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Advances and trends of dairy production in Uruguay

Effect of a multispecies fungal additive on rumen fermentation profile, degradability and kinetic gas production

Bruni, M. [1]; Chilibroste, P. [1]; Casal, A. [1]; Trujillo, A. I. [2]

[1]*Universidad de la República, Facultad de Agronomía, Departamento de Producción Animal, Paysandú, Uruguay*

[2]*Universidad de la República, Facultad de Agronomía, Departamento de Producción Animal, Montevideo, Uruguay*

Editor

Mariana Carriquiry
Universidad de la República, Montevideo, Uruguay

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Correspondence

María de los Ángeles Bruni
mbruni@fagro.edu.uy

Abstract

Two experiments evaluated the effect of a multispecies fungal complex (BP, BIOPREMIX MX®, Ruminal Fermentation Tech, Uruguay). In Experiment 1 (E1), the impact of adding BP to a total mixed ration (TMR) on ruminal fermentation profile and TMR in situ degradation kinetics was assessed. In Experiment 2 (E2), the effect of adding BP to various substrates on in vitro fermentability was examined. In E1, 4 Holstein cows with rumen cannulas were randomly assigned to Control (TMR with forage:concentrate ratio 75:25) or Control + 120 g/cow/d of BP (BP) and received ad libitum TMR for 30 days. Samples of TMR were ruminally incubated to estimate in situ degradation kinetic. Ruminal pH, ammonia, and volatile fatty acids (VFA) concentrations were measured just before feeding, 4 h and 8 h post feeding. In E2, a factorial arrangement included two BP levels (0 -Control or 6.5 g BP per kg dry matter incubated-WBP) and 8 substrates. In vitro gas production kinetics (GPk), dry matter digestibility (IVDMD), methanogenic potential (CH4), partitioning factor (PF), VFA, and microbial crude protein (MCP) were estimated. The BP increased proportion of propionate ($P \leq 0.05$) and reduced ketogenic:glucogenic ratio and Lag phase of NDF ($P \leq 0.05$). WBP tended to increase IVDMD, and substrate affected GPk, IVDMD, CH4, PF, VFA and MCP ($P \leq 0.01$). Overall, BP improved ruminal metabolism favoring a more glucogenic profile, a shortening Lag phase in NDF degradation, and increasing IVDMD.

Keywords: multifungal additive, in situ kinetic, in vitro fermentability

Efecto de un aditivo fúngico multiespecie sobre perfil de fermentación ruminal, degradabilidad y cinética de producción de gas

Resumen

Dos experimentos evaluaron el efecto de un complejo fúngico multiespecies (BP, BIOPREMIX MX®, Ruminal Fermentation Tech, Uruguay). En el Experimento 1 (E1) se evaluó el impacto de agregar BP a una ración totalmente mezclada (TMR) sobre la fermentación ruminal y la cinética de degradación de la TMR. En el Experimento 2 (E2) se evaluó el efecto de agregar BP a varios sustratos sobre la fermentabilidad in vitro. En E1, 4 vacas Holstein con cánulas ruminales fueron asignadas aleatoriamente a Control (TMR con relación forraje:concentrado 75:25) o Control + 120 g/vaca/d de BP (BP),

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y recibieron TMR *ad libitum* durante 30 días. Se incubaron ruminalmente muestras de TMR para estimar la cinética de degradación *in situ*. Se midió pH, concentración de amoníaco y de ácidos grasos volátiles (VFA) en líquido ruminal antes de la alimentación, 4 y 8 h después de la misma. En E2, un arreglo factorial incluyó dos niveles de BP (0 -Control o 6.5 g BP por kg de materia seca incubada -WBP) y 8 sustratos. Se estimaron la cinética de producción de gas in vitro (GPk), la digestibilidad de la materia seca (IVDMD), el potencial metanogénico (CH4), el factor de partición (PF), VFA y la proteína microbiana (MCP). El BP aumentó la proporción de propionato (P ≤ 0,05) y redujo la relación cetogénica:glucogénica y la fase Lag de la FDN (P ≤ 0,05). WBP tendió a aumentar la IVDMD, y el sustrato afectó a GPk, IVDMD, CH4, PF, VFA y MCP (P ≤ 0,01). En general, BP mejoró el metabolismo ruminal favoreciendo un perfil más glucogénico, una fase Lag más corta en la degradación de FDN y un aumento de la IVDMD.

