

# Effects of gradual differences in trypsin inhibitor activity on the estimation of digestible amino acids in soybean expellers for broiler chickens

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**ABSTRACT** The present study investigated the effect of varying trypsin inhibitor activity (**TIA**) in differently processed soybean expellers on apparent prececal amino acid (**AA**) digestibility in male broiler chickens. Two different raw soybean batches were treated using varying processing techniques and intensities. In this way, 45 expeller extracted soybean meal (**ESBM**) variants were created. The processed soybean variants were then merged into a basal diet (160 g/kg crude protein [**CP**]) at 2 inclusion levels (15%, 30%) resulting in 90 different diets plus one basal diet (0.4 mg/g–8.5 mg/g TIA). All diets contained 0.5% titanium dioxide. A total of 5,460-day-old male broilers (Ross 308) were allocated on d 14 to 546 pens (10 birds/pen) after a starter phase (CP 215 g/kg, 14 g/kg Lysine, 12.5 MJ ME/kg). The 91

experimental diets were fed ad libitum until d 22. Subsequently, birds were euthanized and digesta of the terminal ileum was collected for determination of AA digestibility. TIA depressed the prececal digestibility of every single AA significantly in a straight linear fashion ( $P < 0.001$ ). Sulfur-containing AA expressed the strongest suppression by TIA with cystine showing the lowest apparent prececal digestibility measured (10.6% at 23.6 mg/g TIA in raw ESBM). The present data demonstrate that TIA severely depresses digestibility of essential and nonessential AA in a straight linear fashion. On the one hand, this questions the usefulness of defined upper limits of TIA in soy products whereas on the other hand, TIA must be considered when testing raw components for their feed protein value in vivo.

**Key words:** Amino acid, broiler, digestibility, soybean, trypsin inhibitor

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## INTRODUCTION

Soybeans are the most important protein source in livestock feeding. Compared to other plants, soybeans have high contents of oil and protein and a superior amino acid pattern (Clarke and Wiseman, 2005). The nutritional value of most plant materials is limited by the presence of numerous naturally occurring compounds, which interfere with nutrient digestion and absorption (Clarke and Wiseman, 2000). The most important antinutritional substances occurring in soybeans are trypsin inhibitors (**TI**), which can be divided into 2 classes: The Kunitz trypsin inhibitors (**KTI**) and

the Bowman-Birk trypsin inhibitors (**BBI**). The KTI consist of 181 amino acid residues and have a relative molecular weight of 20,100 Da (Koide and Ikenaka, 1973). The reactive site of this inhibitor class is located at residues Arg 63 and Ile 64 and binds primarily trypsin. The KTI have a low content of cysteine and only 2 disulfide bonds. The second class, BBI are rich in cysteine and have seven disulfide bridges, which is the reason for a very dense three-dimensional structure (Odani and Ikenaka, 1973). In comparison to the KTI, the BBI are relatively low in molecular weight (approx. 6,000 Da to 10,000 Da) and have two independent and symmetric binding sites for trypsin and chymotrypsin. The trypsin-reactive site is located on Lys 16 and Ser 17 and the chymotrypsin-reactive site is positioned on Leu 43 and Ser 44 (Odani and Ikenaka, 1973). Both inhibitor classes form stable enzyme-inhibitor complexes on a molar 1:1 ratio (Clarke and Wiseman, 2000).

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Earlier studies have been published showing the negative effect of trypsin inhibitor activity (**TIA**) on growth performance of rats (Grant et al., 1995; Gu et al., 2010), chickens (Clarke and Wiseman, 2005, 2007; Heger et al., 2016), turkeys (Mian and Garlich, 1995), and pigs (Herkelman et al., 1992; Batterham et al., 1993; Zollitsch et al., 1993). Furthermore, TIs cause pancreas hypertrophy and hyperplasia in rats (Abbey et al., 1979; Grant et al., 1995) and chickens (Gertler et al., 1967; Perilla et al., 1997; Pacheco et al., 2014; Hoffmann et al., 2019). In response to the inhibition of digestive processes, the pancreas increases its size and number of acinar cells in order to elevate the secretion of digestive enzymes (Nitsan and Liener, 1976). In this context, it seems like the underlying endocrine signal that facilitates these adaptations is gut-derived cholecystokinin (**CCK**), which responds to a lower influx of free amino acids (**AA**) into the enterocytes of the small intestine (Miura et al., 1997). Due to the increased activity of the pancreas, Lyman and Lepkovski (1957) suggested that the overall depression in growth performance is mainly due to the high endogenous loss of AA and enzymes secreted from the pancreas, since the digestive depression itself could be quite efficiently compensated by the higher pancreatic enzyme secretion.

To avoid depression in performance, the heat-labile antinutritional factors in soybean products have to be sufficiently deactivated. Batterham et al. (1993) recommended to reduce TIA in soybean products for growing pigs to a level of 4.7 mg/g. Similar results were shown in the studies of Clarke and Wiseman (2005, 2007) for poultry. They concluded that TIA in full-fat soybeans should not exceed 4.0 mg/g. On the other hand, it is important to avoid protein denaturation induced heat damage. Pacheco et al. (2014) and Araba and Dale (1990) observed a decline in performance with decreasing protein solubility in potassium hydroxide (**KOH**) below 74 and 70%, respectively, in response to the heat-associated peptide denaturation. Another indicator for heat damage is the concentration of reactive lysine (Fontaine et al., 2007). The  $\epsilon$ -amino group of lysine binds irreversibly with reducing sugars during heat treatment. The balance of adequate denaturation of TI and heat damage is controversially discussed within the available literature: Heger et al. (2016) concluded in their study that growth performance is not impaired even above TIA of 4.0 mg/g, whereas Hoffmann et al. (2019) observed a linear improvement in feed efficiency when gradually decreasing TIA below 1.0 mg/g without impairing growth performance through excessive heat-damage to the protein fraction.

