DETERMINATION OF IN VITRO DIGESTION VALUES OF FEEDS TREATED WITH DIFFERENT LEVELS OF CALCIUM LIGNOSULFONATE

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Abstract. This study aimed to determine the digestion of dry matter (DM) and crude protein (CP) contents of soybean meal (SBM), cottonseed meal (CSM), sunflower seed meal (SSM), barley, wheat, and corn treated with varying levels (0%, 3%, and 6%) of calcium lignosulfonate (CLS) in both the rumen and the small intestine using in vitro techniques. The CLS was applied to all feeds at levels of 3% and 6% during heat treatment at 150°C for 30 min. Digestion data were evaluated in a 3 x 3 factorial trial design, considering 3 levels of CLS and 3 types of feeds separately for protein and grain feeds. The levels of CLS only affected rumen CP degradability (IVRCPD) and total tract CP digestion (TTCPD). However, an interaction between feed type x CLS levels was observed for protein supplements. In vitro DM digestibility (IVDMD), in vitro rumen DM degradability (IVRDMD), and intestinal enzyme CP digestibility (IECPD) were not affected by the levels of CLS in protein feeds. For grain feeds, CLS levels influenced IVRDMD, IVRCPD and TTCPD and feed type x CLS levels interaction were observed for IVRDMD and IVRCPD. As a result, treating protein feeds with CLS did not produce the desired outcomes for in vitro DM and CP digestibility in the rumen, intestine, and whole tract using in vitro techniques.
Keywords: *by-pass protein, degradation, enzyme, grains, protein supplements*

Introduction

The microbial protein synthesized in the rumen, along with some of the bypass protein provided by the feed (during the first phase of lactation and in fast-growing young ruminants), may not be sufficient to meet the protein needs of ruminant animals. In this case, especially the protein source should be high and high quality and the breakdown of the rumen should be reduced. Maillard reactions decrease rumen degradation and increase the availability of soybean meal (SBM) protein (Can and Yılmaz, 2002; Can et al., 2011). However, the cost of xylose as a reducing sugar source affects the widespread use of this method. Since CLS contains xylose and is much more affordable than xylose, it can be utilized to enhance the bypass protein content of feeds. Neves et al. (2007) reported that lignosulfonate treatment did not affect the digestion of DM, crude fat, CP, and NDF, while it only slightly affected the milk fatty acid composition. Similarly, De Marchi et al. (2013) examined the impact of rations containing lignosulfonate-treated sunflower on feed intake, digestion, and ruminal parameters in dairy cows, with and without pelleting. It has been stated that these methods have no effect on sunflower seeds and only grinding will be sufficient. Micek et al. (2020) found that both heat treatment and lignosulfonate treatment at 130°C or higher for 60 min were necessary to decrease the in situ degradability of canola meal CP, lysine, and methionine content. They also concluded that heat treatment alone was insufficient. Recently, cottonseed processing method or the addition of calcium

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lignosulfonate to high-concentrate diets have no effect on the carcass's quality, non-carcass components, or performance of the cull ewes (Viana et al., 2021). In vivo measurements are the most reliable method to assess both rumen and post-rumen digestion of feed CP. In addition to the need for cannulated animals for in vivo measurements, these methods are costly, labor-intensive, and prone to high error rates in calculations due to the use of markers, microbial markers, and animal-related factors to estimate the flow rate of content (Stern et al., 1997). Irshaid (2007) assessed the intestinal digestibility of by-pass protein content in feeds using a fully laboratory-based in vitro enzyme technique. He reported that this technique could be a viable alternative to the mobile bag method.

High milk producing dairy cows and fast-growing young ruminants require high quality rumen protected protein sources. It has not been clearly established whether CLS is successful in increasing rumen CP protection. Therefore, the aim of this study was to determine the digestion of DM and CP contents of SBM, CSM, SSM, barley, wheat, and corn treated with different levels (0%, 3%, and 6%) of CLS in both the rumen and the small intestine using laboratory-based in vitro techniques.

