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Lignosulfonate properties and reaction conditions enhance precipitation and affect ensuing quality of proteins from green biomass juice for monogastric animal feed

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ABSTRACT

This is the first study investigating the effects of lignosulfonate (LS) properties and reaction conditions on the precipitation of proteins from green biomass juice for producing monogastric animal feed. Both the improvement in the protein precipitation yield and the resulting impact on the quality of the protein pellets were investigated with new methods and approaches. High molecular weight (MW) LS gave the highest precipitation yield, 21–33% higher than traditional heat and isoelectric methods. Electrostatic interaction between LS and soluble proteins was observed to be higher in high MW LS than others, and likely improved the precipitation. A response surface design experiment showed that pH was more important than LS concentration for precipitation. The optimum conditions predicted by the model improved precipitation by up to 61% compared to traditional methods. Using high MW LS at near optimum conditions resulted in more LS being retained in the pellet, with a final LS content of up to 60 g/kg dry matter (DM). The improved precipitation yield came at the expense of lower crude protein (CP) content of the pellet. Despite reduced pellet protein purity, the total CP recovery from the juice was improved by up to 800 g/kg DM. The *in vitro* protein digestibility (IVPD) and amino acid composition were not significantly affected by the presence of LS. Using high MW LS at optimum reaction conditions can significantly improve protein precipitation yield from green biomass juice without compromising digestibility. However, finding the compromise between yield and protein purity is necessary to improve feasibility.

Abbreviations: ANOVA, analysis of variance; ATR-FTIR, attenuated total reflectance-Fourier transform infrared; BSA, bovine serum albumin; CCD, central composite design; CP, crude protein; DM, dry matter; IVPD, *in vitro* protein digestibility; LS, lignosulfonate; MW, molecular weight; Org. S, organic sulfur content; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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1. Introduction

The growing meat consumption has led to higher demand for feed protein, especially in the soybean-dependent pork and poultry sectors (Kim et al., 2019). Given the focus on climate impact of the food production, the use of local alternative protein sources as compared to importing soybean is seen as a way to reduce greenhouse gas emissions from land use conversion and transport. Protein extraction from green biomass (e.g. grass and legumes) is under development in the biorefining effort to produce mainly feed proteins and biogas, but potentially also food proteins, fibers, bio-based chemicals and materials (Kamm et al., 2016; Santamaría-Fernández and Lübeck, 2020). This scheme can reduce environmental impact compared to the soybean import (Djomo et al., 2020).

Extracting proteins from green biomass involves fractionation using e.g. a twin-screw press, separating it into juice and pulp fractions (Damborg et al., 2020; Edwards et al., 1975). Recovery of the soluble proteins is challenging due to the dilute nature of the juice (Kumar et al., 2021). Therefore, to obtain a concentrated protein product, precipitation has traditionally been performed either using heat or by adjusting pH to reach the isoelectric point of proteins (Damborg et al., 2020; Edwards et al., 1975). Inclusion of protein pellets from green biomass juice in feed showed good digestibility and effect in tests using rats (Stødkilde et al., 2018), broilers (Stødkilde et al., 2020) and pigs (Stødkilde et al., 2021). As the protein yield determines the sustainability of the process (Djomo et al., 2020), efforts to improve protein precipitation are crucial. Recently, we found that adding lignosulfonate (LS) under acidic conditions enhanced protein precipitation yield compared to the traditional methods (la Cour et al., 2019). However, the factors affecting LS-assisted precipitation of proteins from green biomass juice and the impact on the feed quality are still largely unknown.

Lignosulfonate, a by-product from the sulfite pulping process, is a water-soluble polyelectrolyte with negatively charged sulfonic acid groups (Xu and Ferdosian, 2017). LS has been used to recover proteins from whey (Cerbulis, 1978) as well as wastewater from egg (Xu et al., 2001) and dairy (Kurup et al., 2019) processing plants. While acidic pH and optimal dose are important (Cerbulis, 1978; Kurup et al., 2019), the effect of the properties of LS on protein precipitation has not been investigated. In that respect, knowledge of molecular-level interaction between LS and proteins is still lacking. The fate of LS after protein precipitation is another important aspect, but it is still unknown and barely reported. Although LS in general is considered safe for animals and commonly used in feed, e.g. as pellet binder, the maximum safety recommendation for LS content as feed additive is 10 g/kg dry matter (DM) for chickens and pigs (EFSA FEEDAP Panel et al., 2020). Our previous work estimated the LS content in the produced protein pellets to be as high as 250 g/kg DM after precipitation using a high LS dose at low pH (la Cour et al., 2019), but a rigorous study has not been reported.

The present work aims to determine the effect of LS properties and reaction conditions on the yield and resulting pellet quality of the LS-assisted precipitation of proteins from green biomass juice. Ten LS with different properties, i.e. molecular weight (MW), counter-ion, organic sulfur content (Org. S), and biomass origin were tested. A simple assay was developed to assess the interaction of LS with soluble proteins from green biomass juice. Additionally, the effect of pH and LS concentration on protein precipitation was studied using response surface methodology. The idea was to optimize protein precipitation with reasonably minimum LS dose to prevent high LS buildup in the pellet. Finally, assessment of the protein pellet quality, i.e. residual LS in the pellet, amino acid composition and *in vitro* protein digestibility were examined in relation to the aforementioned factors.

2. Materials and methods

2.1. Lignosulfonate samples

Ten LS samples with different properties were used in this study, with their details indicated in Table 1 as provided by the company (Borregaard Lignotech, Sarpsborg, Norway).

2.2. Plant materials

A mixture of 520 g/kg ryegrass (*Lolium* sp.), 300 g/kg × *Festulolium*, 90 g/kg red clover (*Trifolium pratense*) and 90 g/kg white clover (*Trifolium repens*) from the ForageMax 40 (FM40) seed mix (DLF, Store Heddinge, Denmark) was grown on a DLF experimental field near Store Heddinge, Denmark. The biomass was harvested June 2019 (second cut) and was stored frozen at − 20 °C until use. The DM and crude protein (CP) contents of the biomass were 250 g/kg and 103 g/kg DM, respectively.

Table 1
Properties of the lignosulfonate samples used in the study.

