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In vitro rumen fermentation of by-product from the production of edible mushroom *Pleurotus ostreatus* enriched or not with selenium

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ABSTRACT - The objective of this study was to evaluate *in vitro* effects on ruminal fermentation of by-product from the production of edible mushroom *Pleurotus ostreatus*. The following by-products were tested: (I) non-colonized sugarcane bagasse (control); (II) sugarcane bagasse from non-enriched mushrooms; and (III) sugarcane bagasse from selenium-enriched mushrooms. A semi-automatic *in vitro* system was used. The pH and neutral detergent fiber (NDF) residue were determined at 0, 3, 6, 9, 12, 18, 24, 48, 72, and 96 h of incubation. The ruminal ammonia nitrogen (NH_3-N) concentrations were measured at 0, 6, 12, 24 and 48 h incubation times and the short-chain fatty acids (SCFA) concentrations after 24 h incubation. The pH and NH_3-N were not affected (P>0.05) by the treatments or the treatment × time interaction. However, an effect of incubation time was observed (P<0.05). The production of SCFA was not affected (P>0.05). Non-colonized sugarcane bagasse presented a higher value of the potentially degradable fraction (P<0.05) and a lower value of the undegradable fraction when compared to the other treatments (P<0.05). The results indicate that the by-products do not change the fermentative parameters.

Keywords: agro-industrial waste, fiber, ruminant

1. Introduction

In Brazil, different fiber sources are used in the diet of ruminants. For dairy cows, the main sources of fiber are brachiaria grass (Lima et al., 2021), corn silage (Giombelli et al., 2019) and sugarcane bagasse (Almeida et al., 2018). However, sugarcane bagasse has a high lignin content, which limits fiber utilization by rumen microorganisms (Gunun et al., 2016).

In general, before inclusion in ruminant diets, sugarcane bagasse undergoes chemical treatment to improve its nutritional value. Urea plays a crucial role in this process by enhancing crude protein content through increased nitrogen concentration and solubilizing hemicellulose, which improves fiber digestibility (Ahmed et al., 2013). An alternative is the biological treatment of sugarcane bagasse using *Pleurotus ostreatus*, a white-rot fungi that produce edible mushrooms. Enzymes secreted by

Pleurotus ostreatus degrade lignin, improving nutritional quality, and enhancing its use as a dietary substrate for application in ruminant rations. Thus, by-products generated after harvesting mushrooms grown in an axenic system have lower lignin content and higher crude protein (2.99% in dry matter) than non-colonized sugarcane bagasse (0.39% in dry matter) (Bento et al., 2014).

In addition, when mushrooms grow in lignocellulosic by-products enriched with minerals such as selenium (Se), the substrate has a high content of this essential micronutrient, thus improving the daily feed of animals. Selenium is important to reduce the harmful effects of free radicals and to improve the productive and reproductive performance of animals (Mehdi and Dufrasne, 2016). Positive effects on the digestibility and rumen fermentation were recorded, such as elevated nutrient digestibility (Zhang et al., 2020). Therefore, the use of by-products from the production of edible mushrooms enriched with Se for animal feed could be an important alternative.

This study aimed to evaluate the effects of by-products generated by the production of Se-enriched *Pleurotus ostreatus* mushrooms on *in vitro* ruminal fermentation. We hypothesized that the by-product from the production of Se-enriched mushrooms could be used as an ingredient in ruminant rations without negative effects and could represent an alternative source of fiber and protein for the ruminal microbiota.

2. Material and methods

All procedures were approved by the Ethics Committee on Animal Use of the Universidade Federal de Viçosa (protocol 30/2020), following the guidelines of the National Council for the Control of Animal Experimentation (CONCEA). The experiment was conducted in Viçosa, Minas Gerais (Brazil), at the geographical coordinates 20°45'14" S and 42°52'54" W.