Materials and methods

Calcium lignosulfonate treatment procedure

Samples of SBM, CSM, SSM, barley, wheat, and corn were treated with calcium lignosulfonate (CLS) at two levels (3 or 6 g/100 g DM) and heated at 150°C for 30 min. The nutritional composition of the samples is presented in *Table 1*. Prior to the CLS treatment, all feed samples were ground using a laboratory mill with a 2-mm sieve. After adding the appropriate amount of CLS, distilled water was added to bring the DM content to 70 g/100 g DM. The samples were heated by placing 200 grams of DM in 10 × 15 cm aluminum pans, which were then sealed with aluminum foil and heated for 30 min at 150°C in an oven. The samples were then dried at room temperature for 72 h (Can and Yilmaz, 2002).

In vitro dry matter digestion

Rumen fluid taken from sheep after slaughter was used as the inoculum source. The in vitro DM digestibility of the untreated and treated CLS feed samples was determined in triplicate using the two-stage technique developed by Tilley and Terry (1963).

In vitro ruminal and enzymatic intestinal crude protein digestion

The untreated and treated CLS feed samples were assessed using Dacron polyester bags measuring 15 × 8 cm with a pore size of 40 μm. A 4-gram sample was weighed into each bag and sealed with rubber bands. Three bags for each treatment, totaling 27 bags for each incubation, were suspended in a 5-L plastic fermenter. The fermenter contained 1.5 L of rumen fluid taken from the slaughterhouse and an equal volume of warm, CO_2-reduced in vitro rumen McDougall's buffer solution (Tilley and Terry, 1963) and placed in a water bath at 39°C for 12 h. Following the incubation period, the bags were taken out of the fermenter, given a water wash, and pressed until the wastewater turned clear. After 48 h of drying at 65°C, the bags were weighed. The Kjeldahl technique was used to calculate the amount of nitrogen (N) that was still remaining in the bags (AOAC, 1990).

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Three replicates of each 12-h incubated feed sample residue were weighed into 50-mL centrifuge tubes, with approximately 93.75 mg CP for intestinal protein digestion in each tube. Each tube was then filled with 10 mL of a 0.1 N HCl solution (pH 1.9) containing 1 g/L pepsin, and the tubes were incubated for 1 h at 38°C in a shaking water bath. 0.5 mL of 1 N NaOH was used to neutralize the pH after the incubation. Next, each tube received 13.5 mL of a phosphate buffer (pH 7.8) containing 37.5 mg of pancreatin. After shaking, the tubes were incubated at 38°C. To stop the enzymatic process and precipitate the undigested proteins, 3 mL of a 100% (wt/vol) trichloroacetic acid solution was given to each tube after a 24-h incubation period (Calsamiglia and Stern, 1995; Can et al., 2011).

Table 1. *Chemical composition (%) of untreated and calcium lignosulfonate (CLS) treated feeds*

	DM	**Ash***	**ADF***	**CF***	**CP***
SBM	92.70	7.42	9.38	6.30	47.91
SBM-3 CLS	86.06	7.72	20.11	7.13	46.55
SBM-6 CLS	92.85	8.55	12.74	7.21	46.81
CSM	96.01	7.97	32.02	25.90	22.02
CSM-3 CLS	89.60	8.33	31.63	27.18	21.85
CSM-6 CLS	93.07	9.28	28.54	24.38	23.07
SFM	92.39	7.14	21.98	18.92	34.39
SFM-3 CLS	88.81	7.90	33.02	17.47	33.98
SFM-6 CLS	93.02	8.54	34.38	16.14	34.89
Barley	91.05	3.50	10.44	5.88	11.65
Barley-3 CLS	86.99	4.27	11.08	4.93	11.95
Barley-6 CLS	91.89	5.68	10.73	6.31	12.08
Wheat	91.08	1.70	7.86	2.38	13.71
Wheat-3 CLS	86.98	2.20	8.15	1.33	13.75
Wheat-6 CLS	91.19	3.04	5.26	1.65	14.56
Corn	88.57	1.16	4.40	1.78	9.41
Corn-3 CLS	86.46	2.28	8.58	2.11	9.75
Corn-6 CLS	91.52	3.02	8.36	1.90	10.37

*Dry mater basis; ADF, acid detergent fiber; CF, crude fiber; CP, crude protein; 3 CLS, calcium lignosulfonate treated (3 g/100 g DM); 6 CLS, calcium lignosulfonate treated (6 g/100 g DM)

