Agrociencia Uruguay 2026 | Volume 30 | Article e1848
DOI: 10.31285/AGRO.30.1848
ISSN 2730-5066

Additives and their Effect on Fiber Utilization and Ruminal Fermentation Profile in Grazing Dairy Cows

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Abstract

The objective of this study was to evaluate the effects of sodium monensin (MON) and a phytochemical mixture (PHY) in dairy cows grazing alfalfa and supplemented with total mixed rations (TMR) on the: 1) neutral detergent fiber (NDF) degradability of alfalfa hay (AH), 2) *in-situ* organic matter digestibility (ISDom) of different feeds (four cultivated pastures, AH, pre- and postpartum TMR, and a simulated diet of alfalfa+postpartum TMR), and 3) ruminal fermentation profile. Nine rumen-fistulated Holstein cows were randomly assigned to one of three treatments: control (C), MON (0.30 g/cow/day), or PHY (50 g/cow/day). Additives were incorporated into the concentrate portion of the TMR from 30 days prepartum to 60 days postpartum. NDF degradation kinetics of AH and ISDom were determined between days 56 and 60 postpartum. Rumen fluid was collected at 0, 4, 8, 16, and 24 h after feeding to measure pH, ammonia nitrogen (NH_3-N), volatile fatty acids (VFA), and protozoa counts. Both additives reduced the ISDom of the prepartum TMR ($P<0.05$) and the NDF degradation rate of AH ($P<0.05$) without affecting its potential degradability. They also promoted a more gluconeogenic VFA profile ($P<0.05$) and decreased protozoa counts ($P<0.05$). PHY further reduced NH_3-N concentrations compared with MON and C ($P<0.05$). In conclusion, MON and PHY improved ruminal fermentation efficiency without affecting the potential for NDF degradation and PHY additionally enhanced nitrogen utilization, highlighting its potential as a natural alternative to MON in pasture-based dairy systems supplemented with mixed rations.

Keywords: phytochemicals, monensin, rumen fermentation, in situ degradability, grazing

Editor

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Received *10 Nov 2025*
Accepted *8 Jul 2026*
Published *12 Aug 2026*

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Dorao, C., Cáceres, A., Trujillo, A. I., Casal, A., Mattiauda, D. A., Dall-Orsoletta, A. C., Arturo-Schaan, M., & Bruni, M. Á. (2026). Additives and their effect on fiber utilization and ruminal fermentation profile in grazing dairy cows. *Agrociencia Uruguay, 30*, Article e1848. https://doi.org/10.31285/AGRO.30.1848

Aditivos y su efecto en la utilización de fibra y perfil de fermentación ruminal en vacas lecheras en pastoreo

Resumen

Se evaluaron los efectos de monensina sódica (MON) y de una mezcla de fitoquímicos (PHY) en vacas lecheras consumiendo alfalfa y ración totalmente mezclada (TMR) sobre: 1) degradabilidad de la fibra detergente neutra (FDN) de un heno de alfalfa (HA), 2) digestibilidad *in situ* de la materia orgánica (ISDom) de diferentes alimentos (cuatro pasturas, HA, TMR pre y posparto, y alfalfa + TMR posparto) y 3) perfil de fermentación ruminal. Nueve vacas Holstein fistuladas ruminalmente se asignaron aleatoriamente a uno de tres tratamientos: control (C), MON (0,30 g/vaca/día) o PHY (50 g/vaca/día). Los aditivos se incorporaron al concentrado de TMR desde 30 días antes hasta 60 días después del parto. La cinética de degradación de FDN del HA y la ISDom se determinaron entre los días 56 y 60 posparto. Se colectó fluido ruminal a las 0, 4, 8, 16 y 24 h posalimentación para determinar pH, nitrógeno amoniacal (NH_3-N), ácidos grasos volátiles (AGV) y conteo de protozoarios. Ambos aditivos redujeron la ISDom de TMR preparto ($P<0,05$) y la tasa de degradación de FDN del HA ($P<0,05$), sin afectar su degradabilidad potencial. Asimismo, produjeron un perfil de AGV más gluconeogénico ($P<0,05$), redujeron el número de protozoarios ($P<0,05$) y PHY disminuyó la concentración de NH_3-N en comparación con MON y C ($P<0,05$). Los aditivos mejoraron la eficiencia de fermentación ruminal sin comprometer la degradabilidad potencial de la FDN y PHY, además, mejoró la utilización del nitrógeno, posicionándose como alternativa natural a MON en sistemas pastoriles mixtos.