Palabras clave: aditivo multifúngico, cinética *in situ*, fermentabilidad *in vitro*

Efeito de um aditivo fúngico multiespécie no perfil de fermentação ruminal, degradabilidade e cinética de produção de gases

Resumo

Dois experimentos avaliaramo efeito de um complexo fúngico multiespécie (BP, BIOPREMIX MX®, Ruminal Fermentation Tech, Uruguai). No Experimento 1 (E1) avaliou o efeito da adição de BP a uma ração mista total (TMR) no perfil de fermentação ruminal e na cinética de degradação in situ da TMR. No Experimento 2 (E2) avaliou o efeito da adição de BP a diferentes substratos na fermentabilidade in vitro. No E1, 4 vacas Holstein com cânulas ruminais foram aleatoriamente designadas para Controle (TMR com relação volumoso:concentrado 75:25) ou Controle + 120 g/vaca/d de BP (BP) e receberam TMR ad libitum por 30 dias. As mostras de TMR foram incubadas no rúmen para estimar a cinética de degradação in situ. O pH e concentrações de amônia e ácidos graxos voláteis (VFA) foram medidas no líquido ruminal imediatamente antes da alimentação, 4 horas e 8 horas após a alimentação. Em E2, um arranjo fatorial de níveis de BP (6,5 g BP/ kg MS incubado –WBP ou sem BP -Control) e 8 substratos**.** Foram estimadas a cinética de produção de gases in vitro (GPk), a digestibilidade da matéria seca (IVDMD), o potencial metanogênico (CH4), o fator de partição (FP), os AGV e a proteína microbiana (MCP). O BP aumentou a proporção de propionato (P ≤ 0,05) e reduziu a relação cetogênico:glicogênico e a fase Lag da FDN (P ≤ 0,05). O WBP tendeuaaumentara IVDMD e o substrato afetou, IVDMD, CH4, PF, VFA total e MCP (P ≤ 0,01). No geral, BP melhorou o metabolismo ruminal favorecendo um perfil mais glicogênico, um encurtamento da fase Lag na degradação da FDN e um aumento na IVDMD.

Palavras-chave: aditivo multifúngico, cinética in situ, fermentabilidade in vitro

1. Introduction

Feed additives can be used to manipulate rumen function, increase the level and efficiency of animal performance, and minimize adverse effects of diets on animal health and the environment[(1)]. A variety of products are normally included in the category of feed additives; the most studied in ruminants have been ionophores, yeasts, exogenous fibrolytic, amylolytic, proteolytic enzymes (or mixtures), phytonutrients and nutrients modulators of ruminal microbiota (sugars, amino acids, fatty acids, minerals, and vitamins, among others).

Exogenous enzymes that are widely used in ruminants are typically derived from fungi and bacteria (i.e., species of the genera Trichoderma, Aspergillus, Penicillium, Pseudomonas and Bacillus). These enzymes can be added to the total mixed ration, hay, silages, concentrates, supplements, or premix to increase the availability of nutrients by providing high levels of cellulosic and hemicellulosic activity[(2)(3)].

Several studies have examined the effects of these additives in ruminants growth performance[4] and particularly in feed intake, milk yield and composition, and feed efficiency on lactating dairy cows[5][6][7][8][8][10][11]. Enhanced production efficiency by the use of additives might come from alteration of the rumen microbial population, stabilization of the rumen environment, optimization of ruminal metabolism and/or improvement of cell wall degradation, resulting in higher efficiency of energy utilization and reduction of methane emission. However, the response of animals to dietary enzyme supplementation is highly variable. This variability can be attributable to different factors as: type and activity of enzyme, level and method of enzyme supplementation, type of diet fed, level of feed intake, combination with other additional feed additives, and animal factors such as age, physiological stage, and stress state[5][6][7][8][9].

The multispecies fungal complex additive predominantly consists of a fungal biomass enriched in exogenous multienzyme complexes derived from various fungal strains (including genera such as Trichoderma, Aspergillus, Pleurotus, Pycnoporus, among others). This additive, in general, is combined with other nutrients such as vitamins, minerals, yeast, essential amino acids, urea, and sugars to achieve a functional balance of the ruminal microbiota. In this context, it was hypothesized that dietary supplementation with this multispecies fungal complex additive would improve ruminal fermentation and fiber utilization in dairy cows. To test the hypothesis a combination of an in vivo with an in vitro approach was carried out. The first objective was to evaluate the effects of a multispecies fungal complex added to a total mixed ration (TMR) on ruminal fermentation profile and in situ degradation kinetics of organic matter (OM) and neutral detergent fiber (NDF) of the TMR. A second objective was to evaluate the effects of the multispecies fungal complex added to different substrates on kinetic gas production, in vitro dry matter digestibility (IVDMD), methanogenic potential and indicators of fermentation efficiency.