2.1. Microorganism and culture conditions

The isolate *Pleurotus ostreatus* (Plo 02) used in this study was obtained from the fungal collection of the Laboratory of Mycorrhizal Associations, Department of Microbiology, Universidade Federal de Viçosa. The fungus was cultivated in Petri dishes containing Potato Dextrose Agar (PDA; Merck, Darmstadt, Germany) medium and incubated at 25 °C. After 7 days, the mycelium was used for spawn production in sorghum grains. Before inoculation, the sorghum grains were treated in a 2% (v/v) chlorine solution, washed, and boiled in water for 45 min. After removing excess water, calcium sulfate ($CaSO_4$) and calcium carbonate ($CaCO_3$), were added at a 4:1 ratio. Approximately 100 g of these grains were placed in glass bottles (200 mL) and autoclaved at 121 °C for 60 min (Luz et al., 2013). In each glass bottle containing 100 g of grains, four agar disks with mycelium were inoculated.

2.2. Preparation of the substrate for *Pleurotus ostreatus* cultivation

Sugarcane bagasse was used as a substrate for *Pleurotus ostreatus* cultivation. The sugarcane bagasse was collected from a sugarcane juice shop, at a street market, in Viçosa (Minas Gerais, Brazil). The sugarcane bagasse was initially dried and then ground in a chopper (model DPM-2, Irmãos Nogueira SA Máquinas Agrícolas, Brazil) using a 10-mm sieve for the preparation of the cultivation substrate. The bagasse was immersed in a 2% (m/v) hydrated lime solution ($Ca(OH)_2$) for 16 h to reduce the growth of competing fungi. Subsequently, the material was centrifuged (model BCR01BVD, Britania, Brazil) to remove the excess water.

2.3. Inoculation and enrichment of the substrate with Se

After centrifugation, 1.0 kg of the cultivation substrate was packed in each plastic bag, and 10.0 mL of sodium selenite (Na_2SeO_3) solution at a concentration of 25 mg Se per kg of substrate were added. Then, 150.0 g of inoculum was added. The substrates cultivated with the fungus without Se received

10.0 mL of distilled water (Souza et al., 2022). After inoculation, the bags were tied. The bags containing the inoculated substrate were stored at room temperature for 30 days until complete colonization was achieved. One hundred bags with non-enriched substrate and 100 bags with Se-enriched substrate were prepared.

2.4. Thermal shock, fruiting and harvesting of *Pleurotus ostreatus* mushrooms

After complete substrate colonization, the bags were kept at 10 °C, for 24 h, to induce primordia formation. Next, the bags were transferred to the fruiting room, with 80% relative humidity, at the temperature of 23 °C. After the first cycle of harvesting of the mushrooms, the by-products enriched or not with Se were dried and ground through a 1 mm screen using a knife mill (model TE-650/1, Tecnal, Brazil).

2.5. Quantification of the Se content in the by-products after *Pleurotus ostreatus* mushroom fruiting

To determine Se in the by-products after *Pleurotus ostreatus* mushroom fruiting, the samples of by-products were initially digested in a microwave oven using a diluted mixture of nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) and analyzed by inductively coupled plasma mass spectrometry (ICP-MS).

In the Se-enriched by-product, the concentration was 143 μg Se g^{-1} (143 ppm), whereas in the non-enriched by-products, the Se concentration was below the quantification limit (0.4 μg Se g^{-1}).

2.6. Chemical composition of by-products

The contents of dry matter (DM), crude protein (CP), neutral detergent insoluble protein (NDIP), neutral detergent fiber (NDF), neutral detergent indigestive fiber (NDFi), acid detergent fiber (ADF), ether extract (EE), lignin and ashes in the substrates before *Pleurotus ostreatus* cultivation and in by-products from the production of *Pleurotus ostreatus* were determined using the following methodologies recommended by the National Institute of Science and Technology in Animal Science (Detmann et al., 2012): DM (method INCT-CA G-003/1); CP (method INCT-CA N-001/1); NDIP (method INCT-CA N-004/1); NDF (method INCT-CA F-002/1); NDFi (method INCT-CA F-008/1); ADF (method INCT-CA F-004/1); EE (method INCT-CA G-004/1); lignin (method INCT-CA F-005/1) and ashes (method INCT-CA M-001/1).

