenous AA were assumed to be 0.1215 g/d at the 0% inclusion of the test ingredient and 0.135 g/d at the 50% and 100% inclusion of the test ingredient (calculated assuming basal endogenous Lys of 1.125 mg/kg dry matter intake (An et al. 2020) multiplied by 108 g dry matter intake/d at the 0% inclusion of the test ingredient and 120 g dry matter intake/d at the 50% and 100% inclusion of the test ingredient). The difference in basal endogenous AA of 0.0135 mg/d was subtracted from the digested AA amount that would be measured if feed intake had been unaffected. The steepening effect on the slope was the more pronounced the smaller the difference in ingested AA amount between the 50% and 100% inclusion of the test ingredient was. This is shown in Figure 5a and Figure 5b as examples for higher and lower differences between the ingested AA amount, respectively.

Consequences for the performance of AA digestibility assays

Diet formulation and feeding management should avoid differences in feed intake among experimental diets to achieve the most accurate AA digestibility estimates. The following descriptions are focused primarily on digestibility determined with the regression approach because no other assay was used in the studies amalgamated herein. The possibilities to achieve equal feed intake among diets differ depending on the sampling methods.

When birds are kept in groups prior to digesta sampling, standardizing feed intake by feed restriction is difficult because there would be unequal feed intake among individual animals within a group. It seems probable that the accuracy of measurements is reduced because some birds consume much while less feed is left for others, but there are studies available on this aspect. It therefore seems preferable to achieve similar voluntary feed intake within a group of birds and among dietary treatments by using measures other than feed restriction. Study 1 points to two aspects of diet formulation that make similar voluntary feed intake among dietary treatments probable:

1) The basal diet in an experiment should be formulated to meet the nutrient requirement of the animals because deficient a AA supply can reduce feed intake (Classen et al. 2017). The basal diet contains the lowest AA concentration in an experiment using the regression approach. If the basal diet is AA deficient, the inclusion of the test ingredient decreases the degree of AA deficiency or eliminates it. Hence, the different extent or the absence of AA deficiency in diets contributes to variation in feed intake. Other often described influences on feed intake are variations in ME_N concentrations (Classen et al. 2017). However, provided that the AA concentrations in the basal diet were adequate, there was no difference in feed intake in several studies when cornstarch was replaced with the test ingredient, although ME_N differed widely (e.g., Study 4; Kluth and Rodehutscord 2010). Therefore, the proposition of variation in ME_N being an influence on feed intake and, hence, the accuracy of determined AA digestibility values in experiments using the regression approach seems to be invalidated.

2) Inclusion levels of test ingredients should be defined individually for each test ingredient in a way that avoids feed intake reducing effects. In Study 1, feed intake was reduced when rapeseed cake was included at a level of 200 g/kg to a basal diet adequate in AA concentrations. This probably was a specific effect of such high proportions of rapeseed cake in diets supplied to broiler chickens (Jeroch et al. 2008). It seems advisable to consider recommendations for a maximum inclusion level of feed ingredients in diets that aim to avoid undesirable effects of the feed ingredients, like reduced feed intake. Such recommendations depend on species or age and are available for several types of feed ingredients, including cereal grains, legumes, and oilseed products (e.g., Jeroch and Steinhöfel 2020). Another decision basis for the definition of inclusion levels is the aim to achieve the highest possible ranges of ingested and digestible AA intake. This approach increases the effect size and, hence, the statistical power of comparisons between slopes (Gerrodette 1987). Therefore, the decision for the maximum inclusion level of test ingredients requires a balance between the risk of reduced feed intake and the aim to

maximize effect size. Experiences from experiments (Table 1) show that no decreased feed intake is to be expected at inclusion levels of up to 500 g/kg and 250 g/kg for cereal grains and

Table 1. Compilation of maximum inclusion levels of test ingredients and effects on feed intake in studies using the regression approach to determine the amino acid digestibility of feed ingredients with basal diets adequate in amino acid concentrations.

Test ingredient	Type of poultry	Study	Maximum inclusion level (g/kg)	Feed intake different at highest inclusion of test ingredient compared to basal diet[1]
Cereals and cereal products				
Corn	Broiler chickens	Study 3	500	Increase by 14%
Corn	Laying hens	Zuber and Rodehutscord 2017	500	No
Corn gluten meal	Laying hens	Rezvani et al. 2008a	300	No
Rye	Laying hens	Zuber et al. 2016	500	No
Triticale	Laying hens	Study 8	500	No
Triticale	Laying hens	Study 9	500	No
Wheat	Broiler chickens	Kluth et al. 2009	400	No
Wheat	Laying hens	Zuber and Rodehutscord 2016	500	No
Pulses				
Faba beans	Broiler chickens	Witten et al. 2018	699	Decrease by 7%[2]
Faba beans	Laying hens	Study 11	250	No
Lupins	Laying hens	Study 10	300	No
Peas	Broiler chickens	Witten et al. 2018	699	Decrease by 3%[2]
Peas	Laying hens	Study 10	300	No
Oilseed products				
Rapeseed cake	Broiler chickens	Study 1	200	Decrease by 13%
Rapeseed meal	Laying hens	Rezvani et al. 2012	200	No
Soybean cake	Broiler chickens	Study 4	200	No
Soybean meal	Broiler chickens	Study 2	300	Increase by 10%
Soybean meal	Broiler chickens	Study 3	300	Increase by 16%
Soybean products	Laying hens	Unpublished	300	No
Toasted soybeans	Broiler chickens	Study 1	300	No
Toasted soybeans	Laying hens	Rezvani et al. 2008a	300	No
Others				
Alfalfa	Broiler chickens	Pleger et al. 2021	200	No
Guar meal	Broiler chickens	Study 12	150	Decrease by 4%
Red clover	Broiler chickens	Pleger et al. 2021	200	No
Wheat DDGS[3]	Broiler chickens	Kluth and Rodehutscord 2010	200	No

Table footnotes shown on next page.

Footnotes to Table 1:
[1]Deviation described if significant or if information on significance was not reported.
[2]Information on significance was not reported.
[3]Dried distillers' grains with solubles.

pulses, respectively, while 200 g/kg of rapeseed products or 150 g/kg of guar meal can impose a risk to uniform feed intake.

Increasing feed intake with inclusion of the test ingredients in Study 3 and Study 4 (Table 1) are probably explained by AA deficiency in the basal diet, although the basal diets were formulated to meet the recommendations of the Gesellschaft für Ernährungsphysiologie (1999). The analyzed concentrations of Lys and valine (**Val**) and concentrations of arginine (**Arg**), Lys, phenylalanine (**Phe**), and Val were below the recommendations in Study 3 and Study 4, respectively. This indicates that basal diets need to be formulated with safety margins in AA concentrations unless ingredients of the basal diets are analyzed prior to diet formulation. Although the accuracy of estimated AA digestibility of the test ingredients probably is impaired by the lower intake of the basal diets, the methodological insights gained in Study 3 and Study 4 remained unaffected because all test ingredients were equally affected.

Keeping birds individually – as practiced when cecectomized laying hens are used – enables researchers to standardize feed intake of all experimental animals by restrictive feeding. Decreasing the daily allowance reduced coefficients of variation of the daily feed intake (Figure 6). Of note, incomplete feed intake is almost always recorded even if the troughs are emptied because some feed adhering to the beaks commonly is recorded as feed residual in the drinking water troughs. The advantages of feed restriction for homogenous feed intake are opposed by the possibility that feed restriction might affect the determined AA digestibility (see Section

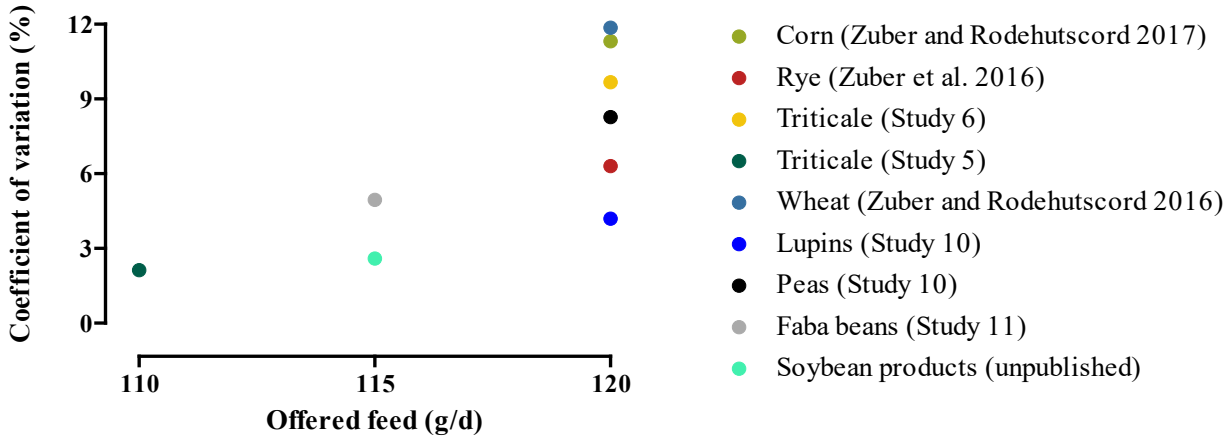

Figure 6. Coefficient of variation of feed intake plotted against the offered amount of feed in studies investigating the amino acid digestibility in cecectomized laying hens.

3.1.1). A solution to this conflict of aims is difficult to find. A probably workable solution is to adjust the daily offered amount of feed during the experiment to about a medium amount of voluntary feed intake determined prior to the experiment.

Test ingredients or the age of the birds may represent an influence on the variation in feed intake. The experiment on soybean products mentioned in Figure 6 was conducted subsequent to the experiment with faba beans. Variation in feed intake differed between the experiments although the same animals were used and the basal diets were composed very similarly. Further, the level of mean feed intake and the variation in feed intake differed between the experiments on corn, rye, triticale, and wheat, although identically formulated basal diets were under investigation and the same animals were used for at least two out of the experiments. This demonstrates that test ingredients determine the voluntary feed intake. Voluntary feed intake therefore needs to be determined using the experimental diets if the amount of feed offered daily during the experiment is aimed to be adjusted to the voluntary feed intake.

The marked effect of AA concentration in the basal diet and feed intake on pc AA digestibility in broiler chickens (Study 1) makes a methodological investigation on this issue in cecectomized laying hens interesting. It may be advisable to conduct an experiment on the effects of offering 110 g, 115 g, 120 g, and an unrestricted amount of feed on the AA digestibility of a test ingredient. The offered amounts of feed might be combined with basal diets formulated to be deficient, adequate, and substantially excessive in AA concentrations in a two-factorial design. The comparison of deficient and adequate AA concentrations in the basal diet and interactions with the offered amount of feed is interesting because AA deficiency in the basal diet was determined as a highly probable influence on feed intake. The comparison of adequate and substantially surplus AA concentrations in the basal diet probably would show whether safety margins in AA concentrations in the basal diet affect the determined AA digestibility.

Feed provisioning protocols in digestibility studies

Intending to avoid influences of feed intake in AA digestibility experiments, feed provisioning protocols that standardize feed allowance have been introduced over the years (Sebastian et al. 1997; Wiseman et al. 2003; Rutherfurd et al. 2007; Ahmed et al. 2014; Koivunen et al. 2015; Asadi Kermani et al. 2017). Other intentions were to standardize the interval between the last meal and digesta sampling or to maximize lumen fill, aiming to achieve a sufficient sample size with a given number of experimental animals. In studies on growing poultry, the feed provisioning protocol often scheduled a short access to feed after feed was withdrawn for

several hours. Digesta was then sampled after a predefined period subsequent to regaining access to feed. The three time periods of first and second withdrawal of and access to feed varied widely among the studies, with time periods in the range of 8–24 h and 0.5–4 h, respectively (e.g., Rutherfurd et al. 2007; Ahmed et al. 2014; Koivunen et al. 2015).

Study 2 demonstrated that how feed is provided prior to digesta sampling can considerably influence the outcomes of AA digestibility experiments on broiler chickens. The determined pc AA digestibility was lowest when digesta were sampled 1 h after broiler chickens regained access to feed after a withdrawal period of 12 h. The pc AA digestibility increased up to the 4 h interval between regaining access to feed and digesta sampling. The interpretation described in detail in Study 2 was that the ingested feed was quickly distributed throughout the previously empty small intestine to facilitate their mixing with digestive enzymes (Lentle and de Loubens 2015). The increasing pc AA digestibility with an increasing interval between regaining access to feed and digesta sampling was explained by more time for digestive and absorptive processes being available for the same portion of ingested feed. This interpretation was supported by measurements of lumen fill, which indicated that only small digesta amounts were transferred to the post-ileal digestive tract up to 4 h between regaining access to feed and digesta sampling. When feed was provided *ad libitum*, pc AA digestibility was considerably lower than with the 4 h protocol. It seemed like the more constant feed intake caused a higher passage rate of the digesta through the digestive tract, leaving less time for digestive and absorptive processes.

The results of pc AA digestibility measurements determined with restrictively fed birds should not be transferred to conditions of *ad libitum* feeding and vice versa. This conclusion results from a calculation based on the data of Study 2. The response in pc AA digestibility to the restrictive feed provisioning protocols can be described by second-order polynomial regressions between pc AA digestibility and hours between regaining access to feed and digesta sampling (R^2=0.924–0.988; see Figure 7a and Figure 7b as examples of the pc digestibility of Lys and threonine (**Thr**), respectively). As the results of the *ad libitum* protocol were within the range of determined pc AA digestibility of the restrictive feed provisioning protocols, the rank of results of the *ad libitum* protocol compared with restrictive feed provisioning protocols can be calculated. This was done by determining the values on the x-axis where the y-value of the polynomial regression equaled the results of the *ad libitum* protocol (dashed arrow in Figure 7a and Figure 7b as examples). Figure 7c shows these values for the pc digestibility of all AA. These values were considerably different between AA with values of the *ad libitum* protocol close to the 2 h protocol for some AA and values of other AA close to the 4 h protocol. This means that data determined with restrictively fed animals should not be transferred to conditions

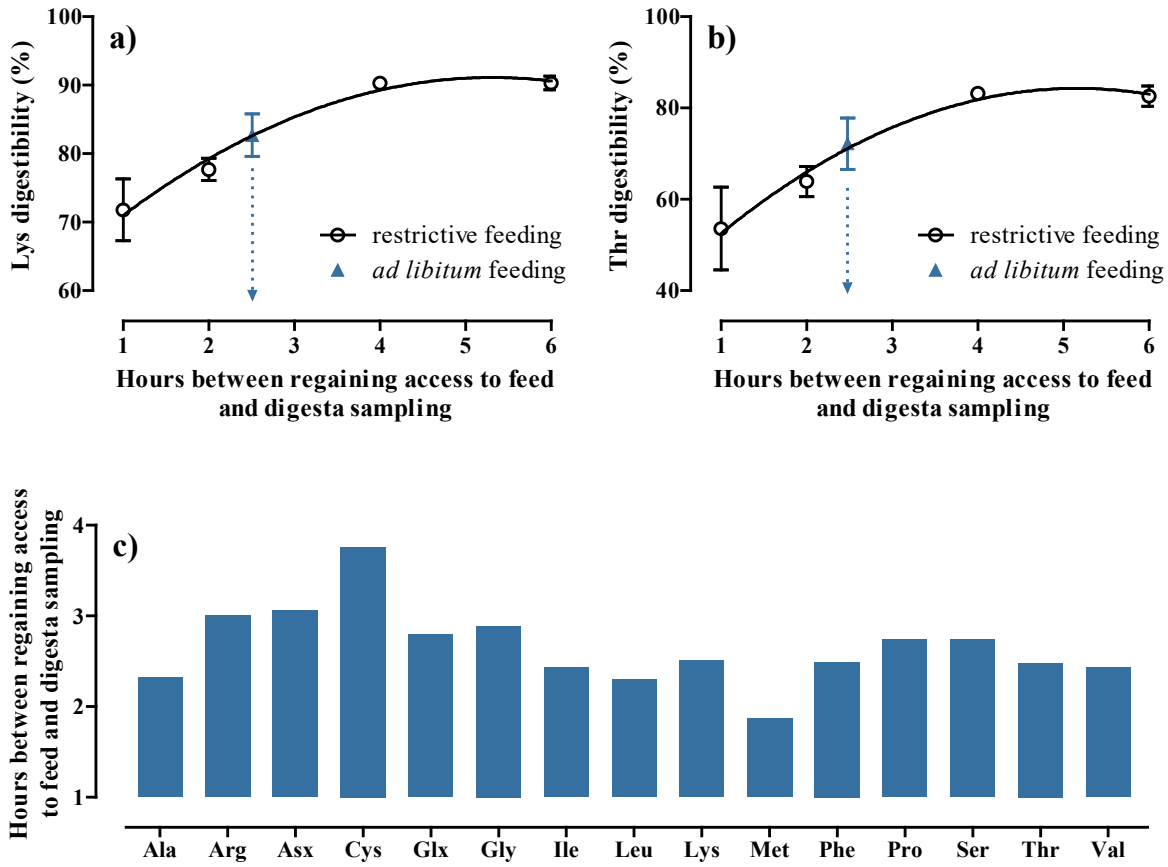

Figure 7. Comparison of the prececal amino acid digestibility of the *ad libitum* protocol compared with the restrictive feed provisioning protocols in Study 2 (details in the text). Panels a) and b) illustrate how placement of the results of the *ad libitum* protocol compared with restrictive feed provisioning protocols was calculated using Lys and Thr as examples. Panel c) shows the placement for all determined amino acids. See the list of abbreviations for abbreviations of amino acids.

of *ad libitum* feeding. Hence, feed-provisioning procedures used in AA digestibility experiments should be similar to those used in practice, which currently is providing feed for *ad libitum* consumption.

Restrictive feed-provisioning procedures might prevail prospectively in practice because several studies have shown that restrictive feeding can increase nutrient utilization without reducing growth performance (Svihus et al. 2010; Moraes et al. 2016; Sacranie et al. 2017; van der Klein et al. 2017). In this case, feed provisioning in pc AA digestibility experiments needs to be adjusted to provide suitable pc AA digestibility values for the practice. A development toward the prevalence of restrictive feeding in practice presumes that the advantage of increased nutrition utilization is considered more important than its disadvantages. Such disadvantages include the risk of reduced flock homogeneity because some animals are able to consume a large quantity of feed while less feed is left for others. This phenomenon can be counteracted

by feeding technology that ensures that the targeted amount of feed is provided to each individual animal, but such a technology is not common at present.

3.1.2 Relevance of particle size

Study 3 showed that grinding corn and soybean meal through a 2- or a 3-mm grid had contrasting effects on pc AA digestibility values calculated for these feeds: More intense grinding increased pc AA digestibility in corn, by an average of 4.5 pp, while pc AA digestibility was decreased in soybean meal, by an average of 5.5 pp. Of note, differences were statistically significant for most AA of soybean meal while no differences were significant for corn although numerical differences were similar. This outcome likely is explained by lower differences in the amounts of ingested and digested AA in corn than in soybean meal, which leads to higher standard errors of the slopes. The extent of the determined differences in digestibility is relevant for feed formulation. Therefore, the particle size distribution or the type of grinding should be stated in pc AA digestibility studies to enable cross-study evaluations. Such cross-study evaluations may enable researchers to identify systematic relationships between particle size distribution and pc AA digestibility. This may enable researchers to derive regressions with pc AA digestibility as the dependent variable and particle size distribution as the independent variable.

Identifying reasons for the contradictory response of pc AA digestibility to grinding intensity between corn and soybean meal is difficult. The lack of identified reasons hinders the aim to make AA digestibility predictable. Explanatory approaches regarding the effects of grinding intensities are partly contradictory or only applicable to one test ingredient. Study 4 included several explanatory approaches in detail. They are summarized briefly as follows: 1) The development and activity of the gizzard as the organ that mainly reduces particle size in poultry as well as retention time in the gizzard depends on grinding intensity (Svihus et al. 2004). This can determine the intensity of gastric proteolysis by pepsin. 2) Finer grinding increases the surface-to-volume ratio of the feed particles. This might increase accessibility of digestive enzymes to the substrate and thereby increase the digestion by particle erosion (Lentle and Janssen 2011). 3) The AA-containing fractions of corn may accumulate up to the terminal small intestine because the digestibility of starch is higher than that of AA (Gehring et al. 2013). Such an effect probably is less relevant in soybean meal because of the high AA concentrations. As a result, the dry matter digestibility and, hence, gut fill of corn in anterior sections of the small intestine would be higher than that of soybean meal. This most likely determines the passage of the digesta through the digestive tract. 4) The heterogeneity of particle sizes differed between

grinding intensities and the two test ingredients. The volume of void spaces in particle agglomerates decreases with heterogeneity of particle sizes because small particles fill voids between larger particles (Lentle and Janssen 2008). This reduces the permeation of particle agglomerates with the fluid phase of digesta and, hence, the encounter of digestive enzymes and the substrate and the transport of absorbable AA to the enterocytes.