Palabras clave: fitoquímicos, monensina, fermentación ruminal, degradabilidad in situ, pastoreo

Aditivos e seu efeito na utilização de fibras e no perfil de fermentação ruminal em vacas leiteiras em pastagem

Resumo

Os efeitos da monensina sódica (MON) e de uma mistura fitoquímica (PHY) em vacas leiteiras consumindo alfafa e uma ração totalmente mista (TMR) foram avaliados quanto a: 1) degradabilidade da fibra em detergente neutro (FDN) do feno de alfafa (FA); 2) digestibilidade in situ da matéria orgânica (ISDom) dos diferentes alimentos (quatro pastagens cultivadas, FA, TMR pré e pós-parto e alfafa + TMR pós-parto) e 3) perfil de fermentação ruminal. Nove vacas holandesas fistuladas no rúmen foram distribuídas aleatoriamente em três tratamentos: controle (C), MON (0,30 g/vaca/dia) ou PHY (50 g/vaca/dia). Os aditivos foram incluídos na fração concentrada da TMR desde 30 dias antes até 60 dias após o parto. A cinética de degradação da FDN do FA e a ISDom foram avaliadas entre os dias 56 e 60 pós-parto. O fluido ruminal foi coletado às 0, 4, 8, 16 e 24 h após a alimentação para determinação do pH, nitrogênio amoniacal (NH_3-N), ácidos graxos voláteis (AGV) e contagem de protozoários. Ambos os aditivos reduziram a ISDom da TMR pré-parto ($P<0,05$) e a taxa de degradação da FDN do FA ($P<0,05$), sem alterar sua degradabilidade potencial. Também promoveram um perfil de AGV mais gliconeogênico ($P<0,05$), reduziram a contagem de protozoários ($P<0,05$) e o PHY diminuiu a concentração de NH_3-N em comparação com MON e C ($P<0,05$). Conclui-se que os aditivos aumentaram a eficiência da fermentação ruminal sem afetar o potencial de degradação da FDN, e que o PHY, adicionalmente, melhorou o aproveitamento do nitrogênio, posicionando-se como alternativa natural à MON em sistemas de pastagens e dietas mistas.

Palavras-chave: fitoquímicos, monensina, fermentação ruminal, degradabilidade in situ, pastejo

1. Introduction

Globally, dairy farmers and the dairy industry are challenged to enhance both milk yield and quality, while also prioritizing animal welfare and reducing environmental impact. The use of feed additives in ruminant nutrition is a widely adopted strategy due to its potential to enhance production efficiency by improving nutrient utilization, as well as mitigating the adverse effects of diets on animal health and the environment (Kholif & Olafadehan,

2021). Among the additives used are various compounds with modulatory action on the ruminal microbiota, such as ionophores, enzymes, specific nutrients, and plant-derived compounds known as phytochemicals (Firkins & Mitchell, 2023).

Monensin is the most commonly used ionophore additive in ruminant diets due to its ability to improve production efficiency (Firkins & Mitchell, 2023). However, its effect can vary depending on factors such as breed, lactation stage, diet composition, and doses (Firkins & Mitchell, 2023; Rezaei Ahvanooei et al., 2023). Under total mixed ration (TMR) feeding conditions, the effect of monensin on the digestive utilization of different nutrients has been inconsistent, with some studies having reported increases in the *in-situ* digestibility (ISD) of neutral detergent fiber (NDF) (Plaizier et al., 2000), acid detergent fiber (ADF) (Plaizier et al., 2000), and nitrogen (Benchaar et al., 2006), whereas others have found no effect (Benchaar, 2016, 2020; Benchaar et al., 2006; Vedovatto et al., 2020). Additionally, several researchers observed no changes in ruminal pH (Benchaar, 2016, 2020; Vedovatto et al., 2020), ammonia nitrogen concentration (NH_3-N) (Benchaar, 2016, 2020), propionate (C3) proportion (Benchaar, 2016; Benchaar et al., 2006), the acetate:propionate (C2:C3) ratio (Benchaar, 2016; Benchaar et al., 2006), protozoa population (Benchaar, 2016, 2020; Benchaar et al., 2006; McGarvey et al., 2019), or methane (CH_4) production (Benchaar, 2020). In contrast, other studies reported a tendency to increases on ruminal pH (Benchaar et al., 2006) and C3 proportion (Benchaar, 2020; Vedovatto et al., 2020), decreases in both the C2:C3 ratio (Benchaar, 2020; Vedovatto et al., 2020) and NH_3-N concentration (Benchaar et al., 2006; Vedovatto et al., 2020), as well as reductions in protozoa population (Dennis et al., 1986) and methanogenic archaea abundance (McGarvey et al., 2019). In dairy cows grazing pastures and supplemented with concentrate, the addition of monensin improved apparent digestibility of dry matter (DM) and NDF without affecting crude protein (CP) digestibility (Kozerski et al., 2017) and CH_4 production (Grainger et al., 2010).

The widespread use of monensin has raised concerns due to the presence of residues in animal-derived products and the potential risk of promoting cross-resistance to antibiotics that are critically important for public health. This latter issue represents a primary concern from a One Health perspective, as the use of ionophores such as monensin may contribute to the selection of resistant bacterial populations with broader implications beyond livestock systems. Consequently, increasing social scrutiny has emerged regarding its inclusion in ruminant diets. In this context, both the European Union (Regulation No. 1831/2003) and Uruguay (Decree No. 332/024) have prohibited the use of antibiotics as growth promoters, thereby driving the search for natural alternatives aimed at modulating ruminal fermentation (Calsamiglia et al., 2005; Olynk, 2012).