The parameter of choice for the determination of the feed protein value is the AA flow at the terminal ileum (Ravindran et al., 1999). This consists of the undigested and unabsorbed feed-borne AA as well as endogenously secreted AA. The pool of endogenously secreted AA is further subcategorized into the basal and specific endogenous losses. While the basal losses are considered to be predominantly affected by the total dry matter intake, the specific AA secretion appears to be affected by the

quantity and characteristics of the protein under study (Angkanaporn et al., 1997; Dänicke et al., 2000; Souffrant, 2001). Hence, when determining feed protein quality as a function of AA flow at the terminal ileum, the specific endogenous AA losses should be considered. However, attempts to quantify endogenous protein secretion yield highly variable results (Donkoh and Moughan, 1999). Therefore, Rodehutscord et al. (2004) proposed an approach by which the digestibility of the dietary protein until the end of the ileum (so called “prececal digestibility”) is estimated through linear regression, as the slope of increasing apparently digested AA until the terminal ileum with gradually increasing dietary AA intake via the feed protein under study. This method is supposed to exclude effects of varying endogenous protein losses, since the slope represents the prececal AA digestibility as affected by the protein source under study.

This study aimed to investigate the effects of differentially treated expeller extracted soybean meal (**ESBM**) and associated finely graded differences in dietary TIA in the feeding of broilers. In particular, the prececal amino acid digestibility according to Rodehutscord et al. (2004) as well as the zootechnical performance of birds was investigated.

## MATERIALS AND METHODS

### *Soybean Processing and Diet Composition*

Raw material for soy processing consisted of 2 homogeneous batches of soybeans. In Batch 1 (breed: Sultana, native TIA: 37.3 mg/g) were conventionally produced soybeans, harvested in Germany, Batch 2 (breed: Merlin, native TIA: 40.5 mg/g) consisted of organically produced soybeans from Romania. These 2 batches were treated equally using 4 different processing techniques (thermal, hydrothermal, pressure, and kilning) at varying processing intensities.

For the thermal treatment, soybeans were moistened and toasted for 40 s at either 115°C or 120°C, respectively. The hydrothermal method comprised the usage of steam at an average temperature of 103°C for about 40 min. The third method included hydrothermal treatment in combination with expander extrusion at intensities varying from 110°C to 130°C for at least one second to a maximum of 5 s. Using the kilning method, antinutritional factors were heat inactivated by hot recirculating air at 130°C up to 190°C with varying duration from 20 to 40 min. After cooling, all differently treated soybeans were mechanically de-oiled. In this way, 46 differently processed ESBM were created. One ESBM variant had to be excluded from the experiment due to hygienic problems, which resulted in 45 experimental ESBMs. Supplementary Table 1 presents the different treatments in detail, which have been already described earlier by Hoffmann et al. (2017). The intention with this processing scheme was to create a wide range of TIA and parameters associated with potential heat-damage to the protein fraction (KOH

**Table 1.** Feed composition of basal diet and experimental diets. The experimental diets contained two different levels of crude protein (CP level 1 = 220 g/kg CP and CP level 2 = 300 g/kg CP) as a result of varying addition of differentially processed expeller extracted soybean meals in exchange for of maize starch.

Ingredients (%)	Basal diet 175 g/kg CP	CP level 1, 220 g/kg CP	CP level 2, 300 g/kg CP
Maize starch	28.0	14.0	-
experimental ESBM	-	15.0	30.0
Soybean oil	4.00	3.00	2.00
Maize	-----	46.2	-----
Solvent extracted soybean meal	-----	10.0	-----
Potato protein	-----	5.00	-----
Titanium oxide	-----	0.50	-----
Mono calcium phosphate	-----	2.51	-----
Sodium chloride	-----	0.51	-----
Limestone	-----	1.15	-----
Mineral feed	-----	0.15	-----
Vitamin premix	-----	0.20	-----
Choline chloride 50%	-----	0.20	-----
L-Lysine HCl	-----	0.62	-----
DL-Methionine	-----	0.20	-----
L-Arginine	-----	0.55	-----
L-Tryptophan	-----	0.03	-----
L-Threonine	-----	0.23	-----

Abbreviations: CP, crude protein; ESBM, expeller extracted soybean meal.

A total of 91 experimental broiler chicken diets were mixed based on the inclusion of either none (control) or one of 45 differently processed expeller extracted soybean meals at two inclusion levels (15%, 30%) in exchange for maize starch.

solubility, reactive lysine) to investigate the antinutritional effect of TIA and its potential interaction with protein damage on the AA utilization within the small intestine. The processing in general was not the objective of the present study and the experimental design would not be appropriate to investigate effects arising from different processing plants. The TIA in ESBM variants ranged from 0.3 mg/g to 23.6 mg/g, KOH soluble crude protein (CP) varied from 64.4 to 97.7% of total CP and reactive lysine from 14.7 to 25.0% of DM. TIA and heat-damage parameters, as well as AA concentrations are presented in [Supplementary Table 2](#). The processed ESBM were then mixed into the basal diet ([Table 1](#)) at either 15% (CP level 1) or 30% (CP level 2) at the expense of maize starch according to [Rodehutscord et al. \(2004\)](#). Thereby, 91 experimental diets were mixed and subsequently pelletized to stabilize particle size distribution. All diets included 0.5% of titanium dioxide (TiO<sub>2</sub>) as an indigestible marker. The TIA in the final feed mixtures varied from 0.4 mg/g in the nonsupplemented basal diet to 8.5 mg/g in the diet containing non-heated ESBM at the highest inclusion rate (30%).

## Animals and Experimental Protocol

The study design was reviewed and approved by the responsible animal welfare officers of the Technical University of Munich (TUM) School of Life Sciences and the Bavarian State Research Center for Agriculture, respectively, and registered and approved by the legal authorities of the District Government of Lower Franconia, Federal State of Bavaria, Germany (registered case no. 55.2-DMS 2532-2-2164). The experiments took place at the Department for Education and Poultry Research, Bavarian State Research Center for Agriculture in Kitzingen (Germany).