The explanatory approaches indicate the relevance of the distribution of particle sizes of the feed. Unfortunately, the particle size distribution or an index for the heterogeneity of particle sizes have only been presented in a few studies on AA digestibility in poultry (e.g., Svihus et al. 2004; Parsons et al. 2006; Pacheco et al. 2013; Singh et al. 2014). This limitation impedes systematic overviews and cross-study analyses at this time. As several indices for average particle size and heterogeneity of particle sizes are used by researchers, presenting results of particle size measurements seems advisable to enable researchers to reevaluate the published literature using their preferred indices for heterogeneity of particle sizes.

Measurements of the particle size distribution in the small intestine in addition to those in the test ingredients or complete diets may reveal further information on the influences on AA digestibility. The particle size distribution in the feed does not accurately determine the particle size distribution in the small intestine due to action of the gizzard (Amerah et al. 2008), particle erosion (Lentle and Janssen 2011), and soluble fractions (Study 9). However, this is a field of research that has not been explored to date.

3.1.3 Relevance of diet compaction

Conflicting results on whether or to which magnitude pelleting (Abdollahi et al. 2011; Roza et al. 2018; Barua et al. 2020) or extruding (Opapeju et al. 2006; Ahmed et al. 2014; Sander et al. 2014; Jahanian and Rasouli 2016) influences AA digestibility have been reported in the literature. Potential mechanisms include an effect of pelleting on feed intake, particle size reduction (especially of large particles), the heat susceptibility of AA, and the chemical effects of heat and pressure, including protein denaturation and Maillard reactions (Svihus et al. 2004; Svihus and Zimonja 2011; Barua et al. 2020). All studies amalgamated in this thesis, except for Study 5 and Study 6, used pelleted diets to avoid effects of feed selection and to facilitate collection of spilled feed, with an aim to obtain the most accurate feed intake measurements. Diets were not pelleted in Study 5 and Study 6 to avoid potential effects of heat during the pelleting process on the activity of supplemented enzymes (Svihus and Zimonja 2011). The relevance of this effect was probably minor because the analyzed phytase activity in Study 7 was very close to the formulated levels. For AA digestibility assays, contradictory effects of

feed compaction on the accuracy of outcomes (precise feed intake measurements versus potential influences on AA digestibility) must be weighed. No clear recommendation for AA digestibility studies appears justified based on the available data at present.

The effect of pelleting has not been investigated when the AA digestibility excluding basal endogenous AA of test ingredients was determined using the regression approach in cecectomized laying hens. Such a methodological investigation should include several test ingredients because the effects of pelleting on apparent pc AA digestibility differed between test ingredients in broiler chickens (Abdollahi et al. 2013).

3.1.4 Relevance of types of poultry

In growing poultry, differences in AA digestibility excluding basal endogenous AA of test ingredients have been described between broiler chickens, turkeys, and Pekin ducks (Kluth and Rodehutscord 2006) and between broiler chickens and Pekin ducks (Kong and Adeola 2013b). In other studies, researchers have described differences in apparent AA digestibility of diets between broiler chickens, ducks and geese (Jamroz et al. 2001, 2002). Comparisons between broiler chickens and laying hens suggest differences in AA digestibility of test ingredients excluding basal endogenous AA (Huang et al. 2006; Adedokun et al. 2009, 2014). Differences in the digestive capacity among avian species might be due to differences in their digestive physiology, like by a genetic variation in AA uptake (Mitchell and Smith 1990), jejunal and ileal villus areas (Mitchell and Smith 1990), intestine length, mucosal properties (Mitchell and Smith 1991; Uni et al. 1995), and digestive enzyme activity (O'Sullivan et al. 1992).

In the literature, studies investigating the apparent pc AA digestibility reported differences between broiler strains. This was described between Cobb 500 broiler chickens and an unspecified Omani strain (Al-Mazooqi et al. 2010; Al-Marzooqi et al. 2011), among three strains described as commonly used in Australia (Ravindran et al. 1999c), and between Ross 308 broilers and a strain designated as commercially not available (Kim and Corzo 2012). In Study 4, differences in apparent pc digestibility between the broiler strains Ross 308 and ISA J-257 were determined for some AA. These strains were chosen to represent fast- and slow-growing broiler strains, respectively. However, no significant and only minor differences in pc AA digestibility excluding basal endogenous AA determined using the regression approach between the strains. This points to differences in basal endogenous AA between the broiler strains. Unfortunately, further studies comparing pc AA digestibility excluding basal endogenous AA between broiler strains are not available. Such studies are needed to verify

whether differences in pc AA digestibility excluding basal endogenous AA among broiler strains generally are of minor relevance.

The aforementioned study results suggest that broiler strains need to be considered when apparent pc AA digestibility is compared. Study 4 indicated that pc AA digestibility excluding basal endogenous AA losses for test ingredients determined using the regression approach might be transferable among strains. If this is verified in further studies, fast-growing broiler strains can be used to determine pc AA digestibility excluding basal endogenous AA losses determined for application in diets formulated for slow-growing broiler strains – as e.g. often used in organic broiler meat production – and vice versa. This approach would reduce the number of animal experiments because feed ingredients do not need to be tested using several broiler strains. Using slow-growing broiler chickens can result in more animals per pooled sample needed to obtain a sufficient sample amount (Ritteser 2016) because the amount of digesta sampled per animal is lower (on average, one third of the amount of the fast-growing strain in Study 4). Hence, using fast- instead of slow-growing broiler strains can reduce the number of experimental animals.

Future studies should investigate whether AA digestibility of test ingredients excluding basal endogenous AA is transferrable among laying hen strains because no such literature is available at present. The transferability of AA digestibility of test ingredients excluding basal endogenous AA between broiler chickens and laying hens might also be examined on a larger scale using the regression approach. This could enable researchers to predict AA digestibility of broiler chickens using data from cecectomized laying hens as model animals. If suitable, this would reduce the number of experimental animals for AA digestibility measurements to a fraction.

3.1.5 Further methodological considerations

Digesta sampling after slaughtering growing poultry

When pc AA digestibility is determined by sampling digesta from the terminal small intestine after slaughtering the animals, the sampled section of the small intestine must be standardized (Kluth and Rodehutscord 2009b). The length of the section to be sampled represents a conflict of aims, as discussed by Kluth et al. (2005): The longer the sampled section of the terminal small intestine is, the higher the risk that digestion was not completed. On the other hand, the sampled amount of digesta is larger the longer the sampled section of the terminal small intestine is. Larger amounts of digesta sampled per animal reduce the number of animals needed to achieve the required amount for analyses (see Section 3.7.1). Considering studies on broiler

chickens (Kluth et al. 2005; Bormann 2019) and laying hens (Rezvani et al. 2008a), the terminal two thirds of the section between Meckel's diverticulum and the end of the small intestine should be sampled. These researchers have found lower AA digestibility up to the proximal compared with the medial and terminal third of the section between Meckel's diverticulum and the end of the small intestine. Hence, sampling digesta from the complete section between Meckel's diverticulum and the end of the small intestine, as is still often found in the literature (e.g., Wu et al. 2020; Watts et al. 2021), leads to underestimated pc AA digestibility values. It should be mentioned that Poureslami et al. (2012) found no significant differences in AA digestibility when the digesta were obtained from the entire section between Meckel's diverticulum and the end of the small intestine or the terminal 15 cm of the small intestine. However, the experimental setup makes differences improbable to be found because parts of the sampled digesta are from the same section of the small intestine in both treatments. Which proportion of the sampled section of the small intestine was the same in the sampling procedures cannot be assessed because Poureslami et al. (2012) did not provide this information. Kluth et al. (2005) recommended not to determine the intestinal section to be sampled based on a fixed length in cm. They argued that such a procedure cannot account for variation in length of the section between Meckel's diverticulum and the end of the small intestine among individual animals, which was 38–62 cm in 21-day-old broiler chickens.

Digesta can be removed from the sampled section of the small intestine by flushing out with distilled water (e.g., Rodehutscord et al. 2004) or by squeezing (e.g., Bandegan et al. 2009). Poureslami et al. (2012) found that the determined pc AA digestibility obtained from flushed out digesta of broiler chickens was ~4 pp higher than pc AA digestibility determined with squeezed out digesta. The authors argued that squeezing out digesta likely expels endogenous AA from mucin and mucosa, as found in a study on pigs (de Lange et al. 1989). Therefore, flushing out the digesta appears more accurate.

Ravindran et al. (2017) recommend freezing digesta immediately after collection. This intends to avoid changes in the AA composition, exemplarily owing to microbial activity or endogenous enzymes present in the digesta. No studies on the influence of time and temperature on the AA composition of digesta of broiler chickens after slaughter are available. However, a study on excreta of cecectomized laying hens showed that the AA composition can change considerably within a few hours after excretion at an ambient air temperature of 19°C and 29°C (Siegert et al. 2021). Further, Künzel et al. (2019b) demonstrated that time between collection of digesta of broiler chickens and freezing affects the composition of inositol phosphate isomers. It therefore appears advisable to use ice-cold water to flush out digesta in AA digestibility

experiments to cool down the digesta as quickly as possible, as performed in experiments on inositol phosphates (Zeller et al. 2015) and pc AA digestibility (Hofmann et al. 2020a).

Experiments on pc AA digestibility determined by sampling digesta from the terminal small intestine after slaughtering the animals presuppose that the sampled digesta originate from the experimental diets only and that the digestive tract is adapted physiologically to the diet. Kluth and Rodehutscord (2010) investigated the duration of prefeeding the experimental diets 7, 5, and 3 days prior to digesta collection. There were no significant differences for apparent pc AA digestibility of the experimental diets and the AA digestibility of dried distillers' grains with solubles as a test ingredient using the regression approach. Numerical differences in apparent pc AA digestibility of the experimental diets were less than 1 pp for most AA among prefeeding durations. Numerical differences in AA digestibility of dried distillers' grains with solubles averaged 1 pp between 7 and 5 days of prefeeding the experimental diets, while the numerical difference between 5 and 3 days of prefeeding averaged 4 pp. Other studies on the duration of prefeeding experimental diets in pc AA digestibility experiments are not available. The numerical differences between 5 and 3 days of prefeeding found by Kluth and Rodehutscord (2010) may have resulted in the recommendation to feed experimental diets 5 days prior to digesta sampling in pc AA digestibility experiments (Ravindran et al. 2017). This appears to be comprehensible but the results of Kluth and Rodehutscord (2010) demonstrate that shorter prefeeding durations are an option when necessary.

Ravindran et al. (2017) recommend the use of 21–35-day-old broiler chickens for experiments, arguing that age-dependent effects on pc AA digestibility are of no relevance after 14 days of age. This view is supported by some studies in the literature (Batal and Parsons 2002; Thomas et al. 2008; Ritteser 2016). In other studies, however, researchers have reported age effects on pc AA digestibility in broiler chickens older than 14 days (Jamroz et al. 2002; Huang et al. 2005; Kim and Corzo 2012; Szczurek et al. 2020; Barua et al. 2021b). The effects of feed ingredients seem to contribute to different conclusions on age effects on pc AA digestibility among studies. The apparent pc AA digestibility of triticale and barley differed between 14- and 28-day-old broiler chickens while no age effect was determined for wheat (Szczurek et al. 2020). Barua et al. (2021b) found that the apparent pc AA digestibility of wheat linearly increased with age while no differences were found for sorghum. This was based on weekly measurements using 7–42-day-old broiler chickens. The age effect was reversed for AA digestibility excluding basal endogenous AA determined on the same days using N-free diets. These AA digestibility estimates declined with age for sorghum while no differences were determined for wheat. Unequivocal conclusions regarding the age of broiler chickens older than

14 days cannot be drawn because the results of the available studies are conflicting. Nonetheless, it appears that age differences were rather small in most studies when broiler chickens were older than 14 days. Hence, the recommendations of Ravindran et al. (2017) to use 21–35-day-old broiler chickens appears suitable until age effects are better understood.

Considerations on the regression approach

Whether two or three experimental diets are needed when the regression approach is used depends on the accuracy of single measurements. Two experimental diets have emerged as sufficient in studies on cecectomized laying hens because of the quantitative excreta collection enables highly precise measurements (see Section 1.4.1) and the risk of deviating feed intake among dietary treatments is relatively low (see Section 3.1.1). Three experimental diets are advantageous in studies when digesta are collected from the terminal small intestine to increase the number of degrees of freedom to counteract the lower precision of single measurements. Alternatively, degrees of freedom could be increased by raising the number of replicates per treatment. A further alternative is to increase the accuracy of single observations by increasing the number of animals per pen. However, these alternatives are disadvantageous when experimental diets cause unexpected unfavorable effects, like divergent feed intake. To give an example, feed intake at the highest inclusion of rapeseed cake was significantly reduced in Study 1, which led to inaccurate AA digestibility estimates. Therefore, Study 1 also includes the more accurate AA digestibility estimates determined without the highest inclusion of rapeseed cake. This would not have been possible if the diet with an equal share of cornstarch and the test ingredient had not been tested.

When the regression approach is used, the effect of the feed ingredient devoid of AA that is substituted at the expense of the test ingredient on the AA digestibility estimates of the test ingredient is unknown. Cornstarch was used as the ingredient devoid of AA in any study using the regression approach so far. Whether increasing dietary carbohydrate concentrations increase endogenous carbohydrase secretion has been reported inconsistently in the literature (Hulan and Bird 1972; Brzęk et al. 2013). Hence, it cannot be ruled out that the cornstarch in diets used for digestibility experiments using the regression approach leads to increased carbohydrase secretion and that the AA contained in the secreted carbohydrase secreted are not reabsorbed. These AA would represent specific endogenous AA of cornstarch. Differences in ingested and digested AA among experimental diets would not only result from undigested AA from the test ingredient and specific endogenous AA due to the test ingredient, but also from specific endogenous AA due to cornstarch. It is difficult to investigate whether such an influence is

relevant because other possible ingredients devoid of AA – like glucose, cellulose, or diamol – might also influence specific endogenous AA (Kluth and Rodehutscord 2009a). The possible influence of ingredients devoid of AA is not limited to the regression approach. Ingredients devoid of AA are also used as fillers when the AA digestibility is determined based on complete diets with the test ingredients as the only source of protein. This particularly applies when test ingredients high in CP are investigated, like peas, soybean meal, or fish meal (Masey O'Neill et al. 2012; Ullah et al. 2016; Siegert et al. 2017).

Experiments on cecectomized laying hens

In experiments on cecectomized laying hens, the number of required excreta collection days and collection intervals have not yet been investigated. Excreta of five (Rezvani et al. 2008b) or four (Study 9) days of collection have been pooled in published studies. Fewer days of collection are favorable in terms of animal welfare, effort, and cost, but this approach bears the risk of less precise measurements. A methodological investigation seems advisable. A possibility is to collect excreta quantitatively for several consecutive days and to process and to analyze excreta of each day separately. This procedure would enable researchers to determine AA digestibility based on individual days and any desired combination of the sampling days.

Concerning collection intervals, excreta have been collected thrice (Rezvani et al. 2008b) or twice (Agbede et al. 2009a) daily. Longer collection intervals reduce effort and may disturb the animals less. More frequent collections promote animal care checks and probably lead to more accurate measurements because feathers, flakes of shed skin, and spilled feed can be separated easier. In addition, the chemical composition of excreta can change after excretion owing to microbial activity and exposure to the environment via numerous mechanisms. Undigested AA or uric acid can be converted to ammonia (Groot Koerkamp et al. 1998), which can be further degraded to nitrite and nitrate (Rasouli-Sadaghiani and Moradi 2014). The AA composition in excreta of cecectomized laying hens can change within a few hours under high airflow at ambient temperatures (Siegert et al. 2021). The relevance of collection intervals should be investigated in a methodological experiment. Possibilities include comparing collections conducted in intervals of 8 h, 12 h, and 24 h, or investigating changes in excreta composition after predefined periods after voiding. Such investigations on the number of collection days and collection intervals are also interesting for the utilization of other nutrients, such as phytate (Agbede et al. 2009b) or ME_N (Zuber and Rodehutscord 2017).

3.2 Variation in amino acid digestibility of feed ingredients

There are differences in AA digestibility among 1) AA within feed ingredients, 2) variants of feed ingredients, and 3) types of feed ingredients. Assay details markedly affect the outcome of AA digestibility studies, making comparisons of digestibility estimates determined using different methods inexpedient. A comparison between studies using the same regression approach in cecectomized laying hens (Figure 8) indicates two consistencies in terms of differences in AA digestibility among types of feed ingredients. First, digestibility of most AA was lowest for rye. Other studies on AA digestibility of rye excluding post-ileal fermentation have not been published, making a verification of a low AA digestibility of rye impossible. Second, among the legume grains, AA digestibility of most AA was lowest in faba beans

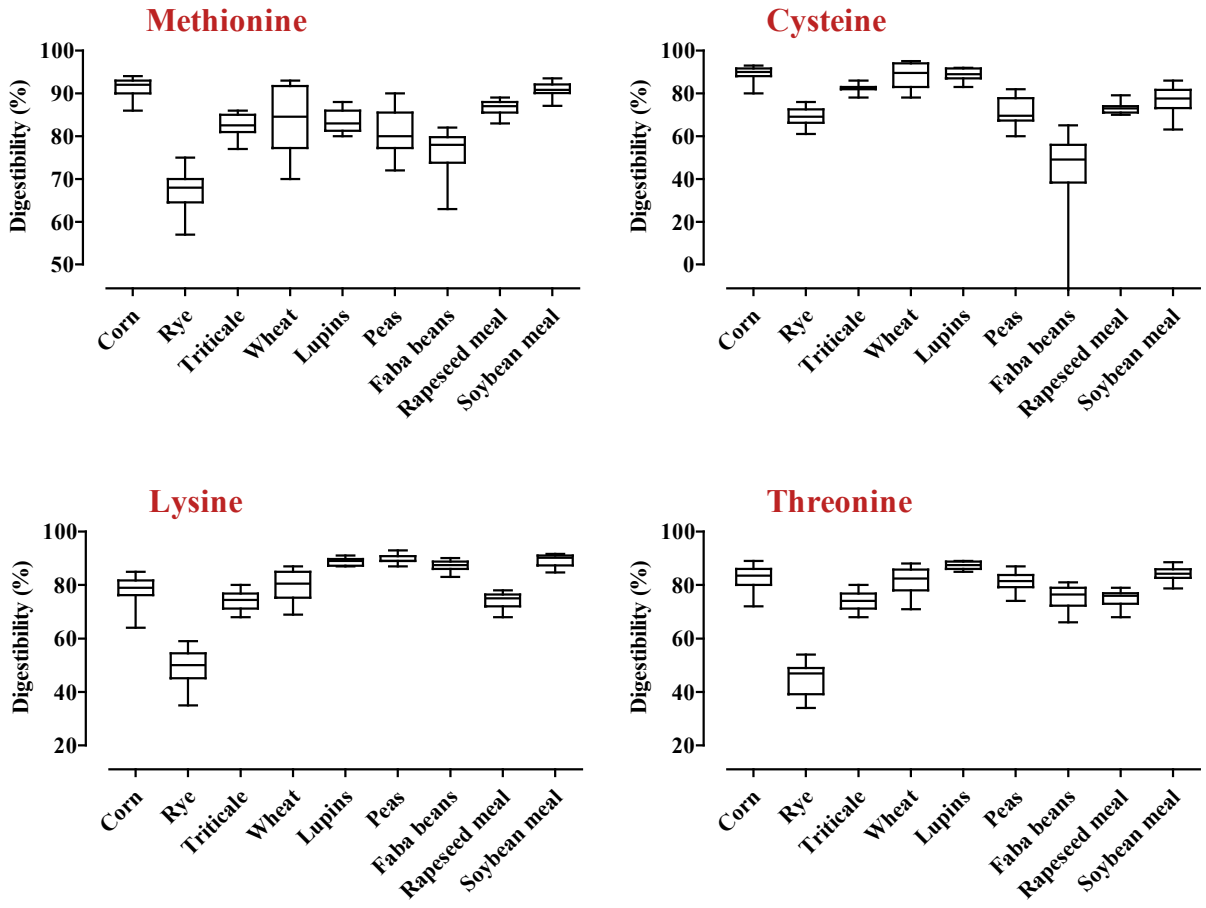

Figure 8. The level of digestibility of selected first-limiting amino acids in studies on cecectomized laying hens investigating the variation within corn (n=20; Zuber and Rodehutscord 2017), rye (n=20; Zuber et al. 2016), triticale (n=20; Study 9), wheat (n=20; Zuber and Rodehutscord 2016), lupins and peas (n=12 each; Study 10), faba beans (n=16; Study 11), rapeseed meal (n=9; Rezvani et al. 2012), and soybean meal (n=18; unpublished results) using the regression approach. The boxes indicate 25% quantiles, the horizontal lines within boxes indicate medians, and the vertical lines extending from boxes indicate the minimum/maximum values. Note that the ordinate has a different scale for each amino acids.

compared with peas and lupins, which represent other commonly used legumes (FAOSTAT 2020). The same ranking of legume grains has also been determined for broiler chickens (Masey O'Neill et al. 2012; Koivunen et al. 2016), turkeys (Palander et al. 2006), and pigs (Jezierny et al. 2011). Further consistencies are not apparent.