The effects of phytochemicals on the digestive utilization of different nutrients are variable and largely dependent on the type and dose of compounds used (Benchaar, Calsamiglia, et al., 2008; Pangesti et al., 2024). The use of cinnamaldehyde plus eugenol or capsaicin showed no effects on the digestibility of DM, organic matter (OM), or NDF (Tager & Krause, 2011). However, other authors using cinnamaldehyde reported reductions of NDF digestibility (Benchaar, 2016; Tager & Krause, 2011) or, when using condensed tannins, decreases in CP degradation (Volpi-Lagreca et al., 2013). In contrast, Cherdthong et al. (2019) found an increase in DM digestibility when pepper leaves were used. Some authors found no effect on the production and proportion of volatile fatty acids (VFA) (Benchaar, 2016, 2020; Benchaar et al., 2006), while others reported reductions in VFA production as well as increases (Agarwal et al., 2009) or decreases (Tekippe et al., 2012) in the C2:C3 ratio. Ruminal NH_3-N concentrations remained unchanged when cinnamon oil and cinnamaldehyde, or cinnamaldehyde plus eugenol (Benchaar, 2016; Tager & Krause, 2011), were used. However, reductions were observed in other studies where diets were supplemented with a mix of cinnamaldehyde, eugenol, and capsicum oleoresin (Geraci et al., 2012), garlic oil (Mahmoud & Salah, 2017) or tannins (Dschaak et al., 2011; Nogueira, 2011), as well as in *in vitro* studies (Pangesti et al., 2024). Ruminal pH often remained invariable (Benchaar, 2016, 2020; Mahmoud & Salah, 2017; Vedovatto et al., 2020), however, other studies have shown that increasing doses resulted in pH increases with essential oils (Macheboeuf et al., 2008) and decreases with condensed tannins (Volpi-Lagreca

et al., 2013). Phytochemicals affect ruminal protozoal populations as high doses tend to reduce protozoa populations, whereas low doses may increase them (Khiaosa-ard & Zebeli, 2014). On the contrary, Tekippe et al. (2011) found no changes in the protozoal population when oregano leaves were used. Furthermore, reductions in CH_4 production have been observed with combinations of cinnamaldehyde, eugenol, and capsicum oleoresin, in both *in vitro* and *in vivo* trials (Khelil-Arfa et al., 2025) or with pepper leaves (Cherdthong et al., 2019). Additionally, the interactions among phytochemicals can be either synergistic or antagonistic, influencing their overall impact on ruminal fermentation (Firkins & Mitchell, 2023; Macheboeuf et al., 2008; Pangesti et al., 2024).

The additive evaluated in the present study consists of a mix of phytochemicals including polyphenols (up to 450 ppm), curcuminoids (up to 280 ppm), piperine (up to 15 ppm), and trans-cinnamaldehyde (up to 1000 ppm) (CCPA Group, Janzé, France). Individual supplementation of these compounds has shown positive effects on ruminal fermentation (Tian et al., 2023) and feed efficiency (Benchaar, McAllister, et al., 2008; Cherdthong et al., 2019).

Given that responses to additives may vary and that most previous studies were conducted under confinement conditions, it is therefore relevant to investigate their effects in dairy pasture-based systems. We hypothesized that use of monensin (MON) and a mix of phytochemicals (PHY) in a mixed diet –combining direct grazing with TMR supplementation– would modulate the ruminal fermentation environment and microbial activity, thereby improving fermentation efficiency while maintaining fiber utilization and organic matter digestibility. The objective of this study was to assess the potential of PHY as an alternative to MON in dairy cows grazing alfalfa and supplemented with TMR. To address this objective, we evaluated the effects of MON and PHY on: (1) the NDF degradability of alfalfa hay (AH); (2) the *in-situ* digestibility of organic matter (ISDom) of four cultivated pastures, AH, pre- and postpartum TMR (TMRpre and TMRpost, respectively), and a simulated diet of alfalfa plus TMRpost; and (3) ruminal fermentation patterns.

2. Materials and Methods

The experiment was conducted at the Estación Experimental Dr. Mario A. Cassinoni (EEMAC) of the Facultad de Agronomía (Paysandú, Uruguay, 32° S, 58° W) of the Universidad de la República (Udelar). Animal procedures were conducted under the approval of the Comité de Ética en el Uso de Animales de Experimentación (CEUA-CHEA, Ethics Committee for Animal Experimentation) of the Universidad de la República, Uruguay (Exp. #020300-000055-22).

