

In vitro ruminal fermentation of processed cottonseed and peanut meals with varying levels of rumen undegradable protein

Fernanda Rigon^{1,2} , Elaine Magnani² , Amanda Regina Cagliari¹ , Kalista Eloisa Loregian¹ , Ana Claudia Casagrande¹ , Bruna Roberta Amâncio² , David Augusto Bittencourt Pereira² , Henrique Almeida Machado Borges² , Hugo Fernando Monteiro³ , Aline Zampar¹ , Marcos Inácio Marcondes^{4*} , Renata Helena Branco² , Pedro Del Bianco Benedeti¹ , Eduardo Marostegan Paula² 

¹ Universidade do Estado de Santa Catarina, Departamento de Zootecnia, Chapecó, SC, Brasil.

² Instituto de Zootecnia, Centro APTA Bovinos de Corte, Sertãozinho, SP, Brasil.

³ University of California, Department of Population Health and Reproduction, Davis, California, United States of America.

⁴ Washington State University, Department of Animal Science, Pullman, Washington, United States of America.

*Corresponding author:
marcos.marcondes@wsu.edu

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Mateus Pies Gionbelli
Ana Clara Baião Menezes

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ABSTRACT - This study aimed to assess the ruminal fermentation of various cottonseed (Exp. 1) and peanut (Exp. 2) meals, each processed using different techniques and exhibiting varying levels of rumen undegradable protein (RUP). Six treatments were evaluated for each feed. For Exp. 1 (cottonseed meal): negative control (conventional meal; Ncontrol), microwave-heated (2 min) with 2% xylose, conventional oven-heated (90 min) with 2% xylose, autoclave-heated (8 min) with 2% xylose, tannin treatment (6% inclusion), and a commercial soybean-based product (Pcontrol). For Exp. 2 (peanut meal): negative control (conventional meal; Ncontrol), microwave-heated (6 min) with 2% xylose, conventional oven-heated (60 min) with 2% xylose, autoclave-heated (24 min) with 2% xylose, tannin treatment (6% inclusion), and a commercial soybean-based product (Pcontrol). An *in vitro* gas production (GP) system was used to evaluate gas production, kinetics, and fermentation patterns, while the *in vitro* organic matter digestibility (IVOMD) was assessed using an *in vitro* incubator system. Both experiments consisted of three consecutive 48-hour fermentation batches. The conventional oven exhibited the lowest fermentation rate and the largest gas pool size ($P<0.01$) in both experiments. Tannin resulted in the lowest total GP at both 24 and 48 hours, IVOMD, and metabolizable energy for both meals ($P<0.01$). Tannin also reduced branched-chain volatile fatty acid in Exp. 1 ($P<0.01$). The Ncontrol and Pcontrol treatments showed the highest estimated methane concentration in Exp. 2 ($P<0.01$). These findings indicate that both conventional oven heating and tannin effectively alter the ruminal fermentation of cottonseed and peanut meals, highlighting their potential as alternative sources of RUP in beef cattle nutrition.

Keywords: feed processing, fermentation pattern, protein sources, ruminal nitrogen metabolism

1. Introduction

Meeting the rumen-undegradable protein (RUP) requirements of high-performance beef cattle is essential to maximize growth, as the microbial protein synthesized in the rumen is insufficient to

meet their metabolizable protein requirements (NASEM, 2016). The diet must provide a substantial amount of RUP, which delivers amino acids directly to the ruminant's small intestine (Haryanto, 2014). However, most protein sources commonly used in beef cattle diets are extensively degraded in the rumen and exhibit variable levels of RUP (Koenig and Beauchemin, 2018). To address this, various processing methods have been developed to enhance the RUP content of protein sources for beef cattle (Koenig and Beauchemin, 2018).

Recent research has demonstrated that the use of tannins (Castro-Montoya et al., 2018; Grazziotin et al., 2020) and heat treatments (Molosse et al., 2023; Rigon et al., 2023), such as microwaving, toasting, autoclaving, and the addition of xylose (Jahani-Azizabadi et al., 2010) in protein sources can increase the RUP content of feed and enhance the flow of amino acids to the small intestine (Chesini et al., 2023). The application of these strategies may contribute to reducing feeding costs and nitrogen excretion in manure (Vanegas et al., 2017), which is desirable from a sustainable perspective. However, to our knowledge, there is a lack of information on the effects of these methods on the kinetics and ruminal fermentation parameters of alternative protein sources, such as peanut and cottonseed meal, which are known to be cost-effective alternative protein sources for ruminants.

Most commercial sources of RUP are based on heat-treated soybean meal (Maxin et al., 2013; Chesini et al., 2023) and typically incur higher costs compared to peanut and cottonseed meals. However, cost-effective protein sources such as cottonseed and peanut meals are widely used as an alternative to soybean meal in beef cattle nutrition, particularly in tropical regions (Pinto and Millen, 2019). These byproducts can serve as viable substitutes for soybean meal without compromising animal performance (Grazziotin et al., 2020). Despite their widespread use, there is a lack of research on processing methods to enhance the RUP content of cottonseed and peanut meals.

To address this gap, our research group conducted a series of studies evaluating the effects of tannin inclusion (0–60 g/kg), xylose treatment (20 g/kg), and heat application via autoclave (8–24 min), conventional oven (30–90 min), and microwave (2–6 min) on the RUP content of these meal (Rigon et al., 2023; Loregian et al., 2023; Molosse et al., 2023). From 50 treatments tested, we selected the eight most effective based on improvements in RUP and intestinal digestibility. For cottonseed meal, autoclaving with xylose (8 min) increased intestine-digested RUP by 102%, conventional oven heating with xylose (90 min) by 151%, microwave heating with xylose (2 min) by 27%, and tannin inclusion (6%) by 32%. For peanut meal, autoclaving with xylose (24 min) increased RUP by 32%, conventional oven heating (60 min) by 36%, microwave heating with xylose (6 min) by 16%, and tannin inclusion (6%) by 8.1%. These treatments were selected for further evaluation due to their promising potential to enhance RUP supply in ruminant diets.