LS[a]	Biomass origin	Counter-ion	MW[a]	Org. S[a]
DP 4531	Softwood	Na^+	Low	= DP 4532
DP 4532	Softwood	Na^+	High	= DP 4531
DP 4533	Softwood	Na^+	>DP 4534	Low
DP 4534	Softwood	Na^+	<DP 4533	High
DP 4535	Softwood	Ca^{2+}	Low	= DP 4540
DP 4540	Softwood	Ca^{2+}	High	= DP 4535
DP 4537	Softwood	Ca^{2+}	= DP 4536	Low
DP 4536	Softwood	Ca^{2+}	= DP 4537	High
DP 4538	Hardwood	Na^+	N/A[a]	N/A
DP 4539	Hardwood	Ca^{2+}	N/A	N/A

[a] LS, lignosulfonate; MW, molecular weight; Org. S, organic sulfur content; N/A, Not available.

2.3. Precipitation of protein

The overall procedure was adapted from the previous studies (Damborg et al., 2020; la Cour et al., 2019). Frozen biomass samples (FM40) were thawed and juiced using a twin-screw press (Angel Juicer 8500 S) with standard housing (Angel Juicer, Busan, South Korea) at 4 °C. Prior to the precipitation experiments, the juice was clarified by centrifuging at 1000 *g* for 2 min at 4 °C. Protein precipitation was performed in triplicates at 30 ml volume in 50 ml polypropylene tubes for each one of the 10 LS samples. In the preliminary test, 1.0 g/L LS was added to the clarified juice, equivalent to dosage of 160 g LS/kg DM CP. Then the clarified juice was precipitated by adjusting the pH to 4 using 6 M HCl. The LS-assisted precipitation yields were compared to the isoelectric (pH adjustment without LS) and heat precipitation methods. The heat precipitation was performed by incubating the 50 ml tubes containing the juice in a water bath until the internal temperature reached 80 °C. After 30 s, the tubes were cooled on ice. After the precipitation, all samples were kept overnight at 4 °C, then centrifuged at 402 *g* for 10 min at 4 °C. The supernatant was decanted, and the resulting pellet was weighed and freeze-dried (Martin Christ Alpha 1–4, Osterode am Harz, Germany).

2.4. Interaction between LS and soluble proteins

To investigate LS-protein interaction, soluble proteins were isolated from the juice (from FM40 biomass and was not clarified) using a modified protocol (Kobbi et al., 2017). The juice was mixed with 0.1 M NaOH (1:5 wt ratio) at 4 °C and was centrifuged at 10,000 *g* for 20 min at 4 °C. The supernatant was dialyzed against demineralized water using Spectra/Por 2 dialysis membrane (MW cut off 12–14 kDa, Repligen Corp., Waltham, MA, USA) overnight at 4 °C. After dialysis, the remaining solution was freeze-dried (Martin Christ Alpha 1–4, Osterode am Harz, Germany), solubilized in 9 g/L NaCl and filtered using 0.22 μm filter. The resulting protein content (1.11 g/L) was determined using Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin (BSA) as standard.

The isolated soluble proteins were then used to study LS-protein interaction using a new method based on performing the Bradford protein assay on the mixture of the proteins with LS, compared to the proteins without LS. The protein solution was diluted using 100 mM Na-acetate buffer pH 5 with or without addition of LS (1.2 g LS/g protein). After 15 min incubation at room temperature, the Bradford protein assay (Bradford, 1976) was performed on the two mixtures. The results were presented as percentage of reduction of absorbance detected in the protein-LS mixture compared to the protein only control. For the soluble proteins without LS, blank correction was performed using the absorbance obtained from the Bradford reagent and buffer. For the soluble proteins with LS, blank correction was performed using the absorbance obtained from the mixture of Bradford reagent and the respective LS in the same buffer.

2.5. Response surface design

A central composite design (CCD) experiment was used to study the effect of pH and LS concentration on protein precipitation. The pH values of 3–5 and concentrations of 0.5–1.5 g/L LS (dose of 80–240 g LS/kg DM CP) were used in the precipitation of the clarified juice using LS DP 4532, which performed the best after the screening study. The center point (pH 4 and 1.0 g/L LS) was done in quadruplicate while the axial and factorial points were done in duplicate (Supplementary material, Table S1). The design was made, and the results on the amount of precipitated crude protein (g/kg DM) and residual LS in the pellet relative to initial amount dispensed (g/kg), were analyzed (model fitted to standard least squares) using JMP Pro 15 (Jones and Sall, 2011; SAS Institute Inc, 2019a).

2.6. Analysis

The N and S contents of the freeze-dried pellets obtained after precipitation of the clarified juice were determined by elemental analysis performed using Vario Macro and Vario Pyro Cube elemental analyzers (Elementar Analysensysteme GmbH, Hanau, Germany). Acetanilide and sulfanilamide were used as standards. Gravimetric measurement was performed on the samples before and after precipitation and freeze-drying to calculate N and S contents on a DM basis. The CP precipitation yield was calculated based on the amount of N recovered before and after precipitation. The residual LS in the pellet was estimated as the difference between the S content of the pellet after LS treatment and the S content of the pellet obtained after isoelectric precipitation at the corresponding pH value. The difference was then used to calculate the approximate amount of LS based on the S content of each LS type.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the supernatant after precipitation was performed using the RunBlue system with RunBlue TEO-Tricine SDS Gels 12% (Expedeon, Cambridgeshire, UK).

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy was performed on selected freeze-dried samples using a Nicolet 6700 FT-IR equipped with a Pike Technologies GladiATR diamond spectrometer (Thermo Scientific, Waltham, MA, USA) operating at a working temperature of 25 °C. Clarified juice, pellets obtained after heat and isoelectric (pH 4) precipitation, pellets obtained after LS-assisted precipitation at center and near optimal point of CCD, as well as the LS DP 4532 were used in the analysis. Five spectra were obtained per sample type. The spectral range was 4000–400 cm^{-1}. The spectral resolution was 4.0 cm^{-1} and each spectrum was based on 64 scans (128 for the background). The IR spectra were normalized using Standard Normal Variate (Barnes et al., 1989).