2.7. Rumen fluid collection

Two rumen-cannulated Nellore females (*Bos indicus*) aged 2 years, weighing 250 kg were used as donors of rumen fluid for the *in vitro* assay. The animals were fed exclusively with sugarcane bagasse and a complete mineral mix. Sugarcane bagasse, mineral mix, and water were provided *ad libitum*. After 15 days of dietary adaptation, the ruminal liquid fraction was collected manually at several points from the liquid–solid interface of the rumen (1.2 L per animal per incubation), in the morning, before feeding. The ruminal liquid was kept in pre-warmed (39 °C) insulated containers and transported to the incubation room within 15 min before each incubation.

2.8. *In vitro* incubation

In a temperature-controlled room (39 °C), the rumen fluid was pooled and filtered through three layers of cheesecloth. Then, 10 mL of rumen inoculum were added under continuous flow of carbon dioxide (CO_2) in a serum bottle containing 250 mg of by-product added with 40 mL of McDougall solution (McDougall, 1948), which totaled 50 mL of inoculum with a ratio of 4:1 between buffer solution and ruminal inoculum (Tilley and Terry, 1963). The pH was adjusted by CO_2 infusion, and the serum bottles were sealed and kept under orbital shaking (40 rpm) (model TE-140, Tecnal®, Brazil) in the temperature-controlled incubation room. For each incubation time (0, 3, 6, 9, 12, 18, 24, 48, 72, and

96 h), one serum bottle per treatment and one blank (10 mL of rumen inoculum was added under continuous flow of CO_2 in a serum bottle containing 40 mL of McDougall solution) were incubated simultaneously, totaling 40 serum bottle per incubation. The incubation procedure was repeated four times, totaling 04 repetitions per incubation time for each treatment. The following treatments were tested: (I) non-colonized sugarcane bagasse; (II) by-product from sugarcane bagasse obtained after the production of non-Se-enriched mushrooms of *Pleurotus ostreatus*; and (III) by-product from sugarcane bagasse obtained after the production of Se-enriched mushrooms of *Pleurotus ostreatus.* For each incubation time, the flasks were removed from incubation for analysis. After opening the flasks, the pH of all replicates was measured. After measuring the pH, samples were aliquoted for analysis of ruminal ammonia nitrogen (NH_3-N) and short-chain fatty acids (SCFA). The remaining volume in the flasks was filtered through crucibles to determine digestibility.

2.9. pH measurements

A previously calibrated digital pH meter was used (Digimed, model DM-2P) for pH assessment. The pH measurements were carried out at times 0, 3, 6, 9, 12, 18, 24, 48, 72, and 96 h of the incubation.

2.10. Determination of the NDF *in vitro* digestibility

The determination of NDF *in vitro* digestibility was evaluated at 0, 3, 6, 9, 12, 18, 24, 48, 72, and 96 hours after incubation. At the end of each incubation time, the serum bottles were removed from the room with controlled temperature, and the contents were filtered in filter crucibles (average porosity between 40 and 60 µm), subjected to vaccum filtration using distilled water at 90 °C. Then, the porous crucibles were placed in polyethylene containers (120 mL), and 50 mL of neutral detergent was added (Mertens, 2002) without the inclusion of sodium sulfide or thermostable α-amylase. The polyethylene containers were autoclaved at 105 °C for one hour, following the method recommended by the National Institute of Science and Technology in Animal Science (Detmann et al., 2012) (INCT-CA F-002/1).