Despite these advances, the effects of increased RUP on ruminal fermentation and energy metabolism remain unclear. Since rumen-degradable protein (RDP) supports microbial growth and fermentation, excessive RUP may limit microbial protein synthesis and reduce fermentation efficiency, potentially impairing fiber digestion and volatile fatty acid (VFA) production, key contributors to the ruminant energy supply (NRC, 2001; Firkins et al., 2007). Therefore, understanding how these RUP-enhancing treatments influence ruminal fermentation is essential. In this next phase of our research, we aimed to evaluate the *in vitro* ruminal gas production (GP) kinetics, fermentation parameters, and *in vitro* dry matter digestibility (IVDMD) of these processed cottonseed and peanut meals using an *in vitro* GP system. We hypothesized that these processed ingredients would exhibit reduced total GP and altered ruminal fermentation compared to unprocessed feed.

2. Material and methods

2.1. Experiment location and ethic committee approval

The experiment was conducted in Sertãozinho, São Paulo, Brazil (21° 17' S, 48° 09' W). The experiment was approved by the Animal Use Ethics Committee of the Instituto de Zootecnia under the protocol n° 249-19.

2.2. Previous study

Three previous studies were conducted to evaluate various techniques for increasing the RUP content of common protein sources used in finishing beef cattle diets (Rigon et al., 2023; Loregian et al., 2023; Molosse et al., 2023). A total of 50 treatments were evaluated using two different protein sources: cottonseed (*Gossypium* spp.) and peanut (*Arachis hypogaea*) meals. These protein sources were subjected to the following processing methods: a) Control treatments (samples without processing); b) Autoclaving at 127 °C with an internal pressure of 117 kPa for different durations (8, 16, and 24 minutes), with and without the addition of xylose (2%); c) Toasting in a drying oven at 150 °C for different durations (30, 60, and 90 minutes), with and without the addition of xylose (2%); d) Microwave browning for different durations (2, 4, and 6 minutes), with and without the addition of xylose (2%); e) A commercial RUP source (SoyPass[®], Nutron Cargill, São Paulo, SP, Brazil); f) Treatment with four different concentrations of tannin solution [Tanfeed[®] 70% Tannic acid (700 g/kg DM): 0, 2.0, 4.0, and 6%]. Both *in situ* (Nocek, 1988) and *in vitro* (Calsamiglia and Stern, 1995; Gargallo et al., 2006) techniques were used to determine rumen-degradable protein (RDP), RUP, and intestinal digestibility of crude protein (CP).

Based on the results of *in situ* degradation kinetics and *in vitro* intestinal digestibility of protein, we selected the eight best processing methods (four for each feed: cottonseed and peanut meals) for further study. Consequently, the two protein sources were individually analyzed in two identical experiments.

2.3. Experimental designs

Experiment 1 (cottonseed meal) aimed to evaluate six different processed cottonseed meals. The treatments included a negative control (Ncontrol) with feed that was not subjected to xylose or heat processing, and five other treatments: feed treated with 2% xylose (Sigma-Aldrich, St Louis, MO, USA) and heated in a microwave oven for 2 minutes (Microwave oven) (Xin and Yu, 2021), feed treated with 2% xylose and heated in a conventional oven for 90 minutes (Conventional oven) (Chesini et al., 2023; Marques et al., 2024), feed treated with 2% xylose and heated in an autoclave for 8 minutes (Autoclave), feed treated with 6% tannin (Tannin; *Acacia mearnsii* extract - TANAC, Montenegro, RS, Brazil), and a commercial soybean-based product used as positive control (Pcontrol; SoyPass[®], Nutron Cargill, São Paulo, SP, Brazil).

For experiment 2 (peanut meal), the peanut meal was evaluated with the following treatments: feed without xylose and heat processing (Ncontrol), feed treated with 2% xylose and heated in a microwave oven for 6 minutes (Microwave oven) (Xin and Yu, 2021), feed heat-treated in a conventional oven for 60 minutes (Conventional oven) (Chesini et al., 2023; Marques et al., 2024), feed treated with 2% xylose and heated in an autoclave for 24 minutes (Autoclave), feed treated with 6% tannin (Tannin), and the same commercial soybean based product, as positive control (Pcontrol).

The experimental design and operational procedures followed the same protocol for both experiments. Thus, a 25-bottle automated *in vitro* gas production system (Ankom Technology, Macedon, NY, USA) equipped with wireless pressure sensors connected to a computer was used to evaluate the ruminal fermentation patterns of the tested ingredients. For the *in vitro* gas production (IVGP) assay, treatments were evaluated in three 48-hour fermentation incubations to assess the gas production profiles and ruminal fermentation parameters. In each incubation, ingredients were individually incubated in 250 mL bottles, which were randomly arranged in the incubator. Each fermentation batch included four replicates of each treatment, plus 1 blank (containing only rumen/mineral/buffer solution), totaling 75 observations.

For the IVDMD assay, a four-jar incubator system (TE-150, Tecnal, Piracicaba, SP, Brazil) was used under rotation and temperature control, in three 48-hour fermentation incubations for each experiment. Each fermenter jar has received three filter bags (F-57, Ankom Technology, Macedon, NY,

USA) per treatment plus three blanks, totaling 21 filter bags per jar, and 63 observations. The IVDMD of cottonseed and peanut meals were performed simultaneously.

2.4. Chemical analysis

All feeds were ground through a 1-mm screen (Wiley mill; Thomson Scientific Inc., Philadelphia, PA). The chemical analyses were performed following the AOAC (2006) methodology. Samples were analyzed for dry matter (DM, method 934.01), ash (method 938.08), crude protein (CP, method 990.13) and ether extract (EE, method 920.85). Organic matter (OM) was calculated as the difference between DM content and ash. The neutral detergent fiber (NDF) followed the methodology proposed by Van Soest et al. (1991) and adapted for the fiber determiner (TE-149, Tecnal, Piracicaba, SP, Brazil). The chemical composition of the ingredients based on cottonseed and peanut meal is presented in Table 1.