The *in vitro* Protein Digestibility (IVPD) of selected pellets was evaluated using the Megazyme assay kit (K-PDCAAS, Megazyme International Ireland Ltd., Bray, Ireland). Digestion for each sample was performed in duplicate and amino acids in each digested sample were further analyzed in duplicate. IVPD was expressed as amino acid concentration measured at the end of digestion relative to the pellet, with correction based on the amino acids Proline, Lysine, Histidine, Arginine contents according to the established

procedure from Megazyme.

Amino acids composition of the selected pellets was determined using a recently established high-throughput procedure in our group using an acid hydrolysis method which can analyze most amino acids in a single run (Dahl-Lassen et al., 2018).

Statistical analysis of the results was performed by executing one-way analysis of variance (ANOVA) using JMP Pro 15 (Jones and Sall, 2011; SAS Institute Inc, 2019b) with post hoc analysis using Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$.

3. Results

3.1. The effect of LS properties on protein precipitation

The preliminary test revealed that addition of all LS samples significantly improved the precipitation of CP compared to the heat and isoelectric methods (Fig. 1). The high MW LS DP 4532 gave the significantly ($P < 0.05$) highest improvement of CP precipitated, 33% and 21% higher than the heat and isoelectric precipitation methods, respectively. The softwood LS DP 4539 gave the lowest improvement, with the corresponding values of 13% and 7% for heat and isoelectric precipitation methods, respectively (Fig. 1).

Statistical analysis indicated that MW, Org. S and biomass origin had significant effect ($P < 0.05$) on the protein precipitation yield, but the type of counter-ion had no significant effect (Table 2). LS with high MW (DP 4532 and DP 4540) had 7–11% higher precipitation yields than LS with low MW (DP 4531 and DP 4535) (Fig. 1, Table 2). Compared to the other LS and the traditional methods, SDS-PAGE analysis also showed better precipitation of especially the low MW proteins (<18 kDa) by the high MW LS DP 4532 (Supplementary material, Fig. S1). Low Org. S LS performed significantly better ($P < 0.05$) than high Org. S LS (1–6% higher), while softwood LS performed significantly better ($P < 0.05$) than hardwood LS (1–13% higher) (Fig. 1, Table 2).

3.2. Interaction of LS with soluble proteins

The concept of the newly developed assay is to incubate the LS with the isolated soluble proteins from the juice prior to subjecting them to Bradford protein assay. The positively-charged amino acids on the proteins will interact with the negatively-charged sulfonic groups on the LS, therefore becoming less available for interaction with negatively-charged sulfonic groups on the Coomassie reagent. This results in reduced absorbance of the mixture following the Bradford protein assay compared to the soluble proteins control without LS. The reduction of absorbance indicates the extent of LS-protein interaction, with higher reduction indicates higher extent of electrostatic interaction. This is a fast and simple assay that can be used to study the electrostatic interaction between proteins and polyelectrolytes. High MW LS DP 4532 and DP 4540, which gave among the highest precipitation yield (Fig. 1), had the highest interaction (Fig. 2). The LS DP 4538 and DP 4539 from softwood, which gave the lowest precipitation yield (Fig. 1), had the lowest interaction (Fig. 2). A similar trend was observed when mixing BSA with LS, again pointing towards higher LS-protein interaction of high MW LS than low MW LS (Supplementary material, Fig. S2). Furthermore, high MW LS constantly had higher LS-protein interaction relative to the low MW LS across different LS dosages, the highest being DP 4532 (Supplementary material, Fig. S3).

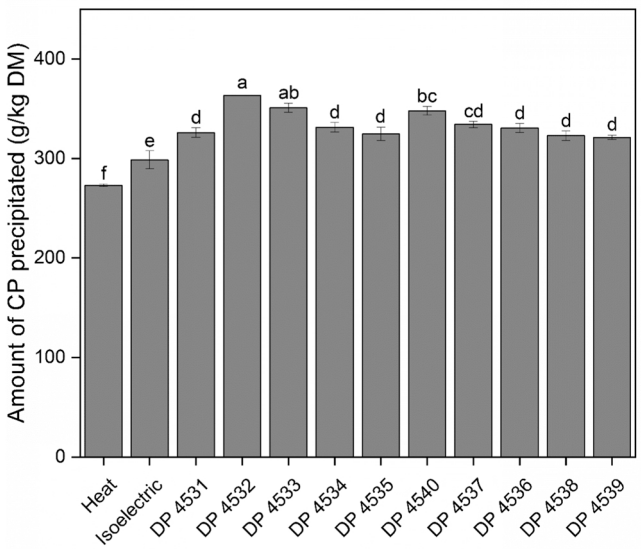

Fig. 1. Amount of crude protein (CP) (g/kg dry matter (DM)) in the clarified juice precipitated by the various lignosulfonate (LS) samples at pH 4 and 1.0 g/L compared to heat and isoelectric (pH 4) precipitation methods. Data points represent average and standard deviation of three replicates (n = 3). Different letters indicate significant statistical difference based on one way analysis of variance (ANOVA, $P < 0.05$).

Table 2
The t-test results on the effect of lignosulfonate properties on crude protein precipitation (g/kg dry matter (DM)).

Parameter	High/Softwood/Na^+		Low/Hardwood/Ca^{2+}		t-test for equality of means		
	Mean	SD[a]	Mean	SD	t	DF[a]	P
MW[a]	356	8.96	325	5.20	7.17	8.01	< 0.001*
Org. S[a]	331	4.17	342	9.82	-2.66	6.74	0.034*
Biomass	339	13.8	322	3.43	5.31	27.9	< 0.001*
Counter-ion	339	16.9	332	10.2	1.43	23.4	0.165

* significant at $\alpha = 0.05$ (two-sided).
[a] SD, standard deviation; DF, degree of freedom; MW, molecular weight; Org. S, organic sulfur content.

3.3. The effect of pH and LS concentration on protein precipitation

DP 4532, which performed best (Fig. 1) and had the highest LS-protein interaction (Fig. 2) was used to study the effect of reaction conditions. The pH in itself had a significant effect on precipitation yield ($P < 0.05$). The highest increase (75%) occurred when going from pH 5 to 4, while the increase was only 14% going from pH 4 to 3 (Fig. 3). Yet, in all cases, the addition of LS still gave significantly higher precipitation than without LS ($P < 0.05$). The improvement of precipitation by LS compared to the isoelectric method was 26% at pH 5, 27% at pH 4 and 20% at pH 3. However, the precipitation at pH 5 was still lower than heat precipitation even with LS added (Fig. 3).

