



EFFECTS OF BROILER CHICKEN AGE AND DIETARY PROTEASE ON THE STANDARDISED ILEAL DIGESTIBILITY OF AMINO ACIDS IN SEEDS FROM TWO LUPIN SPECIES*

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Abstract

The extent to which lupin seeds can replace soybean meal in diets for broiler chickens is limited, and one of the reasons for this may be a lack of careful consideration given to the age-related amino acid availability differences in the feed formulation process. This study aimed to determine and compare standardised ileal digestibility (SID) coefficients of amino acids (AA) in raw seeds of the *Lupinus luteus* (YL) and the *Lupinus angustifolius* (NLL) cultivars as sole sources of AA in the diet of broiler chickens aged 14 or 28 days. An additional purpose was to check the influence of exogenous mono-component protease added to lupin-based assay diets on AA SID in birds at both ages. Each assay diet was offered to six replicate cages. On both sampling days, the contents from the lower half of the ileum were collected for determination of the apparent digestibility values. The SID coefficients were calculated using the age-appropriate basal ileal endogenous AA losses determined from birds fed a N-free diet. Results indicated a substantial advantage of YL over the NLL in SID of Met, Cys, His, Leu, Gly, Asp, Glu, Pro, Tyr and the average of total AA for 14-d-old chickens. With the exception of Cys and Tyr, there was no significant difference between these *Lupinus* species in the SID of AA in 28-d-old birds. The significant age-related differences within species were found only for the seeds of NLL, where the higher SID for Met, His, Ala, Pro, and the averages of total and indispensable AA were noted in birds aged 28 d. The protease increased SID of most AA from *L. luteus* in older broilers, whereas for *L. angustifolius* its efficacy was more pronounced in younger birds, giving the SID values similar to those determined at 28 d on an enzyme-unsupplemented diet. In conclusion, the SID coefficients of indispensable AA determined at 14 or 28 days of age for yellow lupin should not be applied to the precise formulation of starter-type feeds containing seeds of NLL.

Key words: lupins, amino acid, ileal digestibility, age, broiler, exogenous protease

The intensive production of monogastric meats and eggs in the European Union (EU) is highly dependent on imported soybean. In order to reduce this dependency and to mitigate the imbalance between the domestic supply and demand for plant proteins from the poultry and swine sectors, some EU countries, including Poland, are investing considerable efforts to promote and expand home-grown protein crops, especially grain legumes that are adapted to the local agro-climatic conditions (Watson et al., 2017; Jerzak and Śmiglak-Krajewska, 2020). Due to their higher protein content, compared with other grain legumes, lupin species are generally recognised as being the most suitable plant-based protein alternative to soybean meal (SBM) for poultry (Kozłowski et al., 2011; Jeroch et al., 2016). Of the three lupin species grown in Europe, narrow-leaved lupin (*Lupinus angustifolius* L.) and white lupin (*Lupinus albus* L.) are the main cultivated species in northern and southern Europe, respectively, while the production and breeding of yellow lupin (*Lupinus luteus* L.) is currently limited to Poland (Gresta

et al., 2017). Research on the utilisation of lupins in diets for growing broiler chickens reveals, however, the limited extent to which raw lupin seeds can replace SBM in these diets without loss in birds' productive performance. A combination of intrinsic (species, genotype, presence of anti-nutritional compounds) and extrinsic factors (eco-climatic conditions, agronomic management, etc.) has a complex impact on protein digestibility and potential bioavailability of amino acids (AA) in lupins and, therefore, their efficacy in substitution of SBM protein in diet of broilers (Abraham et al., 2019; Hejdysz et al., 2018, 2019).

In addition to the classical methods (soaking, heating, fine grinding), several advanced techniques, such as extrusion or controlled mixed culture fermentation, have been proposed for improving the digestibility of lupin AA through reduction the contents of phytates and oligosaccharides of the raffinose family in seeds (Rutkowski et al., 2016; Zaworska et al., 2017). However, the extra costs associated with applying these technologies call into

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question the financial viability of the use of seeds processed in such ways in poultry feed production. Another, less costly, means to increase the protein value of raw legume seeds is dietary addition of exogenous enzymes (Ravindran, 2013). In spite of the fact that beneficial effect of exogenous proteases on ileal AA digestibility is generally clear and their 'extra-proteinaceous' effects in broiler chickens (e.g. interactions with the digestibility of non-protein dietary components) have been recognised (Cowieson and Roos, 2016; Walk et al., 2018), there is hardly any information regarding the effect of modern microbial proteases on the digestibility of lupins' AA in broilers (Hejdysz et al., 2020).

It is widely accepted that the digestibility of AA in feeds for poultry is a sensitive gauge of the biological availability of these hydrolysed products from protein. Therefore, if the genetic potential of modern broilers is to be attained by feeding with properly balanced diets that ensure an adequate supply of AA for metabolism and growth functions, amassing information regarding their digestibility values from different feed ingredients is of importance. The methodology for determining AA digestibility at the terminal section of the small intestine by sacrificing birds and sampling the ileal contents has gained widespread approval as the most exact approach for assessing the value of dietary proteins for growing broilers (Columbus and De Lange, 2012). A broad consensus has emerged that apparent ileal digestibility (AID) coefficients should be corrected for age-appropriate basal endogenous AA losses, i.e. for the ileal flow of AA of non-dietary origin expected under any feeding situation (Barua et al., 2021 a), which yields more precise values of the standardised ileal digestibility (SID). A growing body of evidence has indicated that the AID and/or SID of particular AA in certain cereals and protein-rich feed materials increase with the advancing age of broiler chickens (Huang et al., 2005; Toghyani et al., 2015; Szczurek et al., 2020; Barua et al., 2021 b), and this suggests that credible ileal digestibility estimates for practical feed formulation should be determined using birds of an age close to the given feeding phase. The present authors are, however, not aware of any published data concerning ileal digestibilities of AA in lupin seeds determined in separate studies involving broilers at various ages or in experiments with chickens younger than three weeks. The extensive and well-described dataset that presently exists regarding ileal AA digestibility in feedstuffs important in broiler nutrition (Blok and Dekker, 2017) includes SID coefficients for lupins that are recalculated from the literature values on AID in birds aged 21–42 days and combined for different species and varietal types.