Table 1 - Chemical composition of ingredients used for cottonseed and peanut meals

Ingredient	Composition (g/kg DM)					
	DM (g/kg)	OM	CP	EE	NDF	RUP (g/kg CP)
Experiment 1 – Cottonseed meal ¹						
Ncontrol	910	937	543	16.4	169	286
Microwave oven	905	938	462	16.4	394	445
Conventional oven	923	941	472	16.4	471	666
Autoclave	899	943	449	16.4	351	461
Tannin	903	937	522	16.4	299	539
Pcontrol	909	936	565	19.4	141	649
Experiment 2 – Peanut meal ²						
Ncontrol	910	946	628	12.8	164	454
Microwave oven	928	947	639	12.8	240	608
Conventional oven	921	946	655	12.8	368	651
Autoclave	910	947	643	12.8	315	605
Tannin	912	948	580	12.8	257	537
Pcontrol	909	936	565	19.4	141	649

DM - dry matter; OM - organic matter; CP - crude protein; EE - ether extract; NDF - neutral detergent fiber; RUP - rumen-undegradable protein.

¹ Ncontrol = conventional cottonseed meal (CM); Microwave oven = CM treated with 2% xylose and heated in a microwave oven for 2 min; Conventional oven = CM treated with 2% xylose and heated in a conventional oven for 90 min; Autoclave = CM treated with 2% xylose and heated in an autoclave for 8 min; Tannin = CM treated with 6% tannin; Pcontrol = commercial soybean-based product.

² Ncontrol = conventional peanut meal (PM); Microwave oven = PM treated with 2% xylose and heated in a microwave oven for 6 min; Conventional oven = PM heat-treated in a conventional oven for 60 min; Autoclave = PM treated with 2% xylose and heated in an autoclave for 24 min; Tannin = PM treated with 6% tannin; Pcontrol = commercial soybean-based product.

2.5. Operational procedures

2.5.1. Ruminal fluid collection and buffer solutions preparation

Rumen fluid was collected from three Nellore steers, each cannulated in the rumen and averaging averaging 550 kg of body weight. The animals were housed in individual pens at an experimental feedlot in Sertãozinho, SP, Brazil (21°17'S, 48°09'W), with free access to water and diets. The steers were fed daily in the morning with a total mixed ration consisting of 60% corn silage and 40% concentrate (ground corn grain, citrus pulp pellet, soybean meal, and mineral mixture). Two hours after feeding (Yáñez-Ruiz et al., 2016), 2,000 mL of rumen fluid were collected from each animal, immediately filtered through four layers of cheesecloth, and kept in pre-warmed (39 °C) thermal bottles for immediate transport to the laboratory. For IVDMD incubation, the rumen fluid collection procedures were similar to those used for the IVGP assay. However, approximately 30 g of rumen solid particles were also added to the fermenter jars.

The IVGP buffer mineral solution was adapted from the methodology described by Menke and Steingass (1988) (Benedeti et al., 2018). The final solution consisted of 25 mL of rumen fluid and 50 mL of buffer mineral solution (1:2 v/v, 75 mL). The IVDM buffer solution was prepared according to McDougall (1948), with the final solution comprising 1,600 mL of buffer solution and 400 mL of rumen fluid for each jar (Benedeti et al., 2018). For both experiments, the rumen fluid was mixed with the buffer mineral solutions in a water bath at 39 °C under anaerobic conditions, maintained by continuous infusion of N₂.

2.5.2. *In vitro* gas production

The samples were introduced into the bottles and hydrated with deionized water to prevent particle dispersion. Each bottle received 0.5 ± 0.05 g (DM basis) of each ingredient. The headspace was continuously flushed with N₂ to maintain anaerobic conditions. After sample incubation, the bottles were closed and placed in a ventilated incubator (EI-450T, ENGCO, Piracicaba, SP, Brazil) under constant heating (39 °C) and agitation (83 rpm) for 48 h. The system was set to read cumulative pressure every five minutes, and data were recorded every 60 minutes.

The cumulative pressure data at 24 and 48 hours were converted to mL according to Tagliapietra et al. (2011), using the formula: $GP \text{ (mL)} = \left(\frac{P_c}{P_o}\right) \times V_o$, in which P_c is the cumulative pressure change (kPa) in the bottle headspace, V_o is the bottle headspace volume (170 mL), and P_o is the atmospheric pressure read by the equipment at the beginning of the measurement. The final GP volumes were corrected for inoculum contribution by subtracting the final GP of the blank bottles. Cumulative pressures were fitted to a one-pool exponential model, as described by Huhtanen et al. (2008), to predict fermentation rate and gas pool size. Metabolizable energy (ME) was calculated according to Menke and Steingass (1988), using the formula: $ME \text{ (MJ/kg DM)} = 1.06 + 0.157GP + 0.084CP + 0.22CF - 0.081CA$, in which GP is the net gas production (mL/200 mg DM) at 24 and 48 h; CP, CF, and CA are crude protein, crude fat and crude ash (% DM), respectively. The *in vitro* organic matter digestibility (IVOMD) was calculated according to Menke and Steingass (1988), as $IVOMD \text{ (g/kg DM)} = 31.55 + 0.8343GP$, in which GP is the net gas production (mL/200 mg DM) at 24 and 48 h.

2.5.3. Ammonia-nitrogen (NH₃-N), pH, volatile fatty acids (VFA), and methane (CH₄)

The solution pH was measured at the beginning and at the end of each 48-hour incubation. At the same time, one subsample was collected from two bottles per treatment to determine VFA and NH₃-N concentrations. The collected material was filtered through four layers of cheesecloth. Then, 0.2 mL of a 500 mL/L H₂SO₄ solution was added to preserve the samples for NH₃-N determination. The NH₃-N concentration was determined using the colorimetric method described by Chaney and Marbach (1962). Total VFA concentrations and profiles were determined using gas chromatography (Nexis CG-2030AF, Shimadzu, Montevideo, Uruguay, equipped with a fused silica capillary column [30 m × 0.53 mm × 0.5 µm film thickness, Supelco, Darmstadt, Germany]) and a flame ionization detector (FID), with Nitrogen as a carrier gas (flow rate of 34.5 mL/min). The CH₄ production was estimated according to Owens and Goetsch (1988) based on VFA stoichiometry using the following equation: $CH_4 = (\frac{1}{2} \text{ acetate}) + (\frac{1}{2} \text{ butyrate}) - (\frac{1}{4} \text{ propionate})$.