2.11. Determination of NH_3-N

The concentration of NH_3-N was determined by the colorimetric method, according to Chaney and Marbach (1962). Absorbance at 630 nm was measured using a spectrophotometer (Multiskan GO; Thermo Scientific, USA). Ammonium chloride (NH_4Cl) was used as the standard. The concentration of NH_3-N was evaluated at 0, 6, 12, 24, and 48 h of incubation.

2.12. Concentrations of SCFA

Concentrations of SCFA were determined by high-performance liquid chromatography (HPLC). Rumen samples were processed according to the methodology described by Siegfried et al. (1984). Analyses were performed on a Dionex Ultimate 3000 Dual chromatograph coupled to a Shodex RI-9 refractive index detector, maintained at 40 °C. The SCFA were separated using a Phenomenex Rezex ROA column maintained at 45 °C. Sulfuric acid (H_2SO_4 5 mmol) at a flow rate of 0.7 mL min^{-1} was used as the mobile phase. For the calibration curve, a series of five 2-fold dilutions were performed from standards, with the following stock concentrations: acetic acid (60 mM), propionic acid (40 mM) and butyric acid (20 mM). The concentrations of SCFA were evaluated after 24 h of incubation.

2.13. Statistical analysis

The pH and NH_3-N values obtained for different incubation times according to a randomized block design in a factorial scheme (treatment × incubation time) were run using the blocking criteria:

$$Y_{ijkl} = \mu + TR_i + T_j + (TRT)_{ij} + B_k + \varepsilon_{ijk},$$

in which μ = the overall mean; TR_i = the fixed effect of level *i* of factor treatment; T_j = the fixed effect of level *j* of factor time; $(TRT)_{ij}$ = the fixed effect of the interaction of level *i* of factor treatment with level j of factor time; B_k = the random effect of level *k* of factor block; ε_{ijk} = random error with mean 0 and variance σ^2.

For each treatment and replication, NDF residues at different times were submitted, through the Gauss-Newton algorithm, to the adjustment of the non-linear model described by Van Milgen et al. (1991).

$$R_t = B \times (1 + \gamma \times t) \times \exp(-\gamma \times t) + U,$$

in which R_t the non-degraded NDF residue at time "*t*" (%), *B* is the potentially degradable fraction (%), *U* is the undegradable fraction (%), and γ is the common rate of lag and degradation (h^{-1}).

The NDF fractional degradation rate was estimated from λ by using the properties of the gamma-2 distribution (Ellis et al., 1994):

$$k = 0.59635 \times \gamma,$$

in which *k* is the fractional degradation rate of a potentially degradable fraction of NDF (h^{-1}).

Then, statistical analyses were performed for each parameter using the PROC GLIMMIX procedure of the SAS software (Statistical Analyzes System) as randomized block design.

$$Y_{ijk} = \mu + TR_i + B_j + \varepsilon_{ijk},$$

in which μ = the overall mean; TR_i = the fixed effect of treatment; B_j = the random effect of level *j* of block; ε_{ijk} = random error with mean 0 and variance σ^2.

The least-square means were considered different when P<0.05 in the Tukey's test.

3. Results

The CP content increased in the by-products obtained after the production of *Pleurotus ostreatus* mushrooms, compared with the non-colonized sugarcane bagasse (Table 1). An increase in NDIP was also observed in the colonized substrates (Table 1). However, when evaluating the difference between CP and NDIP, the amount of available CP was 275% and 328% higher in the colonized by-products without or with Se, respectively. A decrease in lignin content was also observed in the colonized by-products (Table 1). Non-colonized sugarcane bagasse presented a higher value of fraction D (potentially degradable fraction of NDF) and a lower value of fraction I (undegradable fraction of NDF) compared with the colonized by-product (P<0.05; Table 2).