The current study was designed to compare standardised ileal AA digestibility estimates for raw (unprocessed apart from milling) seeds of yellow and narrow-leaved lupin cultivars fed to broiler chickens aged either 14 or 28 days. The basis for choosing these ages were from previously established differences in the apparent digestibility of AA in several common feed ingredients (Huang et al.,

2005). From a practical point of view, the reason behind this choice was also that, in multi-phase feeding programmes recommended by breeding companies, higher proportions of raw materials potentially burdened with a high amount of anti-nutritional compounds are usually included in grower and finisher broiler diets offered from 11 or 25 days of age, respectively. The response of a semi-purified lupin-based diets to the addition of mono-component serine protease included at recommended concentration was also examined.

Material and methods

The experiment was carried out at the experimental poultry farm of the National Research Institute of Animal Production (Balice, Poland) and complied with the EU guidelines for the treatment of animals, including the protection of animals used for scientific purposes (Directive 2010/63/EU) and the rules for the protection of farmed animals at the time of slaughter (Council Regulation No 1099/2009). Since no invasive procedures (i.e. causing pain, suffering or lasting harm) have been planned/performed on living broilers, and all were killed solely for the purpose of using their intestines, according to the Polish law no explicit approval from an ethics committee was required before undertaking the research.

Test lupin seeds and experimental diets

Certified seeds of the yellow lupin cultivar Mister (registered in 2003) and narrow-leaved lupin cultivar Sonet (registered in 1999) were selected for the experiment performed in 2018. Both lupin species/cultivars were grown at the Polish plant breeding station located in the middle west region of the country, and seeds harvested in 2015 were stored under frozen conditions until being used for the digestibility test. Before inclusion into the diets, the whole seeds were ground using a cutting mill (Pulverisette-15; Fritsch GmbH, Idar-Oberstein, Germany) fitted with a 2-mm sieve. In conformity with the basic principle of determining AA digestibility by the 'direct method', the lupins served as the sole source of AA in the semi-purified assay diets prepared in the form of a dry mash and formulated to contain similar amounts of crude protein (CP; $N \times 6.25$). The diets consisted of 52 and 64% of the yellow lupin (YL) and narrow-leaved lupin (NLL) seeds, respectively, to achieve CP level of approximately 20%. The remainder of the diets was composed mainly of maize starch (Sigma-Aldrich, S4126) and dextrose (Merisweet 200; Brenntag Poland Ltd., Kędzierzyn-Koźle, Poland) at a ratio 1:1. Both diets were supplemented with monocalcium phosphate, limestone, sodium bicarbonate and common salt, and identically fortified with vitamins and trace minerals by using a commercial premix. Rapeseed oil was added to reduce the dustiness, and chromium oxide (CAS 1308-38-9) was included as an indigestible marker at 0.5% of the diet. The ingredient composition of the diets is shown in Table 1.

Table 1. Ingredients and analysed crude protein and amino acid concentrations (g/kg as fed) of assay diets used for determination of the apparent ileal amino acid digestibility of lupin seeds

Item	Lupin-based assay diets ¹	
	YL	NLL
Test lupin	520	640
Maize starch	195	135
Dextrose	195	135
Refined rapeseed oil	40	40
Ca(H ₂ PO ₄) ₂	19	19
Limestone	16	16
NaCl	3	3
NaHCO ₃	2	2
Vitamin-trace mineral premix ²	5	5
Indigestible marker (Cr ₂ O ₃)	5	5
Analysed concentration		
crude protein	200.6	199.1
alanine (Ala)	6.3	6.9
arginine (Arg)	24.6	23.2
aspartic acid (Asp)	20.2	19.2
cysteine (Cys)	4.2	2.0
glutamic acid (Glu)	48.7	41.1
glycine (Gly)	7.7	8.2
histidine (His)	5.1	5.3
isoleucine (Ile)	7.4	7.7
leucine (Leu)	15.2	13.2
lysine (Lys)	10.4	9.4
methionine (Met)	1.6	1.5
phenylalanine (Phe)	8.0	7.9
proline (Pro)	7.2	8.3
serine (Ser)	9.7	9.5
threonine (Thr)	5.9	6.7
tyrosine (Tyr)	5.3	7.0
valine (Val)	6.9	7.6

¹The diets were offered without or with the microbial serine protease product added on top (200 mg/kg).

²Premix provided per kilogram of diet: retinol, 3600 mg; cholecalciferol, 125 mg; α -tocopherol, 45 mg; menadione, 3 mg; thiamine, 3.25 mg; riboflavin, 7.5 mg; pyridoxine, 5 mg; cobalamin, 0.03 mg; biotin 0.25 mg; folic acid, 1.5 mg; nicotinic acid, 45 mg; calcium pantothenate, 15 mg; choline chloride, 1000 mg; Fe, 67.5 mg; Zn, 75 mg; Mn, 100 mg; Cu, 17.5 mg; I, 1 mg; Se, 0.28 mg.

YL = yellow lupin cv. Mister; NLL = narrow-leaved lupin cv. Sonet.

The assay diets were fed either unsupplemented or supplemented with chymotrypsin-like microbial serine protease included on top of the recipe, resulting in two dietary treatments within a given lupin species. The protease used in the experiment is an active agent of a solid commercial preparation (Ronozyme® ProAct CT) produced by submerged fermentation of *Bacillus licheniformis* (DSM 19670) containing transcribed genes from *Nocardiopsis prasina*. The enzymatic activity for this protease is measured in PROT units, with 1 unit defined as the amount of enzyme that liberates 1 mmol para-ni-

troaniline (pNA) from 1 mM Suc-Ala-Ala-Pro-Phe-pNA substrate per minute at pH 9.0 and temperature 37°C (EFSA, 2009). Based on the guaranteed analysis of this product (75,000 PROT units/g), the protease was included at the recommended concentration of 15,000 PROT units/kg feed provided when the product was added at 200 mg/kg diet.

