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Effects of dietary supplementation with *Pinus taeda* hydrolyzed lignin on in vivo performances, *in vitro* nutrient apparent digestibility, and gas emission in beef steers

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Highlights

- The effect of *Pinus taeda* hydrolyzed lignin (PTHL) on fattening beef performances was assessed.
- No effect was observed on live performance.
- *In vitro* apparent nutrient digestibility was not affected by PTHL.
- Ruminal propionate production was increased by PTHL
- In vitro CH_4 emission was decreased by PTHL.

Abstract

The aim of this study was to evaluate the effect of a commercially available herbal preparation (Oxyphenol®, I-Green, Padua, Italy) of *Pinus taeda* hydrolyzed lignin (PTHL) on beef fattening performance, *in vitro* rumen apparent digestibility, volatile fatty acid (VFA) and gas production. Forty Limousine steers were randomly divided into two groups of twenty individuals each and reared in two separated pens. The control (CON) group did not receive PTHL whereas the experimental group was supplemented with PTHL (35g/day for each for the first 90 days of the trial and 70g/day for each for other 30 days). The data set of *in vitro* rumen apparent digestibility, VFA and gas production was subjected to analysis of variance (ANOVA) using the GLM procedure in SAS software (SAS 9.4), considering the dietary treatment and the time as fixed effects. The inclusion of PTHL did not affect slaughter performance, with no differences in live weight at slaughter, daily weight gain and carcass dressing percentage between groups. In addition, the apparent digestibility of nutrients tested (dry matter, organic matter, crude fiber, NDF, crude protein and ether extract) was unaffected both by the dietary treatment and by sampling time. Rumen fluid pH was unaffected both by either dietary treatment or the sampling time, showing mean values ranging from 6.53 to 6.61. The acetate concentration was significantly greater for rumen fluid from PTHL group than in CON one, from 30 to 120 days (P<0.01). Propionate production showed higher levels in rumen fluid from the PTHL group at 60, 90 and 120 days of supplement administration

(P<0.001). Conversely, butyrate concentrations showed an opposite trend, with lower content in rumen fluid from the PTHL group, compared with CON group at 30 days (P = 0.0188). Total VFA concentration did not show significant differences because of sampling time (P>0.05), but the dietary effect induced higher values in PTHL group from 30 to 120 days (P<0.05). Finally, rumen fluid from PTHL during fermentation produced less CH_4 relative to CON, with lower values from 30 to 120 days compared to day 0 (P<0.001). Dietary inclusion of PTHL reduced CH_4 production but did not affect apparent digestibility and live and slaughter performance.

Key words: rumen fermentation, *in vitro* apparent digestibility, beef, ammonia nitrogen, VFA, methane

Abbreviations: PTHL, *Pinus taeda* hydrolyzed lignin; VFA, volatile fatty acid; CON, control; ANOVA, analysis of variance; NDF, neutral detergent fiber; GHG, greenhouse gas; TMR, total mix ration; DM, dry matter; CP, crude protein; CF, crude fibre; EE, ether extract; GLM, general linear model; ADF, acid detergent fibre; ADL, acid detergent lignin; TE, trolox equivalents; DW, dry weight; TEAC, trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; SEM, standard error of the mean.

1. Introduction

Methane has a large impact on global warming, being 28-fold more potent as a greenhouse gas (GHG) than carbon dioxide (CO_2) (IPCC, 2014). Ruminants are major producers of methane, accounting for 37% of total GHG from agriculture in the UK (Cottle et al., 2011). Methane is an end product of anaerobic microbial fermentation in the rumen and a significant parameter for the evaluation of both environmental impact and energy costs in animal production (Auffret et al., 2018). Such anaerobic fermentation in the rumen is caused by methanogenic *Archaea* during the disposal of metabolic hydrogen (H_2) (Yang et al., 2017). Consequently, decreasing methanogenesis