The experiment was designed according to the model suggested by [Rodehutscord et al. \(2004\)](#). A total of 5,460 one-day-old male broiler chickens (Ross 308) were obtained from a local hatchery (Brüterei Süd, Regensburg, Germany). Animals were reared with a commercial starter diet (CP 215 g/kg, 14 g/kg lysine, 12.5 MJ ME/kg) fed *ad libitum* from experimental d 1 to 14. On d 15, birds were weighed and randomly allocated to one of 546 pens (10 birds per pen, 1.6 m<sup>2</sup> per pen) equipped with feeder, nipple drinker, and straw beddings. The 91 experimental diets were randomly distributed over pens, yielding an effective sample size of 6 replicates per feeding group. The diets were fed *ad libitum* until d 22 on which birds were weighed individually and euthanized by asphyxiation with carbon dioxide. The animals' body cavities were immediately opened; the section between Meckel's diverticulum and 2-cm anterior to the ileo-cecal-colonic-junction was isolated. The ileal content of two thirds of the terminal section was flushed with distilled water according to the method of [Kluth et al. \(2005\)](#). The digesta was pooled within each pen, frozen at -20°C and freeze-dried for later chemical analyses.

Throughout this study, birds had *ad libitum* access to drinking water (tap water) and the water consumption per pen was monitored continuously.

## Chemical Analyses

Raw soybean batches, ESBM, and experimental diets were analyzed for TIA ([DIN EN ISO 14902:2002-02](#)) and crude nutrient values according to published procedures ([VDLUFA, 2012](#)). Furthermore, ESBM were analyzed for protein solubility in KOH ([DIN EN ISO 14244:2014-02](#)) and reactive lysine according to the homoarginine method applied by [Pahm et al. \(2008\)](#) to assess protein denaturation. AA content of ESBM, experimental diets and digesta were determined by ion-

**Table 2.** Mean values and standard deviation of analyzed nutrient concentration (% DM) and amino acid concentration (% DM) of the control diet and experimental diets CP level 1 and CP level 2 differing in the addition of differentially processed expeller extracted soybean meals in exchange for maize starch.

Nutrient concentration (% DM)	Basal diet 160 g/kg CP	CP level 1, 220 g/kg CP		CP level 2, 300 g/kg CP	
		Mean	SD	Mean	SD
Dry matter	91.2	91.2	0.47	91.2	0.38
Crude protein	15.8	22.7	0.33	29.6	0.81
Crude ash	5.49	6.32	0.18	7.14	0.23
Crude fat	6.65	7.09	0.36	7.62	0.45
Neutral detergent fiber	8.73	11.4	1.25	13.7	1.85
Acid detergent fiber	2.40	3.72	0.63	4.02	0.42
Acid detergent lignin	0.58	0.99	0.39	0.68	0.32
Titanium dioxide	0.54	0.56	0.05	0.55	0.09
Alanine	0.69	0.97	0.01	1.25	0.03
Arginine	1.25	1.77	0.04	2.28	0.07
Aspartic Acid	1.28	2.06	0.04	2.79	0.08
Cystine	0.20	0.30	0.01	0.39	0.01
Glutamic acid	2.00	3.21	0.06	4.39	0.11
Glycine	0.54	0.82	0.01	1.10	0.03
Histidine	0.33	0.50	0.01	0.65	0.02
Isoleucine	0.56	0.85	0.01	1.14	0.03
Leucine	1.26	1.76	0.02	2.26	0.06
Lysine	1.18	1.59	0.03	1.99	0.05
Methionine	0.41	0.49	0.01	0.58	0.01
Methionine + Cystine	0.61	0.79	0.02	0.97	0.02
Phenylalanine	0.68	1.03	0.02	1.35	0.04
Proline	0.82	1.15	0.02	1.49	0.04
Serine	0.64	0.98	0.01	1.31	0.03
Threonine	0.77	1.02	0.01	1.27	0.03
Tryptophan	0.18	0.26	0.01	0.35	0.01
Valine	0.67	0.98	0.02	1.29	0.04

Abbreviations: CP, crude protein; DM, dry matter; SD, standard deviation.

A total of 91 experimental broiler chicken diets was mixed based on the inclusion of either none (control) or one of 45 differently processed expeller extracted soybean meals at two inclusion levels (15%, 30%) in exchange for maize starch.

Values represent analyzed mean values of respective parameters. SD values for expeller extracted soybean meal-supplemented diets (CP level 1, CP level 2) represent variation of analyzed values of each 45 individual diets, respectively, around the respective total mean value.

exchange chromatography referring to [Llames and Fontaine \(1994\)](#). According to this method, the oxidized cysteine-dimer cystine was measured. Additionally, CP content in digesta was analyzed according to [VDLUF \(2012, method 4.1.1\)](#). TiO<sub>2</sub> in diets and digesta were analyzed according to the method of [Brandt and Allam \(1987\)](#). Analyzed crude nutrients and AA concentration of experimental diets are presented in [Table 2](#).

## Statistical Analyses and Calculations

Apparent prececal digestibility coefficients (**DC**) for CP, sum of essential amino acids (**SEAA**), sum of non-essential amino acids (**SNEAA**), as well as for individual AA were calculated using the formula:

$$DC_{AA \text{ diet}} = 100 - \left( \frac{TiO_2 \text{ diet} \times AA_{\text{digesta}}}{TiO_2 \text{ digesta} \times AA_{\text{diet}}} \right)$$

TiO<sub>2</sub> diet and TiO<sub>2</sub> digesta represent the dry matter concentrations of TiO<sub>2</sub> in the diet and digesta and AA<sub>diet</sub> and AA<sub>digesta</sub> are the dry matter concentration of AA in diet and digesta.

The obtained DC were then used to estimate the apparent prececal CP and AA digestibility in regard to the dietary ingested amount of AA at given inclusion level of respective ESBM variants. The prececal AA digestibility from each ESBM was estimated by linear regression technique, using the slope of apparent ileal

AA flux at the terminal ileum in relation to the respective increase in AA intake in response to rising dietary contents of respective ESBM variants ([Rodehutschord et al., 2004](#)). Additionally, the ratio of sulfur-containing AA to lysine was calculated to indicate changes in the biological value and quality of the true protein from different ESBM variants.