2. Materials and methods

The procedures for care and handling of animals employed for this experiment were in accordance with the Guide of the Animal Experimentation Committee of the University of the Republic. Two experiments were carried out at the Experimental Station “Dr. Mario A. Cassinoni” (EEMAC) of Agronomy Faculty (Universidad de la República, Uruguay) in Paysandú, Uruguay (32°S, 58°W).

2.1 In situ degradation and ruminal fermentation profile

2.1.1 Experimental design and treatments

Four first-lactation Holstein cows fitted with ruminal cannulas (KEHL® Industria e Comercio LTDA – ME, Brasil) [BW, 452 ± 33 kg; 2.6 ± 0.3 body condition score (BCS; scale 1-5, Edmonson and others[12]), and 129 ± 35 days in milk (DIM) at the beginning of the study] were utilized in a completely randomized design. The experimental period lasted 30 d, consisting of 20 d for adaptation and 10 d for data and sample collection. The cows were randomly assigned to one of the 2 treatments: 1) TMR without supplementation (Control) and 2) TMR supplemented with 120 g/cow/d of a multispecies fungal complex additive (BP, BIOPREMIX MX®, BIOPREMIX Ruminal Fermentation Tech, Uruguay) top-dressed on the TMR (BP). The most remarkable enzymes that are present in this additive are: endoglucanases, exoglucanases (pure culture or crosses), xylanases, laccases, celobio-hydrolases, beta-glucosidases, hemicellulases and pectinases. Cows were housed in individual covered pens with free access to drinking water and the TMR was offered ad libitum. The TMR was balanced for minerals and vitamins and formulated to cover the nutritional requirements of cows according to NRC[13] recommendations. The TMR was prepared daily before feeding with a vertical mixer (Mixer Mary 55, Uruguay) and placed in the feeders at approximately 9:00 h. After establishing a consistent feeding routine, samples of TMR for both treatments were taken daily for 10 consecutive days and stored at -20 ºC. All samples were pooled to create a

composite sample and then one sub-sample was refrigerated for short-term storage, and the other was dried at 60 ºC in a forced air oven and stored at room temperature for subsequent chemical analysis. The ingredients and chemical composition of the TMR and BP are presented in **Table 1**.

Table 1. Ingredients and nutrient composition (SD in parentheses) of the total mixed ration (TMR) and the multispecies fungal complex additive (BP) (% of DM, unless otherwise stated)

Item	TMR	BP[6]
Ingredients, %[1]		
Sorghum silage	75.6	---
Corn grain, ground	5.6	---
Soybean meal	16.8	---
DDGS[1]	---	8.0
Urea	0.7	18.0
Optigen®[2]	---	3.0
Multi-enzymatic complex	---	0.2
Yeast	---	0.4
Sodium chloride	0.56	10.0
Calcium carbonate	0.34	58.0
Dicalcium phosphate	0.22	---
Magnesium oxide	---	0.4
Mineral and vitamin mix NutriBov®[3]	0.14	---
Mineral and vitamin mix Rovimix®[4]	---	2.0
Sulphur	0.08	---
Nutrient composition, %[5]		
DM, % of amount fed	30.3 (0.7)	93.9
OM	92.2 (0.2)	46.3
CP	12.6 (0.2)	10.6
NDF	49.0 (0.6)	11.0
ADF	25.2 (2.3)	s/d
EE	1.3 (0.1)	4.2
NFC	29.2 (0.24)	20.5

[1]DDGS: dried distiller's grains with soluble; [2]Optigen®: controlled and continual supply of non-protein nitrogen; [3]NutriBov® mix contained: Zn, 12000 mg/kg; Cu, 4000 mg/kg; Se, 40 mg/kg; I, 200mg/kg; Co, 40 mg/kg; Mn, 16000 mg/kg; vitamin A, 1600000 UI/kg; vitamin D3, 320000 UI/kg and vitamin E, 6000 UI/kg. [4]Rovimix® mix contained: Zn, 50000 mg/kg; Cu, 14000 mg/kg; Se, 300 mg/kg; I, 2000 mg/kg; Co, 400 mg/kg; Mn, 72000 mg/kg; Fe, 50000 mg/kg; vitamin A, 5000000 UI/kg; Vitamin E, 20000 UI/kg. [5]DM: dry matter; OM: organic matter; CP: crude protein; NDF and ADF: Neutral and Acid detergent fiber expressed ash-free and alpha-amylase treated; EE: ether extract; NFC: non-fibrous carbohydrates calculated as: 100 – (% NDF + % CP + % EE + % ash). [6]BIOPREMIX MX®, BIOPREMIX Ruminal Fermentation Tech, Uruguay