in the rumen to reduce methane emissions has become an important objective, often driven by feed additives, in particular natural polyphenols (Macheboeuf et al., 2008). On the other hand, the use of natural polyphenols is gaining interest as a means of improving animal health and product quality (Tagliapietra et al., 2013). Recent literature shows that natural polyphenols could be used to reduce oxidative stress in fattening animals because of their antioxidant properties (Kafantaris et al., 2016; Moràn et al., 2012; Zhong et al., 2012). However, some studies indicate that digestibility and rumen bacterial growth can be impaired by these natural substances, but it is unclear if this can be attributed to a time or a dose effect (Tagliapietra et al., 2013). Therefore, it is unclear what factor might reduce methane emissions. Alternatives include a worsening of the digestibility process or an effect on methane production without effects on feed degradation efficiency by the ruminal microbiome. The latter may be due to a selective inhibition of methanogens or ciliate protozoa in the rumen, considering that methanogens are closely associated with ciliate protozoa and reduction in protozoa populations may indirectly affect methanogens (Bodas et al., 2012). This aspect plays a key-role as it contributes in a clear way to evaluate the real effectiveness of polyphenols. Indeed, reduced methane emission due to a worsening of ruminal nutrient digestibility could be a harmful strategy for reducing environmental impact of ruminants productions.

To the best of our knowledge, there is no information on the in vivo dietary inclusion of *Pinus taeda* hydrolyzed lignin (PTHL) in beef cattle and its potential beneficial role. The genus *Pinus* belongs to the family Pinaceae and comprises about 115 species. *Pinus taeda*, commonly known as loblolly pine, is one of several pines native to the South-eastern United States and is the second-most common species of tree in the United States, after red maple. Besides, polyphenolic substances are widely represented in agricultural by-products. Lignins are the most abundant aromatic bio(macro)molecules, with an annual production of 50 megatons, representing an abundant alternative feedstock for making several value-added aromatic chemical compounds and polymers (Buono et al., 2018). PTHL extract is characterized by a set of phenol compounds, including methyl gallate retunoside (about 430 g/kg), vanilline (about 270 g/kg) and quercetine

(about 150 g/kg), that together make up the 85% of its phenol costituents. Therefore, the objective of this work was to assess the effect of a commercially available herbal preparation of PTHL on fattening beef cattle in vitro apparent digestibility and rumen fermentation, volatile fatty acid (VFA), methane (CH_4) and ammonia nitrogen production.

2. Material and methods

2.1. Animals

The protocol for animal research was approved by the Ethics Committee for animal testing–CESA (process number 2-X/17). Forty Limousine steers aged six months, reared in the province of Bari (Italy) were included in the trial. They were randomly divided into two groups of twenty individuals each, reared in two separated pens, equipped with automatic waterers. One group was the experimental one (PTHL; initial mean live weight 339 kg) and the other the control group (CON; initial mean live weight 340 kg). Both groups were fed *ad libitum* with the same TMR (Table 1), for 120 days, until 10 months of age (final weight of 521 kg for PTHL group versus 522 kg for CON). TMR was provided with a mixer wagon once per day, after removal from the manger of the remaining TMR. Only the experimental group received the supplement containing *Pinus taeda* hydrolized lignin (PTHL, Oxyphenol®, I-Green, Padua, Italy). The chemical composition of PTHL and its antioxidant activity are reported in Table 2, obtained according to methods described by Blando et al., 2016 and Gerardi et al., 2015. The PTHL group received 35g/day per head during the first 90 days of the trial and 70g/day per head for last 30 days. The inclusion rate was as per the manufacturer's instructions, based on empirical trials (I-Green, Padua, Italy). The supplement was orally administered to each head of the PTHL group in the self-locking head gate in the feeding front when TMR was unloaded. It was mixed with water to obtain a cream, which was then administered directly in the mouth using a large syringe. All animals were weighed, before and at the end of the trial period, to calculate the mean daily weight gain. Moreover, after slaughtering procedures, dressing percentage was calculated.

2.2. Rumen fluid sampling and fermentation preparation

All the operations of rumen fluid sampling were performed at day 0, when PTHL was added to the experimental group diet and then at 30, 60, 90 and 120 days thereafter. Rumen fluid was obtained, at each sampling time, from 10 randomly chosen animals in each group, using an esophageal tube under mild vacuum from the reticulum near the reticulo-omasal orifice (Tufarelli et al., 2010). Samples of ruminal contents (filtered through eight layers of gauze cloth) were collected in thermos flasks (previously filled with distilled water at 39°C to avoid thermal shock to rumen fluid), insufflating CO_2 into the headspace to ensure the environment remained anaerobic, and taken within 30 min to the laboratory. After transport, the top layer of ruminal contents was discarded, and the remaining portion was mixed and blended under a CO_2 headspace for 1 min to remove any additional particles and/or attached organisms. The combined fluid and contents were strained through 6 layers of cheesecloth to form the *inoculum* for the *in vitro* fermentation (Tufarelli et al., 2010).