2.5.4. *In vitro* dry matter digestibility

The ingredients were individually weighed (0.50 g per bag) into filter bags (F-57, Ankom Technology, Macedon, NY, USA). The bags were then heat-sealed and placed inside each fermenter. After the 48-hour incubation, the bags were washed with cold water and analyzed for DM content (Holden, 1999). The IVDM was calculated as $IVDM, \text{ g/kg DM} = (100 \pm [W_3 - (W_1 \times C_1)] \times 100 / (\frac{W_2 \times DM}{10})) \times 10$, in which W₁ = bag tare weight, W₂ = sample weight, W₃ = final bag weight after *in vitro* incubation, C₁ = blank bag correction (final oven-dried weight/original blank bag weight), DM = g per kg of dry matter.

2.6. Statistical analysis

All results were tested for residual normality and variance homogeneity (Davis and Stephens, 1989). For IVGP of Exp. 1 and Exp. 2, data were collected and analyzed using a randomized block design, using mixed models and considering ingredients as fixed effects and incubation as random, using the following model:

$$Y_{ij} = \mu + T_i + B_j + e_{ij}$$

in which μ is the overall mean, T_i is the fixed effect of treatment i , B_j is the random effect of block (incubation) j , and e_{ij} is the random error with mean 0 and σ^2 .

The bottle averages within each incubation were considered experimental units. When treatment effects were observed, least squared means were compared using the T test (5% significance level). The NLIN procedure of SAS 9.4 was used to estimate the fermentation rate and gas pool size of each replication. Then the parameters of the nonlinear functions were then compared using the same model, and differences were declared at $P < 0.05$. For IVDM, the same model was applied in both experiments, with bag averages within each incubation considered experimental units. All statistical analyses were performed using SAS 9.4 for Windows (Statistical Analysis Institute System, Inc., Cary, NC, USA) through the GLIMMIX procedure, with differences declared at $P < 0.05$.

3. Results

3.1. Experiment 1: cottonseed meal

The Ncontrol treatment exhibited a higher fermentation rate compared to the processing methods ($P < 0.01$; Table 2; Figure 1). Additionally, the Conventional oven treatment had a lower fermentation rate than both Ncontrol and Pcontrol ($P < 0.01$). However, the Conventional oven treatment also showed a greater gas pool size ($P < 0.01$) compared to Ncontrol and Pcontrol, while the Tannin treatment had the lowest value for this parameter ($P < 0.01$). Furthermore, the Tannin treatment also had lower values of total GP (mL/g DM; at 24 and 48 h), ME and IVOMD, in comparison to Ncontrol and Pcontrol ($P < 0.01$).

Feed processing did not significantly affect pH values ($P = 0.31$; Table 3). The Ncontrol produced more total VFA than Pcontrol but was similar to the Microwave oven treatment ($P < 0.01$). In terms

Table 2 - Effects of different processing methods applied to cottonseed meal on ruminal kinetics, total gas production, *in vitro* organic matter digestibility (IVOMD), and metabolizable energy (ME) in a gas production system

Item	Processing methods ¹						SEM	P-value
	Ncontrol	Microwave oven	Conventional oven	Autoclave	Tannin	Pcontrol		
Fermentation rate (h ⁻¹)	0.077a	0.065b	0.025c	0.061b	0.054b	0.077a	0.007	<0.01
Total gas production (mL/g DM)								
24 h	74.4ab	73.7ab	62.0bc	66.6ab	47.5c	80.9a	7.13	<0.01
48 h	92.4a	92.4a	92.3a	82.2ab	60.3b	102a	8.63	<0.01
IVOMD ² (g/kg DM)	469a	469a	469a	453ab	416b	487a	1.44	<0.01
ME ³ (MJ/kg DM)	8.37ab	7.70bc	7.89bc	7.31c	7.18c	8.94a	0.27	<0.01

SEM - standard error mean.

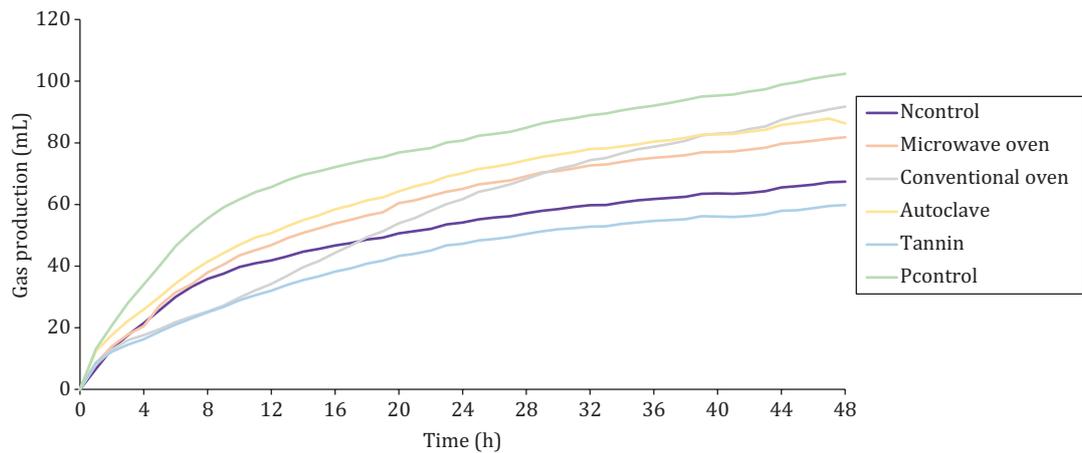
¹ Ncontrol = conventional cottonseed meal (CM); Microwave oven = CM treated with 2% xylose and heated in a microwave oven for 2 min; Conventional oven = CM treated with 2% xylose and heated in a conventional oven for 90 min; Autoclave = CM treated with 2% xylose and heated in an autoclave for 8 min; Tannin = CM treated with 6% tannin; Pcontrol = commercial soybean-based product.

² *In vitro* organic matter digestibility: IVOMD (g/kg DM) = 31.55 + 0.8343GP, where GP is the net gas production (mL/200 mg DM) (Menke and Steingass, 1988).