2.1 Animals, Experimental Design, and Management

Within a larger experiment focused on the effects of the additives on productive performance and the dynamics of ruminal fermentation across the transition period and early lactation (Gonzalez-Chappe et al., 2025), the present study evaluated a subset of nine primiparous Holstein cows fitted with ruminal cannulas to assess the effects on nutrient utilization. At the beginning of the experiment, the cows weighed 569 ± 10.1 kg of body weight and were randomly assigned to one of three treatments: MON (0.3 g/cow/day of sodium monensin), PHY (50 g/cow/day of commercial mix of phytochemicals derived from plants including polyphenols –up to 450 ppm–, curcuminoids –up to 280 ppm–, piperine –up to 15 ppm–, and trans-cinnamaldehyde –up to 1000 ppm–, CCPA Group, Janzé, France), and C (Control), which received no additive. In the MON and PHY treatments, the additives were incorporated into the energy-protein concentrate of the TMR and were applied to each group of cows in each treatment. It is worth noting that, throughout the experimental period, several management practices were implemented to minimize potential animal interactions and within-treatment variability. In the pasture, a high herbage allowance was maintained to reduce grazing competition. During TMR supplementation, feed preparation and delivery were closely monitored on a daily basis, and each cow was allocated 1 m of feed bunk

space to ensure unrestricted and homogeneous access to the assigned diet. Detailed information on feeding management, TMR supplementation pre- and post-calving, and pasture utilization during grazing are provided in Gonzalez-Chappe et al. (2025).

2.2 Feedstuffs, Sampling, and Chemical Analysis

The feeds evaluated in this study that were used in the larger experiment included two TMRs and several forage resources. The TMRpre consisted of 38.7% corn silage, 31.5% barley straw, and 29.8% commercial concentrate, whereas the TMRpost comprised 30.8% corn silage, 13.8% alfalfa hay (AH), and 55.4% commercial concentrate (all proportions expressed on a dry matter basis). The forage resources included four cultivated pastures: alfalfa (A, *Medicago sativa*), tall fescue (FES, *Lolium arundinaceum*), chicory (CH, *Cichorium intybus*), oats (O, *Avena sativa*), and AH. In addition, a simulated diet consisting of TMRpost combined with A pasture (60% TMRpost + 40% alfalfa, TMRpost + A) was evaluated.

All feedstuffs were sampled weekly according to the animals' use of pastures and TMRs, for chemical composition analysis. Pasture samples were collected by simulating grazing. Detailed information on sampling procedures is provided in Gonzalez-Chappe et al. (2025). In all cases, the number of samples analyzed was greater or equal to three. For *in-situ* studies, and considering the relatively low variability in chemical composition, a composite sample was prepared for each feed resource. The AH was used as the standard feed for NDF degradability; a composite sample was prepared by pooling subsamples collected from 10% of the total bales. Samples were dried in a forced-air oven at 60 °C for 48 hours. Two subsamples were ground using a Wiley® mill with different mesh sizes: 5 mm for *in situ* incubations and 1 mm for chemical analyses.

Chemical analyses were performed at the Nutrition Laboratory and Feedstuffs Evaluation of Facultad de Agronomia (Universidad de la República) and included determinations of DM, ash, ether extract, and nitrogen (N), following the methods of the Association of Official Analytical Chemists (AOAC, 2000). The OM was calculated as DM minus ash, and CP was estimated as N × 6.25. The NDF and ADF fractions were analyzed sequentially using Ankom technology (Fiber Analyzer 200, Ankom Technology Corporation, Fairport, NY, USA), without sodium sulfite and with heat-stable amylase, and expressed ash-free (Van Soest et al., 1991). Non-fibrous carbohydrates (NFC) were calculated by difference (National Research Council, 2001). The chemical composition of the forages resources and mixed diets is presented in Table 1.

Table 1. Chemical composition (SD in parentheses) of total mixed rations (TMRs) and forages resources (% of dry matter, DM unless otherwise stated)

	TMRpre	TMRpost	TMRpost+A[1]	AH	A	CH	FES	O
DM, % of amount fed	42.0 (0.81)	46.3 (0.74)	35.7 (1.12)	92.9 (0.98)	20.0 (1.70)	12.8 (1.90)	18.9 (1.81)	15.0 (2.01)
OM	90.6 (1.34)	91.3 (0.44)	82.8 (0.47)	91.6 (0.78)	90.1 (0.51)	85.7 (0.82)	88 (2.88)	88.1 (0.23)
CP	13.2 (1.48)	15.9 (0.96)	15.7 (1.58)	18.1 (1.42)	24.5 (2.52)	24.0 (1.22)	17.3 (1.42)	20.1 (0.56)
NDF	44.2 (2.51)	26.8 (3.44)	26.9 (3.26)	36.9 (2.71)	28.7 (2.98)	26.3 (3.05)	43.5 (2.41)	36.6 (1.22)
ADF	25.9 (1.02)	14.0 (1.50)	23.1 (1.50)	27.3 (1.60)	20.0 (1.50)	18.1 (0.98)	25.4 (1.10)	20.9 (0.89)
EE	1.70 (0.72)	2.30 (0.21)	1.30 (0.22)	2.31 (0.41)	1.05 (0.24)	2.46 (0.38)	1.10 (0.20)	3.32 (0.51)
NFC	33.9 (0.25)	43.5 (0.87)	39.0 (1.01)	34.3 (1.38)	35.8 (1.21)	32.9 (1.29)	26.2 (1.62)	28.1 (0.68)