Birds and housing

One-day-old, feather-sexed Ross 308 chickens (480 birds, sex ratio 1 : 1) were distributed into slatted-floor metal cages (length $\times$ width $\times$ height = 120 $\times$ 65 $\times$ 75 cm) arranged in a three-tier battery in an environmentally controlled broiler house. Each cage was equipped with four nipple-cup drinkers and a trough feeder along the front of the cage. The male and female chicks were kept separately (20 birds/cage from 1 to 7 d and 8 birds/cage from 8 to 21 d) and fed a commercial maize-soybean meal diet in crumble form (220 g/kg CP) until they were assigned to the assay diets. Room temperature was maintained at 32°C during the first four days and gradually reduced to 21°C by the end of the experiment (d 28). The lighting was set to provide continuous fluorescent lighting until 5 days old and then switched to 18 h/6 h light/dark cycle. The birds had free access to feed and water throughout the feeding period and their health status was regularly monitored. At 8 d of age, 288 chickens of similar body weight (average weight across both sexes 262 $\pm$ 28 g) were selected and allotted to 24 clean cages (6 males and 6 females per cage) for the collection of ileal digesta at 14 days of age. At 22 d of age, the remaining 192 birds (average weight 1040 $\pm$ 42 g) were distributed to 24 cages each containing 4 males and 4 females for the collection of digesta at 28 d post-hatch. At each collection time, a single cage was treated as a replicate. Based on a completely randomised design, the cages were allotted to 4 experimental diets (two lupin-based diets containing no enzyme and two lupin-based diets supplemented with protease), with six replicate cages per diet.

Digesta sampling and processing

The birds from both age groups were fed the experimental diets (available *ad libitum*) for 5 consecutive days to allow for enough time for adaptation to the diets before sampling. During this period, four 13-d-old chicks offered the NLL-based diet died. At sampling (14 and 28 d), the chickens had free access to diets for at least 20 min before slaughter to ensure complete filling of the small intestine. All the chickens were stunned cage-wise using a head-only electrical method with a current of 250 mA and 400 Hz (STZ6 apparatus, Koma Ltd, Wilkanowo, Poland) and exsanguinated by a qualified person. Directly after the slaughtering process, the abdominal cavity was opened, and the ileum (portion of the small intestine extending from the vitelline diverticulum to approximately 4 cm proximal to the ileo-caecal junction) was removed. The contents of the lower half of the ileum were gently flushed with deionized water, pooled

within a cage (12, if none died, or 8 birds) and deep-frozen (-20°C).

Sample preparation and chemical analyses

Prior to chemical analyses, the pooled samples of ileal digesta were freeze-dried and ground in a mortar and pestle to a fine powder. Lupin seeds and assay diets were ground using a variable-speed rotor centrifugal mill (Pulverisette-14; Fritsch GmbH, Idar-Oberstein, Germany) to pass through a 0.50 mm diameter sieve for determination of dry matter (DM) and crude nutrients (protein and fibre), and a sieve of 0.08 mm diameter for AA analysis.

The proximate and amino acid analyses of the samples were performed according to AOAC (2000) methods. DM content was determined on the test seeds, assay diets and lyophilised digesta by drying the samples at 103°C for 5 h in a forced air oven (method 935.29). The lupin samples were analysed for total nitrogen (N) by the Kjeldahl procedures (method 955.04D), and crude (CF) and acid detergent (ADF) fibre using the conditions described for methods 978.10 and 973.18, respectively. The AA content of the seed, diet and ileal digesta samples was determined based on the method 982.30E (steps a and b). Before the analyses, all the samples were hydrolysed under nitrogen with 6 N HCl at 110°C for 24 h. For cysteine and methionine determination, the acid hydrolysis was preceded by overnight cold performic acid oxidation and neutralisation with hydrobromic acid. Tryptophan was not analysed. Amino acid concentrations in the hydrolysate were determined by HPLC after post column derivatization with ninhydrin reagent using an AAA-400 analyser (Ingos Ltd, Prague, Czech Republic) and ChromuLan ver. 0.8.1 software (PiKRON Ltd, Prague, Czech Republic) for peak integrations. The chromium contents in the assay diet and ileal digesta samples were measured in triplicates by the method of Saha and Gilbreath (1991) with an Avanta Sigma (GBC Scientific Equipment Pty Ltd, Braeside, Australia) atomic absorption spectrophotometer after wet mineralisation of the samples in $\text{HNO}_3/\text{HClO}_4$ (1 : 1.5) mixture. Non-starch polysaccharides (NSP), divided into total, total non-cellulosic and insoluble non-cellulosic forms, were determined by the enzymatic-chemical method to quantify constituent neutral sugars (Englyst et al., 1982; procedures A, B and C). These were separated and detected as alditol acetates by gas-liquid chromatography, with allose as the internal standard, using a Varian Star 3400 GC equipped with FID and a 30 m Rtx-225 capillary column. The uronic acid content of the samples was determined by colourimetric method at a 450 nm wavelength. Soluble NSP (S-NSP) concentrations were calculated as total non-cellulosic minus insoluble non-cellulosic NSP. The lupin samples were also analysed for the total content of alkaloids by a titration method proposed by Ruiz et al. (1977), with certain modifications. Chloroform extracts of finely ground seeds were titrated with 0.02 N *p*-toluenesulphonic acid (Sigma-Aldrich, T35920) in a combination with the indicator substance. Tetrabromophenol-

phthalein ethyl ester (Sigma-Aldrich, 86778) was used as the indicator solution instead of bromocresol green, and the results were expressed as g of alkaloids per kg of DM.

Calculations and statistical analysis

The coefficients of apparent ileal digestibility (AID) of amino acids were calculated from the formula, whereby the chromium and amino acid concentrations are expressed on DM basis:

$$\text{AID} = 1 - [(Cr \text{ in diet} \times AA \text{ in digesta}) / (Cr \text{ in digesta} \times AA \text{ in diet})]$$

Apparent digestibilities were transformed to the standardised coefficients (SID) by correcting the AID estimates for the ileal endogenous AA losses (IEAA) determined with the nitrogen-free diet feeding method for the respective age groups of chickens. The IEAA values obtained in a parallel experiment testing pea seeds have been reported and extensively discussed elsewhere (Szczurek and Świątkiewicz, 2020). The SID was calculated using the following formula:

$$\text{SID} = \text{AID} + (\text{IEAA, mg/kg DM intake} / \text{AA in diet, mg/kg DM})$$

Statistical evaluation of the results was conducted in accordance with the GLM procedure of Statistica software package (v. 13.3, TIBCO Software Inc., Palo Alto, CA, USA). Pooled digesta samples (one per dietary replicate) served as the experimental unit. Normality of distribution was checked using the Shapiro-Wilk test, and the Levene's test indicated that the variances for all comparisons were equal. Using an alpha level of 0.05 as a criterion for statistical significance, all SID data were analysed by three-way ANOVA with a $2 \times 2 \times 2$ factorial arrangement of treatments. The species of lupin (L): yellow lupin (YL) or narrow-leaved lupin (NLL), age of broilers (A): 14 or 28 d, and protease in the diet (E): absence or presence were tested for main effects and all interactions, and simple contrasts were additionally used to compare the interesting differences between seeds of YL and NLL in chickens of both age groups fed protease unsupplemented diets. Because the significant main effects, and $L \times A$, and $L \times E$ interactions were noted, the subsets of data for YL and NLL were analysed individually. A single degree of freedom contrast (SDFC) was applied to elucidate the overall effect of independent variables (chicken age and protease supplementation), and their level combinations (treatment) means within each of the two lupins tested. All contrasts were considered to be significant at $P < 0.05$.