Table 1 - The contents of dry matter (DM), crude protein (CP), neutral detergent insoluble protein (NDIP), neutral detergent fiber (NDF), indigestible neutral detergent fiber (NDFi), acid detergent fiber (ADF), ether extract (EE), ashes and lignin in the by-products

Item (%)	Treatment		
	Non-colonized sugarcane bagasse	Sugarcane bagasse from of non-enriched mushrooms	Sugarcane bagasse from selenium-enriched mushrooms
DM[1]	90.25	89.76	90.39
CP[2]	1.37	2.95	3.29
NDIP[2]	0.56	0.72	0.70
NDF[2]	69.29	63.80	62.74
NDFi[2]	21.01	32.44	32.24
ADF[2]	52.46	48.83	48.85
EE[2]	0.43	0.20	0.15
Ashes[2]	10.11	7.72	7.65
Lignin[2]	7.00	5.75	5.99

[1] Percentage based on natural matter.
[2] Percentage based on dry matter.

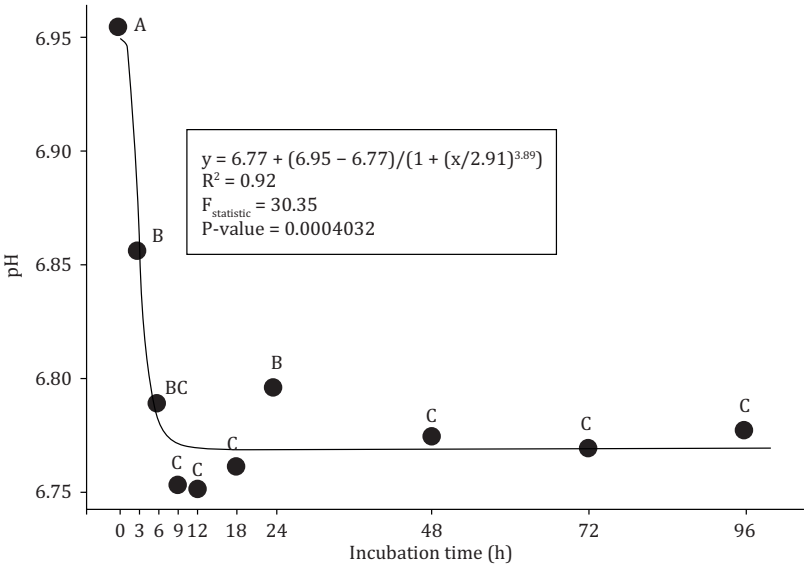
Table 2 - Potentially degradable fraction of NDF (D), undegradable fraction of NDF (I), fractional rate of degradation (k), ruminal pH (pH), ruminal ammonia nitrogen (RAN), acetate, propionate, butyrate and acetate:propionate ratio (A:P) in the by-products

Item	Treatment			SEM	P-value
	Non-colonized sugarcane bagasse	Sugarcane bagasse from of non-enriched mushrooms	Sugarcane bagasse from selenium-enriched mushrooms		
D	66.79a	49.37b	53.57b	2.820	0.010
I	33.20b	50.64a	46.44a	2.820	0.010
k[1]	0.055	0.041	0.041	0.008	0.504
pH	6.80	6.79	6.80	0.010	0.970
RAN[2]	0.048	0.067	0.060	0.040	0.066
Acetate (%)	59.21	51.21	45.20	7.237	0.252
Propionate (%)	33.57	42.55	48.18	5.585	0.292
Butyrate (%)	7.21	6.23	6.61	1.526	0.721
A:P	1.76	1.20	0.94	0.532	0.242

SEM - standard error of the mean.
[1] Data in h^{-1}.
[2] Data in mg dL^{-1}.
Averages in the rows followed by different letters differed at 5% probability.