TMRpre: Prepartum TMR; TMRpost: Postpartum TMR. TMRpost + A[1]: Simulated postpartum diet (60% TMRpost and 40% A); AH: Alfalfa hay, A: Alfalfa pasture; CH: Chicory pasture; FES: Fescue pasture; O: Oat pasture. OM: Organic matter; CP: Crude protein; NDF and ADF: Neutral detergent fiber and acid detergent fiber, analyzed using heat-stable amylase and expressed ash-free; EE: Ether extract; NFC: Non-fibrous carbohydrates estimated as 100 − (% NDF + % CP + % EE + % ash). SD: standard deviation.

2.3 In-Situ Degradability and Digestibility

All determinations were conducted while the cows grazed alfalfa pasture. Between days 56 and 60 postpartum, the *in-situ* degradation kinetics of NDF and ISDom were evaluated using the nylon bag technique (Ørskov et al., 1980). For this purpose, 5 g of DM from each feed sample were placed in polyester bags (Sefar Inc., Switzerland, PET 1000 120-34-W; 10 × 20 cm, with an average pore size of 50 µm). To assess the *in-situ* degradation kinetics of NDF, the bags were filled with AH, in duplicate per animal and per incubation time. Bags were simultaneously introduced into the rumen and retrieved after 4, 8, 16, 24, 48, 72, and 96 hours. Incubation conditions, bag handling, and processing of undigested residues followed the procedures described by Trujillo et al. (2017). The ISDom at 48 h was determined for individual forage resources, TMRs, and TMRpost + A, using the same experimental conditions described above and retrieving the bags after 48 hours. The amount of material disappeared at each incubation time was calculated as the difference between the dry weight before and after incubation.

2.4 Ruminal Fermentation Parameters

On days 56 to 59 postpartum, ruminal fluid samples were collected at 0, 4, 8, 16, and 24 hours after the cows entered the pasture for the determination of pH, NH_3-N concentration, VFA, and protozoa counts, as described by Gonzalez-Chappe et al. (2025).

2.5 Calculations and Statistical Analysis

The data were analyzed using the SAS software (version 9.4; SAS OnDemand for Academics; SAS Institute Inc., Cary, NC, USA).

NDF degradation kinetics data were fitted to the model proposed by Ørskov & McDonald (1979) using the PROC NLIN procedure (nonlinear regression models, iterative Marquardt method):

$$Y(ti) = a + b\,(1 - e^{-ct}),\ t \geq 0$$

Where:

Y(ti) = NDF degradation at time ti;

a = soluble, rapidly degradable fraction (%);

b = insoluble, potentially degradable fraction (%);

c = degradation rate constant (h^{-1}).

Degradation kinetics parameters were analyzed using a mixed-effects model, with treatment as a fixed effect and cow as a random effect. The ISDom values were analyzed using a mixed-effects model including treatment, feedstuff, and their interaction (treatment × feedstuff) as fixed effects, and cow as a random effect. For ruminal parameters, the model included treatment, sampling hour, and their interaction (treatment × sampling hour) as fixed effects, and cow as a random effect.

Mean separation was performed using Tukey's test. Differences were considered statistically significant when $P < 0.05$, and trends were reported when $0.05 \leq P \leq 0.10$. Results are presented as least squares mean ± standard error of the mean (SEM).

3. Results

The effect of treatments on the *in-situ* degradation kinetics of NDF from AH is shown in Table 2 and Figure 1. The additives did not significantly affect the degradation potential of NDF, but they reduced the degradation rate (c) compared to the C treatment (0.06 vs. 0.07 h^{-1}).

Table 2. Effect of additives on *in-situ* degradation kinetics of neutral detergent fiber of alfalfa hay

Degradation kinetics parameters	Treatments			SEM	*P*-value
	C	MON	PHY		
Potentially degradable fraction, b (%)	42.0	43.0	39.0	0.12	0.21
Degradation rate, c (h^{-1})	0.07 a	0.06 b	0.06 b	0.01	0.01

C: Control; MON: Sodic monensin; PHY: Mix of phytochemicals; SEM: Standard error of the mean. Different letters indicate significant differences among treatments (P < 0.05).