³ Metabolizable energy: ME (MJ/kg DM) = 2.20 + (0.1357 × GP₂₀₀) + (0.0057 × CP) where GP₂₀₀ (mL/200 mg of DM incubated) (Menke and Steingass, 1988).

a,b,c - Values in rows with different letters differ significantly ($P < 0.05$).

of VFA profile, the Tannin resulted in lower production of butyrate ($P = 0.02$), *iso*-valerate ($P < 0.01$) and branched-chain VFA (BCVFA) ($P < 0.01$) compared to Ncontrol and Pcontrol. Conversely, Pcontrol had higher levels of butyrate ($P = 0.02$), *iso*-valerate ($P < 0.01$) and BCVFA ($P < 0.01$) than Ncontrol. Processing methods did not affect the concentrations of acetate ($P = 0.62$), propionate ($P = 0.94$), and *iso*-butyrate ($P = 0.23$), nor the acetate:propionate ratio ($P = 0.93$) and $\text{NH}_3\text{-N}$ concentration ($P = 0.19$). The Tannin and Conventional oven treatments had lower IVDMD values ($P < 0.01$; Figure 2A), while Pcontrol had the highest IVDMD value ($P < 0.01$) compared to Ncontrol. Feed processing did not influence the estimated enteric CH_4 values ($P = 0.97$; Figure 3A).



Ncontrol = conventional cottonseed meal (CM); Microwave oven = CM treated with 2% xylose and heated in a microwave oven for 2 min; Conventional oven = CM treated with 2% xylose and heated in a conventional oven for 90 min; Autoclave = CM treated with 2% xylose and heated in an autoclave for 8 min; Tannin = CM treated with 6% tannin; Pcontrol = commercial soybean-based product.

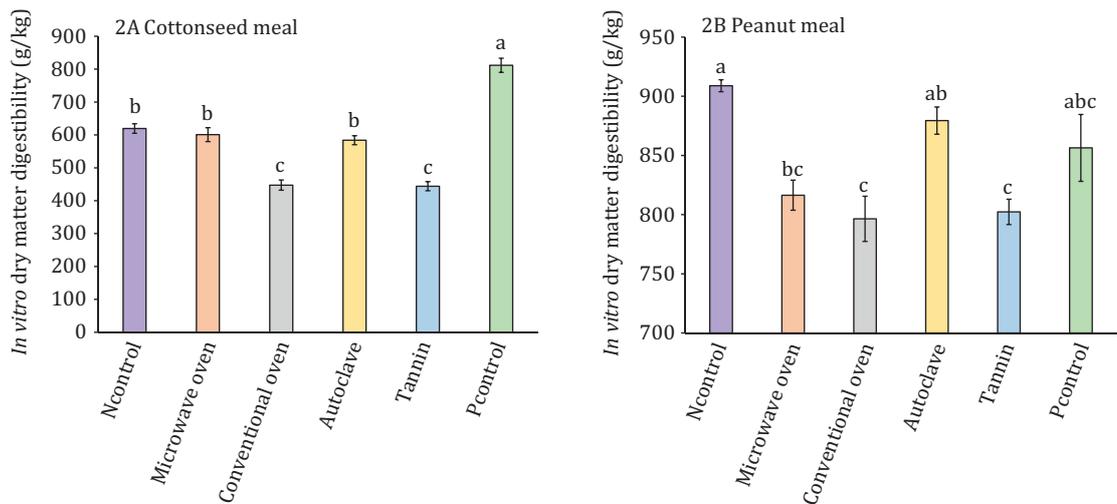
Figure 1 - Ruminal gas production profiles of cottonseed meal treated with different processing methods.

Table 3 - Effects of different processing methods applied to cottonseed meal on ruminal parameters in a gas production system

Item	Processing methods ¹						SEM	P-value
	Ncontrol	Microwave oven	Conventional oven	Autoclave	Tannin	Pcontrol		
Final pH	6.66	6.69	6.67	6.57	6.71	6.62	0.09	0.31
Total VFA (mM/g DM)	241a	220ab	188b	176b	179b	183b	28.1	<0.01
VFA profile (mol/100 mol)								
Acetate	63.8	64.3	64.3	63.7	66	62.7	1.99	0.62
Propionate	21.9	21.6	20.9	21.6	21.2	20.9	1.42	0.94
Butyrate	9.66ab	9.53ab	9.82ab	10.0ab	8.74b	10.9a	0.98	<0.05
<i>Iso</i> -butyrate	1.66	1.62	1.76	1.62	1.39	1.63	0.15	0.23
<i>Iso</i> -valerate	2.91ab	2.84b	3.15ab	2.95ab	2.53b	3.63a	0.35	<0.01
Acetate:propionate	2.92	2.98	3.07	3.03	3.2	3.03	0.35	0.93
Branched-chain VFA	4.58ab	4.47ab	4.92a	4.58ab	3.93b	5.27a	0.49	<0.01
$\text{NH}_3\text{-N}$ (mg/dL)	25.4	24.8	23.4	25	25.6	28.8	2.17	0.19

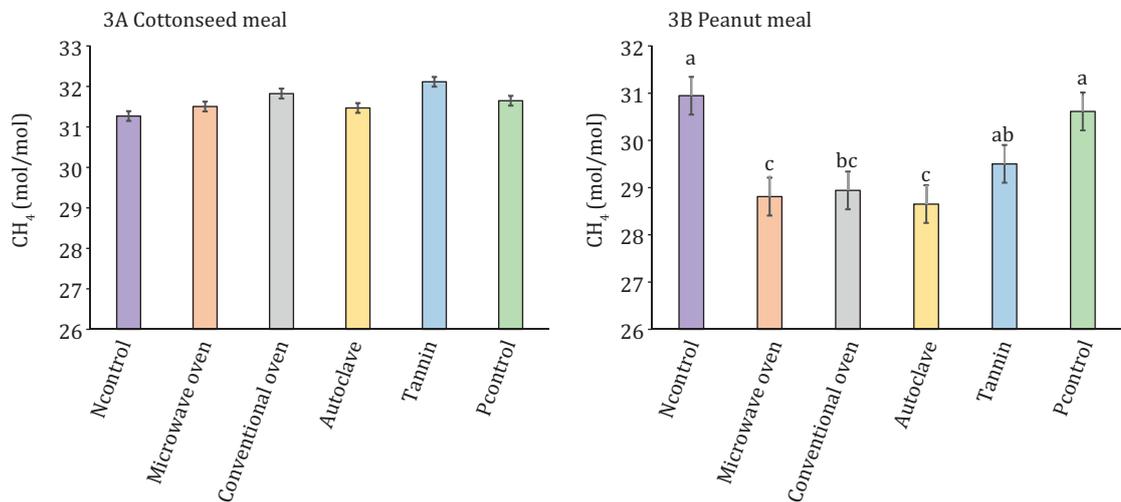
DM - dry matter; VFA - volatile fatty acids; SEM - standard error mean.