Feed analyses and calculations

The untreated and treated CLS feeds were analyzed for DM, CP, crude fibber and crude ash according to the methods outlined by the Association of Official Analytical Chemists (AOAC. 1990). The acid detergent fiber (ADF) content was measured using the method outlined by Goering and Van Soest (1970). Rumen in vitro CP degradability (IVRCPD; % of CP) values were determined using *Equation 1:*

$$IVRCPD = \frac{ICP - RCP}{ICP} \times 100 \quad \text{(Eq.1)}$$

where ICP refers to the initial CP (g) in feed samples and RCP refers to the residual CP in the bags (g) after a 12-h in vitro incubation. The digestibility of intestinal enzyme CP (IECPD, % of CP) was estimated according to *Equation 2:*

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$$IECPD = \frac{RUCP - RCP}{RUCP} \times 100 \quad \text{(Eq.2)}$$

where RCP is the residual CP content (g) of the precipitate and RUCP is the rumen undegradable CP content (g) of feed samples that were weighed into a 50-ml centrifugation tube. The digestibility of total tract CP (TTCPD, % of CP) was determined according to *Equation 3:*

$$IECPD = \frac{(100 - IVRCPD) \times IECPD}{100} + IVRCPD \quad \text{(Eq.3)}$$

with IVRCPD and IECPD as defined above (Can et al., 2011).

Statistical analysis

Data for IVDMD, IVRDMD, IVRCPD, IECPD and TTCPD were evaluated separately for protein and grain feeds using the GLM procedure of SAS (1989). Feed types and CLS levels were arranged in a 3 × 3 factorial design. Treatment sum of squares were arranged factorial and analyzed for the main effects of feed type and CLS level, as well as for the interaction between feed type and CLS level. The Least Significant Differences (LSD) method was used to compare treatment mean separation.

Results and discussion

The effects of untreated and CLS-treated protein feeds on IVDMD, IVRDMD and IVRCPD, IECPD and TTCPD are shown in *Table 2*. There was no interaction between the feed type and CLS treatment for IVDMD (P = 0.2299) and IVRDMD (P = 0.1252). Main effects of feed type and calcium lignosulfonate (CLS) levels on in vitro dry matter digestibility (IVDMD) and in vitro 12 h rumen DM degradability (IVRCPD) for protein feeds are presented in *Table 3*. The effect of feed type on IVDMD was found to be significant (P = 0.0001). IVDMD values were observed to be highest in SBM, followed by SBM, and lowest in CSM. In terms of 12 h IVRDMS, SSM, SBM, and CSM were ranked from highest to lowest. No significant effect of CLS level was observed on IVDMD and IVRDMD (P > 0.05). Similarly, Neves et al. (2007) reported that treating SBM with 30 g/kg DM of lignosulfonate had no effect on the digestibility of DM, ether extract, CP, and aNDF content in an in vivo study. Taghizadeh et al. (2005) found that the in situ DM disappearance of SBM and CSM as a 50.0% and 31.0%, respectively, after 12 h. In this study, the IVRDMD value of untreated SBM was found to be similar to the previously reported value, while the value of untreated CSM was found to be high. The variation in CSM values can be attributed to the processes used during CSM production, the nutrient composition of CSM, rumen and in vitro environments.

There was an interaction between feed type and CLS treatment for IVRCPD, as well as main effects of feed type and CLS levels (P = 0.0001). The IVRCPD values were observed in the following order from highest to lowest: SSM, CSM, and SBM. In terms of CLS level, the highest values were observed in the 6-CLS and untreated control groups, followed by the 3-CLS level. Can et al. (2011) reported in vitro ruminal CP degradability values of 67.4% for SBM and 22.7% for CSM, which differ from the values obtained in this study. It is believed that these variations may be attributed to the fact that the measurements in the study were conducted using a purely enzyme-based in

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vitro method (enzyme versus rumen inoculum) over a longer duration (18 h versus 12 h). In contrast, Taghizadeh et al. (2005) determined that the 12-h in situ CP degradability of SBM and CSM was 33.0% and 65.0%, respectively. These values were found to be similar to the values obtained in this study. Windschitl and Stern (1988) reported that treating SBM with xylose or CLS successfully reduced ruminal protein degradation. In this study, the use of 3-CLS reduced the IVRCPD values, while 6-CLS did not have an effect on it.