2.3. In vitro digestion

In vitro fermentation was conducted for 48 h using the Daisy II incubator system (ANKOM Tech., Fairport, NY). The unit consisted of four incubation vessels with a capacity of 2 L for each. Each vessel contained 1.6 L of buffer solution, 400 mL of rumen liquor, and 25 nylon bags. The buffer solution consisted of 1.33 L buffer A (KH_2PO_4, 10.0 g/L; $MgSO_47H_2O$, 0.5 g/L; NaCl, 0.5 g/L; $CaCl_22H_2O$, 0.1 g/L; and urea, 0.5 g/L) and 266 mL of buffer B (Na_2CO_3, 15.0 g/L and $Na_2S_7H_2O$, 1.0 g/L), mixed in each digestion vessel and the pH was adjusted to 6.8, as reported by the method of Marten and Barnes (1980). Each digestion trial for each animal (each source of rumen fluid) was performed in duplicate. Nylon filter bags (ANKOM F57, ANKOM Tech., Fairport, NY) were rinsed in acetone and allowed to air dry before drying at 100°C for 24 h, after recording dry bag weight (as described by Tufarelli et al., 2010). A total of 0.5 g of administered TMR was put into each bag. The TMR was ground until the particle size reached 2 mm screen using a hammer mill (Pullerisette 19, Fritsch GmbH, Laborgeratebau, Germany). All bags were

weighed again. *In vitro apparent* digestibility was calculated as: (PB-PA)/PB, where PB is the percentage of parameter in the samples before the digestion and PA the percentage of the parameter after digestion, both on an organic matter basis.

2.4. Feed samples chemical analysis

Before and after *in vitro* digestion, the feed samples were analyzed in duplicate. Dry matter (DM) was determined using standard procedures (AOAC, 2005; method 930.15). Ash was determined by standard procedures (AOAC, 2005; method 942.05) using a muffle furnace at 550°C for 16h. Fat was determined using the Soxhlet extraction procedure (AOAC, 2005; Method 991.36), crude protein was determined by Kjeldahl N×6.25 procedures (AOAC, 2005; Method 968.06). Neutral detergent fibre (NDF) were determined with the ANKOM fibre analyser according to Van Soest and Robertson (1985) and were corrected for residual acid-insoluble ash. Sodium sulphite was added to the solution for NDF determination.

2.5. In vitro Gas production and analysis

For gas and volatile fatty acid analysis, an automated pressure transducer system (Ankom Technology, Macedon, NY) was used. It was equipped with 8 different 250 mL bottles. The same buffer solutions described in the "*in vitro* digestion" section were used. Each vessel received 133.3 mL of Buffer A and 26.7 mL of Buffer B. Then, 40 mL of rumen fluid were added. The ground TMR (500 mg) was pre-weighed into each vessel. The head space of each vessel was insuffflated with CO_2 for 2 minutes to ensure anaerobic conditions. Vessels were put in an oscillating water bath (39°C with an oscillating frequency of 45/min), to reproduce movements similar to those found in the rumen, and digestion was simulated for a 48h fermentation period. After this period, vessels were removed from the water bath and placed into an ice bath while gas samples were drawn into evacuated test tubes, as described by Trotta et al. (2018). Gas samples were analyzed for methane production with gas chromatography (Agilent Technologies, Santa Clara, CA) by using the total gas volume at standard temperature and pressure (Xu et al., 2010). Flasks were opened, pH measured and a 1 mL aliquot of the fermentation medium was combined in a 1.5 mL centrifuge tube with 0.1

mL of 500 g/L metaphosphoric acid and 0.1 mL of 85 mM of 2-ethyl butyrate. Samples were centrifuged at 39,000×g, at 23 °C for 15 min. After this, they were processed for VFA concentrations (Xu et al., 2010) using a gas chromatograph with FID (Agilent Technologies, Inc., Santa Clara, CA). For determination of ammonia nitrogen, 2 mL of fluid and 2 mL of trichloro-acetic acid solution (10%, w/v) were mixed to deproteinize the samples and then centrifuged for 5 min at 1,500×g. A volume of 2 mL of supernatant was processed in order to measure the ammonia nitrogen concentration according to the spectrophotometric method (Naziroğlu et al., 2002).