¹ Ncontrol = conventional cottonseed meal (CM); Microwave oven = CM treated with 2% xylose and heated in a microwave oven for 2 min; Conventional oven = CM treated with 2% xylose and heated in a conventional oven for 90 min; Autoclave = CM treated with 2% xylose and heated in an autoclave for 8 min; Tannin = CM treated with 6% tannin; Pcontrol = commercial soybean-based product.
a,b,c - Values in rows with different letters differ significantly ($P < 0.05$).



2A: Ncontrol = conventional cottonseed meal (CM); Microwave oven = CM treated with 2% xylose and heated in a microwave oven for 2 min; Conventional oven = CM treated with 2% xylose and heated in a conventional oven for 90 min; Autoclave = CM treated with 2% xylose and heated in an autoclave for 8 min; Tannin = CM treated with 6% tannin; Pcontrol = commercial soybean-based product.
2B: Ncontrol = conventional peanut meal (PM); Microwave oven = PM treated with 2% xylose and heated in a microwave oven for 6 min; Conventional oven = PM heat treated in a conventional oven for 60 min; Autoclave = PM treated with 2% xylose and heated in an autoclave for 24 min; Tannin = PM treated with 6% tannin; Pcontrol = commercial soybean-based product.
a,b,c - Values with different letters differ significantly ($P < 0.01$).

Figure 2 - The *in vitro* dry matter digestibility of cottonseed and peanut meals treated with different processing methods.



3A: Ncontrol = conventional cottonseed meal (CM); Microwave oven = CM treated with 2% xylose and heated in a microwave oven for 2 min; Conventional oven = CM treated with 2% xylose and heated in a conventional oven for 90 min; Autoclave = CM treated with 2% xylose and heated in an autoclave for 8 min; Tannin = CM treated with 6% tannin; Pcontrol = commercial soybean-based product.
3B: Ncontrol = conventional peanut meal (PM); Microwave oven = PM treated with 2% xylose and heated in a microwave oven for 6 min; Conventional oven = PM heat treated in a conventional oven for 60 min; Autoclave = PM treated with 2% xylose and heated in an autoclave for 24 min; Tannin = PM treated with 6% tannin; Pcontrol = commercial soybean-based product.
a,b,c - Values with different letters differ significantly ($P < 0.05$).

Figure 3 - The enteric CH₄ production of cottonseed and peanut meals treated with different processing methods.

3.2. Experiment 2: peanut meal

Regarding ruminal kinetics, Conventional oven treatment exhibited a lower fermentation rate compared to Ncontrol ($P < 0.01$; Table 4, Figure 4). The Tannin treatment resulted in lower total GP at both 24 and 48 hours compared to Ncontrol ($P < 0.01$). Additionally, the Tannin had lower values for IVOMD ($P < 0.01$) and ME ($P < 0.01$) compared to both Ncontrol and Pcontrol.

The Pcontrol treatment had the lowest pH ($P < 0.01$), while Tannin had the highest pH ($P < 0.01$; Table 5). There were no significant differences among processing methods for total VFA ($P = 0.57$). In terms of the VFA profile, Ncontrol had a lower propionate concentration ($P = 0.01$) and higher values of butyrate ($P < 0.01$), *iso*-valerate ($P < 0.01$), total BCVFA ($P < 0.01$) and acetate:propionate ratio ($P = 0.01$) compared to Pcontrol. Conversely, the Tannin treatment had lower concentrations of butyrate ($P < 0.01$), *iso*-butyrate ($P = 0.04$), *iso*-valerate ($P < 0.01$) and BCVFA ($P < 0.01$) than Ncontrol. The Autoclave

Table 4 - Effects of different processing methods applied to peanut meal on ruminal kinetics, total gas production, *in vitro* organic matter digestibility (IVOMD), and metabolizable energy (ME) in a gas production system

Item	Processing methods ¹						SEM	P-value
	Ncontrol	Microwave oven	Conventional oven	Autoclave	Tannin	Pcontrol		
Fermentation rate (h^{-1})	0.069ab	0.054bc	0.035d	0.050cd	0.066b	0.085a	0.63	<0.01
Gas pool size (mL/g DM)	71.3ab	68.6bc	82.9a	69.8bc	57.8c	60.6bc	9.15	<0.01
Total gas production (mL/g DM)								
24 h	84.3a	69.0b	60.2b	64.4b	62.7b	71.8ab	9.23	<0.01
48 h	108a	97.2ab	96.3ab	92.9ab	86.7b	95.5ab	9.92	<0.01
IVOMD ² (g/kg DM)	495a	477ab	476ab	470ab	460b	474ab	1.59	<0.01
ME ³ (MJ/kg DM)	9.56a	9.33ab	9.39a	8.84bc	8.37c	8.71c	0.31	<0.01

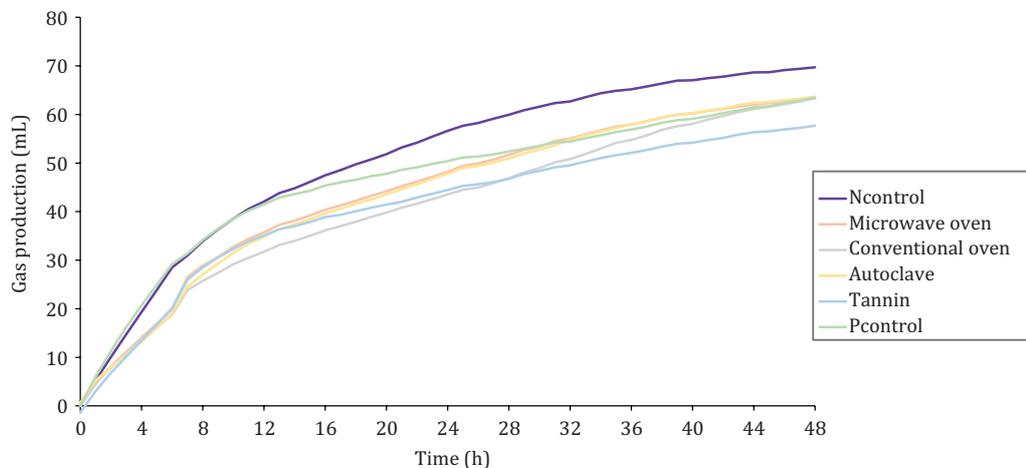
SEM - standard error mean.