Results

Chemical composition of lupin samples

The contents of the analysed crude nutrients, NSP, 17 amino acids and total alkaloids in test lupins are present-

ed in Table 2. The seeds of the YL cv. Mister contained much more crude protein and had higher total concentration of most amino acids, including the essential ones, in their dry matter than seeds of NLL cv. Sonet. The AA profiles showed that CP of yellow lupin exhibited considerably higher concentrations of Cys, Glu and Leu, but distinctly lower concentration of Ser than the CP of narrow-leaved lupin. In comparison with YL, the NLL seeds contained somewhat lower levels of crude fibre and ADF, but had markedly higher concentration of total and S-NSP and over twofold higher concentration of total alkaloids per unit of the dry weight of the sample.

Table 2. Analysed chemical composition¹ of test lupin seeds

Component	Lupin species	
	YL	NLL
Dry matter (DM, %)	89.1	90.2
Crude nutrients (g/kg DM)		
CP (N × 6.25)	433.9	345.4
CF	169.1	154.8
ADF	235.5	218.4
Non-starch polysaccharides (g/kg DM)		
total	295.2	324.6
insoluble ²	71.6	51.5
soluble ²	82.0	173.0
Amino acids (g/kg DM) ³		
Ala	13.7 (3.1)	11.9 (3.4)
Arg	53.1 (12.2)	40.2 (11.6)
Asp	43.6 (10.0)	33.3 (9.6)
Cys	9.0 (2.1)	3.7 (1.1)
Glu	107.1 (24.7)	71.2 (20.6)
Gly	16.7 (3.9)	14.2 (4.1)
His	11.1 (2.5)	9.1 (2.6)
Ile	16.0 (3.7)	13.3 (3.8)
Leu	32.8 (7.6)	22.8 (6.6)
Lys	22.4 (5.2)	16.2 (4.7)
Met	3.4 (0.8)	2.6 (0.8)
Phe	17.2 (4.0)	13.6 (3.9)
Pro	15.5 (3.6)	14.3 (4.1)
Ser	21.0 (4.8)	16.4 (7.5)
Thr	12.7 (2.9)	11.6 (3.4)
Tyr	11.5 (2.7)	12.2 (3.5)
Val	14.9 (3.4)	13.1 (3.8)
total amino acids ⁴	421.8 (97.1)	319.7 (95.1)
indispensable amino acids ⁵	183.5 (42.2)	142.5 (41.2)
Total alkaloids (g/kg DM)	0.22	0.47

¹Data are based on duplicate analyses.

²Determined/calculated as non-cellulosic NSP.

³Values in parentheses are amino acid contents expressed in g per 100 g of CP.

⁴Sum of all the amino acids listed above.

⁵Sum of Arg, His, Ile, Leu, Lys, Met, Phe, Thr and Val – the amino acids, along with tryptophan, traditionally classified as nutritionally essential for poultry.

CP = crude protein; CF = crude fibre; ADF = acid detergent fibre; YL = yellow lupin cv. Mister; NLL = narrow-leaved lupin cv. Sonet.

Digestibility of amino acids

Standardised ileal AA digestibility data for the test seeds, as affected by lupin species, broiler age and dietary protease, are presented in Table 3. Results from the three-way ANOVA showed that, regardless of birds' age and protease supplementation, the effect of species of lupin seeds was statistically significant ($P < 0.001$ to 0.045) for ten individual AA (Arg, Asp, Cys, Glu, Gly, His, Leu, Met, Ser and Tyr) and for the average digestibility of total AA (17AA), with differences in the SID values in favour of the YL cv. Mister. The main effect of protease on the SID was found to be significant for all AA evaluated ($P < 0.001$ to 0.018), with birds fed protease supplemented diets having an increased SID coefficient. The effect of age was significant for Ala, Gly, His, Ile, Leu, Met, Phe, Pro, Val and for the mean digestibility estimates of total AA (17AA) and nine indispensable AA (IAA), where 28-d-old broilers had higher SID ($P < 0.048$) compared with their 14-d-old counterparts. Despite the significant overall (main) effects of the explanatory variables, no significant three-way interaction was observed for any of the AA. However, the significant interactions were found between species of lupin and the age of broilers for His, Leu and IAA, and between lupin species and protease for Cys.

When values determined without supplementary protease were compared using simple contrasts (Table 4), there were marked ($P < 0.001$ to 0.025) differences in favour of the YL cv. Mister in the SID coefficients for 9 individual AA in younger chickens, including (among others) Met, Cys, His and Leu. At the age of 28 days, only the SID of cysteine and tyrosine was higher ($P < 0.05$) in the YL seeds compared with those of NLL cv. Sonet.

Further detailed analysis of the data using SDFC procedure indicated that in the case of YL (Table 5) there were no significant contrasts between treatment combinations in the SID of five AA (Glu, Leu, Phe, Ser and Thr). Of the 17 amino acids analysed, dietary protease generally improved the digestibility of 13 individual AA, irrespective of the age. The significant positive impact of an enzyme was noted in 28-d-old broilers for Ala, Arg, Asp, Cys, His, Ile, Lys, Tyr and Val (contrasts $P < 0.05$). In this approach to comparing the treatment combination means within the NLL seeds (Table 6), the SID coefficients for Ala, His, Met, Pro, and for all indispensable AA measured, were greater ($P < 0.05$) at 28 d than at 14 d, irrespective of the protease supplementation or lack thereof. Protease improved (contrasts $P < 0.05$) the SID of Pro, Tyr, Val, and the average digestibility of total AA and 9 IAA in birds aged 14 days. But except for tyrosine, there were only numerical differences, resulting from its addition, in the SID of the individual AA in 28-d-old broilers. The SID coefficients of most AA were lower for younger birds fed the assay diet without enzyme than for older broilers receiving the protease-supplemented diet (contrasts $P < 0.05$). The exceptions were arginine and cysteine, for which no age and no enzyme effects were observed.