The variation in AA digestibility within one feed ingredients in experiments on cecectomized laying hens using the regression approach was different between feed ingredients and individual AA (Figure 9). The standard deviation of digestibility of 12 out of 16 AA was lowest within lupins and highest within faba beans and rye (tryptophan (**Trp**) not included because it was not analyzed in some of the studies). The standard deviations of Arg digestibility was on a low level and standard deviations of the digestibility of Lys and proline (**Pro**) in the cereal grains corn, rye, triticale, and wheat were on a high and low level compared with the other feed ingredients, respectively. Further consistencies were not detected. The standard deviation of Cys in faba beans is remarkably high because three faba bean variants were considerably lower in AA digestibility compared with the other 13 variants under investigation. The reasons for the low Cys digestibility of the respective variants are not apparent (four winter and four spring faba bean variants each grown in two locations were investigated in this study; the three variants low in AA digestibility were from three different combinations of sowing date and location).

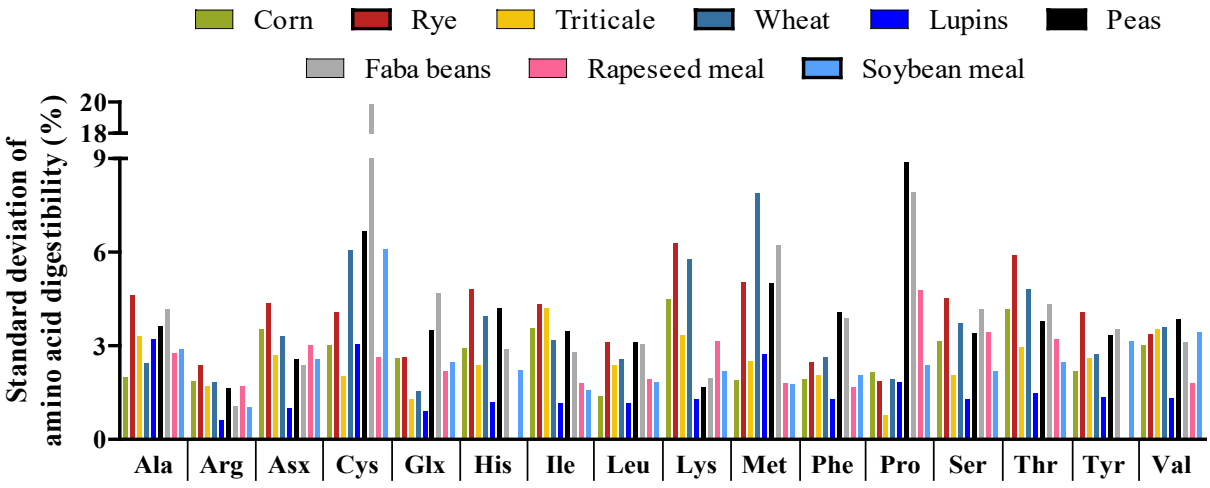

Figure 9. Standard deviation of amino acid digestibility in studies on cecectomized laying hens using different variants of corn (n=20; Zuber and Rodehutscord 2017), rye (n=20; Zuber et al. 2016), triticale (n=20; Study 9), wheat (n=20; Zuber and Rodehutscord 2016), lupins and peas (n=12 each; Study 10), faba beans (n=16; Study 11), rapeseed meal (n=9; Rezvani et al. 2012; Tyr not measured), and soybean meal (n=18; unpublished results) using the regression approach. See the list of abbreviations for abbreviations of amino acids.

The choice of variants of the feed ingredients presented in Figure 8 and Figure 9 differed depending on the aim of the studies. The samples of rye, triticale, and wheat were cultivated

under identical environmental and agronomic conditions, and the conditions were similar among the corn samples (details described in Rodehutscord et al. 2016). Hence, differences among rye, triticale, and wheat samples arose almost exclusively from characteristics of the cereal grain genotypes while genotype characteristics were principal contributors to differences among corn samples. The lupin and pea samples were relevant licensed cultivars from various harvest years. The rapeseed meal samples were obtained from different processing plants and were produced within two months. The soybean meal samples were gathered over a period of one year. Cropping conditions of the lupins, peas, rapeseeds, and soybeans are unknown. For faba beans, identical genotypes grown on two sites were used, enabling the detection of genotype × environment interactions. The described different selection of feed ingredients samples probably influenced the determined variation within feed ingredients but the extend of this influence is unknown.

The relevance of AA digestibility for the digestible AA concentrations in two exemplary practical laying hen diets is shown in a model calculation (Table 2). The practical laying hen diets were chosen based on their ingredient composition so that only ingredients were included for which the variation in AA digestibility was determined using cecectomized laying hens and the regression approach (Figure 8 and Figure 9). The determined ranges of digestible AA concentrations of first-limiting AA in an exemplary diet containing corn, wheat, and soybean meal as ingredients were 1.6 g digestible (Met+Cys)/kg, 2.3 g digestible Lys/kg, and 1.6 g digestible Thr/kg (diet 1a in Table 2). For another exemplary diet containing wheat, peas, and rapeseed meal as ingredients (diet 2a in Table 2), the determined ranges were 1.0 g digestible (Met+Cys)/kg, 1.4 g digestible Lys/kg, and 1.3 g digestible Thr/kg. These ranges appear relevant in relation to the recommended AA concentrations for laying hens by the Gesellschaft für Ernährungsphysiologie (1999). Unfortunately, the Gesellschaft für Ernährungsphysiologie (1999) does not recommend digestible AA concentrations. Assuming 85% AA digestibility, the recommendations of the Gesellschaft für Ernährungsphysiologie (1999) for an average laying hen (1,600 g body weight and 55 g daily egg production) account for 4.6 g digestible (Met+Cys)/kg, 5.3 g digestible Lys/kg, and 3.8 g digestible Thr/kg. The determined differences in digestible AA concentrations relative to the recommended digestible concentrations accounted for 34%, 43%, and 43% for Met+Cys, Lys, and Thr, respectively, for the diet containing corn, wheat, and soybean meal. For the diet containing wheat, peas, and rapeseed meal, differences in digestible concentrations relative to the recommended digestible concentrations accounted for 23%, 27%, and 34% for Met+Cys, Lys, and Thr, respectively.

Table 2. Model calculation of the relevance of variation in amino acid digestibility for digestible amino acid concentrations in practical laying hen diets.

	Met+Cys[1] (g/kg)			Lys[1] (g/kg)			Thr[1] (g/kg)		
	Digestible concentrations	Range	Difference in diet[2,3]	Digestible concentrations	Range	Difference in diet[2]	Digestible concentrations	Range	Difference in diet[2]
Diet 1a) Digestible concentrations calculated with both range in amino acid digestibility and range in analyzed amino acid and dry matter concentrations[4]									
Corn	2.6–4.0	1.4	0.3	1.4–2.5	1.1	0.2	1.9–3.2	1.3	0.3
Wheat	3.3–4.6	1.4[5]	0.5	2.2–3.0	0.8	0.3	2.3–3.4	1.1	0.4
Soybean meal	9.7–13.1	3.3	0.8	23.8–31.3	7.5	1.7	14.4–18.5	4.2[5]	1.0
Sum			1.6			2.3			1.6
Difference in diet relative to recommendations[6]			34%			43%			43%
Diet 1b) Digestible concentrations calculated with range in amino acid digestibility and mean values of analyzed amino acid and dry matter concentrations[4]									
Corn	3.0–3.4	0.4	0.08	1.6–2.1	0.5	0.1	2.2–2.7	0.5	0.1
Wheat	3.3–4.1	0.9[5]	0.3	2.2–2.8	0.6	0.2	2.4–3.0	0.6	0.2
Soybean meal	10.3–12.2	1.9	0.4	25.5–27.6	2.1	0.5	15.6–17.5	1.9	0.4
Sum			0.8			0.8			0.8
Difference in diet relative to recommendations[6]			18%			15%			20%
Diet 2a) Digestible concentrations calculated with both range in amino acid digestibility and range in analyzed amino acid and dry matter concentrations[7]									
Wheat	3.3–4.6	1.4	0.5	2.2–3.0	0.8	0.3	2.3–3.4	1.1	0.4
Peas	3.2–4.2	1.0	0.3	12.8–14.9	2.1	0.7	5.8–7.5	1.7	0.6
Rapeseed meal	10.7–12.5	1.8	0.2	12.2–15.6	3.4	0.4	10.2–13.1	2.9	0.3
Sum			1.0			1.4			1.3
Difference in diet relative to recommendations[6]			23%			27%			34%
Diet 2b) Digestible concentrations calculated with range in amino acid digestibility and mean values of analyzed amino acid and dry matter concentrations[7]									
Wheat	3.3–4.1	0.9[5]	0.3	2.2–2.8	0.6	0.2	2.4–3.0	0.6	0.2
Peas	3.2–4.1	0.8[5]	0.3	13.4–14.4	0.9[5]	0.3	6.0–7.1	1.1	0.4
Rapeseed meal	11.0–12.0	1.1[5]	0.1	12.9–14.8	1.9	0.2	10.7–12.5	1.7[5]	0.2
Sum			0.7			0.8[4]			0.8
Difference in diet relative to recommendations[6]			16%			14%			20%

Table footnotes shown on next page.

Footnotes to Table 2:
[1]See list of abbreviations for abbreviations of amino acids.
[2]In practical diets containing 38.5% wheat, 20% corn, and 23% soybean meal (Bayerische Staatsgüter 2017) or 35% wheat, 35% peas, and 12% rapeseed meal (Bellof et al. 2020) as ingredients containing amino acids in diet 1 and diet 2, respectively.
[3]Two decimal digits presented if value < 0.1.
[4]Values for amino acid concentrations, dry matter concentrations, and amino acid digestibility taken from Rodehutscord et al. (2016), Zuber and Rodehutscord (2017), and Zuber and Rodehutscord (2016) for corn and wheat as well as an unpublished experiment for soybean meal.
[5]Discrepancy between the stated difference and the difference indicated by the range as well as the discrepancy between stated sum and the stated addends caused by rounded values.
[6]Sum of the differences in the diets relative to the recommended concentrations by the Gesellschaft für Ernährungsphysiologie (1999) for laying hens with 1,600 g body weight and 55 g daily egg production; see description in the text.
[7]The values for amino acid concentrations, dry matter concentrations, and amino acid digestibility of wheat, peas, and rapeseed meal taken from Zuber and Rodehutscord (2016), Study 10, and Rezvani et al. (2012), respectively.

These values include both the variation in AA digestibility and the variation in total AA concentrations within the feed ingredients. The contribution of the variation in analyzed concentrations can be excluded by determining the difference in digestible concentrations using the variation in AA digestibility and the mean value for total AA concentrations (diets 1b and 2b in Table 2). In these calculations, the determined differences in digestible concentrations relative to the recommended digestible concentrations accounted for 18%, 15%, and 20% for Met+Cys, Lys, and Thr, respectively for the diet containing corn, wheat, and soybean meal, and 16%, 14%, and 20% for Met+Cys, Lys, and Thr, respectively for the diet containing wheat, peas, and rapeseed meal. Hence, differences in AA digestibility contributed 36–70% to the variation in digestible AA concentrations, with the remainder being attributable to the variation in total AA concentrations.

Whether considering the variation in digestible AA concentrations is justified depends on the claim of precision and efficiency. Considering variation in digestible AA concentrations is imperative if the aim is to meet the requirement of the animals for digestible AA exactly. Taking mean digestibility values for feed ingredients with low variation in AA digestibility – e.g., soybean meal, rapeseed meal, peas, and lupins – into account can be justified when the precision and efficiency receive less attention. Among the investigated feed ingredients, variation in AA digestibility within the cereal grains wheat, triticale, rye, and corn was relatively high. Digestible AA in cereal grains in diets for laying hens contribute about half of the supply with digestible AA. Therefore, taking primarily the variation in AA digestibility of cereal grains into account seems a workable solution when the claim of precision and efficiency is moderate. Taking the variation in AA digestibility in the practice into account presupposes that AA

digestibility of single feedstuff batches is predictable. The predictability of AA digestibility is discussed in Section 3.5.

The ranges of pc AA digestibility in broiler chickens within feed ingredients are similar to the ranges of AA digestibility determined in cecectomized laying hens (Figure 10). Hence, the relevance of considering AA digestibility in feed formulation for precision of feed formulation seems to be on a similar level. Of note, only ranges but not levels of AA digestibility should be compared because different assays were used among studies on broiler chickens.

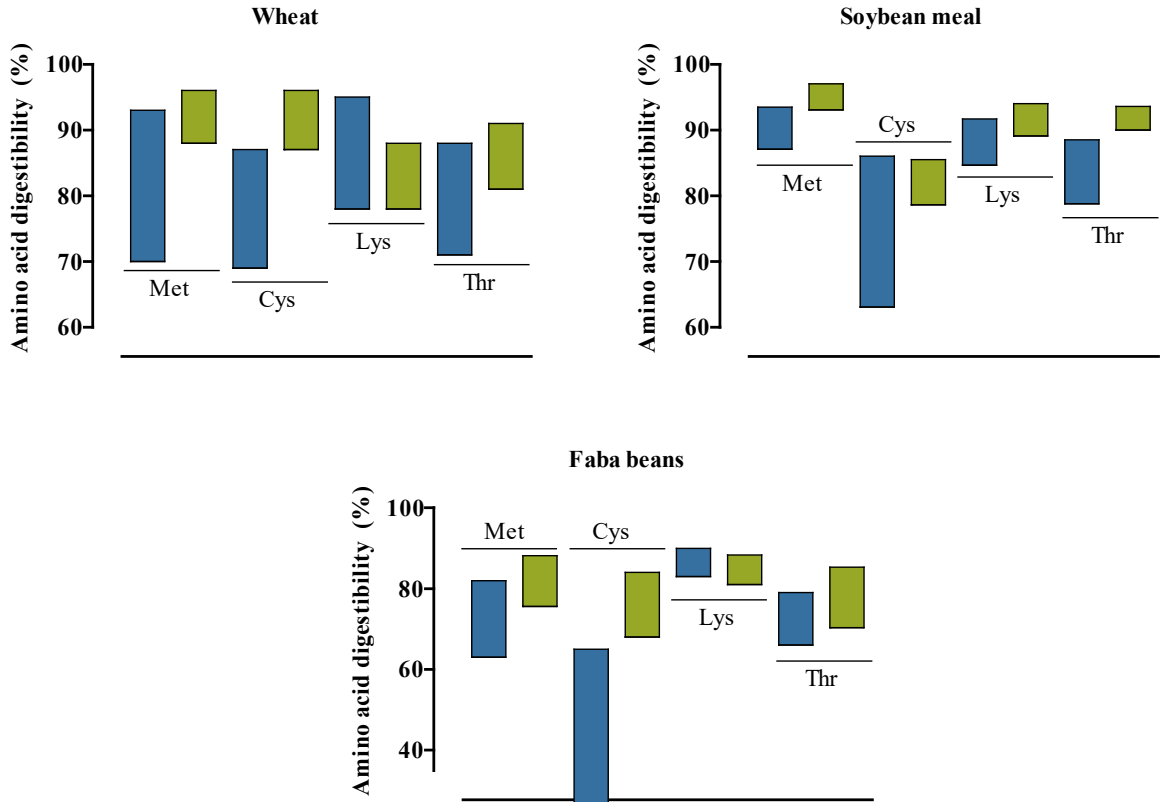

Figure 10. Ranges of amino acid digestibility in cecectomized laying hens (blue) and prececal amino acid digestibility in broiler chickens (green) determined in wheat (laying hens: n=20, Zuber and Rodehutscord 2016; broiler chickens: n=12, Bormann 2019), soybean meal (laying hens: n=18, unpublished results; broiler chickens: n=22, Frikha et al. 2012), and faba beans (laying hens: n=16, Study 11; broiler chickens: n=10, Abdulla et al. 2021). See the list of abbreviations for abbreviations of amino acids.

3.3 Influences of feed composition and feeding management on amino acid digestibility

While Section 3.1 focuses on how to conduct AA digestibility experiments to obtain accurate and representative measurements, this section describes influences on AA digestibility in

poultry feeding. A high number of influences on AA digestibility has been identified, many of which often interact (Lemme et al. 2004).

3.3.1 Feed provisioning

Feed provisioning can considerably influence AA digestibility (Study 2). The decision for using the investigated restrictive protocols in Study 2 was methodologically driven. Those schemes are not applicable for practical feeding purposes. Other studies investigating the effects of more practical feed provisioning protocols on AA digestibility are not available. However, studies have shown that restrictive feeding can increase the gain-to-feed ratio (Svihus et al. 2013; Moraes et al. 2016), PUE (Sacranie et al. 2017), and the utilization of P, phytate, and starch (Sacranie et al. 2017; Kristoffersen et al. 2021) without performance loss compared with birds fed *ad libitum*. Given the results of Study 2, it appears likely that higher AA digestibility contributed to increased PUE observed by Sacranie et al. (2017) in restrictively fed birds. At present, broiler chickens in commercial poultry meat production usually have unrestricted access to feed. This approach may be subject to change in the future when the necessity for a more efficient use of feed ingredients materializes and when restrictive feeding protocols applicable in practical farming are established. This would necessitate additional AA digestibility measurements for feed ingredients because Study 2 revealed that the feed provisioning protocol in AA digestibility experiment should correspond to practical conditions (see Section 3.1.1).

3.3.2 Processing of feed and of feed ingredients

Conflicting effects of grinding feed ingredients have been reported. Studies revealed increasing (Crévieu et al. 1997) and decreasing (Parsons et al. 2006; Valencia et al. 2009; Xu et al. 2015) effects on pc apparent CP digestibility in broiler chickens while others have described no effect (Bhuiyan et al. 2012; Singh et al. 2014). Study 3 demonstrated that more intense grinding can have opposite effects on pc AA digestibility between two test ingredients in one experiment. Another study demonstrated that the effect of particle size distribution of one feed ingredient on pc CP digestibility depended on the particle size distribution of another feed ingredient (Pacheco et al. 2013). This interaction between particle size distribution of two feed ingredients on pc CP digestibility may indicate that particle size effects of one feed ingredient cannot be evaluated without the context of all other ingredients of a diet. This would restrict the additivity of AA digestibility of single feed ingredients in complete diets.

Optimizing particle size distributions of complete diets may be seen as a way to avoid uncertainties in the effects of particle size distributions on AA digestibility. To achieve this, optimized particle size distributions of complete diets need to be determined and must be unerringly produced. As summarized by Lyu et al. (2020), the particle size distribution after grinding is influenced by other determinants in addition to the grid size of the mill. Grinding feed ingredients to predefined particle size distributions can therefore only be achieved approximately and requires broadened understanding of determinants, such as the type, settings, and geometry of the grinder as well as the chemical composition, physical characteristics, and geometry of the feed ingredient. The need for a broader understanding of determinants also includes the effects of feed compaction, like pelleting, on particle size distribution.

As non-additive effects are difficult to implement in feed formulation, particle size effects probably will permanently impede the accuracy of formulating diets that exactly meet the requirement of poultry for digestible AA. Such non-additive effects would contribute to a barely reducible variance between predicted and actual AA digestibility. This irreducible variance can only be met with safety margins in digestible AA supply if the aim is not to risk AA deficiency. The safety margins may or may not be needed by the animals. The consequence is a contribution to unpredictability of PUE because the unutilized N provided with the safety margins will be excreted.

3.3.3 Exogenous protease

The effects of protease supplementation on CP and AA digestibility have been found to be inconsistent. Studies on broiler chickens have shown that pc AA digestibility was increased for all (Stefanello et al. 2016) or some AA (Angel et al. 2011). In other studies, no effects (Kaczmarek et al. 2014) or decreasing effects (Walk et al. 2018) were found. In the few turkey studies, no significant effects for all AA (Boguhn et al. 2011) or no effects for all except for one AA (Vieira et al. 2013) have been described. No studies on laying hens are available. Compiling the results from existing literature indicates that protease supplementation increases AA digestibility in the majority of published studies. However, it appears likely that the conclusions based on published studies are misleading because studies without an effect of enzyme supplementation on AA digestibility are, regrettably, often not published.

The origin and dosage of protease can influence the pc AA digestibility response in broiler chickens. Study 5 compared the effects of three proteases in broiler chickens. The first protease supplemented at the level recommended by the producer resulted in no significant difference in pc digestibility of all AA compared to a diet without protease supplementation. For a second

protease, pc digestibility of all AA was significantly reduced. A significant reduction or no difference compared with the basal diet was found for the third protease. For two of the proteases an eight-fold higher protease supplementation than recommended resulted in no significant change in pc AA digestibility. For the third protease, however, this supplementation increased significantly the digestibility of all AA, by an average of 2.6 pp. A dose-dependent effect of the latter protease was also determined by Angel et al. (2011). In contrast to Study 5, Angel et al. (2011) found the effect of protease supplementation to be fully expressed at the supplementation level recommended by the producer. Classifying distinct protease products with respect to their effectiveness in increasing pc AA digestibility is difficult. Increasing effects of supplementing the proteases that were found not to influence AA digestibility in Study 5 increased pc CP and AA digestibility in other studies (e.g., Liu et al. 2013).