¹ Ncontrol = conventional peanut meal (PM); Microwave oven = PM treated with 2% xylose and heated in a microwave oven for 6 min; Conventional oven = PM heat treated in a conventional oven for 60 min; Autoclave = PM treated with 2% xylose and heated in an autoclave for 24 min; Tannin = PM treated with 6% tannin; Pcontrol = commercial soybean-based product.

² *In vitro* organic matter digestibility: IVOMD (g/kg DM) = $31.55 + 0.8343\text{GP}$, where GP is the net gas production (mL/200 mg DM) (Menke and Steingass, 1988).

³ Metabolizable energy: ME (MJ/kg DM) = $2.20 + (0.1357 \times \text{GP}_{200}) + (0.0057 \times \text{CP})$ where GP_{200} (mL/200 mg of DM incubated) (Menke and Steingass, 1988).

a,b,c - Values in rows with different letters differ significantly ($P < 0.05$).



Ncontrol = conventional peanut meal (PM); Microwave oven = PM treated with 2% xylose and heated in a microwave oven for 6 min; Conventional oven = PM heat treated in a conventional oven for 60 min; Autoclave = PM treated with 2% xylose and heated in an autoclave for 24 min; Tannin = PM treated with 6% tannin; Pcontrol = commercial soybean-based product.

Figure 4 - Ruminal gas production profiles of peanut meal treated with different processing methods.

resulted in a higher $\text{NH}_3\text{-N}$ concentration compared to Ncontrol ($P = 0.03$). Both the Conventional oven and Tannin treatments had the lowest IVDMD values ($P < 0.01$). Ncontrol and Pcontrol had the highest estimated CH_4 emissions ($P = 0.03$; Figure 3B).

Table 5 - Effects of different processing methods applied to peanut meal on ruminal parameters in a gas production system

Item	Processing methods ¹						SEM	P-value
	Ncontrol	Microwave oven	Conventional oven	Autoclave	Tannin	Pcontrol		
Final pH	6.70b	6.72ab	6.70b	6.76ab	6.88a	6.60c	0.03	<0.01
Total VFA (mM/g DM)	200	192	221	222	230	213	24.5	0.57
VFA profile (mol/100 mol)								
Acetate	62.8ab	61.0b	61.6ab	61.4ab	62.4ab	63.4a	1.99	<0.05
Propionate	21.7b	24.7ab	24.7ab	25.0a	24.1ab	22.7ab	1.42	<0.01
Butyrate	9.89a	8.94b	8.57b	8.71b	8.65b	9.14ab	0.98	<0.01
<i>Iso</i> -butyrate	1.64ab	1.80a	1.68ab	1.71ab	1.59b	1.60ab	0.16	<0.05
<i>Iso</i> -valerate	3.88a	3.51a	3.36ab	3.33ab	3.10b	3.10b	0.35	<0.01
Acetate:propionate	3.01a	2.47ab	2.50ab	2.44b	2.58a	2.80a	0.29	<0.05
Branched-chain VFA	5.52a	5.31a	5.05ab	5.05ab	4.69b	4.71b	0.49	<0.01
$\text{NH}_3\text{-N}$ (mg/dL)	26.8b	28.6ab	29.2ab	32.4a	29.9ab	29.6ab	2.43	<0.01

DM - dry matter; VFA - volatile fatty acids; SEM - standard error mean.

¹ Ncontrol = conventional peanut meal (PM); Microwave oven = PM treated with 2% xylose and heated in a microwave oven for 6 min; Conventional oven = PM heat treated in a conventional oven for 60 min; Autoclave = PM treated with 2% xylose and heated in an autoclave for 24 min; Tannin = PM treated with 6% tannin; Pcontrol = commercial soybean-based product.

a,b,c - Values in rows with different letters differ significantly ($P < 0.05$).

4. Discussion

Different types of feed processing to increase RUP can affect the extent of ruminal fermentation and GP (NRC, 2021). Consistent with our hypothesis, tannin treatment resulted in the lowest total GP in both experiments. This outcome may be attributed to the formation of complexes between tannins and proteins via hydrogen bonds (Castro-Montoya et al., 2018). Tannins form stable complexes in the rumen due to the ruminal pH, which prevents ruminal protein degradation (Patra and Saxena, 2011).

Generally, a close correlation is expected between gas production (GP) and the digestibility of organic substrates (Menke and Steingass, 1988). In our study, we observed that tannin treatment also resulted in the lowest IVOMD for both feeds. These results, along with the reduced total GP, may be linked to decreased ruminal protein degradability. A study evaluating the effects of different levels of condensed tannins indicated a reduction in OMD due to the tannins' impact on rumen protein degradation and ruminal fermentation (Koenig and Beauchemin, 2018). This reduction in GP and OMD may also lead to decreased ME of these tannin-treated ingredients, as there is less substrate available for rumen microbes (Menke and Steingass, 1988).

Another factor that could explain these results is the change in the chemical composition of processed feed, specifically the increase in their NDF proportion, which can result in reduced fermentation (Jahani-Azizabadi, 2010). Tannins may also exert toxic effects on ruminal microorganisms, reducing fiber degradation and OM digestibility (Adejoro et al., 2018). The use of condensed tannins may reduce substrate degradation by impairing the attachment of microbes to feed particles and by binding to nutrients and microbial enzymes (Castro-Montoya et al., 2018). Therefore, our results suggest that the use of tannins may affect OM and fiber degradability, resulting in lower GP and ME.