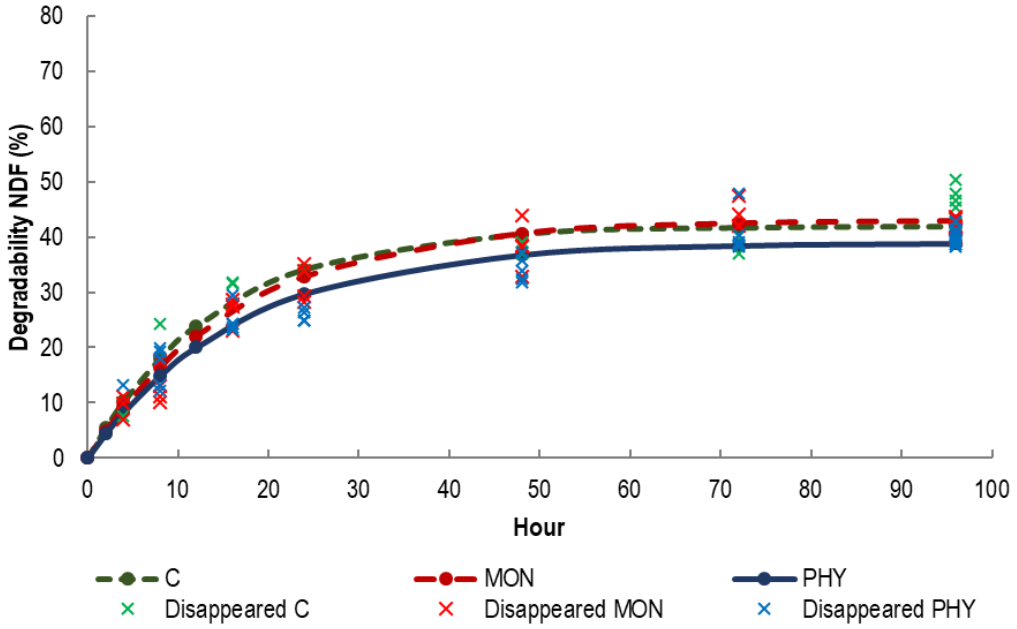

Figure 1. Effect of additives on degradation kinetics of neutral detergent fiber (NDF) of alfalfa hay

C: Control (green); MON: Sodic monensin (red); PHY: Mix of phytochemicals (blue).

Although the treatments had no effect on ISDom, significant differences were observed among feedstuffs (P < 0.05). The highest ISDom values were observed in the O and A pastures, followed by CH. Intermediate values were found in TMRpost and TMRpost + A, while FES and TMRpre showed lower values and the lowest ISDom was recorded for AH (Table 3). The treatment × feedstuffs interaction was also significant (P < 0.05). The TMRpre was the only feed that showed a reduction in ISDom when MON and PHY were used, compared to the C treatment (65.2 and 66.9% vs. 75.5% ± 1.36, respectively).

Additives had no significant effect on ruminal pH; however, there was a significant interaction between treatment and sampling time (P < 0.05, Table 4). Differences between treatments were observed at 0, 16, and 24 h (P < 0.05). The PHY treatment showed higher pH than MON at 0 and 24 h (6.56 and 6.65 ± 0.04 vs 6.37 and 6.39 ± 0.04, respectively), while at 16 h PHY was lower than MON (5.65 vs 5.85 ± 0.04; P < 0.05).

Total VFA concentration did not differ between treatments. Both additives increase (P < 0.05) the molar proportion of C3 and decrease the C2:C3 ratio (P < 0.05). The PHY treatment decreased (P < 0.05) the proportion of C4 compared to C treatment while the MON treatment showed an intermediate value (Table 4).

Table 3. *In situ* organic matter digestibility at 48 h (ISDom) of total mixed rations (TMRs) and forages resources (least square mean of treatments)

Feeds	ISDom (%)
TMRpre[1]	69.2 e
TMRpost[2]	83.1 c
TMRpost + A[3]	81.3 c
Alfalfa hay	62.4 f
Alfalfa pasture	96.6 a
Chicory pasture	93.6 b
Fescue pasture	75.5 d
Oat pasture	97.7 a
SEM	0.79

TMRpre[1]: Prepartum TMR; TMRpost[2]: Postpartum TMR; TMRpost + A[3]: Simulated postpartum diet (60% TMRpost + 40% alfalfa pasture). SEM: Standard error of the mean. Different letters indicate significant differences among feeds (P < 0.05).

Table 4. Effects of additives on ruminal fermentation parameters

	Treatments				*P*-value		
Ruminal parameters	C	MON	PHY	SEM	T	Sh	T*Sh
Ruminal pH	6.38	6.31	6.36	0.02	0.06	<0.001	<0.001
Total VFA, mmol/L	111.6	115.1	113.1	3.49	0.80	<0.001	0.57
Acetate (C2) (%)	62.5	60.3	60.0	0.75	0.55	0.001	0.95
Propionate (C3) (%)	23.4 b	26.3 a	27.3 a	0.66	0.001	0.11	0.74
Butyrate (C4) (%)	12.2 a	11.0 ab	9.87 b	0.31	0.001	<0.001	0.16
Isobutyrate (%)	0.35	0.30	0.29	0.01	0.28	<0.001	0.16
Valerate (%)	1.40	1.29	1.34	0.05	0.49	0.67	0.69
Isovalerate (%)	0.88	0.67	0.67	0.09	0.50	0.002	0.59
Acetate/Propionate ratio (C2:C3)	2.69 a	2.30 b	2.22 b	0.09	0.003	0.025	0.84
NH_3-N (ppm)	74.1 a	80.7 a	57.7 b	1.95	<0.001	<0.001	<0.001
Protozoa × 10^4 cells/mL	11.0 a	5.70 b	3.30 b	1.33	0.003	0.97	0.83

C: Control; MON: Sodic monensin; PHY: Mix of phytochemicals; SEM: Standard error of the mean; T: Treatment; Sh: Sampling hour. Different letters indicate significant differences among treatments (P < 0.05).