The CCD experiment showed that pH had more significant effect on protein precipitation yield than LS concentration (Fig. 4, Supplementary material, Table S2). Within the tested pH range and reasonably low dosages, the optimum condition was pH 3.25 and 1.5 g/L LS (dose of 240 g LS/kg DM CP) (Fig. 4). Model-predicted optimum conditions resulted in about 21% improvement (439 g/kg DM of CP precipitated) compared to the best result in the preliminary study (Fig. 1). Compared to heat precipitation, the improvement corresponded to 61%, while compared to isoelectric precipitation, the improvement was either 28% or 47% at pH 3 or 4, respectively. This vast improvement can also be seen in the SDS-PAGE analysis, where at ca. pH 3 the addition of LS yielded negligible signal (Supplementary material, Fig. S4), indicating that the majority of proteins may have been removed via precipitation.

3.4. Assessment of protein pellet quality

The LS content in the pellet was assessed to estimate the effect of LS on the pellet quality. Based on the preliminary test, the high MW LS had significantly ($P < 0.05$) the highest amount of residual LS in the pellet, with DP 4540 > DP 4532 (Fig. 5). In the case of DP 4540, the amount corresponded to 623 g/kg of residual LS compared to the amount dispensed initially. The lowest one, DP 4539, had only 172 g/kg of residual LS relative to the initial amount (Fig. 5). Changing the parameter to the LS content of the pellet, the highest LS content was 57.5 g LS/kg DM pellet and the lowest LS content was 20 g LS/kg DM pellet in the case of DP 4540 and DP 4539, respectively (Fig. 5).

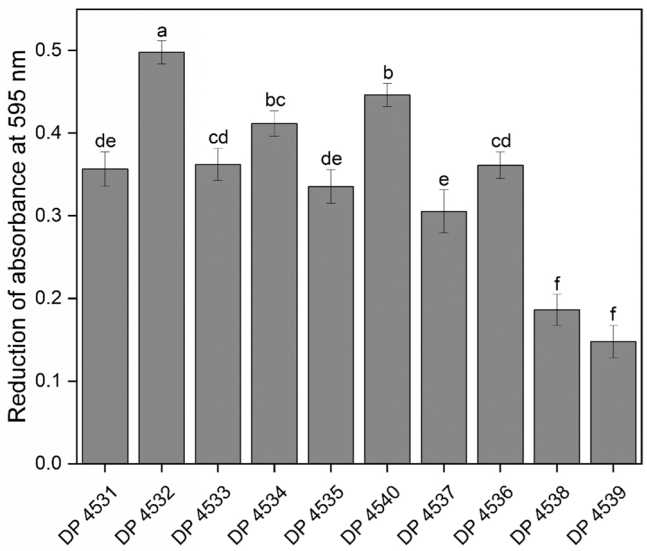

Fig. 2. Relative absorbance reduction of mixture of isolated proteins from green biomass juice with the various lignosulfonate (LS) samples (1.2 g LS/g protein) following Bradford protein assay at 595 nm compared to control without LS. Data points represent average and standard deviation of three replicates (n = 3). Different letters indicate significant statistical difference based on one way analysis of variance (ANOVA, $P < 0.05$).

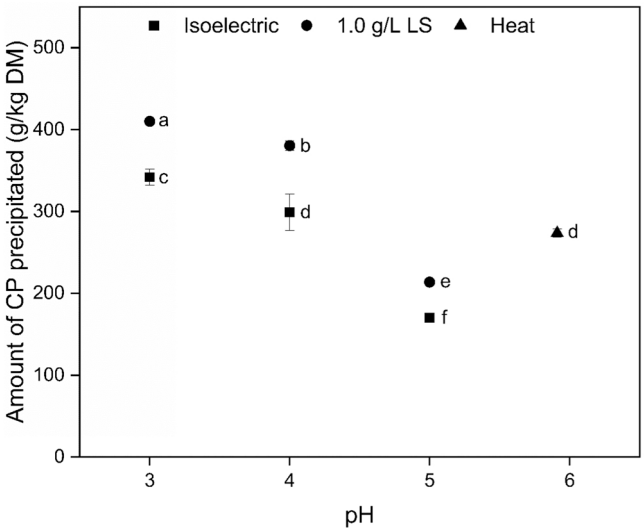

Fig. 3. Amount of crude protein (CP) (g/kg dry matter (DM)) in the clarified juice precipitated by 1.0 g/L lignosulfonate (LS) DP 4532 at various pH values compared to the heat and isoelectric precipitation methods. The data for heat precipitation was placed at pH value of 5.9, the initial pH of the clarified juice. Data points represent average and standard deviation of two replicates (n = 2). Different letters indicate significant statistical difference based on one way analysis of variance (ANOVA, $P < 0.05$).