For statistical analyses, calculated mean values over single pens within experimental runs represented a total of 546 data points. For each experimental diet, a mean value was calculated including the respective 6 replicate values. In this way, 91 values for zootechnical parameters in response to different diets and each 45 data points for the apparent prececal CP and AA digestibility of each ESBM variant were calculated. These values were used for linear regression analysis ( $y = a + bx$ ) applying the variables TIA, KOH-soluble CP and reactive lysine, respectively (The R Project, Version 4.1.0). The threshold of significance was assumed at  $P \leq 0.05$ . Finally, each of 546 pen-wise mean values of partial digestibility data was further used for descriptive statistics.

Our experimental design was planned to reach in any case a minimum statistical power of  $1 - \beta = 0.8$ . The respective power analysis was performed with G\*Power 3.1.9.7 ([Faul et al., 2007](#); [Faul et al., 2009](#)) applying the dataset of [Hoffmann et al. \(2019\)](#) for the determination of the effect size at assumed  $\alpha = 0.05$ , based on zootechnical performance data under comparable experimental conditions.

## RESULTS

### Zootechnical Performance

Broilers were healthy throughout the experimental phase and mortality was <1% and did not correlate to dietary treatments.

In total, zootechnical performance varied considerably between individual pens. Live weight (**LW**) at the end of the experimental phase (d 22) varied from 752 g to 985 g, Total weight gain (**TWG**) and feed conversion ratio (**FCR**) ranged from 313g and 1.50 in the group with the highest dietary TIA (8.5 mg/g) to 539 g and 1.19 in the group with the lowest dietary TIA (0.4 mg/g).

As shown in **Figure 1** and **Table 3**, rising dietary TIA negatively affected final LW, TWG, total feed intake (**TFI**) as well as FCR in a straight linear manner ( $P < 0.001$ ). Accordingly, a stepwise increase of dietary TIA of 1 mg/g depressed LW, TWG, and TFI by 15.0 g, 16.5 g, and 5.7 g, and increased FCR by 0.015.

The dietary amounts of KOH-soluble CP as well as the reactive lysine had no significant effect on zootechnical performance whatsoever (data not shown; see data availability statement).

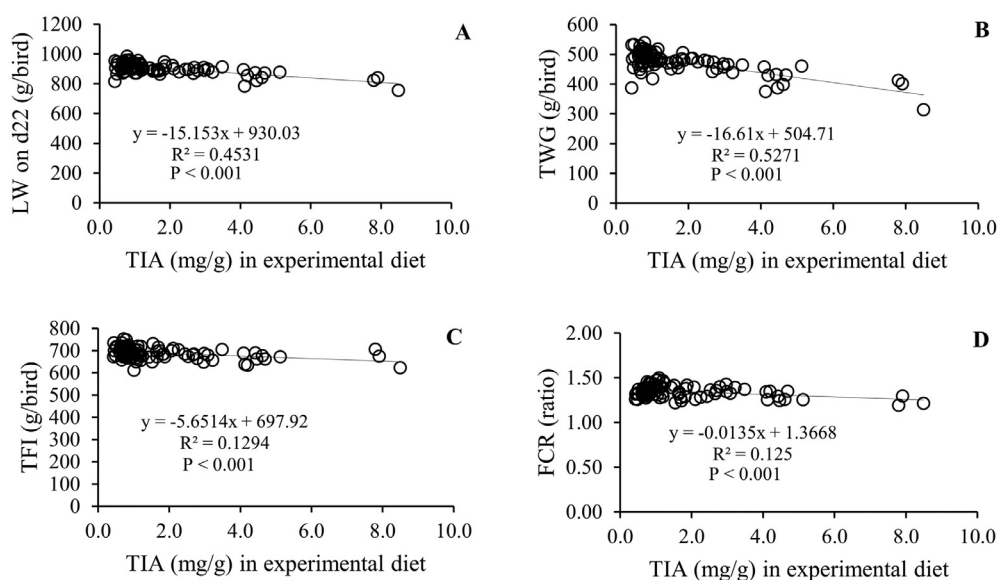
### Partial Prececal Amino Acid Digestibility From Different ESBM Variants

**Table 4** presents descriptive statistics of prececal digestibility of AA and CP arising from the ingestion of different ESBM variants. Like zootechnical performance, partial prececal digestibility varied noticeably

with rising TIA levels. DC of CP, SEAA, and SNEAA varied from 30.33 to 97.21%, 26.76 to 95.12%, and 31.63 to 93.25%, respectively.

According to **Figure 2** and **Table 5**, prececal digestibility of all individual AA as well as of CP of ESBM variants was significantly affected by the respective TIA level in a straight linear fashion ( $P < 0.001$ ). On average, each increase of TIA by 1 mg/g reduced digestibility of CP as well as sums of essential AA and nonessential AA by 1.84, 1.99, and 1.75%, respectively. The magnitude of TIA effects on AA digestibility differed between individual AA. Arginine digestion was least affected by TIA with an average value for all used ESBM of 82% but ranged from 44.8 to 96.5% (**Table 5**). In contrast, the digestibility of cystine was impaired the most by increasing dietary TIA levels, with only 10.59% of prececal digestibility when feeding raw ESBM (23.6 mg/g TIA; please note that the numerically lowest prececal cystine digestibility value was 4.94% at 22.6 mg/g TIA). The measured maximum of prececal cystine digestibility was at 83% (0.3 mg/g TIA), which also fell below the maxima of all other AA ( $\geq 89\%$ ). Furthermore, the ratio of digestible sulfur containing AA to lysine was calculated to characterize the impact of TIA on the biological value of the digestible AA (**Figure 3**). Per unit increase of TIA in ESBM, the ratio of ileal digested sulfuric AA to lysine significantly decreased by 0.0002 (from  $\sim 0.9$  down to  $\sim 0.5$  at 0.3 and 23.6 mg/g TIA;  $P < 0.001$ ).