2.6. Statistical analysis

The data sets of in vivo and slaughter performances were subjected to analysis of variance (ANOVA) using the GLM by SAS software (SAS 9.4), considering dietary treatment as a fixed effect.

The other data were subjected to analysis of variance (ANOVA) using the GLM by SAS software (SAS 9.4), according to the following model:

$$y_{ijk} = \mu + P_i + A_j + (P \times A)_{ij} + \varepsilon_{ijk}$$

where y_{ijk} represents all the investigated patterns as dependent variables; μ is the overall mean; P is the effect of the i^{th} PTHL inclusion in the diet (i= 1, 2), A is the effect of the j^{th} feeding time (j = 1, …5), P × A is the effect of the interaction of the i^{th} dietary treatment and j^{th} feeding time, and ε_{ijk} is the error term. A Tukey's test was applied for post hoc comparison to evaluate the differences between means using SAS (version 9.4) in the same group between feeding times. All the data were expressed as least squares mean. Significance threshold was set at $P< 0.05$.

3. Results

There were no significant differences between the two experimental groups for growth, with similar live weights at slaughter, daily weight gains and carcass dressing percentages. Statistical analysis showed that all the apparent digestibility of the nutrients investigated (dry matter, organic matter, crude fiber, NDF, crude protein and ether extract) were unaffected by dietary treatment or

sampling time. Ruminal pH was un affected by treatment or time, with mean values ranging from 6.53 to 6.61. Acetate production was significantly greater for rumen fluid from the PTHL group, from 30 to 120 days (P<0.01). Moreover, from 90 to 120 days, in the PTHL group, acetate concentrations reached greater levels than at day 0 (P<0.0001). Propionate production showed greater levels in rumen fluid from the PTHL group at 60, 90 and 120 days of supplement administration (P<0.001). At 60, 90 and 120 days, the propionate levels in the PTHL group were greater than at day 0 (P<0.001). During the experiment time, no differences in butyrate concentrations were observed between the groups (P > 0.05); only at 30 days did the CON group show higher concentrations than the PTHL group (P = 0.0188). The acetate/propionate ratio (A/P) showed similar values in both PTHL and CON groups during the trial (P > 0.05). Total VFA concentration tended to increase during the trial in the PTHL group, being noticed higher at 90 and 120 days than at day 0 (P < 0.05). Moreover, from 30 to 120 days, total VFA values in the PTHL group were higher than in the CON group (P < 0.05).

From day 30 until the end of the experimental period, the ammonia nitrogen contents were significantly greater for the PTHL (P<0.01). Moreover, compared to the CON group, these values were higher at 30 days (P<0.01) and from 60 to 120 days (P<0.001). Rumen fluid from PTHL during fermentation produced less CH_4, with lower values from 30 to 120 days compared with day 0 (P<0.01). Moreover, at 30 (P = 0.0080), 60, 90 and 120 days (P<0.001) CH_4 production was lower for the PTHL than for the CON group.

4. Discussion

According to Toumi et al. (2016), the inclusion of substrates rich in phenolic compounds in the diet could improve animal health. Some *in vivo* evidence indicates that supplementation of dairy cow diets with a low level (0.2-7.5 mg/g feed) of a blend of phenols promoted fibre and carbohydrate digestion and increased the efficiency of microbial synthesis (Andrews et al., 2006; Vázquez-Añón and Jenkins, 2007) and animal productivity (Bhatta et al., 2000). According to

Bhatta et al. (2000), these results could be attributed to a shift in the partition of energy, with a higher proportion of nutrients channelled to microbial protein synthesis with phenol feeding.