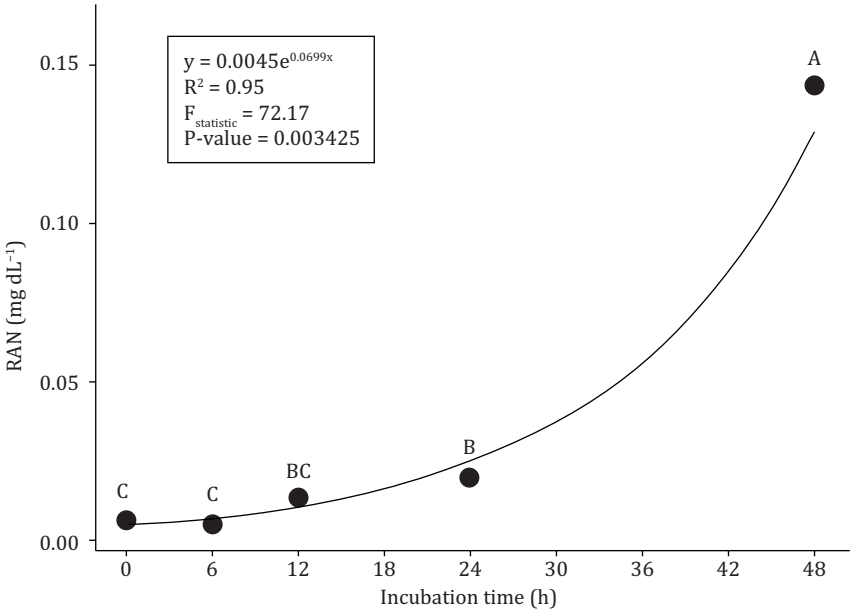
There was no effect of treatment (P>0.05) or treatment × incubation time interaction on pH (P>0.05; Figure 1). However, there was an effect of incubation time on the pH values (P<0.05). Up to 6 h of incubation, pH values decreased, and after this period, they remained constant (Figure 1). The production of ammonia nitrogen *in vitro* increased over time (P<0.05), reaching 0.2125 mg dL^{-1} of ruminal fluid after 48 h of incubation (Figure 2).

No significant differences were found in the production of SCFA (P>0.05) among treatments. However, propionate production increased by 26.7% in colonized non-enriched by-product, and 43.5% in colonized Se-enriched by-products, compared with non-colonized sugarcane bagasse (Table 2).



Points followed by different letters are statistically different by the Tukey test, at 5% probability.

Figure 1 - pH values as a function of the incubation time when the by-products were incubated in rumen liquid.



Points followed by different letters are statistically different by the Tukey test, at 5% probability.

Figure 2 - Ruminal ammonia nitrogen (RAN) values as a function of time, when the by-products were incubated in rumen liquid.

4. Discussion

The increased proportion of CP in colonized by-products may be due to partial degradation of lignocellulosic compounds (Tuyen et al., 2013) and the synthesis of fungal biomass (Kermani et al., 2019). This result is corroborated by the descriptive evaluation of the chemical composition, which shows increased NDFi in the colonized by-products (Table 1). The decrease in fiber degradability may be due to the utilization of compounds, such as hemicellulose and cellulose, during fungal growth (Bento et al., 2014; Zheng et al., 2020). Reduced lignin content was observed in the colonized by-product (Table 1), similar to what was observed by Bento et al. (2014) in by-product from *Pleurotus ostreatus* and *Lentinula edodes* in sugarcane bagasse and by Kasuya et al. (2015) in by-product of the production of *Pleurotus ostreatus* in coffee husk.

The Se supplementation is important for efficient ruminal fermentation and, consequently, for animal improved performance. Wang et al. (2009) evaluated the effect of supplying selenized yeast (0, 0.15, 0.3, and 0.45 mg Se kg^{-1} of dietary DM) in the diet of dairy cows for a period of 30 days. It was observed that increasing levels of selenized yeast in the diet increased total SCFA. In the present study, no increase in acid production was observed, similar to the *in vitro* results obtained by Panev et al. (2013).

The pH value of 6.75, observed after 12 h of incubation, was the lowest during the incubation period. Considering that pH is one of the main factors affecting microbial activity and, consequently, fiber digestibility (Hoover, 1986), the observed pH values (Figure 1) corroborate the lack of treatment effect, since pH values above 6.0 are not expected to affect the production and activity of hydrolytic enzymes (e.g., cellulases and xylanases) produced by fibrolytic microorganisms (Mould and Ørskov, 1983; Morgavi et al., 2000; Mouriño et al., 2001).