Prececal digestibility of CP and AA was not significantly affected by the amount of KOH-soluble CP or reactive lysine, respectively (data not shown; see data availability statement).



**Figure 1.** Effect of trypsin inhibitor activity (TIA) in mg/g in experimental diets for broiler chickens with varying addition of differentially processed expeller extracted soybean meals (ESBM) in exchange for maize starch on live weight (LW) on d 22 in g/bird (A), Total weight gain (TWG) in g/bird (B), total feed intake (TFI) in g/bird (C) and feed conversion ratio (FCR) as feed:gain (D) (see **Table 3** for statistical measures of respective regression models). Data represents zootechnical performance of broiler chickens as affected by 91 different experimental diets expressing individual dietary TIA levels ranging from 0.4 to 8.5 mg/g. Individual dietary TIA was a result of 45 differently treated ESBMs each applied at two inclusion levels (15%, 30%) in exchange for maize starch as well as a control diet without inclusion of ESBM (total of 91 diets). The threshold of significance was set at  $P \leq 0.05$ .

**Table 3.** Effect of trypsin inhibitor activity (mg/g) in experimental diets for broiler chickens with varying addition of differentially processed expeller extracted soybean meals on live weight at d 22 (g/bird), total weight gain (g/bird), total feed intake (g/bird), and feed conversion ratio (feed: gain) using linear regression models ( $y = a + bx$ ;  $x = \text{TIA}$ ).

Item (g/bird)	Intercept			Slope			R <sup>2</sup>
		SE	P-value		SE	P-value	
Live weight on d 22	930	4.4	<0.001	-15.0	1.8	<0.001	0.45
Total weight gain	540	4.2	<0.001	-16.5	1.7	<0.001	0.63
Total feed intake	698	3.9	<0.001	-5.7	1.6	<0.001	0.13
Feed conversion ratio	1.37	0.009	<0.001	0.015	0.004	<0.001	0.17

Abbreviation: SE, standard error of the respective parameter estimate.

The threshold of significance was set at  $P \leq 0.05$ .

Data analysis was based on the response of zootechnical performance parameters of broiler chickens fed 91 different diets with individual TIA levels ranging from 0.4 to 8.5 mg/g (control without expeller extracted soybean meal (ESBM) as well as diets containing one of each 45 differentially processed ESBMs at two inclusion levels (15%, 30%) in exchange for maize starch).

**Table 4.** Descriptive statistics of prececal digestibility (%) of amino acids, crude protein, sum of essential amino acids and sum of nonessential amino acids in differentially processed expeller extracted soybean meals fed to broiler chickens.

Item (%)	Mean	SD	Median	25% Quartile	75% Quartile	Minimum	Maximum
Crude protein	74.3	14.3	79.5	64.1	86.10	30.3	97.2
SEAA	75.6	14.7	81.3	66.0	86.56	26.8	95.1
SNEAA	73.5	13.4	77.8	64.4	83.78	31.6	93.3
Alanine	72.3	16.6	78.7	61.8	84.21	21.6	94.2
Arginine	82.6	10.3	86.7	76.4	90.02	44.8	96.5
Aspartic acid	72.3	12.7	75.3	64.0	82.38	34.9	91.5
Cystine	50.5	19.2	54.4	36.2	67.14	4.94	83.5
Glutamic acid	79.1	11.6	83.5	71.5	87.68	39.6	95.9
Glycine	68.4	14.5	71.9	58.7	80.18	25.2	90.7
Histidine	75.0	13.2	79.7	67.4	84.68	29.6	92.6
Isoleucine	73.7	17.0	80.2	64.1	85.89	17.7	95.5
Leucine	73.7	18.4	81.0	65.2	86.44	15.2	96.1
Lysine	77.3	12.8	81.4	70.3	87.59	34.7	94.7
Methionine	74.8	17.5	81.8	64.7	87.22	17.8	94.6
Methionine + Cystine	62.0	18.2	67.7	49.0	76.43	10.9	89.2
Phenylalanine	77.5	15.4	84.6	68.1	87.75	27.8	98.3
Proline	69.7	15.0	75.0	59.9	80.96	24.0	91.5
Serine	72.0	15.4	77.5	62.7	83.41	26.7	93.5
Threonine	68.0	15.1	72.5	57.6	80.58	23.9	90.6
Tryptophan	69.9	15.0	74.5	60.3	81.37	25.1	90.4
Valine	72.6	16.5	78.9	64.2	84.55	18.0	94.1

Abbreviations: SD, standard deviation; SEAA, sum of essential amino acids; SNEAA, sum of nonessential amino acids.