Polyphenol substances, such as tannins, resveratrol and flavonoids, are reported to have antimicrobial properties (Cobellis et al., 2016; Docherty et al., 2007; Goel and Makkar, 2012; Mallo et al., 2013; Patra and Saxena, 2010). They generally inhibit some microorganisms cytoplasmatic membrane functions, bacterial cell wall or nucleic acid synthesis (Cushnie and Lamb, 2005). Consequently, they have been recognized as potential inhibitors of digestive processes by reducing rumen microbial fermentation. In the current study, PTHL dietary inclusion showed no effects on *in vivo* and slaughtering performances nor on *in vitro* apparent digestibility. Many studies have been conducted into polyphenol inclusion in animal diets, obtaining differing results. In this regard, Liu et al. (2018) observed that the polyphenol extract from *Eucommia ulmoides* did not affect growth performance and apparent digestibility of nutrients in lambs. However, Ma et al. (2017), when supplementing sheep diets with flavonoid extracts from mulberry leaf, observed an increased apparent digestibility of NDF and nitrogen, contributing to greater digestibility of the organic matter. These results were also reflected in higher production of total VFAs. Zhang et al. (2019) reported similar results for short-chain fatty acids production in ewes fed with the addition of natural antioxidants such as resveratrol (a polyphenol) and sanguinarine (a natural alkaloid). Ma et al. (2015) observed that resveratrol increased the proportion of propionate and decreased the molar proportion of butyrate, similar tu PTHL in our study. However, there is not a linear correlation between these two findings, because of the possibility that microbial activity can be improved by some active compounds (such as polyphenols), thus improving VFA production without directly increasing feed digestibility. Similarly, Balcells et al. (2012) registered increased total VFA production in heifers after adding polyphenols to the diet. These authors explained this result with the probable availability of fermentable carbohydrates in the rumen following flavonoid administration. Although our results show an increased total VFA production, with higher levels of acetate and propionate, apparent digestibility of nutrients was unaffected by PTHL. In this regard,

the literature shows that low doses of phenolic compounds do not decrease total VFA levels, whereas higher doses of phenols do (Busquet et al., 2006; Macheboeuf et al., 2008). Polyphenolic compounds can probably express their negative effect by binding and precipitating macromolecules, such as dietary proteins, carbohydrates and digestive enzymes, thus reducing feed digestibility (Bravo, 1998; Wei et al., 2018). Mobashar et al. (2018) reported in their experiment that microbial population without bacteria tend to produce the lowest propionate contents, while protozoa produce acetate and butyrate as major fermentation products. In the present study, propionate-producing bacterial activity was probably improved by PTHL, leading to higher propionate production.

Ruminal pH was constant in both groups and was unaffected by dietary PTHL. It is well known that ruminal pH is severely affected by the rate and extent of diet fermentation and can itself alter microbial fermentation (Wei et al., 2018), though in our case there was no alteration of apparent digestibility. Naziroğlu et al. (2002) stated that pH values of rumen aliquots must be balanced by opposite changes in VFA and lactate values. Although we did not measure lactate, it probably tends to change in opposition to individual VFA changes. Regarding the A/P ratio, our results are in disagreement with data reported by Wei et al. (2018) who found that an increase in red osier dogwood linearly appeared to switch fermentation patterns to more acetate production at the expense of propionate. Probably, when variations in VFA production occur without any variation in fibre and starch digestibility, there is an improvement in microbiota metabolic efficiency. In contrast, we observed increased production of both acetate and propionate in the PTHL group during the trial, with no alteration and no differences in the A/P ratio.

Ammonia nitrogen is one of the major sources for microbial growth and microbial protein synthesis in the rumen (Alexander et al., 2008; Wanapat et al., 2011). The decrease in ammonia nitrogen production and concentration can be related to a lower feed protein degradation in the rumen (Alexander et al., 2008; Liu et al., 2018). We observed that ammonia nitrogen production increased in the PTHL group. Our ammonia nitrogen levels are in agreement with data reported by Naziroğlu et al. (2002) who observed significantly higher ammonia nitrogen contents in the

supplemented groups (with 0.4 and 0.8 mg of vitamin E) as compared with the control batch. These authors found that vitamin E supplementation may increase the numbers of rumen protozoa and ammonia nitrogen levels. In this regard, some rumen protozoa, such as holotrich ciliates, obtain some of their nitrogen from the ingestion and digestion of bacteria. After this, they excrete nitrogenous waste, increasing the ammonia nitrogen contents in the rumen fluid (Naziroğlu et al., 2002). By contrast, when studying the effect of polyphenolic extract from *Eucommia ulmoides* leaf in lamb diet, Liu et al. (2018) observed that high antioxidant inclusion tends to reduce the ammonia nitrogen concentration. Ma et al. (2017), meanwhile, observed that the polyphenols supplementation did not affect ruminal ammonia concentration in sheep. Ammonia is a key metabolite for rumen fermentation, being the principal source of the nitrogen (N) required for microbial protein synthesis (Ma et al., 2017). Changes in ruminal ammonia are often coupled with changes in the duodenal flow of microbial N, indicating less efficient N utilization in the rumen (Ma et al., 2017). However, Liu et al. (2018) stated that this parameter alone is unable to fully account for the protective effect of an antioxidant active substance on the degradation of feed proteins by rumen microbes, given that both feed protein degradation and microbial protein synthesis occurred simultaneously. Lower ammonia concentration can be a common result of rumen defaunation, and is often associated with higher propionate production (Eugene et al, 2004). We did not measure the total protozoa number in this study; however, it can be related to the number of these tend to increase for the effect of PTHL inclusion in the diet. Despite the hypothesis of an increase in protozoa numbers, and therefore ammonia nitrogen concentration, there is not a negative effect on propionate-producing bacteria. They probably increase together with higher propionate accumulation in the rumen. Regarding methanogenesis, CH_4 production was lower in the PTHL group. Methanogenic archaea convert the H_2 and CO_2 produced by a complex community of ciliate protozoa, bacteria and anaerobic fungi to CH_4 (Martin et al., 2010; Morgavi et al., 2010). Some feed additives can act by inhibiting H_2 production or inhibiting the archaea (Knapp et al., 2014; Kumar et