The NH_3-N concentration was affected by treatments, sampling hour, and the interaction between treatment and sampling hour (Table 4). While concentrations were similar among all treatments at the onset of grazing (48.6 ± 3.12 ppm) and increased until 8 h, differences emerged over time among treatments. At 4 h, the PHY treatment resulted in a lower NH_3-N concentration than MON and C (60.9 ± 3.17 vs. 154.5 ± 9.77 and 133.7 ± 8.46 ppm, respectively; P < 0.05). This trend continued at 8 h, where the NH_3-N concentration for PHY was also significantly lower than for MON and C (87.4 ± 4.80 vs. 142.6 ± 9.02 and 127.8 ± 8.08 ppm, respectively; P < 0.05). Thereafter, values decreased, with no significant differences among treatments.

Protozoa count was reduced (P < 0.05) in PHY and MON treatments compared to C (Table 4).

4. Discussion

The proposed hypothesis was partially confirmed, as improvements in ruminal fermentation profiles were observed without compromising the potential fiber degradation of AH or the ISDom of most of the evaluated feedstuffs. However, both the fiber degradation rate of AH and the ISDom of TMRpre were reduced with the use of both additives.

The degradation kinetics of NDF of AH are within the range reported by other authors (Alvir et al., 1998; Trujillo, 2006). Although treatments did not significantly affect the "b" fraction, the numerically lower value observed in the PHY treatment may be biologically consistent with the lower protozoal counts in this group, given the key role of protozoa in the ruminal fibrolytic ecosystem. The slight reduction observed in the rate of NDF degradation is consistent with shifts in ruminal fermentation, including a decreased C2:C3 ratio, decreased C4 concentration, and reduced protozoal count, potentially leading to a detrimental impact on the fibrolytic consortium and, ultimately, on fiber digestion (Newbold et al., 2015). These patterns align with the known effects of monensin on Gram-positive bacteria and propionate production, as well as the antimicrobial action, of phytochemicals. However, other authors have not observed any changes in the NDF degradation rate when using monensin alone or in combination with essential oils (Benchaar et al., 2006) or with other antibiotics (Vedovatto et al., 2020). The observed reduction in the NDF degradation rate of AH could suggest that the degradation rates of other forage resources may follow a similar pattern. If this were the case, a reduction in animal performance might be expected; however, this was not observed (Gonzalez-Chappe et al., 2025).

Both the chemical composition and the ISDom values reflected the differences between the various feed types (Calsamiglia et al., 2005; Jaurena et al., 1994). Among the TMRs, the higher NDF and ADF contents in TMRpre are consistent with its higher inclusion of barley straw, while the substitution of barley straw with AH and the greater concentrate proportion in TMRpost account for its higher ISDom. In this study, we found no effect of the additives on ISDom, consistent with the results reported previously (Benchaar, 2016, 2020; Benchaar et al., 2006; Plaizier et al., 2000; Tager & Krause, 2011; Vedovatto et al., 2020). However, the reduction observed in TMRpre suggests that their impact is influenced by feed type and the high proportion of low-quality fiber.