In the follow-up study (Study 6), the effects of supplemented protease in diets containing different oilseed meals were investigated in broiler chickens. Protease supplementation increased pc digestibility of all AA by an average of 2.1 pp, but no evidence was provided that the efficacy of protease supplementation depends on the protein source used. However, the literature on the effect of diet composition on the efficacy of protease supplementation has shown inconsistent results. The reasons for these divergent results are unclear. Other researchers have determined no effect of protease supplementation on pc CP or AA digestibility in diets with soybean meal or a mixture of soybean meal and canola meal (Toghyani et al. 2017), different origins of full-fat soybeans (Dalólio et al. 2016), or replacement of poultry by-product meal with soybean meal (Mahmood et al. 2017). Different responses to protease supplementation have been reported for diets based on wheat or sorghum (Selle et al. 2016), or different proportions of corn and soybean meal (Freitas et al. 2011).

Several mechanisms for how protease supplementation can increase AA digestibility are discussed in the literature: 1) Protease might complement the own digestive system of the animals. This mechanism could include hydrolysis of peptides that are not hydrolyzed by endogenous enzymes of the animals. The existence of this mechanism is supported by an increased occurrence of free AA as well as di- and tripeptides in the jejunum of broiler chickens when protease was supplemented (Haahr et al. 2019). AA are almost exclusively absorbed as free AA and di- and tripeptides, while absorption of peptides with more than three AA is negligible (Krehbiel and Matthews 2003). However, the higher occurrence of potentially absorbable AA in the jejunum in the experiment of Haahr et al. (2019) did not result in an increased AA digestibility up to the jejunum. Another communication to the same experiment described an increased pc AA digestibility upon protease supplementation (Angel et al. 2020).

The observations of potentially absorbable AA in the jejunum and higher pc AA digestibility may not be causal. Nonetheless, the results of the experiment described by Haahr et al. (2019) and Angel et al. (2020) point to this mechanism being relevant. 2) Protease supplementation might influence the microbiota composition in the digestive tract and AA digestibility would then be influenced by modified microbial activity (see Section 3.3.6 for further explanations). 3) An increase in AA digestibility upon protease supplementation because of reduced endogenous AA has also been proposed (Cowieson and Roos 2016). Among the basal endogenous AA lost, the proportions of aspartic acid/asparagine (**Asx**), Cys, glutamic acid/glutamine (**Glx**), serine (**Ser**), and Thr are relatively high (Kluth and Rodehutscord 2009a). In Study 5 and Study 6, the increase in digestibility upon protease supplementation of these aforementioned AA was not particularly higher than the average of all measured AA (Figure 11). Therefore, these results do not provide evidence that the observed increase in AA digestibility can be explained by a reduction in endogenous AA.

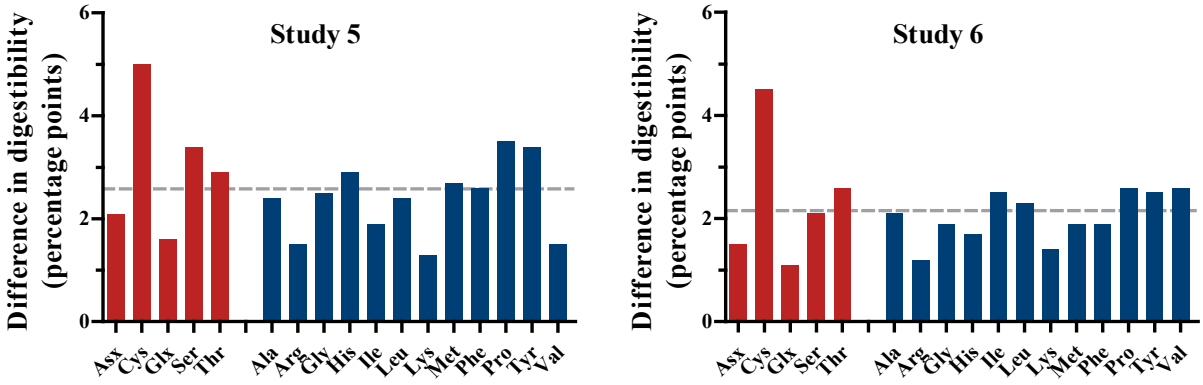

Figure 11. Increase in prececal amino acid digestibility upon protease supplementation in Study 5 (higher dosage of the protease that significantly increased amino acid digestibility) and Study 6. The red and blue bars represent amino acids with a higher or lower proportion in endogenous amino acids, respectively. The dashed lines indicate the median of all amino acids within each panel. See the list of abbreviations for abbreviations of amino acids.

Until now, investigations do not provide an unequivocal indication as to hint which of the mentioned mechanism is pivotal or predominant. Interpretations in the literature might be confounded by protease effects on feed intake (see Section 3.1.1). Feed intake was not influenced in Study 5 and Study 6, supporting evidence that endogenous AA are not the predominant determinant on AA digestibility upon protease supplementation.

3.3.4 Phytate, exogenous phytase, and calcium

Exogenous phytase is primarily used to increase the utilization of plant P, which is mostly present as phytate in the grains, by the animals (Rodehutscord and Rosenfelder 2016). Supplemented phytase can additionally increase AA digestibility. The effects of supplemented phytase on AA digestibility were inconsistent. Similarly to protease, most studies reported increasing effects of phytase supplementation on AA digestibility, but phytase effects on AA digestibility may be overestimated because the probability that investigations determining no effect on AA digestibility are published is lower compared to investigations that found a statistically significant effect of enzyme supplementation. Nonetheless, there are studies that described an increasing effect of phytase supplementation on AA digestibility (e.g., Agbede et al. 2009a; Pirgozliev et al. 2011; Amerah et al. 2014; Sommerfeld et al. 2018b) and others have described no effect (e.g., Sebastian et al. 1997; Snow et al. 2003; Rodehutscord et al. 2004; Agbede et al. 2009b; Manangi et al. 2009) in broiler chickens, turkeys, and laying hens. An exception is the study of Hughes et al. (2009), who determined a linearly decreasing AA digestibility with increasing phytase supplementation for a diet containing dicalcium phosphate in laying hens.

Studies on whether varying responses to phytase supplementation on AA digestibility are caused by the main protein-containing feed ingredients diverge. In Study 6, supplementing 1,500 phytase units (**FTU**)/kg from a 6-phytase produced by genetically modified *Aspergillus niger* increased pc AA digestibility in broiler chickens by an average of all AA of 4.7 pp. However, there was not an interaction between phytase supplementation and different oilseed meals (soybean meal, rapeseed meal, and sunflower meal) as the main protein sources. Consistently, there was no difference in the effect of supplementation of 1,200 FTU/kg phytase from a 3-phytase derived from genetically modified *Aspergillus niger* on pc AA digestibility in broiler chickens between these oilseed meals in another study (Ravindran et al. 1999a). However, Rutherfurd et al. (2002) reported the effects of supplementation of 750 FTU/kg phytase from a 6-phytase derived from genetically modified *Aspergillus oryzae* on pc AA digestibility for rapeseed meal, but not for soybean meal. Possible explanations for these divergent results include the types of methods used to determine AA digestibility. Basal endogenous AA losses, determined in a separate diet containing enzymatically hydrolyzed casein, were considered in AA digestibility calculations in the study by Rutherfurd et al. (2002). However, basal endogenous AA losses were not considered in Study 6 nor in the study by Ravindran et al. (1999a). Differences among studies might also be due to the phytase product and supplementation level used. An influence of feed ingredients is supported by results of

Morales et al. (2013), who determined differences in the efficacy of phytase on protein solubilization in an *in vitro* approach. Based on the available literature, it seems that choice of feed ingredients providing AA has little influence on phytase effects when the phytase dosage is high and mixed feed with several protein-containing ingredients is used.

Dietary Ca seems to influence the response to phytase supplementation in pc AA digestibility studies in broiler chickens. Based on Study 7, there is evidence that 1,500 FTU/kg phytase supplementation can compensate for a decreasing effect of the high Ca concentration on pc AA digestibility. However, an interaction between Ca concentrations and phytase supplementation or the effects of dietary Ca was not determined in other studies (Table 3). Hence, inconsistent consequences of dietary Ca on pc AA digestibility must have been caused by other dietary issues than the dietary Ca concentration per se.

Table 3. Compilation of the results of studies investigating the effects of dietary calcium (Ca) and phytase supplementation on the prececal crude protein or amino acid digestibility in broiler chickens.

Study	Dietary Ca (g/kg dry matter)[2]	Phytase supplementation (FTU/kg)	Effect[1]
Study 7	5.6–8.2	0–1,500	Significant interaction; without phytase, decreasing effect of dietary Ca; increasing effect of phytase with no difference between Ca concentrations
Sommerfeld et al. 2018b	5.9–10.0	0–1,500	No significant interaction; no effect of dietary Ca, increasing effect of phytase
Amerah et al. 2014	5.8–14.8[3]	0–1,000	No significant interaction; decreasing and increasing effect of dietary Ca and phytase, respectively
Walk et al. 2012	7.3–11.7[3]	0–5,000	No significant interaction; no effect of dietary Ca, increasing effect of phytase[4]
Akter et al. 2017	6.8–11.4[3]	0–500	No significant interaction[5]; decreasing and increasing effect of dietary Ca and phytase, respectively

[1]Prececal amino acid digestibility unless otherwise stated.
[2]All studies varied the dietary Ca by the addition of Ca carbonate.
[3]Dietary Ca calculated assuming 88% dry matter of the diets if dry matter was not stated.
[4]Except for two out of 15 AA.
[5]Prececal crude protein digestibility.

Decreased pc AA digestibility at higher dietary Ca concentration is usually explained by an increased pH in the digestive tract because this phenomenon can reduce the efficacy of enzymes, including proteases and phytases (Selle et al. 2009; Chung et al. 2013; Mutucumarana et al. 2014). In studies investigating pc AA digestibility, Ca carbonate was usually used as a Ca source. Ca carbonate is a compound with high and variable buffer and acid-binding capacity

(Kim et al. 2019). The hypothesis in Study 7 was that inconsistent Ca concentration effects on pc AA digestibility are caused by the buffer and acid-binding capacity of feed ingredients. This was tested by replacing Ca carbonate by Ca formate or by adding formic acid to Ca carbonate-containing diets because the buffer and acid-binding capacity of Ca formate is lower than that of Ca carbonate (Lawlor et al. 2005). The buffer or acid-binding capacity, however, does not seem to influence responses to dietary Ca and phytase. Nonetheless, replacing Ca carbonate with Ca formate or by adding formic acid to Ca carbonate-containing diets increased the pc AA digestibility of all AA by 1.1 pp on average.

In the literature, researchers have described no or a decreasing effect while an increasing effect of increasing dietary Ca on pc AA digestibility was not described (Table 3). Some authors have reported an increase in pc AA digestibility in broiler chickens when Ca was supplemented together with mineral phosphate as mono- or dicalcium phosphate (Study 6; Centeno et al. 2007; Pieniazek et al. 2017). Increasing AA digestibility upon monocalcium phosphate supplementation was also observed in cecectomized laying hens (Agbede et al. 2009b). Given that increasing dietary Ca without concurrently increasing mineral phosphate had no effect or decreased pc AA digestibility, it appears probable that the average increase in pc AA digestibility was caused by the mineral phosphate, rather than the Ca contained in the mono- or dicalcium phosphate. Study 6 described two explanatory approaches in detail, which are summarized briefly as follows: 1) More P available for metabolic processes may have enabled higher nutrient absorption due to an increased functionality of membranes and active AA or peptide transporters (Martinez-Amezcua et al. 2006). 2) Mineral phosphate supplementation increased feed intake in Study 6 as in most studies in the literature (summarized by Sommerfeld 2018). The increasing effect on AA digestibility upon mineral phosphate supplementation might therefore represent a consequence of affected feed intake (see Section 3.1.1).

Higher Ca concentrations in diets for laying hens possibly contribute to a generally lower level of AA digestibility in laying hens compared with broiler chickens. This phenomenon was shown by Adedokun et al. (2009) and by a comparison of the results presented by Bormann (2019) and Zuber and Rodehutscord (2016), who investigated the same wheat genotypes in broiler chickens and cecectomized laying hens, respectively. This hypothesis of higher AA digestibility at lower dietary Ca concentrations seems to be contradicted by higher AA digestibility when 31.5 instead of 11.0 g Ca/kg dry matter (assuming 88% dry matter) was fed to laying and broiler chickens hens, respectively (Adedokun et al. 2018). However, these outcomes may also result from the unpractical diet formulation because corn dried distillers' grains with solubles were the only source of protein in the study of Adedokun et al. (2018). It

cannot be ruled out that variation in Ca concentrations may have contributed to the variation in AA digestibility of 20 triticale genotypes in cecectomized laying hens (Study 9) because significant correlation coefficients (P<0.050) between Ca concentrations and the digestibility of nine AA in the range of 0.45–0.69 were determined. The correlation was close to significance for another AA (coefficient 0.44, P=0.053). However, all correlations were positive. This apparently contradicts the hypothesis of higher AA digestibility at lower dietary Ca concentrations. The range of Ca concentrations in the experimental diets accounted for only 0.37 g Ca/kg dry matter in Study 9, which is very low compared with Ca concentration ranges in Table 3. Hence, a causative influence of dietary Ca in Study 9 is rather improbable.

Similarly to protease, different mechanisms may explain phytase supplementation effects on AA digestibility: 1) Phytate can form binary phytate-protein and ternary phytate-protein-cation complexes (Selle et al. 2012). Such complexes make proteins less accessible to digestive enzymes. As phytase hydrolyzes phytate, phytase can prevent formation or contribute to degrade such complexes. Binary protein-phytate complexes are mainly formed in the proventriculus and gizzard because formation is maximized at a pH below 4 and when the pH is below the isoelectric point of the protein. Both conditions usually are achieved in poultry feeding (Krieg et al. 2021). Therefore, long retention times in the crop and phytases with low pH optima are more likely to diminish the relevance phytate-protein complexes. Phytate-protein-cation complexes are mainly formed from phytate-protein complexes at higher pH posterior to the stomachs. This emphasizes the relevance of phytases with low pH optima when complexes limit protein digestion (Sommerfeld 2018). 2) As phytase increases the phosphate digestibility, phytase might increase protein solubility and denaturation because phosphate acts as a kosmotropic agent (Selle et al. 2012). Kosmotropic agents reduce protein solubility by stabilizing the hydrogen bonding network and, hence, inducing stabilization and aggregation of undissolved proteins (Moelbert et al. 2004). This phenomenon would contribute to higher AA digestibility. However, the actual relevance of kosmotropic and chaotropic agents on pc AA digestibility is largely unexplored. 3) Similarly to protease, phytase supplementation might influence the microbiota composition in the digestive tract and AA digestibility would then be influenced by modified microbial activity (see Section 3.3.6 for further explanations). 4) Another possible mechanism is a reduction in endogenous AA secretion upon phytase supplementation, as described before for protease supplementation (see Section 3.3.3). In Study 5, Study 6, and Study 7, the increase in pc AA digestibility gave no indication of a reduced endogenous AA secretion because the increase in pc digestibility of AA with high proportions of endogenous AA overall was not considerably different compared with others

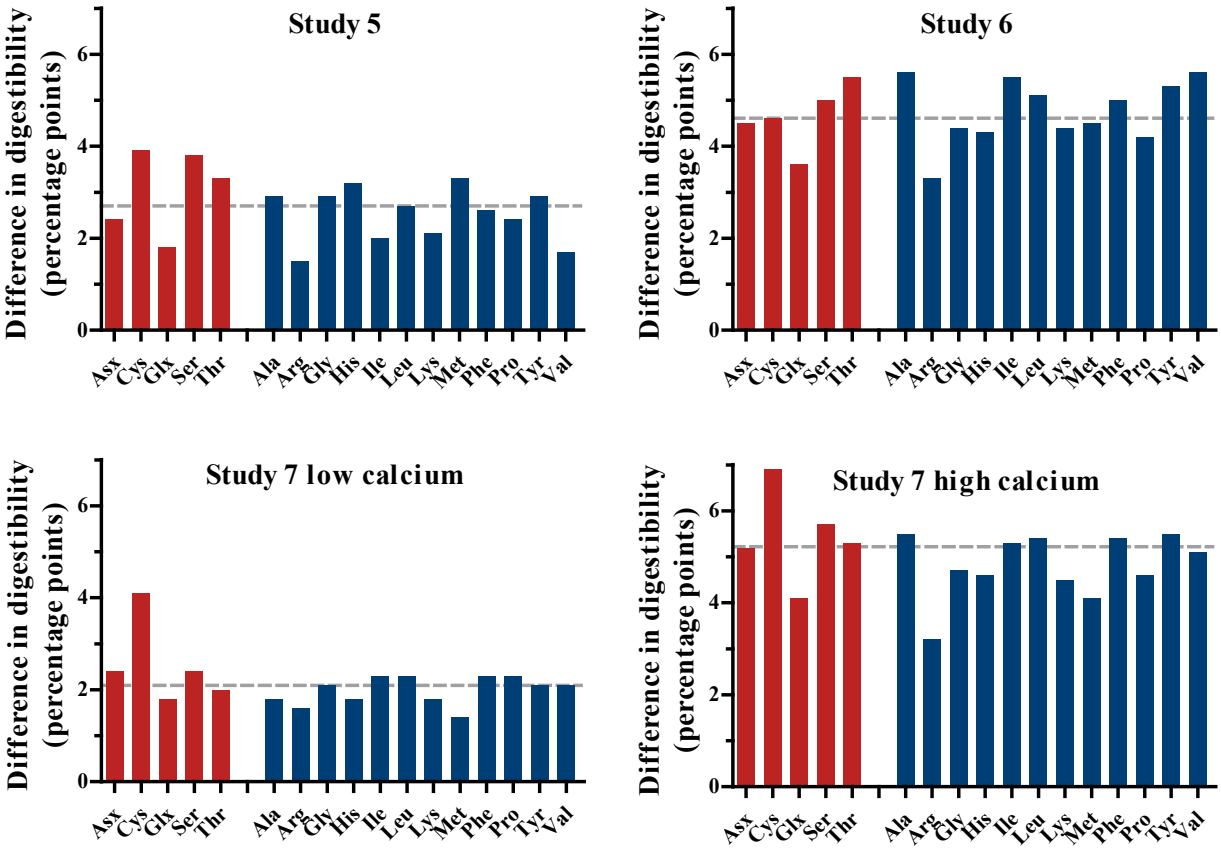

Figure 12. Increase in prececal amino acid digestibility upon phytase supplementation in Study 5, Study 6, and Study 7. The red and blue bars represent amino acids with a higher or lower proportion in endogenous amino acids, respectively (Adedokun et al. 2011; Adeola et al. 2016). The dashed lines indicate the median of all amino acids within each panel. See the list of abbreviations for abbreviations of amino acids.

(Figure 12). However, this does not prove that reduced endogenous AA can be excluded as a mechanism of phytase on pc AA digestibility because phytase supplementation significantly increased feed intake in all of the mentioned studies. As a consequence, endogenous AA losses may be affected by phytase, either directly by the enzyme or by feed intake (see Section 3.1.1), or both.

3.3.5 Non-starch polysaccharides and exogenous carbohydrases

The cell walls of feed ingredients contain non-starch polysaccharides (**NSP**). Arabinoxylans, β-glucans, and cellulose are the main NSP polymers in feed ingredients (Bach Knudsen 2014). Increased digesta viscosity along with reduced nutrient digestibility has been shown in several studies. An early study showing this connection was from Choct and Annison (1992b), where addition of pentosane preparations extracted from a wheat by-product to diets for broiler chickens reduced pc CP digestibility. These pentosane preparations increased digesta viscosity

(Choct and Annison 1992a). Increased digesta viscosity can affect both the access of digestive enzymes to the substrate and reduce the probability that readily absorbable nutrients reach the enterocytes. Additionally, an increasing effect of high digesta viscosity on endogenous AA secretion has been proposed in a study on pigs (Yin et al. 2004). Other explanations often described in the literature are that nutrients are physically entrapped by cell wall structures (Aftab 2012) or NSP influence the microbiota (see Section 3.3.6 for further explanations).