Table 2. *Effects of untreated and calcium lignosulfonate (CLS) treated protein feeds on in vitro dry matter digestibility, in vitro rumen DM and CP degradability, intestinal enzyme CP digestibility and total tract CP digestibility (n = 3)*

	IVDMD (%)	**IVRDMD (%)**	**IVRCPD (% of CP)**	**IECPD (% of CP)**	**TTCPD (% of CP)**
SBM	94.69 ± 1.184	49.70 ± 0.299	35.49 ± 0.383	80.30 ± 0.246	87.30 ± 0.107
SBM-3CLS	91.31 ± 0.821	49.89 ± 0.162	27.93 ± 0.233	83.61 ± 0.870	88.19 ± 0.678
SBM-6CLS	92.75 ± 0.323	48.79 ± 0.856	32.54 ± 1.128	85.12 ± 1.240	89.94 ± 0.959
CSM	55.90 ± 0.656	41.81 ± 0.194	52.85 ± 0.158	59.03 ± 0.302	80.69 ± 0.189
CSM-3CLS	55.80 ± 1.756	43.53 ± 0.898	53.92 ± 0.733	58.85 ± 0.938	81.02 ± 0.666
CSM-6CLS	55.41 ± 1.467	44.75 ± 0.172	57.60 ± 0.132	60.72 ± 1.033	83.35 ± 0.388
SSM	70.41 ± 1.899	59.86 ± 1.177	74.31 ± 0.137	76.57 ± 0.202	93.98 ± 0.212
SSM-3CLS	68.69 ± 2.159	58.16 ± 1.359	66.72 ± 1.081	77.14 ± 2.094	92.36 ± 0.868
SSM-6CLS	73.92 ± 1.475	61.32 ± 1.174	73.28 ± 0.811	71.20 ± 1.960	92.28 ± 0.727
Feed type	0.0001	0.0001	0.0001	0.0001	0.0001
CLS level	0.185	0.2654	0.0001	0.4494	0.0289
Feed type × CLS level	0.2299	0.1252	0.0001	0.0034	0.0124

IVDMD, in vitro DM digestibility (%); IVRDMD, in vitro 12 h rumen DM degradability (%); IVRCPD, in vitro CP degradability (% of CP); IECPD, Intestinal enzyme CP digestibility (% of CP); TTCPD, total tract CP digestibility; SBM, soybean meal; CSM, cotton seed meal; SFM, Sunflower seed meal; 3 CLS, calcium lignosulfonate treated (3 g/100 g DM); 6 CLS, calcium lignosulfonate treated (6 g/100 g DM)

Table 3. *Main effects of feed type and calcium lignosulfonate (CLS) levels on in vitro dry matter digestibility (IVDMD) and in vitro 12 h rumen DM degradability (IVRCPD) for protein feeds (n = 9)*

	Feed type			**CLS level (g/100DM)**			**S.E.M**	**Effects**
	SBM	**CSM**	**SSM**	**0**	**3**	**6**		
IVDMD (%)	92.91^{a}	55.70^{c}	71.01^{b}	73.67	71.93	74.03	1.424	Feed type
IVRDMD (%)	49.46^{b}	43.36^{c}	59.78^{a}	50.46	50.52	51.62	0.948	Feed type

IVDMD, in vitro DM digestibility (%); IVRDMD, in vitro 12 h rumen DM degradability (%); SBM, soybean meal; CSM, cotton seed meal; SFM, Sunflower seed meal; 3 CLS, calcium lignosulfonate treated (3 g/100 g DM); 6 CLS, calcium lignosulfonate treated (6 g/100 g DM); S.E.M, standard error of mean
^{abc}Feed type means within a row with different subscripts differ ($P < 0.05$)

There was an interaction between the feed type and CLS treatment for IECPD ($P = 0.0003$), as well as main effects of the feed type ($P = 0.0001$). The CLS level did not have a significant effect on IECPD ($P = 0.4495$). The IECPD values, from highest to