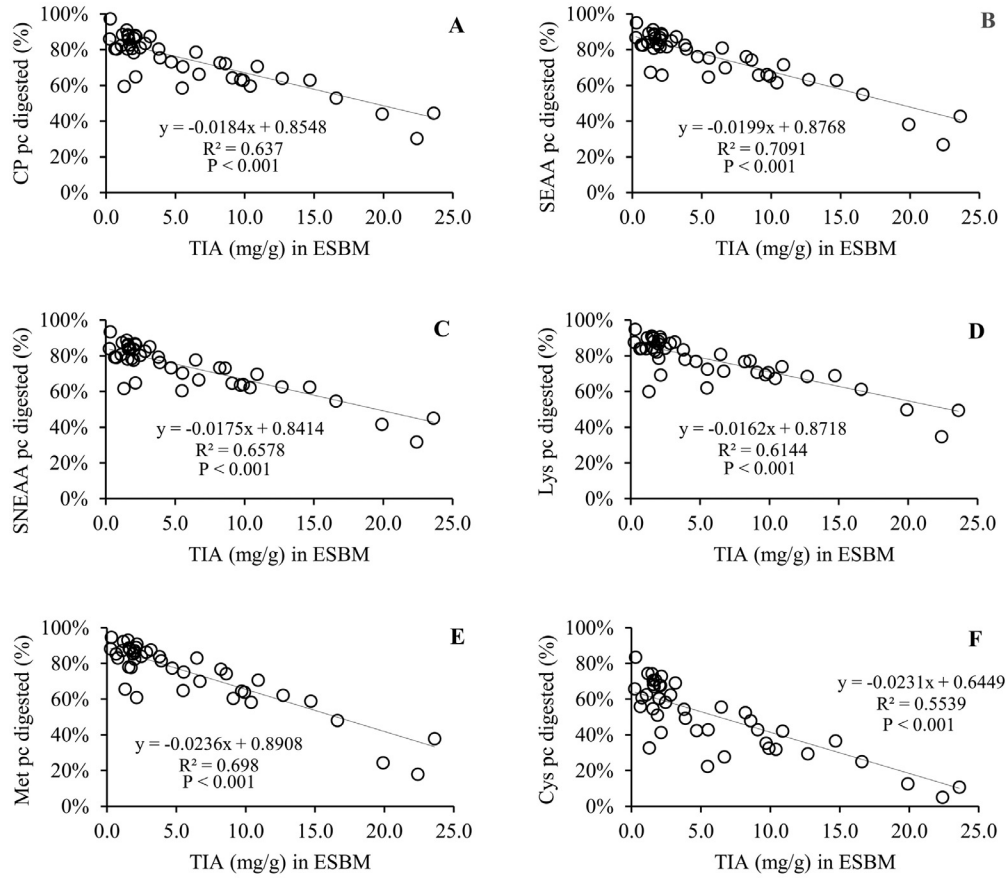
Descriptive statistics are based on prececal crude protein and amino acid digestibility as affected by individual TIA levels of 45 differently processed expeller extracted soybean meals ranging from 0.3 to 23.6 mg/g. The prececal digestibility of crude protein and amino acids from different ESBMs fed to broiler chickens was assessed according to the method of Rodehutschord et al. (2004).

## DISCUSSION

Several studies have been conducted concerning the negative effect of TIA on growth performance in different animal models including rats, pigs, chickens, and turkeys (Herkelman et al., 1992; Batterham et al., 1993; Zollitsch et al., 1993; Grant et al., 1995; Mian and Garlich, 1995; Clarke and Wiseman, 2005, 2007). All these studies gained comparable results: the lower TIA in feed, the better growth performance. Consequently, soybeans are treated with heat and pressure to reduce this antinutritional potential to ensure proper performance and animal wellbeing. Clarke and Wiseman (2005, 2007) claim in their studies to reduce the TIA in full-fat soybeans to 4.0 mg/g is sufficient for broilers. Nevertheless, an overtreatment of soybeans can also lead to decreased growth performance (Araba and Dale, 1990; Pacheco et al., 2014). In the present study, the solubility of CP in KOH varied from 64.4 to 97.7% of total CP and

reactive lysine ranged from 14.7 to 25.0% of total lysine. Neither CP solubility in KOH, nor reactive lysine correlated with growth performance or AA digestibility. This is in good agreement to earlier published data of Herkelman et al. (1991), who gained in their trials the highest chick performance at a minimum of 50% protein solubility. Furthermore, Hoffmann et al. (2019) applied experimental diets comparable to the present study during a whole fattening trial with broiler chickens and did not observe any negative effects of KOH-soluble CP or reactive lysine whatsoever. Therefore, we conclude that protein denaturation by heat treatment of ESBM variants from the present study was negligible and TIA was the dominant antinutritional factor modulating zootechnical performance and protein digestibility.

The aforementioned study of Hoffmann et al. (2019), observed a straight linear decrease of zootechnical performance and especially feeding efficiency during the grower phase of broiler chickens fed diets with finely



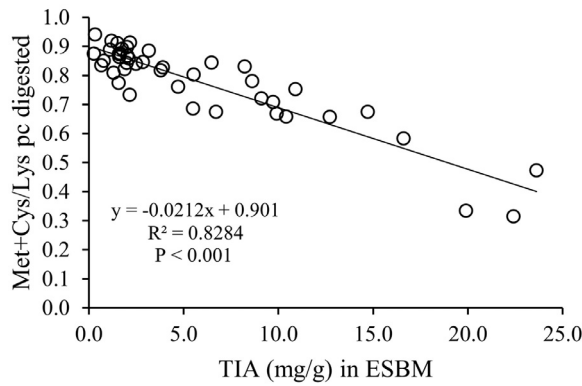
**Figure 2.** Effect of trypsin inhibitor activity (TIA) in mg/g on percentage of prececal (pc) digested crude protein (CP) (A), sum of essential amino acids (SEAA) (B), sum of nonessential amino acids (SNEAA) (C), lysine (Lys) (D), methionine (Met) (E) and cystine (Cys) (F) from differentially processed expeller extracted soybean meals (ESBM) fed to broiler chickens (see Table 5 for statistical measures of respective regression models). Data represents the prececal crude protein and amino acid digestibility estimated according to Rodehutscord et al. (2004) for 45 differently treated expeller extracted soybean meals, as affected by individual TIA levels of individual ESBMs ranging from 0.3 to 23.6 mg/g. The threshold of significance was set at  $P \leq 0.05$ .

**Table 5.** Effect of trypsin inhibitor activity (mg/g) in differentially processed expeller extracted soybean meals fed to broiler chickens on partial prececal digestibility of crude protein, sum of essential amino acids, sum of nonessential amino acids, and individual amino acids using linear regression models (regression model:  $y = a + bx$ ;  $x = \text{TIA}$ ).