Influences of NSP, particularly water-soluble NSP, on AA digestibility are well established. Water-soluble NSP most likely had a substantial contribution to the very low pc AA digestibility of guar meal determined in broiler chickens (Study 12). However, the variation in AA digestibility of 20 genotypes each of triticale (Study 9), rye (Zuber et al. 2016), and wheat (Zuber and Rodehutscord 2016) determined in cecectomized laying hens were not correlated with NSP concentrations. This included total, water-soluble, and water-insoluble NSP, arabinoxylans, β-glucans, and cellulose as well as the non-cellulosic polysaccharide sugar monomers arabinose, xylose, mannose, galactose, uronic acid, and glucose. As variation in NSP concentrations between these genotypes of cereal grains was considerable (Rodehutscord et al. 2016), previously reported effects of NSP on AA digestibility either were an artifact or other influences caused the absence of effects in Study 9, Zuber et al. (2016), and Zuber and Rodehutscord (2016). Artifacts seem highly unlikely given that supplementation of carbohydrases can increase AA digestibility (see corresponding paragraph). It is possible that NSP effects on AA digestibility are less relevant in cecectomized laying hens compared with broiler chickens due to a more mature digestive tract. Supplementation of NSP-degrading enzymes would have no or a smaller effect on AA digestibility in laying hens in this case. Unfortunately, studies investigating carbohydrase supplementation on AA digestibility in laying hens are not available. Some studies have shown effects of NSP-degrading enzymes on other traits in laying hens, for example eggshell quality (Olgun et al. 2018), thus proving that NSP exert a certain relevance in laying hens. The applied analytical procedure of NSP quantifies the cellulose and the non-cellulosic polysaccharide sugar monomers after hydrolysis of the polymers. As discussed in Study 9, this analytical procedure does not allow one to determine the fine structure of polymers. The fine structure can differ considerably, thus altering the physicochemical properties (Izydorczyk and Biliaderis 1995; Collins et al. 2010). Analyzing the fine structure of NSP could possibly help to further explain variation in AA digestibility and, hence, make AA digestibility more predictable.

The physicochemical properties of NSP probably have contributed to the variation in extract viscoelasticity within the genotypes of the cereal grains described in Figure 8. Digesta viscosity

can be estimated from the extract viscosity of feed ingredients because positive correlations between these measures have been found in broiler chickens (Dusel et al. 1997; Maisonnier et al. 2001; Kluth et al. 2009). Nevertheless, there were no significant correlations between extract viscosity at a shear rate of 380 s^{-1} and AA digestibility in Study 9. The shear rate of 380 s^{-1} was chosen because it represents a medium shear rate of extract viscosity measurements reported in the literature (Rodehutscord et al. 2016). The shear rate impact on digesta induced by peristalsis in poultry is unknown but has been estimated below 1 s^{-1} in the small intestine of opossums (Lentle et al. 2005) and rats (de Loubens et al. 2013). With decreasing shear rates, extract viscosity of the 20 triticale genotypes in Study 9 increased, and the differences between the genotypes became more pronounced (Table 4). Consistent with the hypothesis that extract viscosity is an indicator for nutrient digestion and physiologically relevant shear rates are very low, as the shear rate decreased, the negative correlations between extract viscosity and AA digestibility became more pronounced. However, all correlations were clearly not significant, making extract viscosity an unsuitable tool to predict AA digestibility in Study 9.

Table 4. Model calculation on the effect of shear rate (s^{-1}) on extract viscosity (mPa·s) and correlations between extract viscosity and digestibility of first-limiting amino acids of triticale (n=20) in Study 9.

	Extract viscosity					Correlation[3] with digestibility of			
Shear rate	Mean	Min	Max	SD[1]	CV[2]	Met	Cys	Lys	Thr
0.5	10.03	7.05	17.68	2.46	25%	-0.23	-0.08	-0.21	-0.10
1.0	5.52	4.00	9.32	1.23	22%	-0.22	-0.07	-0.20	-0.09
1.5	4.03	2.99	6.54	0.82	20%	-0.21	-0.05	-0.20	-0.08
2.0	3.28	2.49	5.16	0.62	19%	-0.20	-0.04	-0.19	-0.07
2.5	2.84	2.19	4.33	0.50	18%	-0.19	-0.03	-0.19	-0.06
5.0	1.96	1.60	2.69	0.28	14%	-0.12	0.02	-0.15	-0.01
10	1.54	1.28	2.01	0.18	12%	-0.03	0.09	-0.09	0.06
50	1.24	1.03	1.70	0.15	12%	0.11	0.16	0.01	0.13
380	1.26	1.06	1.69	0.14	11%	0.12	0.16	0.02	0.12

[1]Standard deviation.
[2]Coefficient of variation.
[3]None of the correlations was significant ($P \geq 0.33$)

Carbohydrases are often supplemented in poultry diets because endogenous enzymes of the animals do not hydrolyze NSP effectively. The results of the studies on effects of carbohydrase supplementation on AA digestibility are variable and the perception of their effectiveness most likely is overrated because – similarly to protease and phytase – the published literature does not include many investigations determining no effect of carbohydrase supplementation. The

potential of carbohydrase supplementation to decrease digesta viscosity and to increase nutrient digestibility has been described in several publications (Choct and Annison 1992a; Steenfeldt et al. 1998; Kluth et al. 2009). The prevailing NSP in feed ingredients determine which carbohydrase type has the potential to influence AA digestibility, like xylanases in wheat-based diets or β-glucanases in barley-based diets. As an example for wheat, Kluth et al. (2009) investigated pc AA digestibility of three wheat cultivars differing in their soluble NSP concentration in broiler chickens. They found pc AA digestibility was not different between cultivars when a xylanase-containing product was supplemented to the diets. Reduced pc AA digestibility along with increased digesta viscosity was observed when no xylanase was supplemented. By contrast, Bormann (2019) described no effect of xylanase supplementation when 12 wheat genotypes were used. For barley, increased pc CP digestibility upon β-glucanase supplementation in broiler chickens was reported (Wu et al. 2004). In another study on broiler chickens, supplementing an enzyme with β-glucanase as the lead activity to different barley cultivars led to a similar increase in pc AA digestibility for all cultivars (Ravindran et al. 2007).

Over the years, enzyme products have become more diverse, and there has been an increasing number of publications reporting on effects of mixes of different carbohydrases and other enzymes, such as protease or phytase, in diets of very heterogeneous composition (e.g., Rutherfurd et al. 2007; Woyengo et al. 2016; Gallardo et al. 2017). Because application of an enzyme mixture has become common, carbohydrase effects in this area of research are not easy to evaluate. Research has developed into product testing, meaning that treatment effects on certain traits that are relevant for the industry have been determined. However, it has become almost impossible to identify modes of action and to understand the underlying mechanism of a product. Identification of modes of action and aims to increase the efficacy of supplemented carbohydrases would benefit from studies clearly stating which carbohydrase type with which active sites was used. Modes of action might further be clarified by identifying actual impacts of carbohydrases on the chemical structure of NSP in the digestive tract. Such chemical structures have hardly been explored, probably because analyses are technically challenging.

3.3.6 Microbiota in the digestive tract

The microbiota in the digestive tract have the ability to degrade and to synthesize AA and proteins. The AA absorbed by animals can therefore originate from feed protein and *de novo* synthesized AA by the microbiota, which are absorbed when microbial protein is broken down up to the end of the small intestine (Metges 2000). Torrallardona et al. (2003) showed that absorption of essential AA synthesized *de novo* by the microbiota can be considerable in pigs.

AA digestibility can be affected by the microbiota because microorganisms can change the AA composition in the digestive tract, including microbial matter, by synthesizing and degrading AA. Further, the microbiota can increase AA digestibility when microbial protein is more digestible than the ingested protein and vice versa.

The microbiota likely contributes to the effects of dietary interventions on AA digestibility. Based on the high relative abundance of *Lactobacillus* species in the small intestine of broiler chickens, it was estimated that *Lactobacillus* species assimilate 3–6% of the protein ingested by chickens (Apajalahti and Vienola 2016). Therefore, changes in the microbial composition are a probable influence on AA digestibility. Supplementing protease and phytase shifted the relative abundance of *Lactobacillus* species and other genera, but connections between AA digestibility and the relative abundance of genera were not detectable in Study 5. Connections between the relative abundance of microorganisms and AA digestibility may have been undetectable because relative abundances give no hint regarding the functionality. Thus, the functionality of the microbiota was investigated in Study 7. The functionality analysis in Study 7 showed that a high number of metabolic pathways related to protein digestion and absorption as well as the synthesis, degradation, and metabolism of AA were affected by the dietary interventions. Hence, AA concentrations in the content of the digestive tract, including AA in microbial matter, most likely were influenced differently by the microbiota between the dietary interventions. The methodology used in Study 7 (functional prediction based on relative abundance of taxonomic assignation of species determined using the V1–2 region of the 16S ribosomal ribonucleic acid gene) does not allow one to determine the extent to which AA digestibility was affected. Whether determined differences in functionality are quantitatively relevant for AA digestibility therefore cannot be estimated. This should be investigated in the future when appropriate methods are available.

Although artifacts cannot be excluded, it seems probable that the dietary interventions in Study 7 actually affected the functionality of the microbiota. Functionality predictions of microbiota may be criticized 1) because errors can occur when exclusive functions encoded in unknown organisms cannot be revealed (one third of the metagenome sequences lacks a reference genome and about half of the genes miss a known function), 2) because parts of the metagenome sequence reads cannot be assigned to a function, and 3) because abundance of gene sequences does not necessarily mean that the functionality was active (microorganisms may be inactive or dead) (Aßhauer 2015). Another aspect is a susceptibility for artifacts (Belcour et al. 2020). As an example of an artifact, functionality analysis determined metabolic pathways related to photosynthesis as being significantly influenced by dietary interventions in

Study 7. Irrespective of that, the effects of dietary interventions on metabolic pathways related to AA were internally coherent in Study 7: When the prediction of protein digestion and absorption of the microbiota was increased, pathways for AA biosynthesis and AA metabolism were reduced. This is explicable as the need for AA synthesis and AA metabolism probably was lower when AA absorption was higher.

Study 5 and Study 7 cover some dietary influences on the microbiota. In previous studies, numerous dietary influences on the microbial composition were reported, including fat (Wan et al. 2019), NSP (Ivarsson et al. 2014), or trace elements (Skrypnik and Sulibурska 2018). Actual quantitative implications of the influence of the microbiota on pc AA digestibility are hardly understood and the microbiota probably contribute to the unpredictability of AA digestibility. Focusing solely on the relative abundances of microorganisms in the digestive tract does not seem helpful to assess whether the microbiota and AA digestibility are connected because there is no information on causality and the relative abundance of one microorganism depends on the presence of others (Chen et al. 2020). Functional prediction gives more information on causality, but there is currently the risk of drawing wrong conclusions due to artifacts, as discussed by Belcour et al. (2020) and others. Research on the microbiota has made huge advances in the past years, and this trend is expected to continue in the future (Hitch et al. 2021). This presumably will lead to possibilities to assess accurately how the microbiota contributes to AA digestibility and to predict the consequences of dietary treatments on AA digestibility more precisely.

3.3.7 Tannins

Tannins represent a group of antinutritive substances that are relevant for AA digestibility. They are particularly relevant in protein-rich feed ingredients, while concentrations in cereal grains are low (Grela 1996; unpublished results). Tannins have also been described to complex with proteins, including feed proteins and endogenously secreted enzymes like enterokinase and trypsin (Griffiths and Moseley 1980; Blytt et al. 1988). Published studies on broiler chickens suggest a threshold of tannin concentrations of relevance for AA digestibility. For example, no relationship between tannin concentrations and AA digestibility was determined in four pea variants (0.6–1.2 g total tannins/kg dry matter, 0.02–0.05 g condensed tannins/kg dry matter; Kluth et al. 2009), five pea variants (0.1–0.5 g tannins/kg dry matter; Hejdysz et al. 2015), or seven raw or extruded pea variants (0.5–0.8 g tannins/kg dry matter; Konieczka et al. 2014). Other studies found lower CP and AA digestibility with increasing tannin concentrations in two faba bean variants (0–8.2 g tannins/kg dry matter; Woyengo and Nyachoti 2012), three faba

bean variants (0.2–5.6 g tannins/kg dry matter; Smit et al. 2021), or nine pea variants (0.2–12.1 g tannins/kg dry matter; Smulikowska et al. 2001). However, the tannins concentrations were determined using different methods in these studies, making evaluations of the determined concentrations difficult.

In laying hens, Study 10 described significant correlations between AA digestibility and tannin concentrations in peas. The significantly negative correlations were caused by the one color-flowered pea variant under investigation, which featured a higher tannin concentration (3.6 g condensed tannins/kg dry matter) than the 11 white-flowered pea variants (0.1–0.9 g condensed tannins/kg dry matter). There were no significant correlations between AA digestibility and tannin concentrations when the color-flowered variant was excluded from correlation analysis. Higher tannin concentrations in color-flowered compared with white-flowered peas have also been described in the literature (Griffiths 1981; Smulikowska et al. 2001; Hejdysz et al. 2015). This seems to confirm that undesirable effects of tannins can be avoided by the choice of pea varieties (Jeroch and Steinhöfel 2020). The actual effect on the variation in AA digestibility was still not very pronounced because the AA digestibility of the color-flowered variant was in the lower part of the range determined for white-flowered pea variants. Even though higher tannin concentrations and more pronounced variation in tannin concentrations were determined in faba beans (10.6–18.5 g condensed tannins/kg dry matter), there was no relationship between tannin concentrations and AA digestibility in laying hens (Study 11). Of note, Study 10 and Study 11 used the same method of tannin analysis. Study 11 also investigated the effect of dehulling faba beans on AA digestibility. Dehulling almost completely removed tannins to 0.2 g condensed tannins/kg dry matter but had little effect on the digestibility of most AA. Therefore, it appears unlikely that the higher tannin concentrations contributed to the lower level of AA digestibility of faba beans compared with peas (Figure 8). The mentioned studies suggest that tannins are of greater relevance for AA digestibility in broiler chickens than in laying hens. Perhaps age-related features of the digestive tract make tannins more relevant for AA digestibility in broiler chickens than in laying hens.

3.4 Relevance of cropping conditions

3.4.1 Nitrogen availability for plants during cropping

Availability of N to plants during growth, consisting of available N in the soil plus N fertilization (Mengel 1991), is a well-established determinant of AA concentrations in seeds. In the literature, N fertilized at the seeding and tillering stages (Bruckner et al. 1998) as well as at

the tillering stage and the first visible node (Lestingi et al. 2010) increased CP or AA concentrations in triticale but decreased the proportion of most essential AA relative to CP. Similar findings were also described for other crops, including barley (Pomeranz et al. 1977), oats (Eppendorfer 1977), rye (Eppendorfer 1977), sorghum (Mossé et al. 1988), and wheat (Pomeranz et al. 1977). No information on N fertilization at the five-tiller stage, as conducted in Study 5, is provided in the literature. However, the consequences of N fertilization on AA concentrations in Study 5 were similar to N fertilization at other stages: The proportion of most essential and some nonessential AA relative to CP decreased upon N fertilization, which was accompanied by a considerable increase in the proportion of the nonessential AA Glx and Pro relative to CP.

With few exceptions, N fertilization reduced the digestibility of AA in Study 5. For some AA, the main effect of N fertilization was significant while the N fertilization × genotype interaction showed that the magnitude of reduction in AA digestibility differed between genotypes (the few numerical increases in AA digestibility were at most 1 pp). Hence, N fertilization increased AA concentrations but decreased the proportion of first-limiting AA relative to CP and decreased (significantly or numerically) AA digestibility. The higher AA concentration compensated for the lower AA digestibility upon N fertilization in most cases so that the concentration of digestible first-limiting AA was the same or increased marginally. Nonetheless, N fertilization lowered the proportion of digestible first-limiting AA relative to CP. Hence, N fertilization results in more (mainly nonessential) AA provided in concentrations exceeding the requirement of the animals when the supply with first-limiting AA is adjusted to the requirement of the animals. This phenomenon reduces PUE. Low N availability to the plants may therefore be advantageous to achieve high proportions of first-limiting AA relative to CP and high AA digestibility but this practice may hold the risk of low harvest yields. The balance between this conflict of aims is to be determined on a case-by-case basis depending on the wider framework. N fertilization may prospectively consider digestible concentrations of first-limiting AA in the grains together with grain and protein yield per area so that PUE of the entire production chain is optimized. This would particularly come into effect when crops are produced for farm animal feeding because higher N fertilization can be beneficial for other purposes, like quality properties of wheat for baking bread (Guerrini et al. 2020).

The storage protein fractions prolamin and glutelin, which together form the gluten fraction, may reduce AA digestibility because they affect protein solubility (Saint Pierre et al. 2008) and can increase viscoelasticity (Shewry and Halford 2002). Several studies have shown that N fertilization increased the proportion of prolamin and glutelin in the total protein content of

cereal grains and affected the composition of subclasses of prolamin and glutelin (Pechanek et al. 1997; Daniel and Triboi 2000). Further, the effects of N fertilization on the proportions of gluten proteins in total proteins to differ among wheat genotypes (Saint Pierre et al. 2008; Marín-Sanz et al. 2020). The proportion of prolamin and glutelin in the protein of triticale in Study 5 was not measured. However, N fertilization probably also increased the proportion of those proteins in Study 5 because AA analysis showed an increase in the proportion of Glx and Pro relative to CP, and prolamin and glutelin are rich in Glx and Pro (Shewry and Halford 2002; Wieser 2007). The significant correlations between Glx and Pro concentrations and the digestibility of some AA in corn (Zuber and Rodehutscord 2017) might be explained by the consequences of different proportions of storage protein fractions in the total protein. This eventually is contradicted by no such significant correlations determined for triticale (Study 6), rye (Zuber et al. 2016), and wheat (Zuber and Rodehutscord 2016). However, Glx and Pro concentrations are also influenced by the proportions of protein fractions other than prolamin and glutelin in the total protein, thus making AA concentrations an imprecise indicator for the proportions of protein fractions in the total protein. Analyses of protein fractions may be advisable for future studies investigating the N fertilization effects on AA digestibility of feed ingredients.

3.4.2 Phosphorus availability for plants during cropping

Phytate, representing the major storage form of P in plant grains, is known to reduce AA digestibility (see Section 3.3.4). Higher P uptake by the plants has been found to increase phytate concentrations in seeds, including soybeans (Raboy and Dickinson 1987), dry beans (Coelho et al. 2002), oats (Miller et al. 1980), and wheat (Michael et al. 1980). The P uptake is influenced by P fertilization (Feil 2001) and water availability: Kim et al. (2002) found significantly positive correlations between precipitation during the growing period and phytate concentrations in Australian wheat kernels. Bassiri and Nahapetian (1977) described an influence of precipitation with less pronounced differences in phytate concentrations of wheat genotypes grown on dryland when the soil was irrigated.

It appears likely that P availability for the growing plants caused the genotype × environment interactions on AA digestibility of faba beans in Study 11. Study 11 describes lower AA digestibility and higher phytate concentrations of winter compared with spring faba bean genotypes grown on one location, while there was no difference in AA digestibility and phytate concentration of the same winter and spring genotypes grown on another location. Negative correlations between phytate concentrations and AA digestibility have been determined for

several feed ingredients, including faba beans (Hejdysz et al. 2016) and corn (Douglas et al. 2000). Correlations between phytate and AA digestibility were probably causative because the addition of pure phytate reduced AA digestibility when broiler chickens were fed casein, an ingredient without phytate (Cowieson et al. 2006). Therefore, site-specific low P fertilization appears to be a suitable tool to decrease phytate concentrations and, hence, to increase AA digestibility. These effects are opposed by the risk of lower yields at low P uptake (Lickfett et al. 1999; Taliman et al. 2019) and reduced field emergence of crop genotypes selected for low phytate concentrations (Meis et al. 2003). This shows that P fertilization represents a conflict of aims that have to be balanced depending on the framework. Similarly to N fertilization, P fertilization may prospectively consider the consequences on AA digestibility in addition to yield per area to optimize the entire production chain. Supplementing phytase in diets is a known suitable tool to increase the availability of the P contained in phytate to the animals and to minimize the diminishing effects of phytate on AA digestibility (see Section 3.3.4). Phytase supplementation therefore enables higher P fertilization and, hence, higher field emergence and decreased risk of low harvest yields along with unimpaired AA digestibility.