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lowest, were observed in SBM, SSM, and CSM, respectively. Can et al. (2011) reported the IECPD values of SBM and CSM as 77.9% and 60.5%, which are very close to the values obtained in this study. Danesh Mesgaran and Stern (2005) determined IECPD of SBM and CSM as 51.0% and 75.0%, respectively, which are lower values than those obtained in this study. There was an interaction between feed type and CLS treatment for TTCPD ($P = 0.0124$), as well as main effects of feed type ($P = 0.0001$). The TTCPD values, from highest to lowest, were observed in SSM, SBM, and CSM, respectively. Can et al. (2011) reported the TTCPD values of SBM and CSM as 92.82% and 69.51%, respectively, which are very close for SBM and slightly higher for CSM compared to the values obtained in this study. In contrast, de Boer et al. (1987) determined that the total tract CP digestibility of SBM was 98.6% using the mobile nylon bag technique. In the study by Hsu et al. (1977), the in vivo and in vitro total tract CP digestibility of CSM were reported as 85.3% and 81.4% respectively, while the current study found it to be 80.69%. TTCPD values increased with 6-CLS treatment compared to the untreated and 3-CLS treated groups. Similar to the present study, the total apparent digestibility of pelleted diets treated with lignosulfonate-containing sunflower seeds did not differ significantly in terms of DM, OM, CP, EE, NDF, and ADF (De Marchi et al., 2013). Differences in study results can be attributed to the use of different digestibility determination methods, various CLS treatment methods, varying heating temperatures, heating durations and xylose content of CLS.

The impact of untreated and CLS-treated grain feeds on IVDMD, IVRDMD, IVRCPD, IECPD, and TTCPD is presented in *Table 4*. There was no interaction between feed type and CLS treatment for IVDMD ($P = 0.060$). The effect of feed type on IVDMD was found to be significant ($P = 0.0001$). Main effects of feed type and calcium lignosulfonate (CLS) levels on in vitro dry matter digestibility (IVDMD) and total tract CP digestibility (TTCPD, for grain feeds are presented in *Table 5*. The IVDMD values of barley were observed than wheat, and barley grains values ($P < 0.05$). The effect of CLS level was also not observed on the IVDMD of grains ($P = 0.4356$). Jaworski et al. (2015) reported IVDMD value of 93.00% for corn and 92.2% for wheat grains. These values are very close for wheat grain and slightly higher for corn grain compared to the values obtained in this study. Similarly, Nerves et al. (2006) reported that treating SBM with 30 g/kg DM of lignosulfonate had no effect on the digestibility of DM in an in vivo study.

There was a significant interaction between feed type and CLS treatment for IVRDMD and IVRCPD of grain feeds ($P < 0.01$). While IVDMD was lowest for corn grain, it had the highest values for wheat and barley grains. In contrast, the lowest IVRCPD was determined in wheat grain. While the 3CLS level reduced IVDMD, the 6CLS level slightly increased it compared to the 3CLS level. Taghizadeh et al. (2005) determined that the in situ DM disappearance of corn and barley grains after 12 h was 35.0% and 65.0%, respectively. These values are very close for barley grain and slightly higher for corn grain compared to those obtained in this study. In addition, the IVRCPD of corn and barley grain was found to be higher than in the study mentioned above. While the 3CLS level decreased IVRCPD, the 6CLS level increased slightly compared to the 3CLS level and even exceeded the control values in corn. According to Benninghoff et al. (2015), high temperature treatment of wheat, barley, and rye with a Ca-Mg lignosulphonate solution enhanced the percentages of protein that were left undigested in the rumen. Our findings align with those of these researchers.

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Table 4. *The impact of untreated and calcium lignosulfonate (CLS)-treated grain feeds on in vitro dry matter digestibility, in vitro rumen DM and CP degradability, intestinal enzyme CP digestibility and total tract CP digestibility (n = 3)*