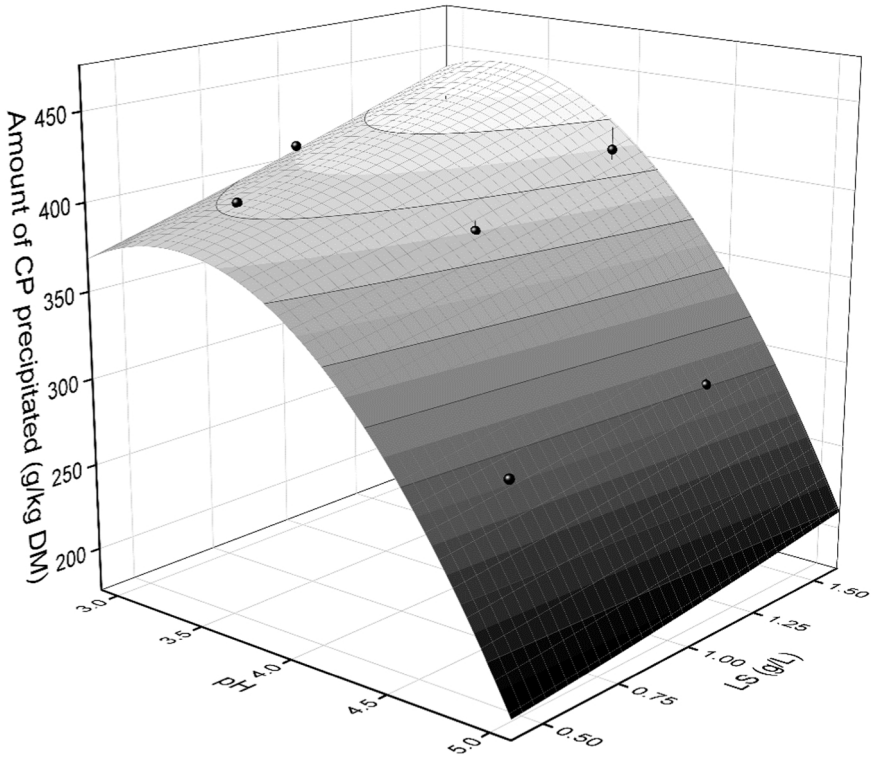

Fig. 4. Response surface model for the amount of crude protein (CP) (g/kg dry matter (DM)) in the clarified juice precipitated at different pH (3−5) and lignosulfonate (LS) DP 4532 concentration (0.5–1.5 g/L LS or dose of 80–240 g LS/kg DM CP). The optimal point was indicated at pH of 3.25 and 1.5 g/L LS by the model, corresponding to 439 g/kg DM CP precipitated. Actual experimental data points are presented as spheres with lines representing standard deviation.

The CCD experiment with DP 4532 also showed that pH had a pronounced effect on the residual LS in the pellet (Fig. 6, Supplementary material, Table S3). Most residual LS was retained in the pellet at pH 3.47 and 0.5 g/L LS (dose of 80 g LS/kg DM CP) with

603 g/kg of residual LS relative to the initial amount dispensed (Fig. 6). This corresponded to a 27% increase in CP precipitation yield compared to the previous result in the screening study (Fig. 1) and 40–50 g LS/kg DM pellet. Therefore, contrary to CP precipitation, when lowering the initial LS concentration, relatively more LS will end up in the pellet.

The purity of the protein pellet and total CP recovery were also crucial in evaluating the precipitation of proteins from green biomass juice. As a result of clarification of the juice, approximately 661 g/kg DM CP was already recovered from the juice as green protein fraction, containing also insoluble fiber. Precipitation of protein from the clarified juice with heat or addition of acid increased the total CP recovery to 708–768 g/kg DM juice (Table 3). Addition of LS further increased the total CP recovery up to 797 g/kg DM juice at reaction conditions near the model-predicted optimal conditions (Table 3). However, the crude protein content in the pellet tended to decrease along with the addition of acid and LS relative to heat and isoelectric precipitation methods. The lowest purity was accordingly found at the best conditions obtained in the preliminary study (DP 4532 in Fig. 1) and the CCD experiment (pH 3.29 – 1.35 g/L LS in Fig. 4), respectively (Table 3). ATR-FTIR spectra indicated that there were likely non-protein compounds precipitated, as indicated by increased signals at 1145 and 1713 cm^{-1} after precipitation with LS (Supplementary material, Fig. S5).

The digestibility of the resulting protein pellet was nevertheless the ultimate indication of feed quality and was measured as *in vitro* protein digestibility (IVPD). The IVPD was slightly reduced from 0.87 in the heat and isoelectric methods to 0.82–0.85 in the treatments with LS (Fig. 7). The middle point (pH 4 – 1.0 g/L LS) and the point closest to the optimum conditions (pH 3.29 −1.35 g/L LS) in the CCD experiment (Supplementary material, Table S1, Fig. 4) were chosen for comparison. However, it is important to note that while the difference in IVPD was 2–6% between the highest and the lowest samples, the difference in their crude protein content was 30–45% (Fig. 7). This indicated that despite the large decrease in crude protein content for pellets with LS compared to other LS-free methods, the proteins were still rather highly digestible with overall IVPD over 0.8. Additionally, the amino acids composition (g/kg DM total amino acid) was relatively unchanged in the treatments and was comparable to soybean meal (Table 4).

4. Discussion

4.1. The effect of LS properties on protein precipitation yield and LS-protein interaction

Comparing LS DP 4532, DP 4533 and DP 4540 that yielded the highest CP precipitation (Fig. 1), it became apparent that all had high MW profiles. DP 4532 and DP 4540 were assigned as high MW, while DP 4533 had higher MW than DP 4534 (Table 1). Thus, it is possible that the improvement seen (Fig. 1, Table 2) was in fact due to high MW alone, which was not found previously (la Cour et al., 2019). Perceptibly, a high MW polyelectrolyte will have higher interaction with the ligand due to more accessible contact points, increasing the chance for adsorption and subsequently precipitation. This is supported by the results from the new assay developed as a part of this study, showing that the high MW LS had higher LS-protein interaction than the low MW LS (Fig. 2, Supplementary material, Fig. S3).

In the assay, the higher reduction of absorbance after Bradford assay indicates that there were less positively-charged amino acid residues on the protein surface that were bound to the negatively-charged sulfonic groups on the Coomassie reagent. This is likely due to the prior binding of the aforementioned amino acid residues with the negatively-charged sulfonic groups of LS. Yet DP 4533 did not have a high LS-protein interaction, in fact lower than DP 4534 (Fig. 2). Possibly, this is due to its charge density as DP 4534 had high Org. S (Table 1). Based on this observation, it could be surmised that, even though charge density correlated with LS-protein interaction, it did not by itself affect precipitation. A combination of charge density and MW is likely needed to promote more precipitation.