2.1.2 Rumen degradation kinetics and fermentation profile

The OM and NDF in situ degradation kinetics of TMR of each treatment were determined using the nylon bag technique[(14)]. Approximately 5 g of dry matter (DM) of TMR refrigerated subsample were weighed into nylon bags and introduced simultaneously in the rumen immediately before the meal and removed sequentially at 4, 8, 12, 30, 48, 72 and 96 h. The incubation conditions, manipulation of undigested residues, and the models and procedures used to fit data were according to Trujillo and others[(15)].

Rumen fluid samples were taken from different portions of the rumen with a manual extraction device and filtered through two layers of cheesecloth. Samples were collected on two consecutive days and on the first day that

the nylon bags were removed before feeding and at 4 and 8 hours after TMR supply. The pH of the filtered ruminal fluid was measured immediately using a portable digital pH-meter (Milwaukee, Model MW102). Aliquots of the filtered ruminal fluid were preserved according to Mattiauda and others[16], centrifuged at 10000x g for 10 min at 4°C and stored at -20°C for subsequent analysis of ammonia (NH_3-N)[17] and volatile fatty acids (VFA)[18] concentrations.

2.2 In vitro gas production profile and fermentation efficiency

2.2.1 Experimental design and treatments

In the in vitro assay, two levels of BP: 0 (Control) and 6.5 g of BP per kg DM (added directly on the substrate, WBP) and 8 substrates were tested in a 2×8 factorial arrangement. Seven substrates were obtained from the dairy cattle farm of EEMAC: TMR (used in the in vivo approach), whole plant sorghum silage, ryegrass silage, alfalfa silage, barley straw, rice straw, and wood chips. The eighth substrate was a native pasture, samples of this substrate were obtained from Campos grasslands at Prof. Bernardo Rosengurtt Experimental Station, Agronomy Faculty in Cerro Largo (32°35'S, 54°15'W). All substrates were oven-dried at 60 ºC for 48 h, grounded in a Wiley mill using 1 mm screen and stored at room temperature in nylon bags for later determination of in vitro gas production and chemical components. The samples (weighing 0.5 g DM) were placed into a nylon bag (4 x 5 cm, 45 ± 3 µm average porosity) and the bags were hermetically sealed. Subsequently, these bags were inserted into 125 ml amber bottles equipped with rubber stoppers. Chemical composition of the substrates is shown in **Table 2**.

Table 2. Nutrient composition (% of DM) of the different substrates

	Composition (%)[1]					
Item	OM	CP	NDF	ADF	EE	NFC
Substrates						
Total mixed ration	92.36	12.52	49.45	27.63	1.33	29.06
Sorghum silage	92.10	5.90	51.50	30.70	2.90	31.80
Ryegrass silage	85.10	10.90	49.60	29.90	3.60	21.00
Alfalfa silage	91.87	14.09	51.54	37.99	2.36	24.18
Native pasture[2]	91.48	12.07	73.27	36.38	0.48	5.66
Rice straw	89.24	2.55	82.27	53.42	1.22	3.20
Barley straw	82.72	2.81	72.10	43.09	0.59	7.22
Wood chips[3]	92.17	1.48	88.95	71.14	0.14	1.61

[1]OM: organic matter; CP: crude protein; NDF and ADF: Neutral and Acid detergent fiber expressed ash-free and alpha-amylase treated; EE: ether extract; NFC: non-fibrous carbohydrates calculated as: 100 – (% NDF + % CP + % EE + % ash); [2]Native pasture from Campos grassland (32°35'S, 54°15'W); [3]Wood chips: forest by-product.

2.2.2 Inoculum

The day of the incubation, inoculum for each treatment was taken from the pair of fistulated cows supplemented (BP) or non-supplemented (Control). Two hours before feeding, 1 L of rumen fluid plus solid fraction was collected from each cow and placed into independent and preheated thermal containers. The rumen fluid was transported to the laboratory, liquefied, and mixed in equal portions and then filtered through four layers of cheesecloth. Forty mL of medium[19] and 10 mL of processed ruminal fluid for each treatment were added into each pre-warm bottle, sealed with stoppers and aluminum seals, and incubated at 39°C in a water bath for 96h. All manipulations were under continuous CO_2 flushing and kept at 39°C.