3.5 Prediction of amino acid digestibility

Although several attempts were made to predict AA digestibility in the studies investigating the variation in AA digestibility within feed ingredients (Study 9, Study 10, and Study 11), none of them was sufficiently accurate. The same conclusion has been drawn in other studies on variation in AA digestibility within feed ingredients that are not amalgamated in this thesis (Zuber et al. 2016; Zuber and Rodehutscord 2016, 2017). The methods of prediction included correlations between AA digestibility and analyzed chemical and physical variables of the feed ingredients and multiple linear regressions using a stepwise forward selection approach that selected chemical and physical variables based on the *P* value of an F-test. The correlations were suitable to explain parts of the variation in AA digestibility determined within test ingredients – for example the influence of tannins on AA digestibility of peas in Study 10 (see Section 3.3.7) – but the accuracy of prediction based on one chemical or physical variable was low overall. The accuracy of AA digestibility prediction using multiple linear regressions was also not sufficient, as judged by low coefficients of determination and high root mean square errors in most cases. This outcome is exemplified for the digestibility of selected first-limiting AA in Table 5. The analyzed composition of the feed ingredients was assigned to several pools of variables according to classes of the analyzed variables. Table 5 shows the pool containing proximate nutrients and commonly analyzed carbohydrate fractions as an example because

Table 5. Descriptions of multiple linear regressions to predict digestibility of first-limiting amino acids in cecectomized laying hens with a pool of analyzed variables in the feed ingredients containing proximate nutrients and commonly analyzed carbohydrate fractions.[1]

Digestibility of	Methionine		Cysteine		Lysine		Threonine	
	Selected variables[2]	Adj. $R^{2\,3}$	Selected variables[2]	Adj. $R^{2\,3}$	Selected variables[2]	Adj. $R^{2\,3}$	Selected variables[2]	Adj. $R^{2\,3}$
Corn (Zuber and Rodehutscord 2017)	CP	0.17	CP, starch	0.24	CP	0.21	CP, starch	0.30
Triticale (Study 9)	CL, $aNDF_{om}$	0.44	None	-	CF, CL	0.25	CF, CL	0.41
Rye (Zuber et al. 2016)	CP, CL, ADL, GE	0.54	CA, CP, CF, GE	0.59	ADL	0.11	CP	0.17
Wheat (Zuber and Rodehutscord 2016)	None	-	None	-	None	-	None	-
Peas (Study 10)	None	-	None	-	$aNDF_{om}$	0.37	None	-
Lupins (Study 10)	CP, ADL, starch	0.86	CP	0.21	CP	0.43	CP	0.24
Faba beans (Study 11)	None	-	None	-	CP	0.22	None	-

[1]The pool contained crude protein (CP), crude fiber (CF), crude fat (CL), crude ash (CA), neutral detergent fiber determined without residual ash and after treatment with α-amylase ($aNDF_{om}$), acid detergent fiber determined without residual ash, acid detergent lignin (ADL) in Study 10 and in the studies on cereal grains, starch, and gross energy (GE).
[2]Variables selected from the pool by a stepwise forward selection approach.
[3]Adjusted coefficient of determination.

those variables were analyzed in all studies compiled in the table. The prediction was very accurate determined in some cases (e.g., Met digestibility in lupins), but there was not a consistent pattern identifiable among AA (apart from some variables being selected often: CP in corn, rye, and lupins; crude fat and crude fiber in triticale). Other variable pools comprising AA concentrations (relative to dry matter and relative to CP), minerals, inositol phosphate isomers, NSP monomers, and antinutritive substances also led to insufficient accuracy of predictions or no identifiable consistent pattern among AA. The overall low predictive quality indicates that very relevant determinants of AA digestibility were not measured or interactions between determinants may have impeded the detection of determinants. Such relevant determinants may include the particle size distribution (see Section 3.3.2), the fine structure of NSP (see Section 3.3.5), protein fractions and their subclasses (see Section 3.4.1), the effects of the microbiota in the digestive tract (see Section 3.3.6), or other chemical constituents or physical characteristics that have not been determined as relevant for AA digestibility so far.

The suitability of an *in vitro* N solubility approach based on Boisen and Fernández (1995) for predicting the AA digestibility of wheat, triticale, rye, and corn was tested in cecectomized laying hens (Study 9; Zuber and Rodehutscord 2016, 2017; Zuber et al. 2016). Predictability was low in all cases. The potential reasons discussed in Study 9 and by Rosenfelder-Kuon et al. (2020) include that *in vitro* approaches lack interactions with the digestive tract of animals and that the use of porcine pepsin and pancreatin to simulate digestive processes may not be adequate for laying hens. In addition, part of the dietary proteins may be digested in the ileum but not absorbed by the intestinal wall, while the *in vitro* approach considers all solubilized N as being digested. However, the *in vitro* approach proved to some extent suitable to classify feed ingredients as low or high in AA digestibility, such as to estimate the low AA digestibility of rye compared with corn, triticale, and wheat in cecectomized laying hens (Figure 13). Hence, this *in vitro* approach is more an attractive tool for screening prior to AA digestibility experiments, e.g., when technical processes in feed ingredient production are optimized. For the future, continued development of *in vitro* approaches optimized to predict AA digestibility

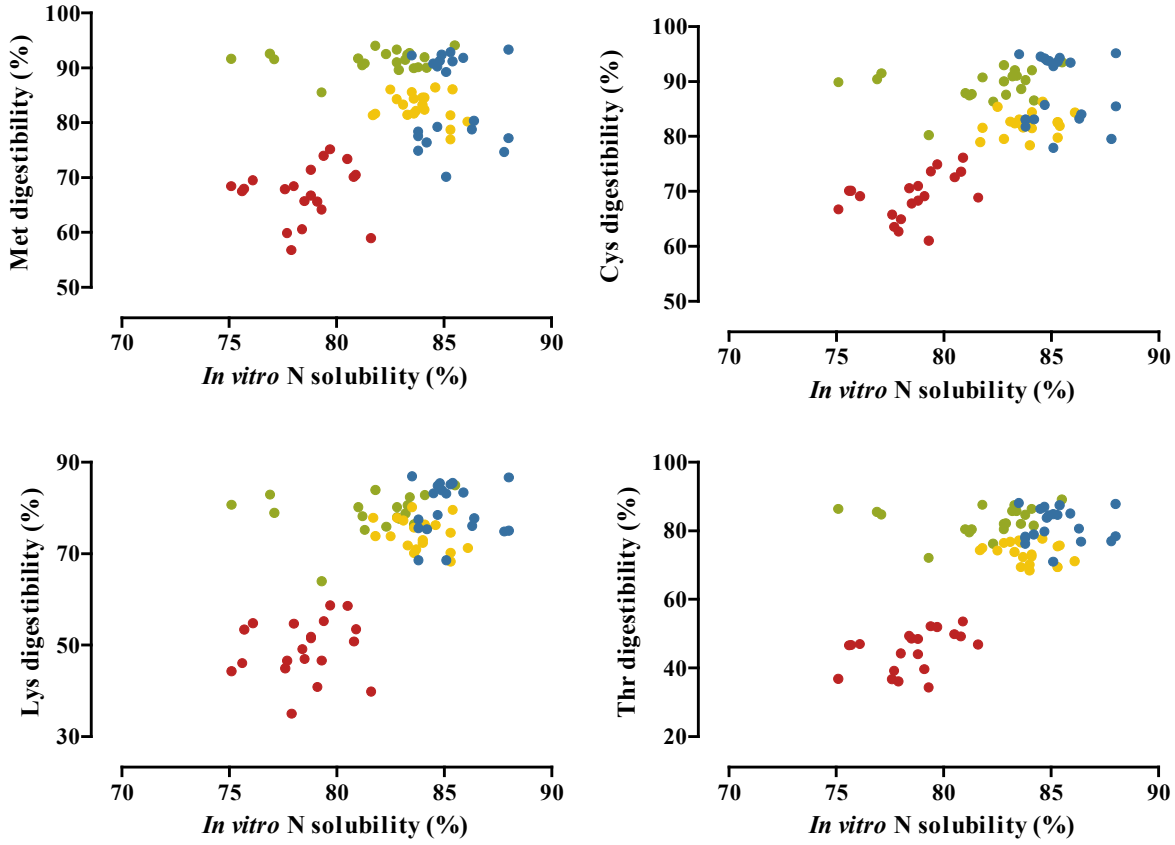

Figure 13. Relationship between the *in vitro* nitrogen (N) solubility (published in Rosenfelder-Kuon et al. 2020) and the digestibility of selected first-limiting amino acids of corn (green; Zuber and Rodehutscord 2017), rye (red; Zuber et al. 2016), triticale (yellow; **Study 9**), and wheat (blue; Zuber and Rodehutscord 2016) in cecectomized laying hens. Note that the ordinate has different scaling for the amino acids. See list of abbreviations for abbreviations of amino acids.

in poultry seems advisable to reduce animal experiments. Future optimization of this *in vitro* approach may include the use of avian enzymes as well as adapted pH and time periods of the process steps to the conditions in the digestive tract of poultry.

Near infrared reflectance spectroscopy (**NIRS**) is an attractive tool to determine characteristics of feed ingredients because measurements are quick, simple, and inexpensive. For AA digestibility, however, the high number of needed reference measurements makes the development of calibrations difficult. Agelet and Hurburgh (2010) described that a minimum of 30 samples are needed for reference measurements for feasibility studies and considerably more samples are needed for robust calibrations. Besides the effort to develop robust calibrations, NIRS predictions may be limited in accuracy because changes in the chemical structure of nutrients that occur during the digestion process are not measured (Shurson et al. 2021). Such calibrations are commercially available, but the underlying reference measurements are not disclosed. It appears highly unlikely that they originate from methodologically uniform experiments, thus decreasing the validity of reference measurements in commercially available calibrations to an unknown extent. Rosenfelder-Kuon et al. (2020) investigated whether NIRS is suitable to predict *in vitro* N solubility, aiming to establish *in vitro* N solubility as a substitute for AA digestibility measurements. However, with coefficients of determination not higher than 0.80, the precision of predictions was only suitable to classify the level of *in vitro* N solubility of feed ingredients. Given the low predictive precision of the *in vitro* N solubility approach for AA digestibility, together with the moderate predictive precision of NIRS for *in vitro* N solubility, the *in vitro* N solubility estimate by NIRS is currently unsuitable to predict AA digestibility. A solution may be found in continued development of *in vitro* approaches that more accurately predict AA digestibility and that can be predicted more accurately using NIRS.

3.6 Consequences of amino acid digestibility on the protein utilization efficiency

Increasing AA digestibility increases PUE provided that the supply of digestible AA remains unchanged, as pointed out in Section 1.3. The present section identifies the extent of the influence of varying AA digestibility on PUE in model calculations. Laying hens are used as an example because the model calculations are based on values of AA digestibility of the feed ingredients shown in Figure 8, which were obtained using cecectomized laying hens. Separate model calculations are needed when AA digestibility is varied by an influence not related to the feed ingredients providing AA, such as exogenous enzymes (AA provided by supplemented

enzymes is negligible), and by selecting variants of feed ingredients providing AA that differ in AA digestibility.

The model calculations were computed for Met+Cys, Lys, and Thr because these AA usually are limiting in this order in poultry. Therefore, the Lys results are only relevant when Met+Cys is not limiting (e.g., when free Met is used), and the Thr results are only relevant when both Met+Cys and Lys are not limiting (e.g., when free Met and free Lys is used). An increase in Met+Cys digestibility by 1 pp in the model calculations means that the Met digestibility and the Cys digestibility increased so that the digestible Met+Cys concentration relative to the total Met+Cys concentration raised by 1 pp.

3.6.1 Influence not related to the feed ingredients providing amino acids

When AA digestibility is varied by an influence not related to the feed ingredients providing AA (such as supplemented enzymes), increasing AA digestibility raises PUE in a linear manner. This increase in PUE is only attributed to the limiting AA in the diet, although supplemented enzymes unselectively increase the digestibility of all AA. An increase in the digestibility of not-limiting AA increases N excretion via the urine and decreases N excretion via the feces. This shift between N excretion via the urine and via the feces does not affect the total N excretion. Hence, PUE remains unaffected.

The extent to which AA digestibility influences PUE depends on the ratio of the concentrations of the limiting AA to CP (AA/CP ratio) in the diet. The influence of the AA/CP ratio on the increase in PUE in pp upon an increase in AA digestibility by 1 pp (δPUE) can be calculated using the following equation:

$$\delta PUE = \frac{a \times b \times 10}{c \times d} \qquad \text{[Eq. 2]}$$

with *a* as the CP accretion (g/d), *b* as the AA/CP ratio (g/g), *c* as the targeted digestible AA concentration of the diet (g/kg dry matter), and *d* as the feed intake (g dry matter/d).

The higher the AA/CP ratio, the more CP not related to the limiting AA can be spared by increasing the digestibility of the limiting AA. The influence of the AA/CP ratio on δPUE can be shown in a model calculation with similar assumptions as in the model calculation shown in Table 2 (targeted digestible concentrations of 5.2 g Met+Cys/kg dry matter, 6.0 g Lys/kg dry matter, and 4.3 g Thr/kg dry matter based on the recommended total concentrations of 6.1 g Met+Cys/kg dry matter, 7.0 g Lys/kg dry matter, and 5.1 g Thr/kg dry matter by the Gesellschaft für Ernährungsphysiologie (1999) for laying hens weighing 1,600 g and 55 g daily egg

production, an assumed 85% AA digestibility on the recommended total AA concentrations because the Gesellschaft für Ernährungsphysiologie (1999) does not recommend digestible concentrations (see Section 3.2), 107 g daily feed intake with 88% dry matter in the feed), 19.2 g CP accretion/kg egg production, and 0 g N accretion in the body tissues. For these assumptions, the relationship between the AA/CP ratio and δPUE upon an increase of 1 pp in AA digestibility according to Eq. 2 can be described as:

$$\delta PUE\ (pp) = 13.4 \times (Met+Cys)/CP, \quad \text{[Eq. 3]}$$

$$\delta PUE\ (pp) = 11.7 \times Lys/CP, \text{ and} \quad \text{[Eq. 4]}$$

$$\delta PUE\ (pp) = 16.1 \times Thr/CP. \quad \text{[Eq. 5]}$$

The influence of the AA/CP ratio within the range found in practical diets on δPUE in this model calculation is shown in Figure 14. For the AA/CP ratios in practical diets like those described in Table 2, increasing AA digestibility by 1 pp raises PUE by ~0.43 pp. Thereby, the baseline level of PUE without enzyme supplementation has no influence on δPUE.

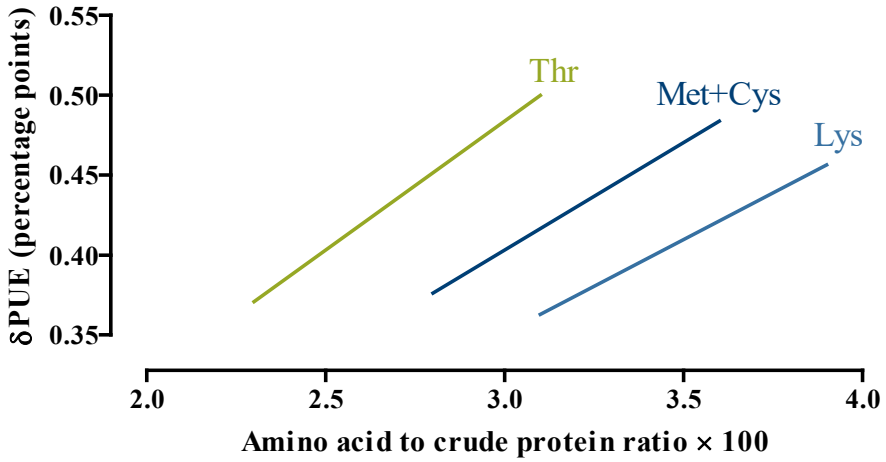

Figure 14. Results of a model calculation on the effects of the ratio between concentrations of first-limiting amino acids and crude protein on the increase in protein utilization efficiency (δPUE) in percentage points upon an increase in amino acid digestibility of 1 percentage point. The values were calculated using Eq. 3 for methionine+cysteine (Met+Cys), Eq. 4 for lysine (Lys), and Eq. 5 for threonine (Thr). The assumptions of the model calculation are described in the text. The medium values of the amino acid to crude protein ratio are the mean values of the practical diets described in Table 2.

The difference in PUE upon enzyme supplementation can be calculated as the difference in AA digestibility multiplied by δPUE. The few available studies investigating enzyme effects on AA digestibility in laying hens determined no increase in AA digestibility (Snow et al. 2003; Agbede et al. 2009b), while many studies reported phytase effects on AA digestibility in broiler chickens. Table 6 shows the potential to increase PUE upon supplementation of phytase and protease in Study 5, Study 6, and Study 7 as studies on enzyme effects amalgamated in this

Table 6. Effects of phytase and protease supplementation on the digestibility of first-limiting amino acids and the resulting potential to increase protein utilization efficiency (PUE). The values are given in percentage points.

	Methionine+Cysteine		Lysine		Threonine	
	Increase in digestibility	Increase in PUE[1]	Increase in digestibility	Increase in PUE[1]	Increase in digestibility	Increase in PUE[1]
Phytase						
Study 5	3.5	1.5	2.2	0.9	3.3	1.4
Study 6[2]	3.4	1.5	4.8	2.1	5.8	2.5
Study 7 low Ca	2.8	1.2	1.8	0.8	2.0	0.9
Study 7 high Ca	5.4	2.3	4.5	1.9	5.3	2.3
Protease						
Study 5	3.8	1.6	1.3	0.6	2.8	1.2
Study 6	2.4	1.0	1.4	0.6	2.6	1.1

[1]Increase in digestibility × 0.43 (see text for the explanation of this value).
[2]Treatments with 1,500 FTU phytase/kg.

thesis. This model calculation assumes that the δPUE value of 0.43 pp determined for laying hens can be transferred to broiler chickens. According to that assumption, the potential to increase PUE varied at a low to moderate level between 0.8–2.3 pp upon phytase supplementation, and ranged at a low level between 0.8–1.6 pp upon protease supplementation. However, other studies have described considerably higher effects of enzyme supplementation on AA digestibility, which would result in a correspondingly higher potential to increase PUE. As an example, phytase supplementation increased the Lys digestibility by 8.3 pp in the study of Amerah et al. (2014). This change would increase PUE by 3.6 pp.

3.6.2 Influence of variation within feed ingredients

When AA digestibility is varied by selecting specific variants of a feed ingredient differing in AA digestibility, the consequences on PUE cannot be determined accurately using an equation. This is because AA digestibility and the AA/CP ratio in feed ingredients vary independently. However, δPUE can be indicated approximatively by a model calculation. This model calculation considers two contributors to the digestible AA concentration of a diet: 1) the feed ingredient for which variants differing in AA digestibility are selected, hereinafter designated the ‘considered ingredient’ and 2) the other AA-containing ingredients in the diet, hereinafter designated the ‘remaining ingredients’.

δPUE in this model calculation is determined by a two-step calculation: First, PUE of diets containing the considered ingredient is calculated for each variant of the considered ingredient. Then, δPUE is determined in a linear regression between PUE of a diet containing the considered ingredient against the AA digestibility of the considered ingredient.

In the first step of this model calculation, PUE is calculated as follows:

$$\text{PUE (\%)} = \left\{ \frac{a}{\left[\left(\frac{b \times f}{d \times e \times 100}\right) + \left(\frac{(c - b) \times 100}{g \times h}\right)\right] \times i} \right\} \times 100 \qquad \text{[Eq. 6]}$$

Eq. 6 states the CP accretion *a* (g/d) in the numerator and the CP supply (g/d) in the denominator. In the denominator, the terms in the left-hand parenthesis (parameters *b*, *d*, *e*, and *f*) describe the CP supply by the considered ingredient, and the terms in the right-hand parenthesis (parameters *b*, *c*, *g*, and *h*) describe the CP supply by the remaining ingredients of the diet. The contribution of the considered ingredient to the digestible AA concentration in the diet *b* (g/kg dry matter) must be set. The difference between the digestible AA concentration in the complete diet *c* (g/kg dry matter) and the set contribution of the considered ingredient to the digestible AA concentration in the diet *b* is provided by the remaining ingredients. Further parameters in the left-hand parenthesis are attributes of the considered ingredient: *d* is the AA concentration of the considered ingredient (g/kg dry matter), *e* is the AA digestibility of the considered ingredient (%), and *f* is the CP concentration of the considered ingredient (g/kg dry matter). For the remaining ingredients in the right-hand parenthesis, additional assumptions have to be made: *g* is the average AA digestibility of the remaining ingredients of the diet (%) and *h* is the AA/CP ratio of the remaining ingredients of the diet. The two parentheses in the denominator of the equation together describe the CP concentration of the diet (g/kg dry matter). The CP concentration in the diet is multiplied by the feed intake *i* (g dry matter/d) for obtaining the CP supply (g/d).

Eq. 6 is the result of arranging the calculations to make Eq. 6 concise. As a consequence, Eq. 6 enables to calculate the protein utilization efficiency with few computations, but comprehensibility is limited. The calculation in the Annex describes the calculation more comprehensibly, but less concisely. The example in the Annex uses the determined CP and Lys concentrations as well as the Lys digestibility of the wheat genotype 'Mulan' (Rodehutscord et al. 2016; Zuber and Rodehutscord 2016).

In the second step, δPUE is determined by using the following linear regression, where PUE determined using Eq. 6 in the first step is the dependent variable on the y-axis and the AA digestibility of the respective variant is the independent variable on the x-axis:

$$\text{PUE (\%) from Eq. 6} = \text{intercept} + \delta\text{PUE} \times \text{AA digestibility (\%)} \qquad \text{[Eq. 7]}$$

The determination of δPUE using linear regressions is necessary because the independently varying AA digestibility and AA/CP ratio of the variants of the considered feed ingredient

creates a scattering of results (visualized in Figure 15). Therefore, δPUE is not unequivocally determined in an equation as in Eq. 2; instead, δPUE is an approximation. The precision of this estimate is expressed by the goodness-of-fit of the linear regression. δPUE was determined for feed ingredients for which investigations on the variation in AA digestibility in cecectomized laying hens were conducted. This included corn (Zuber and Rodehutscord 2017), wheat (Zuber and Rodehutscord 2016), rye (Zuber et al. 2016), triticale (Study 9), lupins (Study 10), peas (Study 10), faba beans (Study 11), rapeseed meal (Rezvani et al. 2012), and soybean meal (unpublished results).