	IVDMD (%)	IVRDMD (%)	IVRCPD (% of CP)	IECPD (% of CP)	TTCPD (% of CP)
Barley	80.75 ± 1.150	67.10 ± 1.299	68.04 ± 1.262	47.42 ± 0.940	83.17 ± 0.965
Barley-3CLS	82.23 ± 0.458	61.76 ± 3.958	44.55 ± 5.739	53.36 ± 2.145	73.93 ± 3.586
Barley-6CLS	81.41 ± 0.832	62.21 ± 2.434	59.06 ± 2.637	44.25 ± 0.837	77.14 ± 1.769
Wheat	91.26 ± 1.279	76.69 ± 2.026	61.94 ± 3.309	61.90 ± 2.733	85.64 ± 0.800
Wheat-3CLS	87.97 ± 0.985	57.27 ± 1.169	26.06 ± 2.024	58.47 ± 1.359	69.34 ± 0.517
Wheat-6CLS	86.04 ± 0.858	59.54 ± 1.835	34.03 ± 2.993	65.18 ± 0.879	77.00 ± 1.464
Corn	87.05 ± 1.484	57.22 ± 1.610	51.83 ± 1.813	59.13 ± 1.593	80.37 ± 0.252
Corn-3CLS	91.76 ± 2.182	52.49 ± 2.405	46.78 ± 2.694	53.22 ± 2.104	75.13 ± 1.533
Corn-6CLS	89.93 ± 2.464	53.50 ± 1.963	54.98 ± 1.900	50.08 ± 1.301	77.48 ± 1.489
Feed type	0.0001	0.0001	0.0001	0.0001	0.8586
CLS level	0.4356	0.0001	0.0001	0.1144	0.0001
Feed type × CLS level	0.060	0.0136	0.0003	0.0005	0.0505

IVDMD, in vitro DM digestibility (%); IVRDMD, in vitro 12 h rumen DM degradability (%); IVRCPD, in vitro CP degradability (% of CP); IECPD, Intestinal enzyme CP digestibility (% of CP); TTCPD, total tract CP digestibility; 3 CLS, calcium lignosulfonate treated (3 g/100 g DM); 6 CLS, calcium lignosulfonate treated (6 g/100 g DM)

Table 5. *Main effects of feed type and calcium lignosulfonate (CLS) levels on in vitro dry matter digestibility (IVDMD) and total tract CP digestibility (TTCPD, for grain feeds (n = 9)*

	Feed type			CLS level (g/100DM)			S.E.M	Effects
	SBM	CSM	SSM	0	3	6		
IVDMD (%)	81.46^{b}	88.42^{a}	89.58^{a}	86.35	87.32	85.79	1.436	Feed type
TTCPD (%)	78.08	77.33	77.66	83.06^{a}	72.80^{c}	77.20^{b}	1.652	CLS Level

IVDMD, in vitro DM digestibility (%); TTCPD, *total tract CP digestibility* (%); SBM, soybean meal; CSM, cotton seed meal; SSM, Sunflower seed meal; 3 CLS, calcium lignosulfonate treated (3 g/100 g DM); 6 CLS, calcium lignosulfonate treated (6 g/100 g DM); S.E.M, standard error of mean
^{abc}Feed type or CLS level means within a row with different subscripts differ ($P < 0.05$)

There was an interaction between feed type and CLS treatment for IECPD ($P = 0.0005$), as well as main effects of feed type ($P = 0.0001$) on grains. The effect of CLS level was also not observed on IECPD ($P = 0.1144$). The IECPD values, from highest to lowest, were observed in wheat, corn, and barley, respectively. IECPD values for corn and barley grains were found to be lower than the values reported previously (Taghizadeh et al., 2015). The intestinal digestibility of corn protein was found to be similar to the in situ mobile bag values reported by Danesh Mesgaran et al. (2005). There was no interaction between feed type and CLS treatment for TTCPD of grains ($P = 0.0505$), and the main effects of feed type were not found to be significant ($P = 0.8586$). TTCPD values of corn and barley were similar mean values of Danesh Mesgaran et al. (2005) obtained with in situ mobile bag, in vitro and 3-step techniques. However, the main effect of CLS level was significant ($P = 0.0001$). The TTCPD values, from highest to lowest, were observed in untreated grains, grains treated with 6CLS, and grains treated with 3CLS, respectively.

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Conclusion

The application of CLS at levels of 3% and 6% in protein feeds, subjected to a heat temperature of 150°C for 30 min, did not yield the desired outcomes for in vitro DM and CP degradability in the rumen, intestine, and whole tract using in vitro techniques. However, some beneficial effects have been observed in the in vitro DM and CP degradability for grain-based feeds. However, these effects are partially diminished when considering the digestibility of CP in the intestines and the total digestive tract. The variation in digestibility values of CP can be attributed to the processes used during the production of oil seed meal, the nutrient composition of feeds, the techniques used to determine digestibility (such as in vivo, in vitro, mobile bag technique, or various combinations of these techniques), and the methods of CLS treatment. However, further research is needed to determine whether treating animal feeds with CLS has an impact on animal growth and yield performance.

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