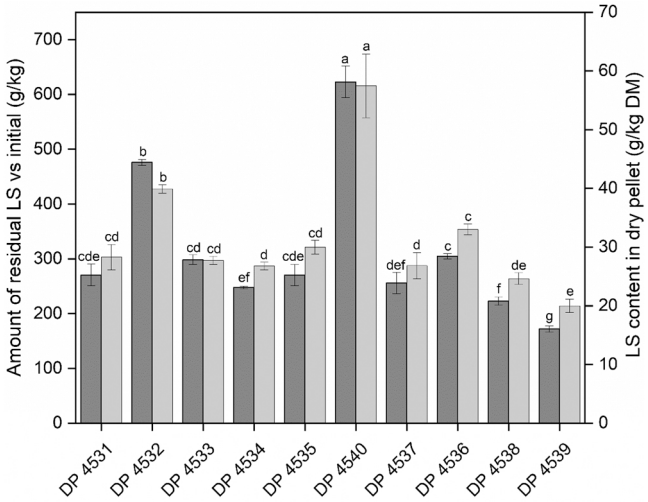

Fig. 5. Estimation of amount of residual lignosulfonate (LS) in the dry pellet compared to the initial amount dispensed (left axis, g/kg) and the content of LS in the resulting protein pellet (right axis, g/kg dry matter (DM)). Data points represent average and standard deviation of three replicates (n = 3). Different letters indicate significant statistical difference based on one way analysis of variance (ANOVA, $P < 0.05$).

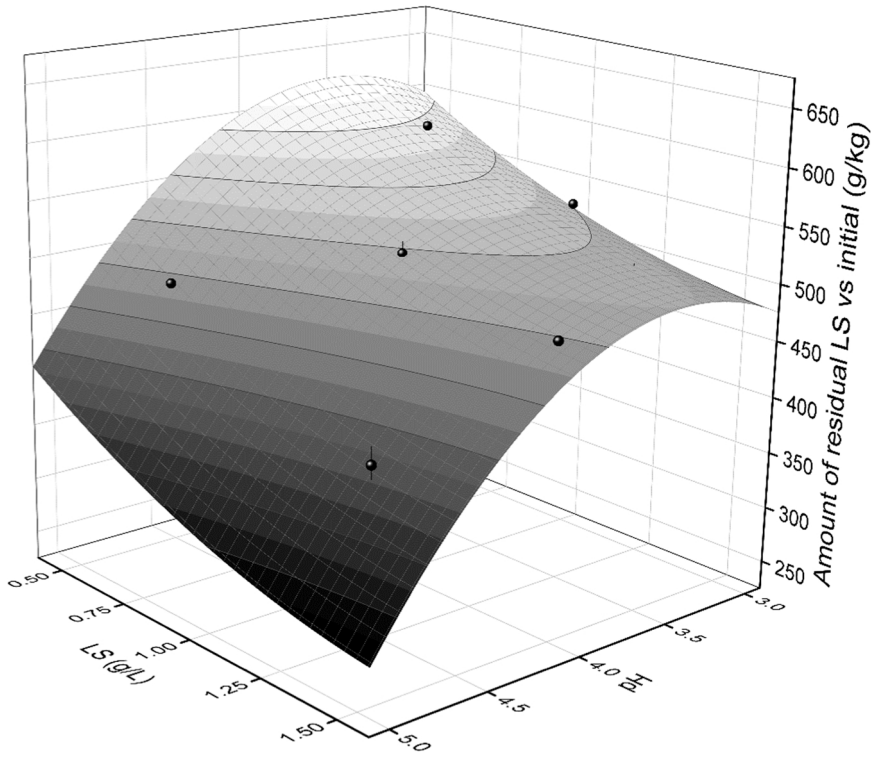

Fig. 6. Response surface model for percentage of initial LS retained in the pellet at different pH (3–5) and lignosulfonate (LS) DP 4532 concentration (0.5–1.5 g/L LS or dose of 80–240 g LS/kg dry matter (DM) crude protein (CP)). The optimal point was indicated at pH of 3.47 and 0.5 g/L LS by the model, corresponding to 603 g/kg LS retained in the pellet relative to the initial amount dispensed. Actual experimental data points are presented as spheres with lines representing standard deviation.

Table 3
Amount of crude protein (CP) in pellet precipitated from clarified juice and total crude protein recovery from the whole juice.

Treatment	CP content (g/kg DM pellet)[1]	Total CP recovery (g/kg DM)[1]	Treatment	CP content (g/kg DM pellet)[1]	Total CP recovery (g/kg DM)[1]
Clarification	175^{kl}	661^{h}	DP 4536[3]	230^{cde}	780^{abc}
Heat	287^{a}	744^{ef}	DP 4538[3]	231^{cde}	777^{abdc}
pH 5[2]	253^{b}	708^{g}	DP 4539[3]	243^{bc}	777^{abcde}
pH 4[2]	253^{b}	753^{bcdef}	pH 5 – 1.0 g/L[4]	220^{defg}	723^{fg}
pH 3[2]	255^{b}	768^{abcde}	pH 4.71 – 0.65 g/L[4]	214^{efgh}	747^{def}
DP 4531[3]	220^{defg}	778^{abcd}	pH 4.71 – 1.35 g/L[4]	201^{gh}	750^{cdef}
DP 4532[3]	196^{hj}	791^{a}	pH 4 – 0.5 g/L[4]	196^{hij}	773^{abcde}
DP 4533[3]	210^{fgh}	787^{a}	pH 4 – 1.0 g/L[4]	176^{ikl}	782^{ab}
DP 4534[3]	230^{cde}	780^{abc}	pH 4 – 1.5 g/L[4]	161^{l}	791^{a}
DP 4535[3]	235^{bcd}	778^{abcd}	pH 3.29 – 0.65 g/L[4]	191^{hijk}	788^{ab}
DP 4540[3]	210^{fgh}	786^{ab}	pH 3.29 – 1.35 g/L[4]	158^{l}	797^{a}
DP 4537[3]	228^{cdef}	781^{abc}	pH 3 – 1.0 g/L[4]	176^{ijkl}	792^{a}

[1] Data points represent average and standard deviation of two to four replicates (n = 2–4) presented on a dry matter (DM) basis. Different letters in each column indicate significant difference ($P < 0.05$).
[2] Isoelectric precipitation at pH 3–5 (Fig. 3).
[3] Preliminary study using DP 4531-DP 4540 at pH 4 and 1.0 g/L lignosulfonate (LS) (Fig. 1).
[4] Data points from the central composite design (CCD) experiment using DP 4532 with pH values and LS concentration.