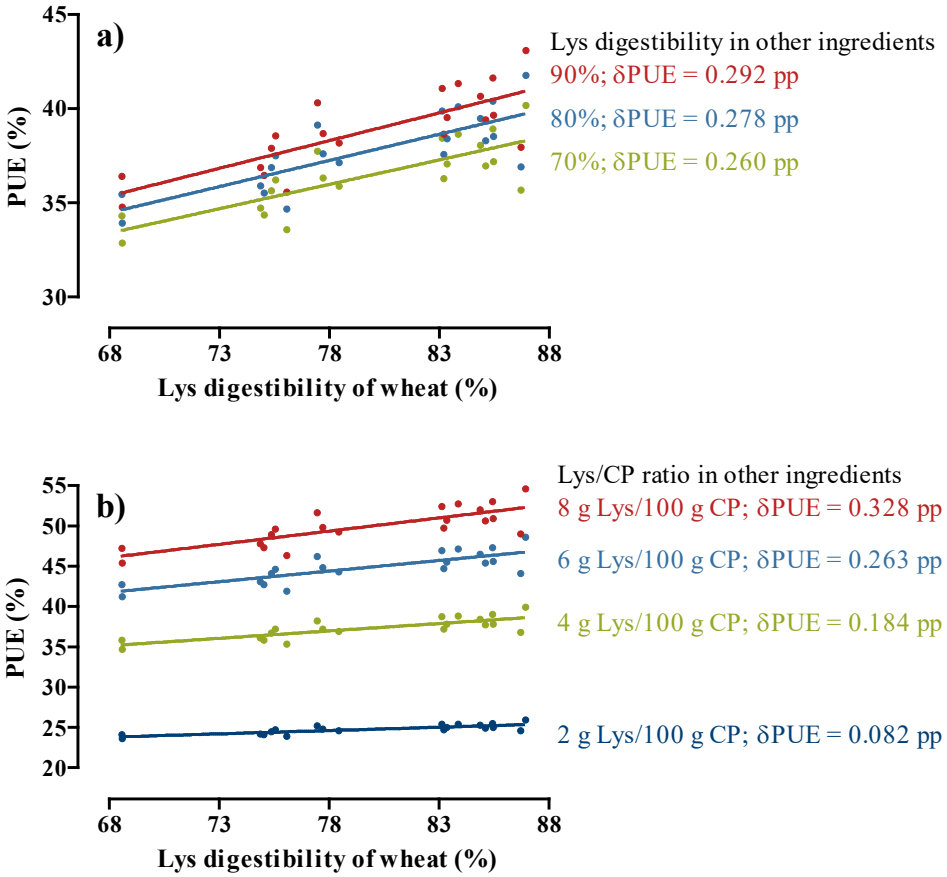

Figure 15. Results of a model calculation on the effects of the lysine (Lys) digestibility (a) and the Lys/crude protein (CP) ratio (b) of other dietary ingredients than wheat as the considered ingredient on the protein utilization efficiency (PUE). The values were calculated using Eq. 7. Of note, 3 g Lys/100 g CP and 85% Lys digestibility of the other ingredients were assumed in a) and b), respectively. CP and Lys concentrations as well as Lys digestibility are taken from Zuber and Rodehutscord (2016). Wheat contributed 2 g digestible Lys/kg dry matter to the diet. Additional assumptions of the model calculation are described in the text. δPUE, presented in percentage points (pp), represents the slopes of the linear regressions.

The following paragraphs describe the consequences of increasing AA digestibility by selecting specific variants of an ingredient on PUE of a diet. Lys is used as an example AA. The same

calculations were also performed using Met+Cys and Thr. The results of those calculations are not presented because the principal conclusions were similar.

The AA digestibility and the AA/CP ratio of the remaining ingredients influenced the determined δPUE of the considered ingredients in this model calculation. δPUE upon selecting variants of a feed ingredient with higher AA digestibility is greater the higher the AA digestibility and the higher the AA/CP ratio of the remaining ingredients, as depicted for wheat as the considered ingredient in Figure 15a and Figure 15b, respectively. Increasing AA digestibility and the AA/CP ratio of the remaining ingredients raised the relative contribution of the considered ingredient to the total unutilized CP provided with the diets. The influence of the AA/CP ratio of the remaining ingredients on δPUE of the considered ingredients was considerable (Figure 15b), while the influence of AA digestibility of the remaining ingredients was low (Figure 15a). Therefore, a constant Lys digestibility of 85% of the remaining ingredients was assumed for all further calculations. By contrast, a uniform Lys/CP ratio for all considered ingredients in Figure 16 and Figure 17 was inadequate because cereal grains are combined with protein-rich components in practical diets and the Lys/CP ratio differs between cereal grains (average 3 g Lys/100 g CP of corn, rye, triticale, and wheat in the studies summarized in Figure 16) and protein-rich components (average 6 g Lys/100 g CP of peas, lupins, faba beans, rapeseed meal, and soybean meal in the studies summarized in Figure 17). Therefore, 6 g Lys/100 g CP was assumed for the remaining ingredients when the cereal grains were the considered ingredients and 3 g Lys/100 g CP was assumed for the remaining ingredients when protein-rich ingredients were the considered ingredients.

The digestible Lys concentration of the diet (provided by the sum of the considered ingredient and the remaining ingredients) in this model calculation was 6.0 g/kg dry matter (see Section 3.6.1). Additional assumptions are the same as described in Section 3.6.1. The results of the model calculation are shown for a contribution of the considered ingredients of 1, 2, 3, and 4 g digestible Lys/kg dry matter to the total digestible Lys concentration of the diet in Figure 16 and Figure 17. As the contribution of the considered ingredients to the digestible Lys concentration in the diet is set, the proportion of the considered ingredients in the diet varies depending on Lys digestibility and the Lys/CP ratio. The resulting PUE may not be on a realistic level in some cases, but the relevant outcome of these calculations is δPUE, which is independent of the general level of PUE.

A result of this model calculation is that whether selecting variants of an ingredient with higher AA digestibility increases PUE depends on the considered feed ingredient (Figure 16 and

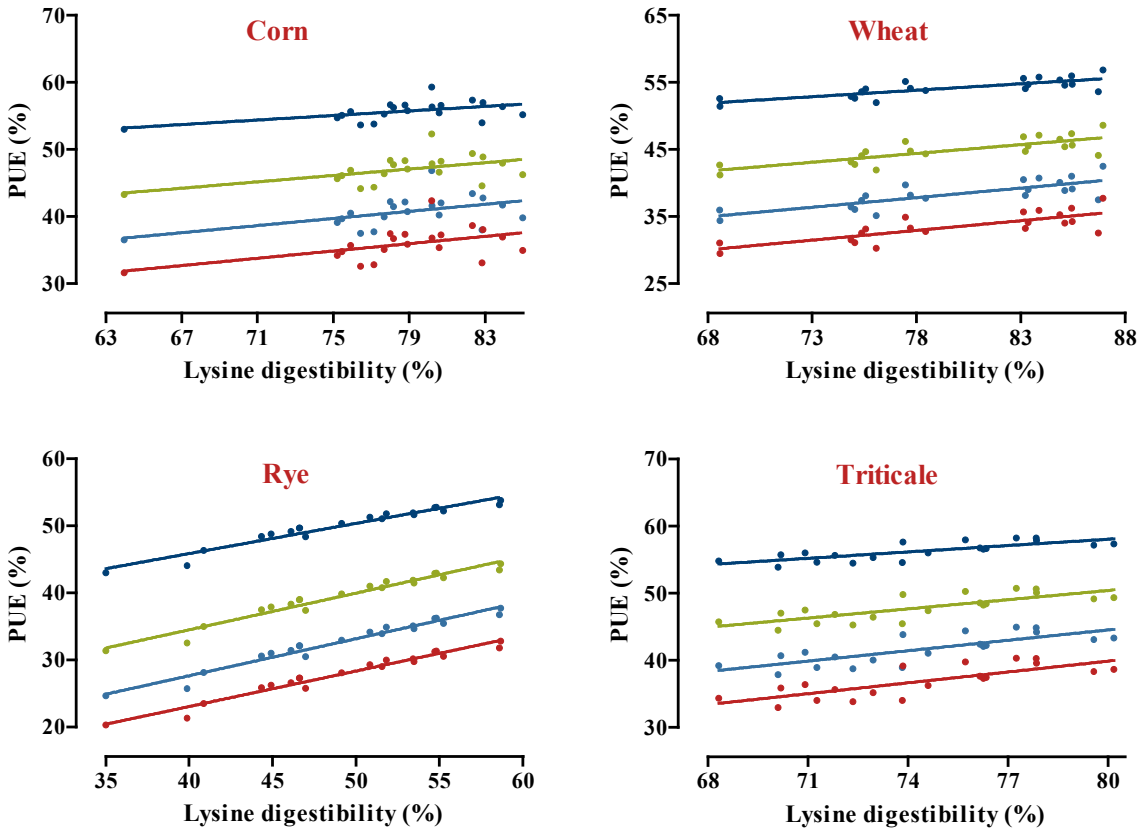

Figure 16. Results of a model calculation on the effects of the lysine digestibility of cereal grains on protein utilization efficiency (PUE) based on Eq. 7. Crude protein and Lys concentrations as well as lysine digestibility are taken from Zuber and Rodehutscord (2017) for corn, Zuber and Rodehutscord (2016) for wheat, Zuber et al. (2016) for rye, and Study 9 for triticale. The dots represent one investigated variant in the respective experiments and the lines represent linear regressions. The digestible lysine concentrations provided in the diets by the considered ingredients were 1 (dark blue), 2 (green), 3 (light blue), and 4 (red) g/kg dry matter. The slopes of the linear regressions represent δPUE in percentage points. Note that the abscissa has a different scale for feed component. Additional assumptions of the model calculation are described in the text. The results of the linear regressions are shown in Table 7.

Figure 17). For most considered ingredients, PUE was elevated with increasing Lys digestibility, as indicated by the positive slopes. In lupins, however, PUE decreased with increasing Lys digestibility. In this case, the variants with higher Lys digestibility featured lower Lys/CP ratios than the variants with lower Lys digestibility. Hence, the effects of higher Lys digestibility on PUE were compensated by the lower Lys/CP ratios. This compensatory effect probably could occur because the range in Lys digestibility in lupins was very low with less than 5 pp.

Estimating the magnitude of the effect of selecting variants of an ingredient by AA digestibility on δPUE is difficult. The constraints are the large number of assumptions in this model calculation and the scattering of single observations caused by the independent variation in the

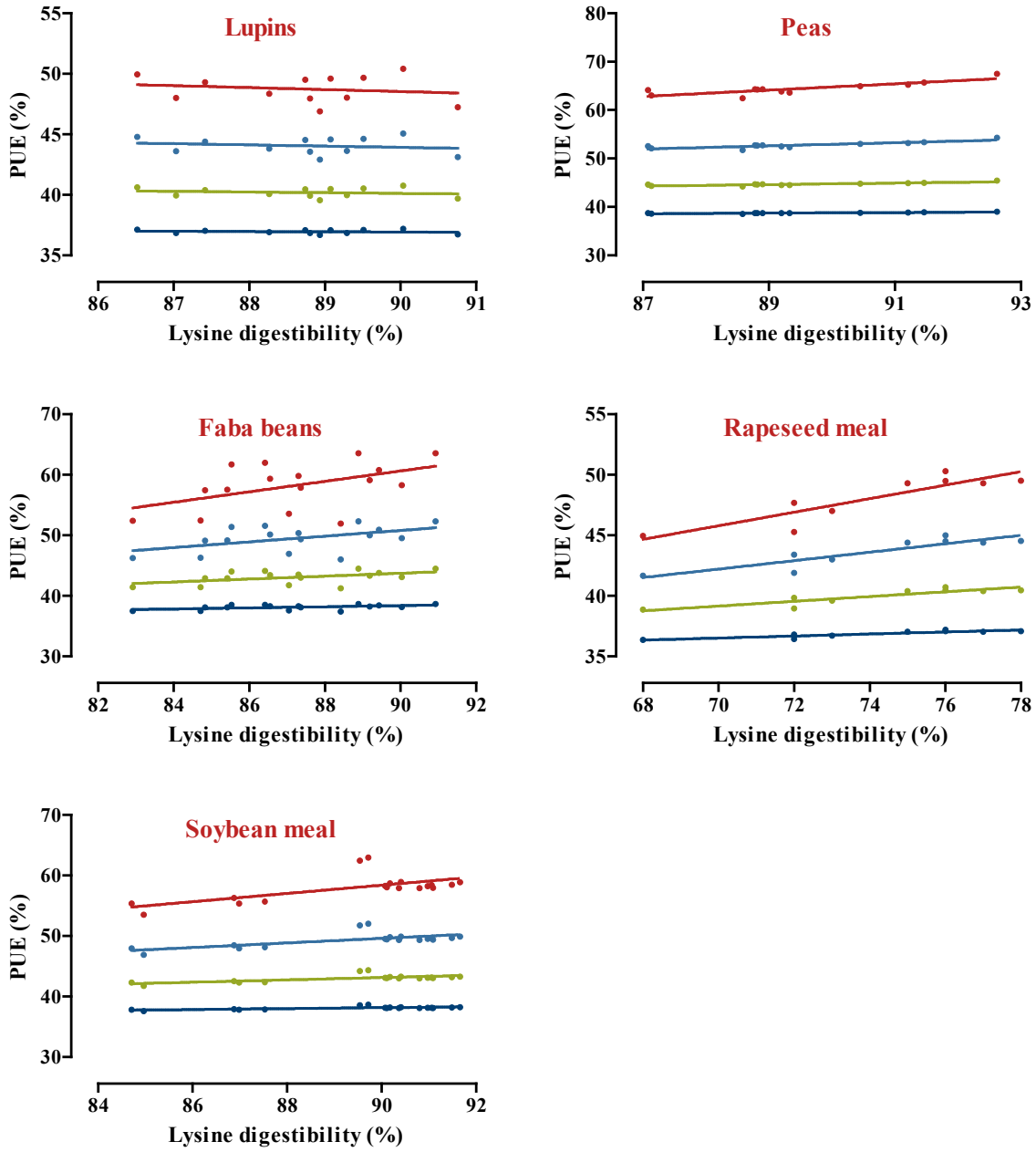

Figure 17. Results of a model calculation on the effects of the lysine digestibility of protein-rich components on protein utilization efficiency (PUE) based on Eq. 7. Crude protein and lysine concentrations as well as lysine digestibility are taken from Study 10 for lupins and peas, Study 11 for faba beans, Rezvani et al. (2012) for rapeseed meal, and unpublished results for soybean meal. The dots represent one investigated variant in the respective experiments and the lines represent linear regressions. The digestible Lys concentrations provided in the diets by the considered ingredients were 1 (dark blue), 2 (green), 3 (light blue), and 4 (red) g/kg dry matter. The slopes of the linear regressions represent δPUE in percentage points. Note that the abscissa has a different scale for feed component. Additional assumptions of the model calculation are described in the text. The results of the linear regressions are shown in Table 7.

Lys/CP ratios and Lys digestibility among variants of a considered ingredient. Table 7 shows δPUE for the assumptions of the model calculation shown in Figure 16 and Figure 17. Based

Table 7. Slopes and goodness-of-fit criteria of linear regressions of the protein utilization efficiency of a diet containing the considered ingredient determined using Eq. 7 against the amino acid digestibility of the considered ingredient. The values in parentheses represent the root mean square error. The values in bold are the range of amino acid digestibility determined for the considered ingredient multiplied by the respective slope. The regressions and model assumptions are described in Figure 16 and Figure 17.

	Contribution of ingredients to the digestible amino acid concentration in the diet (g/kg dry matter)				
	1	2	3	4	R^2 [1]
Corn	0.17	0.24	0.26	0.27	0.25–0.27
	(1.3)	(1.9)	(2.1)	(2.2)	
	3.6	**5.0**	**5.6**	**5.7**	
Wheat	0.19	0.26	0.29	0.29	0.61–0.62
	(0.9)	(1.2)	(1.3)	(1.4)	
	3.5	**4.8**	**5.3**	**5.3**	
Rye	0.45	0.55	0.55	0.53	0.94–0.96
	(0.7)	(0.8)	(0.7)	(0.7)	
	10.7	**12.9**	**13.1**	**12.5**	
Triticale	0.32	0.46	0.52	0.54	0.60
	(0.9)	(1.3)	(1.5)	(1.5)	
	3.8	**5.4**	**6.1**	**6.4**	
Lupins	-0.02	-0.06	-0.10	-0.16	0.03
	(0.2)	(0.4)	(0.7)	(1.2)	
	-0.1	**-0.2**	**-0.4**	**-0.7**	
Peas	0.06	0.15	0.33	0.65	0.70
	(0.1)	(0.2)	(0.4)	(0.7)	
	0.3	**0.9**	**1.8**	**3.6**	
Faba beans	0.09	0.24	0.47	0.86	0.24
	(0.4)	(1.0)	(1.9)	(3.5)	
	0.8	**1.9**	**3.8**	**6.9**	
Rapeseed meal	0.08	0.20	0.35	0.56	0.80
	(0.1)	(0.3)	(0.6)	(0.9)	
	0.8	**2.0**	**3.5**	**5.6**	
Soybean meal	0.08	0.19	0.38	0.68	0.42–0.46
	(0.2)	(0.5)	(0.1)	(1.8)	
	0.5	**1.3**	**2.6**	**4.7**	

[1]Coefficient of determination; same values for the regressions when rounded to 2 decimal digits.

on that, it can be expected that increasing the AA digestibility by 1 pp raises PUE by ~0–0.5 pp.

In addition to δPUE and the contribution of the considered ingredient to the digestible AA concentration of the diet, the potential to increase PUE by selecting specific variants of a feed ingredient depends on the range of AA digestibility. Therefore, the ranges of PUE caused by ingredient variants were calculated by multiplying the range of AA digestibility of each considered ingredient by the respective δPUE (bold values in Table 7). The ranges of PUE caused by ingredient variants diverged considerably with the contribution of ingredients to the digestible amino acid concentration in the diet. In practical diets, about half each of the AA concentration is provided by cereal grains and protein-rich feed ingredients. Assuming that the considered ingredients are the only cereal grains or protein-rich feed ingredients in a diet, this

would correspond to the contribution of ingredients to 3 g digestible Lys/kg dry matter in Table 7. According to that, the range of PUE based on feed ingredient variants differing in AA digestibility was 5.3–13.1 pp in cereal grains and 0–3.8 pp in the protein-rich ingredients. For a wheat-soybean meal-based diet and a corn-soybean meal-based diet, common in Europe and North America, respectively, the ranges of PUE based on feed ingredient variants differing in AA digestibility in this model calculation were 7.9 pp and 8.2 pp, respectively.

3.7 Assessment of the current situation and perspectives

3.7.1 Methodological standardization and advancement

Methodological details of AA digestibility assays can decidedly determine the outcome of experiments, a factor that makes comparisons of digestibility estimates determined using different methods inexpedient, as pointed out in Section 3.2. Hence, the development of a worldwide standard protocol for AA digestibility studies appears desirable. A step toward such a worldwide standard protocol has been achieved by a common communication of recognized experts on AA digestibility research in poultry (Ravindran et al. 2017). However, the communication focuses on the standardized AA digestibility with basal endogenous AA determined using N-free diets, and the regression approach is only mentioned briefly. Arguments in favor of the standardized AA digestibility versus AA digestibility determined using the regression approach apparently did not lead to an agreement among the experts. Therefore, both methods should be equally addressed in one future standard protocol that objectively discusses the advantages and disadvantages of both methods. An alternative is to develop two separate standard protocols, but this endeavor would entail the risk that the aspects of equal relevance for both methods are recommended differently. Two different methods in one standard protocol are adverse for the aim to standardize methods to enhance the comparability of study results, but no agreement among experts appears even more undesirable.

Based on the descriptions in this thesis, some aspects are recommended to be added to or revised in existing methodological communications on AA digestibility experiments (Kluth and Rodehutscord 2009b; Ravindran et al. 2017). The following aspects apply for AA digestibility studies that aim to provide data for practical purposes:

- Procedures that influence AA digestibility differ in practice. Picking out one procedure in AA digestibility experiments is unfavorable for the aim to provide data for the practice. Instead, standard protocols should state which procedures need to be aligned with practical conditions. This approach represents a standardization by which procedures may differ in

experiments instead of one uniform procedure. Mentioning such procedures in a standard protocol enhances the awareness of influences on AA digestibility. Relevant procedures include:

- Feed-provisioning procedures during experiments. This can be *ad libitum* or restrictive feeding.
- Grinding of the test ingredients and other procedures of feed processing. The resulting particle size distribution should be determined in any study on AA digestibility and outcomes of the measurements should be reported.