Table 3. Species affiliation, age of broiler chickens and protease addition main effects on the standardised ileal amino acid digestibility of test lupin seeds

Amino acids	Main effect means						Pooled SEM	Source of variation (P-value)				
	Lupin species (L)		Chicken age (A)		Protease (E)			L	A	E	L × A	L × E ¹
	YL	NLL	14 d	28 d	–	+						
Ala	0.863	0.845	0.841	0.866	0.834	0.873	0.0064	0.109	0.027	0.001	0.067	0.945
Arg	0.954	0.941	0.944	0.950	0.942	0.953	0.0025	0.006	0.184	0.018	0.174	0.478
Asp	0.885	0.863	0.868	0.880	0.863	0.884	0.0039	0.002	0.077	0.001	0.121	0.626
Cys	0.873	0.808	0.837	0.844	0.825	0.855	0.0084	<.001	0.226	0.001	0.509	0.047
Glu	0.940	0.913	0.922	0.932	0.918	0.935	0.0036	<.001	0.076	0.008	0.141	0.338
Gly	0.895	0.862	0.871	0.886	0.865	0.892	0.0048	<.001	0.047	0.001	0.252	0.739
His	0.894	0.867	0.870	0.891	0.870	0.892	0.0046	0.001	0.006	0.004	0.044	0.694
Ile	0.884	0.870	0.868	0.886	0.862	0.891	0.0045	0.075	0.036	0.001	0.061	0.866
Leu	0.897	0.877	0.879	0.896	0.873	0.901	0.0048	0.020	0.048	0.002	0.042	0.599
Lys	0.880	0.867	0.864	0.883	0.858	0.889	0.0053	0.218	0.053	0.003	0.241	0.472
Met	0.847	0.820	0.809	0.858	0.815	0.852	0.0077	0.045	<.001	0.006	0.072	0.603
Phe	0.893	0.881	0.877	0.897	0.876	0.898	0.0045	0.144	0.027	0.011	0.377	0.905
Pro	0.886	0.868	0.865	0.889	0.861	0.893	0.0051	0.039	0.005	<.001	0.116	0.391
Ser	0.873	0.856	0.861	0.868	0.853	0.877	0.0039	0.023	0.291	0.006	0.444	0.949
Thr	0.843	0.832	0.828	0.847	0.820	0.856	0.0057	0.276	0.082	0.001	0.527	0.726
Tyr	0.927	0.862	0.890	0.899	0.881	0.908	0.0056	<.001	0.085	<.001	0.440	0.155
Val	0.850	0.848	0.839	0.860	0.830	0.868	0.0057	0.786	0.041	0.001	0.326	0.982
17AA	0.887	0.863	0.867	0.884	0.862	0.889	0.0044	0.001	0.013	<.001	0.156	0.878
IAA	0.883	0.867	0.864	0.885	0.861	0.889	0.0047	0.059	0.012	0.001	0.049	0.550

¹The remaining interactions (A × E and L × A × E) were not statistically significant ($P^{0.194}$) for any of the amino acids.

YL = yellow lupin cv. Mister; NLL = narrow-leaved lupin cv. Sonet; d = days; – = without enzyme; + = with enzyme; SEM = standard error of the mean; 17AA = average digestibility of all amino acids analysed; IAA = average digestibility of nine indispensable amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Val).

Table 4. Results of contrast testing between the standardised ileal amino acids digestibility of yellow (YL) and narrow-leaved (NLL) lupins determined without dietary supplementation of protease (n = 6) in chickens at 14 or 28 days of age

Amino acid	Contrasts YL vs. NLL			
	P-values for 14 d		P-values for 28 d	
Ala	0.065	4.1	0.748	–0.7
Arg	0.074	1.6	0.727	0.3
Asp	0.003	4.0	0.498	0.9
Cys	0.002	5.6	0.013	5.0
Glu	<.001	4.5	0.077	2.1
Gly	0.003	4.7	0.093	2.5
His	0.002	5.0	0.869	0.2
Ile	0.063	3.1	0.931	0.1
Leu	0.022	4.0	0.582	0.9
Lys	0.273	2.1	0.550	–1.2
Met	0.025	5.9	0.797	0.6
Phe	0.152	2.4	0.928	–0.2
Pro	0.006	4.8	0.926	0.1
Ser	0.132	2.2	0.445	1.1
Thr	0.221	2.5	0.827	0.5
Tyr	<.001	7.6	<.001	6.6
Val	0.335	1.9	0.500	–1.3
17AA	0.004	3.9	0.381	1.1
IAA	0.053	3.1	0.995	0.0

¹Between the SID value for YL and the SID value for NLL, in percentage points.

17AA = average digestibility of all amino acids analysed; IAA = average digestibility of nine indispensable amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Val).

Table 5. Standardised ileal digestibility of amino acids in the seeds of yellow lupin (cv. Mister) as affected by chicken age (14 or 28 days) and supplementation of assay diet with protease