Previous studies using LS as dispersant indeed showed that high MW correlated to better performance and interaction with the ligand (Yang et al., 2008; Zhou et al., 2006).

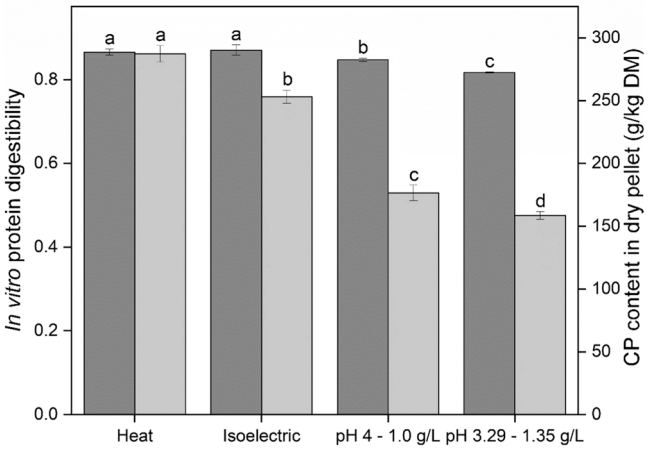

Fig. 7. *In vitro* protein digestibility (IVPD) of selected samples, i.e. the heat and isoelectric (pH 4) methods and two treatments using lignosulfonate (LS) DP 4532 at different pH and LS concentrations (left axis). The pH 4–1.0 g/L data point is the center point and the pH 3.29–1.35 g/L data point is the near optimum point in the central composite design (CCD) experiment. The purity of protein is presented as crude protein (CP) content in dry pellet (right axis, g/kg dry matter (DM)) for comparison. Data points represent average and standard deviation of two to four replicates (n = 2–4). Different letters indicate significant statistical difference based on one way analysis of variance (ANOVA, $P < 0.05$).

Table 4
Amino acids composition and content of the selected pellet samples.

Amino acid	Amino acids composition (g/kg DM total amino acid)[1]					Amino acids content (g/kg DM sample)[1]				
	Heat	Isoelectric pH 4	pH 4 – 1.0 g/L[2]	pH 3.29 – 1.35 g/L[2]	Soybean meal[3]	Heat	Isoelectric pH 4	pH 4 – 1.0 g/L[2]	pH 3.29 – 1.35 g/L[2]	Soybean meal[3]
Alanine	76.8^{a}	79.3^{a}	78.5^{a}	79.3^{a}	46.5	20.3^{a}	17.9^{ab}	12.8^{bc}	11.0^{c}	20.9
Arginine	66.0^{a}	62.6^{a}	60.9^{a}	60.6^{a}	75.9	17.4^{a}	14.1^{b}	9.9^{c}	8.4^{c}	34.1
Glycine	57.1^{a}	56.7^{a}	55.4^{a}	57.0^{a}	45.4	15.1^{a}	12.8^{b}	9.0^{c}	7.9^{d}	20.4
Histidine	23.2^{a}	24.3^{a}	24.5^{a}	22.7^{a}	30.3	6.1^{a}	5.5^{a}	4.0^{b}	3.1^{b}	13.6
Isoleucine	65.6^{a}	62.8^{a}	64.5^{a}	62.2^{a}	52.1	17.3^{a}	14.1^{b}	10.5^{c}	8.6^{d}	23.4
Leucine	104^{a}	105^{a}	99.3^{a}	98.3^{a}	83.7	27.0^{a}	24.0^{b}	16.1^{c}	13.6^{d}	37.6
Lysine	71.4^{a}	72.1^{a}	72.4^{a}	71.1^{a}	67.9	18.8^{a}	16.2^{b}	11.8^{c}	9.9^{d}	30.5
Phenylalanine	67.3^{a}	66.5^{a}	65.8^{a}	65.5^{a}	56.1	17.8^{a}	15.0^{b}	10.7^{c}	9.1^{d}	25.2
Proline	49.9^{a}	47.6^{a}	48.7^{a}	47.6^{a}	51.7	13.2^{a}	10.7^{b}	7.9^{c}	6.6^{c}	23.2
Serine	49.0^{bc}	48.2^{c}	52.9^{a}	52.0^{ab}	48.3	12.9^{a}	10.9^{b}	8.6^{c}	7.2^{d}	21.7
Threonine	52.6^{a}	55.1^{a}	55.8^{a}	56.7^{a}	40.1	13.9^{a}	12.4^{b}	9.1^{c}	7.9^{d}	18.0
Tyrosine	48.1^{a}	47.6^{a}	46.9^{a}	45.5^{a}	38.5	12.7^{a}	10.7^{b}	7.6^{c}	6.3^{d}	17.3
Valine	74.0^{a}	73.3^{a}	73.6^{a}	75.6^{a}	53.5	19.5^{a}	16.5^{b}	12.0^{c}	10.5^{d}	24.0
Aspartic acid	88.6^{a}	89.6^{a}	92.8^{a}	92.8^{a}	119	23.4^{a}	20.2^{b}	15.1^{c}	12.9^{c}	53.3
Glutamic acid	107^{a}	110^{a}	108^{a}	113^{a}	191	28.0^{a}	25.0^{b}	18.0^{c}	16.0^{d}	85.8

[1] Data points represent average of two replicates (n = 2) presented on a dry matter (DM) basis. Different letters in each row indicate significant difference ($P < 0.05$). Total amino acid content is based only on the measured amino acids.
[2] Data points from the central composite design (CCD) experiment using DP 4532 with pH values and lignosulfonate (LS) concentration.
[3] Data obtained from literature (Lagos and Stein, 2017) and recalculated to include only the listed amino acids.