Item (%)	Intercept			Slope			R <sup>2</sup>
	SE	P-value		SE	P-value		
Crude protein	85.5	1.85	<0.001	-1.84	0.21	<0.001	0.64
SEAA	87.7	1.70	<0.001	-1.99	0.20	<0.001	0.71
SNEAA	84.1	1.68	<0.001	-1.75	0.19	<0.001	0.66
Alanine	85.8	1.98	<0.001	-2.22	0.23	<0.001	0.69
Arginine	91.1	1.21	<0.001	-1.39	0.14	<0.001	0.70
Aspartic Acid	81.9	1.73	<0.001	-1.59	0.20	<0.001	0.60
Cystine	64.5	2.76	<0.001	-2.31	0.32	<0.001	0.55
Glutamic acid	88.3	1.45	<0.001	-1.52	0.17	<0.001	0.66
Glycine	79.6	1.91	<0.001	-1.85	0.22	<0.001	0.62
Histidine	85.6	1.61	<0.001	-1.75	0.19	<0.001	0.68
Isoleucine	87.9	1.88	<0.001	-2.34	0.22	<0.001	0.73
Leucine	89.3	2.03	<0.001	-2.56	0.24	<0.001	0.74
Lysine	87.2	1.71	<0.001	-1.62	0.20	<0.001	0.61
Methionine	89.1	2.07	<0.001	-2.36	0.24	<0.001	0.70
Methionine + Cystine	76.3	2.31	<0.001	-2.36	0.27	<0.001	0.65
Phenylalanine	90.4	1.69	<0.001	-2.12	0.20	<0.001	0.74
Proline	81.6	1.86	<0.001	-1.97	0.22	<0.001	0.66
Serine	84.6	1.80	<0.001	-2.08	0.21	<0.001	0.70
Threonine	79.9	1.92	<0.001	-1.96	0.22	<0.001	0.65
Tryptophan	81.5	1.84	<0.001	-1.92	0.21	<0.001	0.66
Valine	86.1	1.93	<0.001	-2.23	0.22	<0.001	0.70

Abbreviations: SE, standard error of the respective parameter estimate; SEAA, sum of essential amino acids; SNEAA, sum of nonessential amino acids. The threshold of significance was set at  $P \leq 0.05$ .

Data analysis was based on prececal crude protein and amino acid digestibility as affected by individual TIA levels of 45 differently processed expeller extracted soybean meals ranging from 0.3 to 23.6 mg/g. The prececal digestibility of crude protein and amino acids from different ESBMs was assessed according to the method of Rodehutscord et al. (2004).



**Figure 3.** Effect of trypsin inhibitor activity (TIA) in mg/g on the ratio of prececal (pc) digested methionine + cystine (Met + Cys) to lysine (Lys) in differentially processed expeller extracted soybean meals (ESBM) fed to broiler chickens. Data represents the ratio of prececal digestible Met + Cys relative to prececal digestible Lys in 45 differentially treated expeller extracted soybean meals (ESBM) as affected by individual TIA levels of said ESBMs ranging from 0.3 to 23.6 mg/g. The prececal digestibility of amino acids from different ESBMs was assessed according to the method of Rodehutsord et al. (2004). The threshold of significance was set at  $P \leq 0.05$ .

graded differences in dietary TIA ranging from 0.3 to 8.7 mg/g. In fact, using ESBM with TIA below 4.0 mg/g further improved feed efficiency by up to 16% until the end of the grower stage. In the present study, we observed a significant linear reduction in LW and TWG. Consistent with Hoffmann et al. (2019), this effect responded in a straight linear fashion indicating further improvement of production efficiency when decreasing TIA in full-fat soybeans below 4.0 mg/g. However, TFI and FCR were less impaired by TIA than LW and TWG, which seems to contradict earlier data at first glance. However, the experimental phase in our trials was set from d 15 to d 22 according to the experimental model of Rodehutsord et al. (2004) whereas Hoffmann et al. (2019) observed the zootechnical performance throughout the whole grower and finisher stages of fattening. This may explain why TFI and FCR were less responsive in the present study. Clarke and Wiseman (2007), who recorded weight gain and feed intake for only 3 d observed also decreased weight gain with rising TIA levels but no significant correlation to feed intake. They concluded that the recording phase of these parameters has to be at least 21 d to gain stable results.

For the estimation of feed protein quality, the determination of prececal digestibility is the most common method today. The method is based on the idea of measuring the “unabsorbed” AA directly in the terminal ileum and estimating the product specific digestibility by calculating the slope of increasing apparently digested amounts of AA at the terminal ileum over a graded increase in the intake of product-specific AA. The advantage of this method is that digesta are collected directly from the terminal ileum, which means there is negligible bias by microbial fermentation and no contamination with renal excretions and other materials. In addition, since the slope predominantly reflects the product-specific AA digestibility, there is no need to correct the data for the endogenously secreted amounts

of AA. This has been demonstrated by Rodehutsord et al. (2004), who observed a linear relationship between product-specific AA intake and quantitative AA flow at the terminal ileum. Kluth et al. (2005) defined the section of the intestine which needs to be collected. Regarding recent literature, many authors have used this or comparable approaches for estimating protein quality in different feedstuffs for broiler chickens (Short et al., 1999; Kluth and Rodehutsord, 2009; Foltyn et al., 2015; Rada et al., 2017).

In the present study, AA digestibility from individual ESBM products showed the same linear response to TIA as growth performance. The higher TIA in the respective soybean product, the lower the associated digestibility. TIA affected the apparent digestibility of every individual AA. The DC of arginine, glutamic acid, phenylalanine, and lysine were the least affected AA. In contrary to those, cystine and methionine showed a markedly increased responsiveness to TIA in ESBM. At 0.3 mg/g TIA arising from ESBM, 83.5% of cystine was apparently digested until the terminal ileum, whereas at TIA of 22.6 mg/g this value dropped to only 4.9%. This is in good agreement with the findings of Clarke and Wiseman (2007), who observed that DC of cysteine and methionine showed the strongest correlation to TIA. In their trials, DC varied from 71.8% at 1.9 mg/g to 34.5% at 14.8 mg/g.