Amino acids	Means for treatment combinations (n = 6)				Pooled SEM	P-values for main effects contrasts (n = 12)	
	14 d –	14 d +	28 d –	28 d +		Age	Protease
Ala	0.844 ab	0.876 ab	0.842 b	0.888 a	0.0084	0.775	0.022
Arg	0.947 ab	0.960 ab	0.946 b	0.962 a	0.0027	0.977	0.010
Asp	0.879 ab	0.889 ab	0.871 b	0.900 a	0.0042	0.845	0.017
Cys	0.847 b	0.890 a	0.857 b	0.897 a	0.0051	0.165	<.001
Glu	0.937	0.942	0.934	0.949	0.0033	0.786	0.132
Gly	0.881 b	0.902 ab	0.884 ab	0.911 a	0.0054	0.521	0.023
His	0.885 b	0.897 b	0.881 b	0.914 a	0.0052	0.472	0.025
Ile	0.870 b	0.891 ab	0.872 b	0.905 a	0.0058	0.451	0.021
Leu	0.885	0.903	0.886	0.915	0.0056	0.553	0.039
Lys	0.859 b	0.892 ab	0.862 b	0.904 a	0.0074	0.568	0.012
Met	0.816 b	0.849 ab	0.847 ab	0.875 a	0.0072	0.032	0.026
Phe	0.880	0.895	0.883	0.914	0.0063	0.346	0.068
Pro	0.873 b	0.888 ab	0.874 ab	0.908 a	0.0062	0.353	0.048
Ser	0.863	0.880	0.861	0.887	0.0054	0.842	0.060
Thr	0.825	0.849	0.829	0.870	0.0079	0.444	0.043
Tyr	0.916 c	0.933 ab	0.917 bc	0.942 a	0.0035	0.383	0.002
Val	0.829 b	0.861 ab	0.835 b	0.877 a	0.0078	0.454	0.017
17AA	0.873 b	0.894 ab	0.876 b	0.907 a	0.0052	0.411	0.010
IAA	0.866 b	0.889 ab	0.871 b	0.904 a	0.0058	0.348	0.018

d = days; – = without enzyme; + = with enzyme; SEM = standard error of the mean; 17AA = average digestibility of all amino acids analysed; IAA = average digestibility of nine indispensable amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Val).

a–c – different letters within rows indicate significant contrasts ($P < 0.05$) between treatment combination means.

Table 6. Standardised ileal digestibility of amino acids in the seeds of narrow-leaved lupin (cv. Sonet) as affected by chicken age (14 or 28 days) and supplementation of assay diet with protease

Amino acids	Means for treatment combinations (n = 6)				Pooled SEM	P-values for main effects contrasts (n = 12)	
	14 d –	14 d +	28 d –	28 d +		Age	Protease
Ala	0.803 c	0.841 bc	0.849 ab	0.886 a	0.0093	0.007	0.022
Arg	0.931	0.938	0.943	0.951	0.0037	0.114	0.305
Asp	0.839 b	0.866 ab	0.862 ab	0.887 a	0.0060	0.049	0.024
Cys	0.791	0.819	0.807	0.816	0.0077	0.443	0.138
Glu	0.892 b	0.916 ab	0.913 ab	0.932 a	0.0052	0.055	0.031
Gly	0.834 b	0.865 ab	0.859 ab	0.887 a	0.0064	0.041	0.013
His	0.835 b	0.866 ab	0.879 a	0.891 a	0.0067	0.005	0.074
Ile	0.839 b	0.874 ab	0.871 ab	0.895 a	0.0069	0.037	0.023
Leu	0.845 b	0.882 ab	0.877 ab	0.905 a	0.0074	0.044	0.021
Lys	0.838 b	0.866 ab	0.874 ab	0.892 a	0.0076	0.039	0.113
Met	0.757 b	0.813 ab	0.841 a	0.871 a	0.0134	0.004	0.062
Phe	0.856 b	0.880 ab	0.885 ab	0.903 a	0.0064	0.032	0.080
Pro	0.825 b	0.874 a	0.873 a	0.901 a	0.0078	0.004	0.003
Ser	0.841 b	0.857 ab	0.850 ab	0.875 a	0.0052	0.186	0.048
Thr	0.800 b	0.839 ab	0.824 ab	0.865 a	0.0081	0.090	0.001
Tyr	0.840 c	0.871 ab	0.851 bc	0.886 a	0.0054	0.142	0.001
Val	0.810 b	0.855 a	0.848 ab	0.878 a	0.0080	0.033	0.012
17AA	0.834 b	0.866 a	0.865 a	0.890 a	0.0063	0.012	0.010
IAA	0.835 b	0.868 a	0.871 a	0.895 a	0.0071	0.015	0.026

d = days; – = without enzyme; + = with enzyme; SEM = standard error of the mean; 17AA = average digestibility of all amino acids analysed; IAA = average digestibility of nine indispensable amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Val).

a–c – different letters within rows indicate significant contrasts ($P < 0.05$) between treatment combination means.

Discussion

The analysed proximate composition of the seeds (CP, CF, ADF) and the concentration of NSP reflected the expected results (YL>NLL and YL<NLL, respectively), being in line with many previous reports pertaining to older and modern Polish lupin cultivars of both species (Alloui et al., 1994; Gdala and Buraczewska, 1996; Sujak et al., 2006; Rutkowski et al., 2015; Sobotka et al., 2016; Książak et al., 2018; Hejdysz et al., 2018, 2019, 2020). With one exception (tyrosine), amino acid contents followed the pattern shown by the CP content. The total content of indispensable AA (g/kg DM) in the *L. luteus* was distinctly higher compared to the amount of these AA determined for the seeds of *L. angustifolius*. This is very consistent with the results of Sujak et al. (2006), Sobotka et al. (2016) and Hejdysz et al. (2019), who within one study have evaluated the same cultivars (Mister and Sonet) for concentration of AA. Moreover, a substantially greater Cys and Leu content in protein of YL seeds in this study is similar not only to what was observed by the above-mentioned authors, but also to the results of an extensive study on chemical composition of lupin cultivars placed on the market and cultivated in Poland before the year 1990 (Wasilewko and Buraczewska, 1999). Jeziorny et al. (2010) maintain that the total alkaloid (TA) content of cultivars of 'sweet' (low-alkaloid) lupins is below 0.26 g/kg DM and below 0.28 g/kg DM for *L. luteus* and *L. angustifolius*, respectively. Yet, from a study involving nine modern German cultivars of sweet NLL conventionally cultivated at one location for three consecutive years (2010–2012), Jansen et al. (2015) reported the average TA level to be 0.66 g/kg air-dry seeds (from 0.21 to 0.94 g/kg across growing seasons). Based on analyses of the material from harvest years 2011–2013, Kaczmarek et al. (2014, 2016) claim that the modern Polish narrow-leaved and yellow lupin genotypes are characterised by very low TA content – far below 0.05 g/kg DM for the ten different cultivars of both species, including Sonet and Mister. It is highly surprising, however, that in other works from the same group of authors the concentration of TA was reported to be around 10-fold higher, amounting to 0.27 or 0.41 g/kg DM for cv. Mister (Rutkowski et al., 2015; Hejdysz et al., 2018, 2019 [harvest year not mentioned]) and to 0.43 g/kg DM for cv. Sonet (Hejdysz et al., 2018, 2019). The present analytical results on TA content of the respective cultivars fit relatively well with the latter values and with those found for Sonet (0.33–0.38 g/kg DM) and Mister (0.26 g/kg DM) in the previous studies of Sujak et al. (2006), Sobotka et al. (2016) and Książak et al. (2018).