4.2. The effect of pH and LS concentration on protein precipitation

The significant effect of pH value was somewhat expected since polyelectrolyte-based protein precipitation relies on electrostatic interaction. A critical pH where LS will be negatively charged and the proteins will be positively charged will maximize the electrostatic interaction and facilitate precipitation (Chen and Berg, 1993; Kurup et al., 2019). The effect of pH by itself was sufficient to promote precipitation, especially at low values (Fig. 3). In the case of protein precipitation from alfalfa juice, the extent of precipitation was also higher at pH 3.5 compared to 4.5 (Miller et al., 1975). However, the isoelectric point of RuBisCO (the most abundant plant protein) was reported to be around 4.4–4.9 (Udenigwe et al., 2017). This indicates that while a pH value of 5 might be enough to initiate protein precipitation, lower pH values of e.g. 4–4.5 as used in both isoelectric and microbial fermentation (Santamaría-Fernández and Lübeck, 2020) are needed to achieve substantial precipitation. Concerning LS concentration, the shape of the response surface graph (Fig. 4) and the statistical analysis (Supplementary material, Table S2) suggested that the optimum concentration could be outside of the tested range. However, higher concentrations of LS can potentially affect the quality of the pellet due to up-concentration of LS, as will be discussed in the next section. Furthermore, dosing a high amount of LS can make the process less feasible economically.

4.3. Assessment of protein pellet quality

The residual LS in the pellet (Fig. 5) correlated with precipitation yield (Fig. 1) and LS-protein interaction (Fig. 2), i.e. it was highest in high MW LS (DP 4532 and 4540). As the interaction improved precipitation, it is likely that LS was co-precipitated. A compromise in choosing LS property is therefore needed to prevent its buildup in the pellet while still improving the yield. Concerning reaction conditions, the CCD experiment (Fig. 6, Supplementary material, Table S3) also indicated that even lower dosages can cause more LS to be retained in the pellet. However, a very low dosage is not realistic to improve precipitation, and at higher dosages the LS content in the pellet will be lower. For comparison, at conditions near optimum CP precipitation (pH 3.29 and 1.35 g/L LS DP 4532), the residual LS was 585 g/kg vs initial amount, which equals to 42.7 g/kg DM LS content in the pellet.

Overall, at the tested conditions, LS did not constitute a substantial fraction of the pellet compared to a previous estimation of 250 g/kg DM when using high dosage (640 g LS/kg DM CP) at pH 3 (la Cour et al., 2019). Therefore, using a reasonably low LS concentration, a high buildup of LS in the pellet can be prevented while still optimizing precipitation by using a low pH. Nevertheless, since the maximum limit of LS content in animal feed is 10 g/kg DM (EFSA FEEDAP Panel et al., 2020), the limit in the pellet was still surpassed for all tested experimental conditions. Therefore, further improvement in the method or additional processing steps will be needed to reduce the LS content to within the recommended limit. Nevertheless, the minor presence of remaining LS can also benefit the pelleting process of the feed by improving the pellet characteristics and production rate due to the role of LS as binder, which is the current use of LS as feed additive (Saleh et al., 2021).

Concerning the reduced protein purity compared to LS-free methods, the LS content of the pellet could be one of the plausible causes. However, as shown here (Fig. 5), the LS content in the pellet was too low to cause the difference. Only 15–45 mg LS was used in each experiment, whereas the amount of dry pellet after precipitation doubled from ca. 200 mg after heat/isoelectric method up to ca. 500 mg with LS addition. Therefore, a likely explanation is that there were also non-protein contaminants precipitated in the pellet. This was reported in the isoelectric precipitation of proteins from alfalfa juice (Miller et al., 1975), but was not consistently found in the more recent work using various green biomass juice (Damborg et al., 2020). ATR-FTIR spectra showed increased signals at 1145 and 1713 cm^{-1} on the pellets after LS-assisted precipitation (Supplementary material, Fig. S5). The peak at 1145 cm^{-1} could indicate cellulose or lignin (Lupoi et al., 2015). Although the LS sample also absorbed at this band, the peak also appeared in the pellet after isoelectric precipitation. The peak at 1713 cm^{-1} was assigned to C═O stretch of carbohydrate origin (Faix, 1991). Since this peak was absent in the LS spectrum, it is speculated to originate from hemicellulose and/or pectin, major constituents of grass cell wall (Vogel, 2008). Thus, the data indicate that the contaminants present in the pellets likely originate from the clarified juice and co-precipitated along with the proteins without substantially affecting their digestibility.

Nevertheless, the IVPD results (Fig. 7) were similar to the feeding test of protein concentrate from white clover performed using rats which also found protein digestibility over 0.8 (Stødkilde et al., 2018). Furthermore, the similar amino acid composition across treatments indicated that the nutritional value was not compromised. Comparable essential amino acids composition (g/kg DM total amino acid) in the pellets to the soybean meal (Table 4) also highlighted their potential suitability as monogastric animal feed. However, it needs to be also noted that the amino acid content in the pellet (g/kg DM sample) will be reduced (Table 4) as the CP content is reduced with increasing high yield treatment (Table 3). This results in a large discrepancy with soybean meal economically in terms of the amount needed to provide a similar quantity of amino acids (Table 4). Altogether, this preliminary work shows that despite the increase in yield, further effort will be needed to increase the purity of the protein in the pellet in order to improve the feasibility of the process. Additionally, dedicated feeding trials on animals will need to be conducted thoroughly in order to ultimately assess the efficacy, quality and safety of the resulting LS-containing feed from green biomass juice.

5. Conclusions

Molecular weight was the most significant lignosulfonate property that improved protein precipitation, likely by increasing electrostatic interaction between lignosulfonate and soluble proteins. Using high molecular weight lignosulfonate at close to model-predicted optimum conditions greatly increased yield, albeit reduced protein purity due to co-precipitation of lignosulfonate and other non-protein contaminants. Nevertheless, the *in vitro* protein digestibility and amino acid composition of the pellet were not severely affected. Identification of the potential contaminants that were precipitated along with the proteins in the juice would provide a better understanding of the process. Efforts to reduce lignosulfonate content in the pellets will be needed to achieve the recommended safety limit for animal feed and possibly to recycle the polyelectrolyte. Regarding applicability, performing *in vivo* digestibility tests of the pellet after treatment with lignosulfonate is needed to reveal its true potential as feed.

CRediT authorship contribution statement

Demi T. Djajadi: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Visualization, Writing – review & editing. **Lívia B. Brenelli:** Formal analysis, Investigation, Writing – review & editing. **Telma F. Franco:** Funding acquisition, Project, administration, Supervision, Writing – review & editing. **Lisbeth G. Thygesen:** Formal analysis, Project administration, Visualization, Writing – review & editing. **Henning Jørgensen:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2022.115212.

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