2.2.3 Kinetic gas production, in vitro dry matter digestibility, methanogenic potential, and fermentation efficiency

The in vitro fermentation was determined using the gas production technique[20]. Pressure and volume of gas were measured at 0, 2, 4, 6, 8, 12, 16, 24, 30, 48, 72 and 96 h post-incubation. During incubation 3 bottles (replicates) for each substrate-treatment were used, and 3 bottles without substrate were incubated as blank per each inoculum. The entire incubation was repeated in two periods (i.e., two runs). Kinetic parameters of gas production were fitted with a nonlinear model[21] as described by Elghandour and others[22].

Following each gas production reading from the bottles, an aliquot of gas was stored in a vacuum vial for later determination of methane concentration. The quantification was done using a gas chromatograph modified for the determination of greenhouse gasses (Agilent 7890B, USA) that was equipped with a stainless-steel column with Hayesep q packing of 80/100 mesh of 12 feet × 1.8 × 2 mm and with a flame ionization detector. FID). N_2 was used as the carrier gas with a furnace temperature of 60 °C, injector temperature of 200 °C and FID of 250 °C and a constant pressure flow of 12 psi. The methanogenic potential was estimated by multiplying the methane concentration and the accumulated gas volume (mL/g OM incubated) and expressed as mg of CO_2 Eq/g OM incubated[23].

At the end of incubation (i.e., 96 h) all bottles were put on ice to stop the fermentation, then the nylon bags with the residues were removed from the bottles, rinsed thoroughly with distilled water, dried at 55 °C for 48 h, and weighed to estimate IVDMD.

To evaluate fermentation efficiency, the partitioning factor (PF), total VFA and microbial crude protein (MCP) were estimated. The PF was determined as the ratio of the amount of digestible DM (aIVDMD) to the volume of gas production (GP) at 96 h[24]. The VFA and MCP were estimated according to the following equations:

VFA = −0.00425 + 0.0222 × GP, where VFA is expressed in mmol per 200 mg DM and GP is the net gas production after 24 h of incubation[25].

MCP = aIVDMD − PF × GP, where MCP is expressed in mg g^{-1} DM, GP is the net gas production after 24 h of incubation, and the PF is the specific partitioning factor for each substrate[24].

2.3 Chemical analysis

All samples from in vivo and in vitro experiments (feedstuffs, in situ and in vitro residues) were ground through a Wiley mill (Thomas Scientific) using 1 mm screen for chemical analysis. Dry matter, ash, ether extract and nitrogen (N) contents were determined according to the procedure of the AOAC[26]. The OM was calculated as DM minus ash, and crude protein (CP) was calculated as N × 6.25. The NDF and acid detergent fiber (ADF) analysis were determined sequentially using an ANKOM200 Fiber analyzer unit based on the procedure described by Van Soest and others[27]. A heat stable amylase was used in the analysis of NDF, and NDF and ADF are expressed without residual ash. The non-fibrous carbohydrates (NFC) were calculated as: 100 – (% NDF + % CP + % EE + % ash).

2.4 Statistical analysis

The data of rumen fermentation profile and parameters of in situ degradation were analyzed using a mixed model in which treatment was considered a fixed effect and the cow as a random effect. The rumen fermentation profile was analyzed before (0 h) and after feeding (until 8 h).

Data of gas production parameters, IVDMD, PF, VFA, MCP and methanogenic potential were analyzed using a mixed model with treatment, substrate and their interaction as fixed effects, and run as a random effect.

All statistical analysis was performed using SAS Academic Edition (SAS OnDemand for Academics, SAS Institute Inc.). To analyze normality of the data, the Proc Univariate was used. Means were separated with the Tukey test and significant differences were considered when P<0.05 and trend when P≤0.10. Values reported are least squares means and its associated standard errors.

3. Results

3.1 In situ degradation and ruminal fermentation profile

There were no significant differences in ruminal pH, NH3-N concentration, and total VFA concentration between cows fed BP and the Control group, neither before nor after feeding. Interestingly, BP cows presented higher (P ≤ 0.05) molar proportion of propionate (P) resulting in a lower (P ≤ 0.05) ketogenic:glucogenic ratio (A+B:P; acetate (A), butyrate (B)) compared to the Control cows, both before and after feeding (**Table 3**).