In vitro gas production is closely related to digestibility and energy feed values for ruminants when incubated with rumen fluid (Menke and Steingass, 1988). Furthermore, many factors can affect the fermentation rates of feedstuffs, such as their chemical composition and processing methods (NASEM, 2016; NRC, 2021; Chesini et al., 2023). Heat processing, for instance, may reduce the fermentation rate of feed due to the Maillard reaction (Francisquini et al., 2017). This chemical reaction forms stable polymers between carbohydrates and proteins, which are resistant to enzymatic attack by ruminal microorganisms (Haryanto, 2014). Additionally, feeds with higher RUP levels can have lower *in vitro* fermentation rates (Paula et al., 2017). Therefore, we expected that ingredients processed with heat techniques would exhibit reduced fermentation and total GP, given their influence on protein protection.

Our results demonstrated that the Ncontrol treatment had a greater total VFA concentration than the conventional oven and autoclave treatments. For cottonseed and peanut meals, Ncontrol had the lowest propionate concentration and the highest butyrate, iso-valerate, BCVFA concentrations, and acetate:propionate ratio. We believe these results may be linked to the lack of processing on cottonseed meal (Ncontrol) and its low NDF content, which can result in a greater fermentation rate and higher total GP. Generally, cottonseed meal only undergoes milling, which contributes to the increase in the total digestible nutrients content of this feed (NRC, 2021; Marques et al., 2024).

The Tannin treatment had the lowest BCVFA concentration for cottonseed and peanut meals, and we believe that this result may be related to possible unavailability of carbohydrates due to their possible complexation with proteins. The BCVFA are the result from the deamination of the branched-chain amino acids valine and leucine, which give rise to iso-butyrate and iso-valerate, respectively (NRC, 2021). These BCVFA are essential for bacteria that break down fibrous carbohydrates (Owens and Basalan, 2016). We believe that the lower BCVFA concentration of tannin also may have contributed to limitations in fibrolytic bacteria growth, the main group of bacteria found in the rumen, which can be explained by the lower total gas production and the smaller gas pool size. Nevertheless, cottonseed and peanut meals have lower leucine and valine concentrations than soybean meal, which can lead to lower BCVFA concentrations.

Enteric CH₄ formation is directly correlated with the degradation of fibrous carbohydrates and acetate production, and it can be considered an energy loss in the ruminal environment (Chaney and Marbach, 1962; Owens and Basalan, 2016). However, enteric CH₄ production is essential for maintaining ruminal fermentation, as it acts as a hydrogen sink (Benedeti et al., 2018). For peanut meal, the Ncontrol and Pcontrol treatments had the highest estimated CH₄ and acetate concentrations, a pattern also observed in the tannin treatment. The processing methods with the lowest acetate values corresponded to the lowest estimated CH₄ values. We believe these results may be due to the greater extent of ruminal degradation of the fibrous carbohydrate fraction in Ncontrol and Pcontrol, as the type of processing should not affect the NDF fraction. These results may also be attributed to the larger gas pool size and total GP of Ncontrol and Pcontrol, leading to increased ruminal VFA and CH₄ synthesis. The ruminal degradability of proteins results in the release of ammonia in the rumen fluid. We believe the lack of response in NH₃-N may have occurred because our study was conducted in a batch culture system, which does not allow for the input of substrates and absorption of compounds such as ammonia (Hristov et al., 2012).

In our study, we observed that the conventional oven and tannin treatments resulted in the lowest IVDMD for both cottonseed and peanut meals. We believe these results may be associated with the effects of heating and the addition of tannins on the feeds, as mentioned earlier. Both treatments can hinder microbial access to feed particles, possibly contributing to lower digestibility values (Adejoro et al., 2018). However, the effects of tannins on IVDMD have been conflicting. However, our results cannot be consolidated yet due to the variation in the profile of the evaluated feeds, and more evaluations of these ingredients are needed. Currently, no information is available on *in vitro* assays using peanut and cottonseed meals in total mixed rations (TMR) for ruminants, highlighting the need for more complex studies with these feeds.

5. Conclusions

Our results suggest that tannin treatment had the most significant effects on the fermentation rate, total GP, IVOMD, ME, IVDMD and total BCVFA concentration for cottonseed and peanut meals, compared to the untreated feeds. The conventional oven treatment also showed a potential to change the fermentation pattern of these feeds. These results represent the possible inclusion of these alternative sources of RUP in beef cattle nutrition as feasible replacements for conventional sources. However, these ingredients must be evaluated in experiments with TMR and *in vivo* studies, especially with peanut meal, due to its higher IVOMD, ME, and IVDMD values.

Data availability

The authors are committed to sharing the data and artifacts supporting the results in the paper upon request.

Author contributions

Conceptualization: Benedeti, P. D. B. and Paula, E. M. **Data curation:** Rigon, F. and Magnani, E. **Formal analysis:** Monteiro, H. F.; Zampar, A. and Marcondes, M. I. **Funding acquisition:** Branco, R. H.; Benedeti, P. D. B. and Paula, E. M. **Investigation:** Rigon, F.; Magnani, E.; Cagliari, A. R.; Loregian, K. E.; Casagrande, A. C.; Amâncio, B. R.; Pereira, D. A. B. and Borges, H. A. M. **Methodology:** Rigon, F.; Magnani, E.; Amâncio, B. R.; Benedeti, P. D. B. and Paula, E. M. **Project administration:** Branco, R. H.; Benedeti, P. D. B. and Paula, E. M. **Resources:** Branco, R. H.; Benedeti, P. D. B. and Paula, E. M. **Supervision:** Branco, R. H.; Benedeti, P. D. B. and Paula, E. M. **Validation:** Benedeti, P. D. B. and Paula, E. M. **Visualization:** Rigon, F.; Marcondes, M. I.; Benedeti, P. D. B. and Paula, E. M. **Writing – original draft:** Rigon, F.; Marcondes, M. I.; Benedeti, P. D. B. and Paula, E. M. **Writing – review & editing:** Magnani, E.; Cagliari, A. R.; Loregian, K. E.; Casagrande, A. C.; Amâncio, B. R.; Pereira, D. A. B.; Borges, H. A. M.; Monteiro, H. F.; Zampar, A. and Branco, R. H.

Conflict of interest

The authors declare no conflict of interest.

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