The trend of increasing ammonia nitrogen concentration is commonly observed in *in vitro* incubations due to the use of the medium protein for microbial protein synthesis (Quigley et al., 1992). The values obtained were less than 5 mg dL^{-1} of ammonia nitrogen in ruminal fluid. According to Satter and Slyter (1974), 5 mg dL^{-1} of ammonia nitrogen in ruminal fluid is considered the adequate minimum

value to support maximum ruminal microbial growth rates. However, under *in vitro* conditions, there is no nitrogen recycling, which further reduces ammonia nitrogen production (Reynolds and Kristensen, 2008).

The low production of ammonia nitrogen can be related to the low CP content of the by-products (Table 1), since under tropical conditions, CP levels below 8% of the DM limit microbial activity (Lazzarini et al., 2009; Sampaio et al., 2010). Thus, supplementing low-quality tropical forage with nitrogen increases ammonia nitrogen production, as reported in other studies. Detmann et al. (2011) verified that the production of ammonia nitrogen from signal grass harvested in the dry season with CP content equal to 4.13% was 0.14 mg dL^{-1} of ammonia nitrogen in rumen fluid. However, when supplemented with urea, a non-protein nitrogen source, the production of ammonia nitrogen increased to 3.77 mg dL^{-1} of ammonia nitrogen in rumen fluid. Ortiz-Rubio et al. (2007) evaluated the supplementation of sugarcane tops with different nitrogen sources in the diet of steers and reported an increased rumen ammonia concentration.

The results indicate that the products generated in the fermentation assays are compatible with the values observed for diets with forage that are commonly supplied to ruminants (Van Soest, 1994).

5. Conclusions

This study demonstrates that the by-product obtained after the production of *Pleurotus ostreatus* mushrooms resulted in improvements in the nutritional composition of sugarcane bagasse, with the potential to optimize ruminal fermentation. Furthermore, the by-product may be a promising alternative to supplement ruminant diets, especially in regions where low-quality forages are common. Future studies could focus on optimizing fermentation conditions to maximize fiber digestibility and selenium bioavailability, as well as evaluating animal performance *in vivo*.

Data availability

The datasets supporting the results of this study was published in the article itself.

Author contributions

Conceptualization: Alves, M. P. **Data curation:** Alves, M. P.; Silva, J. S.; Costa, A. C.; Souza, D. F. and Paixão, R. T. **Formal analysis:** Sampaio, C. B. **Funding acquisition:** Kasuya, M. C. M. **Investigation:** Alves, M. P.; Silva, J. S.; Costa, A. C.; Souza, D. F.; Paixão, R. T.; Sampaio, C. B.; Mantovani, H. C. and Kasuya, M. C. M. **Methodology:** Alves, M. P.; Silva, J. S.; Costa, A. C.; Souza, D. F.; Paixão, R. T.; Sampaio, C. B.; Mantovani, H. C. and Kasuya, M. C. M. **Project administration:** Kasuya, M. C. M. **Resources:** Sampaio, C. B.; Mantovani, H. C. and Kasuya, M. C. M. **Supervision:** Sampaio, C. B.; Mantovani, H. C. and Kasuya, M. C. M. **Validation:** Alves, M. P. and Kasuya, M. C. M. **Visualization:** Alves, M. P.; Silva, J. S. and Kasuya, M. C. M. **Writing – original draft:** Alves, M. P. **Writing – review & editing:** Alves, M. P.; Silva, J. S.; Costa, A. C.; Souza, D. F.; Paixão, R. T.; Sampaio, C. B.; Mantovani, H. C. and Kasuya, M. C. M.

Conflict of interest

The authors declare no conflict of interest.

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