Table 3. Effect of adding of the multispecies fungal complex (BP) to total mixed ration (TMR) on ruminal fermentation characteristics (before and post feeding) in dairy cows

	Treatment[1]			P-value
Item	BP	Control	SEM	T
Before-feeding (0 h)				
pH	6.7	6.7	0.04	0.74
NH_3-N (mg/L)	48.1	53.6	3.85	0.15
Total VFA, mmol/L[2]	108.9	104.8	3.40	0.40
Individual VFA (mol/100 mol)				
Acetate (A)	69.9	71.7	0.88	0.16
Propionate (P)	20.8	18.8	0.59	0.02
Butyrate (B)	9.2	9.4	0.37	0.67
A:P ratio	3.4	3.8	0.16	0.06
A+B:P ratio	3.8	4.36	0.16	0.04
Post-feeding (until 8 h)				
pH	6.6	6.5	0.05	0.25
NH_3-N (mg/L)	104.8	99.9	12.40	0.79
Total VFA[2] (mmol/L)	126.4	133.1	5.90	0.43
Individual VFA (mol/100 mol)				
Acetate (A)	65.8	65.9	0.56	0.96
Propionate (P)	23.5	22.1	0.39	0.02
Butyrate (B)	10.6	12.0	0.21	<0.01
A:P ratio	2.8	3.0	0.07	0.09
A+B:P ratio	3.3	3.6	0.08	0.02

[1]BP: Total mixed ration supplemented with 120 g/cow/d of multispecies fungal complex additive BIOPREMIX MX®, Control: Total mixed ration without supplementation; [2]Total VFA: A+P+B.

The parameters that characterize the in situ kinetic of ruminal degradation of the TMR are presented in **Table 4**. The soluble (a) and potentially degradable (b) fractions or the degradation rates (c) of OM and NDF were not affected by treatments (P>0.05). The model that provided the best fit for the degradation of NDF was a model with a Lag phase, and the value of this parameter was 61% lower (P≤0.05) in BP cows compared to Control cows.

Table 4. Effect of adding the multispecies fungal complex (BP) to total mixed ration (TMR) on the in situ ruminal degradability of OM and NDF in dairy cows

	Treatment[1]			
Item	BP	Control	SEM	P-value
OM[2]				
Soluble fraction (a), g/kg	0.19	0.16	0.013	0.24
Potentially degradable fraction (b), g/kg	0.67	0.66	0.042	0.82
Degradation rates (c), h^{-1}	0.02	0.03	0.005	0.39
NDF[3]				
Potentially degradable fraction (b), g/kg	0.72	0.63	0.05	0.33
Degradation rates (c), h^{-1}	0.02	0.03	0.001	0.33
Lag (h)	0.87	2.21	0.22	0.05

[1]BP: Total mixed ration supplemented with 120 g/cow/d of multispecies fungal complex additive BIOPREMIX MX®, Control: Total mixed ration without supplementation; [2]OM: organic matter, model: a + b (1-Exp $^{(-ct)}$); [3]NDF: Neutral detergent fiber, model: b (1-Exp $^{(-c\,(t-Lag))}$), b corrected by soluble fraction: 0.16 and 0.13 (g/kg) for BP and Control, respectively.

3.2 In vitro gas production profile and fermentation efficiency

The chemical composition of substrates, commonly used in ruminant production systems, showed a wide variation. The CP content ranged from 14.1% in alfalfa silage to 1.4% in wood chips. The NDF and ADF contents ranged from 89 to 49.5% and from 71.1 to 27.6% in wood chips and TMR, respectively. The TMR and whole plant sorghum silage presented higher NFC than ryegrass and alfalfa silages, and it was substantially higher than the other substrates (**Table 2**).

The treatments had no significant effect on the in vitro gas production parameters, PF, VFA, MCP and methanogenic potential, while the IVDMD of WBP tended to increase (4%, P=0.10) compared to Control. The in vitro gas production parameters, IVDMD and indicators of fermentation efficiency were significantly affected (P<0.01) by substrate. Furthermore, a trend (P=0.08) in the interaction between treatment and substrate was found in MCP (**Table 5**), wherein TMR with BP increased 12% compared to Control (data not shown). The differences in gas production kinetic and fermentation efficiency indicators between substrates are shown in **Table 6**.