al., 2014). Because digestibility was unaffected by PTHL, it appears that PTHL directly inhibited the methanogenic archaea and protozoa (Patra et al., 2017).

Conclusions

Supplementing PHTL in the ration of growing steers did not affect *in vivo* and slaughtering performance parameters, such as apparent digestibility, but increased the acetate and propionate production, reducing CH_4 emission. The current study suggests that dietary inclusion of PTHL could be a promising additive for controlling methane production without any negative effects in term of digestibility and production performance. As such, it warrants further investigation. Decreasing the production of CH_4 from ruminants is an important objective, as it would both relieve the greenhouse effect on the planet and improve the utilization efficiency of dietary energy.

Authors declare that they didn't have any relationship with any entity that has a financial interest in the subject matter discussed in this manuscript and so they did not have any conflict of interest.

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Tables

Table 1. Ingredient and nutrient composition of the experimental TMR (g/kg on dry matter basis) fed to beef steers with hydrolyzed lignin of *Pinus Taeda* (PTHL) inclusion and without it (CON) for 120 days.

Ingredients	
Wheat straw	150
Ground maize	440
Soybean meal solvent 44% protein	140
Ground barley	125
Wheat bran	110
Hydrogenated triacylglyceride from palm oil	10
Mineral/vitamin supplement	25
Nutrient Composition	
Dry matter	867.3
Organic matter	852.2
Crude protein	159.2
Crude fiber	92.2
NDF1	256.1
ADF2	101.3
ADL3	26.3
Ether extract	39.4
Ash	40.1

[1]NDF = Neutral-detergent fibre;
[2]ADF = Acid-detergent fibre;
[3]ADL = Acid-detergent lignin.

Table 2. Composition and antioxidant activity of *Pinus taeda* hydrolyzed lignin extract fed to beef steers for 120 days.

Components (g/kg)	
Vanillin	264
Eriodictyol	34
Quercetin	27
Isorhamnetin	16
Rosmarinic acid	14
Quercetin rhamnoside	139
Methyl gallate rutinoside	423
Epigallocatechin-3-methylgallate	15
Ferulic acid derivatives	67
Antioxidant activity (μmol TE[1] g^{-1} DW[2])	
TEAC3	23.9
ORAC4	122.44

[1]TE = Trolox equivalents
[2]DW = dry weight
[3]TEAC = Trolox equivalent antioxidant capacity;
[4]ORAC = oxygen radical absorbance capacity;

Table 3. In vivo and slaughtering performances of *Pinus Taeda* (PTHL) inclusion in the diet of fattening beef steers with hydrolyzed lignin of *Pinus Taeda* (PTHL) inclusion and without it (CON) for 120 days.

	PTHL	CON	SEM[1]	P value
Initial live body weight (kg)	339	340	24.9	0.83
Final live body weight (kg)	521	522	36.5	0.80
Daily gain (kg)	1.49	1.51	0.11	0.91
Dressing percentage (%)	66.7	67	4.28	0.74

Groups: PTHL: group supplemented with *Pinus taeda* hydrolyzed lignin and CON: control group
[1]SEM: Standard error of the means from feeding group

Table 4. Apparent digestibility of dry matter, organic matter, NDF, crude protein and ether extract of beef fed with hydrolyzed lignin of *Pinus Taeda* (PTHL) inclusion and without it (CON) for 120 days.