■ The relevance of similar feed intake among dietary treatments should be pointed out and the possibilities to achieve similar voluntary feed intake should be mentioned. This includes:
- Avoid diets deficient in AA concentrations.
- Avoid high proportions of feed ingredients in the diets that lead to reduced feed intake.
- Restrict the daily offered amount of feed if birds are kept individually and if other aims, like *ad libitum* feed-provisioning, do not exclude this option.

■ Digesta from slaughtered birds should be frozen as quickly as possible. This implies:
- Sample digesta as quickly as possible after slaughtering.
- Use ice-cold water to flush the digesta out of the sampled section of the small intestine.
- Restrict the amount of digesta mixed with flushing water per sample container so that samples quickly freeze to the core.

Methods need to be improved continuously by determining relevant methodological influences on AA digestibility to address welfare concerns of experimental animals and to adapt assays to ensure the outcomes are most suitable for the intended purpose. The following paragraphs describe some suggestions for methodological experiments.

Cecectomized laying hen experiments

The suggestions to investigate the effects of feed intake and AA concentrations in the basal diets and excreta collection intervals for AA digestibility experiments on cecectomized laying hens have already been described in Section 3.1.1 and Section 3.1.5, respectively.

Another methodological aspect worth investigating in terms of animal welfare and effort of future studies is the duration the cecectomized laying hens need to be kept in metabolism units. This includes both feed adaption and excreta collection periods. The required length of excreta collection period may be investigated by quantifying and analyzing the excreta of at least four consecutive days separately. Such an approach would enable to determine the effect of all possible combinations of the sampling days on excreta amount, the excreta composition, and

the AA digestibility. The effect of days of adaptation cannot be investigated using such an approach. Hens must actually spend different time periods in the metabolism units prior to excreta collection to capture such an effect. Such investigations may include microbiota measurements because the adaptation of the microbiota to feed change seems interesting to assess the degree of adaption to the feed.

The finding of more precise AA digestibility measurements when cecectomized laying hens are used compared with sampling digesta from the terminal small intestine of intact laying hens by Rezvani et al. (2008a) may be verified with birds of different ages. Rezvani et al. (2008a) compared an experiment on 29-week-old intact laying hens with an experiment on 46–52-week-old cecectomized laying hens. A similar investigation with hens of the same age, for instance 30, 55, and 80 weeks, seems interesting. Investigating the microbiota in such an experiment seems appealing to capture potential age effects.

Broiler chicken experiments

The optimum number of broiler chickens of which the digesta from the terminal small intestine need to be pooled is currently unknown. This number is defined by the needed sample quantity for analyses and by the need to compensate for the effects of spot-sampling with pooling the digesta of several animals (see Section 1.4.1). From the analytic point of view, the minimum number of animals can be calculated from the needed sample quantity and sampled digesta amount per animal. The needed sample quantity varies among laboratories. For example, in the Animal Nutrition laboratory of the University of Hohenheim, 4.2 g of dried sample is needed to analyze one observation in a pc AA digestibility experiment (including AA, titanium dioxide, and dry matter analyses in duplicates but excluding additional measurements). This sample amount can be reduced to 3.2 g when all possibilities of sample quantity reduction are exhausted. The sampled digesta amount is difficult to anticipate prior to an experiment because the sampled digesta amount per bird and relative to other measures was variable between experiments (Table 8). Further, the section of the digestive tract to be sampled is empty in some animals. Hence, the lowest conceivable digesta amount per bird needs to be assumed to ensure that a sufficient sample amount is obtained. Otherwise, there is the risk that an observation is lost, which reduces statistical power and all animals of the respective observation were sacrificed in vain. The number of birds needed to compensate for the effects of spot-sampling with pooling the digesta of several animals is not known and may be investigated in a future experiment. Such an experiment may, for example, evaluate pooling of sampled digesta of 4

Table 8. Sampled digesta amount of broiler chickens in published prececal amino acid digestibility experiments.[1]

Experiment[2]	1[3]	2	3	4	4	5	6	7	8	9	10	11
Broiler strain	ISA J-257	Ross 308	Ross 308	ISA J-257	Ross 308	Ross 308	Ross 308	Ross 308	Ross 308	Ross 308	Ross 308	Ross 308
Birds per pooled digesta sample[4] (n)	10	10	10	10	10	14	14–17[5]	11	12	10	15	10
Age at slaughter (d)	22	29	21	29	29	23	27	22	22	25	22	24
Body weight at slaughter (g)	508	1,645	821	820	1,167	897	1,558	1,211	741	938	903	877
Dry digesta in pooled samples (g/bird)												
Mean	0.5	1.8	1.1	0.6	1.9	0.8	1.9	1.1	0.8	0.7	1.1	1.3
Min	0.2	1.5	1.0	0.5	1.7	0.5	0.3	0.7	0.3	0.4	0.9	0.8
Max	0.7	2.0	1.2	0.7	2.3	1.0	2.8	1.8	1.2	1.1	1.4	1.8
Dry digesta (g/kg body weight)	0.3	3.0	0.9	0.5	2.3	0.9	1.2	0.9	1.1	0.7	1.3	1.5
Dry digesta (g/kg body weight × proportion of other feed ingredients than cornstarch in basal diets)[6]	0.2	1.3	0.5	0.4	1.8	0.8	-	-	-	-	-	-

[1]Only basal diets of experiments using the regression approach (experiments 1–5) are shown because the lowest digesta amounts are sampled due to the high dry matter digestibility of cornstarch. Among the experiments using the regression approach, the animals were fed *ad libitum* in Study 1, Study 3, and Study 4. Only the *ad libitum* feed provisioning protocol is presented for Study 2.

[2]1: Study 1; 2: Study 2; 3: Study 3; 4: Study 4; 5: Study 12; 6: Sommerfeld et al. 2018b; 7: Sommerfeld et al. 2018a; 8: Künzel et al. 2021; 9: Künzel et al. 2019a; 10: Siegert et al. 2017; 11: Papp et al. 2021.

[3]Basal diet with adequate amino acid concentrations.

[4]Initial number of birds per unit; mortality not considered.

[5]19 birds per unit at beginning of the experimental period; the stated number depended the number of birds per unit used for other measurements.

[6]Presented only for experiments using the regression approach. Presented because the high dry matter digestibility of cornstarch makes a dependency on the proportion of other feed ingredients in basal diets conceivable.

(absolute minimum for 21-day-old Ross 308 broiler chickens in terms of sample quantity), 8, 12, 16, and 20 animals per observation.

The considerably lower number of animals needed in cecectomized laying hen studies compared with studies on broiler chickens make cecectomized laying hens an interesting model animal to evaluate AA digestibility in broiler chickens. This presupposes that AA digestibility data determined with laying hens can be applied to broiler chickens. The available literature does not suggest such a transferability (see Section 3.1.4). As already pointed out in Section 3.1.4, the transferability of AA digestibility of test ingredients between broiler chickens and laying hens might also be examined on a larger scale by using the regression approach. The comparability of the results obtained from such an experiment would partly be restricted when AA digestibility in broiler chickens is determined – as commonly done – by using digesta from the ileum of slaughtered birds and AA digestibility in laying hens is determined using cecectomized birds. AA digestibility may be determined in cecectomized broiler chickens to increase comparability and to obtain very precise measurements. This presupposes that it is considered worth accepting the demanding procedure of cecectomizing approximatively two-week-old broiler chickens. The transferability of the AA digestibility of laying hens and broiler chickens may also be restricted by the age difference. Indeed, age effects were often reported (see Section 3.1.5). Whether AA digestibility is transferable between laying hens and broiler chickens is currently limited because unknown age influences may be determined by comparing broiler chickens and laying hens of the same age. Such studies are not available at present.

3.7.2 Predictability of amino acid digestibility

Previous attempts to predict AA digestibility were not satisfactorily precise (see Section 3.5). A certain degree of predictability based on the analyzed chemical composition with predictors that can be explained causally were described in few studies (Frikha et al. 2012; Rezvani et al. 2012). However, the accuracy of such predictions was usually not very high, with coefficients of variation ≤0.46 reported by Frikha et al. (2012) and an average root mean square error of 2.7% published by Rezvani et al. (2012). Moreover, the predictions could not be validated independently. This probably is explained by the small number of observations in any such data sets as a consequence of the great effort required to obtain AA digestibility measurements. An interesting alternative is computing prediction equations based on several previously published studies by using meta-analytical approaches. Such an approach was conducted for pigs but the accuracy of predictions still was low with coefficients of determination in the range of 0.41–0.68 (Messad et al. 2018). For some feed ingredients, the lack of predictability of AA

digestibility is partly explained by a very low variation in AA digestibility, like soybean meal (Figure 8). In this case, a lack of predictability is of relatively little importance because there is only a small advantage for precisely formulating diets and, hence, increasing PUE.

For the future, predictability of AA digestibility may be increased by the following strategies:

- By continuing to investigate the influences on AA digestibility because very pivotal determinant(s) apparently have not yet been discovered. As pointed out in Section 3.5, these determinant(s) may include the fine structure of NSP, protein fractions and their subclasses, chemical constituents or physical characteristics that have not been determined as relevant for AA digestibility so far, and the microbiota in the digestive tract. As indicated in Section 3.3.6, recent advancements in microbiota research have enabled researchers to gain better assessments of the contribution of the microbiota to AA digestibility. Functionality analyses seem to advance assessability, but determining the quantitative contribution of the microbiota to AA digestibility is still not possible.
- By continuing to determine the relevant methodological influences on AA digestibility to make AA digestibility measurements more precise and more transferrable for the intended purpose of application.
- By standardizing protocols for AA digestibility experiments to reduce potential influences from test facilities. This endeavor would promote the accuracy of meta-analytical approaches.
- By presenting more experimental data in publications or online supplements to publications. An example is feed intake, data for which inexplicably are not presented in the majority of studies despite the clear evidence that feed intake is a determinant of AA digestibility. Presenting more experimental data would also enable future meta-analyses on the influences of AA digestibility that have not yet become apparent.
- By working toward quicker and cheaper AA digestibility experiments to increase the data base of AA digestibility data. This endeavor requires methodological clarifications, including those suggested in Section 3.7.1. Time and cost for wet chemical AA analyses may be reduced by using NIRS (Fontaine et al. 2001; Escuredo et al. 2014) or other methods.
- By reducing the number of experimental animals in AA digestibility experiments without sacrificing precision to sustain the acceptance of such experiments in the public. The suggestions for methodological experiments in Section 3.7.1 may contribute to this goal.
- By intensifying the communication to the public to increase the awareness of how important animal experiments are to achieve goals that are generally conceived as desirable to the public. An opinion communication of the Gesellschaft für Ernährungsphysiologie (2017b)

provides explanations for the necessity of experimental studies with animals in animal nutrition research, including digestibility experiments.

3.7.3 Final assessments

Optimizing diets by approximating the requirement for digestible AA to increase PUE involves considering the changing requirement for digestible AA throughout the production period, increasing knowledge of the requirement of animals for single nitrogenous nutrients, and increasing predictability of AA digestibility (see Section 1.3). Changes in requirements for many AA throughout the production period have been well known for years (Gesellschaft für Ernährungsphysiologie 1999). The limitations for practical application of this knowledge are defined by operational constraints, such as feeding technology and availability of transport and storage capacity for diets with different AA concentrations. In animal nutrition research, progress regarding the potential to increase PUE seems more likely to be found in research on the requirement of animals for single nitrogenous nutrients and on the predictability of AA digestibility. Recent research on the requirement for nonessential AA of up to 21-day-old broiler chickens has enabled to achieve a very high PUE of ~78% (Hofmann et al. 2020b). The difference compared with a PUE of 100% includes an unknown proportion of undigestible protein of about 10% [approximation based on analyzed excreted N minus N in the urine; N in the urine was estimated based on the analyzed ammonia-N and uric acid-N, and a factor of 13% for other nitrogenous components of avian urine described by Scanes (2015)] and an unknown amount of inevitable urinary N loss. Hence, the potential to increase PUE by increasing knowledge on the AA requirement was less than 12%. There is also little room to increase PUE by precisely predicting AA digestibility for the diet used by Hofmann et al. (2020b) because a large proportion of the AA supply was covered by free AA (free AA provided 50 out of 161 g CP/kg to the diet). The digestibility of free AA is complete (Baker 2009). The resulting very high AA digestibility of the diets used by Hofmann et al. (2020b) offers little room for variation in AA digestibility. A personal estimate provides a potential means to increase PUE by less than 3 pp by precisely predicting AA digestibility in such a diet. A higher potential to increase PUE by precisely predicting AA digestibility is to be expected when the level of AA digestibility in a diet is lower, as indicated by the model calculations in Section 3.6. Nonetheless, increasing knowledge on the requirement for nitrogenous nutrients is more most likely important for increasing PUE than precisely predicting AA digestibility – irrespective of the level of AA digestibility of the diets.

In practice, there are limitations in increasing PUE – besides economical constraints – regarding the possibility to mix diets with matching calculated and actual AA concentrations because analyzed AA concentrations of feed ingredients in mixing batches are not available. This phenomenon is illustrated by a deviation of up to 1.1 pp of dietary CP in a practical experiment on CP reduction (Lemme et al. 2019). Such uncertainties in diet formulation largely compensate for the potential to increase PUE by a precise predictability of AA digestibility compared with using table values for AA digestibility of feed ingredients. It appears probable that rapid analysis tools, such as in-line stream NIRS devices (Penz 2017), prospectively enable to estimate AA concentrations of feed ingredients in real-time and to adjust diet composition automatically to achieve aspired dietary AA concentrations. This procedure may include AA digestibility if determinable with the prediction tool. Ideally, better predictability of AA digestibility has already been achieved until current inaccuracies in practical diet formulation are overcome.

Higher AA digestibility often is considered desirable, but whether higher AA digestibility is actually advantageous depends on the wider framework. Increasing AA digestibility has the potential to raise PUE, but only when an extra supply with digestible AA because of higher AA digestibility is coupled with adapted dietary total AA concentrations to meet the requirement for digestible AA. Otherwise, higher AA digestibility causes an oversupply of digestible AA, which results in increased AA catabolism, and the N contained in catabolized AA has to be excreted via the urine. In comparison, the N contained in unabsorbed AA is excreted via the feces (Simon 2020). As pointed out in Section 1.2, the urinary N is the fraction of excreted N of particular environmental relevance because N volatilization from urinary N is more pronounced than N volatilization from fecal N. Hence, digestible AA oversupply caused by increased AA digestibility has no effect on PUE, but it increases N volatilization. Higher AA digestibility is disadvantageous in such a case. Therefore, designating changes in AA digestibility upon dietary interventions using judgmental statements such as ‘improved’, ‘better’, ‘superior’, ‘worse’, or ‘poor’, as often found in the scientific literature, seems inadequate.

A maximized PUE is to be pursued when the major aim of feed formulation is to reduce water consumption and the consequences of excreted nitrogenous compounds on the environment or health. When the major aim of feed formulation is global food security, a maximized PUE is only beneficial if feed ingredients are used that are inedible by humans. Lower PUE can be accepted for the aim of global food security when components hardly consumed by humans are used because other components can be consumed directly by humans without transformation

losses. Using feed ingredients that are hardly consumed by humans for farm animals increases the human-edible output in the form of animal-based food per human-edible input. This approach ultimately would lead to higher availability of food for humans (Wilkinson 2011; Ertl et al. 2015; Windisch 2021). This principle is untouched when PUE is low because components hardly consumed by humans with low AA digestibility – like guar meal (Study 12) – are used. This fact does not limit the necessity of knowledge of the digestible AA requirement of animals and predictability of AA digestibility to avoid inefficient use of feed and the downsides of unnecessary urinary N excretion, including environmental and health issues and surplus water consumption.

4 Summary

One aim of poultry nutrition research that has been pursued for decades is to decrease the ingested protein relative to the protein accreted in animal body weight or eggs, which is described in the key figure 'protein utilization efficiency'. Increasing protein utilization efficiency aims to ensure global food and water security and to minimize the effects of excreted nitrogenous compounds on the environment and the health of animals and humans. Protein utilization efficiency can be increased by adjusting the supply of digestible amino acids to animals relative to the requirement for digestible amino acids. The predictability of amino acid digestibility of feed ingredients is a prerequisite to achieve this goal. This habilitation thesis puts knowledge gained from studies on methods of amino acid digestibility determination, influences on amino acid digestibility, and variation in amino acid digestibility within feed ingredients into the context of predictability of amino acid digestibility. Methodological, diet-related, and animal-related influences that considerably determine amino acid digestibility are presented and evaluated. This includes feed intake, feed provisioning, feed processing, chemical composition of feed ingredients, feed enzymes, and microbiota in the digestive tract. Cropping conditions influencing amino acid digestibility are also addressed. The gained insights may contribute to make amino acid digestibility more predictable in the future. Recent attempts to predict amino acid digestibility, however, have not been sufficiently accurate to fulfill the aim of being able to formulate diets according to the requirement for digestible amino acids in practice. Suggestions for future strategies to work toward a more accurate predictability of amino acid digestibility are included. Model calculations show that increasing amino acid digestibility can considerably raise protein utilization efficiency. When amino acid digestibility is increased by an influence not related to the feed ingredient providing amino acids (e.g., supplemented enzymes), increasing amino acid digestibility by 1 percentage point raises the protein utilization efficiency by ~0.43 percentage points. An increase in protein utilization efficiency of up to 0.5 percentage points can be expected when amino acid digestibility is increased by selecting variants of a feed ingredient for higher amino acid digestibility. The thesis concludes with a critical examination of the general perception that higher amino acid digestibility and maximized protein utilization efficiency are advantageous. Situations in which lower amino acid digestibility and smaller protein utilization efficiency provide benefits are discussed.

Annex

Example calculation of the PUE according to Eq. 6 using determined CP and Lys concentrations as well as the Lys digestibility of the wheat genotype 'Mulan' (Rodehutscord et al. 2016; Zuber and Rodehutscord 2016). In summary, the following values for equation parameters were used in the example calculation. The PUE in the example calculation is carried out in 6 steps.

Parameter in Eq. 6	Value	Description
a	6.6 g/d	CP accretion (value explained in Section 3.6)
i	94.2 g DM/d	Feed intake (value explained in Section 3.6)
CP supply by the considered ingredient (left-hand parenthesis in the denominator)		
b	2 g/kg DM	Contribution of the considered ingredient to the digestible Lys supply of the complete diet (value explained in Section 3.6)
f	132 g/kg DM	CP concentration of the considered ingredient (Rodehutscord et al. 2016)
d	3.75 g/kg DM	Lys concentration of the considered ingredient (Rodehutscord et al. 2016)
e	86.9 %	Lys digestibility of the considered ingredient (Zuber and Rodehutscord 2016)
CP supply by the remaining ingredients (right-hand parenthesis in the denominator)		
c	g/kg DM	Digestible Lys concentration in the complete diet (value explained in Section 3.6)
g	85%	Average Lys digestibility of the remaining ingredients (value explained in Section 3.6)
h	6 g/g	Lys to CP ratio of the remaining ingredients (value explained in Section 3.6)

Step 1: Digestible Lys concentration in the considered ingredient

	86.9	% Lys digestibility of the considered ingredient (parameter *e*)
×	3.75	g Lys/kg DM in the considered ingredient (parameter *d*) / 100
=	3.26	g digestible Lys/kg DM in the considered ingredient

Step 2: CP supply by the considered ingredient

	2	g digestible Lys supply by the considered ingredient/kg DM of the complete diet (parameter *b*)
/	3.26	g digestible Lys/kg DM in the considered ingredient (calculated in Step 1)
=	0.613	proportion of the considered ingredient in the diet on a DM basis
×	132	g CP/kg DM in the considered ingredient (parameter *f*)
=	80.9	g CP supply by the considered ingredient/kg DM of the complete diet

Step 3: CP supply by the remaining ingredients

	5.23	g digestible Lys/kg DM in the complete diet (parameter *c*)
-	2	g digestible Lys supply by the considered ingredient/kg DM of the complete diet (parameter *b*)
=	3.23	g digestible Lys supply by the remaining ingredients/kg DM of the complete diet
/	85	% Lys digestibility of the remaining ingredients (parameter *g*) × 100
=	3.80	g Lys/kg DM in the remaining ingredients
/	6	Lys to CP ratio of the remaining ingredients (parameter *h*) × 100
=	63.3	g CP supply by the remaining ingredients/kg DM of the complete diet

Step 4: CP concentration of the complete diet

	80.9	g CP supply by the considered ingredient/kg DM of the complete diet (calculated in Step 2)
+	63.3	g CP supply by the remaining ingredients/kg DM of the complete diet (calculated in Step 3)
=	144.2	g CP/kg DM of the complete diet

Step 5: CP intake

	94.2	g DM feed intake/d (parameter *i*)
×	144.2	g CP/kg DM of the complete diet (calculated in Step 4) / 1000
=	13.6	g CP intake/d

Step 6: PUE

	6.6	g CP accretion/d (parameter *a*)
/	13.6	g CP intake/d (calculated in Step 5) × 100
=	48.5	% PUE

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