Table 5. Effect of adding the multispecies fungal complex (BP) to different substrates on in vitro gas production kinetics, digestibility of the dry matter at 96 h, methanogenic potential and fermentation efficiency indicators

	Treatment[1]			*P*-value[2]		
Item	WBP	Control	SEM	T	S	T×S
GPmax (mL/g OM)[3]	242.7	229.5	6.34	0.16	<0.01	0.12
c (h^{-1})[4]	0.021	0.023	0.002	0.32	<0.01	0.76
IVDMD (%)[5]	49.79	47.73	0.87	0.10	<0.01	0.29
PF (mg/mL)[6]	2.36	2.38	0.03	0.65	<0.01	0.56
VFA (mmol/200 mg DM)[7]	0.41	0.41	0.01	0.77	<0.01	0.11
MCP (mg/g DM)[8]	285.8	269.9	21.4	0.13	<0.01	0.08
CO_2eq (mg/g OMi)[9]	107.1	107.9	10.8	0.88	<0.01	0.35

[1]WBP: Total mixed ration with 6.5 g of multispecies fungal complex additive BIOPREMIX MX® per kg of dry matter, Control: Total mixed ration without supplementation; [2]T: treatment, S: substrate, T×S: interaction treatment per substrate; [3]GPmax: asymptotic GP (mL/g DMof organic matter); [4]c: rate of GP (h^{-1}), model: GPt= GPmax (1-EXP(-c*t)); [5]IVDMD: in vitro dry matter digestibility; [6]PF: partitioning factor; [7]Estimated volatile fatty acid; [8]Estimated microbial crude protein; [9]Methanogenic potential (mg of CO_2 eq /g of organic matter incubated)

Table 6. In vitro gas production kinetics, digestibility of the dry matter at 96 h, fermentation efficiency indicators and methanogenic potential of the different substrates

Item	GPmax[1] (mL/g OM)	c[2] (h^{-1})	IVDMD (%)	PF[3] (mg/mL)	VFA[4] (mmol/200 mg DM)	MCP[5] (mg/g DM)	CO_2 eq[6] (mg/g OMi)
TMR	318 a	0.024 ab	76.6 a	2.7 a	0.624 a	389.6 a	152.9 ab
Sorghum silage	285 ab	0.021 b	57.1 b	2.2 b	0.485 c	306.1 b	135.6 bc
Alfalfa silage	234 c	0.035 a	59.7 b	2.6 a	0.583 ab	249.1 b	136.3 abc
Ryegrass silage	287 bc	0.028 ab	74.7 a	2.8 a	0.560 b	394.4 a	175.1 a
Native pasture[7]	184 c	0.021 b	50.0 c	2.7 a	0.327 d	254.8 b	80.8 d
Barley straw	287 ab	0.011 c	48.7 c	2.7 a	0.283 d	313.8 b	94.4 d
Rice straw	292 ab	0.012 c	44.2 d	2.2 b	0.277 d	295.7 b	104.4 cd
Wood chips[8]	76 d	0.028 a	7.8 e	1.1 c	0.169 e	18.9 c	30.9 e
SEM	13.5	0.002	1.78	0.07	0.0172	24.82	13.2

[1]GPmax: asymptotic GP (mL/g of organic matter); [2]c: rate of GP (h^{-1}), model: GPt=GPmax (1-EXP$^{(-c*t)}$); [3]PF: Partitioning factor; [4]Estimated volatile fatty acid; [5]Estimated microbial crude protein; [6]Methanogenic potential (mg of CO_2 eq/g of organic matter incubated); [7]Native pasture from Campos grasslands (32°35'S, 54°15'W); [8]Wood chips: forest by-product. Means with different letters in the same column differed with P <0.05.

4. Discussion

Our hypothesis was partially confirmed, revealing that the multispecies fungal complex additive contributed to enhancing the ruminal fermentation profile; however, the effects were less pronounced in ruminal fiber utilization, IVDMD and in the improvement of microbial protein synthesis. Additionally, the in vitro approach indicated a significant substrate effect on all response variables.

The addition of the multispecies fungal additive in our study increased the molar proportion of P, decreased the A+B:P ratio and showed a tendency to decrease the A:P ratio both before and after feeding. These results are consistent with the decrease in the A:P ratio in supplemented dairy cows with exogenous fibrolytic enzymes reported by other authors[28][29]. Our results also align with the numerical decreases in both A:P ratio and A+B:P ratio reported[30], and with the significant increases in the molar P proportion and decreases in A:P ratio obtained from in vitro studies[31]. A facilitated nutrient release, promoted by the multispecies fungal additive and the consequent shift towards a more glucogenic fermentation profile, would provide increased energy supply contributing to enhanced microbial growth.