One explanation for the generally negative impact of elevated dietary TIA levels on AA digestibility could be that TI bind irreversibly on the digestive enzymes trypsin and chymotrypsin and thereby impair protein digestion. This suicidal binding of TI was shown on several occasions, latest by Brugger et al. (2015) who deactivated trypsin inhibition arising from maize kernels by stepwise increase with porcine trypsin *in vitro*. Foltyn et al. (2015) measured the trypsin activity in the jejunum and discovered a reduction in enzyme activity when feeding raw full fat soybeans to chickens for 4 d. This period is comparable to that from the present study. Hence, it appears plausible that the activity of trypsin and chymotrypsin was negatively affected by KTI and BBI from ESBM of the present study. This conclusion may vary under experimental conditions comprising longer periods of treatment feeding, since the organism tends to adapt over time to the antinutritive stimulus by increased pancreatic secretion to satiate the binding sites of the inhibitor pool, thereby providing a surplus of active trypsin and chymotrypsin within the small intestinal lumen (Lyman and Lepkovski, 1957; Nitsan and Liener, 1976). Recent studies investigated the potential of an exogenous protease supplement to counteract the detrimental effect of purified soybean TI addition to soybean meal-based as well as nitrogen-free diets. Under nitrogen-free conditions, the purified TI increased the endogenous amino acid losses, which was not affected by the protease supplement (Aderibigbe et al., 2021). On the contrary, protease treatment compensated for diminishing effects of TI on performance and nutrient utilization under conditions of soybean meal feeding (Aderibigbe et al., 2020), although the



effect appeared to be independent of the TI thereby questioning a direct interaction with the enzyme. Given the proven suicidal interaction of TI with trypsin *in vitro* (Brugger et al., 2015), it would be interesting to repeat those studies with a protease more closely resembling (chymo)trypsin.

BBI from soybeans contain many disulfide bonds and are consequently rich in cysteine (Odani and Ike-naka, 1973). Accordingly, the higher amounts of sulfur-containing AA at the terminal ileum may also derive from an enrichment of BBI with further increase in the dietary proportion of ESBM with high TIA. Besides that, high endogenous losses of cysteine and methionine may further explain the high concentrations of sulfuric AA at the terminal ileum. Trypsin and chymotrypsin are rich in sulfur-containing AA (Nitsan and Liener, 1976). Peptide hormone CCK promotes the exocrine pancreatic synthesis and secretion of the digestive enzymes in response to increasing TIA in the small intestinal lumen. Fölsch et al. (1978) reported that continuous injections with CCK increased the pancreatic weight of rats. They could also prove a hypertrophy and hyperplasia of the gland. Furuse et al. (1990) observed in their survey a prompt increase of plasma CCK after 15 min, when feeding rats with soybean trypsin inhibitor. In the present study, we used similar ESBMs as Hoffmann et al. (2019). In their feeding trials, a linear increase of pancreatic weight with rising TIA levels in feed was observed at the end of the finishing phase. Therefore, it is plausible that there was also increased activity of the exocrine pancreas in the present study; although presumably to a lower extent given the short experimental duration. At this point, it is not possible to estimate which of the above discussed potential causes had the strongest effect on apparent prececal digestibility of sulfur-containing AAs. Hence, this issue must be addressed in follow-up studies.

Increasing TIA from ESBM did not just decrease prececal protein utilization from a quantitative perspective but also affected the qualitative value of the available protein. Specifically, the ratio of apparently digested sulfur-containing AAs to lysine decreased linearly from ~0.9 to ~0.5 with increasing TIA, which was due to the aforementioned disproportionately higher digestive depression for cysteine compared to other AA and especially lysine. In other terms, the degree of TIA reduced the biological value of the absorbed true protein. The biological value of feed protein reflects how close the spectrum of amino acids represents the ideal protein for growing organisms. The closer the AA spectrum in feed resembles the ideal protein at a given stage of the production cycle, the lesser total CP and true protein is necessary to meet the animals demand (van Milgen and Dourmad, 2015). Hence, an increasing dietary TIA by usage of poorly processed ESBM further increases the necessity to either increase total ESBM in complete feed or to balance the AA spectrum by adding crystalline AA. Both strategies increase feed costs and thus impair the economic success of poultry production.

In conclusion, varying dietary TIA from the usage of differentially processed ESBM variants negatively affected zootechnical performance and prececal AA digestibility in a straight linear fashion. Every single AA was affected but cysteine showed the lowest values over the whole range of applied TIA. To the best of our knowledge, this is the first study to show significant effects of TIA <4.0 mg/g in soy products on prececal AA digestion. The linearity of the observed effects questions the suitability of defining upper limits for TIA in broiler feed. In contrast, the KOH-soluble CP as well as the amounts of reactive lysine in respective diets did not affect the investigated parameters. This suggests that TIA could be decreased close to zero under the present conditions without promoting negative effects through excessive denaturation or formation of maillard products. In addition to the quantitative effects of TIA on feed protein utilization, it was further observed that the quality of the digestible spectrum of AA decreased significantly. This was evident by a marked change in the ratio of sulfur-containing AA to lysine. Overall, these findings suggest that the TIA of dietary components should be considered when determining their protein value *in vivo*. Future studies should identify strategies to deal with residual TIA in complete feed for broilers to stabilize performance and improve wellbeing. The described consequences for the assessment of feed protein value for broilers arising from gradual increase in dietary TIA could likely be translated to non-ruminating species in general, including pigs and humans.

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Data availability: Raw data and nonsignificant data can be obtained from the corresponding author upon request.

Author contributions: **S. Kuenz:** Formal analysis, Investigation, Writing – Original Draft, Visualization, Project administration **S. Thurner:** Conceptualization, Funding acquisition, Resources, Project administration, Supervision **D. Hoffmann:** Conceptualization, Investigation **K. Kraft:** Conceptualization, Investigation **M. Wiltafsky:** Investigation **K. Damme:** Resources **W. Windisch:** Conceptualization, Writing – Review & Editing, Supervision **D. Brugger:** Conceptualization, Writing – Review & Editing, Supervision.

## DISCLOSURES

The authors have nothing to disclose.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.101740.

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