Pattern	Group	Time (days)					SEM[1]	P-values		
		0	30	60	90	120		Group	Time	Group × Time
Dry matter	PTHL	0.56	0.54	0.53	0.53	0.52	0.06	0.79	0.82	0.62
	CON	0.55	0.53	0.53	0.50	0.50				
Organic matter	PTHL	0.58	0.58	0.57	0.57	0.58	0.08	0.62	0.66	0.42
	CON	0.57	0.57	0.56	0.57	0.58				
Crude fiber	PTHL	0.41	0.40	0.39	0.39	0.38	0.06	0.76	0.10	0.07
	CON	0.40	0.41	0.39	0.39	0.38				
NDF[2]	PTHL	0.43	0.44	0.43	0.43	0.43	0.05	0.58	0.71	0.49
	CON	0.45	0.44	0.45	0.43	0.42				
Crude protein	PTHL	0.55	0.54	0.54	0.56	0.55	0.06	0.62	0.75	0.58
	CON	0.55	0.55	0.54	0.54	0.53				
Ether extract	PTHL	0.37	0.39	0.38	0.38	0.38	0.05	0.56	0.58	0.49
	CON	0.37	0.38	0.37	0.37	0.38				

[1]Standard error of the means;
[2]NDF = Neutral-detergent fibre;
Groups: PTHL: group supplemented with *Pinus taeda* hydrolyzed lignin and CON: control group

Table 5. pH and volatile fatty acids (VFA) (mmol/L), ammonia nitrogen (mmol/L) and CH_4 production (ml) of rumen aliquots of beef steers fed with hydrolyzed lignin of *Pinus Taeda* (PTHL) inclusion and without it (CON) for 120 days.

Pattern	Group	Time (days)					SEM[1]	P-values		
		0	30	60	90	120		Group	Time	Group × Time
pH	PTHL	6.54	6.57	6.57	6.61	6.57	0.06	0.51	0.40	0.25
	CON	6.58	6.57	6.53	6.60	6.57				
Acetate	PTHL	69.1^{A}	$76.6^{AB, X}$	$76.3^{AB, X}$	$82.1^{B, X}$	$82.2^{B, X}$	4.24	<0.001	<0.001	<0.001
	CON	70.2	66.6^{Y}	68.6^{Y}	67.4^{Y}	71.3^{Y}				
Propionate	PTHL	16.2^{A}	16.3^{AB}	$19.8^{B, X}$	$20^{B, X}$	$19.9^{B, X}$	1.85	<0.001	<0.001	<0.001
	CON	17.1	16.7	17.4^{Y}	18.1^{Y}	17.3^{Y}				
Butyrate	PTHL	8.96	8.54^{x}	9.67	9.7	9.76	0.93	0.002	0.21	<0.001
	CON	9.57	10.6^{y}	10.3	10.2	10.5				
A/P	PTHL	4.41	4. 8	3.9	4.3	4.21	0.08	0.26	0.29	0.013
	CON	4.27	4.07	3.78	3.78	4.11				
Total VFA	PTHL	90.6^{A}	$99.4^{AB, x}$	$100^{AB, x}$	$105^{B, x}$	$107^{B, x}$	5.37	0.001	0.001	<0.001
	CON	91.8	86.4^{y}	90.5^{y}	89.8^{y}	92.4^{y}				
Ammonia Nitrogen (mmol/L)	PTHL	16.8^{A}	$26.1^{B, X}$	$28.3^{B, X}$	$29.4^{B, X}$	$28.2^{B, X}$	1.72	<0.001	<0.001	<0.001
	CON	17.4	18.6^{Y}	17.8^{Y}	19.3^{Y}	19.2^{Y}				
CH_4 production (ml)	PTHL	6.84^{A}	$4.59^{B, X}$	$4.29^{B, X}$	$4.12^{B, X}$	$4.21^{B, X}$	0.04	<0.001	<0.001	<0.001
	CON	6.72	6.08^{Y}	6.19^{Y}	6.03^{Y}	6.42^{Y}				

[1]Standard error of the means from feeding time;
Different superscript letters in the same line show statistical differences (A, B: $P < 0.01$)
Different superscript letters in the same row, for each investigated pattern, show statistical differences (X, Y: $P < 0.01$; x, y: $P < 0.05$)
Groups: PTHL: group supplemented with *Pinus taeda* hydrolyzed lignin and CON: control group