On the other hand, we observed a significant reduction in the Lag phase of NDF degradation in supplemented cows. An earlier onset of NDF degradation would be showing better conditions for fiber utilization. However, other studies[8][29][30] did not observe any improvement in degradation of TMR in supplemented cows with exogenous fibrolytic enzymes, suggesting that in some instances enzyme supplements may interact with microbial metabolism rather than simply increase feed degradation in the rumen[5] or modify feed utilization throughout the entire gastrointestinal tract[32]. Particularly, Beauchemin and others[32] proposed that the enzymes may be released into the ruminal fluid and pass through rapidly before they can exert their full effect, potentially impacting digestion, and nutrient absorption in the small intestine when enzymes are added to the TMR just before feeding. It should be noted that we added the multispecies fungal complex to the TMR in this manner, which could account for the slight effect on TMR degradation.

The more glucogenic fermentation profile, the earlier initiation of NDF degradation and the high stability observed in the ruminal profile (data not shown) may have provided more energy supply for ruminal microbial fermentation.

In this context, an increase of microbial protein could be expected in supplemented cows. Even though we did not measure microbial protein synthesis, an estimation of microbial protein showed a 12% increase in the TMR with BP in the in vitro approach. An increase in ruminal microbial protein synthesis was also reported in dairy cows supplemented with exogenous enzymes[28].

It is important to highlight that, in parallel, while the in vivo experiment was running, individual DM intake (DMI) and productive performance of a group of 8 first lactation cows that were supplemented (BP) and non-supplemented (Control) were recorded. Although the number of animals is not sufficient for statistical analysis, the supplemented cows showed increases in DMI (8%; 18.2 vs. 16.8 kg DM/day), milk yield (19%; 22 vs. 18.5 kg/day), milk protein (18%; 0.77 vs. 0.65 kg/day), and milk lactose (17%; 1.14 vs. 0.97 kg/day) compared to non-supplemented cows (Control). Consistent with these findings, a farmlet experiment conducted by our research group involving 24 cows managed in a pasture-based system and supplemented or not with BP showed a significant increase in milk yield (11%; 22 vs. 19.8 kg/day). In that sense, results reported in a recent review[9] provided further support for the positive impact of exogenous enzyme supplementation on dairy cows performance and milk solids.

In the in vitro approach, chemical composition and digestibility of the substrates are within the reported ranges with variations possibly attributed to differences in varieties, environmental conditions, growth stage at harvest and processing techniques[33][34][35][36]. All response variables (kinetic gas production, methanogenic potential, PF, VFA and MCP) were significantly affected by substrate, which is in line with the nutrient content and inherent characteristics of the substrates evaluated.

Analyzing the most contrasting substrates, wood chips showed the lowest extent of GP and methanogenic potential. This can be explained by their very low IVDMD and high NDF and ADF content[37][38][39]. In addition, the difficulty of ruminal microbes to attach to the particles of this substrate could also contribute to its limited fermentation[40]. On the contrary, TMR presented the highest GP value and one of the highest methanogenic potentials. Although the latter may seem contradictory, when estimating the methanogenic potential per gram of truly digestible substrate (data not shown) the values are reversed, with wood chips having the highest methanogenic potential and TMR the lowest.

In general, GPmax showed a positive relationship with IVDMD and/or the NFC content, and a negative one with the NDF concentration and its quality[37][38]. A higher PF, as an index of partitioning of microbial biomass and fermentation gasses, indicates that a greater proportion of DM is utilized for synthesizing microbial biomass[24]. Conversely, the lowest PF values were associated with lower digestibility, and probably with specific characteristics of the cell wall or other compounds that were not analyzed in the chemical analysis. The TMR presented the best fermentation efficiency indicators in all the variables evaluated in the in vitro approach, which could be attributed to the higher concentration of NFC and lower concentration of NDF and ADF. This would lead to a more glucogenic fermentation profile, coinciding with our in vivo results as well as those reported by other authors[28][36].

The lack of a response to the addition of the multispecies fungal complex may suggest an absence of limitations in fibrolytic microorganisms, their enzymes, or nutrients within the rumen inoculum used in this study. Additionally, the trend showing a 4% improvement in IVDMD aligns with findings from other in vitro studies[5][31][37][38][41].

5. Conclusions

The addition of a multispecies fungal complex to a TMR improved the ruminal fermentation profile, possibly due to increased availability of energy and precursors for both ruminal and animal metabolisms. Further research is needed to explore the effectiveness of these complexes across diverse fibrous substrates.

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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

María Bruni: Conceptualization; investigation; resources; project administration; formal analysis; writing – original draft, writing – review & editing.

Pablo Chilibroste: Funding acquisition; project administration; conceptualization; formal analysis; writing – review & editing.

Alberto Casal: Resources; writing – review & editing.

Ana Inés Trujillo: Conceptualization; investigation; resources; project administration; formal analysis; writing – original draft, writing – review & editing.

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