For the ruminal fermentation parameters, the most notable changes were an improvement in fermentation efficiency, reflected in a more glucogenic profile, a reduction in NH_3-N concentration and greater stability, and a decrease in protozoal populations. The increase in the molar proportion of C3 and decrease in C2:C3 ratio observed with both additives are consistent with previous reports (Benchaar, 2020; Benchaar, Calsamiglia, et al., 2008; da Silva et al., 2019; Gonzalez-Chappe et al., 2025; Tekippe et al., 2012). The combination of compounds in the PHY treatment may have exerted a broader and more stable antimicrobial pressure on butyrate-producing bacteria, resulting in lower butyrate concentrations compared to the C group. This may indicate improved metabolic efficiency, contributing to a more glucogenic fermentation profile. This shift could help explain the increases in milk and lactose yield reported by Gonzalez-Chappe et al. (2025), implying enhanced energy utilization efficiency and potentially reduced CH_4 emissions (Kholif & Olafadehan, 2021; Li et al., 2014; Suybeng et al., 2020). The lack of effects of the additives on pH and total VFA aligns with findings reported in literature (Benchaar, 2016, 2020; Gonzalez-Chappe et al., 2025; Khelil-Arfa et al., 2025; Tager & Krause, 2011; Tekippe et al., 2011). Fluctuations in NH_3-N concentration throughout the day reflect the temporal feeding pattern of the cows, with increases following grazing on alfalfa pasture, which is characterized by a high proportion of rapidly degradable nitrogen fractions (Cajarville et al., 2006; Khalili & Sairanen, 2000), and subsequent declines after access to the TMRpost, which provides readily fermentable energy. The potential ability of PHY to reduce these ammonia peaks while maintaining high ISDom for alfalfa pasture suggests that it improves nitrogen utilization efficiency without hindering the digestion of high-quality forage. This effect could be attributed to several factors, including the presence of phenolic compounds (Mahmoud & Salah, 2017; Nogueira, 2011; Tian et al., 2023; Zhu et al., 2021), reduction in hyper-ammonia-producing bacteria and protozoa (da Silva et al., 2019; Firkins & Mitchell, 2023; Macheboeuf et al., 2008; Suybeng et al., 2020), inhibition of microbial colonization to feed particles (Patra & Saxena, 2009), and an enhanced microbial protein synthesis (Mahmoud & Salah, 2017; Tian et al., 2023; Zhu et al., 2021). The reduction in protozoal count observed with both additives was consistent with their lipophilic properties, that affect membrane permeability, and with previous findings under both *in vivo* and *in vitro* conditions using similar additives (Dennis et al., 1986; Firkins & Mitchell, 2023; Soltan et al., 2018; Tian et al., 2023; Ye et al., 2018). This reduction may contribute to reduced CH_4 production, as methanogenic archaea are

known to establish and maintain symbiotic associations with certain protozoa (Honan et al., 2022; Sallam & Abdelgaleil, 2010). Although the sample size is limited, as is inherent to mechanistic studies involving ruminal cannulated cows, this study provides valuable insights into how additives modulate the ruminal environment under pasture-based systems. Overall, both additives similarly modified ruminal energy fermentation by reducing the C2:C3 ratio; however, their effects on nitrogen metabolism differed. Although ionophores such as monensin can reduce ruminal NH_3-N concentration (Benchaar et al., 2006; Marques & Cooke, 2021; Vedovatto et al., 2020), their effects may depend on diet composition, dosage, and microbial adaptation, potentially limiting long-term efficacy (Gonzalez-Chappe et al., 2025; Marques & Cooke, 2021). In the present study, which focused on 60 days postpartum, reduced ruminal NH_3-N concentration, whereas MON treatment did not. However, as reported by Gonzalez-Chappe et al. (2025), at 30 days postpartum both MON and PHY reduced concentrations, likely reflecting differences in mechanisms of action or in microbial adaptation.

An additional consideration when interpreting these results relates to the experimental design. Although treatments were applied at the group level, response variables were measured at the individual animal level to characterize biological responses associated with the study objectives. We acknowledge that this design may limit the independence of observations and, consequently, the strength of statistical inference. Nevertheless, several management practices were implemented throughout the experiment to minimize within-group variability and potential animal interactions, including maintaining a high herbage allowance during grazing, ensuring unrestricted access to supplementation through adequate feed bunk space, and closely monitoring feed preparation and delivery. Similar experimental approaches have been adopted in grazing and dairy cattle research when logistical and biological constraints limit the feasibility of complete experimental replication (de Melo et al., 2024; Mattiauda et al., 2013; Mendina et al., 2024). Therefore, the results should be interpreted with appropriate caution and viewed primarily as evidence of biologically relevant treatment responses under commercial grazing conditions.

5. Conclusions

The results of this study indicate that both monensin and the mix of phytochemicals modified ruminal fermentation patterns by promoting a more glucogenic profile and reducing protozoal populations, suggesting improved energy utilization efficiency. Meanwhile, they did not affect the potential NDF degradation of alfalfa hay or the digestibility of most evaluated feedstuffs, which appeared to be associated with fiber characteristics and mediated by changes in the ruminal microbial ecosystem.

In addition, the phytochemical mix reduced ruminal NH_3-N concentration, suggesting greater efficiency in dietary nitrogen utilization, which could translate into potential environmental benefits. Therefore, the phytochemical mix appears to be a viable alternative to monensin in dairy pasture-based systems. Nevertheless, further studies are required to assess its long-term impact, with a focus on elucidating the specific effects and mechanisms of action on the ruminal microbiota.

Transparency of Data

Available data: The entire data set that supports the results of this study was published in the article itself.

Author Contribution Statement

	C Dorao	A Cáceres	AI Trujillo	A Casal	DA Mattiauda	AC Dall-Orsoletta	M Arturo-Schaan	MÁ Bruni
Conceptualization			■					■
Data curation	■	■						■
Formal analysis			■					■
Funding acquisition						■	■	■
Investigation	■	■		■	■			■
Methodology			■	■				■
Project administration				■				■
Resources				■				■
Software								■
Supervision				■				■
Validation	■	■	■	■				■
Visualization	■		■					■
Writing – original draft	■	■	■					■
Writing – review and editing		■	■			■	■	■

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