Our data obtained from feeding protease-supplemented assay diets point to a clear advantage of the YL over the NLL as a source of digestible (SID) AA for younger 14-d-old broiler chickens, including *inter alia* Met, Cys, His, Leu and Gly. This observation was not *surprising* in view of the considerable difference between the concentration of NSP in the test seeds. In this

context, it should be mentioned here that the calculated contents of S-NSP (on as-fed basis) originating from lupins in the assay diets amounted to 38 g/kg for YL-based diet and to 100 g/kg for NLL-based diet. It is well recognised that dietary soluble NSP interfere with nutrient digestion, absorption and the gut ecosystem in monogastric animals, and that their anti-nutritive effects in poultry are linked primarily to increased viscosity of digesta within the small intestine (Choct et al., 2010; McDonald et al., 2001; Konieczka and Smulikowska, 2018). The results reported by Hejdysz et al. (2018) indicated a positive linear relationship between the dietary level of S-NSP and viscosity of digesta in the ileum of broilers fed diets with increasing amount of NLL (cv. Sonet), which was not the case for the YL (cv. Mister). Moreover, these authors found a similar association between an increasing S-NSP level in the NLL-containing diets and excretion of sialic acid – a component of oligosaccharide chains of mucin – considered as an indirect indicator of endogenous AA losses (Lien et al., 2001). Although digesta viscosity was not measured in the present study, it appears that increased viscosity of intestinal contents was the main factor implicated in reducing the extent of protein digestion and absorption of AA from NLL seeds in younger chickens, and may explain in large part the differences in AID and, as a consequence, the SID estimates between the test lupins. However, this effect was not seen in older broilers and generally lower AA digestibilities for NLL were noted in birds aged 14 d than in those at d 28, which suggested that, with advancing age, chickens could withstand increased viscosity in the digesta induced by soluble NSP from *L. angustifolius*. This assumption seems reasonable given the results from broilers aged between 15 and 45 d obtained by Petersen et al. (1999), who indicated a strong linear reduction in the gut digesta viscosity with age in birds fed the barley-based diet for 5 d prior to measurements. According to Choct and Annison (1992), better resilience of older chickens to viscous NSP may be connected to higher activity or stability of the intestinal microbiota. In a review by Adedokun et al. (2011), it was concluded that the level of dietary fibre and its form (insoluble or soluble) may explain some of the increases in both basal (diet-independent) and diet-specific IEAA losses in monogastrics resulting, *inter alia*, from the augmented mucin output in the digesta. With regard to the adverse effects of soluble NSP from cereals on the AID of dietary AA in broilers, it has been demonstrated that such effects are mainly due to direct suppression of AA absorption and an increase in specific EAA flow (Angkanaporn et al., 1994). Considering that Pro, Asp, Glu and Gly are one of the most abundant amino acids in mucin (Lien et al., 2001; Adedokun et al., 2007), it appears that lower digestibility coefficients for these AA in NLL at 14 d, relative to the YL, could be linked to their greater proportions in the total pool of endogenous AA in ileal contents. However, it cannot be ruled out that the adverse effect of a higher level of total alkaloids in NLL compared to YL seeds, which roughly corresponded

to 0.27 vs. 0.10 g of TA/kg assay diet, was also a factor contributing to the lower AA digestibility in 14-d-old chickens. Study conducted by Olkowski et al. (2001) revealed that feeding young broilers with 'sweet' NLL (400 g/kg diet) containing less than 0.10 g of TA per kg seeds led to a pronounced alteration of systemic physiological functions, evidenced by a more than doubled content of cytochrome P-450 in the liver microsomes, and resulted in remarkably depressed feed consumption.

To date, there are no other experimental data on the SID of amino acids in lupins determined with broiler chickens. Furthermore, only a few feeding trials were devoted to the measurement of the apparent ileal digestibility of AA in lupins (the Australian NLL samples) as the only source of dietary protein, two of which have been performed on birds aged 6 weeks (Ravindran et al., 2002, 2005), and in one experiment chickens at 4 weeks post-hatch have been used (Toghyani et al., 2020). In studies of Ravindran et al. (2002, 2005) the AID coefficient of IAA averaged 0.820 (from 0.760 for Thr to 0.900 for Arg), which is an intermediate value relative to 0.806 and 0.846 found in this study (data not presented here) for *L. angustifolius* with birds at 14 and 28 d, respectively. And the current AID data for NLL at 28 d (e.g. 0.796 for Met, 0.859 for Lys, 0.945 for Arg; not shown) are in high accordance with the values determined at distal ileum by Toghyani et al. (2020), but unfortunately the authors did not provide any analytical results regarding the concentration of anti-nutritional factors in test seeds or diet. The AID of amino acids in lupins has been extensively evaluated in several studies with a 'substitution method' exploiting the calculated difference in digestibility between the basal (maize-soybean meal) and assay diet (a mixture of the basal and the test lupin seeds at a ratio of 75:25 or 70:30). Specifically, in an experiment testing three New Zealand cultivars of NLL (with the S-NSP content between 29 and 32 g/kg DM) Nalle et al. (2011) found no significant differences in AA digestibility among lupin variants in 35-d-old broilers and obtained the mean AID coefficients of 9 indispensable AA (0.840–0.850) that are very close to values determined in the present study for a cultivar of *L. luteus* Mister with birds at both ages (0.844 on average, 0.939–0.940 for Arg and 0.779–0.810 for Met; data not shown). In two independent studies on chickens aged 3 weeks Kaczmarek and co-workers investigated the AID of AA in seeds of four modern NLL and three modern YL cultivars bred in Poland, including Sonet and Mister. Although the NSP had not been analysed in their experiments, the authors found a significant negative relationship between viscosity of water extracts (WEV) of ground lupin samples and the AID of some amino acids in NLL and of the majority of AA in yellow lupins, despite the relatively small differences between WEV values (1.2–1.8 cP). For narrow-leaved variants, they reported the AID of eight essential AA measured (Thr, Val, Ile, Leu, Phe, His, Lys, Arg) averaging 0.811, with a value of 0.803 for Sonet cv. (Kaczmarek et al., 2014). Similarly, the mean AID value of IAA for *L. lu-*

teus differed among three cultivars from 0.782 (Mister) to 0.837 (Lord), with a digestibility range between 0.734 (Thr) and 0.892 (Arg) (Kaczmarek et al., 2016). In light of the above, it is surprising that in a study lately published Hejdysz et al. (2020) reported an exceptionally low AID coefficient, being between 0.586 and 0.702, for as many as five out of 15 AA (the values for Met and Cys were not disclosed) in seeds of both the YL and NLL modern Polish cultivars (Perkoz and Kalif) tested by the 'substitution method' with 3-wk-old broilers. The AID values averaged for IAA analysed in that study were also low (0.745 and 0.747, respectively), unfortunately, the reasons for this have not been elucidated by the authors. When considering the potential applications of the present results from protease-unsupplemented diets, it is worth stressing that the SID of AA determined at d 28 in both lupin species were, except for Lys in YL and Cys in NLL, generally higher than the 'universal' SID coefficients elaborated by the CVB researchers (Blok and Dekker, 2017) for lupins named as 'all qualities'. And since their appraisal has been based on considerably greater basal IEAA loss values than those determined in our investigation, this mismatch could be fully ascribed to lower average apparent ileal digestibility coefficients calculated from published studies and subsequently recomputed into SID values.

Only a few extensive studies with broilers have been conducted that were aimed at determining the ileal (apparent) digestibilities of AA in diets composed solely of plant-based ingredients in the presence or absence of the same mono-component protease as that used in this experiment, without admixture of other enzymes. These studies concluded that responses to this specific protease could be divergently affected by the choice of feed materials and their proportions, depending on the dose applied (Borda-Molina et al., 2019). When supplemented at the recommended level (15,000 PROT units/kg feed), this protease improved Met and Glu digestibility determined with 35-d-old-broilers for a diet containing 29% wheat, 30% maize, 24% SBM and 8% rapeseed meal (Rada et al., 2016), and increased significantly the AID of Arg, Lys, Thr, Val, Asp, Cys and Ser in 3-wk-old birds fed diet comprising 65% maize and 23% SBM (Angel et al., 2011). Barekatin et al. (2013), in turn, in broilers at this age, noted an increase in AID only for methionine when this protease was added to a maize-based diet containing 30% of sorghum distillers' dried grains with solubles (sDDGS).

In an already mentioned study (Hejdysz et al., 2020), the influence of a commercial product containing viable spores of *B. licheniformis* and its protease (600,000 units/g) on the AID of AA in YL and NLL has been examined using the assay diets composed of 30% of lupin seeds and 70% of basal feed mixture formulated from maize, SBM and fish meal as the main feedstuffs. A positive effect of this enzyme product (0.5 g/kg feed) has only been found for the YL (diet) where the AID values of Lys, Ser and Val were improved. It is curious, how-

ever, that the authors reported a concurrent highly significant increase in the ileal digestibility of CP from seeds of both lupin species after the addition of this preparation. In the experiment herein, protease supplementation improved the SID of most AA from yellow lupin in older broilers, whereas for narrow-leaved lupin its efficacy was more pronounced in younger birds, giving the SID values similar to those determined at 28 d on an enzyme-un-supplemented assay diet. There appears to be no obvious explanation for the discordance between these observations, although there is a possibility that this difference in the α -galactoside content between the yellow and NLL seeds might have contributed to the diverse efficacy of an enzyme product used in this experiment, depending on chickens' age. It is suggested that the presence of these water-soluble oligosaccharides causes an increase in the osmolarity in the small intestine and affects the flow behaviour of nutrients and enzymes in the intestinal lumen (Martínez-Villaluenga et al., 2008). Unfortunately, the current study did not determine the concentration of α -galactosides in the test lupins. On the other hand, some authors postulate that the beneficial effects of exogenous proteases may be related, in part, to the removal of structural proteins in the cell wall, allowing greater microbial hydrolysis of NSP (Colombatto and Beauchemin, 2009). Barekatin et al. (2013), who used the same protease at a dose similar to that in the present study, demonstrated significant drop in viscosity of the ileal contents of young chickens fed with high dietary level of sDDGS. Data from the broiler study reported by Olukosi et al. (2015) provide some support for the hypothesis of an indirect effect of subtilisin-like serine protease in the disappearance of insoluble and soluble NSP fractions of the maize-SBM-based diet. What is more, the same authors observed that protease reduced the post-caecal flow of glucose and galactose from the resistant oligosaccharide fraction. But it is clear that further studies on this topic are needed to ascertain, *inter alia*, whether these specific benefits of exogenous proteases addition can be achieved on the NSP-rich diets that contain a substantial proportion of lupins.

Conclusions

Data from the present study pointed to an unequal ability of broiler chickens at 14 d of age to digest protein and absorb AA from the tested lupins, and the presence of non-starch polysaccharides, particularly in soluble form, could substantially contribute to such differences. The current results also suggest that, with the exception of cysteine and tyrosine, there is no significant difference between these two lupin species in the digestibility of AA in 28-d-old chickens. Nevertheless, in terms of concentrations of the standardised ileal digestible AA in lupin protein (g/100 g of CP) as an indication of the quantities of amino acids that are available to the broilers aged 4 weeks, the relative similarity of the tested lupins is not apparent. Generally, considering age-related differences in the SID values of IAA between species, the lupins test-

ed can be ranked as follows: day 14, NLL (Sonet) was less than YL (Mister); day 28, NLL (Sonet) was equal to YL (Mister), and differences that occurred within species were appreciable only for the seeds of NLL. Therefore, when a digestible AA system is used, the SID coefficients of indispensable AA determined at 14 or 28 days of age for yellow lupin are not applicable to the formulation of starter-type feed mixtures containing seeds of NLL. The mono-component protease selected for applying in this study had a beneficial effect on the SID of AA, and the magnitude of improvement in individual AA digestibility to this product varied depending on lupin species (cultivar) and bird age. However, it appears that this type of microbial protease may be successfully used to increase the overall quantity of nutritionally essential AA available for absorption from narrow-leaved lupins fed to younger birds, and from yellow lupins when fed to the physiologically more mature broilers aged 28 days.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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